

ANALYSIS OF THE VALUES OF OXIDATIVE STRESS PARAMETERS IN SALIVA OF CHILDREN WITH GINGIVITIS

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Early detection and analysis of the values of the parameters of oxidative stress, malondialdehyde (MDA) and reactive carbonyl groups (RCG) in saliva, as possible biochemical markers in the diagnosis and prognosis of periodontal disease, may be of particular importance in children. For this reason, the aim of this study was to examine the levels of lipoproteins in the saliva of children without gingivitis and with gingivitis, as well as the degree of gingival inflammation.

The testing was conducted in 120 children aged 12.2 years, with permanent dentition. Gingival index by Löe-Silness was used for the gingival estimation. A modified method with thiobarbituric acid was used for the determination of MDA in unstimulated saliva. Colorimetric reaction with 2.4 dinitrophenylhydrazine (2.4 DNPH) was applied for the determination of RCG.

Results of the analysis of the average values of prooxidizer in the saliva of the patients in a study and the control group showed as statistically significantly higher in the patients of the study group, in RCG concentration (UMW=667.5, $z=-4.137$, $p<0.001$) as well as in the level of MDA (UMW=452.5, $z=-5.44$, $p<0.001$). The results of the analysis of the MDA level showed an increase in average values with increasing degree of gingival inflammation with statistical significance between the groups confirmed by the Kruskal-Wallis test ($\chi^2KW=32.45$, $p<0.001$) but not by the Mann-Whitney test. Results of the analysis of concentration of carbonyl groups in patients with varying degrees of gingival inflammation showed an increase with statistically significant differences in the values of this parameter among all groups of patients ($\chi^2KW=45.23$, $p<0.001$) and by the Mann-Whitney test the highest among the patients with healthy gingiva and patients with severe gingival inflammation (UMW=113.00, $z=-4.98$, $p<0.001$).

The presence and increase in the parameters of oxidative stress of malondialdehyde and carbonyl groups in the saliva of children with gingivitis is in accordance with the existence and severity of periodontal disease. These biochemical parameters may be an important diagnostic and prognostic biomarkers of paradontium health state. *Acta Medica Medianae* 2016;55(2):12-18.

Key words: children, gingivitis, malondialdehyde, reactive carbonyl groups

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Introduction

The composition of saliva reflects the nature and extent of the reaction of an organism to the periodontal infection. The analysis of the composition of saliva can contribute to an easier assessment of the risk of the disease, but also to the early diagnosis of gingivitis and periodontal di-

sease. For this reason, it has a special significance as an important biological material for new diagnostic tests.

Oxidative stress plays an important role in the etiopathogenesis of periodontal disease (1). One of the major pathophysiological mechanisms of oxidative damage to biomolecules is lipid peroxidation of polyunsaturated fatty acids of cell membranes (2). During the process of lipid peroxidation, primary highly reactive intermediates occur: alkyl radicals, conjugated dienes, peroxy radicals, alkoxy radicals, as well as lipid hydroperoxides. By the degradation of these primary products, secondary products of lipid peroxidation are formed: short-chain hydrocarbons and aldehydes. The end product of lipid peroxidation is a short-chain malondialdehyde which can react with free SH and NH² groups of amino acids, peptides, proteins, nucleotides, and phospholipids, leading

to covalent modifications of the macromolecules, thereby altering their structure and function (3). The intensity of the process, as well as the possibility of partial or total damage reparations, depends on the prooxidant or antioxidant environment in which the process takes place (4, 5).

Another important pathophysiological mechanism of oxidative damage, almost impossible to separate from lipid peroxidation, with regard to the unity of lipoprotein interactions in cell membranes, is a process of oxidative protein modification. Oxidation of unsaturated fatty acids and delipidation of membrane structure reduces the fluidity of cell membranes, and the oxidative modification of protein disrupts the interaction of a lipoprotein and changes the structural and functional properties and the movement of membrane proteins. The lipid peroxidation process changes the affinity of the interaction of the protein with lipids, essential process for the performance of a large number of functions of membrane proteins and homeostasis of cells as a whole (6). The consequences are numerous, and the degree of oxidative modification of a protein can be determined by the amount of free amino acids, if they are not produced in any other way in the cell (alanine in erythrocytes), or through the determination of sulfhydryl groups (methionine sulphoxide and bityrosyl). The determination of reactive carbonyl groups in amino acid residues is an important indicator of oxidative protein modification (3).

The cells destructed by oxidative damages of cell membranes during the development of gingivitis induce the increased release and activity of cytosolic and mitochondrial enzymes, as well as the level of malondialdehyde and carbonyl groups. Based on the results of recent studies, elevated levels of these oxidative stress parameters in saliva can be considered a biochemical marker of functional periodontal condition (7).

Aim

The aim of this study was to determine the value and to compare the parameters of oxidative stress, malondialdehyde and reactive carbonyl groups, in the saliva of children without gingivitis and with gingivitis, as well as to examine the correlation between the values obtained with the degree of gingival inflammation.

Method

Testing was conducted in 120 children, aged from 11.5 to 12.9 years, of approximately equal gender prevalence, with the completed permanent dentition. The study included children without the existence of acute or chronic general diseases in personal anamnesis.

Clinical overview of the gingival and saliva sampling was carried out early in the morning, before breakfast and after brushing the teeth. Unstimulated mixed saliva was taken in a manner

that patients collected saliva for a period of 5 to 10 minutes in a sterile laboratory glass.

To assess the gingival condition, gingival index by Loe and Silness was used (8). Clinical examination of gingiva, according to the aforementioned index, included the assessment of the gingiva by inspection, palpation and gingival probe for vestibular, mesial, oral and distal sides of each present tooth. Inspection and palpation determined color, consistency, size, existence of tumefactions and spontaneous gingival bleeding. The existence or absence of gingival bleeding upon provocation was determined by a round-tip probe, set parallel to the longitudinal axis of the tooth, and the power used for probing was equal to the probe weight.

After the applied gingival index, the gingival condition was expressed numerically, 90 patients had gingival inflammation (gingival index from 0.1 to 3.0), and they were in the study group. There were 30 patients with mild gingivitis (gingival index of 0.1 to 1.0), 30 with moderate (gingival index from 1.1 to 2.0) and 30 patients with a strong gingival inflammation (gingival index from 2.1 to 3.0). The control group consisted of 30 patients with no signs of gingival inflammation (gingival index 0). In this manner, four groups of patients who gave samples of saliva were formed.

For the determination of MDA in saliva, a modified method with thiobarbituric acid (TBA) was used (9). Saliva was combined with 5% butylated hydroxytoluene and TBA solution, and was incubated at 100°C. The resultant colored product was read at a wavelength of 535 nm and recalculated through molar extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ for MDA, and the concentration was expressed in $\mu\text{mol/l}$.

To determine the total protein of saliva due to the small protein concentration in saliva, a modified method with Poncey S reagent with a standard curve of various concentrations of protein was used (10). To determine reactive carbonyl groups, colorimetric reaction with 2,4 dinitrophenylhydrazine (2,4 DNPH) was used, which according to the type of Schiff's base formed 2,4 dinitrophenylhydrazine reactive carbonyl derivatives. Conversion was performed at a molar extinction coefficient for DNPH reactive carbonyl derivatives of $22 \times 10^3 \text{ l/mol/cm}$, and the concentration of carbonyl groups was expressed as protein $\mu\text{mol/g}$ (11).

Tested parameters were processed with statistical methods of descriptive and quantitative analysis (SPSS 14.0 for Windows 2003). The distribution of frequencies was examined by Shapiro-Wilk test. Provided that non-parametric tests were applied, the data are presented as median and interquartile differences. To test the statistical significance between the examined groups, rank analysis was used (Kruskal-Wallis ANOVA), and to compare two groups of data a nonparametric Mann-Whitney Rank Sum Test was used. For all applied statistical tests, the levels of statistical

significance were determined (p) of 0.05, 0.01 and 0.001. Survey results were analyzed and presented in tables (Excel 2003).

Results

Results of the analysis of average values of prooxidants in the saliva of the patients in the study and the control group by Mann-Whitney test showed significantly higher values in the patients of the study group, as well as in the concentration

Table 1. Average values of prooxidant parameters (carbonyl groups and MDA) in the saliva of patients without gingivitis and with gingivitis

Parameter	Without gingivitis $\bar{X} \pm SD$	With gingivitis $\bar{X} \pm SD$
Carbonyl groups	1.68±1.15	2.58±1.19***
MDA	0.43±0.24	1.13±1.22***

*** $p < 0.001$

of carbonyl groups (UMW=667.5 $z = -4.137$, $p < 0.001$), and in the level of MDA (UMW=452.5, $z = -5.44$, $p < 0.001$) (Table 1).

Results of statistical analysis of the value of the concentration of carbonyl groups in patients with varying degrees of gingival inflammation, presented in Table 2, showed that they were lower in patients with healthy gingiva (1.68±1.15) compared to the patients with mild inflammation (1.74±0.78). Patients with moderate gingival inflammation had higher average value of this parameter (2.45±0.58) compared to patients with

mild inflammation and those with healthy gingiva. Patients with severe gingival inflammation had higher average value of carbonyl group concentration (3.53±1.32) in relation to all groups of patients.

The results showed a marked heterogeneity in the values of the concentration of carbonyl groups in patients with healthy gingiva, and mild and severe gingival inflammation (CV>30%).

Testing the significance of differences in the concentration of carbonyl groups between the examined groups by Kruskal-Wallis test showed a statistically significant difference in the values of this parameter among all groups of patients ($\chi^2_{KW} = 45.23$, $p < 0.001$). Mann-Whitney test revealed a statistically significant difference between patients with healthy gingiva and groups of patients with mild gingival inflammation (UMW=210.0, $z = -3.55$, $p < 0.001$). A statistically significant difference was found between patients with healthy gingiva and groups of patients with moderate gingival inflammation (UMW=123.5, $z = -4.83$, $p < 0.001$), and between patients with healthy gingiva and groups of patients with severe gingival inflammation (UMW=119.00, $z = -4.89$, $p < 0.001$). Notwithstanding the increase in average values of MDA levels with the increase in the inflammation degree, there was no significant difference between the groups of patients with mild to moderate gingival inflammation, mild and severe, and moderate and severe gingival inflammation.

Results of statistical analysis of the value of the concentration of carbonyl groups in patients with varying degrees of gingival inflammation

Table 2. Average values of MDA levels in patients with various degrees of gingival inflammation

	MDA ($\mu\text{mol/l}$)		
	$\bar{X} \pm SD$	Median (interquartile difference)	C_v (%)
Healthy gingiva	0.43±0.24 ^{a,b,c}	0.35 (0.23-0.58)	60%
Mild inflammation	0.72±0.32	0.78 (0.49-0.90)	44.44%
Moderate inflammation	1.17±1.14	0.82 (0.64-0.99)	97.45%
Severe inflammation	1.49±1.68	0.79 (0.65-1.42)	44.40%

^a healthy vs mild inflammation, $p < 0.001$

^b healthy vs moderate inflammation, $p < 0.001$

^c healthy vs severe inflammation, $p < 0.001$

Table 3. Average values of carbonyl groups concentration in patients with varying degrees of gingival inflammation

	Carbonyl groups ($\mu\text{mol/g}$ of protein)		
	$\bar{X} \pm SD$	Median (interquartile difference)	C_v (%)
Healthy gingiva	1.68±1.15 ^{a,b}	1.20 (0.95-1.88)	68.45%
Mild inflammation	1.74±0.78 ^{c,d}	1.74 (1.03-2.23)	44.82%
Moderate inflammation	2.45±0.58 ^f	2.48 (1.97-2.80)	23.67%
Severe inflammation	3.53±1.32	3.09 (2.43-4.54)	37.39%

^a healthy vs moderate inflammation, $p < 0.001$

^b healthy vs severe inflammation, $p < 0.001$

^c mild vs moderate inflammation, $p < 0.001$

^d mild vs severe inflammation, $p < 0.001$

^f moderate vs severe inflammation, $p < 0.001$

presented in Table 3 showed that they were lower in patients with healthy gingiva (1.68 ± 1.15) compared to the patients with mild inflammation (1.74 ± 0.78). Subjects with moderate gingival inflammation had higher average value of this parameter (2.45 ± 0.58) compared to subjects with mild inflammation and those with healthy gingiva. Group of patients with severe gingival inflammation had a higher average value of carbonyl group concentration (3.53 ± 1.32) in relation to all groups of patients.

The results showed a marked heterogeneity in the values of carbonyl groups concentration in groups of patients with healthy gingiva, mild and severe gingival inflammation ($CV > 30\%$).

Significance of the difference in values between the carbonyl groups concentration and groups examined by Kruskal Wallis test showed a statistically significant difference in the values of this parameter among all groups of patients ($\chi^2_{KW} = 45.23, p < 0.001$) (Table 3).

Mann-Whitney test showed that there is a statistically significant difference between patients with healthy gingiva and groups of patients with moderate gingival inflammation (UMW=180.5, $z = -3.98, p < 0.001$), as well as between the patients with healthy gingiva and patients with severe gingival inflammation (UMW=113.00, $z = -4.98, p < 0.001$). A statistically significant difference was found between patients with mild and moderate gingival inflammation (UMW=212.5, $z = -3.51, p < 0.001$), as well as the patients with mild and severe gingival inflammation (UMW=98.5, $z = -5.197, p = 0.001$). There was a statistically significant difference in the values of this parameter between the patients with moderate and severe gingival inflammation (UMW=226.5, $z = -3.3, p = 0.001$).

Discussion

As the composition of saliva reflected the nature and extent of the reaction of the organism to periodontal infection, our results confirmed the existence of higher values of prooxidant parameters in saliva in the presence of an inflammatory process, located in the soft tissues of the tooth supporting apparatus, which corresponds to the initial stage of periodontal disease, gingivitis (12).

The process of lipid peroxidation is the most extensively studied process of cell damage in conditions of oxidative stress, and its end product, malondialdehyde, is known as a biochemical marker of the degree of oxidative damage to cell membranes (13, 14). Intensified process of lipid peroxidation is present in over one hundred diseases, and in some cases, represents one of the immediate causes of the basic disease (15). Numerous studies point to the intense process of lipid peroxidation with periodontal disease (16-18). Analysis of MDA levels in saliva of people with gingivitis or periodontal disease prove higher values of this prooxidant parameter compared to the saliva of healthy patients (4). Except in saliva, in the presence of periodontal disease, the in-

crease in MDA levels was detected in the serum as well as in gingival fluid, with the largest increase recorded in gingival fluid, then saliva, and the lowest the serum of the diseased (14). MDA level in the gingival fluid is more frequently than saliva the subject of interest for researchers, for more truthful display of the condition of periodontal tissue, because of direct contact with them (19), but the simplicity of sampling saliva assigns it an attribute of the most examined oral fluid for diagnostic purposes (20, 21).

Test results of MDA levels in saliva of children with gingivitis, obtained in this study, confirmed the expected increase in the level of MDA in the existence of inflammatory process in gingiva and in accordance with the results of other authors (17). The results were difficult to compare because the studies in which patients were children, and with the existence of a general disease, are rare. In most of these studies, subjects were adult patients, mostly smokers or with some general diseases (22-24). Acute or chronic general disease, often corresponding medicaments for the treatment of this disease, significantly modify the health condition of the supporting apparatus of teeth and other oral structures. The consequence is a qualitative and quantitative alteration in the composition of saliva, thus MDA level in saliva, when it is not a valid indicator of just the gingival condition (25).

Analysis of MDA level relative to the degree of gingival inflammation in our study showed no significant difference. A possible explanation should be sought in the fact that it involved the children with the newly established permanent dentition and gingival changes mainly in the initial stage, which makes the clinical assessment of gingiva difficult. Poor clinical picture at the initial stage of gingivitis reduces the possibility of realistic evaluation and makes it even more subjective. This interpretation is confirmed by the wide range of values of tested parameters within the groups ($CV > 30\%$) (Table 2).

The severity of this problem suggests the impossibility of defining the differences between normal histologically and clinically healthy gingiva. The complexities of this problem is supported by the knowledge that in strictly controlled experimental conditions, histologically normal gingiva is characterized by the intact epithelium and complete absence of inflammatory cell infiltrate in the lamina propria, which is clinically observed as gingiva of pale pink color and firm consistency that fills the interdental space (26). Therefore, clinically healthy gingiva, according to some authors, still contain inflammatory infiltrate, which probably occurs even during the eruption. It is composed of neutrophils and lymphocytes, and mechanisms that will lead to clinical manifestations of the disease, as well as the length of their operation are not known (27). Options for the selection of the relevant test for accurate assessment of the condition of periodontal tissues increase with age, as the frequency and severity of clinical gingivitis

grow. However, studies in which periodontal disease are classified according to the severity of the clinical picture are scarce and conflicting results. While one group of authors who have classified the subjects according to the severity of the disease recorded a significant increase in markers of lipid peroxidation only in patients with advanced disease (16), other authors conclude that the increase in oxidative stress parameters are in accordance with the severity of periodontal disease (28). These results may be joined by our results that there are significant reductions in MDA levels after the conservative periodontal treatment, which significantly changes the clinical picture (7).

Besides the evident increase in MDA levels with increasing degrees of inflammation, significantly higher values of the tested prooxidant in the saliva of adult patients registered in studies by other authors should also be noted (29). The lowest average value of the examined parameter was in the group of patients with healthy gingiva (0.43 ± 0.24). If we bear in mind that the patients in this study were children, with just completed permanent dentition, this difference can be explained by the extension of pathological processes in adults, i.e. the number of affected teeth, and thus which part of the gingiva is affected (localized or generalized gingivitis, marginal, papular or diffuse gingivitis) or the entire periodontal disease, as well as during the inflammatory process (acute, subacute, or chronic). The fact that the intensity of the process and the values of associated biomarkers of oxidative stress, as well as the possibility of partial or full repair of the resulting damage, depend on the prooxidative, i.e. anti-oxidative conditions in the environment in which the process takes place, has been demonstrated both *in vitro* (5) as well as *in vivo* (4).

Another important pathophysiological mechanism of oxidative damage in inflammatory diseases, such as gingivitis, is a process of oxidative modification of proteins, causing a disruption in the structure of proteins and the consequent abnormalities in physico-chemical properties. They lead to irreversible conformational changes, particularly of long-lived proteins, such as collagen, elastin, fibronectin and laminin. One effect is the oxidative modification of proteins and increased proteolytic susceptibility. Determination of reactive carbonyl groups in amino acid residues is an im-

portant indicator of oxidative modification of proteins (30).

Results of the analysis of the values of prooxidant parameters in the saliva of patients with gingivitis showed significantly higher values for both the MDA level and carbonyl groups concentration ($p < 0.001$) (Table 1).

Lipid peroxidation and oxidative modification of proteins represent two independent processes. Their interaction is reflected in the fact that products of lipid peroxidation, such as MDA, can be reacted with amino groups of the protein, building intermediate compounds. With regard to the unity of lipoprotein interactions, already violated by the intensified process of lipid peroxidation, which was proved by an increase in the MDA level, the increase of the value of carbonyl groups concentration in patients with gingivitis was expected.

The increase in carbonyl groups concentration in the saliva of children was in accordance with the severity of gingival inflammation, as well as the increase of MDA level, but unlike it, it is significantly important among all groups of patients ($p < 0.001$) (Table 3).

The explanation may be sought in the fact that the protein modification is quick and linear and is considered the parameter of oxidative modification of biomolecules more sensitive than lipid peroxidation. Likewise, the degradation of oxidatively modified protein may not always be correlated with the degree of modification thereof, considering that some of the proteins upon oxidative modification are more susceptible to proteolytic degradation, and their aggregation degree is variable. Due to the consequences of oxidative protein modification that become more susceptible to proteolytic degradation, this process is given a name of "universal signal proteolysis" (31).

Conclusion

Values of the analysis of the parameters of oxidative stress in saliva, malondialdehyde and carbonyl groups show the presence and increase in accordance with the severity of periodontal disease. Such knowledge indicates the possibility of practical application of the analyzed parameters of saliva in the diagnosis of the risks of periodontal disease.

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ANALIZA VREDNOSTI PARAMETARA OKSIDATIVNOG STRESA U PLJUVAČKI DECE SA GINGIVITISOM

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Rana detekcija i analiza vrednosti parametara oksidativnog stresa, malondialdehida (MDA) i reaktivnih karbonilnih grupa (RKG) u pljuvački, kao mogućih biohemijskih markera u dijagnostici i prognozi oboljenja parodontijuma, može imati poseban značaj u dečjem uzrastu. Iz tog razloga, cilj ovog rada bio je da se ispituju vrednosti navedenih parametara u pljuvački dece bez gingivitisa i sa gingivitisom, kao i u odnosu na stepen inflamacije gingive.

Ispitivanje je obavljeno kod 120 dece stare 12,2 godine sa stalnom denticijom. Za procenu stanja gingive upotrebljen je gingivalni indeks po Löe-Silnessu. Za određivanje MDA u nestimulisanoj pljuvački korišćena je modifikovana metoda sa tiobarbiturnom kiselinom. Za određivanje RKG primenjena je kolorimetrijska reakcija sa 2,4 dini-trofenilhidrazinom (2,4 DNPH).

Rezultati analize prosečnih vrednosti prooksidanasa u pljuvački ispitanika studijske i kontrolne grupe pokazali su statistički značajno više vrednosti kod ispitanika studijske grupe, kako u koncentraciji RKG (UMW=667,5 $z=-4,137$ $p<0,001$) tako i u nivou MDA (UMW=452,5, $z=-5,44$, $p<0,001$). Rezultati analize nivoa MDA pokazali su porast prosečnih vrednosti sa porastom stepena inflamacije gingive sa statističkom značajnošću između grupa potvrđenom Kruskal Valisovim testom ($\chi^2KW=32,45$, $p<0,001$), ali ne i Man-Vitnijevim testom (Mann-Whitney test). Rezultati analize koncentracije karbonilnih grupa kod ispitanika sa različitim stepenom inflamacije gingive pokazali su porast sa statistički značajnim razlikama u vrednostima ovog parametra između svih grupa ispitanika ($\chi^2KW=45,23$, $p<0,001$), a Man-Vitnijevim testom najveću između ispitanika sa zdravom gingivom i ispitanika sa jakom inflamacijom gingive (UMW=113,00 $z=-4,98$, $p<0,001$).

Prisustvo i porast parametara oksidativnog stresa malondialdehida i karbonilnih grupa u pljuvački dece sa gingivitisom u skladu je sa postojanjem i težinom parodontalnog oboljenja. Ovi biohemijski parametri mogu biti važan dijagnostički i prognostički biomarker stanja zdravlja parodontijuma. *Acta Medica Medianae* 2016;55(2):12-18.

Ključne reči: deca, gingivitis, malondialdehyd, reaktivne karbonilne grupe

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