Original Article

8-Isoprostane: A salivary oxidative stress biomarker for oral submucous fibrosis and oral squamous cell carcinoma

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Abstract

Background: 8-isoprostane is one of the stable oxidative stress marker formed by the lipid peroxidation of arachidonic acid. It is present in detectable quantities in all biological fluids. Elevation of 8-Isoprostane has been reported in various neurological, cardiological disorders, and periodontal diseases.

Aim: The present study was conducted to estimate and compare the level of 8-isoprostane in plasma and saliva in patients with oral squamous cell carcinoma (OSCC), oral submucous fibrosis (OSMF), and in controls. The study also aimed to find out if 8-isoprostane can be used as an effective oxidative stress marker in evaluating the disease progression in OSCC.

Materials and Methods: Plasma and salivary samples were taken from 10 cases each of clinically diagnosed OSMF, clinically and hisotpathologically diagnosed cases of OSCC and controls. The samples were subjected to 8-Isoprostane ELISA procedure and analyzed. Statistical analysis was performed using the SPSS software.

Results: The levels of 8-isoprostane in plasma showed an average increase from normal to OSMF to OSCC but was not statistically significant. The variations in the level of salivary 8-isoprostane were found to be statistically significant ($P = 0.037$) suggesting that there is a gradual increase in levels of isoprostane from controls to OSMF to OSCC.

Conclusion: The results showed that the concentration of isoprostane in saliva showed a progressive and steady increase from control through OSMF to OSCC indicating that saliva could be used as an effective diagnostic tool in estimating tumor markers. Large scale studies correlating with other potentially malignant oral disorders are required to ascertain the role of 8-Isoprostane as an ideal tumor marker.

Keywords: ELISA, isoprostane, oral squamous cell carcinoma, oral submucous fibrosis, oxidative stress, plasma, saliva

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INTRODUCTION

Reactive oxygen species (ROS) are the free radicals and the oxidants that are derived exogenously due to pollution, radiations, chemicals, pathogens, etc., and endogenously as metabolic intermediates or end products. Reactive oxygen species (ROS) encompasses a wide range of molecules including superoxide, hydrogen peroxide, hydroxyl radical, and peroxynitrite.[1,2] In physiological conditions, ROS is

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produced in picomolar concentrations[3] and are usually removed rapidly because they cause damage to nucleic acids, proteins, and lipids of the cellular and extracellular matrix leading to cellular dysfunction and eventual cell death.[4] Oxidative stress is considered as disruption of redox signaling and control[5,2] leading to many diseases such as cancer, cardiovascular diseases,[6] neurological disorders,[7] renal disorders,[8] liver disorders,[9] and degenerative disorders.[10,11]

The role of ROS in potentially malignant oral disorders is still under research. Oral submucous fibrosis (OSMF) is a potentially malignant disorder which is predominantly seen in Asian countries, prevalence being more in India where more than 5 million people have been reported to be suffering from this premalignant condition.[12] Under alkaline conditions (due to the presence of lime) areca nut ingredients release ROS.[13] OSMF has one of the highest rates of malignant transformation among potentially malignant oral lesions and condition in the range of 7%–13%.[14,15] For the transformation from premalignancy to malignancy oxidative stress plays a major part which has been established by authors.[16] The development of human cancer is multifactorial and ROS are found to be involved in both initiation and promotion of multistep carcinogenesis.[17] In India and South– East Asia, the most common malignant neoplasm of the oral cavity is the oral squamous cell carcinoma (OSCC) and it accounts for up to 40% of all malignancies.[18] The etiology of OSCC includes various carcinogens in tobacco and related products such as polynuclear aromatic hydrocarbons, and nitrosamines which exposes the oral epithelium to ROS.[19]

The most widely used markers for oxidative stress are malondialdehyde, exhaled volatile alkanes, and lipid hydroperoxides.[20] However, these markers are of limited value as they lack sensitivity and or specificity.[21] Morrow and Roberts (1991) discovered isoprostanes which are prostaglandin such as substances produced *in vivo* primarily by free radical induced peroxidation of arachidonic acid. They are synthesized in cell membrane and are released into the circulation by the action of phospholipases. They circulate in the plasma and are uptaken and metabolized by the tissues.[22] 8-iso-PGF2α also known as 15 F2t isoprostane/8-isoprostane is a major isoprostane and is thought to be associated with oxidant injury. They are formed only after endogenous antioxidants are exhausted.[23] Isoprostane is now emerging as a gold standard oxidative stress marker[24] and its advantages over other oxidative stress markers are that they are chemically stable, specific products of peroxidation, present in detectable amounts in all normal tissues and biological fluids[25] including exhaled breath condensate[26] and their levels increase substantially in oxidant injury. They are used as the disease marker in tissue fibrosis, prostate, and lung cancer therefore suggesting the use of 8-isoprostane as a potential marker in oral cancer and OSF.[27]

The aim of the study is to understand the role of ROS and assess the potential risk of malignant transformation of OSMF into OSCC by measuring the levels of 8-isoprostane in plasma and saliva in patients with OSMF and OSCC and comparing it with the control group.

MATERIALS AND METHODS

The study was approved by the Institutional Ethical Committee of SRM Dental College, Ramapuram, Chennai. Patients for the study were recruited from the Outpatient Department of SRM Dental College and Referrals from Private Dental clinics.

- Control patients (Group I) – 10 cases
- OSMF (Group II) – 10 cases
- OSCC (Group III) – 10 cases.

The inclusion criteria include clinically diagnosed OSMF patients, clinically and histopathologically diagnosed cases of OSCC, normal patients without any associated habits of smoking, alcohol, and chewing and patients belonging to the age group of 15–65 years. Both males and females are included in the study. The exclusion criteria are OSMF patients who are currently under treatment, OSCC patients who have undergone treatment or currently undergoing treatment, patients with a history of systemic illness, patients with other oral premalignant disorders, patients with infections and inflammatory conditions of oral cavity. Complete case histories and verbal and written consent were obtained from all patients enrolled in the study. Blood and salivary samples were obtained from patients in all three groups and tissue samples were obtained from patients in Group III and diagnosis was confirmed by histopathology.

Five milliliter of unstimulated saliva was collected in a sterile uricup and transferred to a sterile centrifuge tube. 2.5 ml of peripheral blood were drawn from patients using standardized phlebotomy procedures and transferred into an ethylenediaminetetraacetic acid coated centrifuge tube. The samples were centrifuged in a cooling centrifuge at 3000 rpm for 15 min at 4C and the resultant supernatant and plasma was separated into 2 ml aliquots and stored at −80C for further analysis. ELISA procedure was performed using 15-Isoprostane F2t Elisa Kit (Product Number–EA 84) manufactured by Oxford Biomedical Research, USA, and Wash Well Plate ELISA washer and Readwell touch

Automatic ELISA plate analyzer version 3.30 manufactured by Robonik Company. The procedure was done after proper standardization and calibration.

RESULTS

A total of 30 patients were taken for the study and the level of isoprostane was identified and quantified using ELISA and statistical analysis of the data obtained was done using SPSS software (SPSS Inc., IBM 2009). The descriptive statistics were calculated for the individual groups and comparison between individual groups and the overall comparison in both plasma and saliva was done using Mann–Whitney test[28] and Kruskal Wallis test.[29]

The age-wise data indicate that the distribution of the samples was between 20 and 70 years with more number ($n = 10$) of patients above the age of 50 years. In the three groups, the number of males ($n = 19$) were more than the number of females ($n = 11$) [Table 1]. The mean levels of 8-isoprostane in plasma was found to increase from normal group to OSMF to OSCC [Table 2 and Chart 1]. The levels were not statistically significant (Mann–Whitney test $P = 0.284$) [Table 3]. The average 8-isoprostane level in saliva was found to be 3.2 ng/mL, 5.5 ng/mL, and 8.5, respectively for the normal, OSMF, and OSCC groups [Table 4 and Chart 2]. The comparison between the control group and the OSCC group revealed that the salivary levels were statistically significant with a $P = 0.015$. On comparing all the three groups the variations in the level of isoprostane in saliva was again found to be statistically significant with a P value ($P = 0.037$) suggesting that there is a gradual increase in levels of isoprostane from controls to OSMF to OSCC indicating the role of oxidative stress in the progression of OSCC [Table 5].

Table 1: Distribution of demographic variables by group

Variable	Control	OSMF	OSCC
Age			
20-29	3 (30.0)	8 (80.0)	0
31-39	1 (10.0)	1 (10.0)	1 (10.0)
40-49	3 (30.0)	1 (10.0)	2 (20.0)
≥50	3 (30.0)	0	7 (70.0)
Sex			
Male	5 (50.0)	8 (80.0)	6 (60.0)
Female	5 (50.0)	2 (20.0)	4 (40.0)

OSMF: Oral submucous fibrosis, OSCC: Oral squamous cell carcinoma

Table 2: Levels of 8-isoprostane in plasma in the three groups

Serial number	Control (ng/mL)	OSMF (ng/mL)	OSCC (ng/mL)
1	562.300	585.6	444.5
2	4.223	469.1	289.5
3	0.002	583.3	412.3
4	593.700	337.7	441.9
5	627.800	251	522.4
6	513.000	269	476.9
7	39.910	249.5	767.3
8	0.030	1.163	591.0
9	109.800	578.7	395.7
10	514.000	1.666	587.3
Mean	296.471	332.673	492.880

OSMF: Oral submucous fibrosis, OSCC: Oral squamous cell carcinoma

Table 3: Comparison of 8-isoprostane level in plasma between the 3 groups

Group	Mean (ng/mL)	Mann-Whitney test	*P*	Kruskal-Wallis test	*P*
Control	296.477	0.221	0.853	2.519	0.284
OSMF	332.673				
Control	296.477	0.983	0.353		
OSCC	492.883				
OSMF	332.673	1.736	0.089		
OSCC	492.883				

OSMF: Oral submucous fibrosis, OSCC: Oral squamous cell carcinoma

Table 4: Levels of 8-isoprostane in saliva in the three groups

Serial number	Control (ng/mL)	OSMF (ng/mL)	OSCC (ng/mL)
1	2.80	19.96	4.094
2	3.243	4.837	4.361
3	2.120	3.717	16.860
4	2.464	4.242	3.134
5	3.494	4.728	3.171
6	4.027	4.784	9.884
7	4.799	2.600	4.140
8	4.128	3.360	4.402
9	2.523	2.517	31.69
10	2.169	4.769	3.623
Mean	3.177	5.551	8.536

OSMF: Oral submucous fibrosis, OSCC: Oral squamous cell carcinoma

DISCUSSION

OSMF is a chronic insidious disease affecting the oral cavity. The risk of malignant transformation is relatively very high even after the control of tobacco use. OSCC is the most common head and neck malignancy accounting for about 40%–50% of all cancers in India.[30] ROS exert a key role affecting several hallmarks of cancer.[31] Isoprostanes are metabolites of lipid peroxidation which are a sensitive and specific oxidative stress marker. They also have numerous biologic effects thereby functioning as pathophysiologic mediators of oxidative stress.[1,23] The levels of 8-isoprostane were studied in OSMF and OSCC and compared with normal individuals and the plasma levels of isoprostane showed variations within the control group. The reason for this variation could be attributed to various factors such as age, gender, dietary pattern, physical exercise, body mass index, psychological stress, and smoking and alcohol consumption.[1,32-40]

Isoprostane has been studied in various pathologic conditions such as periodontitis, atherosclerosis, systemic sclerosis and in malignancies such as prostate cancer and

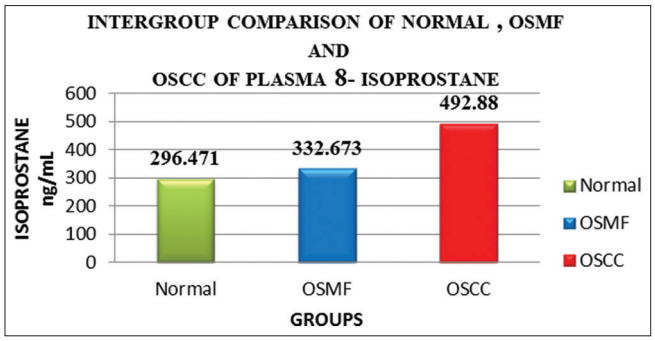

Chart 1: Intergroup comparison of plasma 8-isoprostane

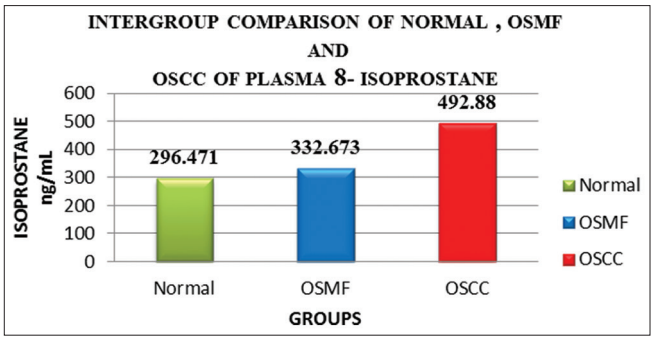

Chart 2: Intergroup comparison of saliva 8-isoprostane

Table 5: Comparison of 8-isoprostane level in saliva between the three groups

Group	Mean (ng/mL)	Mann-Whitney test	*P*	Kruskal-Wallis test	*P*
Control	3.177	1.965	0.052	6.601	0.037
OSMF	5.554				
Control	3.177	2.416	0.015		
OSCC	8.536				
OSMF	5.554	0.227	0.853		
OSCC	8.536				

OSMF: Oral submucous fibrosis, OSCC: Oral squamous cell carcinoma

esophageal squamous cell carcinoma.[41,42] This study is one of its kind where salivary levels of 8-isoprostane has been estimated and both saliva and plasma isoprostane levels have been studied in potentially malignant oral disorders in comparison with OSCC. The isoprostane levels in plasma varied between the three groups. The mean value of plasma isoprostane was showing a gradual increase from the control group (296.471 ng/mL) to OSMF group (332.673 ng/mL) to OSCC group (492.880 ng/mL). This reflects that the essential level of cellular oxidation which occurs as a result of aerobic metabolism is significantly altered in the test groups when compared to the normal individuals.[38]

Many oxidative stress markers have been studied in different cancers and were found to be increased in cancer patients when compared to the normal group. The results of the present study were similar to the previous studies such as Beevi *et al*. in 2004,[43] and Manoharan *et al*. in 2005,[44] and Raghavendra *et al*. in 2010.[45] These studies suggest that in OSCC elevated levels of oxidative stress marker may be due to the increased formation of free radicals and inadequate clearance of free radicals by the cellular antioxidants such as G-SH and superoxide dismutase activity resulting in increased levels of oxidative stress. The mean plasma levels of isoprostane when compared between the three groups (control, OSMF, and OSCC) showed an increase from normal to OSMF to OSCC but the values were not statistically significant when compared individually (Control vs. OSMF $P = 0.853$, Control vs. OSCC $P = 0.353$, OSMF vs. OSCC $P = 0.089$ respectively) as well as in overall comparison ($P = 0.284$).

Studies using saliva as the biological sample in oral cancers have increased greatly in the recent years as saliva is a medium which can be easily collected and that it is in contact with the lesion makes the measurement of tumor marker in saliva as an alternative to plasma testing as put forth by Shpitzer *et al*. 2009.[46] Hence, it is interesting to study the levels of salivary isoprostane in comparison with plasma. However, no studies have been done to evaluate the isoprostane levels in PMODs in comparison with OSCC. Similar to the levels of plasma isoprostane, the mean salivary levels of isoprostane also showed alterations between the three groups which was statistically significant in the overall comparison ($P = 0.037$). The levels were highest in the OSCC group with mean of 8.536 ng/mL followed by the OSMF group with 5.551 ng/mL and then the control group with the value of 3.177 ng/mL.

The elevated levels of isoprostane in saliva of patients with OSCC compared to control group could be attributed to the fact that a synergistic deleterious interaction exists between saliva and toxic metabolites which may result in the rapid destruction of biological macromolecules. The reaction between redox-active metals in saliva and low reactive free radicals is responsible for the lethal synergistic effect. When exposed to these metabolites, saliva loses its antioxidant capacity and becomes a potent pro-oxidant milieu as found by Nagler *et al*. 2006.[47]

SUMMARY AND CONCLUSION

Isoprostane levels were found in detectable quantities in saliva and plasma in OSMF, OSCC, and control groups. The mean concentration of isoprostane in plasma and saliva was increased in OSCC when compared to OSMF and control groups. The control group showed the least levels. This indicates that oxidative stress plays a role in initiation and progression of OSCC and localized

alteration in the oxidative status occurs in the tumor and its microenvironment. The salivary isoprostane levels in our study showed a significant increase in OSCC group when compared to OSMF and control groups than the plasma isoprostane levels; indicating saliva can be used as an alternative diagnostic tool but more studies with larger samples have to be done to evaluate the variations among the normal individuals, in tobacco-related PMOD's such as leukoplakia, erythroplakia, in conditions like Lichen planus in addition to OSCC. Even among the PMODs, the samples can be divided into various groups according to the degree of dysplasia and in the same way in OSCC; the subgroups can be made according to the histological grading and then evaluated for the levels of isoprostane. This can help us to determine the sensitivity and specificity of isoprostane as an effective oxidative stress biomarker in PMODs, OSCC, and other oral lesions.

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Conflicts of interest

There are no conflicts of interest.

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