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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

Objectives: Superoxide dismutase (SOD) is an antioxidant enzyme that acts against superoxide, an oxygen radical, released in inflammatory pathways and causes connective tissue breakdown. In this study, SOD activities in gingiva and gingival crevicular fluid (GCF) from patients with chronic periodontitis (CP) and periodontally healthy controls were compared.

Material and Methods: Twenty-six CP patients and 18 controls were studied. In patients, teeth with moderate-to-severe periodontal breakdown and $\geqslant 5$ mm pockets that required full-thickness flap surgery in the right or left maxillary quadrant, and in controls, teeth scheduled for extraction for orthodontic reasons were studied. After the clinical measurements (probing depth, clinical attachment level, gingival index, gingival bleeding index, plaque index), GCF samples were collected. Tissue samples were harvested from the same teeth, during flap operation in patients and immediately after tooth extraction in controls. SOD activities were spectrophotometrically assayed.The results were statistically analysed.

Results: Gingival SOD activity was significantly higher in the CP group than in controls ($p < 0.05$). No significant difference was found in GCF SOD activity between the groups ($p > 0.05$). Correlations between gingival and GCF SOD activities were not statistically significant in CP and control groups ($p > 0.05$).

Conclusion: In CP, SOD activity seems to increase in gingiva, probably as a result of a higher need for SOD activity and protection in gingiva in CP than in periodontal health, while not significantly changing in GCF, suggesting a weak SOD activity in GCF in periodontal disease state. The weak correlation between gingival and GCF SOD activities suggests distinct actions of these SODs.

Key words: antioxidant mechanism; chronic periodontitis; gingiva; gingival crevicular fluid; superoxide; superoxide dismutase

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The antimicrobial activities of monocytes and polymorphonuclear leucocytes (PMNLs) have been broadly characterized as being either oxygen-dependent or oxygen-independent systems (Halliwell 2000). Oxygen-dependent systems include the production of reactive oxygen species (ROS). ROS is a collective term, which includes oxygen-derived free radicals: superoxide (O_2^-), hydroxyl (OH), and nitric oxide (NO), and non-radical derivatives of oxygen; hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCL) (Chapple 1997, Halliwell 2000, Waddington 2000). Following stimulation by bacterial antigen, PMNLs produce O_2^- via the metabolic pathway of the ''respiratory burst'', during phagocytosis (Curnutte & Babior 1987, McCord 1993, Gutteridge 1994, Chapple 1997, Halliwell 2000, Waddington 2000). Several inflammatory cells, fibroblasts, vascular endothelial cells and osteoclasts also

produce ROS (Meier et al. 1990, McCord 1993, Steinbeck et al. 1994, Halliwell 1996, Chapple 1997). ROS serve as agents highly toxic to the internalized microbial agent; however, they can also lead to extracellular structure degradation (Bartold et al. 1984, Chapple 1997, Bauer & Bauer 1999, Halliwell 2000, Waddington 2000).

O_2^- attacks a number of biological events and is converted to the more potent H_2O_2, OH, and singlet oxygen (Gutteridge 1994, Chapple 1997, Halliwell 2000, Waddington 2000). The reaction of NO with O_2^- produces the damaging OH radical via an intermediate peroxynitrite ion, which is also able to damage biological molecules (Chapple 1997, Halliwell 2000, Waddington 2000).

Protection against such species is provided by antioxidants (AOs), many of which are released locally at sites of inflammation by PMNLs and other cells. In healthy organisms, the balance is maintained among oxidants and AOs. Under pathological conditions, the balance may be tilted towards the oxidative side (Halliwell & Gutteridge 1990, Gutteridge 1994, Halliwell 1996, Chapple 1997, Kelly 1998).

Superoxide dismutase (SOD) is one of the AO enzymes in protecting the cell against the deleterious effects of ROS, ensuring that O_2^- is efficiently converted to H_2O_2 (Curnutte & Babior 1987, Halliwell & Gutteridge 1990, McCord 1993, Gutteridge 1994, Fridovich 1995, Waddington 2000). Body extracellular fluids contain extremely low levels of SOD activity (Gutteridge 1994). Although extracellular SODs (EC-SOD) contain copper (Cu) and zinc (Zn), they are very different from intracellular Cu ZnSOD that EC-SODs have a much higher relative molecular mass and possess attached carbohydrate (Halliwell & Gutteridge 1990).

Excess production of ROS and the resultant oxidative stress contribute significantly to tissue damage in many diseases such as rheumatoid arthritis (RA), diabetes, AIDS, and cancer (Curnutte & Babior 1987, Halliwell 1996, Chapple 1997, Kelly 1998, Bauer & Bauer 1999). More recently, ROS have been implicated in the pathogenesis of periodontal disease. Several studies have shown increased generation rate of ROS from peripheral blood PMNL in rapidly progressive (RPP) (Shapira et al. 1991, 1994), juvenile (Asman 1998), and chronic adult periodontitis (Whyte et al. 1989, Kimura et al. 1993, Gustaffson & Asman 1996, Fredriksson et al. 2003). *Porphyromonas gingivalis* lipopolysaccharide has been shown to cause a dose-dependent increase in O_2^- production by stimulated neutrophiles from RPP patients (Shapira et al. 1994).

In the literature, there are only very few studies corcerning the relationship between SOD and inflammation in the oral cavity or periodontium, and the results are conflicting. A significant reduction of SOD activity was found in gingival tissue adjacent to deep periodontal pockets (Ellis et al. 1998). Similar SOD activities were found in periapical granuloma and healthy gingiva (Marton et al. 1993). A remarkably higher SOD activity was shown in irreversible pulpitis, suggesting that SOD activity increases with the progression of inflammation (Tulunoğlu et al. 1998). Studies concerning ROS and/or AO mechanisms in oral fluids are also limited (Guarnieri et al. 1991, Moore et al. 1994, Chapple et al. 1997). It was observed that part of the gingival fluid PMNL activation to release O_2^- was inhibited in chronic adult periodontitis patients in comparison to healthy controls (Guarnieri et al. 1991). The aim of the present study was to determine SOD activity levels in gingiva and gingival crevicular fluid (GCF) from patients with chronic periodontitis (CP) and periodontally healthy controls and to analyse the correlation between gingival and GCF SOD activities.

Material and Methods

Clinical studies

Study groups

Forty-four individuals (26 patients, 18 controls) were included in the study. The patient group consisted of 14 women and 12 men (mean age 38.4 years; range 31–52 years) who applied to Hacettepe University Faculty of Dentistry, Department of Periodontology for periodontal problems and were clinically and radiographically diagnosed as CP patients according to the criteria currently accepted (Armitage 1999). The teeth which required full-thickness periodontal flap surgery in right or left maxillary quadrant ($\geqslant$4 teeth) with $\geqslant$5 mm periodontal pockets, gingival inflammation and 50% periodontal bone loss were included in the study. The control group included 13 women and five men (mean age 24.77 years; range 22–29 years) who presented first or second premolars scheduled for extraction for orthodontic reasons. They presented periodontal health and good oral hygiene with no clinical signs of gingival inflammation (bleeding on probing, hyperaemia and oedema), deep pockets, or tooth mobility as were determined by clinical and radiographical examinations and the related clinical indices. Special care was taken to select the controls among persons who had good periodontal status, and oral hygiene. All of the subjects had the following criteria: were non-smokers, had no history of a systemic disease, no antibiotic or anti-inflammatory drug treatment within the last 6 months, and no history of a periodontal treatment (including scaling) within the last 6 months. The subjects were informed about the study and their approval obtained. The protocol of the study was reviewed and approved by the ethics committee of Hacettepe University.

Clinical measurements

The periodontal status of the subjects was determined by measuring probing depth (PD) and clinical attachment level (CAL), and by recording gingival index (GI) (Löe & Silness 1963), gingival bleeding index (GBI) (Ainamo 1975) and plaque index (PI) (Silness & Löe 1964) values. Full-mouth periapical radiographs were taken from the patients in order to determine the periodontal bone loss. All of the clinical and radiographical examinations were performed by one examiner who was not blinded, a periodontist (Ph.D.), in the periodontology department. The unit of measurement/analysis was per subject.

Sampling of GCF

In patients, GCF was collected from teeth that presented $\geqslant$5 mm PD and 50% alveolar bone loss in right or left maxillary quadrant ($\geqslant$4 teeth) requiring surgery for pocket elimination. To avoid irritation, sampling was performed 1 week after the clinical measurements were performed, in a separate seance, in the morning between 08:00 and 10:00 hours. The area was isolated with cotton rolls, with attention to eliminate salivary contamination, and gently air dried. The samples were collected by standardized Periopaper strips (Ora Flow Inc., Amityville, NY, USA) using Brill's (1962)

deep intra-crevicular technique. The strips were inserted into the pockets until a slight resistance was felt and held in the sulci for 5 s with delicate care to avoid irritation of pocket/sulcus epithelium. Any paper contaminated with blood was discarded and collection was repeated in another point. Twelve strips were used for each quadrant of each person. Thus, standardization was provided by using equal numbers of strips and keeping them in the pockets/sulci in equal durations for each person (both in patient and control groups). In controls, first or second premolars in maxilla, to be extracted for orthodontic reasons, were selected for sampling. They presented no signs of clinical gingival inflammation and alveolar bone loss. The same procedure of GCF sampling was performed in controls. The paper strips were placed within aluminum foils and stored in tightly closed tubes at -20°C until analysis.

Sampling of gingiva

In patients, tissue samples were harvested during full-thickness flap operation from the same teeth from which GCF samples were obtained. After the initial treatment (scaling and polishing), modified Widman flap procedure was performed for pocket elimination and debridement. The tissues, excised by internal bevel, sulcular, and interproximal incisions were used as gingival samples for analysis of SOD. Routine periodontal treatment and oral hygiene procedures were continued after surgery. In controls, the same teeth from which GCF samples were obtained were selected for tissue sampling. The gingiva surrounding the socket was excised in small pieces, immediately after the extraction of the indicated teeth for orthodontic reasons. All the gingival tissue samples were washed in saline solution immediately after excision and dried with filter papers. Tissue samples were stored in firmly wrapped sterile Eppendorf tubes at -20°C until the analysis.

Laboratory studies

SOD activity was assayed based on reduction of nitroblue tetrazolium (NBT) by O_2^- anion produced by xanthine – xanthine oxidase. The formazan that is formed subsequent to electron transfer to NBT by xanthine oxidase was spectrophotometrically determined at 560 nm. One unit (U) of SOD is defined as the amount of protein that inhibits 50% of the rate of NBT reduction (Sun et al. 1988).

The paper strips which absorbed GCF were put in glass tubes and treated by 1 ml, 20 mM tris-HCl buffer (pH 6.5). The samples were kept at $+4^{\circ}$C for $\frac{1}{2}$ h and then vortexed. The extracts were then transferred to tubes and stored frozen until work time.

Tissue samples (20–50 mg), washed out from contaminated blood with saline solution at $+4^{\circ}$C were dried by filter papers. one milliliter of 20 mM tris-HCl buffer (pH 6.5) was added after they were weighed by an electronic scale. The samples were homogenized in equal amounts of cold distilled water by using a Potter Elvehzem homogenizer (Janke & Kunkel KG, Staufen, Germany) with a teflon pastle for 3 min. The homogenates were centrifuged at 1500 × g for 30 min. to remove debris. Clear upper supernatant fluids were taken and the assays were carried out in this part. All the procedures mentioned above were performed at $+4^{\circ}$C.

SOD activities in GCF extracts and tissue homogenate/supernatants were spectrophotometrically determined by the method of Sun et al. (1988). To adapt this method (which was used on blood plasma in that study) to tissue homogenates; we referred to a previous study, which had assayed SOD activity in gingival tissue extracts (Ellis et al. 1998). The results are given as total activity: U, for GCF and U/ml homogenate for gingiva.

Statistical analysis

The homogeneity of variances was tested by Levene test. To compare the groups, the variances which were not homogeneous were analysed 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 analysed 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 CP and control groups. All of the clinical parameters were significantly higher in the CP group than the control group ($p<0.05$) (Table 1). Since the results belong to sampling areas only, in the control group, the values of clinical parameters were 0.00 ± 0.00 except PD (only maxillary right or left, first or second premolars).

Laboratory findings

SOD activity in gingiva

Mean gingival SOD activity levels were 16.59 ± 1.71 U/ml homogenate in the CP group and 3.73 ± 0.22 U/ml homogenate in the control group. The CP group showed significantly higher gingival SOD activity level than the control group ($p<0.05$). The results revealed that SOD activity in inflamed gingiva from periodontal disease sites of CP patients was significantly higher than

Table 1. Comparison of clinical parameters between chronic periodontitis (CP) and control groups

Parameter (sampling area)	Group n CP:26 Control:18	$\bar{x}$ ± SEM (sampling area)	Min.	Max.	*U*	*P*
PD	CP	3.80 ± 0.09	2.97	4.94	0.0	0.0001*
	Control	1.26 ± 0.06	0.64	1.66		
CAL	CP	4.60 ± 0.15	3.52	6.43	0.0	0.0001*
	Control	0.00 ± 0.00	0.00	0.00		
GI	CP	1.72 ± 0.07	0.70	2.25	0.0	0.0001*
	Control	0.00 ± 0.00	0.00	0.07		
GBI	CP	0.94 ± 0.04	0.20	1.00	0.0	0.0001*
	Control	0.00 ± 0.00	0.00	0.00		
PI	CP	1.85 ± 0.11	0.71	3.00	0.0	0.0001*
	Control	0.00 ± 0.00	0.00	0.00		

Mann–Whitney – *U*-test:

*The difference is statistically significant compared with the control group ($p<0.05$). $\bar{x}$, arithmetic mean. SEM, standard error of mean; PD, probing depth; CAL, clinical attchment level; GI, gingival index; GBI, gingival bleeding index; PI, Plaque index.

Table 2. Comparison of gingival superoxide dismutase activities between chronic periodontitis (CP) and control groups

Group	*n*	$\bar{x} \pm$ SEM (U/ml homogenate)	Min.	Max.	*U*	*P*
CP	26	16.59 ± 1.71	0.51	27.43	72.0	0.0001*
Control	18	3.73 ± 0.22	1.59	5.17		

Mann–Whitney – *U*-test:
*The difference is statistically significant between the groups ($p<0.05$). $\bar{x}$, arithmetic mean. SEM, standard error of mean.

Table 3. Comparison of gingival crevicular fluid superoxide dismutase activities between chronic periodontitis (CP) and control groups

Group	*n*	$\bar{x}$ ± SEM (Unit = U)	Min.	Max.	*t*	*P*
CP	26	0.88 ± 0.08	0.21	1.68	0.933	0.356
Control	18	1.02 ± 0.13	0.10	2.20		

t-test for independent samples: The difference is not statistically significant between the groups ($p>0.05$). $\bar{x}$, arithmetic mean. SEM, standard error of mean.

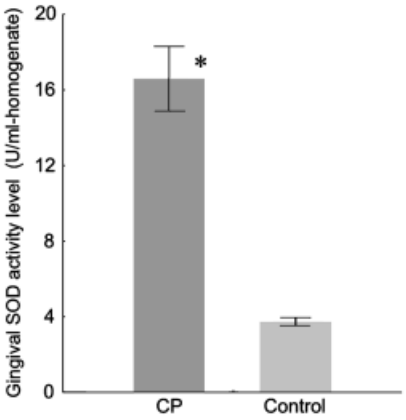

Fig. 1. Gingival superoxide dismutase (SOD) activity levels in groups. *The difference is statistically significant compared with the control group ($p<0.05$). Chronic periodontitis group, $n = 26$; Control group, $n = 18$.

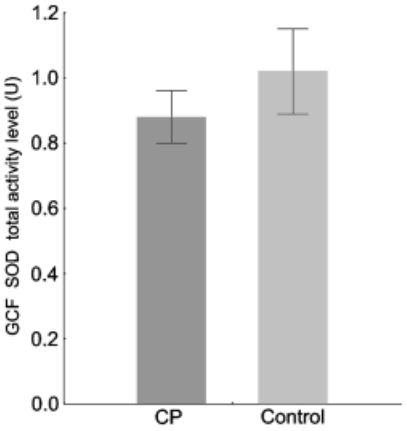

Fig. 2. Gingival crevicular fluid superoxide dismutase total activity levels in groups. The difference is not statistically significant ($p>0.05$). Chronic periodontitis group, $n = 26$; Control group, $n = 18$.

that of healthy gingiva in controls (Table 2, Fig. 1).

SOD activity in GCF

Mean total SOD activities were 0.88 ± 0.08 U in CP group and 1.02 ± 0.13 U in the control group. Although control group showed higher GCF SOD activity level than CP group, the difference was not statistically significant ($p>0.05$). The results indicated that GCF SOD activity did not differ significantly between periodontal disease and health (Table 3, Fig. 2).

Correlations

The correlations between gingival and GCF SOD activities and between clinical parameters and SOD activities were analysed by simple correlation analysis. Correlations between gingival and GCF SOD activities were not statistically significant in either group ($p>0.05$). Negative, weak, and statistically non-significant correlations were found between these parameters in both groups. Pearson's correlation coefficients and significance levels were as follows: in the CP group, $r = -0.266$, $p = 0.188$; and in the control group, $r = -0.280$, $p = 0.260$. It was seen that the relationship between gingiva and GCF was weak with respect to SOD activity levels. 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 relationship among these parameters (data are not given).

Discussion

The present findings indicated higher SOD activity in inflamed gingiva from CP patients than in healthy gingiva from controls.This finding seems to confirm several findings in the medical literature about oxidant – AO balance. It has been shown that SOD activity increases directly after occurrence of oxidative stress (Godin & Wohaieb 1988, Redl et al. 1993). Local production of glutathione, an important AO, is reported to be raised in pulmonary alveolar lining fluid in smokers (Cantin et al. 1989).

Studies on SOD in relation to periodontal tissues or the oral cavity are limited and the results are conflicting. In contrast to our finding, ROS have been shown to depolymerize gingival hyaluronic acid and proteoglycans and it has been presupposed that the balance between ROS and the presence of SOD is disturbed (Bartold et al. 1984). Ellis et al. (1998) found a significant and progressive reduction in SOD activity within gingiva adjacent to deeper pockets. Marton et al. (1993) showed that SOD activities were similar in periapical granuloma and healthy gingiva. However, consistent with our finding, another study has shown that SOD activity increases with the progression of inflammation in pulp (Tulunoğlu et al. 1998). The human periodontal ligament has been shown to possess the enzyme SOD, which might afford biological protection against ROS, particularly O_2^-, during the inflammatory response (Jacoby & Davis 1991). Bacterial LPS was shown to stimulate O_2^- release from gingival fibroblast, suggesting that the induction of SOD may represent an important defence mechanism of the fibroblast during inflammation (Skaleric et al. 2000). 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 (Yamamoto et al. 2001). In the present study, increased gingival SOD activity level in CP seems to support these findings. Pryor (1986) suggested that the availability of SOD has provided a tool that allows testing physiological processes for the involvement of O_2^-. In the present study, increased SOD activity level in inflamed gingiva from CP patients may indicate the increased O_2^- generation by PMNLs invaded at the disease site. This increase

in O_2^- production may have led to the occurrence of oxidative stress, which in turn caused an increased need for SOD production to establish the ROS-AO balance to protect the tissue.

The present findings indicated that although GCF SOD activity was higher in periodontal health than in CP, the difference was not statistically significant, suggesting that SOD activity in GCF does not significantly change in the periodontal disease state. Several studies have analysed SOD in synovial fluid from patients with RA whose pathogenesis resembles that of periodontitis. ROS–AO interactions take part in the pathogenesis of these diseases. These studies have provided contrary findings. Blake et al. (1981) could find no SOD activity in the synovial fluid of RA patients, while lgari et al. (1982) found fourfold higher SOD concentration in synovial fluid from RA patients. Marklund et al. (1986) found that synovial fluid SOD activity in RA patients was significantly lower than that in healthy controls. Our finding is in contrast with these findings, while it is consistent with the finding of Biemond et al. (1984) who observed no significant difference in synovial fluid SOD activities between RA patients and controls. Similar to our finding, Guarnieri et al. (1991) have shown that the AO activity (SOD activity) able to scavenge O_2^- in GCF did not differ between controls and CP patients. Also, studies have indicated that total salivary AO activity remains at the same level (similar to our finding) in periodontal disease (Moore et al. 1994) or is reduced (Chapple et al. 1997). It is known that SODs are mainly found in cells and tissues and there is only a minor activity in extracellular fluids (Chapple 1997, Bauer & Bauer 1999). This may be one of the possible reasons for low GCF SOD activity in CP in the present study. Another possibility may be that a supression on SOD production may have occured in GCF/PMNLs because of the oxidative damage caused by the increased ROS/O_2^- generation (Marklund et al. 1986, Guarnieri et al. 1991).

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 (Layik et al. 2000). 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 (Griffiths et al. 1998), 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 is achieved by sampling all sites for the same length of time (Lamster et al. 1986, 1988). Therefore, we preferred the standardization on collection time basis, rather than GCF volume basis for analysis of GCF SOD activity, and expression of total activity for data presentation instead of concentration. Although less than those from the patients, detectable amounts of GCF were collected from the controls in 5 s because of several factors, e.g., the sampling method (deep intra-crevicular method), subclinical inflammation in the area and the stimulation of GCF because of the unavoidable irritation of sulcus epithelium. Some of the controls did not present any GCF flow in the sampling site. These persons were excluded from the study.

We also analysed the relationship between gingival and GCF SOD activities, however, we could not find a significant correlation. Based on this finding, it may be speculated that the activities of SODs in gingiva and GCF are independent from each other, or that SOD activity (maybe AO capacity) of gingiva does not completely flow to GCF. In conclusion, SOD activity seems to increase in gingiva in CP. This increase may be because of the occurrence of increased oxidative stress, and the increased need for oxidant/AO balance and protection. Since GCF SOD activity does not seem to significantly change (and/or increase) in CP patients in comparison with healthy controls, it may be considered that SOD defense in GCF may be weak in CP and it is doubtful whether this low SOD activity in GCF can protect against ROS.

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