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Original Research article

A STUDY TO EVALUATE THE ROLE OF ANTIOXIDANTS AND IRON AS DIAGNOSTIC MARKERS IN ORAL CANCER

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ABSTRACT

Oral cavity (OC) and oropharyngeal (OP) cancers afflict about 500,000 people globally. Dental health care practitioners need to understand the causes, diagnosis, and treatment of oral cavities and oropharyngeal cancer.The constant and direct exposure of oral mucosal cells to tobacco product chemical carcinogens such as polynuclear aromatic hydrocarbons and nitrosamines causes the generation of free radicals/reactive oxygen species.Changes in salivary antioxidant and micronutrient levels may prove to be an important factor in the pathogenesis of oral premalignant and malignant disorders. The present study aimed to evaluate the levels of Vitamin C, MDA and iron levels in oral cancer, pre-malignant group and the oral cancer group. 15 oral cancer patients, 30 premalignant condition patients, and 15 normal controls were analyzed in the study. Among the 30 patients of the premalignant conditions- group (Group S1) 12 were diagnosed with oral submucous fibrosis, and 18 of them were diagnosed with oral leukoplakia. The oral cancer patients (Group S2) were segregated based on the TNM system from stages I to IV. The oral submucous fibrosis patients were subdivided into four grades. The oral leukoplakia patients were categorized into homogenous and speckled types. A detailed history was obtained from the patients. After obtaining informed consent, saliva samples will be collected from patients who have not consumed any food or any form of tobacco 2 hours before collection. Following a thorough mouth rinse using distilled water, saliva is allowed to accumulate in his or her mouth for 5 minutes. Accumulated saliva is collected by the spit method.5ml of blood sample will also be collected from these patients, Serum was separated by Centrifugation. Collected serum and saliva were stored at -80°C until further analysis. The serum and salivary samples were then analyzed for Vitamin C, iron, and malonaldehyde (lipid peroxidation)according to the standardized protocol.In the present study, a total of 60 samples were screened for Vitamin C, Iron, and MDA in both serum and saliva of normal control, premalignant, and oral cancer patients. We found that there was a decreased level of Vitamin C and iron in both serum and saliva of premalignant and oral cancer patients. MDA level was higher in premalignant, and oral cancer patients and there was a significant increase in the level of MDA in oral cancer patients. Understanding antioxidants can help reduce the incidence of oral cancer in its early stages. More study is needed before this supplement may be recommended as an adjuvant treatment.

Key Words: Oral Cancer, Leukoplakia, Antioxidants, MDA

INTRODUCTION

Oral cavity (OC) and oropharyngeal (OP) cancers afflict about 500,000 people globally. Dental health care practitioners need to understand the causes, diagnosis, and treatment of oral cavities and oropharyngeal cancer. The five-year survival rate has increased from 50% to 65% during the past 30 years. Improved survival is attributed to surgical factors such as microvascular repair, clearer grounds for elective neck dissection, and liberal treatment of the contralateral neck. Various treatments, including combined chemoradiation, intensity-modulated radiation therapy, targeted therapy, and immunotherapy, have helped improve outcomes[1].The Globocan 2020 study focuses on the overall burden of cancer incidence and mortality as a cause of premature death. Lip and oral cavity cancer accounts for around 0.37 million new cases and 0.17 million deaths[2]. Oral cancer, an epithelial disease, has a complex aetiology that includes genetic, epigenetic, behavioural (tobacco/areca nut/cigarette/alcohol), and microbiological variables that differ by geography or ethnic group. Several histological changes have been reported in normal mucosa as oral cancer progresses. These changes include hyperplasia, dysplasia, carcinoma in situ, and, eventually, mouth cancer[3,4].

A white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer" [5]. Several attempts have been made to identify leukoplakia since the presence of other oral white lesions complicates proper identification and classification, leading in uncertainty and ambivalence in consistent reporting of this lesion [6]. Shanbhag et al. proposed using the term "white lesion" rather than "white plaque" since other oral lesions may interfere with the identification of leukoplakia due to common etiological and clinical aspects.The new definition of leukoplakia was proposedas "a predominantly white, irreversible, non-scrapable lesion of the oral mucosa that cannot be characterized clinically or histopathologically as any other lesion/disease and has increased risk of cancer occurrence than its normal counterpart and is usually associated with consumption of tobacco, betel quid, and alcohol, but otherwise can be of idiopathic in nature"[7].

The constant and direct exposure of oral mucosal cells to tobacco product chemical carcinogens such as polynuclear aromatic hydrocarbons (PAH) and nitrosamines causes the generation of free radicals/reactive oxygen species (ROS). Free radicals are molecules with an unpaired electron in their exterior orbit and are hence, extremely reactive. ROS include superoxide anion radicals (O−2), hydroxyl radicals (HO), hydroperoxyl (HO2), peroxyl (ROO), alkoxyl (RO), and hydrogen peroxide (H2O2) . ROS and reactive nitrogen species (RNS) have a positive impact on cellular responses and immunological function at low to moderate levels. However, at greater levels, ROS causes a variety of diseases[8].OS interactions with biological molecules harm the lipid biomembrane and the sulfhydryl linkages of proteins and carbohydrates[9]. The bio-membrane lipid peroxidation damage is caused by the abstraction of hydrogen from unsaturated fatty acids. The produced free radicals start a chain reaction that leads to total degradation of the cellular membrane, which is critical in cancer development. Furthermore, the degradation of these peroxidized lipids occurs quickly and produces reactive carbon molecules such as lipid hydroperoxides (LHP) and malondialdehyde (MDA). These byproducts act as a sign of lipid peroxidation. These lipid peroxidation products can influence cell proliferation and tumour progression by stimulating the signal transduction system. Furthermore, they serve as co-carcinogenic

agents, exhibiting their high cytotoxicity[10].Iron is an essential nutrient, and iron deficiency is a prevalent cause of malnutrition worldwide. The observation of a high incidence rate of alimentary tract cancer among Swedish women in the early twentieth century led to the hypothesis that insufficient iron consumption could induce oral cancer. Many of these ladies had Plummer-Vinson (Patterson-Kelly) syndrome, which is defined by nutritional inadequacies, especially iron deficiency. The inclusion of iron supplements in Swedish meals resulted in a decrease in Swedish mouth cancer incidence rates[11].In 2015, the International Head and Neck Cancer Epidemiology (INHANCE) group showed that taking vitamin C from food can prevent oral cancer [12], and later in 2017 [13], they found that taking more fibre might reduce the incidence of head and neck cancer [14].The study also attempts to determine the changes in micronutrient levels in the saliva. There is also a need to evaluate whether the changes in antioxidant and micronutrient levels in serum correlate to that of saliva. Changes in salivary antioxidant and micronutrient levels may prove to be an important factor in the pathogenesis of oral premalignant and malignant disorders. Salivary antioxidant and micronutrient evaluation could soon be a crucial marker for early detection of oral cancer thereby enabling better management of this life-threatening disease entity.Hence the present study aimed to evaluate the levels of Vitamin C, MDA and iron levels in oral cancer, pre-malignant group and the oral cancer group.

METHODOLOGY

Ethical Clearance:

This study was approved by the Ethics committee of A.B.Shetty Memorial Institute of Dental Sciences, Deralakatte, Mangalore. 60 patients reporting to the Department of Oral Medicine and Radiology A.B.Shetty Memorial Institute of Dental Sciences, Deralakatte, Mangalore were recruited for the study after taking informed consent.

Sample collection

15 oral cancer patients, 30 premalignant condition patients, and 15 normal controls were analyzedin the study.Among the 30patients of the premalignant conditions- group (Group S1) 12 were diagnosed with oral submucous fibrosis, and 18 of them were diagnosed with oral leukoplakia.The oral cancer patients (Group S2) were segregated based on the TNM system from stages I to IV. The oral submucous fibrosis patients were subdivided into four grades. The oral leukoplakia patients were categorized into homogenous and speckled types.A detailed history was obtained from the patients. After obtaining informed consent, saliva samples will be collected from patients who have not consumed any food or any form of tobacco 2 hours before collection. Following a thorough mouth rinse using distilled water, saliva is allowed to accumulate in his or her mouth for 5 minutes. Accumulated saliva is collected by the spit method.5ml of blood sample will also be collected from these patients, Serum was separated by Centrifugation. Collected serum and saliva were stored at -80°C until further analysis.The serum and salivary samples were then analyzed for Vitamin C, iron, and malonaldehyde (lipid peroxidation).

Estimation of Vitamin C

Standardisation: The standardization protocol should be carried out as given

below Plot the optical densities of the standard solutions on a graph

against their respective concentrations.

Sl.No.	Vol. of std.VitC(mL)	Conc. ofstd. Vit C(mL)	Vol.of5%TCA(mL)	Vol. ofDTCreg(mL)		Vol. of4.5MH_2SO_4(mL)	O.Dat512nm
B	0.0	0.0	1.0	0.4mL	INCUBATE AT 60^oC INWATERBATHFOR60MINUTES	2mL	
1	0.2	0.4	0.8	0.4mL		2mL	
2	0.4	0.8	0.6	0.4mL		2mL	
3	0.6	1.2	0.4	0.4mL		2mL	
4	0.8	1.6	0.2	0.4mL		2mL	
5	1.0	2.0	0.0	0.4mL		2mL	

100 µL of the sample (serum/Saliva) was taken in a clean test tube. 900 µL of 5% TCA was added to it and allowed to precipitate proteins for about ten minutes andcentrifuged.500 µL of the supernatant was taken and transferred into another test tube.To this 200 µL of DTC reagent added, plug the tube and incubated the mixture at 60 o C for60 min in a water bath.Simultaneouslya blank with 1 mL of TCA and 200 µL of DTC reagent was also maintained under similar conditions. The reaction mixture was cooled following 60 minutes of incubation, in an ice bath. 1mL of 4.5M sulphuric acid added to it and cooled to room temperature. Optical density was measured at 540 nm against blank.

Estimation of Iron

Procedure Standardization:

Working standard (Ammonium ferrous sulfate) prepared in aliquots of 0.1, 0.2, 0.3, 0.4,

0.5mL respectively in different test tubes.The volume of solution was made up to 1mL by adding deionized water.2 ml of the chromogen solution was added to all the tubes. Placed at room temperature for 5minutes. The optical density of all the reaction mixtures was measured Spectro photometrically at 535nm against the blank replacing the working standard with deionised water.The obtained optical densities was then plotted on a graph with the concentrations on theX-axis and the respective optical densities along the y-axis.

Estimation of iron in sample

100 µL of the sample (serum/saliva)was taken in clean microcentrifuge tube and makeup to 250µL with deionized water.Added 500 µL of protein precipitating solution.Centrifuged the mixture at 2000 rpm for 10 minutes.500 µL of the supernatant was taken and added to 500 µL of the chromogen solution. The optical density immediately (within 10 minutes) measured at 535nm against a blank treated ina similar way as the test wherein the sample is replaced with the deionized water

Calculation

The concentration of iron in the sample is obtained by plotting the optical densities of the test

against the standard graph. The obtained concentration is then multiplied by 2.5 (dilution factor).

Estimation of MDA

MDA has frequently been measured in serum thiobarbituric acid – reactive substances (TBAARS) assay ccording to Kei (1978). Here TBA reacts with MDA to form pink 2:1, maximally at 532 nm. This colored complex was measured by a spectrophotometer. The optical densities of the test samples is directly proportional to the concentration of MDA in the sample and calculated by the plotting

against the standard graph and multiplied by the respective dilution factors the final concentration is expressed as µM/L.

Statistical analysis

Statistical analysis was carried out using). Results were expressed as mean ± Standard Deviation (SD). All data collected from experiments were performed in three replicates and analyzed using HSD Tukey and ANOVA test. A value of P *P < 0.05, **P < 0.01, ***P < 0.001, and **** P < 0.0001 are considered statistically significant.

RESULTS AND DISCUSSION

A total of 60 samples were screened for Vitamin C, Iron, and MDA in both serum and saliva of normal control, premalignant, and oral cancer patients. Among them were 15 patients of group S2, which encompassed 12 male and 3 female patients. Majority of the patients of group S2 belonged to stage I oral cancer. Four patients had Stage II and four of them had Stage III. Only 2 subjects had Stage IV oral cancer. Most of the patients in group S2 were tobacco users (13), and only 2 female patients were nontobacco users. Group I encompassed 12 patients with OSMF and 18 with oral leukoplakia. Five patients with OSMF were in grade I condition. Two patients each in grade II and IV condition. And 3 subjects in grade III condition. Oral leukoplakia patients were subdivided into homogenous and speckled varieties. The majority of the patients (12) oral leukoplakia patients were diagnosed with a homogenous variety of leukoplakia and 6 of them had the speckled variety.15 controls were enrolled in the study during the first half year out of them 9 subjects were tobacco users and 6 non-tobacco users. Only one female subject was a tobacco user. Among the non-tobaccousers, 3 subjects werefemales.

Serum analysis of Vitamin C showed that in group S2 was 1.06667 ± 0.790539 mg/dL, group S1 1.30000±0.4568322 mg/dL, and in group C 1.48889 ±0. 4728754 mg/dL.The mean values of serum iron levels in group S2 were75.2667 ± 41.7141289 µg/dl, group S1 was 94.529412 ± 521321849 µg/dl and group C was 98.4444 ± 13.5932990 µg/dl.The mean values of serum malonaldehyde in group S2 were 1.56667 ± 0.825549 µM/L, group S1 was 0.77826 ± 0.5230829 µM/L and group C was 0.655556 ± 0.150923 µM/L (Table 1).

Table 1: Mean values of Vitamin C, Iron and MDA in the serum sample

Parameter	Control	Group S1 (Premalignant)	Group S2 (Oral cancer)
Vitamin C (mg/dL)	1.48889 ± 0.4728754	1.30000±0.4568322	1.06667 ± 0.790539
Iron (µg/dl)	98.4444 ± 13.5932990	94.529412 ± 52.1321849	75.2667 ± 41.7141289
MDA (µM/L)	0.655556 ± 0.150923	0.77826 ± 0.5230829	1.56667 ± 0.825549

Saliva analysis of Vitamin C showed that in group S2 was 0.46667 ± 0.1926361mg/dL, group S1 was 0.695238 ± 0.7736324mg/dl and in group C 0.833333 ± 0.5230649mg/dL. The mean values of salivary iron levels in group S2 were75.0000 ± 50.1611688 µg/dl, group S1 was 85.91667 ± 50.0444499 µg/dl and group C was 88.1111 ± 21.4385427µg/dl. The mean values of salivary malonaldehyde in group S2 were 0.440000 ± 0.2501428 µM/L, group S1 was 0.40000 ±0.2533980 µM/L and group C was 0.30000 ±-0.1581139 µM/L.

Table 2: Mean values of Vitamin C, Iron and MDA in the saliva sample

Parameter	Control	Group S1 (Premalignant)	Group S2 (Oral cancer)
Vitamin C (mg/dL)	0.833333 ± 0.5230649	0.695238 ± 0.7736324	0.46667 ± 0.1926361
Iron (µg/dl)	88.1111± 21.4385427	85.91667 ± 50.0444499	75.0000 ± 50.1611688
MDA (µM/L)	0.30000 ± 0.1581139	0.40000 ±0.2533980	0.440000 ±0. 2501428

Intercomparison of the three groups using the one-way ANOVA revealed that there was no significant difference in the serum vitamin C, serum iron and MDA levels (Table 3).

Intercomparison of the three groups using the one-way ANOVA revealed that there was no significant difference in the serum vitamin c and serum iron levels. However, serum MDA levels showed a significant difference (P= 0.001) in all three groups (Table 3). The groups were further subjected to multiple comparisons using the HSD Tukey test (Table 4). The serum MDA levels of the oral cancer group differ significantly from the precancer group as well as the controls. There is also a significant difference in the levels of serum MDA levels between precancer and control.

Table 3- Intercomparison of Group S2, group S1 and Group C for serum vitamin C, serum Iron and serum MDA levels with ANOVA test.

Parameters	P value
Serum Vitamin C	0.459
Serum iron	0.526
Serum MDA	0.001**

Table 4- Intercomparison of group S2, group S1 and Group C for salivary vitamin C, saliva Iron and saliva MDA levels with ANOVA test.

Parameters	P value
Serum Vitamin C	0.093
Serum iron	0.137
Serum malonaldehyde	0.377

DISCUSSION

Oral cancer is a major global issue in public health. Oral carcinomas often develop from premalignant lesions. Most oral malignancies are squamous cell carcinomas. WHO defines "oral squamous cell carcinoma" as an epithelial neoplasm with varying degrees of squamous differentiation and a propensity for early and extensive lymph node metastasis. It typically affects alcohol and tobacco users in their fifth and sixth decades of life[15].

Free radicals are constantly produced in our bodies.3 The mitochondria in cells generate free radicals when they consume oxygen for energy production. Once formed, highly reactive radicals can initiate a chain reaction. Free radicals have a strong affinity for lipids, proteins, and DNA. Antioxidants neutralize radicals by giving electrons, halting the electron-taking process. Antioxidant nutrients are stable in both forms and do not become free radicals by donating electrons[16].

In the present study, a total of 60 samples were screened for Vitamin C, Iron, and MDA in both serum and saliva of normal control, premalignant, and oral cancer patients. We found that there was a decreased level of Vitamin C and ironin both serum and saliva of normal control, premalignant, and oral cancer patients. MDA level was higher in premalignant, and oral cancer patients and there was a significant increase in the level of MDA in oral cancer patients. Previous studies by et al have shown a significant decrease in the level of vitamin C in oral cancer patients compared to the control group [17]. Low vitamin C levels dramatically increase the risk of oral cancer. Vitamin C levels were also found to be associated with tumour grades of the patient, hence measuring vitamin C in the blood of oral cancer patients may be useful in determining the tumour grade. Tobacco users, particularly gutkha chewers, have reduced serum levels of the antioxidant vitamin C. Low antioxidant nutrients, notably vitamin C, and tobacco use can be considered key risk factors in the progression of oral cancer. Previous studies have also shown the decreased level of iron in the serum of oral cancer patients[18]

Previous studies have also shown the decreased level of MDA in the serum of oral cancer patient [19, 20]. Increased serum malondialdehyde in oral cancer and precancer would be a useful marker for both preventive and clinical intervention, and it may warrant further exploration for early detection, treatment, and prognosis.

CONCLUSION

Understanding antioxidants can help reduce the incidence of oral cancer in its early stages. Recent clinical research indicates that antioxidants may benefit oral leukoplakia and other precancerous lesions. The role of natural foodstuffs, such as fruits and vegetables, in preventing disease is yet unclear. More study is needed before this supplement may be recommended as an adjuvant treatment.

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Authors Contribution

All authors made equal contributions in the Conception and design, acquisition, analysis and interpretation of the data. All authors approved the final version of the manuscript to be submitted.

Conflict of Interest: None

Ethical approval: The study is approved by the ethics committee of A.B Shetty Memorial Institute of Dental Science. Informed consent was taken before sample collection.

Data Availability

Author declare that all the data are available within the manuscript.

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