THE EVALUATION OF SALIVA OXIDATIVE AND ANTIOXIDATIVE MARKERS' LEVELS IN ADOLESCENTS WITH GINGIVAL INFLAMMATION

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PROCENA NIVOA OKSIDATIVNIH I ANTIOKSIDATIVNIH MARKERA PLJUVAČKE KOD ADOLESCENATA SA ZAPALJENJEM GINGIVE

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ABSTRACT

Periodontal disease is highly prevalent and affects 10%–15% of the world population. Salivary markers of oxidative stress and antioxidant status represent promising tool for research of oral diseases. Given the importance of reactive oxygen species (ROS) in the pathogenesis of periodontal disease, the aim of this study was to determine the association between oxidative stress parameters and periodontal disease gingivitis in adolescents. The study included a consecutive sample of 80 male and female participants referred to the Institute of Dentistry, Kragujevac, Serbia, as a result of periodontal problems or for routine control, aged 18 to 21, with definitive dentition as an inclusion criterion. Patients were divided into three groups depending on their gingival inflammation status. The first group was control group (n=20) with no gingival inflammation, the second group was with mild gingival inflammation (n=19), the third group was with moderate gingival inflammation (n=21) and the fourth group was with severe gingival inflammation (n=20). Oxidative stress parameters were measured in unstimulated whole saliva samples (superoxide anion radical, hydrogen peroxide, nitric oxide, index of lipid peroxidation, reduced gluthatione, catalase and superoxide dismutase). We have found increased levels of hydrogen peroxide and reduced glutathione in the saliva of patients with moderate levels of gingival inflammation, while the other markers were not significantly affected. In conclusion, oxidative stress plays a central role in the pathogenesis and the determination of oxidative and antioxidative levels could be a potent tool in controlling the development of gingivitis.

***Keywords**: gingivitis, adolescents, oxidative and antioxidative markers, unstimulated saliva.*

SAŽETAK

Periodontalna oboljenja su veoma česta i javljaju se kod 10%-15% svetske populacije. Pljuvačni markeri oksidativnog stresa i antioksidativnog statusa su od velike pomoći u istraživanju oralnih oboljenja. Zbog važnosti reaktivnih kiseoničnih vrsta (ROS) u patogenezi periodontalnih oboljenja, cilj ovog istraživanja je bio da se odredi povezanost parametara oksidativnog stresa sa periodontalnim oboljenjem gingivitisom, kod adolescenata. Uzorak od 80 muškaraca i žena pregledanih u Zavodu za stomatologiju Kragujevac, ili zbog periodontalnih problema, ili zbog rutinske kontrole , uzrasta izmedju 18 i 21godina i kompletiranom stalnom denticijom kao uključujućim kriterijumom, je korišćen u istraživanju. Pacijenti su bili podeljeni u tri grupe u zavisnosti od stepena izraženosti zapaljenja gingive.Prva grupa je bila kontrolna grupa (n=20) bez zapaljenja, druga je imala blago zapaljenje (n=19), treća grupa je imala zapaljenje srednjeg stepena(n=21), i četvrta je bila sa najtežim stepenom inflamacije (n=20). Parametri oksidativnog stresa su bili izmereni u uzorcima nestimulisane pljuvačke (superoksid anjon radikal, vodonik peroksid, azot monoksid, indeks lipidne peroksidacije , redukovani glutation , katalaza i superoksid dizmutaza). Otkrili smo povišene nivoe vodonik peroksida i redukovanog glutationa u pljuvački pacijenata sa srednjim stepenom zapaljenja, dok drugi markeri nisu bili mnogo izmenjeni. U zaključku, oksidativni stres ima veliku ulogu u patogenezi i odredjivanje oksidativnih i antioksidativnih nivoa bi moglo biti moćno orudje u kontrolisanju razvoja gingivitisa.

Ključne reči: *gingivitis, adolescenti, oksidativni i antioksidativni markeri, nestimulisana pljuvačka.*

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INTRODUCTION

Periodontal disease is highly prevalent and affects 10%–15% of the world population (1). The inflammatory and immune response induced by subgingival plaque is the most important factor in the development of this disease. Subgingival plaque composition and biota have been the subject of several studies, since the presence of different bacteria subtypes has been found to be associated with periodontal status deterioration, greater pocket depth, and higher bleeding indices (2, 3).

Gingivitis and periodontitis are two distinct chronic inflammatory processes belonging to the spectrum of periodontal diseases of the oral cavity affecting the tooth supporting tissues in response to bacterial accumulation. In contrast to periodontitis, gingivitis is initiated only after a few days of inadequate oral hygiene procedures by local plaque deposits adjacent to the highly vascularized gingival tissues. Gingivitis is a superficial inflammatory affection and is not destructive towards the surrounding connective and bone tissues and completely declines with the initiation of adequate oral hygiene procedures (1-3).

Evidence has shown an association between ROS and gingivitis (4-8). This is important because both ROS in the pathogenesis of periodontal disease and the different composition of the biotain periodontal pockets are related to periodontitis (4,5). Periodontal disease can be described as one of the predominant polymicrobial infections of humans (6). Strong evidence of periodontal etiology has been demonstrated for *Porphyromonas gingivalis* (PG), *Aggregatibacter actinomycetemcomitans* (Aa), *Treponema denticola* (TD) and *Tannerella forsythia* (TF) (7).

Still, gingivitis development mechanisms are not well understood. The disorder is probably multifactorial, and it is characterized by the generation of ROS (4-6) by activated phagocytes at the gingival sulcus (5-7) which have the ability to initiate the destruction of connective tissue. ROS are generated during mitochondrial oxidative metabolism as well as in cellular response to xenobiotics, cytokines and bacterial invasion. Oxidative stress refers to the imbalance due to excess ROS or oxidants over the capability of the cell to mount an effective antioxidant response (8).

Salivary markers of oxidative stress and antioxidant status represent promising tools for research of oral diseases (9). Oxidative stress represents disbalance between the production of various ROS and the activity of endogenous antioxidative defense system (ADS) (9). These reactive molecules are reported to be capable of inducing periodontal tissue destruction. The ability of the host to scavenge ROS is regarded as a key protective mechanism against inadvertent ROS mediated host tissue damage (9, 10). Oxidative stress has been involved in pathogenesis of more than two hundred chronic and acute diseases (9-11). However, the connection between the increased production of ROS and gingival inflammation in adolescents is still very poorly investigated. Given the importance of ROS in the pathogenesis of periodontal disease, the aim of this study was to determine the association between oxidative stress parameters and periodontal disease gingivitis in adolescents.

PATIENTS AND METHODS

Compliance with Ethical Standards

All patients signed an informed consent before the initiation of the study. The study was approved by the Ethics Committee of the local institution (Institute for Dentistry, Kragujevac) in compliance with ethical standards.

Study Group

A consecutive sample of 80 male and female individuals referred to the Institute of Dentistry, Kragujevac, Serbia, as a result of periodontal problems or for routine control, between 18 and 21 years old and with definitive dentition, as inclusion criteria, were included in the study. Patients were divided into three groups depending on their gingival inflammation status. First group was control group (n=20) with no gingival inflammation, second group was with mild gingival inflammation (n=19), third group was with moderate gingival inflammation (n=21) and fourth group was with severe gingival inflammation (n=20). The degree of gingival inflammation was evaluated and have had clinically proven gingivitis, by commonly applied index of WHO (12). This study included adolescents with gingival inflammation except the examinees in the control group, and excluded adolescents without any other acute or chronic disease. The study also excluded patients who, for any reason, did not have completed permanent dentition. None of the patients wore orthodontic appliance, because orthodontic therapy could change the composition of saliva significantly. All clinical measurements were performed by the same investigator.

Collection of unstimulated whole saliva

Oxidative stress parameters were measured in unstimulated whole saliva samples, which were collected in the morning, after at least 12 hours of fasting before clinical examinations and bacterial collection. Subjects were instructed to allow saliva to pool in the bottom of the mouth and drain it into a collection tube when necessary and not to swallow any saliva for the duration of the collection. Before the analysis, saliva was centrifuged at 4.000 ×g for 10 min at 4°C to eliminate cell debris and the supernatant was aliquoted and stored at −80°C until the analysis (13).

Oxidative markers from saliva (determination of superoxide anion radical (O2-), hydrogen peroxide (H2O2), index of lipid peroxidation (TBARS) and nitrites (NO2-))

The level of superoxide anion radical (O2-) was measured NBT (nitroblue tetrazolium) reaction in TRIS-buffer combined with saliva samples and read at 530 nm (14).

The protocol for measurement of hydrogen peroxide (H2O2) was based on oxidation of phenol red in the presence horseradish peroxidase (POD). 200 µl sample with 800 µl PRS (phenol red solution) and 10 µl POD were combined (1:20). The level of H2O2 was measured at 610 nm (15).

Nitrite (NO2-) was determined as an index of nitric oxide production with Griess reagent. 0.1 ml 3N PCA (perchloric acid), 0.4 ml 20 mM EDTA (ethilenediaminetetraacetic acid) and 0.2 ml saliva were put on ice for 15 min and then centrifuged 15 min at 6000 rpm. After pouring supernatant, 220 µl K2CO3 was added. Nitrites were measured at 550 nm. Distilled water was used as a blank probe (16).

The degree of lipid peroxidation in saliva was estimated by measuring thiobarbituric acid reactive substances (TBARS) using 0.4 ml 1% TBA (thiobarbituric acid) in 0.05 NaOH mixed with 0.8 ml of saliva, incubated at 100°C for 15 min and measured at 530 nm. Distillated water was used as a blank probe. TBA extract was obtained by combining 0.8 ml saliva and 0.4 ml TCA (trichloroacetic acid) and then samples were put on ice for 10 min, and centrifuged for 15 min at 6000 rpm (17).

Antioxidative markers from saliva (determination of reduced glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT))

The level of reduced glutathione (GSH) was determined based on GSH oxidation with 5.5- dithio-bis-6.2-nitrobenzoic acid, using method by Beutler (18).

SOD activity was determined by the epinephrine method of Beutler. A hundred µl supernatant and 1ml carbonate buffer were mixed, and then 100 µl of epinephrine was added. Detection was performed at 470nm (19).

CAT activity was determined according to Aebi. Supernatants of saliva were diluted with distilled water (1:7 v/v) and treated with chloroform-ethanol (0.6:1 v/v) to remove haemoglobin and then 50 µl CAT buffer, 100 µl sample and 1 ml 10 mM H2O2 were added to the samples. Detection was performed at 360 nm (20).

Statistical analysis

Results are expressed as means ± standard deviation (SD). All results shown are expressed as mean and 95% confidence interval. Statistical comparisons between groups were assessed by Mann-Whitney or Kruskal-Wallis tests. The independent variables were age, gender (as confounding variables) and the different oxidative markers. All p values were two-tailed and probability values of less than 0.05 were considered to be statistically significant. Statistical analyses were performed using SPSS software (IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.).

RESULTS

Basic characterics of study population

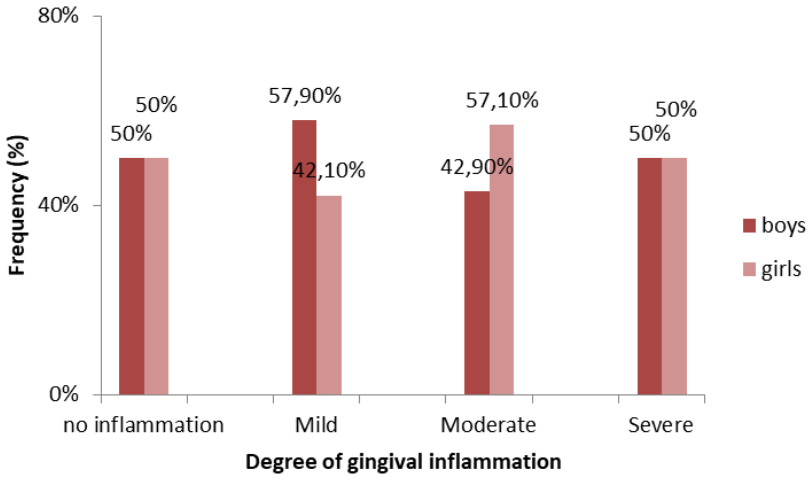
Among 80 subjects in this study, 40 participants (50%) were female, and 40 participants (50%) were male. The study included adolescents from 18 to 21 years old, and the average age was 19.15±0.66 in the first group, 19.10±0.66 in the second group, 19.26±0.70 in the third group and 19.18±0.60 in the fourth group (Table 1). The distribution of participants according to the degree of gingival inflammation is shown in Table 1.

Table 1. Basic characteristics of the study population and the distribution of the degree of gingival inflamma-tion among the study population

		Without	**Mild**	**Moderate**		
Number of participants		20	19	21	**Degree of gingival inflammation**	*p*
Gender	Male	10 (50%)	11 (57.9%)	**Severe**	10 (50%)	[b]*p*=0,825
	Female	10 (50%)	8 (42.1%)	12 (57.1%)	10 (50%)	
Age		19.15±0.66	19.10±0.66	19.26±0.70	19.18±0.60	[a]*p*=0,857
(X±SD (Med, min-max)		(19.25; 18-27)	(19.0; 18-21)	(19.50; 18-21)	(19.1; 18.1-21)	

**p; at-test; bχ 2-test*

Figure 1. Frequency of basic characteristics of the study population and the distribution of the degree of gingival inflammation among the study population in percent (%)



O_2^- values

Values of superoxide anion radical were not significantly higher in the experimental groups compared to the control (p>0.05). Also, in comparing levels of this marker among groups with present inflammation, statistical difference was not proved (p>0.05) (Table 2).

H_2O_2 values

Values of H_2O_2 were significantly higher in the group with moderate inflammation comparing to the control, mild and severe inflammation group (p<0.05). When comparing experimental groups among themselves, the values of this marker were again higher in the group with moderate gingival inflammation compared to the groups with mild and severe inflammation (p<0.05) (Table 2).

NO_2^- values

Values of nitrites were not significantly higher in the experimental groups compared to the control (p>0.05). When comparing experimental groups among themselves, the values of this marker were higher in the group with severe inflammation compared to the group with mild and moderate inflammation and compared to the control group, but not significantly (p>0.05) (Table 2).

TBARS values

Values of TBARS were not statistically affected in the groups with gingival inflammation compared to the control (p>0.05). In the group of patients with severe inflammation levels of TBARS were increased but not significantly (p>0.05) (Table 2).

Table 2. Levels of prooxidative and antioxidative markers from saliva samples during different of gingival inflammation among the study population

Parameters	Degree of gingival inflammation				*P value*
	Without	Mild	Moderate	Severe	
TBARS (µmol/ml)	3.53±2.36 (3.73; 0.11-7.25)	3.42±2.21 (3.89; 0.17-7.22)	3,63±2,86 (4,09; 0,10-7,22)	4,38±2,20 (4,90; 0,61-7,01)	[a]p=0,594
NO (nmol/mol)	14,45±8,11 (11.46; 2.47-33.69)	14,23±7,05 (13.11; 5.55-28.68)	13,59±6,16 (11.52; 5.89-25.43)	14,96±5,92 (14.93; 6.56-33.70)	[a]p=0.935
O_2^- (nmol/mol)	2.39±2.78 (1.48; 0.33-10.22)	2.81±3.78 (1.98; 0.33-17.78)	1.68±0.99 (1.65; 0.33-4.28)	1.93±1.36 (1.65; 0.0-5.60)	[b]p=0.639

Parameters	Degree of gingival inflammation				*P value*
	Without	**Mild**	**Moderate**	**Severe**	
H_2O_2 (nmol/mol)	0.47±0.27 (0.53; 0.023-0.967)	0.31±0.34 (0.14; 0.046-1.16)	0.30±0.25 (0.23; 0.023-0.967)	0.28±0.30 (0.18; 0.023-1.382)	**[b]p=0.047***
SOD (U/mg protein)	35.39±40.52 (20.20; 0.0-146.52)	35.13±83.19 (16.28; 0.0-374.40)	56.20±106.29 (16.28; 0.0-463.98)	25.64±45.92 (16.28; 0.0-211.64)	[b]p=0.714
CAT (U/mg protein)	140.45±86.54 (125.6; 28.75-408.5)	173.52±122.91 (134.4; 25.75-545.0)	173.60±80.59 (157.75;96.0-381.0)	192.05±130.47 (158.12; 25.75-564.5)	[a]p=0.493
GSH (U/mg protein)	478.74±391.28 (356.14; 83.73-1842)	367.98±225.27 (293.06; 83.73-711.7)	707.73±201.73 (586.12; 83.73-2051)	537.97±445.41 (397.72; 83.73-1716)	**[b]p=0.012***

**p<0.05; [a]ANOVA; [b]Kruskal Wallis test*

GSH values

Values of GSH were significantly changed in the group with moderate inflammation compared to the control, and compared to the groups with mild and severe inflammation ($p<0.05$). Also, among all experimental groups, levels of this marker were significantly changed ($p<0.05$) (Table 2).

SOD values

Values of SOD were not significantly changed in the group with inflammation compared to the control ($p>0.05$). Also, among all experimental groups, levels of this marker were not significantly changed ($p>0.05$) (Table 2).

CAT values

Similarly to SOD, values of CAT were not significantly changed in the group with inflammation compared to the control ($p>0.05$). Also, among all experimental groups, levels of this marker were not significantly changed ($p>0.05$) (Table 2).

DISCUSSION

The present study was aimed at assessing potential connection between oxidative stress and gingival inflammation at different clinical stages. This topic was very poorly investigated in reliable databases, especially having in mind that our study population consisted of adolescents.

The medical significance of oxidative stress has become increasingly recognized to the point that it is now considered to be a component of virtually every disease process. More recently, evidence has also emerged for a crucial role of ROS in periodontal tissue destruction (1-5). ROS are described as oxygen free radicals and other non-radical oxygen derivatives involved in oxygen radical production (7). They are involved in normal cellular metabolism and continuously generated by the cells in most tissues. Another category of substances called antioxidants exist in the cells and can effectively delay or inhibit ROS-induced oxidation (2). Under physiological conditions, ROS are effectively neutralized by antioxidants, which prevent ROS-mediated tissue damage. When an inflammation happens, ROS production is drastically increased mainly due to cells of innate immune system, e.g., neutrophils and macrophages during the process of phagocytosis via the metabolic pathway of the “respiratory burst” (9). Subsequently, high levels or activities of ROS cannot be balanced by the antioxidant defense system, which leads to the oxidative stress and tissue damage (8). ROS can directly cause tissue damage, involving lipid peroxidation, DNA damage, protein damage, and oxidation of important enzymes; meanwhile, they can function as signaling molecules or mediators of inflammation (1-9).

In the first part of the study, we examined the potential connection between superoxide anion radical and hydrogen peroxide production during different levels of gingival inflammation in adolescents aged 18 to 21. O_2^- and H_2O_2, as a very reactive oxygen species, are responsible for various oxidative stress-related damages of organs and tissues. It is well-known that neutrophils are the most abundant blood white cells and belong to the first defense line against bacterial infection. During an infection, after the initiation of the host response by pathogenic biofilm, neutrophils become the most common inflammatory cells gathering in periodontal

tissue and gingival sulcus and they are believed to be the predominant source of ROS in periodontitis (8, 9). Following the stimulation by pathogens, neutrophils produce O_2^- via the metabolic pathway called "respiratory burst" catalyzed by NADPH oxidase during phagocytosis (4, 9). O_2^- can be released into phagosomal and extracellular environment and then converted to different radical and non-radical derivatives, such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), hydroxyl radical (OH•) and singlet oxygen ($^1O_2$).

In our study, values of O_2^- were not significantly higher in the experimental groups compared to the control ($p>0.05$). Also, in comparing levels of this marker among groups with present inflammation, statistical difference was not proved ($p>0.05$) (Table 2). On the other hand, values of H_2O_2 were significantly higher in the group with moderate inflammation compared to the control, mild and severe inflammation group (Table 2). Previous clinical study also confirmed connection between increased ROS production and gingival inflammation. Given the close relation between inflammation and oxidative stress, the role of ROS and antioxidant systems in the pathogenesis of periodontal tissue injury and gingival inflammation has regained attention in the last years (21-23).

Furthermore, we examined the influence of gingival inflammation on index of lipid peroxidation and nitric oxide in saliva samples of participants. None of the mentioned markers were influenced in patients with gingival inflammation compared to patients without gingivitis (Table 2). In our study, local oxidative response to mild and severe inflammation was weak or undetected, but moderate inflammation induced significant increase of hydrogen peroxide in saliva.

Lamster and Novak described that oxidants can also lipid peroxidation, induction of pro-inflammatory cytokines, and hydrolytic enzymes, such as, MMPs, as well as inactivation of protease inhibitors. Additionally, H_2O_2 overproduced extracellularly can even pass through biologic membranes freely and act as intracellular second messengers, activating a variety of signal transduction pathways (24).

Various studies have pointed toward the axiom that the reactive oxygen species and antioxidants are in dynamic equilibrium and any disturbance in one would lead to changes in the other (25).

Studies have confirmed that the inflammatory response in gingivitis is associated with an increased local and systemic oxidative stress and compromised antioxidant capacity (25). The results of our study partially confirmed previous research, and during the elevated levels of hydrogen peroxide, we found elevated levels of GSH in the same group of participants and during the moderate degree of gingivitis.

Numerous studies showed higher level of antioxidant markers in saliva of gingivitis patients compared with that of healthy controls as well as their significant association with clinical periodontal parameters (26).

One of the few studies about oxidative status in the state of gingival inflammation in adolescents is the study by *Marton et al*. They reported about the level of malondialdehyde, a stable end-product of lipid peroxidation which was induced by reactive oxygen intermediates and the activity of two potent antioxidant enzymes, superoxide dismutase and glutathione peroxidase from tissue homogenates of 22 surgical periapical granuloma specimens. In their study, malondialdehyde levels were significantly higher and glutathione peroxidase activity was significantly lower in periapical granuloma samples than in healthy gingival tissue homogenates, which were used as controls. The activity of superoxide dismutase was similar in periapical granuloma and in the control samples. The results of the mentioned study indicated an altered balance between the production and the elimination of toxic oxygen metabolites in chronic apical periodontitis, and it was concluded that reactive oxygen intermediates, which are being produced by activated phagocytic cells abundantly present in periapical granulomas, can contribute to periapical tissue injury and bone loss in this disease (27).

Brock et al reported that the levels of antioxidant markers were significantly higher in adult gingivitis patients than in healthy periodontal controls. They concluded the involvement of ROS in periodontal pathology and reported that it would be modulated by *in vivo* antioxidant defense systems (28).

Literature data did not suggest differences of oxidative status in youth and adult period, but suggested potential mechanisms by which gingivitis induces ROS production. Namely, ROS are able to induce the activation of the key matrix myeloperoxidases (MMPs) in periodontal tissues, such as MMP-8 and MMP-9, through direct enzyme oxidation, although indirect mechanisms involving intracellular signaling cannot be precluded. MMP-8 and MMP-9 are both promising periodontal and apical disease biomarkers which cooperatively hydrolyze type I collagen, a key step in periodontal supporting tissue loss. PMN-derived myeloperoxidase (MPO) catalyzes HOCl release and besides its antimicrobial effects, it has been reported to oxidatively activate latent proMMP-8 and -9 in vitro and inactivate tissue inhibitor of metalloproteinase (TIMP)-1. Ex vivo studies suggest that oxidative activation of MMP-8 and MMP-9 represents the dominant mechanism in destructive periodontal lesions as well as in gingivitis (29-32).

Previous clinical studies, as well as our results, suggest that the antioxidant capacity of gingivitis patients is both qualitatively and quantitatively distinct from that of saliva, plasma and serum. Whether changes in the redox status is, in gingivitis is undoubtedly present predisposition to or the results of ROS-mediated damage. Probably, the type of sample for determination of markers of redox status may be a reason for partial differences of our results with the results of some previous studies (33, 34).

Also, it is important to notice that presence of different types of bacteria could differently influence on markers of oxidative stress by different mechanism (35).

Limitation of our study is that our study is focuses on the increased local oxidative stress in gingivitis, and specifically on the relationship between the local and systemic biomarkers of oxidative stress. Also, the relationship between gingivitis and systemic inflammation, and the effects of periodontal therapy on oxidative stress parameters are not still evaluated and discussed in this stage of our study.

CONCLUSION

In conclusion, oxidative stress plays a central role in the pathogenesis and the determination of oxidative and antioxidative levels could be a potent tool in controlling the development of gingivitis. Further studies are needed to unravel the complex effects of ROS in gingival tissue breakdown and their associated systemic diseases, as well as the potential contributions of adjuvant antioxidant therapies.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interests relevant to the manuscript.

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