Original Article

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Dentistry Section

Recurrent Aphthous Stomatitis: An Assessment of Antioxidant Levels in Plasma and Saliva

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ABSTRACT

Introduction: Recurrent Aphthous Stomatitis (RAS) is a common oral mucosal disorder that affects 20% of the population worldwide. Factors such as trauma, stress, genetic, hypersensitivity, nutrition, immune disturbance and hormonal imbalance may disturb the oxidant and antioxidant balance of an organism and precipitate RAS, but the relationships are poorly understood.

Aim: The purpose of this study was to evaluate the antioxidant status in plasma and saliva of patients with RAS.

Materials and Methods: Forty patients with RAS and forty healthy individuals were included in the study. The levels of antioxidants such as Superoxide Dismutase (SOD), Glutathione Peroxidase (GSHPx) Catalase (CAT) and Uric Acid (UA) were measured in plasma and saliva. Statistical analysis was performed to compare the two groups using independent t-test and ANOVA.

Results: Decreased SOD levels were observed in plasma amongst RAS patients ($p < 0.03$) whereas, increased levels were observed in their saliva ($p < 0.001$) compared to the control group. A significant difference ($p < 0.001$) was noticed in GSHPx levels: RAS patients exhibited higher levels in plasma but decreased in saliva compared to the control group. CAT activities and UA levels in saliva ($p = 0.015$ and $p < 0.001$ respectively) were observed to be significantly higher in RAS patients. Within the RAS group elevated plasma SOD level ($p < 0.006$) was found in patients with major ulcers whereas, an increased plasma UA ($p < 0.01$) level was observed in patients with minor ulcers.

Conclusion: The non-equilibrium antioxidant levels observed in both plasma and saliva indicate the antioxidant status of the body is disturbed in patients with RAS.

Keywords: Antioxidants, Catalase, Glutathione peroxidise, Recurrent aphthous stomatitis, Superoxide dismutase, Uric acid

INTRODUCTION

RAS is a common but poorly understood disorder that affects almost 20% of the population worldwide [1,2]. RAS is characterised by recurring ulcers confined to the oral mucosa with no other signs of disease. The aetiologic and predisposing factors of RAS such as trauma, stress, genetic, hypersensitivity, nutrition, immune disturbance and hormonal imbalance can disturb the oxidant and antioxidant balance of the organism, which can accelerate the formation of free radicals [3]. It is suspected that the free radicals precipitate the formation of ulcers. Antioxidants block the oxidation process by neutralizing the free radicals. The physiological role of antioxidants is to reduce damage to cellular components that arise due to the consequence of reactions involving free radicals [4]. The antioxidant enzymes such as SOD, GSHPx, CAT, serve as the primary line of defence in destroying free radicals [5]. Uric acid is also a powerful antioxidant and is a scavenger of singlet oxygen and radicals [6].

Recently, studies have shown that saliva has its own antioxidant system including UA, superoxide dismutase, glutathione peroxidase and catalase. Uric acid constitutes around 70% of total antioxidant capacity of saliva [7,8]. However, there is a lack of research regarding plasma and salivary antioxidant status with recurrent aphthous stomatitis [8]. This study aimed to assess the level of antioxidants (superoxide dismutase, glutathione peroxidase, uric acid and catalase) in the plasma and saliva of patients with RAS. Correlations were performed to test the relationship between the levels of antioxidants: and a) the duration of current RAS; b) the number of years of experiencing RAS; and c) with each of the different types of RAS.

MATERIALS AND METHODS

Selection of patients and controls: A cross sectional study was carried out at the department of Oral Medicine and Radiology, Meenakshi Ammal Dental College, Chennai, India, over a period of one year from March 2014 to February 2015. This study was approved by the Institutional review board and the ethics committee. Written consent was obtained from all study subjects. The study group comprised of forty patients based on the previous studies done [8] in the age group 15-40 years diagnosed with aphthous stomatitis, who were selected on the basis of history and clinical features,and who agreed to take part. A similar group of forty individuals (appearing in the Dental Outpatients Department), with age and gender matched with study group and no history of RAS, were selected as the control group. Patients with any systemic disease, who were receiving any therapeutic regimen during the past three months and those with a history of tobacco habits or alcoholism were excluded from the study.

Data collection: Demographic details of the subjects such as name, age, gender, occupation and communication address were recorded. The study group patients were interviewed and the responses recorded. RAS is classified with respect to frequency and duration of ulcers as Type A, B and C [9] according to the history and presentation. Patients were asked the duration in days of the current ulcer and the number of years that they had experienced similar ulcers. They were examined clinically and their RAS status recorded. The classification of RAS presentation was based on the WHO classification with respect to size, shape, site and healing as Minor (Mi RAS), Major (Ma RAS), Herpetiform (HU) ulcers [10]. The number of ulcers present and their location was also recorded.

Plasma and saliva preparation: The blood samples were collected under aseptic protocol by vein puncture. Venous blood (2 cc) was withdrawn with a 5 ml disposable syringe and a 24 gauge disposable needle in to a plain vacutainer containing heparin as anticoagulant. The blood was centrifuged at 1000 g for 10 minute at 40^{0}C to obtain plasma, which was stored in small aliquots at -200^{0}C. Unstimulated saliva samples were obtained from the subjects after rinsing their

mouth with water. Subjects were asked to spit to a volume of 5 ml in a sterile plastic container which was stored at –200°C.

Assessment of antioxidant levels: The levels of antioxidants such as SOD, GSHPx, CAT and UA were measured in the plasma and saliva samples and compared between the study and control groups.

Assay of SOD activity: The SOD level was assessed in samples by the Marklund S and Marklund G method [11]. Twenty units of heparin were used to anticoagulate 1 ml of blood to obtain heparinised blood. These blood cells were suspended in 1.5 ml of saline and centrifuged for 10 minutes at 3000 rpm. Further the cells were haemolysated and centrifuged. Optical density was measured using spectrophotometer at 340 nm.

Assay of GSHPx activity: GSHPx level was determined using the method described by Pagila DE and Valentine WN [12]. GSHPx catalyses the oxidation of glutathione by means of cumene hyperperoxide. In the presence of glutathione reductase and NADPH the oxidized glutathione is converted immediately to the reduced form with the concomitant oxidation of NADPH to NADP. The decrease in absorbance was measured at 340 nm using a spectrophotometer.

Assay of CAT activity: The catalase level was measured in samples using the method adopted by Cimen MY et al., [13]. One unit of catalase decomposes 1 µmole of H_2O_2 in a minute at pH 7.0 at 25°C. While the concentration of H_2O_2 falls from 10.3 mM to 9.2 mM, the absorbance decrease due to disappearance of $H_2O_2$was measured at 240 nm using a spectrophotometer [14].

Assay of UA activity: The method proposed by Fossati P et al., was used to assess the concentration of uric acid [15]. Uricase converts uric acid to allantoin and hydrogen peroxide. The hydrogen peroxide formed reacts with a phenolic compound and 4-aminoantipyrine by the catalytic action of peroxidise to form a red coloured quinoneimine dye complex. The intensity of the colour formed is directly proportional to the amount of uric acid present in the sample [16]. The change in absorbance was measured at 546 nm.

STATISTICAL ANALYSIS

Statistical analysis was performed using independent t-test to find the significance of the difference in levels of antioxidants between the study and control group in plasma and saliva. The independent t-test was done to find the significance of the different levels of antioxidants in patients having single and multiple ulcers. Independent t-test was performed to compare mean values of antioxidant levels between minor and major type of ulcers among the study group. Pearson correlation was performed to analyse the correlation of antioxidant levels with respect to the duration of current ulcers in days and recurrence of ulcers in years.

RESULTS

Demographic data: The study group comprised 40 subjects, of which males were nineteen (N = 19) and females were twenty one (N = 21). The 40 subjects in control group, consisted of 20 males and 20 females. The minimum age was 16 in both the groups, whereas the maximum age was 40 and 39 in groups A and B respectively. The highest number of cases was in the age group of 21-30 years for both Group A and Group B. The mean age was found to be 26.2 and 26.8 years for group A and B respectively.

Clinical features: In the study group, twenty one (52.5%) out of 40 patients had single ulcers while nineteen (47.5%) patients had multiple ulcers. Of the patients with multiple ulcers, nine (22.5% of the study group) had two ulcers and ten (25%) had three or more ulcers. The most common clinical presentation of RAS was Type A, which was observed in 60% of cases followed by 27% of Type B and 13% of Type C. Out of 40 patients, 29 had Mi RAS, 10 had Ma RAS and one patient had HU ulcers. Single ulcers were observed most commonly in the lower labial mucosa (38%) followed by buccal mucosa (24%). The other sites were upper labial vestibule, lower labial vestibule, tongue and gingiva with an occurrence of 9.5% each. Multiple ulcers occurred most commonly in combinations of the following sites: lower labial mucosa, buccal mucosa, ventral surface of the tongue, tip of the tongue, buccal vestibule and gingival.

	SOD (U/mg protein)	GSHPx (U/mg protein)	CAT (U/mg protein)	UA (mg/dl)
Plasma				
RAS (n = 40)	1206.2±195.9	21.94±2.38	197.86±10.1	3.40±0.71
Control (n = 40)	1314.4±237.7	18.27±2.42	196.44±16.77	3.63±0.80
P-value	0.03	<0.001	0.65	0.19
Saliva				
RAS (n = 40)	1.53±0.23	1.58±0.27	1.0001±0.21	4.57±1.64
Control (n = 40)	1.02±0.41	1.89±0.37	0.892±0.18	2.44±1.03
P-value	<0.001	< 0.001	0.015	<0.001

[Table/Fig-1]: Plasma and saliva SOD, GSHPx, CAT activities and UA levels of RAS patients and controls[1].
[1] SOD - superoxide dismutase; GSHPx - glutathione peroxidase; CAT – catalase; UA - uric acid; RAS – recurrent aphthous stomatitis. Independent t-Test analysis was performed. Values are expressed as mean ±SD.

Analysis of antioxidants: [Table/Fig-1] shows the activities of SOD, GSHPx, CAT and UA concentration levels in plasma and saliva of RAS patients and controls. A decreased SOD level was observed with patients in plasma ($p < 0.03$) whereas an increased SOD level was noticed in saliva ($p < 0.001$) with RAS patients. The p-values ($p < 0.001$) obtained for GSHPx activities in plasma and saliva indicate a significant variation between the two groups. An elevated GSHPx level was found in the plasma of RAS patients but decreased GSHPx level was observed in the saliva of RAS patients. In plasma no statistically significant difference ($P > 0.05$) was found for CAT levels and UA levels between patients and controls. However, the CAT activities and UA levels in saliva ($P = 0.015$ and $P < 0.001$ respectively) were observed to be significantly different between the study and control groups. The CAT levels and UA levels in saliva were found to be higher with RAS patients than the control group.

Correlation of antioxidants with respect to duration of ulcers: Pearson correlation was performed to analyse the correlation of antioxidants with respect to the duration of the current ulcers in days and with respect to the number of years the patients had experienced RAS.

Duration of Ulcers (Days): A positive correlation was observed for SOD ($C = 0.174$, $p = 0.283$) and GSHPx ($C = 0.122$, $p = 0.455$) in plasma, whereas a negative correlation was observed for SOD ($C = -0.091$, $p = 0.578$) and GSHPx ($C = -0.008$, $p = 0.959$) in saliva. A negative correlation was noticed for the antioxidants UA and CAT in both plasma and saliva. The p-value of all antioxidants in both plasma and saliva indicates that the correlation between the antioxidant values and the duration of ulcers in days was statistically insignificant.

Number of years having recurrent ulcers: A negative correlation was found between the duration of recurrence of ulcers for the antioxidant SOD ($C = -0.074$, $p = 0.650$) in plasma, whereas a positive correlation ($C = 0.264$, $p = 1.0$) was noticed in the value of SOD in saliva. A positive correlation was observed for the antioxidants GSHPx and UA in both plasma and saliva whereas a negative correlation was observed for CAT in both plasma and saliva. The p-value of all antioxidants in both plasma and saliva indicates that the correlation between the antioxidant values and the duration of recurrence of ulcers in years was statistically insignificant except for GSHPx in

saliva. This suggests that people with a long lasting experience of RAS are likely to have higher levels of GSHPx in their saliva.

Comparison of antioxidants between single and multiple ulcers: Statistical analysis was performed using independent t-test to find the significance of the different levels of antioxidants between single and multiple ulcers. The mean values of SOD, CAT and UA in plasma were observed to be higher in patients with multiple ulcers, compared to patients with single ulcers, whereas the values in saliva were found to be higher in patients with single ulcers. However, the mean value of GSHPx in plasma was found to be higher in patients with a single ulcer, whereas in saliva it was higher in patients with multiple ulcers. However, the p-values indicate that the differences in antioxidant levels between single and multiple ulcers were not significant.

Comparison of antioxidants among the types of ulcers: One-way Analysis of Variance (ANOVA) was performed to determine the difference in the antioxidant parameters with respect to the three types of ulcer. The p-values indicate that the variation in levels of antioxidants across the type of ulcers was insignificant. Independent t-test was performed to compare mean values between minor and major type of ulcers excluding the single case of herpetiform ulcers. The p-values indicate that the variations in antioxidant levels between the major and minor ulcers were not significant except for SOD and UA in plasma for which the p-values were 0.006 and 0.010 respectively. The antioxidant levels in plasma and saliva of RAS patients with minor and major ulcers are depicted in [Table/Fig-2].

Type of ulcers	SOD (U/mg protein)	GSHPx (U/mg protein)	CAT (U/mg protein)	UA (mg/dl)
Plasma				
Minor (n = 28)	1155.9±187.3	21.99±2.61	197.69±9.75	3.56±0.77
Major (n = 11)	1344.2±160.4	21.86±1.93	198.68±11.65	3.05±0.39
p-value	0.006	0.88	0.79	0.01
Saliva				
Minor (n = 28)	1.563±0.231	1.62±0.31	0.972±0.21	4.74±1.63
Major (n = 11)	1.454±0.212	1.49±0.09	1.066±0.20	4.12±1.74
p-value	0.18	0.06	0.21	0.30

[Table/Fig-2]: The antioxidant levels in plasma and saliva of RAS patients with minor and major ulcers[2].
[2]SOD - superoxide dismutase; GSHPx - glutathione peroxidase; CAT – catalase; UA - uric acid; RAS – recurrent aphthous stomatitis. Independent t-Test analysis was performed. Values are expressed as mean ±SD. * statistical significance.

DISCUSSION

Recurrent aphthous stomatitis is a commonly occurring oral mucosal disorder characterized by recurring ulcers that affect 20% of the worldwide population [1,4]. The role of antioxidants in RAS is attracting attention because the levels of antioxidants such as superoxide dismutase, glutathione peroxidise, catalase and uric acid in plasma and saliva seem to vary more with RAS patients compared to healthy individuals [7]. The antioxidant defence system of the mammalian cells is believed to reduce the oxidative damage of the cells and allows them to survive in an aerobic environment [7,8]. Several authors have observed that the first episodes of RAS occur most frequently during the second decade of life [17-19]. Reflecting this, the number of RAS patients in the age group of 21-30 years in this study was relatively high. In the literature, the number of females presenting with RAS is higher than the number of males and this study included slightly more female patients [20-22].

A decreased level of SOD level in plasma may be due to its high consumption which leads to overproduction of H_2O_2 as a result of the dismutation reaction [3]. The SOD level is high in saliva and low in plasma; this indicates that these antioxidant molecules (or molecules leading to the synthesis of MRNA for antioxidants) are likely to be transported from plasma to the body fluids in the areas of occurrence of ulcers [7]. Similar results have been reported in the literature by various authors [3,7,8,13].

The reason that GSHPx level in saliva was low in this study is believed to be inhibition resulting from a high concentration of H_2O_2 in the lesion area. Generally, H_2O_2 is thought to have high concentration at the site of the lesion but its diffusion in plasma is limited. The elevated GSHPx level in plasma is due to the lower concentration there of H_2O_2 [7]. In saliva, during the detoxification of increased H_2O_2 by GSHPx, consumption of chemically reduced GSH also increases. Insufficient GSH may have decreased the level of GSHPx in the saliva of RAS patients [8]. Further more, the plasma antioxidant system elements in the organism are likely to be deported to an area where oxidative stress is localized which is a subject yet to be studied. Similar results have been reported by Karincayoglu Y et al., and Saxena et al., [7,8] but contradictory results have been reported by Cimen MY et al., and Arikan S et al., [13,23].

Catalase has been recognized as the secondary antioxidant enzyme in peroxidative defence [3]. It hydrolyses H_2O_2 into H_2O and O_2. In this study, catalase was found to be insignificant in the plasma of RAS patients, this is similar to the results obtained by Momen Beitollahi J et al., [3]. The catalase acts only after attaining an optimum concentration of hydrogen peroxide; below which catalase has no significant role [24]. However, catalase levels were found to be significant in the saliva of RAS patients which is in agreement to Karincayoglu Y et al., and Saxena S et al., [7,8]. The observed increase in CAT level could be due to the higher level of hydrogen peroxide formation.

Uric acid represents the end product of endogenous purine metabolism. It acts as an effective free radical scavenger as well as an inhibitor of lipid peroxidation [25]. This study suggests that UA in saliva plays actively as an antioxidant against free radicals. This is in accordance with the literature that uric acid constitutes around 70% of total antioxidant capacity of saliva [8,9]. Similar results have been obtained by Saxena S et al., [8]. However, the role of UA in plasma was found to be insignificant, which is similar to the results reported by Karincayoglu Y et al., whereas Gurel A et al., found elevated UA level in plasma [7,26].

The correlations of the antioxidant levels in plasma and saliva with the duration of ulcers in days were not found to be statistically significant. This indicates that the level of antioxidants does not vary with respect to the duration of ulcer in days. Similarly, the correlations of the antioxidants in plasma and saliva with respect to the number of years of experiencing ulcers were found to be insignificant except for GSHPx level in saliva. This indicates that the GSHPx level in saliva increases with the number of years of having RAS.

The comparison of antioxidant levels between the single and multiple ulcers as well as between the types of ulcers (Type A, B, C) revealed no significant correlations. The comparison of antioxidant levels between minor and major ulcers reveals that the production of SOD is high in major ulcer and plays a significant role due to its characteristics as powerful enzymatic antioxidants to fight against free radicals. Uric acid in plasma tends to lead to the increased production of minor ulcers. However, UA does not play significantly in the severity of lesions. Comparisons of antioxidant levels with single and multiple ulcers, types of ulcers, duration of present ulcers as well as the frequency of recurrence of ulcers are not found in the literature to our best of knowledge. More studies have to be performed to find out the influence of these antioxidants with respect to the nature of recurrent aphthous stomatitis.

LIMITATION

Neither the present nor previous studies could definitely conclude whether the antioxidant system impairment in RAS patient's causes

aphthous or the aphthous ulcers in RAS patients causes antioxidant impairment (or possibly both are due to some other causal factor). Hence, the relationship between RAS and antioxidants needs to be extensively studied. Therefore, it is proposed to carry out controlled, multi-centre prospective studies on large case series of RAS.

CONCLUSION

RAS is one of the most common oral mucosal diseases. This study provides information on the activity of antioxidant levels in plasma and saliva of patients with RAS. It can be concluded that the equilibrium in the antioxidant system in plasma and saliva is altered in RAS patients.

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