IMMUNOHISTOCHEMICAL DETECTION OF 8-HYDROXY DEOXYGUANOSINE (8-OHdG) - A BIOMARKER OF OXIDATIVE DNA DAMAGE IN ORAL SUBMUCOUS FIBROSIS

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in partial fulfilment of the requirements
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BRANCH VI
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MELMARUVATHUR – 603319.

ORAL PATHOLOGY AND MICROBIOLOGY

CERTIFICATE

This is to certify that **Dr.CHANDRAMOHAN.K,** Post Graduate student (2015-2018) in the Department of Oral Pathology and Microbiology, Adhiparasakthi Dental College and Hospital, Melmaruvathur – 603319, has done this dissertation titled **"IMMUNOHISTOCHEMICAL DETECTION OF 8-HYDROXY DEOXYGUANOSINE(8-OHdG) - A BIOMARKER OF OXIDATIVE DNA DAMAGE IN ORAL SUBMUCOUS FIBROSIS"** under our direct guidance and supervision in partial fulfilment of the regulations laid down by **THE TAMILNADU DR.M.G.R MEDICAL UNIVERSITY**, Chennai – 600032 for **MASTER OF DENTAL SURGERY - (BRANCH-VI) ORAL PATHOLOGY AND MICROBIOLOGY** degree examination.

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TITLE OF THE DISSERTATION	**IMMUNOHISTOCHEMICAL DETECTION OF 8-HYDROXY DEOXYGUANOSINE (8-OHdG) - A BIOMARKER OF OXIDATIVE DNA DAMAGE IN ORAL SUBMUCOUS FIBROSIS**
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DURATION OF THE COURSE	3 YEARS
NAME OF THE GUIDE	DR.M.DEVI,MDS.,
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I hereby declare that no part of the dissertation will be utilized for gaining financial assistance or any promotion without obtaining prior permission of the Principal, Adhiparasakthi Dental College and Hospital, Melmaruvathur – 603319. In addition, I declare that no part of this work will be published either in print or in electronic media without the guides who has been actively involved in dissertation. The author has the right to reserve for publishing the work solely with the permission of the Principal, Adhiparasakthi Dental College and Hospital, Melmaruvathur – 603319

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ABSTRACT

BACKGROUND: Oral submucous fibrosis(OSMF) is one of the common potentially malignant disorder prevailing in India. The primary etiological factors include tobacco and are canut which contain numerous Reactive Oxygen Species (ROS). The imbalance between ROS and antioxidant status creates oxidative stress. Reactive oxygen species attack guanine bases in DNA and form 8-Hydroxy Deoxyguanosine(8-OHdG) which can be detected in patients who have diseases associated with oxidative stress. The oxidative DNA damage produced by oxidative stress may induce malignant transformation. The aim of the present study is to detect the expression of 8-OHdG in OSMF and compare the expression within the different grades and normal oral mucosa.

AIM: To study the Immunohistochemical expression of 8-OHdG in oral submucous fibrosis.

MATERIALS AND METHODS: A total of 30 samples were examined for the immunohistochemical expression of 8-OHdG. The control group includes 10 formalin fixed paraffin embedded tissue blocks of normal buccal mucosa. The study group includes 20 cases of formalin fixed paraffin embedded tissue blocks of oral submucous fibrosis (5cases of very early, early, moderately advanced, advanced cases of OSMF). 3 micron thickness sections were made from each sample and stained with 8-OHdG antibody. The expression is scored and

analysed statistically. The results were statistically analysed using Kruskal-Wallis and Mann - Whitney U test

RESULTS: Statistically significant difference exists in the intensityof 8-OHdG expression between the study groups. Increased intensity in the oral submucous fibrosis samples were observed when compared with normal mucosa. The p value obtained was less than 0.001 which was highly statistically significant. Staining intnesity was also compared with different grades of OSMF, the p value obtained was less than 0.01 which was statistically significant.

CONCLUSION: Our present study is the first attempt to evaluate the expression of 8-OHdG in tissue samples that revealed the role of free radicals and oxidative DNA damage which enhances the initiation and progression of Oral submucous fibrosis. Further research with a larger sample size, clinicopathologic correlation and long term followup will shed more light on the pathogenesis of Oral submucous fibrosis. It will also be useful for the development of new therapeutic stratergies targeting treatment modalities for the oral submucous fibrosis.

KEY WORDS: Oral submucous fibrosis, Oxidative DNA damage,8-OHdG,Immunohistochemistry

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ABBREVIATIONS

OPMD	-	Oral Potentially Maligant Disorder
IARC	-	International agency for Research on Cancer
OSMF	-	Oral Submucous Fibrosis
OSCC	-	Oral squamous Cell Carcinoma
8-OHdG	-	8-Hydroxy Deoxyguanosine
ROS	-	Reactive Oxygen Species
RNS	-	Reactive Nitrogen Species
HPLC	-	High Performance Liquid Chromatography
GC-MS	-	Gas Chromatography- Mass Spectrometry
LC-MS	-	Liquid Chromatography-Mass Spectrometry
MDA	-	Malaonaldehyde
SOD	-	Superoxide Dismutase
GSH	-	Glutathione
TAC	-	Total Antioxidant Capacity
DPX	-	Dibutyl Phthalate Xylene
DAB	-	Diaminobenzidine
HRP	-	Horseradish Peroxidase
IHC	-	Immunohistochemistry
DNA	-	Deoxyribonucleic acid
TGF	-	Transforming growth factor
PDGF	-	Platelet derived Growth factor
HSP	-	Heat Shock Protein
MMPs	-	Matrix metalloproteinases

INTRODUCTION

Oral potentially malignant disorders(OPMD) are the ones that are described as “Not all lesions and conditions may transform to cancer rather than a morphological alteration amongst which some may have an increased potential for malignant transformation". It conveys that not all precancerous lesions and conditions may transform to cancer, rather some may have increased chance for malignant transformation[1]. Precancerous lesions like leukoplakia, erythroplakia and palatal lesions in reverse smoking and Precancerous conditions like oral submucous fibrosis (OSMF), actinic keratosis, lichen planus and discoid lupus erythematosus that were previously recognized as precancer by WHO in 1978 are now categorised as potentially malignant disorders[2]. In Indian subcontinent, leukoplakia and oral submucous fibrosis are the most common potentially malignant disorders caused largely by tobacco smoking and areca nut consumption. Areca nut has been declared as a known human carcinogen by International agency for research on cancer (IARC) expert group in 2003[3].

WHO ranks tobacco smoking as one of the ten important risk factors to health.Tobacco contains 400 tumorigenic chemical agents including carbon monoxide, nicotine, and aromatic hydrocarbons. Tobacco and areca nut also contains numerous free radicals and reactive oxygen species important of which includes singlet oxygen (O_2), hydroxyl radical (OH^-), and nitric oxide (NO).

Oral submucous fibrosis being a premalignant condition and associated with carcinogens like tobacco was thought to have some relation with Reactive oxygen species (ROS).

Reactive oxygen species are free radicals associated with the oxygen & their equivalents and have stronger reactivity with other molecules than with molecular oxygen[4]. Reactive oxygen species usually indicate the following four species: Superoxide anion(O_2^-), Hydrogen peroxide(H_2O_2),Hydroxyl radical (OH^-), Singlet oxygen (O_2). It is established that ROS are involved in a variety of biological phenomenon such as mutation, pre-cancer & carcinogenesis, aging, atherosclerosis and inflammation.

Reactive oxygen species attack guanine bases in DNA easily and form 8-Hydroxydeoxyguanosine (8-OHdG) by hydroxylation of guanine base. 8-OHdG can cross the cell membrane, it is usually detected in the urine, serum or saliva of patients who have diseases associated with oxidative stress. In nuclear and mitochondrial DNA, 8-OHdG is one of the predominant agents of free radical induced oxidative lesions. It has been used widely in many studies as a biomarker for measurement of endogenous oxidative DNA damage including cancer and other degenerative diseases. The level of8-OHdG is generally regarded as a biomarker of mutagenesis consequent to oxidative stress[5].

In healthy individuals the production of reactive oxygen species is approximately balanced with antioxidant defense system. The serious imbalance between reactive species production and antioxidant defense system is termed as oxidative stress and the damage produced is called oxidative damage.

Tobacco-smoking, radiation, air-pollution, ultra-violet radiation, mitochondrial damage, chronic inflammation and chronic systemic diseases lead to uncontrolled oxidative damage which may progress to produce unrepairable DNA damage eventually leading to mutation and carcinogenesis[6]. The oxidant and antioxidant status in potentially malignant disorders serve as an early biomarker tool to analyze the level of oxidative damage and any further progression of DNA damage. The most commonly produced base lesion results from oxidation of guanine base, which is often measured as an index of oxidative DNA damage, is 8-hydroxydeoxyguanosine (8-OHdG)[7].

Various oxidative stress markers have been studied in oral submucous fibrosis and antioxidants are being routinely used for the treatment of OSMF, though they reduce the severity of disease, reversal of fibrosis is still an enigma.

With this fact in mind a more advanced oxidative stress marker 8-hydroxydeoxyguanosine (8-OHdG) was decided to be studied in oral submucous fibrosis by Immunohistochemistry (IHC) method.

AIM & OBJECTIVES

Aim:

To analyze the Immunohistochemical expression of 8-hydroxy deoxyguanosine (8-OHdG) in oral submucous fibrosis

Objectives:

1. To evaluate the immunohistochemical expression of 8-OHdG in oral submucous fibrosis.

2. To compare the immunohistochemical expression of 8-OHdG in different grades of oral submucous fibrosis.

3. To compare the expression of 8-OHdG in oral submucous fibrosis and normal oral mucosa.

4. To compare the expression of 8-OHdG in different layers of epithelium in oral submucous fibrosis.

REVIEW OF LITERATURE

POTENTIALLY MALIGNANT DISORDERS:

In 1978 World Health Organization categorized 'Precancerous' into precancerous lesions and conditions[2].

A precancerous condition is 'a generalised state associated with a significantly increased risk of cancer'.

Precancerous conditions are:

Oral Submucous fibrosis (OSMF)

Actinic keratosis

Lichen planus

Discoid lupus erythematosus

Concept of precancer:

The concept of denoting 'precancerous' is based on the evidence that:

1. In longitudinal studies, areas of tissue with certain alterations in clinical appearances identified at the first assessment as 'precancerous' have undergone malignant change during follow-up[1].
2. Some of these alterations, particularly red and white patches, are seen to co-exist at the margins of over the oral squamous cell carcinomas.

3. A proportion of these may share morphological and cytological changes observed in epithelial malignancies, but without any frank invasion.
4. Some of the chromosomal, genomic and molecular alterations found in clearly invasive oral cancers are detected in these presumptive 'precancer' or 'premalignant' phase.

In 2005, WHO Working group has made an attempt to unify the terminology, and it was termed as 'epithelial precursor lesions'[3].

In 2005 the current working group coordinated by the WHO collaborating Centre, did not favour subdividing precancer to lesions and conditions and the consensus view was to refer all clinical presentations that carry a risk of cancer under the term **'potentially malignant disorder' t**o reflect their widespread anatomical distribution[1].

The term 'potentially malignant disorders', as it conveys not all lesions and conditions described under this term may transform to cancer, rather, there is a family of morphological alterations amongst which some may have an increased potential for malignant transformation.

ORAL SUBMUCOUS FIBROSIS (OSMF)

DEFINITION

Oral submucous fibrosis is defined as "An insidious chronic disease affecting any part of the oral cavity and some times the pharynyx. Although occasionally preceded by and/or associated with vesicle formation, it is always associated with juxta epithelial inflammatory reaction followed by fibro-elastic change of the lamina propria, with epithelial atrophy leading to stiffness of the oral mucosa and causing trismus and inability to eat"[7].

HISTORY

Oral submucous fibrosis can be traced back to antiquity, over thousands of years and the condition was first described in ancient Indian manscript by Sushruta around 400 B.C. He described this disease as 'VIDARI'[8] and the patients suffered from narrowing of mouth, burning sensation and pain.

In 1952 **Schwartz**[9] was the first person to describe a fibrosing condition in five Indian females from Kenya for which he coined the term "atrophiaidiopathicatropicamucosaeoris".

In 1953 **Joshi**[10] an ENT surgeon described this condition in palate and pillars of fauces.

Su *et al*in 1954[11] coined the term as "Idiopathica scleroderma of the mouth".

Pindborg and Sirsat[7] in 1954 coined the term as oral submucous fibrosis.

EPIDEMIOLOGY:

An ethnic basis is predominantly seen in OSMF. It is found predominantly in Asians or Asians settled in other countries. They tend to adhere to habits very strongly. In recent years high occurrence of OSMF is observed in many parts of India like Bihar, Madhya Pradesh, Gujarat, and Maharashtra.

An epidemiology assessment of prevalence of OSMF among Indian villagers, based on baseline data, recorded a prevalence of 0.2% (n= 10,071) in Gujarat, 0.4% (n=10,287) in Kerala, 0.04% (n= 10,169) in Andhra Pradesh, and 0.07% (n=20,388) in Bihar. The prevalence among 101,761 villagers in the state of Maharashtra was 0.03%.[12]

In 1980 **Gupta *et al***, calculated the incidence rate of OSMF in Ernakulum, Kerala district and found it to be 8 for men and 19 for women per 100,000[13].

According to the systematic review conducted by **Rajeshwar N.** Sharan and co-workers in 2012 to assess the association of betel nut with carcinogenesis, India recorded the largest betel nut (BN) chewing

population in the world with about 20–40% of the population in India[14].

ETIOLOGY:

Various etiological factors are proposed by various authors.

QUID:

Quid is a "substance or mixture of substances, placed in the mouth or chewed and remains in contact with the mucosa, usually containing one or both of the two basic ingredients, tobacco and/or areca nut, in raw or processed form".

Su in 1954 observed that tannic acid and arecoline content of betal nut together with influence of lime causes oral submucous fibrosis[15].

Sirsat and Khanolkar in 1962 suggested that OSMF is caused by Capsaicin[7].

In 1970 **Abrol and Krishnamoorthi** suggested that OSMF is caused by genetic predisposition[16].

Renni*et al* in 1982 demonstated experimental study on Hamster with Iron deficiency anemia and found quantitative histological changes in oral epithelium[17].

Canif.JP. Harvey.W in the year 1986 proposed two etiological factors in the development of OSMF, which are genetic predisposition and betel nut chewing[18].

Sinor P.N in the year 1990 suggested that areca nut is the most probable causative agent[19].

Khanna.J.N and Andrade.N.N in the year 1995 depicted the role of areca nut in the pathogenesis of OSMF[20].

W.M.Tilakaratne*et al* in the year 2005 established the role of autoimmunity in OSMF[21].

PATHOGENESIS:

Harvey W *et a*l in 1986 proposed that alkaloids in areca nut stimulate fibroblast proliferation and collagen synthesis leading to OSMF[22].

Canif. J.P *et a*l in 1986 proposed two etiological factors in the development of OSMF, which are, genetic predisposition and betel nut chewing. They propose that human leukocyte antigen (HLA) antigens A10, DR7 together with autoantibodies constitute an autoimmune basis for OSMF[23].

They also stated that an alkaloid in areca nut stimulates collagen synthesis and proliferation of buccal mucosal fibroblasts. Tannin that is present in betel nut increase the resistance of collagen to degradation which further enhances fibrosis.

Meghji S *et al* in 1987 found that clonal selection of fibroblasts with a high amount of collagen production during the long-term exposure to areca quid ingredients paves pathogenesis for OSMF[24].

Ma RH *et al* in 1995 said increase in collagen cross- linkage is caused by up-regulation of lysyloxidase by OSMF fibroblasts[25].

Shah N and Sharma PP in 1998 found that chewing of areca nut/quid or pan masala was directly related to OSMF. Also, pan masala was chewed by comparatively younger age group and was associated with OSMF changes earlier than areca nut/quid chewing. However, chewing or smoking tobacco with various other chewing habits did not increase the risk of developing OSMF. It was also found that frequency of chewing rather than the total duration of the habit was directly correlated to OSMF[26].

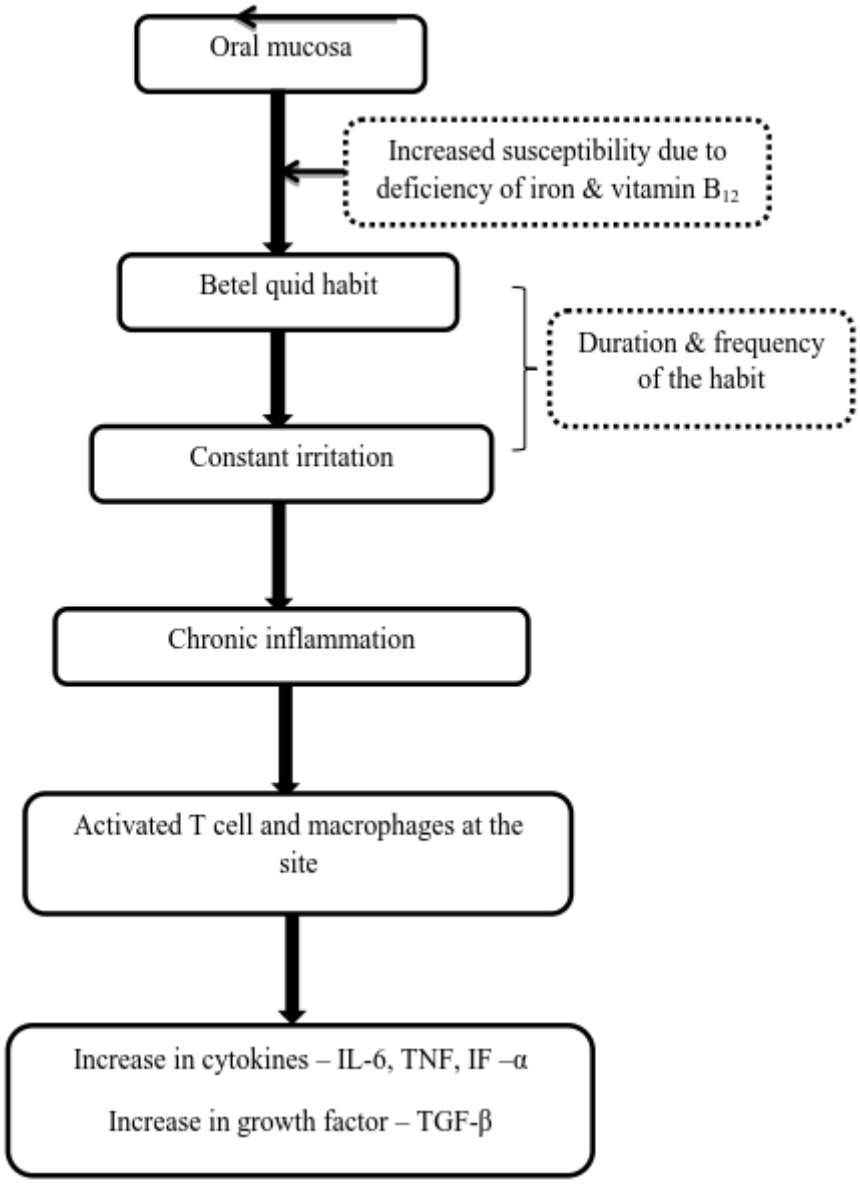
Jeng JH *et al* in 2001 state that it appears that areca nut toxicity is not completely due to its polyphenol, tannin and alkaloid content. The factor responsible for areca nut carcinogenicity awaits further clarification. Reactive oxygen species produced during auto-oxidation

of areca nut polyphenols in the betel quit-chewer's saliva, are crucial in the initiation and promotion of oral cancer. Nitrosation of areca alkaloids also produces areca nut specific nitrosamines that have been demonstrated to be mutagenic, genotoxic and are capable of inducing tumors in experimental animals. Arecaidine and areca nut extract are further suggested to be tumor promoters. Antioxidants such as glutathione and N-acetyl-L-cysteine can potentially prevent such areca nut-elicited cytotoxicity. Further studies are needed to delineate the metabolism of areca nut ingredient and their roles in the multi-step chemical carcinogenesis, in order to enhance the success of the future chemoprevention of oral cancer and oral submucous fibrosis[27].

Rajalalitha P and S Vali in 2005 review was aimed to high molecular events involved in the overproduction of insoluble collagen and decreased degradation of collagen occurring via exposure to betel quid and stimulation of the TGF-β pathway, and elucidate the cell signalling that is involved in the etiopathogenesis of the disease process[28]. They published the following schematic presentation:

Rajalalitha P and Vali S in 2005 described the initial events of disease processes:

Initial events of disease process



Oral mucosa, which is in direct contact with betel quid due to the habit, is the site of constant irritation. This results in a chronic inflammatory process characterized by the presence of inflammatory cells like T cells and macrophages. These cells release and/or stimulate the synthesis of various cytokines and growth factors. IL6: Interleukin 6: TNF: Tumor necrosis factor : ILα :Interferon alpha: TGFβ- Transforming growth factor -beta.

MOLECULAR PATHOGENESIS:

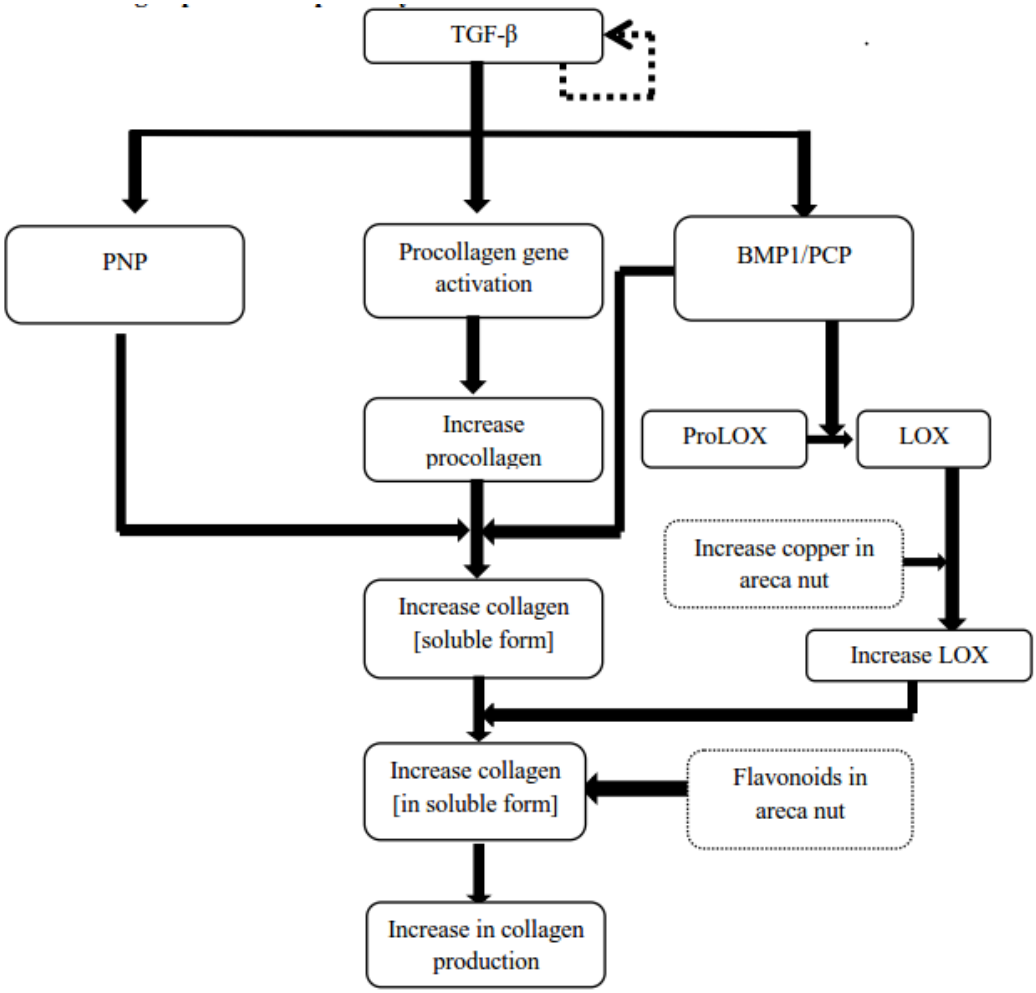
(Rajalalitha P and Vali S in 2005)

Oral sub mucous fibrosis results from increased production of collagen by fibroblasts. In addition to this there is decreased breakdown leading to accumulation of excessive amount of collagen[28].

Increased Collagen Production:

Stimulation of collagen synthesis occurs through different mechanism. Under the influence of areca nut, fibroblasts differentiated into phenotypes that produce more collagen. The alkaloids present in areca nut, arecadine and arecoline are responsible for this. Arecoline gets converted into arecadine which is the active metabolite. There is dose dependent increase in production of collagen by fibroblasts under the influence of these factors. Various cytokines are increased in oral submucous fibrosis like Transforming growth factor (TGF), Platelet derived growth factor (PDGF) and Basic fibroblast growth factor (bFGF). These are fibrogenic growth factors that stimulate collagen production. Another cytokine that has anticollagen effect is Interferon (IFN), this is decreased in OSMF.

Collagen production pathway : (Rajalalitha P and Vali S in 2005)



Collagen production pathway as regulated by TGF-ß

TGF-ß is a growth factor, which has autocrine activity. This activates the procollagen genes, resulting in production of more pro-collagen. It also induces the secretion of PCP and PNP, both of which are required for the conversion of Pro-collagen to collagen fibrils. In OSF, there is increased cross linking of the collagen, resulting in increased insoluble form.This is facilitated by increased activity and production of a key enzyme LOX. PCP/BMP1 and increased copper (CU) in BQ stimulate LOX activity, a key player in the pathogenesis of this disease. The

flavonoids increase cross linking in the collagen fibers.[Pro-Lox: pro-lysyloxidase: Lox: Lysyloxidase: PNP: Pro-collagen N-proteinase: PCP: Pro-collagen C-proteinase: BMP1: bone morphogenic protein [1]

There are three main events in this pathway:

1. Activation of procollagen

The activation of procollagen genes by TGF- β is causes by an increased expression of procollagen genes and hence increases the collagen level in OSMF.

2. Elevation of procollagenproteinase levels

Elevation of procollagenproteinases cleaves C-terminal which plays an essential role in pathogenesis of OSMF.

3. Upregulation of lysyloxidase (LOX activity).

Up-regulation of lysyloxidase by enzyme lysyloxidase. This is a copper dependent enzyme and plays a key role in collagen synthesis and its cross linkage.

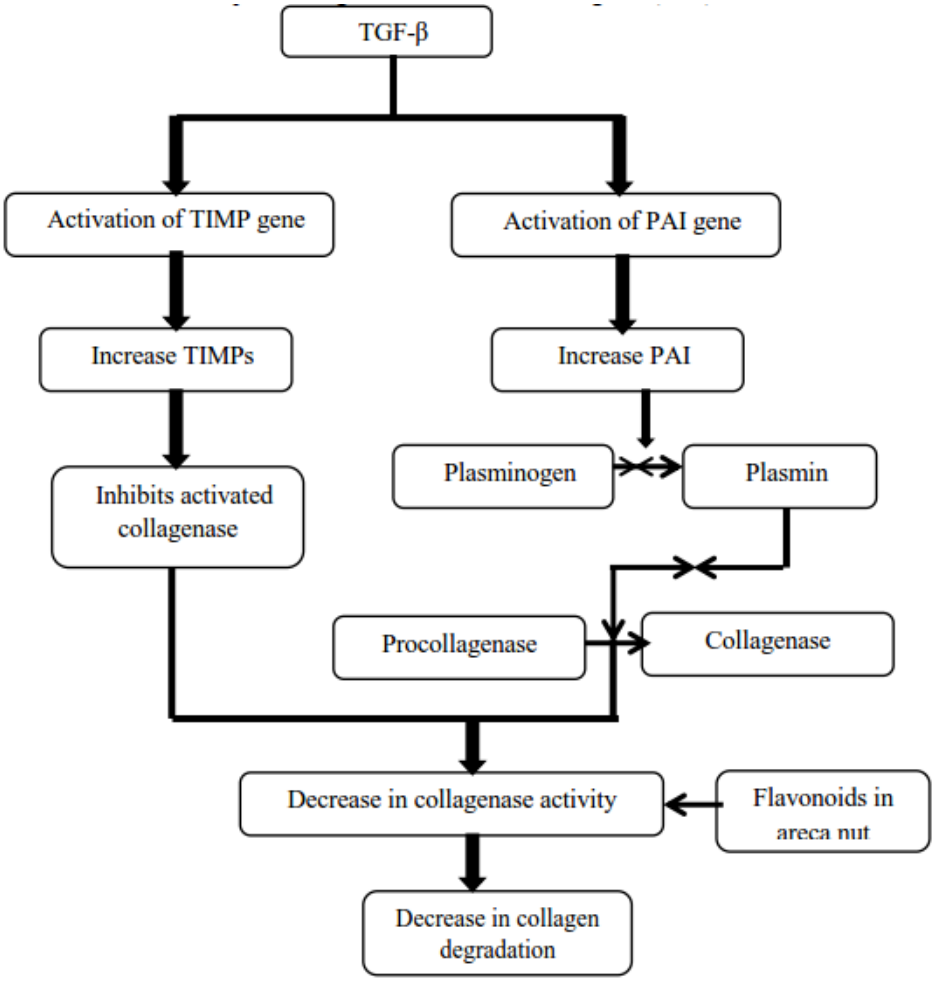
Collagen degradation pathway: (Rajalalitha P and Vali S in 2005)

There are two main events that are modulated by TGF – β:

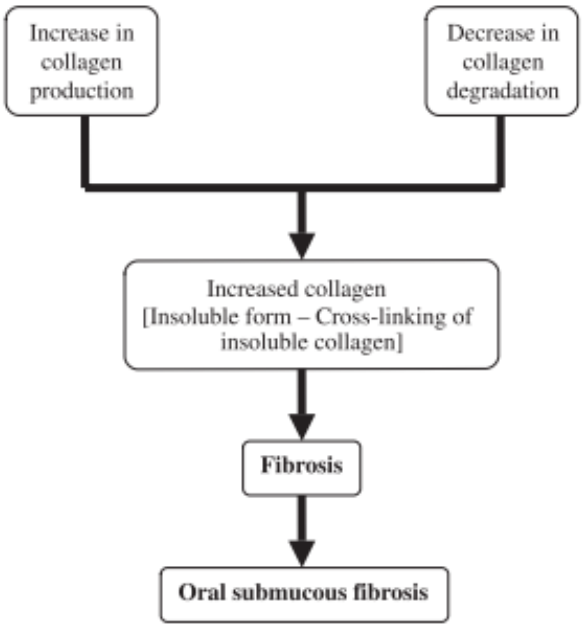
Activation of tissue inhibitor of matrix metalloproteinase gene (TIMPs).

Activation of plasminogen activator inhibitor gene (PAI).

Collagen degradation pathway

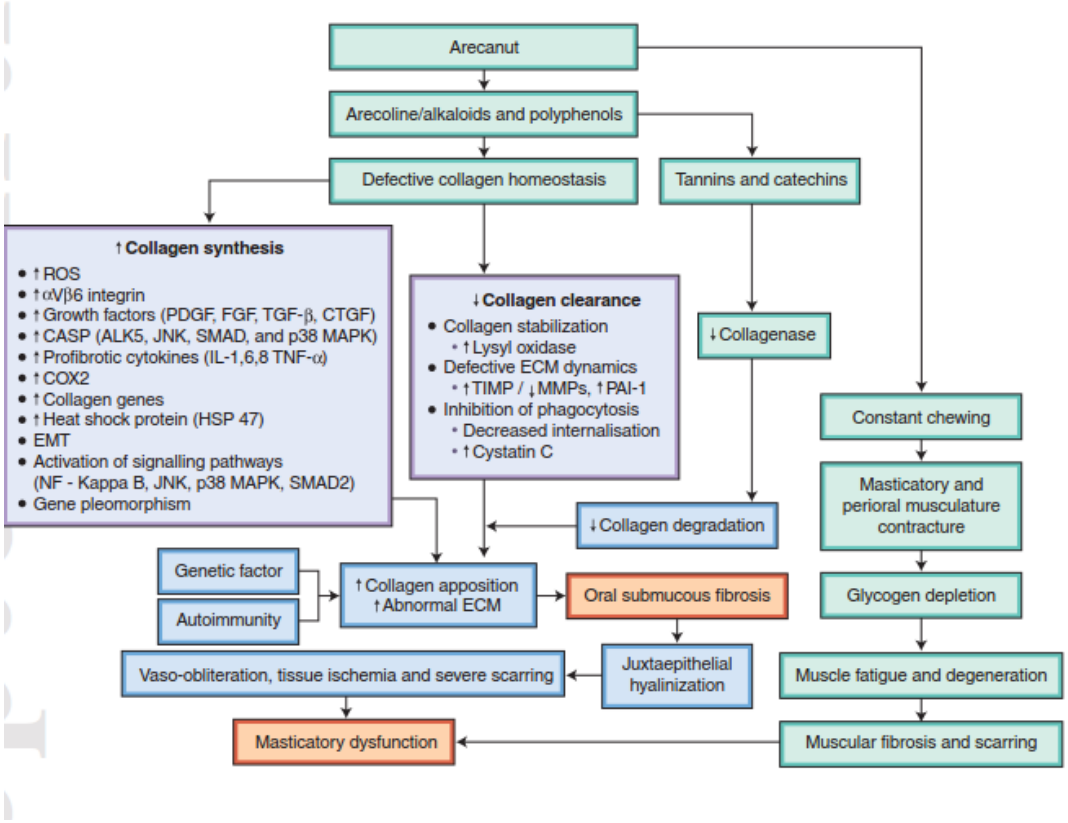


Overall effect of activated TGF – β pathway:



There is an increase in collagen production and cross-linking (insoluble form) along with a decrease in collagen degradation. This produces an increased collagen deposition in the subepithelial connective tissue layer of the oral mucosa leading to OSMF.

Pathogenesis of OSMF described by **Gururajarakeri*et al*** in 2017.



The pathogenesis of OSMF can be viewed under three broad categories-Defective collagen homeostasis theory, Genetic theory, Autoimmunity theory. It is reasonable to hypothesize an abnormality in collagen production and degradation as a possible basic mechanisim in the development of OSMF. Many pathways are involved leading to fibrosis, trismus and malignant transformation in some patients[29].

CLINICAL FEATURES:

Clinical features of OSMF vary depending upon its severity and progression[12].

Early OSMF:

Burning sensation in the mouth while taking spicy foods, appearance of blisters especially on the palate, ulceration or recurrent generalised inflammation of the oral mucosa, excessive salivation, defective gustatory sensation and dryness of the mouth.

Advanced OSMF:

As the disease progresses, the oral mucosa becomes blanched and slightly opaque, and white fibrous band appear.

Vertical fibrous bands are present.

The fibrous tissue in the faucial pillars ranges from a slight submucosal accumulation in both pillars to a dense fibrosis extending deep into the pillars with strangulation of the tonsils. Circular bands present around the entire rimaoris.

With progressing fibrosis, the stiffening of certain areas of mucosa occurs leading to difficulty in opening the mouth, inability to whistle or blow out a candle and difficulty in swallowing.

CLASSIFICATION:

Classification based on mouth opening and its clinical features: Various classifications for OSMF based on various criteria have been proposed. Few widely accepted classifications are mentioned below[30].

Pindborg JJ (1989) divided OSMF into three stages:

Stage I : Stomatitis includes erythematous mucosa, vesicles, mucosal ulcers, melanotic mucosal pigmentation and mucosal petechiae.

Stage II : Fibrosis occurs in healing vesicles and ulcers, which is the hallmark of this stage.

- Early lesions shows blanching of the oral mucosa.
- Older lesions include vertical and circular palpable fibrous bands in the buccal mucosa and around the mouth opening or lips.
- This results in the mottled marble like appearance of the mucosa because of the vertical thick, fibrous bands in association with a blanched mucosa.

Specific findings include reduction of mouth opening, stiff and small tongue, blanched and leathery floor of the mouth, fibrotic and depigmented gingiva, and rubbery soft palate with decreased mobility, blanched and atrophic tonsils, shrunken bud like uvula and sunken cheeks.

Stage III : Sequelae of OSMF are as follows:

- Leukoplakia is found in more than 25% of individuals with OSMF.
- Speech and hearing deficit may occur because of involvement of tongue and the eustachian tube.

Lai DR *et al* in 1995 divided OSMF based on interincisal distance as follows:

Group A: >35 mm

Group B: Between 30 - 35 mm

Group C: Between 20 - 30 mm

Group D: <20 mm

Khanna JN and Andrade NN (1995) developed a classification system for surgical management of OSMF.

Group I : Very early cases: Common symptom is burning sensation in the mouth, acute ulceration and recurrent stomatitis and not associated with mouth opening limitation.

Group II : Early cases: Buccal mucosa appears mottled and marble like, widespread sheets of fibrosis palpable, interincisal distance of 26 - 35 mm.

Group III : Moderately advanced cases:Trismus, interincisal distance of 15 to 25 mm, buccal mucosa appear pale & is firmly attached to underlying tissues, atrophy of vermilion border, vertical fibrous bands

palpable at the soft palate, pterygomandibularraphae and anterior faucial pillars.

Group IVA : Advanced cases: severe trismus, interincisal distance of less than 15 mm, thickened faucial pillars, shrunken uvula, restricted tongue movement, presence of circular band around entire lip and mouth.

Group IVB : Advanced cases: presence of hyperkeratoticleukoplakia and/or squamous cell carcinoma.

Ranganathan K *et al* (2001) classified OSMF based on mouth opening as follows:

Group I : only symptoms, with no demonstrable restriction of mouth opening.

Group II : limited mouth opening, 20 mm and above.

Group III : Mouth opening less than 20 mm.

Group IV : OSMF advanced with limited mouth opening. Precancerous or cancerous changes seen throughout the mucosa.

Rajendran R (2003) reported the clinical features of OSMF as follows:

Early OSMF: Burning sensation in the mouth. Blisters especially on the palate, ulceration or recurrent generalized inflammation of oral mucosa, excessive salivation, defective gustatory sensation and dryness of mouth.

Advanced OSMF: Blanched and slightly opaque mucosa, fibrous bands in buccal mucosa running in vertical direction.

Palate and faucial pillars are the areas first involved. Gradual impairment of tongue movement and difficulty in mouth opening.

Kiran Kumar *et a*l (2007) categorized three clinical stages of OSMF on the basis of mouth opening as follows:

Stage I : Mouth opening >45 mm.

Stage II : Restricted mouth opening, 20 - 44 mm.

Stage III : Mouth opening <20 mm.

Chandramani More *et al* (2011):

Stage 1 (SI) : Stomatitis and/or blanching of oral mucosa.

Stage 2 (S2) : Presence of palpable fibrous bands in buccal mucosa and/or oropharynx, with /without stomatitis.

Stage 3 (S3) : Presence of palpable fibrous bands in buccal mucosa and/or oropharynx, and in any other parts of oral cavity, with/without stomatitis.

Stage 4 (S4) : A. Any one of the above stage along with other potentially malignant disorders, e.g. oral leukoplakia, oral erythroplakia, etc.

B. Any one of the above stage along with oral carcinoma.

Functional staging:

Ml : Interincisal mouth opening up to or greater than 35 mm.

M2 : Interincisal mouth opening between 25 - 35 mm.

M3 : Interincisal mouth opening between 15 - 25 mm.

M4 : Interincisal mouth opening less than I5 mm.

HISTOPATHOLOGICAL FEATURES:

- Histopathological features of OSMF varies depending upon its clinical and functional staging[14].
- Juxta-epithelial inflammation including edema is present.
- Large fibroblast & inflammatory infiltrate are present in the underlying connective tissue.
- In advanced stages collagen bundles with early hyalinization and contain chronic cell types such as lymphocytes and plasma cells.
- Formation of thick band of collagen and hyalinization extending into submucosal tissues and decreased vascularity.
- Epithelial lining becomes thin and looses melanin or become hyperkeratotic.
- Inflammation and fibrosis of minor salivary glands.
- Muscle degeneration occurs.

Pindborg JJ and Sirsat SM (1966):

Very early stage: Finely fibrillar collagen dispersed with marked edema. Plump young fibroblast containing abundant cytoplasm. Blood vessels are dilated and congested. Inflammatory cells, mainly polymorphonuclear leukocytes with occasional eosinophils are found. Moderate number of plump young fibroblasts are present. Dilated and congested blood vessels. Inflammatory cells are primarily lymphocytes, eosinophils and occasional plasma cells.

Moderately Advanced stage: Collagen is moderately hyalinised and thickened collagen bundles are separated by slight residual edema. Fibroblastic response is less marked. Blood vessels are either normal or compressed. Inflammatory exudate consists of lymphocytes and plasma cells.

Advanced stage: Collagen is completely hyalinised. A smooth sheet with no separate bundles of collagen is seen. Edema is absent. Hyalinised area is devoid of fibroblasts. Blood vessels are completely obliterated or narrowed. Inflammatory cells are lymphocytes and plasma cells.

Khanna JN and Andrade NN (1995) developed a classification system for surgical management of OSMF:

Group I: Fine fibrillar collagen network interspersed with marked edema, blood vessels dilated and congested, large aggregate of plump young fibroblasts present with abundant cytoplasm. Inflammatory cells mainly consist of polymorphonuclear leukocytes with few eosinophils. The epithelium is normal.

Group II: Juxta-epithelial hyalinizalion present, collagen present as thickened but separate bundles, blood vessels dilated and congested, young fibroblasts seen in moderate number, inflammatory cells mainly consist of polymorphonuclear leukocytes with few eosinophils and occasional plasma cells. Flattening or shortening of epithelial rete-pegs evident with varying degree of keratinization.

Group III: Juxta-epithelial hyalinization present, thickened collagen bundles, residual edema, constricted blood vessels, mature fibroblasts with scanty cytoplasm and spindle-shaped nuclei, inflammatory exudates which consists of lymphocytes and plasma cells, epithelium markedly atrophic with loss of rete pegs, muscle fibers seen with thickened and dense collagen fibers.

Group IV: Collagen hyalinized smooth sheet, extensive fibrosis, obliterated the mucosal blood vessels, eliminated melanocytes, absent fibroblasts within the hyalinised zones, total loss of epithelia rete pegs, presence of mild to moderate atypia and extensive degeneration of muscle

Kiran Kumar *et al* (2007) proposed histological grading as follows:

Grade I : Loose, thick and thin fibers.

Grade II : Loose or thick fibers with partial hyalinization.

Grade III : Complete hyalinization.

MALIGNANT TRANSFORMATION:

The precancerous nature of OSMF was first postulated by Paymaster, who described the development of a slow-growing squamous cell carcinoma in one third of OSMF cases seen in the Tata Memorial Hospital, Bombay. This precancerous potential was also emphasized by other authors, based on clinical and epidemiological grounds[31].

Murti*et al* in 1985 conducted a follow- up study in OSMF patients for a period of 17 years in Eranakulam District, Kerala. They found that oral cancer developed in five (7.6%) patients[32].

Maher R *et al* in 1994 conducted a case control study in Pakistan and found that an increased risk was observed for areca nut chewing. This habit when practiced alone appeared to have the highest risk, followed by pan with or without tobacco. Logistic regression and discriminate analysis showed that daily consumption rates appeared to be more important with respect to risk than lifetime duration of habit. Tobacco habits were more prevalent amongst those 15 cases who presented with concurrent carcinoma and OSMF. Authors concluded that areca nut chewing has a causal relationship with OSMF and additional tobacco insult may be necessary for subsequent carcinoma development[33].

Trivedi*C et al* in 1999 have studied the immunolocalization of Lysyloxidase from 13 subjects with OSMF were examined. Strong positive staining was observed in 7/13 OSMF samples in the cytoplasmic processes of fibroblasts and extracellularly in the upper third of the lamina propria. Furthermore, lysyloxidase was found to co-localize in the areas stained strongly for collagen and elastin by histochemical stains. Examination of squamous cell carcinoma tissues showed localization of lysyloxidase adjacent to invading epithelial islands as evidence of a stromal reaction both in carcinomas arising from OSMF and in squamous cell carcinoma from non-OSMF cases[34].

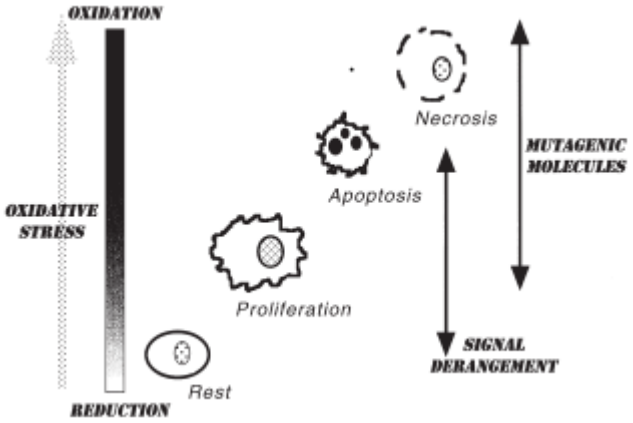
These findings suggest that upregulation of lysyloxidase may be an important factor in the pathogenesis of OSMF and in the early stromal reaction of oral cancer. Recent research has generated sufficient evidence to implicate betal nut as well as betel quid, with or without tobacco, as a suspected carcinogen to humans and a major risk factor for the prevalence of oral potentially malignant disorders. Betel nut without tobacco was recognized as a group I carcinogen to human by the International Agency for Research on Cancer (IARC) and World Health Organization (WHO) in 2004.

Epidemiological studies have shown that the process of carcinogenesis in OSMF occurs by generation of Reactive Oxygen Species (ROS). Reaction of the ROS with cellular DNA results in oxidative damage which is considered to be crucial in cancer development.

8-hydroxy-2-deoxyguanosine (8-OHdG): A Critical Biomarker of Oxidative Stress

Oxidative stress represents a deregulation of the homeostasis between the reactive oxygen species and the mechanisms of detoxification and repair. Reactive oxygen species (ROS) are produced during the metabolic cycle of every aerobic cell and consist of an atom of oxygen and an unpaired electron. ROS react with the most important structures of cells and particles altering their biological function . Similarly, reactive nitrogen species (RNS) create toxic products through their interaction with cells and particles[35].

Significance of oxidative stress in cellular biology



It is now well accepted that relatively low levels of oxidative stress promotes cellular proliferation. Apoptosis and Necrosis produced depends on the dose and duration of the exposure[4].

Both ROS and RNS play important roles not only in the.process of energy production, lipid peroxidation, oxygenation, nitration, and

nitrozylation of proteins and of DNA, but also in the body's response to catecholamines. The particles are inactivated by natural antioxidants (e.g. beetroot). Excessive production of ROS and RNS results in both oxidative and nitric stress both of which are involved in various pathological conditions typical of neoplasms, neurodegenerative disorders, viral, toxic, or inflammatory processes[35].

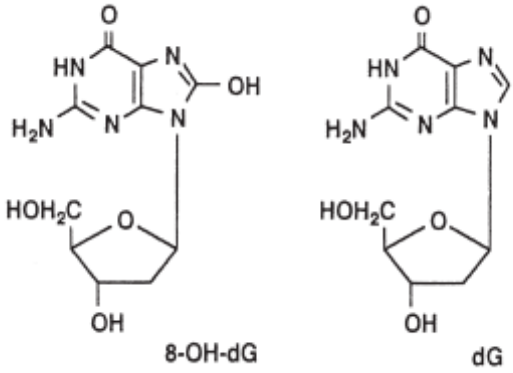
Reactive oxygen species (ROS) are formed continuously in living cells of aerobic organisms as part of the physiological processes, metabolic, and other biochemical reactions. These endogenously produced ROS and oxygen-free radicals have important physiological functions, but because of their reactive nature can cause oxidative damage to lipids of cellular membranes, proteins,and DNA. Also, exogenous factors, such as UV radiation, tobacco smoke,asbestos, and carcinogenic substances, can produce ROS under various conditions[36].

The most important oxygen-free radical causing damage to basic biomolecules (proteins, membrane lipids, and DNA) is the hydroxyl radical (HO). The HO attacks DNA strands, the interaction of HO with the nucleobases of the DNA strand, such as guanine, leads to the formation of C8-hydroxyguanine (8-OHGua) or its nucleoside form deoxyguanosine (8-hydroxy-2-deoxyguanosine). Initially, the reaction of the HO addition leads to the generation of radical adducts, then by one electron abstraction, the 8-hydroxy-2-deoxyguanosine (8-OHdG) is formed.. The 8-OHdG undergoes keto-enoltautomerism, which favors

the oxi-dized product 8-oxo-7,8-dihydro-2-deoxyguanosine (8-oxodG). In the scientific literature 8-OHdG and 8-oxodG are used for the same compound[36].

Although the other nucleobases of DNA react with HO• in a similar manner, the 8-oxodG lesion is the most abundant DNA lesion because it is relatively easily formed and is promutagenic, and therefore a potential biomarker of carcinogenesis.

Structure of 8-OHdG



Oxidative stress markers of three categories[36]

1. Modifiedmolecules by ROS: 8-hydroxyguanine,4-hydroxy-2-nonenol,malondialdehyde
2. Antioxidant enzymes and molecules: glutathione(GSH), GSH peroxidase, superoxide dismutase, DNA repair enzymes, heat shock protein
3. Transcription factors: c-myc, c-fos, c-jun

Among these categories modulation of transcription factors are associated with relatively low levels of oxidative stress and difficulty in visualization in paraffin-embeded tissue is anticipated.

Molecules or enzymes in the second category are difficult to evaluate as a difference in the measurement timing may lead to a difference in the amount.

Therefore the first category molecules are most appropriate as possible targets for visualization of ROS induced damage. Furthermore dosage effect is expected to be proportional; the products accumulate and will be detected precisely at the place where the free radical reaction took place[36].

In recent years, the 8-OHdG lesions can be detected and analyzed with high sensitivity by high-performance liquid chromatography (HPLC), gas-chromatography-mass spectrometry (GC-MS), and liquid chromatography-mass spectrometry-mass spectrometry (LC-MS-MS) and by immunohistochemical methods and single cell gel electrophoresis. Except for the nicking based and some immunoassays , all of the chromatographic techniques require extraction of DNA before enzymatic digestion whereas GC-MS requires acid hydrolysis[37]
Determination and analysis of 8-OHdG can be performed in animal organs and in human samples (urine, human organs, leukocyte DNA) as a biomarker of oxidative stress, aging, and carcinogesis.[36]

REMOVAL OF FREE RADICALS:

Antioxidants are the scavenging system which acts against free radicals. Antioxidants according to the definition given in 1989 by Halliwell and Gutteridge, are substances which slow down the rate at which something decays because of oxidization. The enzymatic antioxidants like Superoxide dismutase, Glutathione peroxide, Catalase and non-enzymatic antioxidants like vitamin A and vitamin E either block or inactivate the free radicals[38].

AshishAggarwal in 2011 estimated the serum beta carotene levels in patients with oral submucous fibrosis in India. Degree of oxidative damage in OSMF was assessed by estimation of serum beta carotene levels using Bradley and Hornbeck method and the levels were 67µg/dl, 56.40 µg/dl and 51.33µg/dl in groups I, II and III respectively and the normal level was 93.02µg/dl. They also concluded that OSMF patients treated with beta-carotene showed significant reduction in disease severity and progression towards malignancy[39].

ShubhaGurudath*et al* in 2012 estimated the level of Superoxide dismutase and Glutathione peroxidase in Oral Submucous Fibrosis, Oral leukoplakia and Oral cancer. Superoxide dismutase and Glutathione peroxidase levels were decreased in OSMF and Oral leukoplakia when compared to normal individuals. They stated that imbalance in antioxidant enzyme may be considered as one of the factors responsible for pathogenesis of cancer and may serve as a potential biomarker and therapeutic target to reduce the malignant transformation in Oral Premalignant lesions and conditions[40].

ROLE OF OXIDATIVE STRESS & DNA DAMAGE, VARIOUS OXIDATIVE STRESS MARKERS IN PATHOGENESIS OF ORAL SUBMUCOUS FIBROSIS

Uikey*et al* in 2003 estimated serum antioxidant enzymes superoxide dismutase and glutathione peroxidase in oral submucous fibrosis patients. They estimated using Ciba corning express plus autoanalyser for spectrometry. From this they concluded that decreased SOD and GPx when compared to normal individuals[41].

Soma Gupta *et al* in 2004 did a study to estimate Lipid peroxidation product, malonaldehyde (MDA) and antioxidants in plasma and erythrocytes of 34 cases of oral submucous fibrosis (OSMF) of different grades with equal number of healthy controls to evaluate the association of reactive oxygen species (ROS) and OSMF. While plasma MDA was found to be significantly higher in patients (3.3. + 0.4 nmole/ml, P < 0.001) as compared to controls (2.4 +_ 0.5 nmole/ml), plasma beta carotene and vitamin E levels were found to be decreased significantly in patients (81.7 + 14.3 i~g/100 ml, P<0.001; 9.3 + 0.9 mg/L, P<0.01 respectively) with respect to healthy controls (110 _+ 20.8 l~g/100ml and 10.1 _+ 1.2 mg/L). The decrease in beta-carotene and vitamin E was found to be more significant in OSMF grade II and III than in grade I. After 6 weeks of oral administration of beta-carotene and vitamin E, patients showed increase in plasma level o f these two antioxidants along with decrease in MDA level associated with clinical improvement[42].

SuryakantMetkari*B et al* in 2007 estimated the serum level of Lipid Peroxidation product Malondialdehyde, antioxidant- Superoxide Dismutase (SOD) and vitamin A in relation to clinical and histopathological grading of OSMF. 5 ml of fasting venous blood, was collected from antecubital vein. The sample was clotted at room temperature for 2 hours and centrifuged at 3000 rpm for 10 mins[43].

Serum MDA, SOD and vitamin A level were estimated in spectrophotometer using Kei Satoh method and he concluded that MDA level is increased and SOD level and vitamin A level are decreased and have positive correlation with clinical grade of OSMF.

BalwantRai*et al*in 2010 to investigate whether the anti precancer activities assigned to curcumin are mediated through an anti oxidant and DNA protection mechanism. Patients with oral leukoplakia, Oral submucous fibrosis or Lichen planus, and healthy individuals aged between 17-50 (n-25 for each group) yrs were included in the study. Salivary and serum oxidative markers such as Malonadehyde (MDA), 8-hydroxydeoxyguanosine (8-OHdG), vitamins C and E measured just prior to the intake of Curcumin, after one week of curcumin intake and following clinical cure of precancerous lesions. Serum and salivary salivary vitamins C and E showed increases, while MDA and 8-OHdG levels showed decreases in patients with oral leukoplakia, oral submucous fibrosis and Lichen planus after intake of curcumin for all categories of precancerous lesions. Based on these results, they

concluded that curcumin mediates its anti-pre-cancer activities by increasing levels of Vitamins C and E, and preventing lipid peroidation and DNA damage[44].

K.S.C Bose *et al* in 2012 conducted the study to evaluate the role of oxidative stress in causation and progression of OSMF by measuring the levels of nonenzymatic antioxidants in OSMF patients. They selected 27 newly diagnosed OSMF patients of both sex with age group between 23 to 40 years and the same number of age and sex matched healthy individuals were selected as control group. In both the groups they measured plasma non enzymatic antioxidants like vitamin A. E, C and reduced glutathione. Total antioxidant activity was also assessed in both the groups. They observed a very low levels of plasma non-enzymatic antioxidants ($p < 0.001$) and at the same time a very poor antioxidant activity ($p < 0.001$) in OSMF patients when compared to controls. They concluded that consumption of tobacco or areca quid creates an oxidative stress environment which might plays a major role in the causation of OSMF[45].

Shishir*et al* in 2012 did clinicopathological and biochemical study to estimate the Malondialdehyde levels in oral submucous fibrosis. They suggested positive correlation between erum and salivary MDA levels, but negative correlation to tissue levels, by using TBARS assay. There was increased level tissue MDA in grade I and II and decreased level of MDA in the advanced histopathological grades of Oral sub mucous

fibrosis due to utilization of Malondialdehyde in cross linking of collagen[46].

Vaishalishende*et al* in 2013 did a correlative study of salivary nitrate and nitrite in tobacco related oral squamous carcinoma and submucous fibrosis. The study was undertaken to detect nitrate and nitrite factor in saliva of cases with oral carcinoma, oral submucous fibrosis and in normal individuals. Oral carcinoma patients showed a significant increase in salivary nitrate and nitrite level compared to control group, whereas OSMF patients did not show a significant value. They study concluded that oxidative and nitrosative stress contributes to the development of oral carcinogenesis through deoxyribonucleic acid (DNA) damage[47].

Poorani*et al* in 2014 conducted the study to estimate the level of MDA in OSMF and compared its level with healthy individuals. The study revealed a statistically significant increase in serum MDA level in OSMF in comparison with normal healthy individuals. They concluded that evaluation of serum MDA level may be regarded as potential biochemical parameters for evaluating the oxidative stress and disease process of Oral submucous fibrosis[48].

Jasdeepkaur*et al* in 2015 did a study to analyze oxidative DNA and lipid damage using salivary 8-hydroxy-2-deoxyguanosine (8-OHdG), malondialdehyde (MDA), and vitamins C and E in oral lichen planus

lesions, oral leukoplakia, oral submucous fibrosis, oral squamous cell carcinoma (SCC), and controls and to determine the value of salivary biomarkers in the diagnosis of oral pre-cancer and cancer patients. Unstimulated saliva was collected from a group of patients diagnosed with 40 oral squamous cell carcinoma (OSCC), 40 oral lichen planus lesions, 40 oral leukoplakia, 40 oral submucous fibrosis, and from a control group of healthy age- and gender-matched individuals. Salivary 8-OHdG, MDA, and vitamins C and E were measured.Results was Squamous cell carcinoma and pre-cancer patients showed significantly higher levels of salivary 8-OHdG and MDA and lower levels of vitamins C and E when compared to levels in healthy normal subjects. This study indicates the presence of oxidative DNA and lipid damage in pre-cancerous and SCC patients. They concluded that detection of salivary 8-OHdG, MDA, vitamin C, and vitamin E can act as suitable diagnostic biomarkers of oral pre-cancer and cancer.[49]

ChandniShekhawat*et al* in 2016 done a biochemical study to determine the effect of betel quid chewing on serum and salivary total antioxidant capacity (TAC) in Oral sub mucous fibrosis (OSMF). A total no of 60 subjects reporting to the Department of Oral Medicine and Radiology were enrolled in the study. Study Group comprised of 45 individuals with the clinical diagnosis of OSMF. Study group was further divided into 3 groups of 15 each (Mild, Moderate, Severe), according to the clinical grading of OSMF using validated criteria modified from different literature reviews. They found that, mean

serum TAC of all study groups was significantly less than that of control subjects, with least in the severe form of OSMF patients reflecting the increase in free radical damage with the progression of the disease. The decrease in the TAC may be due to poor antioxidant system and excessive free radical formation leading to the utilization of antioxidants by the affected tissues or in combating the oxidative stress in circulation[50].

ROLE OF OXIDATIVE STRESS & A BIOMARKER OF OXIDATIVE DNA DAMAGE IN OTHER ORAL & SYSTEMIC DISEASES.

JanneLeinonen*et al* in 1997 investigated oxidative DNA damage in patients with non-insulin-dependent diabetes mellitus (NIDDM) by urinary 8 -OHdG assessments. They determined the total urinary excretion of 8 -OHdG from 24 h urine samples of 81 NIDDM patients 9 years after the initial diagnosis and of 100 non-diabetic control subjects matched for age and gender. The total 24 h urinary excretion of 8-OHdG was markedly higher in NIDDM patients than in control subjects (68.2 ± 39.4 /ig vs. 49.6 ± 37.7 fig, P = 0.001). H i g h glycosylated hemoglobin was associated with a high level of urinary 8 -OHdG . The increased excretion of urinary 8 -OHdG is seen as indicating an increased systemic level of oxidative DNA damage in NIDDM patients[51].

Naoyuki Sugano et alin 2003 did a study for the detection of streptococcus anginosus and 8-OHdG in saliva in patients with periodontitis. Salivary levels of S. anginosus were measured by real-time PCR. S. anginosus was detected in 28 out of 38 (73.7%) of subjects. The 8-OHdG level was significantly higher in patients positive for S. anginosus than in patients negative for the bacterium. Accumulation of reactive oxygen species which may result from the continuous generation of ROS associated with chronic inflammation has been reported in human preneoplastic lesions and in cancerous tissue. They hypothesis that oxidative damage due to s.anginosus infection could be a driving force that leads from chronic inflammation to head and neck cancer[52].

Zimnoch L1 ***et al***in 2004 conducted an Immunohistochemical study on paraffin sections, obtained from 377 surgically resected gallbladders with chronic cholecystitis. Immunohistochemical reaction was conducted on deparaffinized sections, using a monoclonal antibody against 8-hydroxydeoxyguanosine (8-OHdG), a biomarker of oxidative DNA damage. The expression of 8-OhdG (8-OhdG index) was determined by counting the number of positive-cells among 200 epithelial cells of gallbladder mucosa (the mean of two calculations at 200x magnification for each case).They found that, In chronic Cholecystitis, the expression of 8-hydroxy-deoxyguanosine, a biomarker of oxidative DNA damage, is increased[53].

Nomoto*et al* in 2008 did a study to clarify the possible role of oxidative stress in hepatocytes in nonalcoholic fatty liver disease(NAFLD), the hepatic expression of 8-hydroxydeoxyguanosine (8-OHdG), a good marker of oxidative DNA damage, was immunohistochemically investigated in nonalcoholic steatohepatitis (NASH) and steatosis. In double immunostaining, the cytoplasmic fine granular 8-OHdG expression was considered to reflect 8-OHdG–positive mitochondrial DNA affecting oxidation stress. Immuno reactivity for 8-OHdG was observed only in the cytoplasm with a fine granular pattern (1 of 13 cases, 8%), only in the nucleus (6 of 13cases, 46%), and in both the cytoplasm and the nucleus (5 of 13 cases, 38%). They proposed herein that evaluation of cytoplasmic 8-OHdG may be a sensitive diagnostic marker of early nonalcoholic fatty liver disease events[54].

Daniela Murtas*et al* in 2009 investigated whether 8-OHdG, human 8-oxaguanine DNA -glycosylase (hoGG1), glucose -6-phophate dehydrogenase (G6PD) expression in tumor tissues might be a predictor of survival in melanoma patients. They stated that involvement oxidative DNA damage in the process of melanoma pathogenesis and 8-OHdG expression in nuclei of tumor cells could be useful as an early independent prognostic marker in patients with primary cutaneous melanoma[55].

Kumar *et al* in 2012 did a study to determine the salivary antioxidant capacity (TAC, GSH), free radicals (ROS, RNS) and oxidative DNA damage to understand the involvement of these biologic indexes in head and neck squamous cell carcinoma. The study included 100 consenting SCCHN patients and 90 matched healthy controls and was approved by the Ethical Committees for Clinical Research. All cases were newly diagnosed, biopsy proven squamous cell carcinoma of head and neck (SCCHN) and received no chemo-/radiotherapy. Their findings clearly demonstrate a significant role of oxidative stress in squamous cell carcinoma of head and neck[56].

Tsai *et al* in 2011 did a study to evaluate the intensity of cytosol expression of 8-OHdG in normal renal tubules is associated with the severity of renal fibrosis. THis study demonstrated that the cytosol expression of 8-OHdG in normal tubules was associated with the severity of renal fibrosis[57].

Xia Xu*et al* in 2013 performed immunohistochemistry study to determine the 8-OHdG level in Serous ovarian carcinoma using hogg1 and p53. 48 high-grade papillary serous carcinomas (HG-SOC), 24 low-grade papillary serous carcinomas (LG-SOC), 20 serous cystadenomas,and 16 non-tumor control ovaries was tested. Increased 8-OHdG level and decreased expression of hOGG1 in tumor were found in HG-SOC but not LG-SOC. They concluded that Increased 8-OHdG

level in tumor DNA was significantly associated with poorer overall survival and progression-free survival in serous ovarian carcinoma[58].

Rajeev Arunachalam*et al* in 2015 evaluated the salivary levels of 8-OHdG to examine the oxidative DNA damage in thirty individuals with clinically healthy periodontium and thirty individuals with chronic periodontitis. 8-OHdG levels in saliva were estimated by ELISA. They found that increase in mean value of 8-OHdG in chronic periodontitis when compared to healthy group. They concluded that elevated 8-OHdG levels should be taken as evidence of Impaired DNA repair which was reflected in a biological fluid as a result of inflammatory reaction[59].

MATERIALS AND METHODS

STUDY DESIGN AND PATIENT SELECTION:

This study was conducted on the archival formalin fixed, paraffin embedded tissues obtained from the Department of Oral Pathology, Adhiparasakthi Dental College and Hospital, Melmaruvathur. The study group included 20 cases of histologically diagnosed oral submucous fibrosis(Group1). Control group includes biopsies from the normal buccal mucosa adjacent to the site of surgery during the surgical removal of third molar in 10 patients (Group2).

Two subsequent sections, each 3µ thickness were cut for each sample, from formalin fixed, paraffin embedded tissues of histologically diagnosed Oral submucous fibrosis and normal buccal mucosa.

MATERIALS

PARAFFIN BLOCKS:

Paraffin embedded tissues of histologically confirmed cases of oral submucous fibrosis were used in the study.

EQUIPMENTS:

- Microtome (Thermo scientific, MICROM HM340E)
- Paint brush
- Disposable microtome blades
- Hot plate

- Hot water bath
- PathnSitu positively charged slides
- Pressure cooker (5 Liters)
- Measuring Jars
- Coplin Jars
- Electronic Timer
- Absorbent wipes
- Coverslip for slides
- Binocular Light Microscope (Olympus CX21i)
- Micropipette
- Rectangular steel trough
- Induction stove
- Incubator (Hitech Equipments)
- Liquid repellent slide marking pen
- Deparaffinization stainless steel staining trough and rack
- pH meter (E1 digital pH meter)
- A DELTA PLAN2 AP40 Trinocular Light Microscope with camera Head

ANTIBODIES:

1. Primary antibody

   8-Hydroxy deoxyguanosine [Rabbit polyclonal antibody] – 8-OHdG (Bioss ANTIBODIES Biotechnologies Private Limited) (IHC-P-1:100-500)

2. Secondary kit (PolyExcel HRP/DAB Detection System) - PathnSitu Biotechnologies Private Limited

a) PolyExcel H_2O_2

b) PolyExcel Target Binder

c) PolyExcel Poly HRP

d) PolyExcel stun DAB – Chromogen

e) PolyExcel stun DAB – Buffer

REAGENTS:

- Tris-EDTA Buffer – 50X concentration (PathnSitu Biotechnologies Private Limited)
- Immuno wash Buffer – 25X concentration (PathnSitu Biotechnologies Private Limited)
- Distilled water
- Xylene
- Absolute alcohol (Isopropyl Alcohol)
- Alcohol 90% (Isopropyl Alcohol)
- Alcohol 70% (Isopropyl Alcohol)
- Harris Hematoxylin
- Mountant (Dibutyl Phthalate Xylene)

METHODOLOGY:

- Formalin fixed paraffin embedded tissues were sectioned at 3µm and mounted on charged slides and kept for overnight incubation at 37^{o}C
- Incubated at 60 – 70 ^{o}C for 1 hour
- Deparaffinized by 2 changes of xylene 10 minutes each
- Hydrated through descending grades of alcohols as follows:
- Absolute alcohol – 1 change, 5 minutes
- 90% alcohol – 5 minutes
- 70% alcohol – 5 minutes
- Washed in distilled water, 2 changes, 2 minutes each
- Antigen retrieval done for 15- 20 minutes (upto 2 whistles in pressure cooker)
- Cooled for minimum of 30 minutes
- Washed in distilled water, 2 changes, 2 minutes each
- Washed in PBS / Sodium citrate buffer for 2 minutes
- Circles were marked enclosing the section using liquid repellent pen
- Endogenous peroxidase blocking was done by adding PolyExcel H_2O_2 on the section, keep for 5 minutes
- Washed in wash buffer for 5 minutes, 3 changes
- Incubate slides with diluted primary antibody (1:100) overnight at 4 c with gentle agitation.
- Washed in wash buffer for 5 minutes, 3 changes

- PolyExcel Target Binder reagent was added and incubated for 12 minutes
- Washed in wash buffer for 5 minutes, 3 changes
- Polyexcel HRP was added and incubated for 12 minutes
- DAB solution was prepared (1 ml of DAB buffer + 1 drop DAB chromogen, mix well)
- Washed in wash buffer for 5 minutes, 3 changes
- Working DAB chromogen was added and kept for 2-5 minutes, then washed in distilled water.
- Counterstained with hematoxylin for 30 seconds
- Washed in running tap water for 5 minutes
- Dehydrated through successive changes of alcohol and clear with xylene
- Dried and mounted with DPX

POSITIVE CONTROL:

Positive control section includes Breast Ca for 8-OHdG and was treated in the same manner as the test groups.

NEGATIVE CONTROL:

One section of test sample was selected and treated in the same manner as the test groups except that, the primary antibody 8-OHdG was omitted.

ANALYSIS OF IMMUNOREACTIVITY OF 8-OHdG:

Ten random fields were selected at 40x magnifications. Sections were scored for stain intensity and scaled as, No stain-0, Mild staining-1, Moderate staining-2,intense staining-3

Figure 1: Primary and Secondary Antibody kit

Primary antibody 8-OHdG

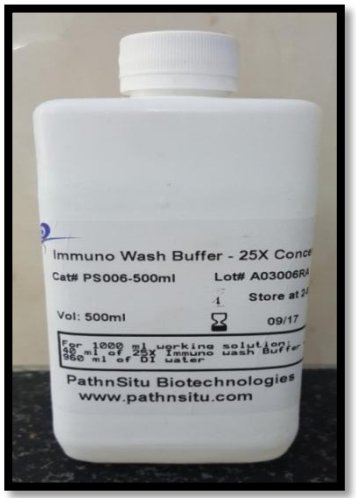
[Rabbit Polyclonal antibody]

Secondary kit DAB Chromogen and DAB buffer

[H2O2, Target Binder, Poly HRP]

Figure 2: Reagents

Tris – EDTA



Wash Buffer

Hematoxyilin

Figure 3: Equipments

Deparaffinization stainless steel staining trough and rack

Micropipette

Incubator

Reagent blocker

Microtome

Microscope

pH meter

Moist chamber

Electronic Timer

Induction stove and Pressure cooker

RESULTS

A retrospective cross sectional Immunohistochemistry study was carried out on 30 archival tissue blocks including 20 histopathologically diagnosed OSMF cases and 10 normal oral mucosa to study the expression of 8-OHdG- a biomarker of Oxidative DNA damage.20 osmf cases were divided into 4 subgroups (5 in each group)- Very early, early, moderately advanced, advanced according to histopathological grading system by Pindborg et al.

Two pathologists observed the expression and Cronbach α test was applied to analyze the interobserver variablity . On evaluation and comparison of staining intensities between different grades of OSMF , parameters given by Observer 2 was statistically not significant. Thus the evaluation made by Observer 1 was subjected to further statistical analysis.

EVALUATION & COMPARISON OF STAINING INTENSITIES BETWEEN DIFFERENT GRADES OF OSMF

In OSMF, among very early group , about 80%(4) of cases exhibited mild expression of antigen.

Remaining one case showed moderate expression.

In early group 60%(3) of cases showed moderate expression of antigen. Remaining 40%(2) of cases showed mild expression.

In moderately advanced group 40%(2) of cases exhibited intense expression of antigen. About 60%(3) of cases exhibited moderate expression.

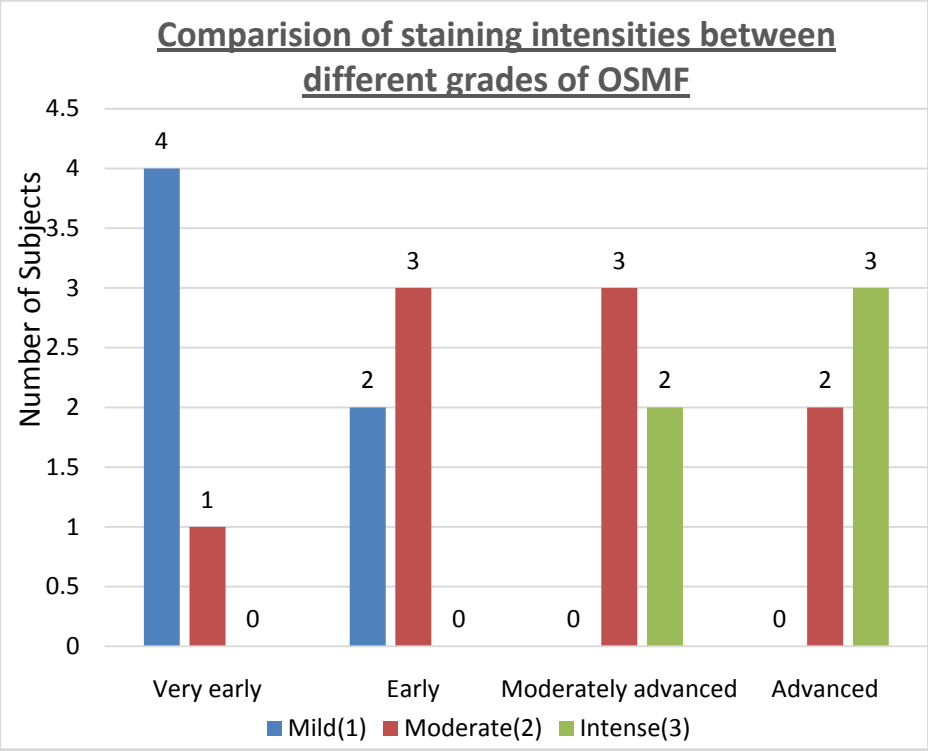
Among Advanced group, 60%(3) of cases exhibited intense expression.40%(2) of cases showed moderate expression of antigen.

Kruskal Wallis test was performed for comparing staining intensities between different grades of osmf and p value obtained was <0.01 which was statistically significant.(TABLE 1, GRAPH 1)

TABLE 1- Evaluation &Comparision of staining intensities between different grades of OSMF

Groups	No Of Cases (n)	Scores			Median staining intensity	IQR	p Value
		Mild(1)	Moderate(2)	Intense(3)			
Very early	5	4(80%)	1(20%)		1	0.5	<0.01
Early	5	2(40%)	3(60%)		2	1	
Moderately advanced	5		3(60%)	2(40%)	2	1	
Advanced	5		2(40%)	3(60%)	3	1	

GRAPH 1- Evaluation &Comparision of staining intensities between different grades of OSMF



EVALUATION &COMPARISON OF 8-OHDG EXPRESSION IN OSMF AND NORMAL ORAL MUCOSA

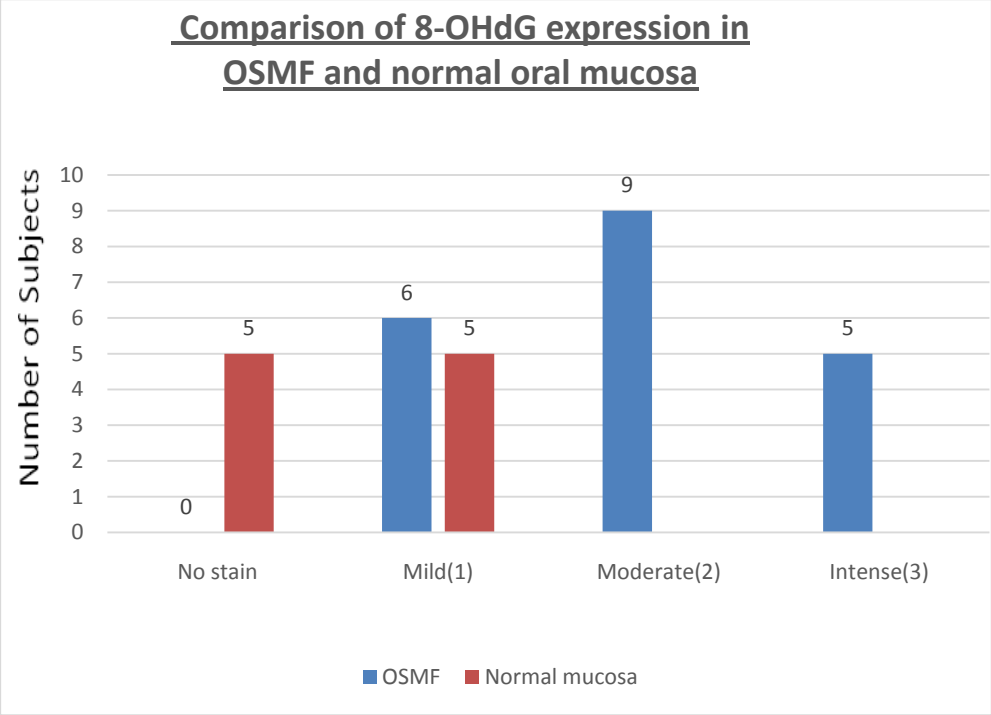
In OSMF group, all the cases showed retention of antigen. Its expression varied from one case to another and also within the same tissue section. Among 20 osmf cases , 30% (6 out of 20) of cases showed mild expression, 45% (9 out of 20) cases showed moderate expression, 25% (5 out of 20) of cases exhibited an intense expression . In normal oral mucosa about 50%(5 out of 10) of cases exhibited mild expression. Remaining 50% (5 out of 10) of cases exhibited no retention of antigen.

To compare the staining intensity between osmf group and normal oral mucosa, statistical analysis Mann-Whitney U was performed. The p value was < 0.001 which was highly statistically significant.(TABLE 2, GRAPH 2)

TABLE 2- Evaluation &Comparison of 8-OHdG expression in OSMF and Normal oral mucosa

Groups	No Of Cases (n)	Scores				Median staining intensity	IQR	p Value
		No Stain(0)	Mild(1)	Moderate(2)	Intense(3)			
OSMF	20		6(30%)	9(45%)	5(25%)	2	1.75	0.001
Normal oral mucosa	10	5(50%)	5(50%)			0.5	1	

GRAPH 2- Evaluation &Comparison of 8-OHdG expression in OSMF and Normal oral mucosa



COMPARING THE EXPRESSION OF 8-OHDG IN DIFFERENT LAYERS OF EPITHELIUM IN OSMF:

Among 20 cases of OSMF, 10%(2) exhibited expression in basal/parabasal layer only.40%(8) of cases exhibited expression till spinous layer of epithelium. Remaining 50%(10) expression in all layers. To compare the expression, statistical analysis was done applying McNemar test. The p value obtained was 0.36 Which was statisticallly not significant.(TABLE 3)

TABLE 3- Comparing the Expression of 8-OHdG in different layers of Epithelium In OSMF:

Observer 1	Observer 2			p Value
	Only Basal /Parabasal	Till spinous	All layers	
Only Basal/Parabasal	1	1	0	<0.36
Till spinous	0	7	1	
All layers	0	0	10	
Total	1	8	11	

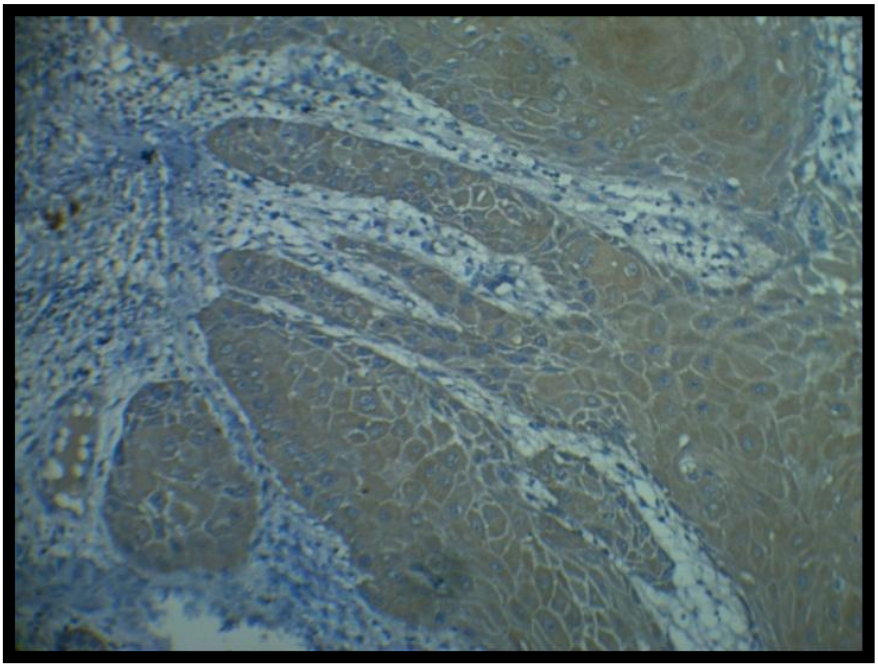


FIGURE 4a : Low power view(10X) showing expression of 8-OHdG in Positive control(OSCC)

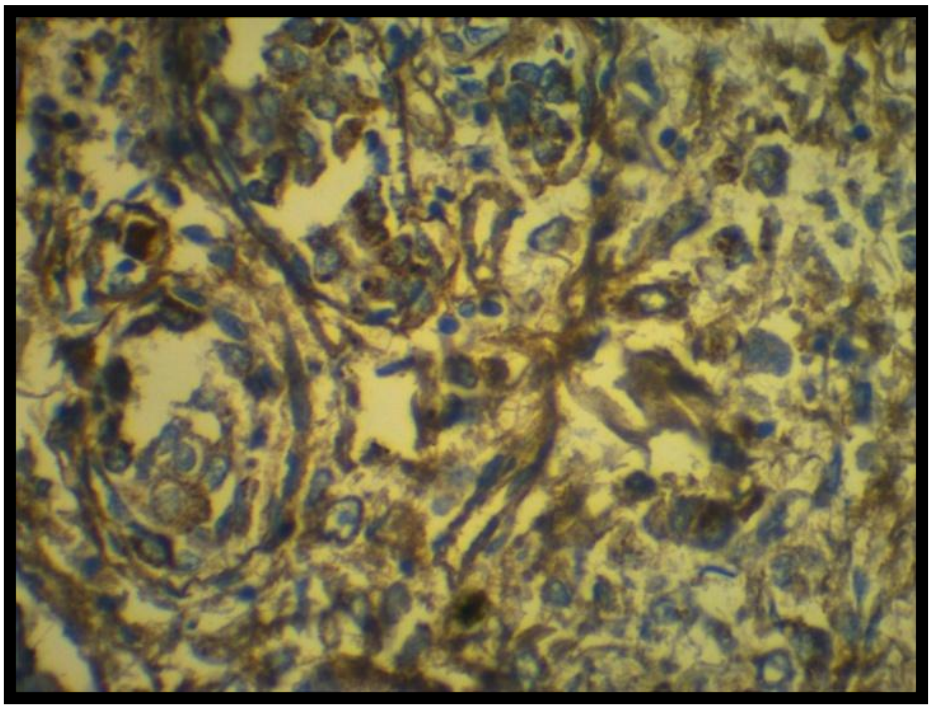


FIGURE 4b : High power View(40X) showing Expression of 8-OHdG in Positive control (Breast ca)

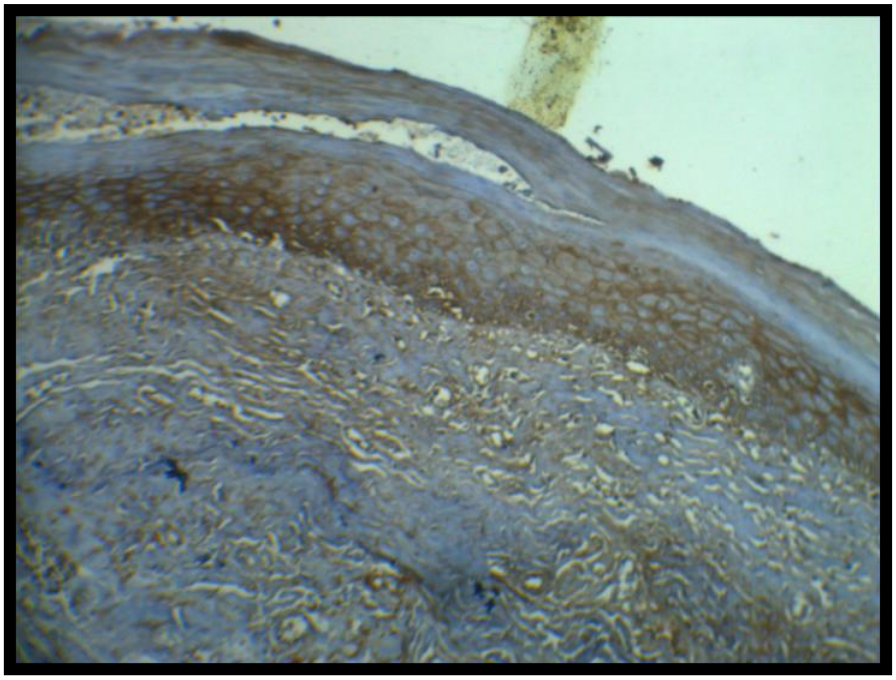


FIGURE 5a: Low power view (10X) showing mild expression of 8-OHdG in very early stage of OSMF

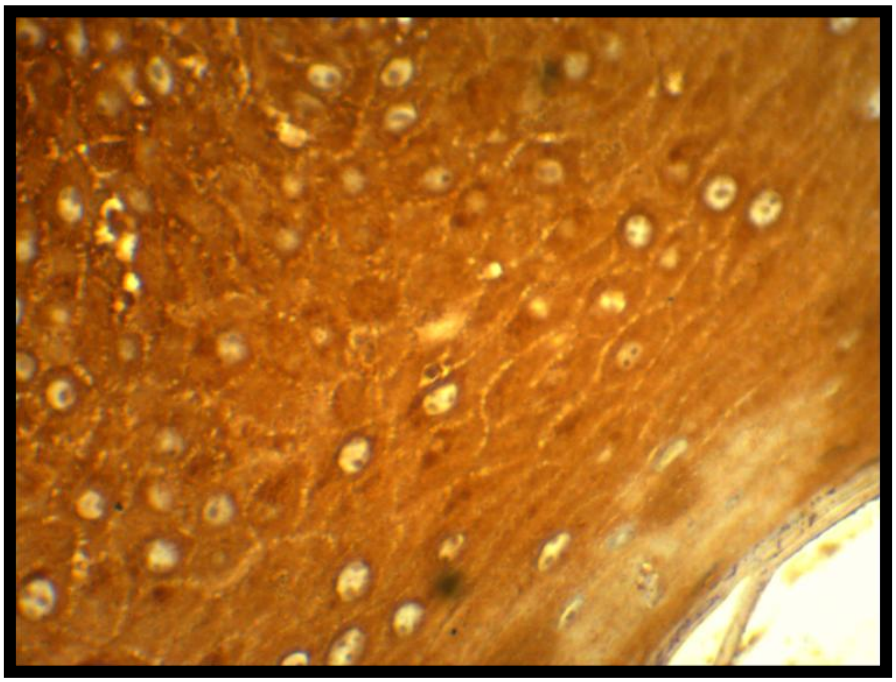


FIGURE 5b: High power view (40X) showing mild expression in very early stage

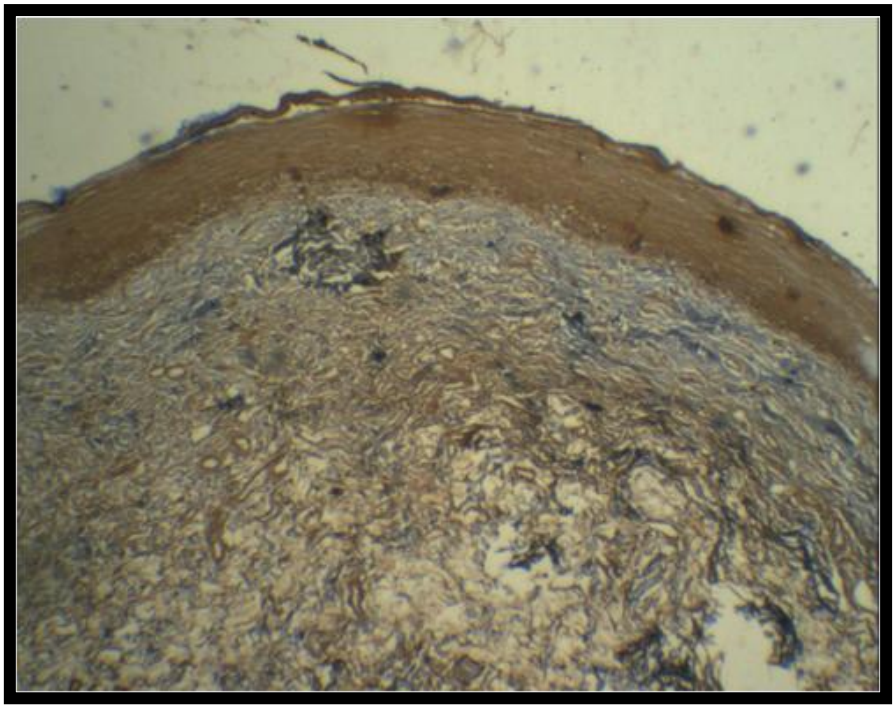


FIGURE 6a: Low power view (10X) exhibiting moderate expression of 8-OHdG in Early stage of OSMF

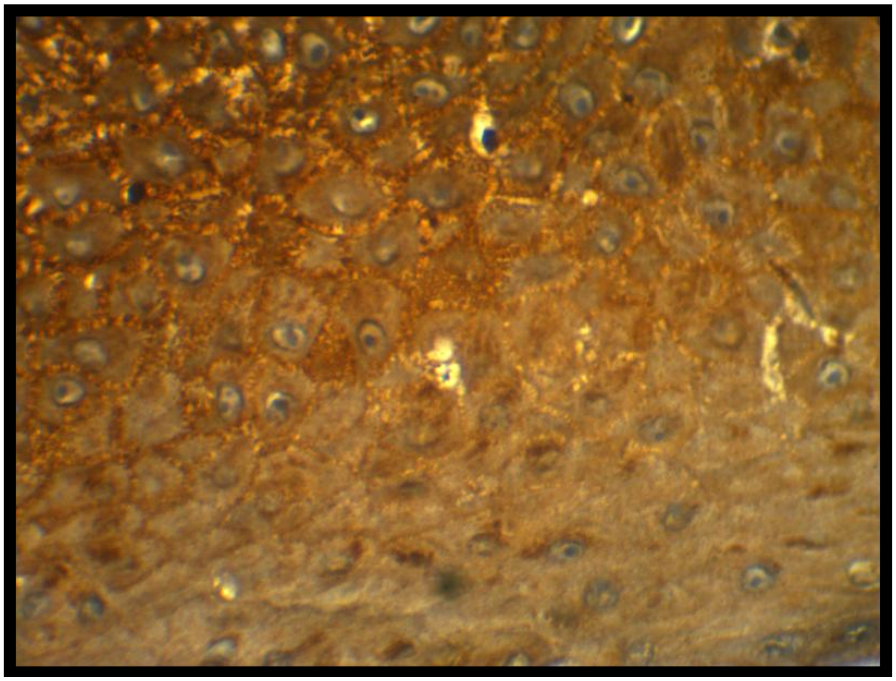


FIGURE 6b: High power view (40X) exhibiting moderate expression in Early stage of OSMF

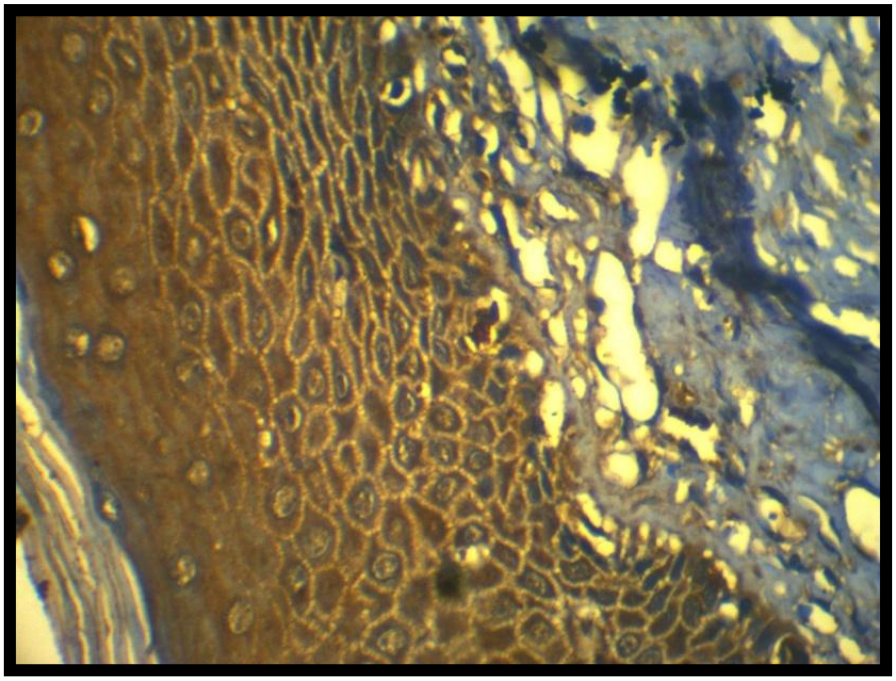


FIGURE 7: High Power view (40X) showing Intense expression of 8-OHdG in Moderately advanced OSMF

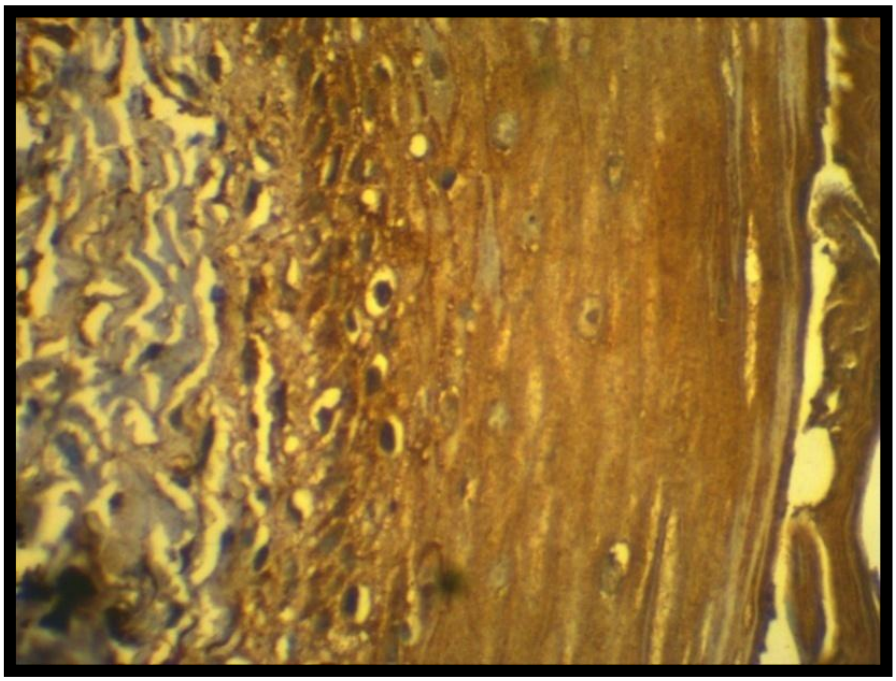


FIGURE 8: High power view (40X) showing Intense expression of 8-OHdG in Advanced stage of OSMF

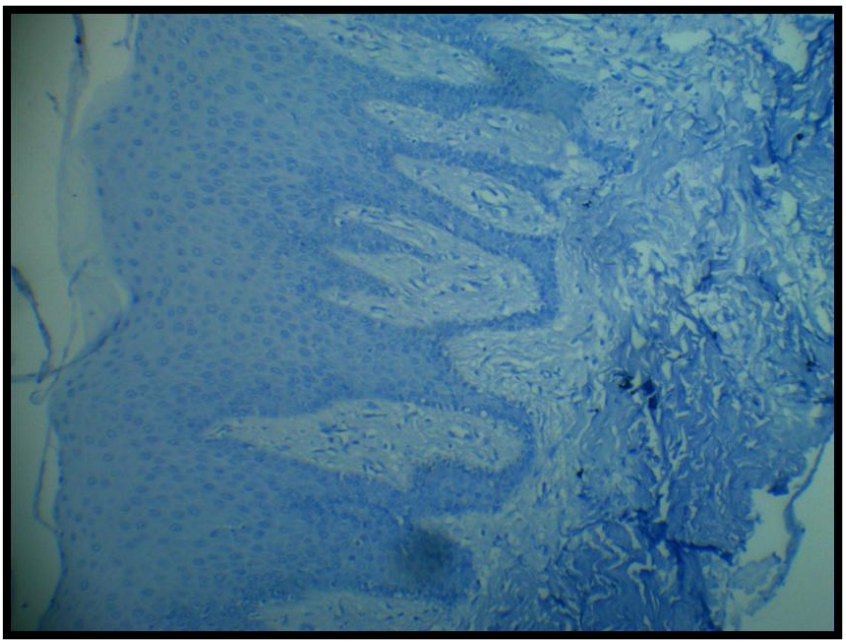


FIGURE 9: Low power view (10X) showing expression of 8-OHdG in normal oral mucosa

DISCUSSION

Oral potentially malignant disorders (OPMD), convey that not all lesions and conditions described under this term may transform into cancer, rather than there is a family of morphological alteration amongst which some may have an increased potential for malignant transformation. OPMD has the prevalence rate of 0.2-0.5%[12] with malignant transformation rate of 7.6%[32].

Oral submucous fibrosis, which was previously known as premalignant condition is now categorized under OPMD. **Sinor.P *et al*** suggested that arecanut is the most probable causative agent[19]. Arecanut has been declared as a known human carcinogen by International agency for research on cancer expert group in 2003.Numerous reactive oxygen species and reactive nitrogen species in the arecanut stimulate the carcinogenic effect in the cell.

Oxidative stress, is a process that occurs when there is failure of body's endogenous antioxidant defenses, to scavenge the free radical species. Oxidative stress can lead to oxidative DNA damage. The consquences of such DNA damage includes cell death, mutagenesis and carcinogenesis. Although it is very difficult to measure these free radical species because of very short half life, the products of these free radical species causes damage to DNA, lipids, proteins and these are considered as good markers of oxidative stress[60].

8-OHdG, a compound formed as a result of reaction between DNA and ROS, is an important biomarker of oxidative DNA damage. Very few studies on OSMF, OSCC and other oral lesions associated with oxidative stress are done using 8-OHdG as a biomarker.

The present retrospective study was carried out to study the immunohistochemical expression of 8-OHdG in oral submucous fibrosis. The study aimed to evaluate and compare its expression within the different grades of OSMF and also within normal oral mucosa.

The study consisted of two groups- Histopathologically diagnosed archival tissue blocks of 20 OSMF cases and 10 Normal oral mucosa. The OSMF cases were further divided into 4 subgroups (5 in each group- Very early, Early, Moderately Advanced, Advanced) according to histopathological grading system given by **Pindborg *et al.***

Two pathologists observed and evaluated the expression and Cronbach α test was applied to analyze the interobserver variability. On evaluation and comparison of 8-OHdG expression in OSMF, parameters given by Observer 2 was statistically not significant. Thus the evaluation made by Observer 1 was subjected to further statistical analysis.

Out of 20 cases of OSMF, all the cases exhibited positive cytoplasmic expression as in positive controls. In OSMF, among very

early group, about 80%(4) of cases exhibited mild expression of antigen and remaining one case showed moderate expression. In early group 60%(3) of cases showed moderate expression of antigen and remaining 40%(2) of cases showed mild expression. In moderately advanced group 40%(2) of cases exhibited intense expression of antigen and about 60%(3) of cases exhibited moderate expression. Among advanced group, 60%(3) of cases exhibited intense expression.40%(2) of cases showed moderate expression of antigen. (Table 1, Graph 1)

Kruskal Wallis test was performed for comparing staining intensities between different grades of OSMF and p value obtained was <0.01 which was statistically significant.

The study results are in accordance with the study done by **Pi Tsai *et al***[57] to evaluate the intensity of cytosol expression of 8-OHdG in renal fibrosis. He concluded that intensity of 8-OHdG expression was associated with severity of renal fibrosis.

Kumar *et al*[56] did a study to determine the salivary antioxidant capacity (TAC, GSH), free radicals (ROS, RNS) and oxidative DNA damage (8-OHdG) to understand the involvement of these biologic indexes in head and neck squamous cell carcinoma. He concluded that increased 8-OHdG in the habituate group (Smokers, chewers) along

with other oxidative stress markers suggest a strong contribution towards increased DNA oxidation by free radicals.

The results of the above studies can be correlated with our study that intense expression suggest a strong contribution toward the increased oxidative DNA damage with advanced grades of OSMF.

The cytoplasmic expression of 8-OHdG in OSMF is not in accordance with the study done by **Zimnoch *et al***[53] in chronic cholecystitis. The performed IHC study revealed a strong nuclear expression of 8-OHdG in the epithelial cells of the gallbladder mucosa in chronic cholecystitis..

In the present study, all the cases of OSMF showed immunoreactivity in the cytoplasm, because mitochondrial DNA is not covered extensively by proteins such as Histones, it may be more susceptible to excited oxygen than nuclear DNA. In addition mitochondrial DNA may be less efficient in repairing DNA damage and replication errors than the nucleus.

The phenomenon of cytoplasmic staining in the present study is similar to results obtained from previous study conducted by **Nomoto *et al***[54] in nonalcoholic fatty liver diseases. They proposed that the cytoplasmic fine granular expression may be a sensitive diagnostic biomarker of early non alcoholic fatty liver disease events.

In the present study the expression of 8-OHdG in OSMF was compared with Normal oral mucosa. Out of 20 cases of OSMF 30%(6) of cases exhibited mild expression, about 45%(9) cases exhibited moderate, & remaining 25%(5) cases exhibited intense expression of 8-OHdG.Among 10 cases of normal oral mucosa, 50% of cases exhibited mild positive expression, remaining 50% of cases exhibited negative expression. The reason for mild expression of 8-OHdG in normal buccal mucosa may be due to the presence of inflammation as the archival tissue blocks were obtained from the buccal mucosa of impacted third molar region.(Table 2, Graph 2)

On comparing the expression of 8-OHdG between OSMF and Normal oral mucosa, statistical analysis Mann-Whitney U test was performed. The p value obtained was <0.001 which was statistically highly significant.

The results in the present study are in accordance with the study done by **Canakci *et al***[61], to evaluate the levels of 8-OHdG and its relationship with antioxidant enzymes in periodontitis patients. They found higher 8-OHdG levels in periodontitis patients compared to that of healthy controls and concluded that higher 8-OHdG levels reflect increased oxygen radical activity during periodontal inflammation.

Similar findings are observed by **Jasdeep kaur *et al***[49], on evaluation of the salivary 8-OHdG levels in OSMF, OSCC and they

found significantly higher levels of salivary 8-OHdG in OSMF, OSCC compared with healthy normal subjects.

Pi Tsai *et al* [57] stated that an overproduction of ROS and downregulated expression of cellular antioxidant enzymes impairs DNA reparative mechanism which results in formation of 8-OHdG .The expression of 8-OHdG is an important biomarker of any chronic degenerative disease.

In the present study, the expression of 8-OHdG was compared with different layers of epithelium in OSMF. Out of 20 cases of OSMF, 55%(11) of cases exhibited expression of 8-OHdG in all the layers of epithelium. About 40%(8) of cases exhibited expression till spinous layers and remaining 5%(1) of case exhibited expression only in Basal /parabasal layer. The p value obtained was <0.36 which was statistically not significant.(Table 3)

Zimnoch *et al* [53] did a immunohistochemical study on chronic cholecystitis using a Monoclonal antibody against 8-OHdG, a biomarker of oxidative DNA damage. Increase in 8-OHdG expression was found in chronic cholecystitis and they concluded that the expression of 8-OHdG is associated with inflammation degree and disease duration.

Jasdeep kaur *et al* [49] studied the presence oxidative DNA damage in precancerous and SCC patients by evaluating the salivary 8-OHdG

levels. The study results revealed significantly higher levels of 8-OHdG when compared to normal subjects. They concluded that detection of 8-OHdG in oral precancerous lesions and cancer can act as a suitable diagnostic biomarker of oxidative DNA damage.

Our present study is the first attempt to evaluate the expression of 8-OHdG in tissue samples of OSMF to know the role of free radicals and oxidative DNA damage which enhances the initiation and progression of oral submucous fibrosis.

SUMMARY AND CONCLUSION

The aim of the study was to analyze the immunohistochemical expression of 8-OHdG in oral submucous fibrosis. A total of 20 samples of oral submucous fibrosis & 10 samples of normal mucosa were included in the study and the tissue samples were taken from the archival tissue blocks. Immunoexpression of 8-OHdG was studied by qualitative analysis of intensity.

From the present study done with 8-OHdG, following conclusions were drawn:

- Statistically significant difference exists on comparing the staining intensities between different grades of Oral submucous fibrosis.
- On comparing the expression of 8-OHdG between OSMF and normal oral mucosa, statistically high significant results exist.
- On comparing the expression of 8-OHdG in different layers of epithelium in OSMF the results were not statistically significant.
- Our present study is the first attempt on tissue samples by Immunohistochemical investigation, to visualize oxidative DNA damage in Oral submucous fibrosis and expression of 8-OHdG can be considered as a biomarker of considerable specificity.
- In Oral submucous fibrosis, the expression of 8-OHdG is also associated with degree of inflammation and disease duration.

- Further studies with a larger sample size, clinicopathological correlation, long term follow up will shed more light on the pathogenesis of Oral submucous fibrosis.
- This study will also be useful for the development of new therapeutic strategies targeting treatment modalities for Oral submucous fibrosis.

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ANNEXURE

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BIOSS ANTIBODIES

bs-1278R-TR

[Primary Antibody]

8-OHdG Polyclonal Antibody

Size: 20ul
Conc: 1ug/ul

Host:	Rabbit
Target Protein:	8-OHdG/8 Hydroxyguanosine
Clonality:	Polyclonal
Isotype:	IgG
Source:	KLH conjugated 8-OhdG
Purification:	Purified by Protein A.

Applications:
IHC-P(1:100-500)
IF(IHC-P)(1:50-200)

Cross Reactive Species:
Others
(8-OHdG)

Storage: Aqueous buffered solution containing 1% BSA, 50% glycerol and 0.09% sodium azide. Store at -20°C for 12 months.

Background: 8-Hydroxydeoxyguanosine (8OHdG) is a modified base that occurs in DNA due to attack by hydroxyl radicals that are formed as byproducts and intermediates of aerobic metabolism and during oxidative stress. There is increasing evidence to support the involvement of free radical reactions in the damage of biomolecules that eventually lead to several diseases in humans, such as atherosclerosis, cerebral and heart ischemia-reperfusion injury, cancer, rheumatoid arthritis, inflammation, diabetes, aging, and neurodegenerative conditions, such as Alzheimer's disease.

For research use only. Not intended for diagnostic or therapeutic use.

PI 0041, Rev. D DCN: 2665 Effective Date: 05/24/2017

Bio SB
BIOSCIENCE FOR THE WORLD

IVD
For In Vitro Diagnostic Use

ImmunoDetector Protein Blocker/Antibody Diluent

Intended Use *For In Vitro Diagnostic Use*

Summary And Explanation ImmunoDetector Protein Blocker/Antibody Diluent is used to dilute ascites, supernatants, purified antibodies, and polyclonal antibodies. The reagent is designed to minimize the non-specific reaction that may be caused by non-specific antibody interactions and encourages specific antigen-antibody binding.

Presentation ImmunoDetector Protein Blocker/Antibody Diluent contains phosphate TBST, pH 7.6, with bovine serum albumin, and preserved with sodium azide as an anti-microbial. It is provided in liquid form ready-to-use.

Availability

Catalog No.	Concentration	Volume
BSB 0113	Ready-to-use	15 mL
BSB 0040	Ready-to-use	50 mL
BSB 0041	Ready-to-use	100 mL
BSB 0114	Ready-to-use	200 mL
BSB 0115	Ready-to-use	1000 mL

Storage Store at 2-8°C **Stability** 3 years

The ImmunoDetector Protein Blocker/Antibody Diluent is stable at room temperature for up to **3** years from when originally produced (see expiration date on product label). This product is stable up to the expiration date on the label. Do not use this product after the expiration date. Adhere to all local laws when disposing of this product.

Preparation of Working Solutions

The ImmunoDetector Protein Blocker/Antibody Diluent is a ready-to-use working solution and requires no further preparation.

Recommended Protocol

When diluting antibodies, add antibody to the diluent, not diluent to the antibody. Addition of the antibody to the mixing vessel before the diluent can cause contamination of the diluent if multiple dispenses are necessary.

PathnSitu

Rev: A
Release Date: 03/13/2014
IVD

PolyExcel HRP/DAB Detection System-TWO STEP
Universal kit for Mouse and Rabbit Primary Antibodies

Intended Use: For *In vitro* diagnostic use

PolyExcel detection system is intended to use with primary antibodies raised against **mouse** and **rabbit** for the qualitative identification of antigens by light microscopy in normal and pathological paraffin-embedded tissues, cryostat tissues or cell preparations.

Summary and Explanation: PathnSitu's highly sensitive and specific PolyExcel two step detection system is non-biotin, micro-polymer based detection system which significantly reduce or shows no back ground on tissues containing high levels of avidin, biotin ex: Kidney, Liver and lymphoid tissues. This system is based on an HRP labeled polymer, which is conjugated with secondary antibodies.

Principal of procedure: Incubating the specimen for 5–10 minutes with H_2O_2 quenches any endogenous peroxidase activity. The specimen is then incubated with respective diluted mouse or rabbit primary antibody, followed by incubation with the PolyExcel Target Binder for 10 minutes then followed by a PolyExcel HRP labeled polymer using recommended 10 minutes incubation. Staining is completed by 5–10 minutes incubation with 3,3'-diaminobenzidine (DAB) substrate-chromogen which results in a brown-colored precipitate at the antigen site (DAB is a potential carcinogen; Please take appropriate precautions).

Kit Contents:
PathnSitu PolyExcel detection kit supplied as 3 pack sizes. Details below:

Description	Cat# / Pack Size	Kit Contents
PolyExcel HRP/DAB Detection System	PEH002-6ml	PolyExcel H2O2 PolyExcel Target Binder PolyExcel PolyHRP PolyExcel Stunn DAB Substrate Buffer PolyExcel Stunn DAB Substrate Chromogen
	PEH002-50ml	
	PEH002-100ml	

Materials required but not supplied:
1. Positive charged slides (PathnSitu Cat# PS011-72)
2. Control Tissues
3. Xylene
4. Isopropyl alcohol
5. DI Water
6. Hematoxylin
7. Cover glass
8. Mounting media
9. Antigen retrieval buffers (PathnSitu Cat# PS007, PS008, PS009)
10. Immuno wash Buffer (PathnSitu Cat# PS006)

INSTITUTIONAL ETHICS COMMITTEE AND REVIEW BOARD

ADHIPARASAKTHI DENTAL COLLEGE AND HOSPITAL

Melmaruvathur, Tamilnadu-603019

An ISO 9001:2008 certified institution. Accredited by NAAC with "B" grade.
Recognised by DCI, New Delhi. Affiliated to: The Tamil Nadu Dr. M.G.R. Medical University, Chennai.

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This ethical committee has undergone the research protocol submitted by **CHANDRAMOHAN.K** Post Graduate Student, Dept of **ORAL PATHOLOGY** under the title "**IMMUNOHISTOCHEMICAL DETECTION OF 8-HYDROXY DEOXYGUANOSINE (8-OHdG) - A BIOMARKER OF OXIDATIVE DNA DAMAGE IN ORAL SUBMUCOUS FIBROSIS",** Reference No**: 2015-MD-Br VI-DEV-05/APDCH** under the guidance of **DR.M.DEVI, MDS.,** for consideration of approval to proceed with the study.

This committee has discussed about the material being involved with the study, the qualification of the investigator, the present norms and recommendation from the Clinical Research scientific body and comes to a conclusion that this research protocol fulfils the specific requirements and the committee authorizes the proposal.

Date:

CHAIR PERSON

- Inform IEC/IRB immediately in case of any issue(s) / adverse events.
- Inform IEC/IRB in case of any change of study procedure, site and investigator.
- Annual report to be submitted to IEC/IRB.
- Members of IEC/IRB have right to monitor the trial with prior intimation.