Original Article

Estimation of Salivary Antioxidant level in patients with Oral Potentially Malignant Disorders and Oral Squamous Cell Carcinoma

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Abstract

Background: Antioxidants are intimately involved in prevention of cellular damage which is the common pathway for cancer and a variety of diseases. Saliva is the first biological fluid met by external substances and hence it denotes the first line of defense against the oxidative stress. This study was undertaken to estimate the antioxidant levels in saliva of individuals with oral potentially malignant disorders and oral squamous cell carcinoma and to compare it with that of healthy individuals. **Material and Methods:** A Cross sectional study was carried out using 123 samples comprising of 43 from patients with oral squamous cell carcinoma and 40 each from patients with oral potentially malignant disorders & healthy individuals. Salivary uric acid level, salivary peroxidase level and nitrate level were measured using a colorimeter and compared among the three groups. **Results:** A statistically significant difference was obtained while comparing the levels of salivary uric acid, salivary peroxidase and salivary nitrate level in each group by using ANOVA and Turkey's multiple comparisons test. **Conclusion:** The current study's results showed that individuals with oral squamous cell carcinoma and oral potentially malignant disorders had certain disruptions in their salivary enzymatic and non-enzymatic antioxidant defense.

Keywords: Oral Potentially Malignant Disorders; Oral Squamous Cell Carcinoma; Salivary Uric Acid; Salivary Peroxidase; Salivary Nitrate; Antioxidant.

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Introduction

The body maintains an oxidant-antioxidant balance under normal physiological circumstances. Stress, hormone imbalances, cardiovascular diseases, and neurodegenerative conditions can all cause disruptions to it, which can result in oxidative stress (OS), causing cellular damage.[1] Antioxidants are present in all bodily fluids and tissues to help combat the impacts of free radicals. Saliva is one of the body's earliest defences against oxidative stress, and an increasing number of proof-of- principle studies have been conducted using saliva to monitor systemic diseases and conditions linked to oxidative stress.[2] The salivary antioxidant system, like other biological systems, is made up of a variety of chemicals and enzymes. The two most significant ones are the water-soluble peroxidase enzyme and the uric acid molecule.[3]

In order to avoid the production of free radicals, the glutathione system is one of the most important groups of enzymes, which consists of glutathione, glutathione reductase (GSH), glutathione peroxidase (GPx), and glutathione S-transferase (GST). Uric acid (UA), on the other hand, functions as a non-enzymatic antioxidant.[4] Since saliva is in direct contact with the oral mucosa and therefore with the lesions like oral squamous cell carcinoma (OSCC), its molecular assessment is easy, efficient, and non- invasive.

According to estimates, oral and pharyngeal cancer rank as the third most prevalent cause of death in developing nations and the ninth most common cause of cancer worldwide. A further estimate is that, with the exception of nasopharyngeal carcinoma, 56% of the world's oral and pharyngeal cancer burden is from Asia.[1] One of the malignant tumors that might lead to life-threatening consequences is oral squamous cell carcinoma. DNA damage, mainly because of products of oxidative stress like reactive oxygen species, is a frequent mutagenic agent that triggers carcinoma. High levels of dietary nitrate have long been considered risk factors in the development of various types of cancer in humans. The analysis of salivary and dietary nitrate and nitrite has been used as a measure of exogenous nitrate burden as well as an indicator of risk for upper aerodigestive tract cancers.[5] Antioxidant capacity is a biomarker often used in order to investigate oxidative stress in many pathological conditions. Studies have been conducted to evaluate salivary oxidative stress in oral cancer and potentially malignant disorders.[6] Anti-oxidants have been widely used as dietary supplements and have been investigated for the prevention of diseases such as cancer, coronary heart disease, oral potentially malignant diseases and

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conditions.[2,3] The main objective of this study was to investigate the changes in antioxidant levels in the saliva of individuals with oral potentially malignant disorders and oral squamous cell carcinoma and whether these parameters can be used as a non–invasive biomarker in predicting the premalignant and malignant changes of the oral cavity.

Materials and Methods

Following Institutional Ethics Committee approval, a cross-sectional study was carried out., using 123 samples; out of which 40 were from healthy individuals, 40 from patients with oral potentially malignant disorders (oral leukoplakia, oral lichen planus, and oral sub mucous fibrosis) and 43 from patients with oral squamous cell carcinoma. Collection of saliva was done after provisional diagnosis of oral squamous cell carcinoma (OSCC) , oral potentially malignant disorders (OPMD) , and subsequent incisional biopsy was performed to confirm the clinical diagnosis. The samples were also taken from healthy individuals without any deleterious oral habits. Unstimulated whole expectorated saliva from each subject was collected into sterile tubes after a single mouth rinse with 15 ml of distilled water to wash out the exfoliated cells. The collected sample was placed in refrigerator immediately and then centrifuged at 3000 rpm and 2 ml of supernatant was used for analysis. Uric acid, peroxidase, and nitrogen species in saliva were estimated by colorimetric method under different nanometers.

For the estimation of peroxidase, 2 ml of the supernatant was taken and the enzyme DTNB (Dithionitrobenzoic acid) was added to it. The colorimetric change induced by the reaction between the enzyme and substrate, dithiobis 2 nitrobenzoic acid in the presence of mercaptoethanol was read at a wavelength of 412 nm. For estimation of uric acid, 2 ml of the supernatant saliva was taken and 0.5 ml of urease enzyme was added to it. Uric acid is transformed by urease to allantoin and then hydrogen peroxide was added to it, which under the catalytic influence of peroxidase, oxidizes the chromogen (4-aminophenazone) to form a red compound with the intensity of colour which is proportional to the amount of uric acid present in the sample, was read at wavelength 546 nm. For the estimation of nitrogen species, 2 ml of the supernatant was taken. Tin metal powder was added to it and then sulphanilamide and N (-naphthyl) ethylenediamine were added. The purple-colored azo compound then formed was measured colorimetrically at 540 nm.

Results

Analysis and Comparison of Uric Acid levels among three categories

While comparing the OPMD and OSCC groups, the average difference in uric acid level was 0.67 and the 95% confidence interval (CI) was 0.5 to 0.9 [Table 1, 2]. This 95% CI also does not include zero and also the 95% CI of mean uric acid levels between the two groups does not overlap with each other [Figure 1-A, Figure 1-B]. So, there is a significant difference in uric acid levels between OPMD and OSCC and similarly, in the case of normal Vs OPMD and normal Vs OSCC. [Table 2].

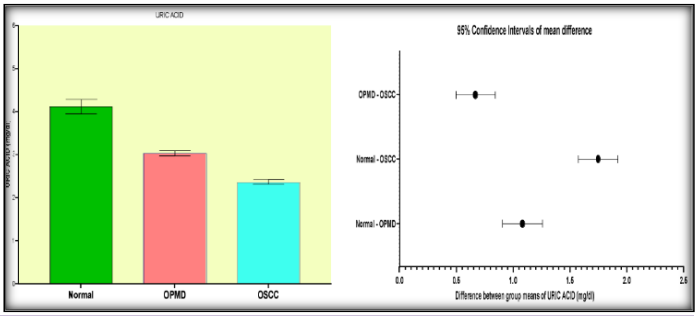

Figure 1: A-Bar diagram showing uric acid levels in 3 categories. B: Mean uric acid levels and 95% CI of mean difference in uric acid levels.

Table 1: Mean uric acid levels

	N	URIC ACID (mg/dl)						
		Mea n	Standard Deviation(sd)	Standard error(se)	95% CI for Mean		Min.	Max.
					Lower (L)	Upper (U)		
Normal	40	4.12	0.52	0.08	3.95	4.29	2.83	5.06
OPMD	40	3.04	0.18	0.03	2.98	3.10	2.64	3.39
OSCC	43	2.37	0.18	0.03	2.32	2.43	2.02	2.71
Total	123	3.15	0.79	0.07	3.01	3.30	2.02	5.06
F			289.36					
P value			<0.0001					

Table 2: Mean difference in uric acid levels by inter-comparison of groups

Turkey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	SE of diff.	p value
Normal vs. OPMD	1.08	0.9044 to 1.256	0.074	<0.001
Normal vs. OSCC	1.747	1.573 to 1.921	0.073	<0.001
OPMD vs. OSCC	0.6662	0.4933 to 0.8390	0.073	<0.001

Analysis and comparison of Nitrate levels among three groups: The average difference in nitrate level between normal and OPMD was 4.599 and 95% CI was between -6.086 to -3.11 [Table 3, 4]. In the case of normal Vs OSCC, the mean difference in nitrate level was -30.5 with a 95% CI of -31.97 to -29.03. In the case of OPMD Vs OSCC, the mean difference in nitrate level was between -25.9 and the 95% CI was between -27.37 to -24.44 [Figure 2-A, Figure 2-B]. In all these cases the 95% Confidence Intervals of the mean difference of nitrate levels does not include zero and while comparing the groups, the 95% CI of mean nitrate levels does not overlap each other. Hence there is a significant difference

between the groups while comparing nitrate levels [Table 4].

Table 3: Mean nitrate level

	N	Nitrate LEVEL (µmol/l)						
		Mean	sd	se	95% CI for Mean		Min	Max
					L	U		
Normal	40	34.17	1.07	0.17	33.83	34.51	33.01	36.43
OPMD	40	38.77	2.43	0.38	38.00	39.54	36.14	49.35
OSCC	43	64.67	4.03	0.61	63.43	65.91	56.78	71.11
Total	123	46.27	13.88	1.25	43.80	48.74	33.01	71.11
F				1428				
P value				<0.001				

Table 4: Mean difference in Nitrate levels by inter comparison of groups

Turkey's multiple comparisons test	Mean Diff.	SE of diff.	95.00% CI of diff.	p
Normal vs. OPMD	-4.599	0.6269	-6.086 to -3.111	<0.001
Normal vs. OSCC	-30.5	0.6196	-31.97 to -29.03	<0.001
OPMD vs. OSCC	-25.9	0.6157	-27.37 to -24.44	<0.001

Analysis and comparison of Peroxidase Levels among three groups: The average difference in Peroxidase level between normal and OPMD groups was 19.75 with a 95% Confidence Interval of mean difference between 15.56 to 23.94 [Table 5,6]. In comparing Normal Vs OSCC the average difference in Peroxidase level was 64.4 with a 95% CI between 60.26 to 68.55. In OPMD Vs OSCC, the average difference in Peroxidase level was 44.65 with a 95% CI of mean difference of 40.54 to 48.77. In all these cases the 95% Confidence Intervals of mean difference of Peroxidase levels do not include zero and while comparing the groups 95% CI of mean Peroxidase level does not overlap each other [Figure 3-A, Figure 3-B]. Hence there is a significant difference between the groups while comparing Peroxidase levels. [Table 6].

Table 5: Mean Peroxidase levels

	N	PEROXIDASE (mU/ml)						
		Mean	sd	se	95% CI for Mean		Min	Max
					L	U		
Normal	40	384.84	1.55	0.24	384.35	385.34	382.09	388.42
OPMD	40	365.09	6.01	0.94	363.19	366.99	353.98	376.89
OSCC	43	320.44	12.05	1.84	316.73	324.15	296.32	349.58
Total	123	355.98	28.32	2.54	350.94	361.01	296.32	388.42
F			721.1					
P value			<0.0001					

Table 6: Mean difference in Peroxidase levels by inter comparison of groups

Turkey's multiple comparisons test	Mean Diff.	SE	95.00% CI of diff.	P
Normal vs. OPMD	19.75	1.766	15.56 to 23.94	<0.001
Normal vs. OSCC	64.4	1.745	60.26 to 68.55	<0.001
OPMD vs. OSCC	44.65	1.734	40.54 to 48.77	<0.001

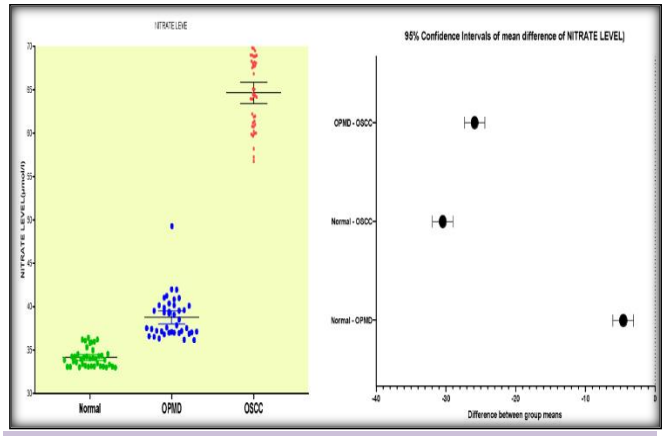

Figure 2: A: Scattered diagram showing nitrate levels in three groups. B: Mean nitrate level and 95% CI of mean difference of Nitrate level.

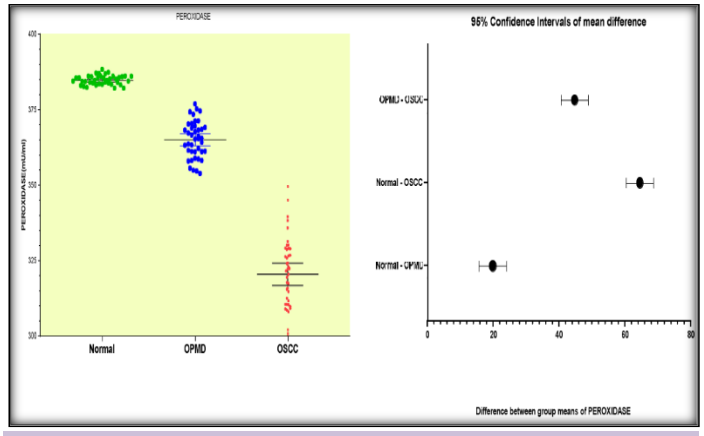

Figure 3-A: Scattered diagram showing peroxidase levels in three groups. B: Mean Peroxidase level and 95% Confidence Intervals of mean difference of Peroxidase.

Discussion

Research indicates that saliva possesses a diverse range of molecular and microbial analytes. These analytes have established the basis for the field of salivary diagnostics and consequently prompted studies that have resulted in the identification of saliva-based biomarkers for conditions spanning from infectious diseases to cancer. Among the more recent advances is a series of studies revealing the capacity to measure uric acid (UA) in saliva. UA is produced during the breakdown of purine nucleotides.[3] It was reported that uric acid accounts for more than 85% of the total antioxidant capacity of the human unstimulated and stimulated saliva. Oral Peroxidase (OP) is a significant enzymatic antioxidant salivary enzyme. The role of salivary peroxidase is the reduction of Hydrogen Peroxide (H2O2), the product of the metabolism of oral bacteria, in the presence of ions thiocyanate (SCN-). Compared to healthy people, oral cancer patients had higher salivary nitrate and nitrite levels as well as higher nitrate reductase activity.[7]

The present study aimed to evaluate whether there was any significant variation in the level of salivary antioxidants-peroxidase, uric acid, and nitrate in patients with oral potentially malignant disorders and oral squamous cell carcinoma when compared with healthy individuals by using colorimetric method. The results showed significant differences and this was in accordance with studies conducted by Lyudmila V. Bel'skaya *et. al* in 2020, Joanna Giebułtowicz *et. al* in 2011.[8,9]

The most important finding of this study was that the patients with OSCC had a reduced salivary antioxidant capacity. The peroxidase level in patients with OPMD was 95% of that of healthy individuals with a mean difference of 19.75 ($P < 0.001$) whereas the level in patients with OSCC was 83.3% of that of healthy individuals with a mean difference of 64.4 ($P < 0.001$). This statistical data shows that there was a reduction in salivary peroxidase levels from healthy individuals to patients with OPMD and from OPMD to patients with OSCC. In a study conducted by Bahar, Gideon Feinmesser *et al*,[10] in 2007 they saw a significant reduction in salivary peroxidase levels in patients with OSCC compared with that of healthy individuals.

The uric acid level obtained in patients with OPMD was 73.78% of that of the mean value obtained in healthy individuals with a mean difference among the group of 1.08 ($P< 0.001$). While comparing normal with that of OSCC patients, the level of uric acid obtained in OSCC patients was 57.5% of that of uric acid level in healthy individuals with a mean difference of 1.747 ($P<0.001$). The mean difference among OPMD and OSCC was 0.66 ($P<0.001$). This was in full agreement with a similar study conducted by Priya Tiwari, Nidhi Khajuria,[11] in estimating the uric acid level in OPMD and OSCC patients and they got a significant reduction in uric acid level in OSCC patients in comparison to patients with OPMD.

The level of nitrate obtained in patients with OPMD was 22.87% higher than that of the value obtained for healthy individuals. The mean difference of the two groups was - 4.99 ($P<0.001$). The nitrate level was increased from that of healthy individuals to patients with OPMD. In the case of patients with OSCC, the level was 48.2% higher than the value obtained for healthy individuals. The mean difference of the two groups was -30.5 ($P< 0.001$). The mean difference among OPMD and OSCC groups was -25.9 ($P<0.001$). This result was in accordance with that obtained in a study conducted by R Metgud, C.Anandani *et.al* in 2014.[12]

In similar studies conducted by Kumar A, Pant MC,[13] in 2012 for evaluating the RNS (Reactive Nitrogen Species) in patients with OSCC, a higher value was obtained for the study groups in comparison to the healthy controls. In a study conducted by L'ubomíraTóthová, and Natália Kamodyová on salivary markers of oxidative stress in oral diseases in 2015 they pointed out that excessive production of RNS can lead to oxidative stress. Oxidative stress has been implicated in the etiology and pathogenesis of several oral diseases including dental caries, periodontitis, and oral carcinomas.[14]

ROS/RNS can induce oxidative damage to cellular components with serious pathophysiological consequences.[15] Similar to oxidative stress, nitrosative stress is defined as the pro-oxidant RNS bias in the ratio of reactive nitrogen species to antioxidants. Biomacromolecules suffer when nitric oxide (NO) is produced in excess or in an inappropriate manner. Because NO has a high level of reactivity, it combines with other reactive species, such as superoxide, to generate more reactive compounds that do harm.[16] Oxidative stress is associated with inflammation and carcinogenesis.[17]

Results of the current study revealed some disturbances in enzymatic and non- enzymatic antioxidant defense in the saliva of patients with oral cancer and oral potentially malignant disorders. The levels of antioxidant enzyme peroxidase and the levels of uric acid decreased in patients with OPMD and OSCC whereas the salivary nitrate level increased in these groups. Majority of similar studies showed the same results. However, there were slight differences in the range of values obtained. These apparent discrepancies may be partly due to different laboratory methods used in individual studies. The differences in sample collection such as passive and stimulated saliva collection, variations in the assays used for measuring antioxidant levels, and differences in the kits, reagents, and instruments can have varying sensitivities and specificities. The discrepancies in storage conditions such as storage temperature, duration and presence of preservatives could also be a reason for such results.

Conclusion

The present study showed that there was a significant decrease in the levels of salivary uric acid and salivary peroxidase in patients with oral potentially malignant disorders and oral squamous cell carcinoma when compared to healthy individuals. But in the case of salivary nitrate level, the result was reversed. A higher salivary nitrate levels were measured in patients with oral squamous cell carcinoma and, a slightly lesser value was obtained in patients with oral potentially malignant disorders. The lowest value was obtained for the samples of healthy individuals. This may indicate that along with other diagnostic parameters, the levels of antioxidants: uric acid, salivary nitrate level and salivary peroxidase in saliva can be used as a diagnostic indicator in

patients with oral potentially malignant disorders and oral squamous cell carcinoma. The result obtained emphasizes the need for more long-term clinical studies with larger sample sizes to be conducted in this regard for the assessment of the levels of antioxidants in saliva and to assess their impact on the diagnosis of OPMDs and OSCC and also to prevent the ongoing transition of various oral premalignant lesions and conditions into malignant degenerations.

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Conflicts of interest
There are no conflicts of interest.

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