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D. DARCZUK[1], W. KRZYSCIAK[2], P. VYHOUSKAYA[3], B. KESEK[1], D. GALECKA-WANATOWICZ[1], W. LIPSKA[1], T. KACZMARZYK[4], M. GLUCH-LUTWIN[5], B. MORDYL[5], M. CHOMYSZYN-GAJEWSKA[1]

SALIVARY OXIDATIVE STATUS IN PATIENTS WITH ORAL LICHEN PLANUS

[1]Department of Periodontology and Oral Medicine, Jagiellonian University Medical College, Cracow, Poland;
[2]Department of Medical Diagnostics, Faculty of Pharmacy, Jagiellonian University Medical College, Cracow, Poland;
[3]Student Scientific Association at the Department of Medical Diagnostics, Jagiellonian University Medical College, Poland;
[4]Department of Oral Surgery, Jagiellonian University Medical College, Cracow, Poland;
[5]Department of Pharmacobiology, Faculty of Pharmacy, Jagiellonian University Medical College, Cracow, Poland

Reactive oxygen species (ROS) are involved in the pathogenesis of many inflammatory diseases, including oral lichen planus. Therefore, determining the salivary markers of oxidative stress is an excellent alternative approach to diagnosing oral cavity diseases. The objective of our study was to provide preliminary validation and determination of the salivary markers of oxidative stress in both patients with reticular and erosive forms of oral lichen planus as well as in healthy individuals without any oral lesions. In total, 62 patients with oral lichen planus (OLP) were enrolled in the study, including 31 with the reticular form of lichen planus (44.63 ± 11.05 years) and 31 with erosive forms (40.43 ± 10.05 years), who had never been treated for their disease. The control group comprised 30 individuals without any oral lesions (42.12 ± 12.22 years). We determined the saliva levels in glutathione (GSH), total antioxidant capacity (TAC), and thiobarbituric acid reactive substances (TBARS). The mean saliva levels of GSH and TAC were significantly lower ($P < 0.01$) in OLP patients compared to the control group. The mean levels of salivary TBARS were higher in both OLP groups (reticular and erosive) compared to the control group ($P = 0.01$). The lower saliva levels of GSH and TAC in patients with OLP indicate that free radicals and the resulting oxidative damage may play an important role in the pathogenesis of OLP lesions. In conclusion, monitoring the oxidant-antioxidant status of saliva may serve as an efficient and less intrusive marker for determining stages of disease development in patients with OLP.

Key words: *antioxidants, glutathione, oral lichen planus, saliva, oxidative markers, reactive oxygen species, total antioxidant capacity, thiobarbituric acid reactive substances*

INTRODUCTION

Oral lichen planus (OLP) is a chronic disease of the mucous membrane in the oral cavity. It occurs in approximately 0.1 – 4% of the adult population (1), and is slightly more common in women than in men (2). In approximately half of all cases, changes in the oral cavity may be accompanied by lichen planus lesions on the skin, nails and genitals. Clinically, during the course of the disease, a lace-like network of slightly raised grey-white lines (Wickham's striae) can be discerned on the oral mucosa, sometimes accompanied by erosions, ulcerations, blisters or atrophy (2). OLP lesions are usually located bilaterally in a more or less symmetrical pattern. Most often, i.e. in 80 – 90% of cases, changes are located on the buccal mucosa (2).

The histological criteria for OLP include the presence of a well-defined band-like zone of cellular infiltration in the lamina propria and signs of necrolysis in the basal cell layer (3). The etiopathogenesis of OLP is not fully understood. The role of specific and non-specific immune mechanisms is pronounced, a fact which characterizes the disease as a T-lymphocytic inflammatory response to the antigen located in the keratinocytes of the basal layer of the epithelium, which in turn leads to their apoptosis (1, 4).

Recently, researchers have focused on the role of oxidative stress in the pathomechanism of OLP (5). Enzymatic and non-enzymatic anti-oxidants act as a physiological barrier against reactive oxygen species (ROS) and cytokine-mediated toxicity (6). As a result of the prolonged release of ROS, the level of antioxidants decreases during cases of chronic inflammation and carcinogenesis (7). Oxidative stress participates in the pathogenesis of numerous diseases, e.g. ischemic stroke, Parkinson's disease, some skin diseases, and tumors (8, 9). In the case of the oral cavity, it may possibly play a role in recurrent aphtous stomatitis (RAS) and periodontitis (10, 11). Some observations suggest that oxidative stress may also be involved in the etiology of OLP, as it is dependent on T lymphocytes (12).

As a consequence of oxidative stress, OLP is accompanied by, inter alia, increased lipid peroxidation, which is expressed in higher concentrations of its products, such as malondialdehyde (MDA) belonging to the thiobarbituric acid reactive substances group (TBARS) and 4-hydroxy-2-nonenal (4-HNE) (13). The role of these compounds in OLP is not fully understood, and recently their participation has been shown to consist in a signal

transfer, inter alia in apoptosis, *via* their influence on the way Bcl-2 and Bax are linked, and also in the role played by these compounds in mitochondrial regulation and regulation dependent on CD8+ lymphocytes, which in turn occurs through their influence on nuclear factor kappa B activity (NF-κB) (14, 15).

Thus, oxidative stress may constitute a significant pathogenetic mechanism in OLP and its complications. Its harmful role stems from its influence on the basic mechanisms of signal transfer and transduction, resulting in the dysfunction of keratinocytes and their impaired apoptosis (16). Antioxidant therapy strategies aimed at protecting keratinocytes against damage, independent of disease progression, can be based on knowledge of the molecular aspects of oxidative stress in numerous diseases, including OLP (17). Some researchers suggest that oral use of antioxidants or antioxidant medications may successfully inhibit increased oxidative stress, and thus may help achieve clinical improvement (18).

So far, only a few studies have been published assessing oxidant-antioxidant status in patients with selected clinical forms of OLP in comparison with healthy individuals without symptoms of the disease (12, 13). Furthermore, although the role of oxidative stress in patients with OLP has often been documented, the majority of published studies do not provide any validation of the methods used in different biological materials. This issue should be reconsidered in laboratory conditions. The selected oxidant-antioxidant markers used to monitor oxidative stress is undisputed, and it appears to assume different aspects in different forms of the disease, particularly those with a varied clinical picture.

It should be pointed out that saliva samples are widely available for collection and further diagnosis and constitute a potential future tool for screening tests, not only for diseases of oral cavity. Cavity but also in systemic conditions, due to the documented correlation observed between salivary markers and the clinical state of a patient and the severity of the disease (19, 20).

The objective of this study was to provide preliminary validation and determination of salivary markers of oxidative stress, together with total salivary protein levels, in patients with reticular and erosive forms of OLP as well as in healthy individuals without any oral lesions.

MATERIALS AND METHODS

Study groups

The study was performed in accordance with the Helsinki Declaration of 2008. All the participants gave their informed consent for the study. The protocol was approved by the Bioethics Committee of the Jagiellonian University in Cracow (No. KB/112/b/2014).

The participants were recruited from among OLP patients treated at the University Dental Clinic in Cracow, Poland. The diagnosis was made by a dentist who enrolled participants on the basis of their medical and dental histories, and this diagnosis was confirmed by a histopathological examination.

In total, 92 patients were enrolled in the study. In accordance with the clinically and histopathologically modified WHO diagnostic criteria of OLP (21), 62 patients with OLP were divided into a reticular oral lichen planus (RLP) group and an erosive oral lichen planus (ELP) group. A specialist examined the patients for clinical signs and symptoms.

Thirty-one patients, in whom distinctive white lacy lines surrounded by an erythematous border, usually asymptomatic, were observed, were qualified for the RLP group (16 women, 15 men; aged 31 – 62 years). In the majority of cases, the lesions were located on the mucous membrane of the cheeks (80%), and less often on the tongue (13%) or the gums (7%).

Thirty-one patients qualified for the ELP group (16 women, 15 men; aged 31 – 62 years). The selection criterion for the patients with the erosive form of OLP was the presence of erosions or ulcerations within erythematous changes. These lesions were located in the mucous membrane of the cheeks (50%), edges of the tongue (17%) or simultaneously in both sites (33%).

A group of 30 healthy volunteers (20 women, 10 men; aged 30 – 57) served as the control group.

The exclusion criteria were as follows: aged below 18 or above 80 years, pregnancy, diabetes, cigarette smoking, periodontal disease, caries, epithelial dysplasia and inflammatory lesions of the oral mucosa. Antibiotics, non-steroid, anti-inflammatory medications, corticosteroids, and vitamin intake within the last 3 months also resulted in a patient's exclusion from the study.

Saliva preparation for the oxidant-antioxidant tests

Unstimulated saliva samples (4 ml) were collected in sterile plastic tubes using the Salivette® Cotton Swab system (Sarstedt, Nümbrecht, Germany). The biological material was collected in the morning hours, between 9:00 and 11:00 AM. The subjects rinsed their mouths with tap water for 30 s and expectorated it before the saliva was collected. The samples were taken before any treatment was undertaken that might have led to bias in the results. The exclusion criteria included diseases affecting the quality and quantity of the saliva, such as asthma, enteritis, arthritis, Sjogren syndrome, and sinusitis, as well as the use of certain medications. In accordance with the study protocol, the patients did not eat, drink, brush their teeth, smoke or chew gum for a minimum of 2 hours prior to the sampling.

The samples were placed in ice and transported to the laboratory for further processing within a period of no more than 2 hours.

The saliva was centrifuged at 900 g for 10 minutes at a temperature of 4°C. Then the entire filtrate was transferred to sterile 1.5 ml micro test-tubes (Eppendorf type) and frozen at –80°C until analysis.

To determine TBARS levels, the saliva was transferred to 1.5 ml micro test-tubes by adding a 15 µl 0.15 mol/l solution of butylated hydroxytoluene (BHT) in ethanol per 1 ml of saliva so as to prevent lipid peroxidation during sample storage (22). The contents of the test tubes were shaken and stored at a temperature of –80°C until the TBARS determinations were performed. The determinations were performed within one month of material collection.

After collecting 90 saliva samples, one of each copy of the material divided into portions was defrosted and analyzed using the control materials.

To validate the individual methods, cumulative saliva was used for both the study group and the control groups.

Thiobarbituric acid reactive substances (TBARS)

TBARS was determined on the basis of the Aust method (23) as outlined in the *Current Protocols in Toxicology* (24), combined with the Gutteridge modification (25).

Saliva was used in the experiment. The following Sigma reagents (Sigma Aldrich, USA) were employed: 3.75% trichloroacetic acid (TCA), 0.025 mol/l hydrochloric acid (HCl), 0.0925% thiobarbituric acid (TBA) and 0.03% butylated hydroxytoluene (BHT). The working solution was obtained by dissolving the stock reagent, TBA/TCA/HCl in water (on the day of determination), thereby obtaining 3.5 ml TBA/TCA/HCl + 10.5 ml H_2O + 0.21 ml BHT. 1,1,3,3-tetramethoxypropane was

used as the standard, which hydrolyzes in an acidic environment at a stoichiometric ratio to TBARS. Hydrolysis was conducted in 0.05 mol/l hydrochloric acid at room temperature for 10 minutes, then standard solutions of 1,1,3,3-tetramethoxypropane were prepared in a range of 10 – 50 nmol/ml. The solution of the studied sample was mixed with the working reagent at a 1:1 ratio (v/v). The ratio of the studied sample to the working solution was 150 µl to 150 µl. The contents of the test-tubes were mixed for 10 seconds with the aid of a microshaker, and then heated in a boiling water bath for 15 minutes. After this, the test-tubes were instantly cooled and remained in ice for 10 minutes. The test-tubes were centrifuged for 10 minutes at 4000 × g at room temperature. Then, 250 µl of the organic-butanol layer was carefully transferred to the wells of a black, 96-well plate (OptiPlate-96F Black, Perkin Elmer). Fluorimetric measurements were taken at an excitation wavelength (Ex) of 536 nm and an emission wavelength (Em) of 549 nm. Readings of the results were taken with a FLUOstar Omega spectrophotometer (BMG Labtech, Germany) after 10 minutes.

Total antioxidant capacity (TAC)

The TAC of saliva for reducing Fe^{3+} ions was determined using the Benzie and Strain method (26). The TAC of saliva was determined by measuring its capacity to reduce Fe^{3+} ions to Fe^{2+} ions, which in the presence of tripyridyl triazine (TPTZ) results in the formation of an Fe^{2+}-TPTZ complex with an intense, blue color.

Saliva was used in the experiment together with the following reagents from Sigma-Aldrich, USA: 100 ml of 0.3 mol/l acetate buffer at pH = 3.6, 10 ml of TPTZ 0.01 mol/l dissolved in 10 ml 0.04 mol/l of an HCl solution at a temperature of 70°C, and 10 ml of ferric chloride (III) 0.02 mol/l $FeCl_3 \cdot 6H_2O$. Iron sulfate (II) 0.001 mol/l in concentrations: 0.1 – 1.0 mmol/l was adopted as the standard. The working solution was prepared on the day of determination, and this contained 100 ml of acetate buffer, a 10 ml TPTZ solution, and 10 ml of ferric chloride (III). The wells of the 96-well plate were filled in the following order: 15 µl of the studied sample, a blank test with distilled water or the standard solution. Then, 300 µl of the working substrate solution was added and the contents were mixed. Absorbance was measured at a temperature of 37°C and at a wavelength of 593 nm. The results were read after 10 minutes on a FLUOstar Omega spectrophotometer (BMG Labtech, Germany).

Total glutathione (GSH)

The GSH, i.e. total glutathione in the saliva, was determined using the Ellman method (27). Total glutathione GSH in the saliva was measured spectrophotometrically, based on the reaction of thiols with the chromogenic DTNB (5,5'-dithiobis-2-nitrobenzoic acid), whereby the formation of the yellow dianion of 5-thio-2-nitrobenzoic acid (TNB) is measured (28, 29).

The following reagents from Sigma-Aldrich, USA, were employed: 100 ml of phosphate buffer (NaH_2PO_4) 0.2 mol/l at pH = 8.2, 10 ml of DTNB 10 mmol/l dissolved in 100 ml of phosphate buffer, 0.2 mol/l at pH = 8.2, and 10 ml of trichloroacetic acid (TCA) 2.5%. GSH 0.001 mol/l in concentrations of 10 – 100 µmol/l was used as the standard. The working solution was prepared on the day of determination, and it contained a 150 µl 0.2 mol/l phosphate buffer at pH = 8.2; 40 µl 10 mmol/l DTNB and 110 µl supernatant (deproteinized saliva). For deproteinizing purposes, 30 µl of TCA was added to 90 µl of saliva, shaken in a vortex and centrifuged for 5 minutes at 5000 rpm. The supernatant was transferred to the wells of a 96-well plate, placed in ice, with the stopwatch being switched off when the supernatant was being added. A 2.5% TCA was used to obtain blank tests. Absorbance at the wavelength λ = 412 with regard to the blank test was read precisely 5 minutes after introducing the supernatant. Absorbance was measured at room temperature. The readings were made on a FLUOstar Omega spectrophotometer (BMG Labtech, Germany).

Total protein levels

Total protein (TP) levels were determined in both saliva samples (TP-SA). The protein concentrations of the samples were determined on the basis of the bicinchonic acid (BCA) method in accordance with the manufacturer's (Sigma-Aldrich, USA) instructions, with bovine serum albumin (BSA) adopted as the standard. The principle behind the BCA method entails reducing Cu^{2+} to Cu^{+}, whereby Cu^{+} ions then react in an alkaline medium (in which the emerging complex is stable) with bicinchonic acid (BCA), which gives it a violet coloring. Absorbance is measured at a wavelength of λ_{max} = 562 nm. Compared to the Lowry method, which is based on a similar principle, the bicinchonic acid method is less dependent on the amino acid composition and fewer interfering substances such as urea and guanidine chloride affect the measurement. The working solution was prepared by mixing reagents A (BCA, Na_2CO_3, and sodium tartrate $Na_2C_4H_4O_6$ in 0.1 M NaOH) and B (4% $CuSO_4 \cdot 5H_2O$) at a ratio of 50:1 (19 ml A + 0.38 ml B) until a green color appeared. A standard solution of bovine serum albumin BSA in a concentration of 1.0 mg/ml (in 0.15 M NaCl with 0.05% of sodium azide NaN_3 added as a preservative) was dissolved in a solution of 0.15 NaCl with the aim of achieving different concentrations of BSA within the following range: 200 – 1000 mg/ml. An assay was made for standards, blind samples and study samples. With this aim in mind, a 25 µl standard solution/test solution (saliva) or a 0.15 M NaCl solution was mixed as a blind sample together with a 200 µl working solution. The blind sample was prepared for each series in 10 repetitions. After adding all the reagents, the plate was sealed with protective film in order to eliminate the evaporation of reagents. The reagents were incubated for 30 minutes at a temperature of 37°C. Then the plates were cooled to room temperature (over a time not exceeding 1 hour). The absorbance of each study sample was determined at a wave length of λ_{max} = 562 nm at a temperature of 37°C. A calibration curve was plotted and a simple linear regression equation determined. The concentration of protein was calculated on this basis and expressed in mg/ml.

Method validation

To confirm the validity of the methods for determining TBARS, GSH and TAC in saliva, validation was performed according to European standardization guidelines (30, 31).

To evaluate calibration, 8 scales of standards were prepared and variability coefficients were determined for each concentration. Differences in precision regarding extreme concentrations in Snedecor's F test were not significant. The determined correlation coefficients (r) amounted to between 0.99 and 0.99 and these were higher than the assumed limit value $r \geq 0.99$. The correlation coefficient r differed significantly from zero. Linearity characterized the entire range of the calibration curve. The slope coefficients (indicating the sensitivity of the method) were statistically significant.

To evaluate absorbance, fluorescence scatter around the standard curve was determined together with residual standard deviations. The standard deviations of the Sm method, which show the precision of the calibration, were satisfactory and amounted to 0.00377 (for TAC) and 0.551 (for GSH). The calibration variability coefficients (0.773 – 1.54%) were lower than the limit value of 3 – 5%.

Table 1. Validation parameters for colorimetric determination of TBARS with 2-thiobarbituric acid, spectrophotometric determination of total antioxidant capacity expressed as TAC and spectrophotometric determination expressed as total glutathione GSH in saliva.

	TBARS	**TAC**	**GSH**
Coefficients of variation, %	11.7 – 17.6	2.3 – 8.4	2.3 – 9.3
Correlation coefficient *r*	0.99	0.99	0.99
Slope factor	2040	0.868	0.00386
Residual standard deviation *Sy*	943.6	0.00327	0.00213
Standard deviation of the method *Sm*	0.00463	0.00377	0.551
Coefficient of variation of calibration, %	1.54	0.773	1.19
Limit of detection *Ld*	0.482 mmol/ml	0.01182 mmol/l	1.734 µmol/l
Limit of quantification *Lq*	1.608 mmol/ml	0.03940 mmol/l	5.779 µmol/l
Repeatability, %	5.6 – 13	2.3 – 15.3	3.7 – 9.3
Average recovery factor for the entire range, %	60.5	82.6	105
The average recovery factor for the different	54.2 – 64.8	69.9 – 90.0	99.5 – 108
Average precision of recovery for the range	9.06	9.25	7.26
Average relative error of recovery	39.5	17.4	4.76

The detection limit and the qualification limit were both determined (calculated in accordance with the IUPAC recommendations) (34-36). The repeatabilities for four concentration levels amounted to between 2.3% and 15.3%, whereas the mean recovery coefficients ranged from 60.5% to 105% (from 54.2% to 108% for individual ranges). The compatibility of the parameters obtained *via* the validation process with the desired values confirmed the validity of the methods used in the present study. All the validation parameters are presented in *Table 1*.

Statistical analysis

The statistical evaluation of the results was based on regression equations determined for 8 standard series, with the result expressed in nM/mg protein for TBARS, mM/mg protein for TAC, and µM/mg protein for GSH. The descriptive statistics were presented and a compatibility analysis was performed. The results were expressed as median ± upper and lower quartiles. Since TBARS, TAC and GSH concentration levels were not normally distributed, statistical comparisons were made between groups using the Kruskal-Wallis test. A value of $P < 0.05$ was considered statistically significant. Superscripts a, b, c denote groups differing in Bonferroni-corrected Mann-Whitney tests. An analysis of covariance (ANCOVA) was performed to assess the connections between TBARS, TAC and GSH on the one hand and the forms of the disease on the other, and was corrected to take into account the effect of confounding factors (age, sex).

The statistical analysis was performed using R 3.2.3. (R: Development Core Team, 2009; A language and environment for statistical computing; Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.).

RESULTS

Study group characteristics

The RLP group consisted of 16 women and 15 men (with a mean age of 44.63 ± 11.05), whereas the ELP group comprised 16 women and 15 men (with a mean age of 40.43 ± 10.05). The control group included 20 women and 10 men (with a mean age of 42.12 ± 12.22). The ages of the patients in the groups with OLP did not differ significantly from the ages of the individuals in the control group. There were no significant differences in terms of sex between the study and control groups.

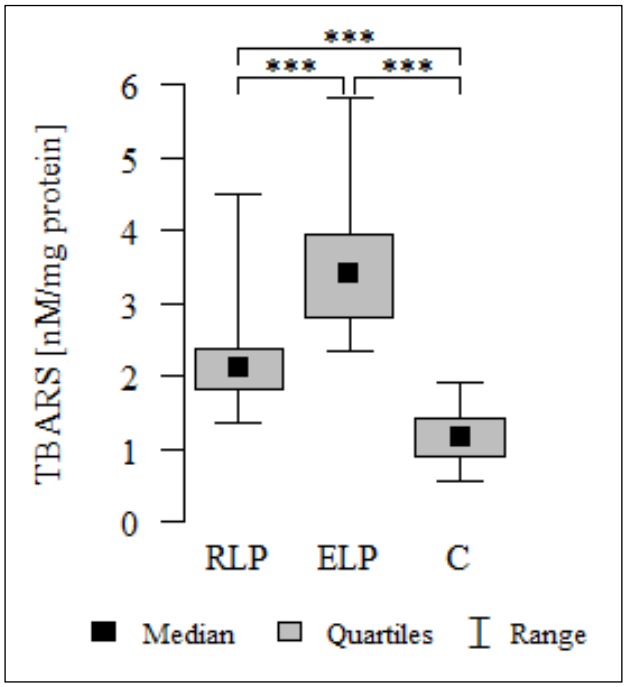

Fig. 1. Concentration of TBARS in the saliva of both patient groups with oral lichen planus and controls. The median and interquartile range (box), and percentile 5 – 95% range (whiskers) are shown. The dashed lines denote reference intervals in the saliva; P-values are given for inter-group comparisons (***$P < 0.01$ *post-hoc* Mann-Whitney test with Bonferroni correction). RLP, reticular patients with oral lichen planus; ELP, erosive patients with oral lichen planus; C, control without lesions.

TBARS, TAC, GSH and protein concentrations in the saliva

Inter-group differences in TBARS concentrations in saliva were statistically significant, i.e. they were higher in the RLP and ELP groups than in the control group (*Table 2, 5*). Inter-group differences in TAC and GSH concentrations in saliva were statistically significant, i.e. they were higher in the control group than in the RLP and ELP groups (*Tables 3-5*). The TBARS, TAC and GSH concentrations varied between the groups (patients with RLP and ELP) (*Figs. 1-3*).

The highest TBARS concentrations were recorded in the saliva of patients with ELP median (lower quartile - upper quartile) at 3.42 (2.41 – 5.18) nM/mg of protein (*Fig. 1*), whereas

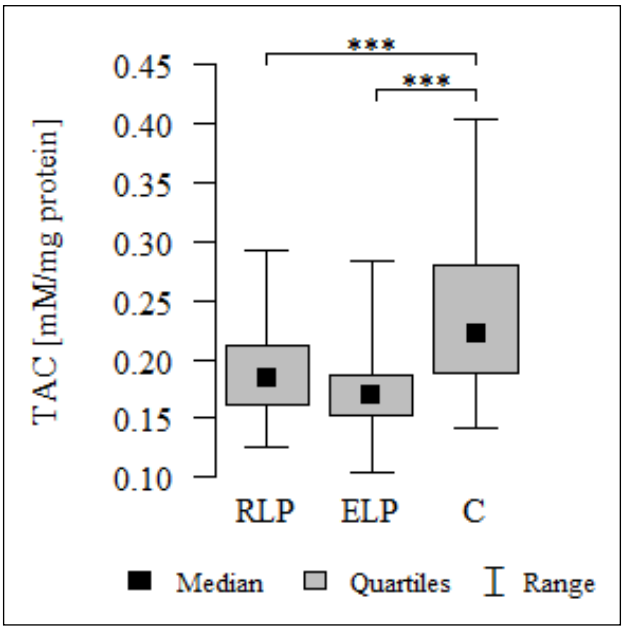

Fig. 2. Concentration of TAC in the saliva of both patient groups with oral lichen planus and controls. The median and interquartile range (box), and percentile 5 – 95% range (whiskers) are shown. The dashed lines denote reference intervals in the saliva; P-values are given for inter-group comparisons (***P < 0.01 *post-hoc* Mann-Whitney test with Bonferroni correction). RLP, reticular patients with oral lichen planus; ELP, erosive patients with oral lichen planus; C, control without lesions.

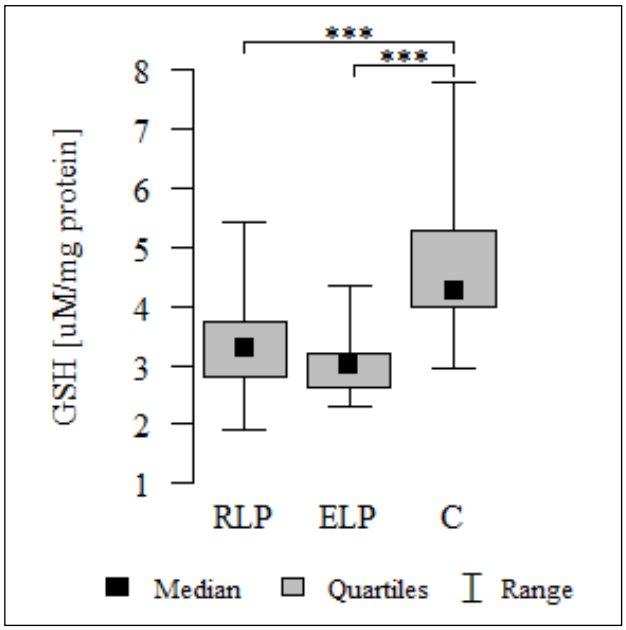

Fig. 3. Concentration of GSH in the saliva of both patient groups with oral lichen planus and controls. The median and interquartile range (box), and percentile 5 – 95% range (whiskers) are shown. The dashed lines denote reference intervals in the saliva; P-values are given for inter-group comparisons (***P < 0.01 *post-hoc* Mann-Whitney test with Bonferroni correction). RLP, reticular patients with oral lichen planus; ELP, erosive patients with oral lichen planus; C, control without lesions.

TAC and GSH concentrations were the lowest in this patient group: 0.17 (0.14 – 0.24) mM/mg protein for TAC (*Fig. 2*) and 3.0 (2.42 – 4.0) µM/mg protein for GSH, respectively (*Tables 3* and *4*; *Fig. 3*). The differences were statistically significant in the case of the above-mentioned parameters (P < 0.01) (*Table 5*).

Inter-group differences in protein concentrations in saliva were statistically significant, i.e. 1.88 (1.36 – 2.32) mg/ml in RLP and 1.70 (1.40 – 1.92) mg/ml in ELP, and thus lower in these groups than in the control group 1.93 (1.51 – 2.44) mg/ml.

There was no significant correlation between concentrations of the studied markers on the one hand, and the age and gender of the study participants on the other. The linear regression model showed that age and gender did not affect the TAC, TBARS and GSH (age variable P = 0.87 and gender variable P = 0.97 for TAC value; age variable P = 0.74 and gender variable P = 0.40 for TBARS value; age variable P = 0.88 and gender variable 0.59 for GSH; multifactorial ANOVA).

DISCUSSION

Although the OLP pathomechanism is not entirely understood and the etiology of the disease is unknown, the oxidative stress accompanying numerous diseases is believed to play a significant role (37-38).

The study showed considerable differences (P < 0.05) between the mean concentrations of total antioxidant capacity expressed as TAC in patients with oral lichen planus compared to the control group. They were 21% lower in the case of the reticular form and about 34% lower in the erosive group. Similarly, in the case of GSH the average concentrations in patients with oral lichen planus were 31% and 41% (the reticular and erosive groups) lower, respectively, than in the control group (P < 0.05).

With regard to thiobarbituric acid reactive substances (TBARS), a statistically significant difference (P < 0.05) was observed between all three tested groups. The results for the reticular form were 86% higher in relation to the control group, and 166% higher in the case of the erosive form.

The erosive form, which has a potential capacity for malignant transformation, is characterized by more intense inflammation and pain than the reticular form. Histopathological studies confirmed more advanced pathological changes in patients with E-OLP (39). The low concentration of antioxidant defense markers and high levels of TBARS indicate increased severity of oxidative stress in tested lichen planus patients, particularly those with the erosive form of the disease.

After reviewing the severity of oxidative stress in patients with OLP in the available literature, we tried to compare the results we obtained with the results of other researchers.

Our results were consistent with those from earlier studies (40, 41). Statistically significant differences were observed for all three parameters between the different groups of patients and the healthy subjects. A similar relationship was observed not only in the case of saliva, but also for serum and tissue homogenate (42).

A study by Ergun *et al.* revealed no statistically significant differences between the average concentration of TBARS in serum in patients with OLP and the control group (3.00 ± 0.92 versus 2.68 ± 0.60 nmol / ml) (43). A lack of any published studies on the concentration of TBARS in various forms of lichen planus prevents any comparison of the results.

Studies conducted by Shirzad *et al.* (12) and Lopez-Jornet *et al.* (9) strongly emphasize the role played by oxidative stress and lipid peroxidation products, among others TBARS, in tissue destruction in OLP. These authors devote considerable attention to the practical aspect of studies and the preventive role

Table 2. Average TBARS concentrations in oral lichen planus - results and review of the study.

TBARS concentration nM/ml mean ± S.D. (increase compared to control group)			Significance level of average differences	Material	Method	Source
Control Group	RLP	ELP				
2.18 ± 0.48 nM/ml	4.07 ± 0.61 (86%)	5.81 ± 0.98 (166%)	P < 0.01	Saliva	Fluorimetric - Buge & Aust (50)	Our study
1.18 ± 0.36 nM/mg of protein	2.27 ± 0.71 (92%)	3.49 ± 0.87 (195%)	P < 0.01	Saliva	Fluorimetric - Buge & Aust (50)	Our study
1.5 ± 0.1 nM/ml	2.49 ± 0.30 (66%)		P < 0.01	Saliva	Spectrophotometric - Esterbauer (51)	(9)
0.15 ± 0.11 nM/ml		0.49 ± 0.30 (227%)	P < 0.01	Saliva	Spectrophotometric - Buge & Aust (50)	(12)
0.44 (0.19 – 0.70) nM/mg of protein	2.67 (0.26 – 3.40) (507%)		P < 0.01	Tissues	Fluorimetric - Conti (52)	(40)
0.219 ± 0.054 nM/ml	0.760 ± 0.536 (247%)		P < 0.05	Serum	Spectrophotometric - Esterbauer (51)	(5)
3.08 ± 0.98 nM/ml	30.16 ± 4.6 (879%)		P < 0.01	Serum	Spectrophotometric - Cayman Kit	(53)
0.25 ± 0.04 nM/mg białka	0.31 ± 0.025 (24%)		P < 0.05	Saliva	Spectrophotometric - Biosystems Kit	(54)
3.42 ± 1.44 nM/ml	5.59 ± 2.05 (63%)		P < 0.01	Saliva	Spectrophotometric Esterbauer (51)	(13)
1.47 ± 0.37 nM/ml	2.03 ± 0.81 (38%)		P < 0.05	Saliva	Fluorimetric	(41)
2.68 ± 0.60 nM/ml	3.00 ± 0.92 (12%)		P > 0.05	Serum		

Table 3. Average GSH concentrations in oral lichen planus - results and review of the study.

GSH concentration nM/ml Mean ± S.D. (decrease, compared to control group)			Signifi-cance of average differences	Material	Method	Source
Control Group	RLP	ELP				
8.58 ± 0.60 nM/ml	5.94 ± 0.50 (31%)	5.06 ± 0.37 (41%)	P < 0.01	Saliva	Spectrophotometric - Ellman (55)	Our study
9.56 (6.5 – 12.5) nM/ml	2.3 (1.25 – 5.70) (76%)		P < 0.01	Tissues	Spectrophotometric - Ellman (55)	(40)
472.13 ± 54.27 nM/ml	378.26 ± 1.50 (20%)		P < 0.05	Serum	Spectrophotometric - Ellman (55)	(5)

performed by oxidant-antioxidant status monitoring. One hypothesis suggests a connection between the pathophysiology of OLP and inflammatory processes, which activate inflammatory cells so as to release ROS. On the other hand, lichen planus is a delayed form of sensitivity reaction, during which T lymphocytes are activated and pro-inflammatory cytokines released. These cytokines stimulate keratinocytes to increase ROS production (44). Thus, our study demonstrated

Table 4. Average TAC concentrations in oral lichen planus - results and review of the study.

TAC concentration µM Mean ± S.D. (decrease, compared to control group)			Signifi-cance of average differences	Material	Method	Source
Control Group	RLP	ELP				
440.97 ± 63.85 µM	350.19 ± 92.14 (21%)	292.6 ± 35.64 (34%)	P < 0.01	Saliva	Spectrophotometric - Benzie (56)	Our study
241.036 ± 0.074 µM/mg of protein	192.252 ± 0.04 0 (20%)	174.643 ± 0.03 3 (28%)	P < 0.01	Saliva	Spectrophotometric - Benzie (56)	Our study
1.17 ± 0.07 µM	1.09 ± 0.06 (7%)		P < 0.01	Serum	Spectrophotometric - Cayman Kit	(53)
1240.0 ± 0.16 µM/mg of protein	800.0 ± 0.15 (35%)		P < 0.05	Saliva	Spectrophotometric - Biosystems Kit	(54)
205.0 ± 0.02 µM	136.0 ± 0.01 (34%)		P < 0.01	Saliva	Spectrophotometric - Benzie (54)	(13)
791.43 ± 183.95 µM		297.23 ± 149.72 (63%)	P < 0.01	Saliva	Spectrophotometric - Benzie (56)	(12)
591.43 ± 103.95 µM	567.23 ± 89.72 (4%)		P < 0.05	Saliva	Spectrophotometric - Benzie (56)	(9)
2037.0 ± 0.1382 µM	1054.0 ± 0.3013 (48%)		P < 0.05	Serum	Spectrophotometric - Koracevic (57)	(5)
586.00 ± 104.79 µM	561.86 ± 71.41 (4%)		P < 0.05	Saliva	Spectrophotometric - Benzie (56)	(41)
796.10 ± 153.07 µM	716.19 ± 77.66 (10%)		P < 0.05	Serum		

Table 5. Adjusted ratios of reduced glutathione, total antioxidant capacity and malondialdehyde. Data represent medians. Superscripts a, b, c denote groups differing in Mann-Whitney tests with Bonferroni correction. No statistically significant differences were observed in groups with the same letter. GSH, reduced glutathione; TAC, total antioxidant capacity; TBARS, thiobarbituric acid reactive substances. *P-value obtained by Kruskal-Wallis test adjusted for age, and gender.

	GSH (µM)	P*	post-hoc**	TAC (mM)	P*	post-hoc**	TBARS (nM)	P*	post-hoc**
RLP (n = 32)	3.29	P < 0.01	a	0.18	P < 0.01	a	2.12	P < 0.01	a
ELP (n = 30)	3.00		a	0.17		a	3.42		b
C (n = 30)	4.26		b	0.22		c	1.70		c

that protein lipid and nucleic acid oxidation intensifies as a result of the reduced antioxidant capacity of antioxidant systems. The results of the present study are consistent with the studies mentioned above, which assert the absence of any balance between antioxidants and ROS in OLP.

There is no data in the literature regarding the differences in oxidant-antioxidant equilibrium between different forms of oral lichen planus. Only one study examined total antioxidant capacity and MDA levels in the saliva of patients with the erosive form of the disease in relation to a control group (12). Other researchers focused on patients diagnosed with OLP, but without further dividing the condition into its different forms. The reason may be the difficult differential diagnosis of the disease, which often requires not only assessing the symptoms and anamnesis, but also performing a histopathological examination. In addition, the prevalence of OLP forms other than the erosive and reticular types is not high. This clearly increases the value of this work, because the results demonstrate different levels of oxidative stress in patients with OLP.

In the present study, we tried to identify changes in the concentration of selected parameters of the oxidant-antioxidant balance, depending on different OLP forms. Studies on selected

subgroups of patients (suffering from reticular and erosive forms of the disease) proved the validity of antioxidant status monitoring in patients with OLP. A comparison of selected redox markers in the saliva of patients with reticulate and erosive forms of OLP indicates that oxidative stress is greater in the erosive form of OLP than in the reticulate form. Oxidant-antioxidant control monitored by measuring selected salivary markers may serve as an important tool in efforts to prevent this disease and its progression. Therefore, it is vitally important to determine elements of antioxidant defense in those systems susceptible to oxidative stress induced by high risk behavior. On the other hand, antioxidant therapy is developing a new significance as a therapy, not only simply by temporarily decreasing inflammation, but also through its positive, long-term effects.

Saliva as a facilitating fluid is also the important medium for exchange of the different micro elements as well as taking part in the oxidative stress and antioxidizing activity. So it's important to conduct studies in varying disciplines with respect to understanding the process of releasing microelements into saliva which might help effective pharmacological and enhancing host resistance therapy (45-47).

Therefore, we recommend conducting further studies to determine the therapeutic results of antioxidant therapy so as to be able to elaborate new strategies for OLP treatment.

One of the problems encountered in the present study was that of comparing the results (in particular, the absolute values of TBARS concentrations) with those from other published studies, due to the diversity of the methods that were employed in them (*Tables 2-4*) (48-51). Such a comparison was also impeded by differences in the methods and periods of material storage. In none of the papers cited above was an addition of BHT used to limit *in vitro* lipid peroxidation. Another problem was that such factors as blood contamination, glucose content and especially the saliva collection method can determine the heterogeneity of the sample, and can be a potential source of bias. The literature revealed that saliva can often be contaminated with blood, especially in patients with periodontitis (52, 53). Diabetes mellitus and periodontitis were exclusion criteria in our study and this fact should not be ignored during the study planning process.

Another limitation of our study is the absence of any comparison between the results of different saliva collection systems. The Salivette saliva collection system significantly altered the determined concentrations of several oxidative stress markers compared with measurements of unstimulated whole saliva samples (i.e., TBARS concentrations were higher in unstimulated whole saliva) (54).

In addition, it is worth noting that TBARS levels differ from accepted trends in lipid peroxidation in men (55). The dynamics of the salivary TBARS levels did not differ between the sexes in our research. However, intraindividual variability is visible, and therefore the differences between genders in endogenous TBARS levels are not excluded. The dynamics of change and thus, more significant differences in observed oxidative stress parameters within the sexes cannot be excluded when increasing a group's size. An additional evaluation of the TBARS level in plasma as well as an increase in the size of study groups could confirm or exclude the obtained data.

Attention must also be paid to limitations regarding the use and interpretation of some tests, given their selection procedures and the small number of individuals covered by them. When determining the required sample number, an analysis of test power may constitute a helpful tool when planning further studies. An analysis of the duration and treatment of lichen planus, pro-inflammatory cytokines or other biochemical markers would make it possible to formulate more comprehensive conclusions on the role of oxidative stress in the pathogenesis of OLP. Although it has not conclusively resolved the problem, the present study validates and confirms the need for further research in this field.

In summary, both an imbalance in salivary markers of total antioxidant capacity (a decrease in TAC and GSH) and an increase in oxidative damage markers (TBARS) were observed in patients with OLP, in its reticular and erosive forms. Further studies are recommended to clarify whether these markers play an important part in the pathogenesis of OLP lesions.

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Author's address: Prof. Maria Chomyszyn-Gajewska, Department of Periodontology and Oral Medicine, Jagiellonian University Medical College, 4 Montelupich Street, 31-155 Cracow, Poland.
E-mail address: mdgajews@cyf-kr.edu.pl