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Research Article

BIOCHEMICAL STUDY OF ANTIOXIDANT-OXIDANT INDEX IN ORAL POTENTIALLY MALIGNANT DISORDERS AND ORAL SQUAMOUS CELL CARCINOMA

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ABSTRACT

The aim of present work was to evaluate and compare the salivary levels of nitric oxide, vitamin C, Total Sialic acid and GSH in cases of Oral Potentially Malignant Disorders (OPMDs) and Oral Squamous Cell Carcinoma (OSCC) and healthy controls. The mean nitric oxide level was 27.34±5.51 µmol/l in OSCC group, 22.5±2.33 µmol/l in OPMD group and 10.11±0.88µmol/l in control group. The glutathione reductase activity in control patients was found to be 0.0915 U/ml under optimal pH, temperature and K_m. In OPMD group, the GR activity was found to be 0.0515 U/ml. Similarly, the activity in the OSCC group was found to be 0.0292 U/ml. The total sialic acid (TSA) in the saliva of control patients was found to be 41.241±5.3312μg/mL. In the case of OPMD patients, it was 64.25±4.33μg/mL and in the OSCC patients it was, 79.60±6.93 μg/mL., The levels of salivary vitamin C and glutathione were significantly reduced and those of nitric oxide and sialic acid were raised in patients having OPMD's and oral squamous cell carcinoma. The anti-oxidant to oxidant index (AOI) was measured between NO and vitamin C; NO and GSH; total sialic acid and vitamin C and lastly, total sialic acid and GSH. Thus, the findings of the present study indicate that estimation of Vitamin C, NO, GSH and sialic acid can be suitably used and could assist in the early diagnosis of potentially malignant disorders and oral cancer using saliva.

Keywords: Salivary levels, Nitric oxide, Vitamin C, Total Sialic acid, GSH, Oral Squamous Cell Carcinoma.

1. INTRODUCTION

Worldwide, oral cancer accounts for 2%-4% of all cancer cases. In a number of regions, the frequency of oral cancer is higher, reaching the 10% of all cancers in Pakistan, and around 45% in India [1-2]. In 2004-2009 over 300,000 new cases of oral and oropharyngeal cancer were diagnosed worldwide. Throughout the similar time period, over 7,000 affected individuals died of these cancers [3]. Oral cancer includes a group of neoplasms affecting any region of the oral cavity, pharyngeal regions and salivary glands. However, this term tends to be used interchangeably with oral squamous cell carcinoma (OSCC), which represents the most frequent of all oral neoplasms. It is estimated that more of 90% of all oral neoplasms are OSCC [4]. However, since not everyone who engages in these high-risk behaviors develops oral SCC, and because oral SCC can be idiopathic, there must be person-specific genetic and environmental causes that either guard against the development of oral SCC or predispose to or even encourage the development of oral SCC. The Indian subcontinent has the largest occurrence and prevalence of oral SCC, which is exacerbated by the very common practices of chewing tobacco, betel quid, and areca-nut chewing. Tobacco, alcohol, betel quid, and areca nut have mutagenic effects that are dependent on dosage, frequency, and time of use, and are accelerated and exaggerated by the combined use of two or more of these agents. However, since not everyone who engages in these high-risk behaviors develops oral SCC, and because oral SCC can be idiopathic, there must be person-specific genetic and environmental causes that either guard against the development of oral SCC or predispose to or even encourage the development of oral SCC.

Despite advancements in treatment methods, OSCC morbidity and mortality rates have remained relatively unchanged over the past 30 years. Percentages of morbidity and mortality in males are 6.6/100,000 and 3.1/100,000 respectively, while in females the same percentages are 2.9/100,000 and 1.4/100,000 [5]. Furthermore, OSCC is becoming more popular among young white people aged 18 to 44, especially among

white women [6]. Patients of OSCC have a 40-50 percent probability of surviving for five years. Despite the ease with which the oral cavity can be examined for medicinal purposes, OSCC is normally detected in its advanced stages. The two prominent factors are an erroneous original diagnosis and the patient's or attending physician's negligence [7]. The aim of present work was to evaluate and compare the salivary levels of nitric oxide, vitamin C, Total Sialic acid and GSH in cases of Oral Potentially Malignant Disorders (OPMDs) and Oral Squamous Cell Carcinoma (OSCC) and healthy controls.

2. MATERIAL AND METHODS

2.1. Material

Data was collected non-invasively from patients visiting the Jawaharlal Nehru cancer center, Bhopal and Dept. of Oral Medicine and Radiology, RKDF Dental College and Research Centre, Bhopal. The study was performed in the Department of Biochemistry, RKDF Dental College and Research Centre, Bhopal after obtaining approval from the university ethical committee.

2.2. Methods

2.2.1. Method of collection of data

Saliva from 20 patients with OSCC, 40 patients with OPMDs and 20 healthy subjects in the age group of 35 to 75 years was analyzed for levels of nitric oxide, vitamin C, Total Sialic acid and GSH using spectrophotometry.

Study group 1: Patients who were histopathologically diagnosed with OSCC (n = 20)

Study group 2: Patients who were histopathologically diagnosed with OPMD (n = 40)

Control group: Normal healthy individuals with clinically normal oral mucosa (n = 20)

All the patients were examined using the mouth mirrors and probes under the artificial light. Their history and clinical findings were recorded using the standard pro-forma after informed consents.

2.2.2. Sample Collection of Saliva

Five ml of unstimulated salivary sample was collected from each patient after rinsing and spitting with normal saline (0.9% v/v) for a period of one minute between 10 am to 12 pm to avoid circadian variations. Two milliliters of saliva was collected and transferred for biochemical analysis.

2.2.3. Processing of saliva samples

Saliva samples were immediately centrifuged (1000 g, 10 minutes) at 4°C to remove cell debris. The resulting supernatants were immediately transferred to 4 separate aliquots, The sample was centrifuged for about 15 minutes at 16,000 rpm for 5 minutes to remove the cellular components.

i. First group of aliquots were used for estimating Vitamin C
ii. Second group of aliquots were used for estimating NO
iii. Third group of aliquots were used for estimating Sialic acid

Fourth group of aliquots were used for estimating GSH

2.2.3.1. GSH

A method proposed by Carlberg and Mannervik (1975) [8] and modified by Mohandas et al. (1984) [9] was adopted to investigate GR. To 1.65ml of phosphate buffer, 0.05ml of 1mM GSSG, 0.1ml of 1mM β-NADPH and 0.1ml of 0.5mM EDTA were added. To the reaction mixture 0.05ml of saliva was added and vortexed for 10s. A decrease in the OD/30 seconds for about 3 minutes was monitored at 340nm by using Rolex UV Spectrophotometer. A blank solution was used without saliva. The enzyme activity was expressed in U/ml [8].

2.2.3.2. Nitrites

Nitric oxide (NO) was measured in terms of its products, nitrite (NO_2) and nitrate (NO_3), by the Griess method [10]. Values are expressed as μmol/L saliva.

2.2.3.3. Vitamin C

The serum levels of vitamin C were estimated by the phenyl-hydrazine spectrophotometry method as done by Lowry OH et al. [10]. Estimation of Ascorbic acid by Dinitrophenyl Hydrazine (DNPH) method [9-10].

2.2.3.4. Trichloroacetic acid

Vitamin C or Ascorbic acid is a good reducing agent. It undergoes conversion to its oxidized form dehydro-ascorbic acid which is reversible. Both these forms coupled with DNPH to yield an osazone which in turn gives a yellow color with sulfuric acid. Copper in the DNPH reagent functions as a catalyst and the intensity of the color is read at 520 nm.

2.2.3.5. Sialic acid

The saliva was centrifuged at 3000 rpm for 15 min. The protein-bound sialic acid in saliva was measured by thiobarbituric acid (TBA) method described by Skoza and Mohos [11].

2.3. Antioxidant-Oxidant Index (AOI)

AOI will calculated by calculating the ratio between the levels of nitric oxide, Vitamin C, total sialic acid and GSH peroxidation levels.

Post hoc Bonferroni's test analysis was used for the comparison of the two study groups to the control group. Correlation between the groups was done using Pearson's correlation coefficient test Nitric oxide free radicals (NO•), one of the free radical implicated in carcinogenesis, is a short lived free radical generated from l-arginine by the enzyme Nitric Oxide Synthase (NOS) [13]. The tendency of nitric oxide to bind with superoxide anions to form peroxynitrite, an oxidizing free radical that can induce DNA fragmentation and lipid peroxidation, contributes to its toxicity [14]. Angiogenesis, apoptosis, cell cycle, penetration, and metastasis are only a few of the cancer-related activities that nitric oxide affects. However, nitric oxide has been found to have a simultaneous effect in carcinogenesis, with both tumoricidal and carcinogenic effects [15]. Nonenzymatic antioxidants (e.g., Glutathione (GSH), vitamins C and D) and enzymatic antioxidants (e.g., Superoxide Dismutase (SOD), Glutathione Peroxidase (GPx), glutathione reductase, catalase) are classes of molecules that function as antagonists to oxidant species inside the body [16].

Vitamin C, also known as ascorbate or ascorbic acid (AA), is a ketolactone with a molecular weight of 176.13 g/ml and is regarded as one of the strongest reducing agents and a radical scavenger. Ascorbate inhibits the development of carcinogenic nitrosamines by reducing nitrates. Ascorbate-induced cytotoxicity appears to be mainly mediated by aggregation of H_2O_2 [16]. Calculating AOI to estimate antioxidant and oxidant levels in OPMD and OSCC could serve as an analytical biomarker for disease progression tracking. As a result, the current research was intended to measure the AOI in OPMD and OSCC patients by measuring salivary amounts of nitric oxide, vitamin C, total sialic acid, and GSH. There would also be an important correlation between NO and vitamin C levels and tumor staging. These levels might represent the tumour load and may help in assessing the severity of disease and also in the staging of HNSCC patients. The data is expressed as mean ± SD. The statistical significance of the results will be analyzed using post hoc Bonferroni's test.

3. RESULTS AND DISCUSSION

The study comprised of 80 subjects, there were 27 females (34%) and 53 males (66%) in the study group. (Graph 1). There were fifty-three males (controls-10; OPMD-30; OSCC-13) (Graph 2) and twenty-seven females (controls-10; OPMD-10; OSCC - 07; Graph 3). The mean age was 36.4±1.58 years in control group, 39.30±10.57 years in OPMDs and 49.25±15.58 years in OSCC. Out of forty OPMD patients, five (12.5%) were without any habit, eighteen (45%) with tobacco chewing, ten (25%) with smoking and seven (17.5%) with both tobacco chewing and smoking habits. Among twenty OSCC patients, one (5%) was without any habit, six (30%) with habit of tobacco chewing, four (20%) with smoking and nine (45%) with both smoking and tobacco chewing. Among forty OPMD patients, in twenty-five (62.5%) cases, the leukoplakia was present, oral submucous fibrosis was present in ninecases (22.5%) and lichen planus was present in six (15%) cases. Among twenty OSCC patients, in n=7 (35%) cases, lesions were present on buccal mucosa, in n=6 (30%) patients was present on tongue, in n=3 (15%) cases was present on tonsil, n=2 (10%) was present on alveolus, n=1 (5%) was present on palate and n=1 (5%) was present on retromolar trigone.

3.1. Descriptive Statistics of Variables

The data was recorded and analyzed statistically using SPSS software version 20.0 using one way ANOVA and post hoc Bonferroni's tests. Mean of controls and patients were compared using Student's t-test. The difference was considered statistically significant when p-value were 0.001 or less. The mean salivary vitamin C level was 30.32±4.34 µmol/l in OSCC group whereas; it was 38.20±8.45 µmol/l in OPMDs group and 48.76± 2.60µmol/l in control group. The mean nitric oxide level was 27.34±5.51µmol/l in OSCC group, 22.5± 2.33µmol/l in OPMD group and 10.11±0.88 µmol/l in control group. The glutathione reductase activity in control patients was found to be 0.0915 U/ml under optimal pH, temperature and K_m. In OPMD group, the GR activity was found to be 0.0515 U/ml. Similarly, the activity in the OSCC group was found to be 0.0292 U/ml. The total sialic acid (TSA) in the saliva of control patients was found to be 41.241±5.3312 μg/mL. In the case of OPMD patients it was 64.25±4.33 μg/mL and in the OSCC patients it was, 79.60±6.93 μg/mL As seen from the graphs above, the levels of salivary vitamin C and glutathione were significantly reduced and those of nitric oxide and sialic acid were raised in patients having OPMD's and oral squamous cell carcinoma. The anti-oxidant to oxidant index (AOI) was measured between

NO and vitamin C; NO and GSH; total sialic acid and vitamin C and lastly, total sialic acid and GSH. The following indices are tabulated below with the corresponding values.

Our results were consistent with the grouped analysis between various salivary biomarkers as can be from the tabulated data above. There was significant increase in AOI [NO/Vit.C] from control group (0.023), OPMDs (0.167) and OSCC group (0.279 and AOI [NO/GSH] from control group (0.012), OPMDs (0.122) and OSCC group (0.289). Similarly the increase was seen in AOI [TSA/Vit.C] from control group (0.093), OPMDs (0.467) and OSCC group (0.812) (Table 4). The AOI [TSA/GSH] showed increase from control group (0.052), OPMDs (0.351) and OSCC group (0.842) On comparing salivary vitamin C levels and GSH levels with those of the nitric oxide and sialic acid, the difference was highly significant ($p<0.003$). Though the levels were not significant between OPMD and OSCC group ($p>0.001$).

Table 1: Mean age distribution of the patients

Total No. of patients	Minimum age of Patient (Yrs.)	Maximum age of patient (Yrs.)	Mean age (Yrs.)	S.D.
80	35	75	57.8	11.98

Table 2: AOI in controls and study groups

Variable	Group	n	Mean	Std. Deviation	p-value
AOI NO/VIT.C	Control	20	0.207	0.023	<0.001
	OPMD	40	0.589	0.166	
	Cancer	20	0.901	0.279	

**ANOVA, p-value <0.001 considered statistically significant*

Table 3: AOI in controls and study groups

Variable	Group	n	Mean	Std. Deviation	p-value
AOI NO/GSH	Control	20	0.11	0.012	<0.001
	OPMD	40	0.436	0.122	
	Cancer	20	0.936	0.289	

**ANOVA, p-value <0.001 considered statistically significant*

Table 4: AOI in controls and study groups

Variable	Group	n	Mean	Std. Deviation	p-value
AOI TSA / VIT.C	Control	20	0.845	0.093	<0.001
	OPMD	40	1.687	0.466	
	Cancer	20	2.625	0.812	

**ANOVA, p-value <0.001 considered statistically significant*

Table 5: AOI in controls and study groups

Variable	Group	n	Mean	Std. Deviation	p-value
AOI TSA/GSH	Control	20	0.45	0.052	<0.001
	OPMD	40	1.247	0.351	
	Cancer	20	2.72	0.842	

**ANOVA, p-value <0.001 considered statistically significant*

Table 6: Pairwise comparison of AOI index in control and study groups

Dependent variable	(i) Group	(J) Group	Mean Difference (i-J)	p-value
AOI NO/VIT.C	Control	OPMD	-0.382*	0.001
		Cancer	-0.694*	0.001
	OPMD	Control	0.382*	0.001
		Cancer	-0.312*	0.001
	Cancer	Control	0.694*	0.001

**Post-hoc Bonferroni's analysis with ANOVA, p-value <0.001 considered statistically significant*

Table 7: Pairwise comparison of AOI index in control and study groups

Dependent variable	(i) Group	(J) Group	Mean Difference (i-J)	p-value
AOI NO/GSH	Control	OPMD	-0.326*	0.001
		Cancer	-0.826*	0.001
	OPMD	Control	0.326*	0.001
		Cancer	-0.500*	0.001
	Cancer	Control	0.826*	0.001

**Post-hoc Bonferroni's analysis with ANOVA, p-value <0.001 considered statistically significant*

Table 8: Pairwise comparison of AOI index in control and study groups

Dependent variable	(i) group	(J) group	mean Difference (i-J)	p-value
AOI TSA/VIT.C	Control	OPMD	-0.842*	0.001
		Cancer	-1.420*	0.001
	OPMD	Control	0.842*	0.001
		Cancer	-0.938*	0.001
	Cancer	Control	1.420*	0.001

**Post-hoc Bonferroni's analysis with ANOVA, p-value <0.001 considered statistically significant*

Table 9: Pairwise comparison of AOI index in control and study groups

Dependent variable	(i) group	(J) group	mean Difference (i-J)	p-value
AOI TSA/GSH	Control	OPMD	-0.797*	0.001
		Cancer	-2.270*	0.001
	OPMD	Control	0.797*	0.001
		Cancer	-1.473*	0.001
	Cancer	Control	2.270*	0.001

4. CONCLUSION

OSCC increases oxidative stress and may trigger mutations, suggesting that it may play a role in the initiation and development of multistage carcinogenesis. Understanding the function of reactive oxygen species (ROS) as key mediators in signaling pathways may open up new avenues for pharmacological intervention. Thus, the findings of the present study indicate that estimation of Vitamin C, NO, GSH and sialic acid can be suitably used and could assist in the early diagnosis of potentially malignant disorders and oral cancer using saliva.

Conflict of interest

None declared

5. REFERENCES

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