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Original Article

Dentistry Section

Assessment of Oxidant-Antioxidant Status and Stress Factor in Recurrent Aphthous Stomatitis Patients: Case Control Study

SHERIN ZIAUDEEN[1], RATHY RAVINDRAN[2]

ABSTRACT

Introduction: Despite its vast occurrence, the aetiology of Recurrent Aphthous Stomatitis (RAS) still remains unknown and its aetiology is multifactorial. The factors believed to be associated with the aetiology of RAS, may disturb the equilibrium of oxidant-antioxidant status of the organism and may accelerate the formation of free radicals, resulting in Oxidative Stress (OS). Psychological stress is believed to act as a triggering factor or modifying factor for RAS.

Aim: To find whether oxidant-antioxidant status and psychosocial stress play a role in the pathogenesis of RAS.

Materials and Methods: The study was conducted on 60 subjects over a period of one year (August 2014-August 2015) equally divided into two groups-patients with RAS and healthy controls.

Psychosocial stress was analyzed by using Recent Life Changes Questionnaire (RLCQ). Saliva was analyzed to evaluate Superoxide Dismutase (SOD) and Glutathione Peroxidase (GSHPx) activities, Malondialdehyde (MDA) and Uric Acid (UA) levels in both the study and the control groups, using UV spectrophotometry.

Results: The mean value of salivary SOD and MDA was increased while the activity of GSHPx and UA decreased in the study group when compared to the controls; the difference being statistically significant ($p<0.005$). The mean RLCQ stress score was also found to be increased in the RAS group, which showed elevated levels of mental stresses when compared to physical stresses. No significant association was observed between SOD, MDA, GSHPx and UA with high levels of stress score ($p>0.05$). In the study group, no correlation was observed between the study variables and gender, the number of ulcer episodes in one year, the number of ulcers per episode or the duration of ulcers.

Conclusion: This study shows that salivary antioxidant levels show a significant difference in response to OS in RAS patients. An increase in levels of psychosocial stress is seen associated with patients with RAS indicating its role as a modifying or triggering factor in the initiation of RAS.

Keywords: Aphthous ulcers, Glutathione peroxidase, Malondialdehyde, Superoxide dismutase, Uric acid

INTRODUCTION

RAS or Recurrent Aphthous Ulceration (RAU) is one of the most common ulcerative diseases of the oral mucosa that presents as recurrent, shallow, painful round ulcers with an erythematous halo and yellowish-grey pseudomembranous base [1].

The aetiology of RAS is largely unknown, however several factors have been proposed as possible causative agents. These include local factors such as trauma, microbial factors, nutritional factors, immunologic factors, genetics, psychosocial stress, allergy and hormonal changes [1]. All of these factors can disturb the oxidant-antioxidant equilibrium of the individual and thereby, trigger the formation of free radicals. The OS that occurs as a result of an increase in the concentrations of these free radicals can suppress the activities of immune system causing cellular damage. To counteract this OS, mammalian cells have the antioxidant system that includes both enzymatic and non enzymatic activities. The enzymatic antioxidants are the SOD, Catalase (CAT) and GSHPx; and the non enzymatic antioxidants include vitamins A, E, C, melatonin, UA and glutathione [2].

Psychosocial stress has also been reported to have association to the onset of RAS. Stress can influence the immunity of the individual through innervations of the central nervous system, immune system or the neuroendocrine immune pathways by release of hormones such as cortisol. An increase in stress is therefore known to create an OS state contributing to cell damage [3]. The present study has been undertaken to assess whether OS and psychosocial stress play a role in the pathogenesis of RAS.

MATERIALS AND METHODS

In this case control study, Group I (study group) consisted of 30 patients diagnosed as having RAS and Group II (control group) consisted of 30 healthy volunteers. The study participants were selected by simple random sampling from the patients attending the outpatient department of Azeezia Dental and Medical College, Kollam, Kerala, India, fulfilling the inclusion and exclusion criteria. The study was initiated after obtaining ethical clearance from institutional ethics committee. A detailed case history was taken after obtaining written informed consent from all the participants. The subjects were also asked to complete the distributed RLCQ questionnaire to quantify the stressful life events experienced in a period of six months. Patients, above 18 years, with history of RAS within the past year were included in the study. Subjects with any systemic diseases (eg., celiac diseases, Crohn's disease, ulcerative colitis, AIDS), vitamin and microelement deficiencies, food allergies, under a therapeutic regimen or supplementary vitamins, history of surgery or trauma for the past two months, with a history of alcoholism, tobacco smoking and chewing habit and those having a partial or full dentures and ulcers caused due to trauma were excluded from the study.

Unstimulated saliva was collected from the patient between 8.00 am and 10.00 am using passive drool method. About 10 minutes prior to saliva collection the patients were asked to rinse their mouth with water and then spit out or swallow the saliva already present in the mouth. After a few minutes of relaxation, they were asked to lean forward and drool the produced saliva into a vial

using a custom made saliva collecting funnel. The saliva containing vials were placed in an ice carrier box and transferred to laboratory for biochemical analysis. Saliva samples were stored at -20°C until analysis. Saliva samples were centrifuged at 3000 rpm for 10 minutes at 4°C. The supernatants were collected and subjected to spectrophotometric analysis (using Spectrophotometer; Agilent Cary 60) for estimation of SOD, MDA, GSHPx and UA.

Estimation of Superoxide Dimutase Activity

Fifty microlitres (50 µl) of saliva samples were added to a test tube containing 3 ml of reaction mixture [50 mM potassium phosphate buffer (7.8), 45 µM methionine, 5.3 mM riboflavin, 84 µM NBT and 20 µM potassium ferric cyanide]. The tubes were incubated at 25°C for 10 minutes and read on spectrophotometer at 600 nm [4].

Estimation of Malondialdehyde

Fifty microlitres (50 µl) of saliva samples were mixed with 500 µl of 70% of alcohol and 1 ml of 1% Thiobarbituric Acid (TBA). Then all the tubes were kept in boiling water bath for 20 minutes. After cooling to room temperature 50 µl of acetone was added to all the test tubes and read absorbance at 535 nm in spectrophotometer. The MDA values were compared with a standard MDA graph [5].

Estimation of Gluathione Peroxidase

Fifty microlitre (50 µl) of saliva samples were added to a test tube containing 3 ml of reaction mixture [1mM of β-NADPH + 1mM sodium azide solution, 200 mM reduced glutathione]. Mixed by inversion and equilibrated to 25°C and monitored the absorbance at 340 nm until constant. The tube containing 3 ml reaction mixture and 50 µl of phosphate buffer (pH 7) was taken as blank. A 50 µl of 0.042% of hydrogen peroxide was added to these tubes. It was immediately mixed by inversion and the decrease in absorbance was recorded at 340 nm for approximately five minutes [2].

Estimation of Uric Acid

UA concentration was measured in the saliva of the participants, using a kit supplied by Erba diagnostics [Cat No: BLT00062; Pack Name: UA SINGLE 200; Packaging (content): R1: 4x50 ml and R2 standard: 1x5 ml]. To 20 µl samples, 1 ml working reagent was added. Working reagent was prepared by making the vial and Aqua-4 to attain the room temperature (15-30° C) and then adding Aqua-4 (100 ml) to the contents of each vial and mixing gently to completely dissolve. Working reagent and sample were mixed and incubated for five minutes at 37° C. At the same time blank and standard solution were taken. Absorbance of the standard and each test was read at 546 nm against reagent blank [2].

Calculating the RLCQ Stress Score

All the subjects were asked to choose and tick the events in the questionnaire that has been experienced by them in a period of six months. The numbers of the Life Changes Units (LCU) were totalled for the final score. A score of 300 or more indicated high stress in the life of the individual [6].

STATISTICAL ANALYSIS

Data analysis were performed using the software, statistical package SPSS (version 20.0). The descriptive statistics for each of study variables for both the study and control groups were calculated. Unpaired student's t-test was used to compare the study variables among themselves and with patient's characteristics such as gender, ulcer episodes in one year, ulcer per episode, duration of ulcer episodes. In all the analysis significance level were taken to be 0.05 (p-value<0.05). Pearson's correlation was used to compare between study variables and the ulcer experience to find out the probability of occurrence of aphthous ulcers in relation to the variables.

RESULTS

In the present study, out of the 60 subjects, 23 were males and 37 were females. The mean age in the control and study group were found to be 30.93 and 33.13 respectively.

The mean values of salivary oxidants and antioxidants were compared to the gender, ulcer episodes in one year, number of ulcers per episode and duration of ulcer episodes of all the cases. No significant association was noted between the mean values of SOD (p=0.437), MDA (p=0.723), GSHPx (p=0.519) and UA (p=0.530) with gender; between SOD (p=0.464), MDA (p=0.941), GSHPx (p=0.079) and UA (p=0.067) and ulcer episodes in 1 year; and between the mean values of SOD (p=0.987), MDA (p=0.738), GSHPx (p=0.408) and UA (p=0.506) with the duration of ulcer episodes.

Although a statistically significant association was observed between the mean value of UA and ulcers per episode (p=0.038), no association could be noted with SOD (p=0.980), MDA (p=0.541) and GSHPx (p=0.605).

The mean and standard deviation of SOD, MDA and stress score was highest for RAS group than the controls; whereas, it was lowest for GSHPx and UA [Table/Fig-1]. Both physical and mental stresses were highest in RAS group than the controls.

On comparing the salivary oxidant-antioxidant levels and stress score between study and control groups, the difference of each variable between the groups were statistically significant (p=0.001). The difference between the mean value of mental stresses in the study and control group was statistically significant (p=0.015). Whereas, the difference in physical stresses of the study and control group was statistically insignificant (p=0.069) [Table/Fig-2].

In the study group, 18 subjects were positive for high levels of stress (RLCQ stress score>300) whereas, only four subjects showed higher levels of stress in the control group. Rest of the subjects showed lower levels of stress score (RLCQ stress score <300) [Table/Fig-3]

No significant association was observed on comparing the mean values of SOD, GSHPx and UA with high levels of stress score in the study group and in the control group. On comparing the mean value of MDA with high levels of stress score in the study group, a significant association was observed (p=0.011). However, no significant association could be noted in the control group (p= 0.288) [Table/Fig-3].

Pearson's correlation between the study variables and the presence of RAS showed a positive correlation between stress, SOD and MDA indicating an increase in probability of occurrence of RAS proportional to an increase in the variables. However, Pearson's

Groups	SOD (Mean±SD)	MDA (Mean±SD)	GSHPx (Mean±SD)	Uric Acid (Mean±SD)	RLCQ Stress Score (Mean±SD)
Group – I (Study)	1.27±0.48	1.45±0.55	1.60±0.39	3.99±1.59	306.17±74.92
Group – II (Control)	0.72±0.41	0.95±0.28	2.19±0.62	5.79±1.61	228.27±87.85
p-value	0.000*	0.000*	0.000*	0.000*	0.000*

[Table/Fig-1]: Comparison of salivary oxidant, antioxidant levels and stress factor between study and control groups.
* Unpaired student's t-test

Stress score	Physical stress (Mean±SD)	Mental stress (Mean±SD)
Group-I (Cases)	81.97±53.61	224.20±87.26
Group-II (Control)	59.30±39.94	168.97±83.54
p-value	0.069	0.015*

[Table/Fig-2]: Comparison of physical and mental stress between study and control groups.
* Unpaired student's t-test

RLCQ STRESS SCORE	GROUP I (CASES)					GROUP II (CONTROLS)				
	No.	SOD (Mean)	MDA (Mean)	GSHPx (Mean)	UA (Mean)	No.	SOD (Mean)	MDA (Mean)	GSHPx (Mean)	UA (Mean)
< 300	12	1.35	1.46	1.75	4.21	26	0.73	0.83	2.24	5.84
>300	18	1.22	1.44	1.50	3.86	4	0.61	0.78	1.84	5.41
p value		0.196	0.011*	0.261	0.961		0.475	0.288	0.378	0.562

[Table/Fig-3]: Comparison of salivary oxidant and antioxidant levels with stress score.
* Unpaired student's t-test, (*p<0.05 significant)

Study Variables	p-value (with Presence of RAS)	Pearson's Correlation
RLCQ Stress Score	0.000	0.437
SOD	0.000	0.534
MDA	0.000	0.594
GSHPx	0.000	- 0.575
UA	0.000	- 0.491

[Table/Fig-4]: Pearson's correlation between study variables and presence of RAS.

correlation was negative for GSHPx and UA suggesting that the probability of occurrence of RAS increases with a decrease in value of the variables [Table/Fig-4].

DISCUSSION

The aetiologic factors of RAS may disturb the oxidant/antioxidant equilibrium of the individual, thereby accelerating the formation of Reactive Oxygen Species (ROS). Oxidative stress (OS) occurs when the concentrations of ROS increase above physiologic values, leading to cellular damage [2]. Various antioxidant systems are produced by the body to detoxify the ROS. This imbalance between the free radicals and antioxidants are believed to cause many inflammatory pathologies [7]. Assessment of the levels of the antioxidant enzyme activities such as those of SOD, CAT, GSHPx and the non enzymatic activities of Vitamin A, E, C, melatonin, UA and glutathione help to evaluate the antioxidant status of the individual [2].

Primary defense against ROS is by catalytic removal of ROS by antioxidant enzymes. SODs are a family of metalloenzymes found in all aerobic organisms and are the first enzymes to be involved in antioxidant defense. They catalyse the dismutation of superoxide to hydrogen peroxide, three forms of SODs are present in mammalian tissues each with a specific subcellular location and different tissue distribution. They are Copper Zinc SOD (CuZn-SOD); Manganese SOD (Mn-SOD); and Extracellular SOD (EC-SOD). While CuZn-SOD is found in the cytosol and organelles of all mammalian cells, Mn-SOD are present in the mitochondria of cells. EC-SOD is a copper and zinc containing SOD that is detectable in extracellular fluids [8]. During OS, cell responds to reactive oxygen metabolites with SOD. SOD protects the cell from damage caused by superoxide (O_2^-) and hydroxyl radical, releasing H_2O_2 in the process. While SOD lowers the steady state level of O_2^-, catalase and peroxidases do the same for H_2O_2.

Glutathione peroxidase also forms the first line of defense against OS, which in turn requires glutathione as a co-factor. It detoxifies peroxides, by acting as an electron donor in the reduction reaction producing glutathione disulphide as an end product [9]. The activity of the enzyme is however dependent on the constant availability of reduced glutathione [8].

Saliva also contains non enzymatic antioxidants such as UA, albumin and ascorbic acid. UA appears to be the dominant antioxidant present in saliva, responsible for 70% of the total antioxidant capacity [10]. UA scavenges radicals; being converted in the process to allantoin [8]. UA has been found to be an important salivary biomarker with clinical importance in monitoring the OS [11].

While UA is the most important antioxidant molecule in saliva, MDA is a suitable biomarker of endogenous DNA damage. Reactive oxygen metabolites lead to destruction and damage to cell membranes by lipid peroxidation, which results from the oxidation of membrane-associated polyunsaturated fatty acids of phospholipids. MDA is the principal end product of polyunsaturated fatty acid peroxidation and is a good marker of free radical mediated damage and OS.

The present study showed a female predominance since male subjects having tobacco smoking or chewing habits and alcohol consumption were excluded. No correlation was observed between the study variables and gender, the number of ulcer episodes in one year and the duration of ulcers. This was comparable to the study by Akoglu G et al., [12].

In the present study, the mean value of salivary SOD and MDA was increased while the activity of GSHPx and UA decreased in the study group when compared to the controls. The infiltration of immune cells into the lesional area must have resulted in an increase in concentration of free radicals. More of SODs are needed to bring about dismutation of the superoxide radicals which resulted in its higher levels. The dismutation reaction results in the over production of H_2O_2. While GSHPx functions to detoxify the H_2O_2, reduced glutathione gets consumed in the process. The unavailability of reduced glutathione may have reduced the levels of GSHPx in the saliva.

These results were in accordance with studies by Saxena S et al., and Karincaoglu Y et al., [2,13] however, UA levels were found to be raised in the study by Saxena S et al., which was believed to be due to a transfer of the antioxidant molecules from the plasma to the site of the ulcer for better function [2]. The results obtained were contrary to that of Momen Beitollahi J et al., who noticed a decrease in SOD and an increase in GSHPx. The decrease in SOD activity was suggested to be due to its increased consumption, leading to over production to H_2O_2. GSHPx being the dominant antioxidant participating in getting rid of H_2O_2, the increased amount may be due to genetic control mechanisms and feedback effects of H_2O_2 on mRNA expression. They reported that although changes in SOD activity are important, the other defenses such as GSHPx and CAT do not seem to play a primary role in the aetiopathogenesis [14].

Due to a decrease in salivary GSHPx and UA levels, the combined antioxidant potential is impaired in the individual. This impairment may have activated the peroxidation reactions which explain the increase in MDA levels. Also, the increase in levels of MDA may be due to its increased formation or inadequate clearance of ROS by the antioxidants. This increased scavenging by the lipid peroxides may decrease the levels of antioxidant enzymes such as GSHPx and UA [15]. Contrary to this result, Khademi H et al., found no significant difference in the levels of MDA between RAS patients and normal controls. Variation in method of saliva sampling, genetic divergence of the population or some other acquired or environmental situation, may be the reason for this variation in result [16].

However, in the study by Cağlayan F et al., [7], no significant difference was found between OS parameters between RAS and controls, suggesting that ROS may not play a role in its aetiology.

There is also evidence of psychosocial stress as a triggering or modifying factor for RAS episodes. Studies by Galecki P et al.,

and Bouayed J et al., have linked a relationship between OS and anxiety [17,18]. Psychosocial stress has shown to induce immunoregulatory activity as well as an effect on OS. In the present study, the mean RLCQ stress score was increased in RAS when compared to the controls. Also, high level mental stresses were obtained than physical stresses. Stress could be a triggering or modifying factor for the occurrence of RAS. Similar results were shown by Huling LB et al., De Barros Gallo C et al., and Soto Araya M et al., who found a higher level of psychological stress among the RAS group [19-21].

No significant association of stress was observed with the levels of salivary oxidants and antioxidants in the RAS group. The mean values of SOD, MDA, GSHPx and UA were raised in RAS patients with increased stress. This was similar to results shown by Galecki P et al., [17]. On the contrary, Reginald B and Kiran G found a decrease in salivary peroxidases levels in highly stressed RAS patients [3]. They stated that due to increased stress levels, the body's regulatory mechanism would have failed leading to an imbalance in the oxidant/antioxidant activity that further resulted in decreased enzyme levels.

The probability of occurrence of RAS was found to be related to the levels of stress, oxidant and antioxidant markers. A higher chance of occurrence of aphthous ulcers is predicted to occur with an increase in stress, SOD and MDA, and a decrease in levels of GSHPx and UA. These findings emboss the importance of psychological stress, oxidant-antioxidants status in the aetio-pathogenesis of aphthous ulcers, suggesting antioxidant as a treatment modality. However, further study to evaluate the outcome of RAS after treatment with antioxidants is recommended.

LIMITATION

The present study was a case control study which evaluated the parameters at one point of time.

CONCLUSION

In the present study, the mean values of SOD and MDA in the study group showed a significant increase when compared to the control group, whereas, GSHPx and UA showed a significant decrease in the study group. The patients with RAS also showed a significant increase in stress levels, with high levels of mental stresses when compared to physical stress. Salivary antioxidant levels show a significant difference in response to OS, which might in turn be related to psychological stress, in RAS patients. A higher chance of occurrence of aphthous ulcers was predicted to occur with an increase in stress, SOD and MDA, and a decrease in levels of GSHPx and UA.

REFERENCES

[1] Akintoye SO, Greenberg MS. Recurrent aphthous stomatitis. Dent Clin North Am. 2014;58(2):281–97.
[2] Saxena S. Assessment of plasma and salivary antioxidant status in patients with recurrent aphthous stomatitis. RSBO. 2011;8(3):261–65.
[3] Reginald B, Kiran G. Aphthous ulcers, salivary peroxidase and stress: Are they related? J Oral Maxillofac Pathol. 2015;19(1):37.
[4] Misra HP, Fridovich I. Superoxide dismutase: A photochemical augmentation assay. Archives of Biochemistry and Biophysics.1977;246(22):6886-90.
[5] Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analytical Biochemistry. 1979;95(2):351-58.
[6] Miller MA. Rahe RH. Life changes scaling for the 1990s. Journal of Psychomatic Research. 1997;43(3):279–92.
[7] Cağlayan F, Miloglu O, Altun O, Erel O, Yilmaz AB. Oxidative stress and myeloperoxidase levels in saliva of patients with recurrent aphthous stomatitis. Oral Dis. 2008;14(8):700–704.
[8] Young I, Woodside J. Antioxidants in health and disease. J Clin Pathol. 2001;54(3):176–86.
[9] Iannitti T, Rottigni V, Palmieri B. Role of free radicals and antioxidant defences in oral cavity-related pathologies. J Oral Pathol Med. 2012;41(9):649–61.
[10] Kondakova I, Lissi EA, Pizarro M. Total reactive antioxidant potential in human saliva of smokers and non-smokers. Biochem Mol Biol Int. 1999;47(6):911–20.
[11] Miricescu D, Greabu M, Totan A, Didilescu A, Rădulescu R. The antioxidant potential of saliva: Clinical significance in oral diseases. Molecules. 2011;4:5
[12] Akoglu G, MetinA, Kilinc F, Pektas SD, Isikoglu S, Akbas A, et al. Total serum oxidant/antioxidant status and arylesterase activity in recurrent aphthous stomatitis. Ann Dermatol. 2013;25(3):273–77.
[13] Karincaoglu Y, Batcioglu K, Erdem T, Esrefoglu M, Genc M. The levels of plasma and salivary antioxidants in the patient with recurrent aphthous stomatitis. J Oral Pathol Med. 2005;34(1):7–12.
[14] Momen-Beitollahi J, Mansourian A, Momen-Heravi F, Amanlou M, Obradov S, Sahebjamee M. Assessment of salivary and serum antioxidant status in patients with recurrent aphthous stomatitis. Med Oral Patol Oral Cirugía Bucal. 2010;15(4):e557–61.
[15] Shree S, Kumar K, Itagappa M, Ramesh G. Study of oxidative stress and antioxidant status in oral cancer patients. Int J Oral Maxillofac Pathol. 2013;4 (2):2–6.
[16] Khademi H, Khozeimeh F, Tavangar A, Amini S, Ghalayani P. The serum and salivary level of malondialdehyde, vitamins A, E, and C in patient with recurrent aphthous stomatitis. Adv Biomed Res. 2014;3:246.
[17] Gałecki P, Szemraj J, Bieńkiewicz M, Florkowski A, Gałecka E. Lipid peroxidation and antioxidant protection in patients during acute depressive episodes and in remission after fluoxetine treatment. Pharmacol Reports Pr. 2009;61(3):436–47.
[18] Bouayed J, Rammal H, Soulimani R. Oxidative stress and anxiety. Oxid Med Cell Longev. 2009;2(2):63-67.
[19] Huling LB, Baccaglini L, Choquette L, Feinn RS, Lalla RV. Effect of stressful life events on the onset and duration of recurrent aphthous stomatitis. J Oral Pathol Med. 2012;41(2):149-52.
[20] De Barros Gallo C, Mimura MAM, Sugaya NN. Psychological stress and recurrent aphthous stomatitis. Clin Sao Paulo Braz. 2009;64(7):645-48.
[21] Soto Araya M, Rojas Alcayaga G, Esguep A. Association between psychological disorders and the presence of oral lichen planus, Burning mouth syndrome and recurrent aphthous stomatitis. Med Oral Órgano of Soc Española. 2004;9 (1):1-7.

PARTICULARS OF CONTRIBUTORS:
1. Postgraduate Student, Department of Oral Pathology, Azeezia College of Dental Science and Research, Kerala University of Health Sciences, Kollam, Kerala, India.
2. Professor and Head, Department of Oral Pathology, Azeezia College of Dental Science and Research, Kerala University of Health Sciences, Kollam, Kerala, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Dr. Rathy Ravindran,
Professor and Head, Department of Oral Pathology, Azeezia College of Dental Science and Research,
Kerala University of Health Sciences, Kollam-691005, Kerala, India.
E-mail: rathyravindran27@gmail.com

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