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ENZYMATIC ANTIOXIDANT CAPACITY AND SALIVARY OXIDATIVE STRESS REDOX MARKERS: VERIFICATION OF METHODS FOR FURTHER INVESTIGATION

ВЕРИФИКАЦИЈА НА МЕТОДИ ЗА ОДРЕДУВАЊЕ ЕНЗИМСКИ АНТИОКСИДАТИВЕН КАПАЦИТЕТ И НИВО НА САЛИВАРЕН ОКСИДАТИВЕН СТРЕС ПРЕКУ РЕДОКС МАРКЕРИ

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Abstract

Introduction: Saliva, as complex mixture of fluids, is an interesting alternative diagnostic tool which contains many immunoglobulins, enzymes, lactoferrin, lysosomes and histamines. Despite the role of oxidative stress in numerous systemic diseases, explaining the mechanisms which oxidative stress can contribute to pathology, makes the saliva screening methods specific and more sensitive in terms of evaluation of the biomarker level. **Material and methods:** This observational study included 20 participants (10 smokers and 10 non-smokers). Saliva samples were collected in the period between 9 and 12 hours in the morning, and centrifuged (15 minutes per 10000 g at 4oC). The supernatant was kept at -20oC until further analyses that included analysis of salivary enzymatic antioxidant capacity (activity of antioxidant enzymes superoxide dismutase- SOD, catalase-CAT and glutathione peroxidase-GPx) and the level of lipid peroxidation marker for oxidative stress – malondialdehyde (MDA). **Results:** Salivary levels of SOD were higher in smokers than non-smokers group and the average activity of GPx was with significant lower value in smokers group. There was noted positive finding in the imbalance of salivary enzymatic antioxidant activity due to the presence of free radicals in cigarette smoke group. The level of salivary redox biomarkers did not vary based on gender. **Conclusion:** According to our findings, the increased salivary levels of MDA in smokers as the result of process of peroxidation, which indicates oxidative stress, contributing to the increased levels of free radical–mediated oxidative damage of lipids, definitely supports the hypothesis that oxidative damage in smokers is due to their active exposure directly to saliva. **Keywords:** oxidative stress; saliva; malondialdehyde; superoxide dismutase; catalase.

Апстракт

Вовед: Плунката во чиј состав влегуваат значителен број компоненти, претставува ветувачки медиум и алтернативна дијагностичка алатка за детекција на голем број заболувања. И покрај улогата на оскидативниот стрес во голем број системски нарушувања, го прават овој метод поспецифичен и посензитивен за верификација на патолошки процеси. **Материјал и методи:** Во оваа опсервациска студија беа вклучени 20 испитаници (10 пушачи и 10 непушачи). Беше собрана нестимулирана плунка, во периодот од 9 до 12 часот наутро, и истата беше центрифугирана (15 минути на 10 000g, при 4 oC). Супернатантот беше чуван на -20 °C до понатамошните анализи кои вклучуваа анализа на ензимскиот антиоксидативен капацитет на плунката (активност на антиоксидативните ензими супероксид дизмутаза-SOD, каталаза и глутатион пероксидаза- GPx) и на степенот на липидна пероксидација – преку маркерот за нивото на оксидативен стрес – малондиалдехид (MDA). **Резултати:** Пушачите имаа значајно повисоко ниво на SOD активност во плунката во споредба со непушачите, додека пак просечната активност на GPx беше пониска кај пушачите. Забележана е позитивна корелација во нарушениот баланс помеѓу активностите на антиоксидативните капацитети на ензимите во плунката поради присуството на слободни радикали во цигарите. Имајќи ја предвид просечната возраст и половата дистрибуција како коваријанта при анализата на податоците за саливарен редокс, многу малку или речиси не се разликуваа. **Заклучок:** Индицирајќи ја состојбата на оскидативен стрес, забележано е зголемено ниво на MDA кај пушачите, процес посредуван од слободни радикали преку формирање липидни пероксиди, чија улога дефинитивно ја потврдува хипотезата за оксидативниот дисбаланс кај пушачите, кој се должи на директната исложеност на плунката и оралната празнина со цигарите. **Клучни зборови:** оксидативен стрес; плунка; малондиалдехид; супероксид дисмутаза; каталаза.

Introduction

According to the Global Cancer Observatory (GLOBOCAN), which is an online database providing global cancer statistics, 2019-2020 is considered the period when COVID-19 outbreak was declared a pandemic. Hence, on a global level, a total of 377.713 new cases of oral cell carcinoma were registered worldwide [1,3,5-16]. The geographical distribution of this type of carcinoma is of variable parameters, epidemiologically distributed: Asia - 248.360, Europe - 65.279, North America - 27.469, Latin America and the Caribbean - 17.888, Africa - 14.286, Oceania - 4.431[3-5, 8-11]. Early detection and screening of premalignant and malignant diseases in oropharyngeal region can have a significant influence on the patients' mortality and morbidity. Smoking, as a stress factor, impacts the absorption of harmful components in the human body such as: nitric oxide, carbon monoxide, nicotine, cadmium, methanol and polycyclic carbohydrate compounds[1,3,5,6,9,10]. These components or their metabolic compounds most commonly activate the biological macromolecules that further influence the level of salivary oxidative stress and generate free radicals that are responsible for tissue damage. Saliva is the first defense barrier that comes in contact with foreign bodies or gasses (such as cigarettes, for e.g.). The activity of salivary antioxidants in smokers can be disturbed in terms of cumulative stress caused by the decline of immune cells and metabolic processes in the gingival fluid. Saliva, as a medium, and its chemical composition are the most suitable constituent components that play a big role in the determination of the individual antioxidant capacity and status in the oropharyngeal cavity of smokers[1-6,10,11,14-16]. Damages to the oral mucosa include the initial stage of formation and colonization of reactive processes that take place at the level of cellular and extracellular matrix[9,13,14,17-21]. Significant values of increased salivary antioxidative stress have been detected in oral carcinoma compared to patients with other benign epithelial lesions[2-5,10,17,19]. Diagnosis of malignancy through saliva, evaluation of the level of salivary and serum biomarkers, and determining the redox status have seen enormous progress by confirming or rejecting most of its constituent parameters, especially when it comes to oral squamous cell carcinoma. The aim of this study was to determinate the methods for SOD, CAT and CPx activity following MDA concentrations in smokers and non-smokers group, representing the antioxidative status and oxidative stress levels which will be an important part from the perspective of using saliva for further research.

Material and method

Subjects and experimental design

This observational study included 10 smokers (group P, with a smoking history not less than 10 years, from 5 to 10 cigarettes per day) and 10 non-smokers (group K as a control group), who were selected by a simple, non-randomized method. In general, subjects were healthy and received no therapy for systemic diseases in the last 3 months. All of them were informed about the objectives of the study, that is, about the analyses of their saliva; they were guaranteed anonymity, and they filled out a written informed consent. Subjects were required not to consume food or water for at least 4 hours prior to collecting saliva samples. Smokers were forbidden to smoke for at least 2 hours prior to taking saliva samples. Unstimulated saliva was collected; each subject was asked to spit saliva in a 15 ml sterile vial. The procedure was performed in a standing position in the period between 9 and 12 hours in the morning. The saliva was centrifuged to remove squamous cells and different cellular fragments (15 minutes at 10000 g at $4^{o}C$, Universal 320 centrifuge, Hettich Lab Technology, Germany). The supernatant was kept at $-20^{o}C$ until further analyses that included analysis of salivary enzymatic antioxidant capacity (activity of antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase), and the level of lipid peroxidation marker for oxidative stress – malondialdehyde. All biochemical analyses were conducted at the Institute of Biology, Faculty of Natural Sciences and Mathematics - Skopje.

Determination of superoxide dismutase (SOD) activity

The activity of superoxide dismutase was determined by the kinetic method described by Marklund and Marklund (1974). It is based on the ability of superoxide dismutase to inhibit pyrogallol autooxidation in an alkaline environment. The reaction mixture contained 50 mM Tris-HCl, pH 8.65, 1 mM diethylenetriaminepentaacetic acid (DETAPAC) and a saliva sample. The reaction was started by adding pyrogallol (final concentration of 0.2 mM), and the absorption was measured kinetically at a wavelength of 420 nm ($25^{o}C$) for 3 minutes (Model 680 Microplate Reader, Bio-Rad Laboratories, USA). One unit of activity (U) is defined as the amount of the enzyme (from the sample) necessary to perform 50% of inhibition of pyrogallol oxidation.

Determination of catalase (CAT) activity

The catalase activity was measured by the method of Claiborne (1985). The reaction mixture contained 50 mM of potassium phosphate buffer (pH 7.0), 19 mM H_2O_2, and a saliva sample. The reaction was initiated by adding H_2O_2, and the absorption changes were measured at a wavelength of 240 nm for 30 seconds (every 5 seconds) at $25^{o}C$. In such conditions, molar absorption coef-

ficient for H_2O_2 was 43.6 M^{-1} cm^{-1}. One unit of activity corresponds to the amount of the enzyme that performs conversion of 1 µmol H_2O_2 in 1 minute.

Determination of glutathione peroxidase (GPx) activity

The activity of superoxide dismutase was determined by the modified method of Lawrence and Burk (1976). The reaction mixture contained 50 mM potassium phosphate buffer, pH 7.0, 1 mM sodium azide, 2 mM GSH, 0.2 mM NADPH, 1 U/ml GR, 1.5 mM cumene hydroperoxide and a saliva sample. The reaction was started by adding cumene hydroperoxide, and the absorption change originating from the NADPH oxidation was monitored at a wavelength of 340 nm for 3 minutes. The reaction took place at 25°C, in the presence of glutathione reductase and reduced glutathione. One unit of activity is defined as the amount of the enzyme (from the sample) that catalyzes oxidation of 1 µmol of NADPH for 1 minute.

Determination of malondialdehyde (MDA) concentration

The concentration of malondialdehyde in saliva was determined by the modified method of Yagi (1998). Solutions of 50% trichloroacetic acid (TCA), and 1.3% thiobarbituric acid (TBA) (dissolved in 0.3% NaOH) was added to the saliva sample. After 20 minutes of incubation at 90-95° in a water bath, samples were instantly chilled/cooled in ice and then centrifuged (10 min. per 4000 g). The supernatant absorption was measured at a wavelength of 535 nm. 1,1,3,3-tetraethoxypropane was used as a standard.

Statistical analysis

Data in the figures are presented as mean ± standard deviation (SD). Normal distribution of data was verified with Kolmogorov-Smirnov test. The statistical analysis for comparison of the means of the examined parameters between the groups was made with the Student's t-test for independent groups of samples. The correlation between the variables was analyzed with the Pearson's test. All analyses were made with the statistical package GraphPad Prism, version 8.0.0. (GraphPad Software, San Diego, CA, USA). Values of $p<0.05$ were considered to be statistically significant.

Results

Twenty patients, including 10 non-smokers (4 males and 6 females) and 10 smokers (6 males and 4 females) were included in this study. The statistical distribution according to gender in both groups (K-non-smokers and P-smokers) was almost equal. The mean age of participants in non-smokers was 48.4 years (aged 37-61) and 49.1 years (aged 40-59) for the second group (smokers). The age wasn't observed as covariant, then we respectfully and mathematically output to verify that there is no statistically significant difference between the groups.

Table 1. Distribution according to the patients' gender and mean age (**K** - nonsmokers, **P** - smokers).

Parameters/ Group	K	P
Number	10	10
Gender (m/f)	4/6	6/4
Mean age	48,4 ± 7,9 (37-61)	49,1 ± 6.3 (40-59)

The analysis showed that salivary levels of SOD were higher in the smoker's group than in the non-smokers group (p=0,0008) (Figure 1.). On the other hand, the average activity of GPx had a significantly lower value in smokers compared to non-smokers group (p=0,0001) (Figure 1). Similarly, the mean activity of catalase (CAT) in saliva among the participants in the smokers group was observed to be lower than in the non-smokers group, but there was no evidence of statistical significance between the two groups. Thus, there was positive finding in the imbalance of salivary enzymatic antioxidant activity, and due to the presence of free radicals in the smokers group, increased levels of salivary MDA are observed in smokers (p=0,0102) (Figure 1), and consequently induces oxidative stress.

The data obtained in this study show negative correlation between SOD and MDA (r = -0,4313, p=0,0567), CAT and MDA (r = - 0,5726, p=0,0083),), GPx and MDA (r = -0,4818, p=0,0315) (Figure 2). So, there is a marked increase of SOD, GPx and MDA salivary levels in group of participants that smoked cigarettes, contributing to increased levels of free radical–mediated oxidative damage of lipids, supporting the hypothesis that oxidative damage in smokers is due to their active exposure directly to saliva.

Fig 1. show the results evaluated for total antioxidant capacity of saliva and lipid peroxidation biomarkers for evaluating oxidative stress.

Figure 2 presents the statistical correlation between every mathematical parameter which represents the total

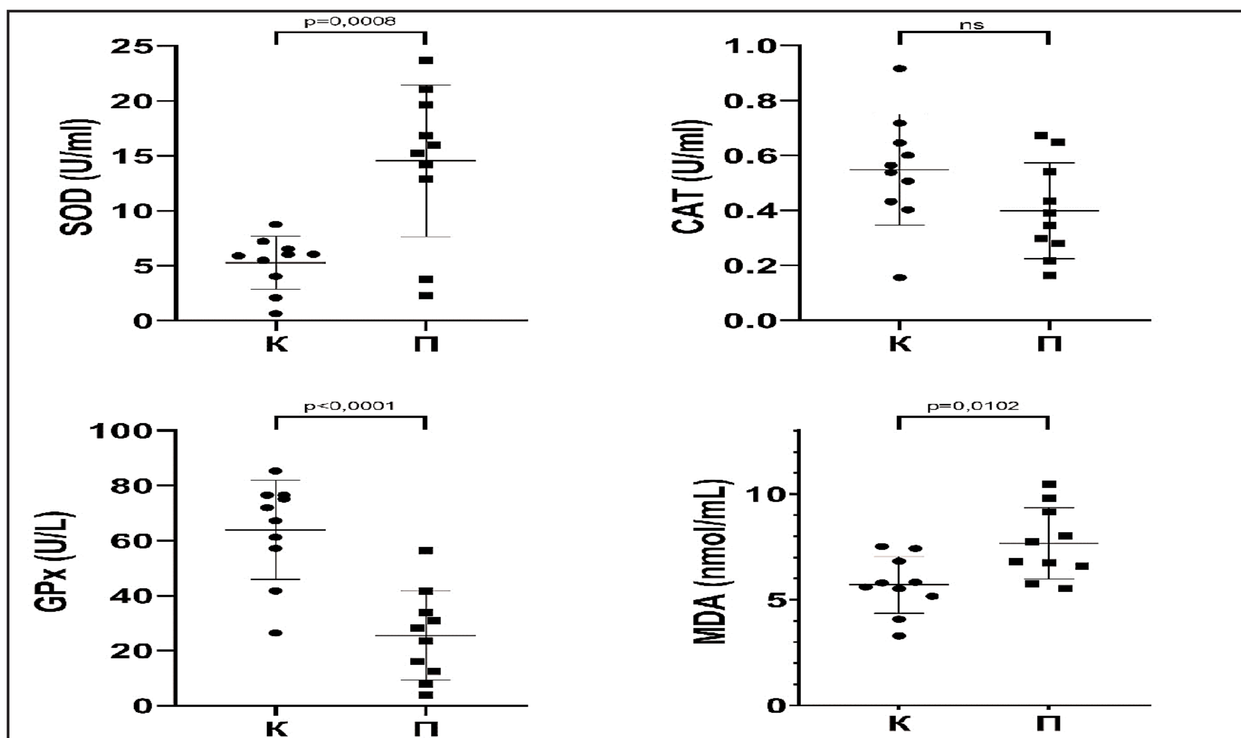

Figure 1. Salivary total antioxidant capacity and oxidative stress in smokers and non-smokers. (mean ± SD; К – non-smokers, П – smokers; SOD, CAT, GPx, MDA).

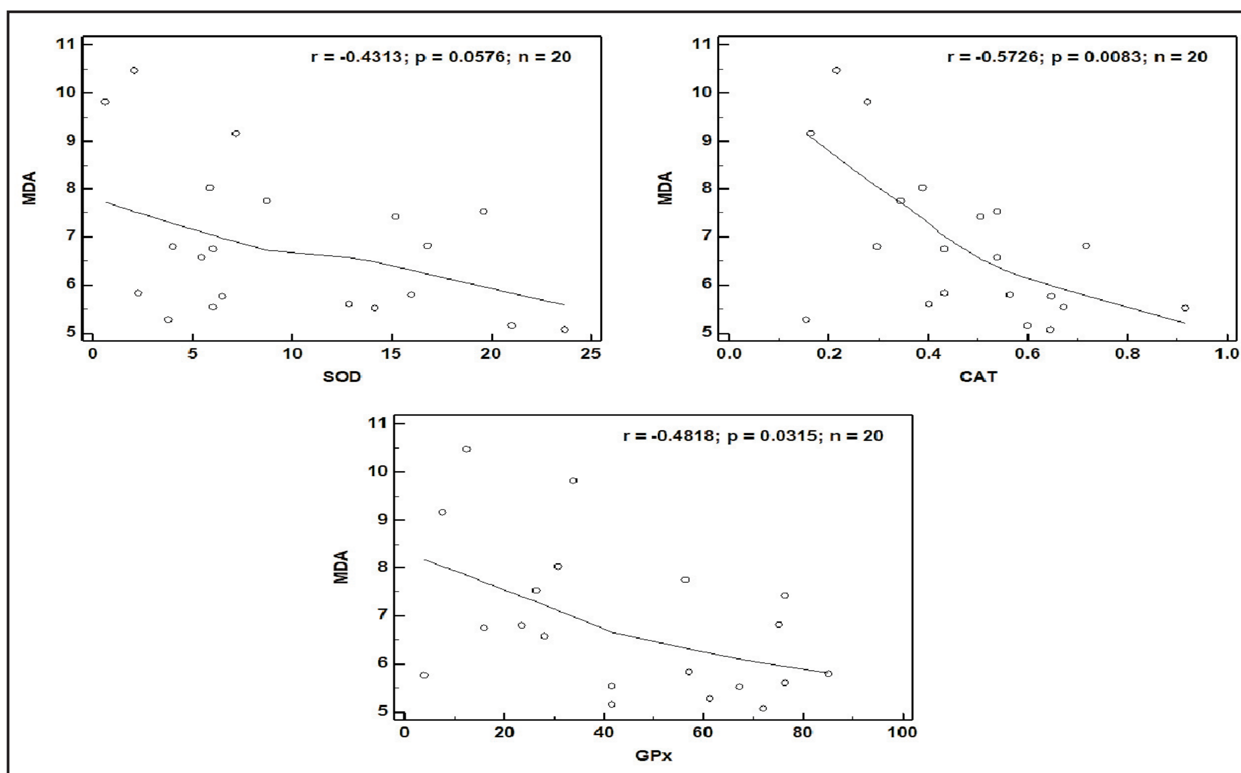

Figure 2. Correlation between parameters that explains the oxidative stress level in smokers and non-smokers group. (mean ± SD; К – non-smokers, P – smokers; SOD, CAT, GPx, MDA)

oxidative status, and lipid peroxidation biomarker for oxidative stress in both groups.

Discussion

Cigarette consumption is connected with many diseases including malignant neoplasms in the oral cavity or the mouth, causing and showing some imbalance between antioxidants in saliva[1-5, 18, 20]. We also know that tobacco contains more than 4500 toxic substances which can cause programmed cell death, related with necrosis and structural mitochondrial defects, especially in airway cells called A549[15-19]. The harmful effects of smoking results in production or clearance of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px), and malondialdehyde (MDA) as a lipid peroxidation marker[18, 20]. All these products of reactive oxygen species and its effects could be beneficial for monitoring and progression of some oral diseases, including primary oral squamous cell carcinoma. The primary objective of this study was to evaluate the total antioxidative capacity, and the salivary markers of oxidative stress in oral diseases in two health groups (smokers and non-smokers, participants without any disease or cardiovascular issue). Also, sample of saliva was taken with methods for unstimulated saliva because several studies show that the activity of antioxidant enzymes or total antioxidative capacity in unstimulated saliva is higher compared with others group.

In our study, we observed increased levels of SOD saliva in tobacco users compared with non-smokers, and significant decreased GPx activity in the same group. The reviewed literature indicates that smokers are more likely to suffer from progressive elevation in SOD[17-21]. The increased SOD levels in healthy smokers-participants, measured in the present study, showed us that the salivary MDA levels (as an indicator of the degree of lipid peroxidation in the saliva of smokers), at baseline, were significantly higher for the same group. Similarly, we observed negative correlation between SOD and MDA levels among participants, resulting in a pathway progress of cells with tissue destruction by oxidative stress. Our findings are in agreement with Balasubramaniam A and Arumugham MI[15], V Sosa[16], Maciejczyk M[17], Vo TTT[18], Forni C et al.[19] In this research, MDA levels were found to be increased with overproduction of oxidants, leading to oxidative imbalance, and decreased results for GPx, which can be attributed to the different ingredients in cigarettes. In another study, Dhama K et al.[20] explained that various interactive species outcomes with arterial and venous metabolic circulation stress. Research in these field should give us accurate results for identification of potent biological marker, objectively measured and evaluated for different kind of mouth disease, including oral squamous cell carcinoma.

Conclusions

Cigarette smoking is associated with increased lipid peroxidation, generating free radicals in the mouth that are responsible for imbalance and oxidative stress. Furthermore, our results suggest that the collection and analysis of saliva samples may be an alternative tool for further analysis of the role of antioxidants and oxidative stress in more specific mouth disorders, such as oral squamous cell carcinoma, understanding its relation and disturbance between free radicals produced, and the capability of the antioxidant system.

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