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Salivary analysis of reactive oxygen species in oral submucous fibrosis and oral squamous cell carcinoma patients

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Abstract

In this in vitro study, evaluation of biochemical diagnostic markers like prooxidants, free radicals, total protein content and trace elements has been carried out. Significant elevation in the level of lipid peroxides ($p<0.001$) in Oral Squamous Cell Carcinoma (OSCC) was found when compared to Oral Sub Mucous Fibrosis (OSMF) patients. Conjugated dienes ($p<0.001$) were found to be increased in OSMF compared to OSCC patients. There were increased levels of hydroxyl and hydrogen peroxides radicals ($p<0.001$) in OSMF compared to OSCC patients. An elevated level of trace elements ($p<0.001$ & $p<0.05$) was found in OSMF than OSCC patients. High level of zinc & iron ($p<0.001$) were found in OSCC patients. Total protein content and trace elements alone were found in normal healthy individuals. No detectable amount of prooxidants and free radicals were observed in normal healthy individuals. This shows that the above parameters may be used as good biochemical diagnostic marker for the detection of oral cancer at early stages of cancer development.

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Introduction

Chemical compounds and reactions capable of generating potential toxic oxygen species / free radicals are referred to as pro - oxidants. On the other hand, compounds and reactions disposing off these species, scavenging them, suppressing their formation or opposing their actions are called antioxidants. In a normal cell, there is an appropriate pro oxidant - antioxidant balance. This balance can be shifted to pro oxidant when productions of free radicals are increased and which results in serious cell damage (Irshad and Chaudhuri, 2002). In comparing salivary CA 125 concentrations among healthy controls, women with breast cancer, found a significant elevation of salivary CA 125 concentrations among ovarian cancer, their results suggested that it is a better diagnostic value than the comparable serum assay (Di *et al.,* 1990). **Genetic biomarker isolated in saliva predicted oral cancer (David and Wong, 2005). The above words made the people to focus on saliva to find out biochemical markers for oral cancer** and precancerous stages. Human saliva is an attractive diagnostic fluid because saliva collection is less invasive than the collection of blood for serum / plasma analyses Len, 2005. In addition blood concentrations of many components are reflected in saliva. A number of studies have suggested that salivary proteins may be potentially valuable for diagnosis or prognosis of human disease like oral cancer (Negri *et al.,* 1988). The analysis of saliva, like blood-based analysis, plays a significant role in identifying individuals before and after treatment (Copeland, 1974). Saliva is normally secreted in the salivary glands continuously at about 500 ml per day but and this can be stimulated by masticatory or gustatory activity. Diagnosis of precancerous stages of oral cavity is a difficult task due to several reasons. The most important is lack of symptoms. Therefore, developments of simple, accurate, rapid and cost effective methods to detect neoplastic lesions are required. Over the past few decades, saliva based tests for a range of purposes have been developed seems to play a promising role in diagnosis of many diseases. A number of studies have been suggested that salivary proteins may be potentially valuable for diagnosis of human diseases, such as oral cancer. The main objective of this work is to screen the levels of pro-oxidants, free radicals and total protein in both Oral Squamous Cell Carcinoma (OSCC) and Oral Sub Mucous Fibrosis (OSMF) patients. In addition the work also accomplished with the estimation of trace elements in saliva. These biochemical parameters probably may be used as a diagnostic marker in early stages of oral cancer.

Materials and methods

Study design and case selection

A total of60 cases among which 20 cases of OSMF (age 30 ± 10 years), 20 cases of OSCC (Cancer of buccal mucosa and alveolus - age 30 ± 10 years) attending outpatient Department of Oral Medicine and Radiology, Sri Ramachandra Medical College and Research Institute (Sri Ramachandra University), Chennai, were selected along with equal number (n=20) of age and sex matched individuals (Control) were included in this study. Patients were selected for this research on conformation with physical examination and histo–pathological conformation by the dentist.

Sample collection and storage

Resting saliva samples were collected from healthy individuals, OSMF and OSCC patients. 4ml of saliva was collected and centrifuged at 2000 rpm for 10 minutes. Supernatant was collected in a sterile disposable container. Saliva samples were analyzed within 7 days period. Samples were stored at - 20° C until analysis.

Estimation of lipid peroxides

Tubes containing aliquots of 100 µl of distilled water, 20 µl of saliva and 500 µl of 10 % trichloro acetic acid in a test tube was added and mixed. About 1ml of thiobarbutyric acid was added, mixed and heated at

100° C for 30 minutes. The absorbance was read at 532 nm against the blank using a UV - Spectrophotometer. Total lipid peroxide is expressed as nano moles / per mg protein (Ohkawa *et al.,* 1979).

Estimation of conjugated dienes

5ml of ethanol - ether mix was added to 100 µl of sample. The contents were mixed well and centrifuged at 2000 rpm for 10 minutes. Supernatant was discarded. The pellet was air dried to remove the traces of ethanol - ether mixture. The pellet was re - suspended in 3 ml of methanol. The absorbance was read at 213 and 233 nm against the blank using a UV – Spectrophotometer. The conjugated dienes is expressed as units / ml of saliva (Klein, 1970).

Estimation of hydroxyl radicals

The basic reaction mix of hydroxyl radicals production was prepared consisting of 30 µl of phosphate buffer, 20 µl of sodium azide, 20 µl of dimethyl sulfoxide, 10 µl of NADH, 10 µl of ferric EDTA and 20 µl of sample. Incubate the reaction mixture at 37° C for 20 minutes. The reaction was arrested by adding 200 µl of TCA. Mixed well and centrifuged at 2000 rpm for 10 minutes (Cederbaum and Cohen, 1984).

Estimation of hydrogen peroxide

The reaction mixture was prepared by taking of 40 µl of KCl, 40µl of $MgCl_2$, 40 µl of methanol, 10 µl of catalase, 10 µl of NADH and 20 µl of sample. Incubate the reaction mixture at 37° C for 20 minutes. Adding 200 µl of TCA arrested the reaction. Mix well and centrifuge at 2000 rpm for 10 minutes. Supernatant was used for the estimation of formaldehyde. The level of hydrogen peroxide production is expressed as nanogram / mg protein (Hilderdrandt *et al.,* 1978).

Estimation of formaldehyde

Hydroxy radicals in the form of formaldehyde were estimated by this method. Equal volume of supernatant and Nash reagent was added and incubated at 58° C for 8 minutes. Cool the sample to room temperature. The absorbance was read at 412 nm against a blank using a UV - Spectrophotometer. The level of hydroxyl radical production is expressed as nanogram / mg protein (Nash, 1953).

Estimation of total protein

An aliquot of sample (20 µl) was made up to 1ml with distilled water. 1.1ml of alkaline copper reagent was added to all the tubes including blank. Blank containing 1ml of water and standard containing aliquots of bovine serum albumin were also treated similarly. After mixing, the contents were incubated at 37° C for 10 minutes. 100 µl of freshly prepared Folin - Ciocalteu's reagent was added and incubated at 37° C for 20 minutes. The blue color developed was read at 640 nm using a UV - Spectrophotometer. The protein levels were expressed as µg/ml of sample (Lowry *et al.,* 1951).

Estimation of micronutrients by atomic absorption spectroscopy

Two milliliter of each sample were diluted to 20 ml with distilled water and stored at 4° C until of analysis. Concentration of elements like copper, calcium, iron, manganese, magnesium, zinc, sodium, potassium were determined by using Perkin - Elmer 2380 atomic absorption spectrophotometer equipped with a hallow cathode lamps of elements and flames such as air - acetylene flame, acetylene - nitrous oxide flame. The instrument initially calibrated with the standard solutions at appropriate wavelengths (Chicharro *et al.,* 1999) (Table 1).

Nebuliser gas flow rate, iron lens voltage, hallow cathode lamp emission, flames selection, resolution, were optimized using standard solutions. 3 sets of each 1.5 ml of standard solutions with appropriate concentrations were injected into nebulizer for 30 minutes. Then the injected port was flooded with laboratory grade Milli Q water. 20 ml of each diluted sample were injected into the nebulizer after each wash and the absorption spectrum for each element was

recorded. ppm (mg/l) value of each element was determined and graphed.

Table 1. Table shows the wavelengths and concentrations of standard (three concentrations for each elements in ppm level) elements.

Element	Cathode	Wavelength (nm)	Flame	Linear working range (ppm)
Calcium	Ca	422.7	Air - nitrous oxide	1.0, 1.5, 2.0
Iron	Fe	248.3	Air - acetylene	2.0, 4.0, 6.0
Potassium	K	766.5	Air - acetylene	0.6, 1.2, 1.8
Magnesium	Mg	285.2	Air - acetylene	0.2, 0.4, 0.6
Sodium	Na	589.6	Air - acetylene	0.4, 0.8, 1.2
Zinc	Zn	213.9	Air - acetylene	0.5, 1.0, 1.5
Copper	Cu	324.7	Air - acetylene	1.0, 2.0, 3.0
Manganese	Mn	279.5	Air - acetylene	0.8, 1.6, 3.2

Statistical data

Statistics for the data obtained was performed with ANOVA and student't' - test. The level of significance was studied using student't' - test. Values are expressed as mean ± SD.

Results

The lipid peroxides level was significantly increased ($p< 0.001$) in OSCC (Group-II) when compared to OSMF (Group-I) patients. There was no detectable amount of lipid peroxides were observed in normal healthy individuals (Fig 1). The level of conjugated dienes was estimated in both OSMF and OSCC patients. It was found that conjugated dienes level is high in OSMF then OSCC cases ($p< 0.001$) (Fig 2). The hydroxyl radicals level was elevated in OSMF (Group-I) when compared to OSCC (Group-II) patients. Significant elevation was observed in OSMF ($p< 0.001$). There was no detectable amount found in normal healthy individuals (Fig. 3). Levels of hydrogen peroxides were estimated in saliva of both OSMF (Group-I) and OSCC (Group-II) patients. The hydrogen peroxide levels were elevated in OSMF compared to OSCC patients. But no significant elevation was observed.

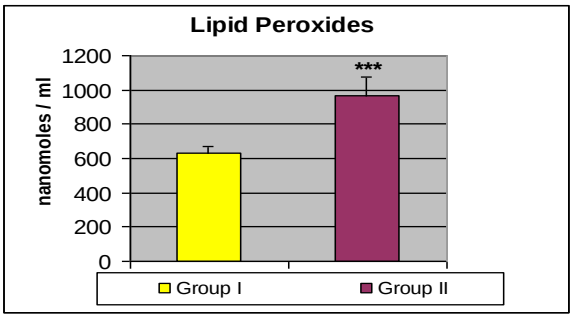

Fig. 1. Levels of Lipid Peroxides in OSMF and OSCC patients. Values were expressed as mean + SD (n=20). Group-I (OSMF), Group-II (OSCC).

There was no detectable amount of hydrogen peroxide found in normal healthy individuals (Fig 4). The protein levels were significantly elevated in OSCC ($p< 0.01$) and OSMF ($p< 0.01$) patients when compared to normal healthy individuals (Fig 5).

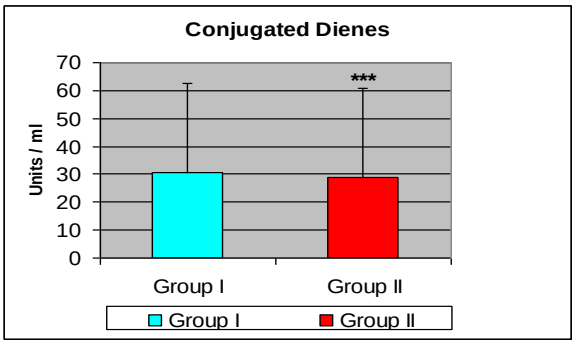

Fig. 2. Levels of Conjugated Dienes in OSMF and OSCC patients. Values were expressed as mean +SD (n=20), Group-I (OSMF), Group-II (OSCC).

The levels of trace elements such as copper ($p<0.05$) (Fig 6), calcium ($p<0.001$) (Fig 7) and magnesium ($p<0.001$) (Fig 8) were elevated in OSMF (Group-II) compared with normal healthy individuals (Group-I). Zinc ($p<0.001$) (Fig 9), iron ($p<0.001$) (Fig. 10) levels were found to be increased in OSCC (Group-III) compared to normal healthy individuals, but iron content was not found in detectable amount in normal healthy individuals. Hence, iron levels were compared

only with OSMF (Group-I) patients, the significant increase of iron ($p<0.001$) was found in OSCC patients (Group-II). A significant increase of sodium (Fig 11) was found in OSCC patients ($p<0.001$) when compared to normal healthy individuals. Level of potassium (Fig 12) was found to be increased ($p<0.001$) in OSMF patients compared to normal healthy individuals.

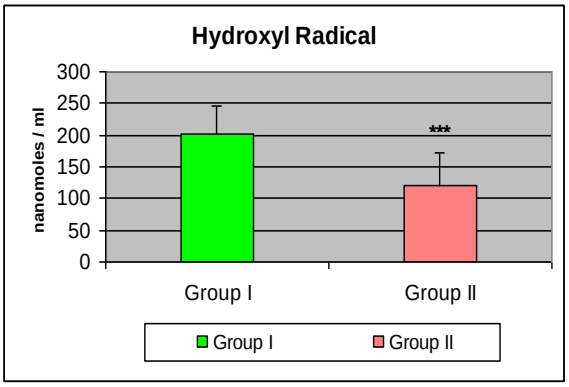

Fig. 3. Levels of Hydroxyl radicals in OSMF and OSCC patients. Values were expressed as mean +SD (n=20), Group-I (OSMF), Group-II (OSCC).

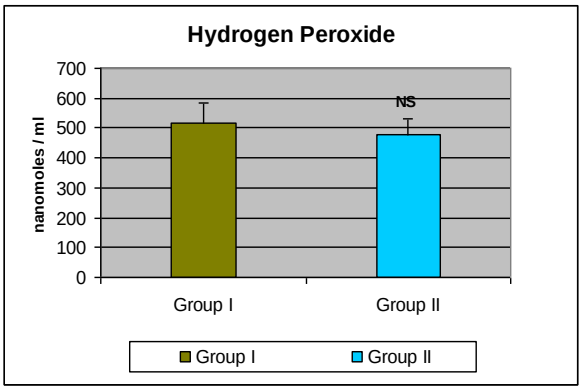

Fig. 4. Levels of Hydrogen peroxide in OSMF and OSCC patients. Values were expressed as mean +SD (n=20), Group-I (OSMF), Group-II (OSCC).

Discussion

Analysis of micronutrients shows that there is an elevated level of copper; calcium and magnesium were found in OSMF (Group-II) compared with OSCC (Group-III) patients. High levels of zinc were found in Oral Squamous Cell Carcinoma patients (group-III). The rate of occurrence of Oral carcinoma and other precancerous stage like OSMF is still increasing even though there is an existence of sophisticated diagnostic and therapeutic approaches. Early detection is thus the most important feature to improve the long - term prospects of patient suffering from this type of cancer.

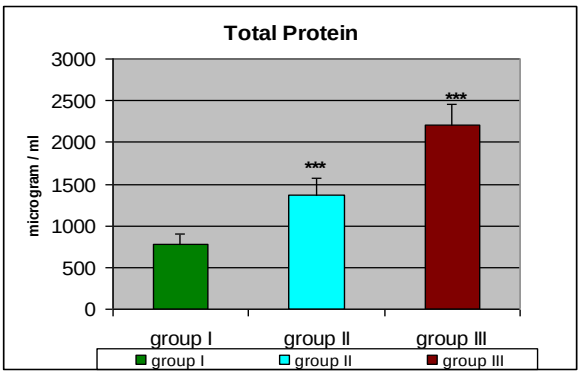

Fig. 5. Levels of total protein in OSMF and OSCC patients. Values were expressed as mean +SD (n=20), Group-I (Normal), Group-II (OSMF), Group-III (OSCC).

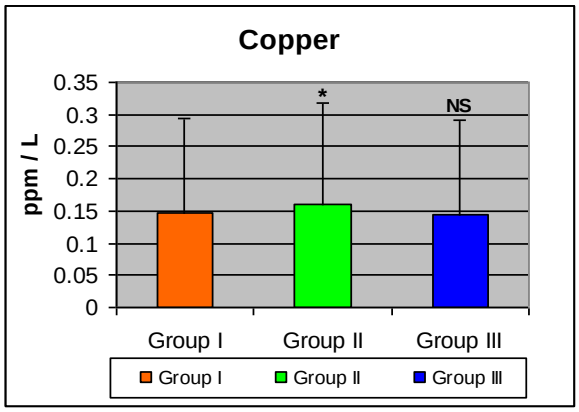

Fig. 6. Levels of Copper in OSMF and OSCC patients. Values were expressed as mean +SD (n=20), Group-I (Normal), Group-II (OSMF), Group-III (OSCC).

Various studies have been carried out to screen the clinical usefulness of certain biochemical and physiological changes for early diagnosis and management of oral cancer. Among these, the most commonly studied parameters are: lipid peroxides, conjugated dienes, hydroxyl radicals, hydrogen peroxide, total protein content, micronutrients which might be probably helpful to detect oral cancer in early stages. In addition, the cancer samples also analyzed to give a comparative data between precancerous and

cancerous conditions. Free radicals and micronutrients found prevalently in many different sites including, body fluids, tissues, and even in cell membranes that are the reactive oxygen species, which are found in significant amount relatively to disease progression.

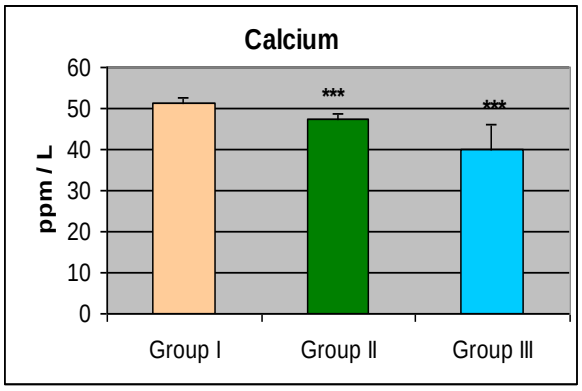

Fig. 7. Levels of calcium in OSMF and OSCC patients. Values were expressed as mean +SD (n=20), Group-I (Normal), Group-II (OSMF), Group-III (OSCC).

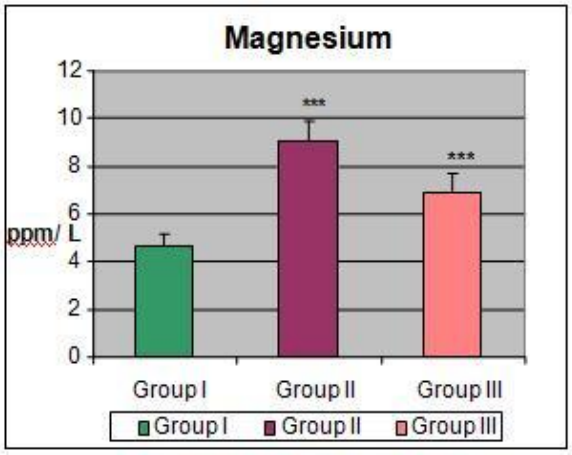

Fig. 8. Levels of Magnesium in OSMF and OSCC patients. Values were expressed as mean +SD (n=20), Group-I (Normal), Group-II (OSMF), Group-III (OSCC).

People working in cancer research have got significant experience with free radical biology, which are more important in the phenomenon of tumor metastasis and angiogenesis. Lipid peroxides are the important free radical, which includes polyunsaturated fatty acids, which leads to membrane destruction. These lipid peroxides, and lipid hydro peroxides are easily scavenged or neutralized by certain anti oxidant enzymes like glutathione peroxidase, a non - enzymatic antioxidant like vitamin - C, vitamin - E and reduced glutathione. They scavenge lipid peroxides and this will be immediately metabolized. In case of cancer condition more lipid peroxides will be produced which find less sufficient amount of these antioxidants to neutralize them. Hence lipid peroxides in turn generate lipid hydro peroxides and causes membrane damage. These free radicals are more in cells when compared to their levels found in other body fluids. Lipid peroxides level was estimated in both OSMF and OSCC cases.

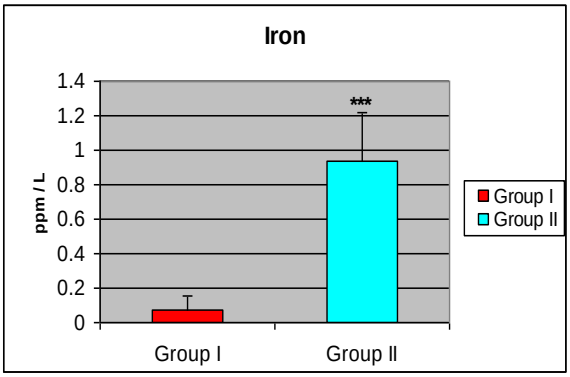

Fig. 9. Levels of Zinc in OSMF and OSCC patients. Values were expressed as mean +SD (n=20), Group-I (Normal), Group-II (OSMF), Group-III (OSCC).

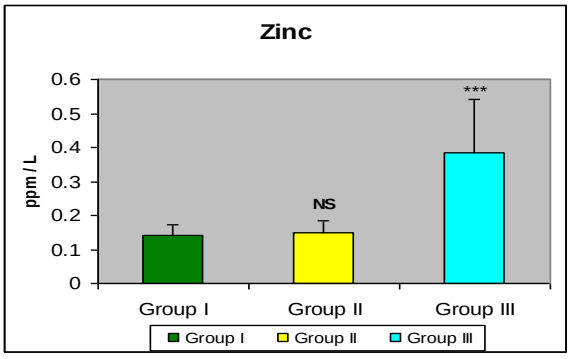

Fig. 10. Levels of Iron in OSMF and OSCC patients. Values were expressed as mean +SD (n=20), Group-I (OSMF), Group-II (OSCC).

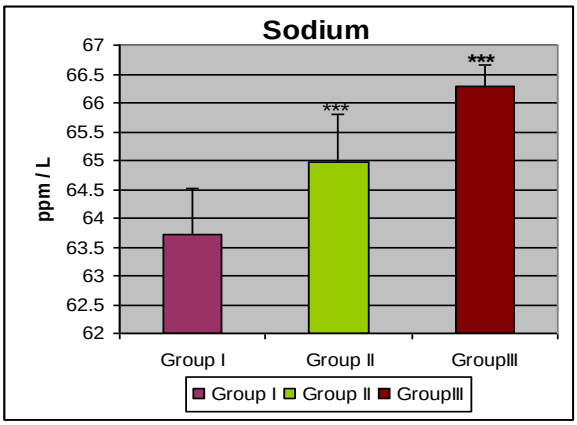

Fig. 11. Levels of Sodium in OSMF and OSCC patients. Values were expressed as mean +SD (n=20), Group-I (Normal), Group-II (OSMF), Group-III (OSCC).

The level of lipid peroxides was significantly increased in OSCC when compared to that of OSMF cases. This is due to per oxidation of polyunsaturated fatty acids of cell membrane that results in the formation of DNA adduct. Higher lipid peroxides and lipid hydro peroxides either cause DNA mutation or mis pairing which might leads to or which determine the severity of the disease progression to oral cancer and periodontal diseases, dental caries and other oral diseases (Guyton and Kensler, 1993).

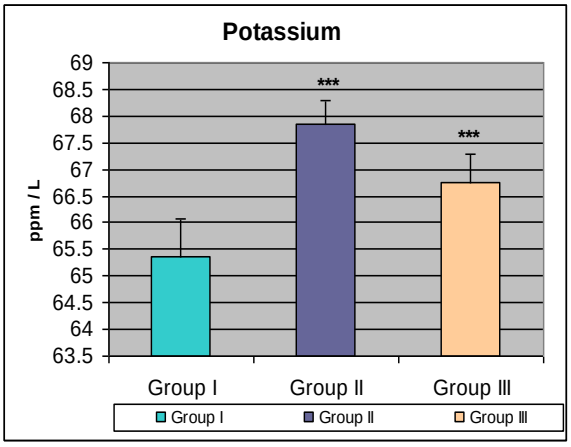

Fig. 12. Levels of Poassium in OSMF and OSCC patients. Values were expressed as mean +SD (n=20), Group-I (Normal), Group-II (OSMF), Group-III (OSCC).

Our present study showed high lipid peroxide content in OSCC patients were co insides well with above findings. High levels of conjugated dienes are due to increased H° ion abstraction; less amount of malondialdehyde is generated in OSMF cases. But incase of OSCC, H° ion abstraction is comparatively less, which generates more amount of lipid peroxides and malondialdehyde, which shows the low levels of conjugated dienes in OSCC and high conjugated dienes levels in OSMF patients. Conjugated dienes can be considered as an important biochemical marker to detect early stages of oral carcinoma (Schoneich, 1967).

The high rates of polyunsaturated fatty acids of membrane damage results the elevated levels of low molecular weight conjugated dienes in OSMF were noticed in this study and also no detectable amount of conjugated dienes was observed in normal healthy individuals shows a good diagnostic marker for oral precancerous stages. Hydroxyl radicals are generated in the presence of high iron/copper contents. This increased hydroxyl radical targets the lipid bilayer of cell membrane predominantly which causes peroxidation and hydro peroxidation of lipids, inturn leads to cell damage (Cerutti *et al.,* 1994). In OSCC cases, the present study showed low levels of hydroxyl radical might be due to binding of these radicals with nucleic acids, forming DNA adducts. Increased hydroxyl radical was observed in the saliva of OSMF patients which might be due to high copper content in OSMF was noticed in the present study. Hydrogen peroxide is very sensitive and it can be easily degradable.

The degraded products react with metal ions, which have a strong effect on the oxidation and reduction process. Increased hydroxyl radicals are due to excess amount of hydrogen peroxide conversion in OSMF patients when compared to OSCC patients (Datta *et al.,* 2000). It was found that hydrogen peroxide is high in OSMF than OSCC which might be due to over production of hydroxyl radicals, indicates that both

hydrogen peroxide and hydroxyl radicals levels are interdependent.

There was no hydrogen peroxide level in normal healthy individuals. Elevated levels of hydrogen peroxide might be due to increased copper content in the saliva of OSMF patients. This was found in our present study. Protein levels in the body can helps to determine the useful diagnostic marker hemorrhages, polyneuritis and trauma conditions (Elaine, 2003). A number of studies have suggested that salivary proteins may be potentially valuable for diagnosis or prognosis of human diseases, such as oral cancers including salivary gland tumors (Armstrong *et al.,* 1991). All these evidences showed that the ultimate rise in salivary protein level in both OSMF and OSCC cases. A significant increase in protein level in OSCC compared to OSMF and normal healthy individuals were observed in the study.

A significant increase of sodium was found in OSCC patients compared to normal healthy individuals. This might be due to increased activity of sodium ion channels that liberates more sodium ions outside the cell compared to OSMF patients. Level of potassium was found to be increased in OSMF patients compared to normal healthy individuals. This might be due to the hyperactivity of potassium dependent ATPases, release of more amounts of potassium ions through membrane channels in OSMF patients. Increased copper level is due to continuous exposure of areca nut (High copper content), which leads to more amount of copper levels in saliva of OSMF cases.

The copper level in areca nut was high, when chewing tobacco with betel quid will enhance the formation of OH° radical from H_2O_2. These hydroxyl radicals enhance the lipid per oxidation that ultimately cause DNA adducts in the cell, which in turn leads to cancer. Copper content enhance the lysyl oxidases, that enhance the cross - linking of collagen fibers in oral mucosal tissues (Rucker *et al.,* 1996). High calcium and magnesium content were found in saliva of oral sub mucous fibrosis cases due to membrane damage that leaks out calcium and magnesium ions from cytoplasm, endoplasmic reticulum and nucleus. Hence this pays a way to high calcium and magnesium contents in the saliva. The iron level also enhances the formation of hydroxyl radicals, which was elevated in OSMF, that in turn develops carcinogenesis by DNA adducts formation (Chessman and Slater, 1993). High levels of zinc was found in OSCC, due to the utilization of copper by copper dependent super oxide dismutase present in saliva, which could displays an imbalance between zinc and copper levels in saliva.

The above results shows that these biochemical analysis of free radicals are good diagnostic marker for the detection of oral cancer at an early stages of cancer development.

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