

Evaluation of Salivary Total Antioxidant Capacity in Smokers with Severe Chronic Periodontitis

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Abstract

Background: Smoking leads to changes in immune reaction and total antioxidant capacity. Smoking, through dysfunction of antioxidant system, plays an important role in the pathogenesis of inflammatory diseases.

Objectives: The current study aimed at comparing salivary antioxidant capacity in smokers and non-smokers with severe chronic periodontitis.

Patients and Methods: In this case-control study, among patients referred to Zahedan School of Dentistry without any systemic diseases, 64 patients with severe chronic periodontitis, including 27 smokers as the case group and 37 non-smokers as the control group, were selected. Stimulated salivary samples were collected and total antioxidant levels were evaluated through the Ferric reducing antioxidant power (FRAP) technique. All analyses were performed by SPSS (Ver.18) using Student's t-test, Mann Whitney test or analysis of variance (ANOVA) with significance level of 0.05 in all of the tests.

Results: Total antioxidant capacity in smokers ($378.43 \pm 207.34 \mu\text{M}$) with severe chronic periodontitis was significantly lower than non-smokers ($698.30 \pm 231.86 \mu\text{M}$) ($P = 0.0001$). Smoking at a rate of more than 10 cigarettes per day caused a significant decrease in total antioxidant capacity as compared to less than 10 cigarettes a day ($P = 0.009$), although there was no significant relationship between total antioxidant capacity and duration of smoking ($P = 0.23$).

Conclusion: smoking has an influence on antioxidant status in patients with severe chronic periodontitis. Smoking could be effective in development and progression of inflammation process through changes in oxidative stress. These findings confirm the benefits of smoking-cessation in improvement of antioxidant levels in periodontitis.

Keywords: Smoking, Periodontitis, Antioxidants, Saliva

1. Background

It has been confirmed that there is a direct relationship between oxidative stress and pathologic conditions or diseases. Assessment of the elements effective in oxidative stress on body's fluids, such as saliva, could be beneficial for monitoring of progression and treatment of some diseases (1).

Smoking produces large amounts of reactive oxygen species (ROS), which have an influence on normal cellular function and causes changes in the inflammation markers (2). Oxidative stress, which is produced through serious imbalance between the generation of reactive oxygen species and antioxidant protection, is effective in the pathogenesis of inflammatory conditions, such as periodontal diseases (3). On the other hand, periodontitis is a chronic infective disease, in which local and systemic factors, including diabetes and smoking change response of

the immune system to local agents, such as dental plaque, and consequently affect the progression of the disease (4, 5).

Studies revealed that smoking is the second major risk factor for periodontal diseases; the possibility of chronic periodontitis in smokers is 4 times more than non-smokers (6). Smoking makes the periodontal tissue respond atypically to microbial pathogens (5). It seems that smoking plays an important role in the pathogenesis of periodontal diseases through an increase in oxidative stress, induction of an imbalance between oxidative stress and antioxidant capacity and dysregulation of innate and adaptive immune system (7).

Some researchers have paid attention to evaluate effects of cigarette on development and progression of this disease via assessment of oxidative stress in various bio-fluids.

Different studies have measured anti-oxidant levels in smokers. Abdolsamadi's study demonstrated that salivary superoxide dismutase enzymes (SODs) and Glutathione Peroxidase (GSH-Px), an antioxidative parameter, in smokers are less than non-smokers, although there was not any difference between 2 groups, according to salivary uric acid levels (8). In another study, Malondialdehyde (MDA) levels were significantly higher in smokers with periodontitis as compared to non-smokers. Patients with periodontitis had more salivary lipid peroxidation than healthy controls (7).

It was determined that tobacco abuse affects the development of periodontal disease through free radicals and other reactive particles, which destroy cellular structures.

One study compared oxidative stress markers in male smokers and non-smokers with chronic periodontitis and revealed that smokers with periodontitis had concurrently higher periodontal clinical parameters and higher systemic oxidative stress (9).

Also, another study demonstrated that serum total oxidant status levels in smokers and non-smokers were reduced after periodontal treatment; however, there was no significant difference in serum total antioxidant status (TAS) levels in both groups after treatment. Also, this study showed that total antioxidant status of gingival crevicular fluid (GCF) in smokers did not show any significant difference after treatment, yet TAS levels were raised in the non-smoker group after periodontal treatment. These results indicate differences between different body fluids regarding the oxidative/antioxidative system (10).

Furthermore, studies confirmed that smoking has a dose-dependent effect on oxidative parameters. As indicated by Agnihotri's study, which compared average levels of superoxide dismutase (SOD) in the GCF and saliva of smokers and non-smokers with periodontitis, there was a significant reduction in superoxide dismutase levels in smokers compared to non-smoker subjects. Moreover, it was proved that there was a gradual decrease in the levels of SOD of the GCF and saliva from light smoking to heavy smoking (11).

Review of previous studies suggests that assessment of oxidative stress plays an important role in evaluation of destructive effects of smoking on various body tissues.

2. Objectives

To the best of our knowledge, in Iran, similar studies have not been done on antioxidant levels in smokers with periodontitis. As cultural and racial factors are important elements in smoking habit and periodontal diseases, this study aim to evaluate total salivary antioxidant of smokers and non-smokers with periodontitis in South-East of Iran.

3. Patients and Methods

3.1. Subject Selection

Sixty-four patients with severe chronic periodontitis were enrolled in this descriptive-analytic case-control study. All subjects were selected among patients, who referred to Zahedan school of dentistry for dental care. Among them, patients, who were older than 30 years old and had confirmed periodontist, were divided to 2 groups: 27 smokers (case group) and 37 non-smokers (control group), both groups were matched, according to age and severity of periodontal diseases.

Inclusion criteria included:

1. Severe periodontitis with attachment loss ≥ 5 mm in at least 30% of teeth,
2. Gingival bleeding during gentle probing, which indicated active periodontal disease (12).

Exclusion criteria included:

1. Pregnancy
2. Alcoholism or any drug addiction, including opioids, smokeless tobacco, etc. during the past 2 years
3. Using antioxidant drugs during in the past 3 months (13)
4. History of trauma or surgery during the past 4 weeks (13)
5. Auto-immune diseases, such as arthritis rheumatoid, Sjögren's syndrome, etc. (13)
6. AIDS/HIV (13)
7. Inflammatory conditions
8. Juvenile periodontitis
9. Malignancy
10. Oral diseases, including ulcers, red and white plaques, tumors, etc.

Subjects, who smoke at least 3 cigarettes per day for at least 6 months, were included in the case group and people, who did not have any history of smoking, were included in the control group. People, who gave up smoking, were excluded from the study.

The protocol of this study was approved by the ethics panel of Zahedan University of Medical Sciences. Moreover, the subject's rights were protected and an informed consent was obtained from the study participants.

Firstly, all subjects were placed under precise periodontal examination by a periodontist and periodontal pocket depth was measured using a tiny probe (PCP-UNC 15, Hu Friedy, Leimen, Germany). Clinical attachment loss and bleeding on probing were evaluated. Information about the subjects' periodontal condition and smoking habit was recorded in the study check-list.

3.2. Salivary Sample Collection

Saliva sampling was performed according to the standard technique at 9 to 11 am. Subjects were asked not to eat, drink, smoke, brush or use any hygienic device during at least 90 minutes before sample collection (14). Stimulated saliva was collected after chewing tasteless, sugarless gum for 1 minute (15). The patients were asked to bend their heads forward and spit the saliva into a 50-mL centrifuge tube without swallowing.

The saliva samples were sent to the biochemical laboratory while they were kept on dry ice. In the laboratory environment, their temperature was raised to 4°C. Next, the saliva samples were centrifuged (4000 rpm, 10 minutes) to separate the cells and debris.

The samples were kept at -80°C until the analysis time. This temperature is necessary for preventing the salivary proteins from the degeneration.

For evaluation of total antioxidant capacity, the samples were washed with distilled water and 10% nitric acid solution. Acetate buffer was prepared through mixing 16 mL of acetic acid and 3.1 grams of sodium acetate. The solution volume was then elevated to one liter and its pH was set to 3.6 by adding distilled water. The prepared solution included a mixture of iron chloride (2.5 mL) and 2, 4, 6-tripyridyl-s-triazine (TPTZ) (2.5 mL) added to acetate buffer (25 mL) (16).

The antioxidant capacity of saliva samples were analyzed by Ferric reducing antioxidant power (FRAP) assay using ENGLAN ultropec 3000 biotech and pharmacia spectrophotometer. In this method, the total antioxidant capacity of saliva was assessed by the ability of saliva for reduction of ferric (Fe+3)-to-ferrous (Fe+2) in the presence of a (Fe+2)-stabilizing legend, such as TPTZ. The Fe+3-TPTZ complex could change to Fe+2-TPTZ by a reducing agent in the acidic environment. The Fe+2-TPTZ could be observed at 593 nm wavelength because of its intense blue color. The blue color could show the speed of production of Fe2-TPTZ complex and reducing ability of salivary samples (16, 17).

At the beginning of the reaction, 30 µL of saliva, which had been centrifuged at 4°C, was added to the solution. The rate of absorption was evaluated at the start of the reaction and 8 minutes later.

The difference between primary and final absorption was assessed by a standard curve. During the laboratory process, temperature of the environment was 37°C.

3.3. Statistical Analysis

The data were entered in the SPSS software (Ver.18). The analyses were conducted using 3 appropriate tests: independent t test, Man Whitney or analysis of variance (ANOVA) test. The significance level was set at 0.05 in all tests.

4. Results

Sixty four patients with severe chronic periodontitis, including 27 smokers (23 males and 4 females) and 37 non-smokers (13 males and 24 females) were enrolled in this study. The average age of the case and control group was 43.40 ± 8.13 and 43.28 ± 6.34 , respectively. There was no significant difference between the 2 groups (t test; $P = 0.942$).

Although the percentage of bleeding on probing (BOP) in smokers was more than non-smokers, the difference was not significant in the 2 groups ($P > 0.05$). The BOP in smokers was 95.01 ± 5.14 (mean \pm SD) while in non-smokers this was 93.05 ± 20.11 . Also, there was no significant difference between the 2 groups regarding probing pocket depth (PPD) ($P > 0.05$); the mean of PPD was greater in smokers compared to non-smokers (5.62 ± 0.23 mm and 5.20 ± 0.12 mm, respectively) ($P > 0.05$).

Total antioxidant capacity of stimulated saliva in the case group was determined in this study:

The TAOC in smokers was 378.43 ± 207.34 µM and in non-smokers, this was 698.30 ± 231.86 µM. The difference was significant (t test; $P = 0.0001$) (Table 1).

Table 1. Demographic and Clinical Data of the Smoker and Non-Smokers

Periodontitis Patients	Non-Smoker (n = 37)	Smoker (n = 27)
Gender (m/f)	13:24	23:4
Average age, y	41.2 ± 6.33	43.6 ± 7.23
Smoking, cigarettes per day	0	15.2 ± 5.3

There was no statistically significant association between salivary TAOC and duration of smoking habit (ANOVA; $P = 0.235$) (Table 2); however, total salivary antioxidants in patients with periodontitis, who smoked more than 10 cigarettes a day, was significantly less than patients, who smoked up to 10 cigarettes per day (t test: $P = 0.009$) (Table 3).

Table 2. Comparison of Total Salivary Antioxidant According to Duration of Smoking Habit

Duration of Smoking Habit	Number	Mean \pm SD, µM	P Value
Less than 6 years old	11	461.18 ± 175.77	0.235
Between 6 and 12 years old	10	319.99 ± 123.07	
More than 12 years old	6	324.13 ± 330.24	

Although TAOC in 30- to 45-year-old subjects, including smokers and non-smokers (566.26 ± 249.23 µM) was

Table 3. Comparison of Total Salivary Antioxidant According to the Number of Cigarettes Smoked Daily

The number of Cigarettes Per Day	Number	Median, μM	Percentiles		P Value
			25th	50th	
Less than 10 cigarettes	13	468.00	359.83	553.67	0.009
More than 10 cigarettes	14	243.10	113.51	390.08	

higher than subjects older than 45 years old ($522.44 \pm 312.80 \mu\text{M}$), there was no significant difference between subjects younger and older than 45 years old, according to total salivary antioxidant (t test: $P = 0.84$). Moreover, among both age groups, non-smokers had higher total salivary antioxidant level than coeval smokers (t test: $P = 0.001$ and $P = 0.003$; respectively for ≤ 45 and > 45 years old) (Table 4).

Table 4. Comparison of Total Salivary Antioxidant in Smokers and Non-Smokers According to Age

Age	Number	Mean \pm SD, μM (TAOC)	P Value
30 - 45 smoker	17	403.56 ± 167.7	0.001
Nonsmoker	23	699.37 ± 226.58	
Higher than 45 smoker	10	328.18 ± 275.13	0.003
Nonsmoker	14	696.62 ± 248.59	

The TAOC in the 36 evaluated males was $498.96 \pm 258.18 \mu\text{M}$ and in the 28 females was $645.88 \pm 71.71 \mu\text{M}$. The analysis showed that levels of total salivary antioxidants in male subjects were significantly lower than female subjects (t test; $P = 0.035$), although this result could be related to higher number of male smokers compared to female smokers. In each gender, there was a significant difference between smokers and non-smokers according to TAOC (t-test: $P = 0.001$ and $P = 0.02$; respectively for males and females) (Table 5).

Table 5. Comparison of Total Salivary Antioxidant in Smokers and Non-Smokers According to Gender

Gender	Number	Mean \pm SD, μM (TAOC)	P Value
Male Smoker	23	379.2 ± 222.45	0.001
Nonsmoker	13	710.86 ± 166.01	
Female Smoker	4	374.02 ± 98.36	0.02
Nonsmoker	24	691.2 ± 265.23	

5. Discussion

In this study, total salivary antioxidant levels of smokers and non-smokers with periodontitis were assessed.

Smoking and periodontitis compromised the antioxidant capacity of saliva and other body fluids. They increased enzymes related to oxidative stress.

Antioxidant agents could be categorized to 3 groups: one group prevents the formation of free radicals; the second group is radical-scavenging antioxidants, which eliminate free radicals and consequently inhibit destruction related to free radicals; and the third group includes DNA repairing enzymes (18).

After increased production of ROS in the oxidative stress process, higher levels of free-radical-scavenging antioxidants are consumed, therefore, researchers assess components of antioxidant activities for evaluation of oxidative stress (19, 20).

Although assessment of salivary total antioxidants activity is not a relevant indicator of antioxidant activities of body fluids, measurement of several antioxidants is time-wasting, complicated and expensive; as a result, TAOC index, which shows accumulative effect of all antioxidants in the bio-fluids, is assumed to be an appropriate indicator (21, 22).

Antioxidants activities are modified through variant factors, including nutrition, stress, physical activity and smoking. In fact, smoking leads to an imbalance between reactive oxygen species and antioxidant levels and consequently induces oxidative stress (23).

On the other hand, smoking is one of the most important factors in development and progression of periodontal diseases; prevalence of periodontitis in cigarette smokers is 6 to 6.2 times more than non-smokers. Nicotine changes immune reaction, gingival inflammation, and oxidant and antioxidant capacity (7, 24). Several studies about oxidant and antioxidant activity and effective factors (such as Reactive Oxygen Species and lipid peroxidation products) have been done, which emphasized that saliva is useful in the diagnosis of conditions, which affect oxidant-antioxidant system, such as periodontal diseases and pre-malignant lesions.

In the present study, salivary total antioxidant capac-

ity of smokers was significantly lower than non-smokers. This result was similar to Guentsch's study, which revealed that antioxidant levels of smokers involved with periodontitis were lower than non-smoker subjects, while MDA levels (the last product of oxidative stress) were considerably higher in smoker subjects than non-smokers (7). Their study confirmed that smoking leads to increased oxidative stress, as well as an imbalance between oxidants and antioxidants (7). Also, Rai et al. concluded that total glutathione of saliva was significantly higher in smokers with periodontitis in comparison with non-smokers (25). They demonstrated that salivary total glutathione concentrations were significantly decreased after some periodontal treatments, such as scaling and root planning in smokers as well as in nonsmokers involved with periodontitis (25). There were some similarities according to study subjects in Rai's study and the present study, yet in the current study the entire salivary antioxidant capacity was evaluated, which was lower in smokers than non-smokers involved with periodontitis. Furthermore, Abdolsamadi et al. with evaluation of antioxidant concentrations in healthy smokers and non-smokers revealed that there was a decrease in salivary antioxidant levels in smokers (8). This result was comparable with the present study; however, in the current study all subjects were involved with periodontitis. Moreover, Ahmad's study showed a significant decrease in some salivary antioxidant factors in smokers as compared to non-smokers. They concluded that salivary antioxidants are relevant factors for evaluation of oxidative stress in smokers. Also, there was an indirect correlation between salivary antioxidant levels and duration of smoking habit (26), although in the present study, duration of habit did not have any significant influence on salivary antioxidant capacity.

There is some controversy between the results of different studies regarding the levels of various elements and enzymes related to oxidative stress. Diken's study revealed that the activity of glutathione in erythrocytes was not influenced by smoking (27). Moreover, Zappacosta et al. demonstrated that smoking causes a decrease in concentration of salivary glutathione; however, they found that there was no significant difference between smokers and non-smokers regarding uric acid concentrations and total antioxidant capacity (28). On the other hand, Reddy et al. revealed a significant decrease in salivary and serum superoxide dismutase enzymes in smokers as compared to non-smokers (29). Also, Kanehira et al. concluded that concentrations of salivary thiocyanate and copper/zinc superoxide dismutase were enhanced in smokers while levels of other enzymes, such as peroxidase and glutathione peroxidase, were lower in these subjects (30). However, this study evaluated these elements in only elderly people in contrast

to the current study.

It seems that with aging, oxidative stress increases while antioxidants activity, which plays an important role in some inflammatory diseases, does not show any significant difference (31). In agreement with this theory, Balkan et al. revealed that antioxidant activities in elderly subjects (61 to 85 years old) were lower than young people (32).

Antioxidant system is very complicated and includes different types of extra and intra-cellular molecules and enzymes in biofluids, such as plasma and saliva.

In some studies, antioxidant capacity of serum and gingival crevicular fluid in smokers and cases with periodontitis were assessed (23, 33). Serum and gingival crevicular fluid TAOC in smokers and non-smokers involved with periodontitis were estimated in one study, which revealed that gingival crevicular fluid and serum TAOC decrease in smokers as compared to non-smokers (23), the results of that study was similar to the present study in which smokers had lower levels of TAOC in comparison with non-smokers; however, different body fluids were evaluated in these two studies.

In another study, Toker et al. evaluated the effects of smoking on interleukin- 1β levels and total oxidant and antioxidant levels of gingival crevicular in periodontal diseases. The interleukin- 1β levels in gingival crevicular fluid were significantly higher in smokers than non-smokers (3).

These conflicting results of studies could be related to a variety in sample sizes, the number of cigarettes per day, type and duration of smoking habit, assessment of various antioxidant elements in different studies, various kits or laboratory techniques, different structure of the studies and different age groups and other properties of subjects. It seems that according to most studies' results, smoking changes antioxidant capacity, yet the mechanism of these alterations have not been well known (34).

There is a challenge about the exact correlation between smoking, periodontitis, and oxidative stress. Smoking could affect the antioxidant system through different mechanisms. Smoking causes an increase in oxidative stress and production of free radicals with stimulation of interleukin- 1β . In oxidative stress, an imbalance between production of free radicals and antioxidant levels leads to an increase in ROS levels in the biological environment. Overproduction of ROS causes destruction of some bio-molecules, including DNA, amino acids, carbohydrates, lipids, and disturbance in structural organization and cell function (35-37).

Bakhtiari et al. confirmed that oxidative stress from smoking was not reduced even after using anti-oxidant drugs, including vitamin C (38). On the other hand, in an animal study, the activities of some antioxidant enzymes, including superoxide dismutase, catalase, and glutathione

peroxidase activities, were increased in rats exposed to cigarette smoke. The mentioned study showed that using supplementation of antioxidants before smoking attenuated the harmful effects of cigarette on the pulmonary system; however, it was not enough to protect the organs completely (39).

The current study revealed that salivary TAOC was higher in female than male subjects. Brock's study showed that salivary antioxidants capacity in healthy males was greater than females; however, there was no difference according to gender in patients with periodontal diseases. In contrast, GCF antioxidant concentrations in males were lower than females in both healthy and patient groups; however, the difference was not significant (15). In another study, Scully confirmed that mean levels of total antioxidant capacity were significantly lower in females as compared to males, regardless of periodontal problem, although, they revealed that periodontal diseases was associated with a decrease in salivary antioxidant capacity (17). It seems that some conditions, such as periodontitis, could change antioxidants capacity in both genders.

Various results of these studies could be related to simultaneous presence of periodontitis and smoking in our study's subjects. Furthermore, fewer female smokers enrolled in the current study in comparison with male smokers had some influence on the results.

Moreover, in the present study, salivary TAOC in subject, who smoked more than 10 cigarettes per day was lower than other smokers. It is logical to claim that harmful effects of smoking on antioxidant system are based on daily dose. Review of other studies showed that smoking is a major risk factor for acceleration of periodontal diseases, and this effect is dose-dependent (40, 41).

Briefly, it is logical to claim that smoking could effect oxidative-anti oxidative system in patients with periodontitis, although the exact mechanism is not well known.

5.1. Conclusions

In the present study, among subjects involved with periodontitis, smoking caused some reduction in antioxidant capacity. This effect of smoking on antioxidant activity was dose-dependent. In respect to effects of cigarette on oxidative stress, smoking-cessation efforts could be effective in treatment of periodontal diseases through an increase in antioxidant capacity.

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Footnotes

Authors' Contribution: Design, study concept and supervision: Masoomeh Shirzaei; Acquisition of data and administrative, technical, and material support: Mohammad Aiub Rigi Ladiz; Analysis and interpretation of data: Javid Dehghan Haghighi; Drafting of the manuscript and critical revision of the manuscript for important intellectual content: Zohreh Dalirsani.

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