IMMUNOHISTOCHEMICAL STUDY OF EXPRESSION OF MYOFIBROBLASTS IN ORAL SUBMUCOUS FIBROSIS, ORAL SQUAMOUS CELL CARCINOMA AND NORMAL MUCOSA

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CERTIFICATE

This is to certify that this dissertation titled "**IMMUNOHISTOCHEMICAL STUDY OF EXPRESSION OF MYOFIBROBLASTS IN ORAL SUBMUCOUS FIBROSIS, ORAL SQUAMOUS CELL CARCINOMA AND NORMAL MUCOSA**" is a bonafide dissertation performed by **A.SIVACHANDRAN** under our guidance during the postgraduate period 2009-2012.

This dissertation is submitted to THE TAMILNADU DR. M.G.R MEDICAL UNIVERSITY, in partial fulfillment for the degree of **MASTER OF DENTAL SURGERY** in **ORAL PATHOLOGY AND MICROBIOLOGY, BRANCH VI**. It has not been submitted (partial or full) for the award of any other degree or diploma.

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Introduction

Oral Submucous fibrosis (OSF) is an insidious, chronic disease affecting any part of the oral cavity and sometimes the pharynx. Although occasionally preceded by and/or associated with vesicle formation, it is always associated with a juxta-epithelial inflammatory reaction followed by a fibroelastic change of the lamina propria, with epithelial atrophy leading to stiffness of the oral mucosa and causing trismus and inability to eat[1]. There are connective tissue changes present in oral submucous fibrosis. The early stage is characterized by a finely fibrillar collagen, dispersed with marked edema. The fibroblastic response is produced by fibroblasts which are plump of young cells with abundant cytoplasm. In later stages the collagen present in connective tissue is moderately hyalinized, the amorphous change starting from the juste-epithelial basement membrane. Occasionally, thickened collagen bundles are still seen separated by slight residual edema. The fibroblastic response is less marked and the fibroblasts are mostly having elongated spindle-shaped nuclei and scanty cytoplasm. Oral epithelium in the affected areas is markedly atrophic as compared with the thickness of normal oral epithelium. The rete pegs are completely lost. The buccal mucosa is normally non-keratinised showing atrophy probably secondary to the connective tissue changes.[1]

The malignant transformation of OSF was suggested by Murti PR et al (1985) Oral submucous fibrosis patients were followed-up for a period of 17 years (median observation 10 yr) in Ernakulam District, Kerala, India. Oral cancer developed in five (7.6%) patients. The malignant transformation rate in the same sample was 4.5% over a 15-yr observation period (median 8 yr). These findings impart a high degree of malignant potential of oral submucous fibrosis.[2]

In wound healing extracellular matrix is primarily produced by myofibroblasts which are contractile cells expressing α-smooth muscle actin (α-SMA). Myofibroblasts play an important role in fibrosis, the relationship between myofibroblasts and oral submucous fibrosis was established.[3]

It is now recognized that the tumor microenvironment makes significant contribution to tumor progression. Activated fibroblast endothelial cells, inflammatory cells and various extra cellular matrix components are parts of this microenvironment. Most of the activated fibroblasts turn into α-smooth muscle actin expressing myofibroblast that often represents the majority of tumor stromal cells.[4]

Stromal elements play a key role in growth and development of different neoplasms and premalignant conditions. Myofibroblasts are the major components and they present in stromal tissue during carcinogenesis processes. An increase in the number of αSMA-positive myofibroblasts change the distribution pattern during oral carcinogenesis process which can be an expression of their role in tumor invasive characteristics.[5]

Thus, it is necessary to understand the molecular mechanism of oral submucous fibrosis and oral cancer progression. Myofibroblasts are responsible for producing a variety of factors that are involved in the fibrotic processes. Interruption of their development, recruitment or activation could provide a unique therapeutic approach to combat fibrosis. The purpose of this study was to evaluate and expression of myofibroblasts in normal mucosa, oral submucous fibrosis and oral squamous cell carcinoma.[5]

AIM

The aim of the study was to evaluate expression of myofibroblasts in oral submucous fibrosis and oral squamous cell carcinoma

OBJECTIVES

To evaluate the expression of myofibroblasts

- In Oral Submucous Fibrosis
- Oral Squamous Cell Carcinoma
- Normal mucosa

STUDY SETTING

The study was conducted in Department of Oral and Maxillofacial Pathology, Ragas Dental College and Hospital, Chennai, using paraffin embedded tissues.

It is a cross-sectional study done to evaluate the expression of myofibroblasts in oral submucous fibrosis, oral squamous cell carcinoma and normal buccal mucosa using immunohistochemistry in formalin fixed, paraffin embedded tissue specimens.

NULL HYPOTHESIS:

Myofibroblasts are not expressed in both oral submucous fibrosis and oral squamous cell carcinoma.

ALTERNATIVE HYPOTHESIS:

Myofibroblasts are expressed in both oral submucous fibrosis and oral squamous cell carcinoma.

STUDY SAMPLE SIZE

The study material comprised of 50 formalin fixed, paraffin embedded tissue specimens (archival blocks). The samples are divided into 3 groups namely: Group I, Group II and Group III.

Group I: 20 histopathologically confirmed oral submucous fibrosis tissue specimens.

Group II: 20 histopathologically confirmed oral squamous cell carcinoma tissue specimens.

Group III: 10 normal buccal mucosa tissue specimens.

METHODOLOGY

1. Tissue samples Oral submucous fibrosis(n=20), 2 Oral squamous cell carcinoma(n=20) and Normal mucosa (n=10) were taken from archival blocks.

2. Four micron thick sections were cut and used for routine hematoxylin and eosin (H & E) staining and immunohistochemical (IHC) staining.

3. This project was approved by The Institutional Review Board (IRB) of Ragas Dental College and Hospital, Chennai and patient consent was taken.

HEMATOXYLIN & EOSIN STAINING

REAGENTS

- Harri's hematoxylin
- 1% acid alcohol
- Eosin

PROCEDURE

- The slides were dewaxed in xylene and hydrated through graded alcohol to water.
- The sections on the slides were flooded with Harry's hematoxylin for 5 minutes.

- The slides were washed in running tap water for 5 minutes.
- The slides were differentiated in 1% acid alcohol for 5 minutes.
- The slides were washed well in running tap water for 5 minutes.
- The tissue sections on the slides were then stained in eosin for 30 seconds.
- The slides were washed in running tap water for 1 minute.
- The slides were then dehydrated through alcohol, cleared, mounted and viewed under light microscope (LM).

IMMUNOHISTOCHEMISTRY (IHC)

ARMAMENTARIUM

- Microtome
- Autoclave
- Hot air oven
- Slide warmer
- Couplin jars
- Measuring jar
- Weighing machine
- APES coated slides
- Slide carrier
- Aluminium foil
- Micro-pipettes
- Toothed forceps

- Electronic timer
- Beakers
- Rectangular steel tray with glass rods
- Sterile gauze
- Cover-slips
- Light microscope

REAGENTS USED

1. Conc. HCl
2. Laxbro soln.
3. APES (3 amino propyl tri ethoxy silane)
4. Acetone
5. Citrate buffer
6. Phospho Buffer Saline (PBS)
7. 3% H_2O_2
8. Deionized distilled water
9. Nuclear Fast Red
10. Absolute alcohol

11. Xylene

ANTIBODIES USED

1. Primary antibody – α-smooth muscle actin

Biogenex –synthetic NH2 terminal decapeptide of α-smooth muscle actin, Mouse monoclonal category and IgG2a immunoglobulins.

2. Secondary antibody – Biogenex-super sensitive IHC detection system kit (Poly Horse Radish Peroxidase -pretitrated anti-species immunoglobulins labeled with enzyme polymer, super enhancer reagent, anti mouse monoclonal negative control serum, and liquid DAB- Diamino-benzidine-chromogen)

IHC PROCEDURE

PRETREATMENT OF THE SLIDES

- The slides were first washed in tap water for few minutes
- The slides were then soaked in detergent solution for 1 hour
- After 1 hour, each slide was brushed individually using the detergent solution and were transferred to distilled water.
- The slides were washed in two changes of distilled water.
- The slides were washed in autoclaved distilled water.
- The slides were immersed in 1 N HCL (100 ml HCl in 900 ml distilled water) overnight.

- The following day slides were taken out of acid and washed in two changes of autoclaved distilled water.
- All the slides were then transferred to slide trays, wrapped in aluminium foil and baked in hot air oven for 4 hours at 180 degrees centigrade.

APES (3 Amino propyl tri ethoxy silane) coating

Slides first dipped in couplin jar containing acetone for 2 minutes

Dipped in APES for 5 minutes

Dipped in two changes of distilled water for 2 minutes each

Slides left to dry

PREPARATION OF PARAFFIN SECTIONS

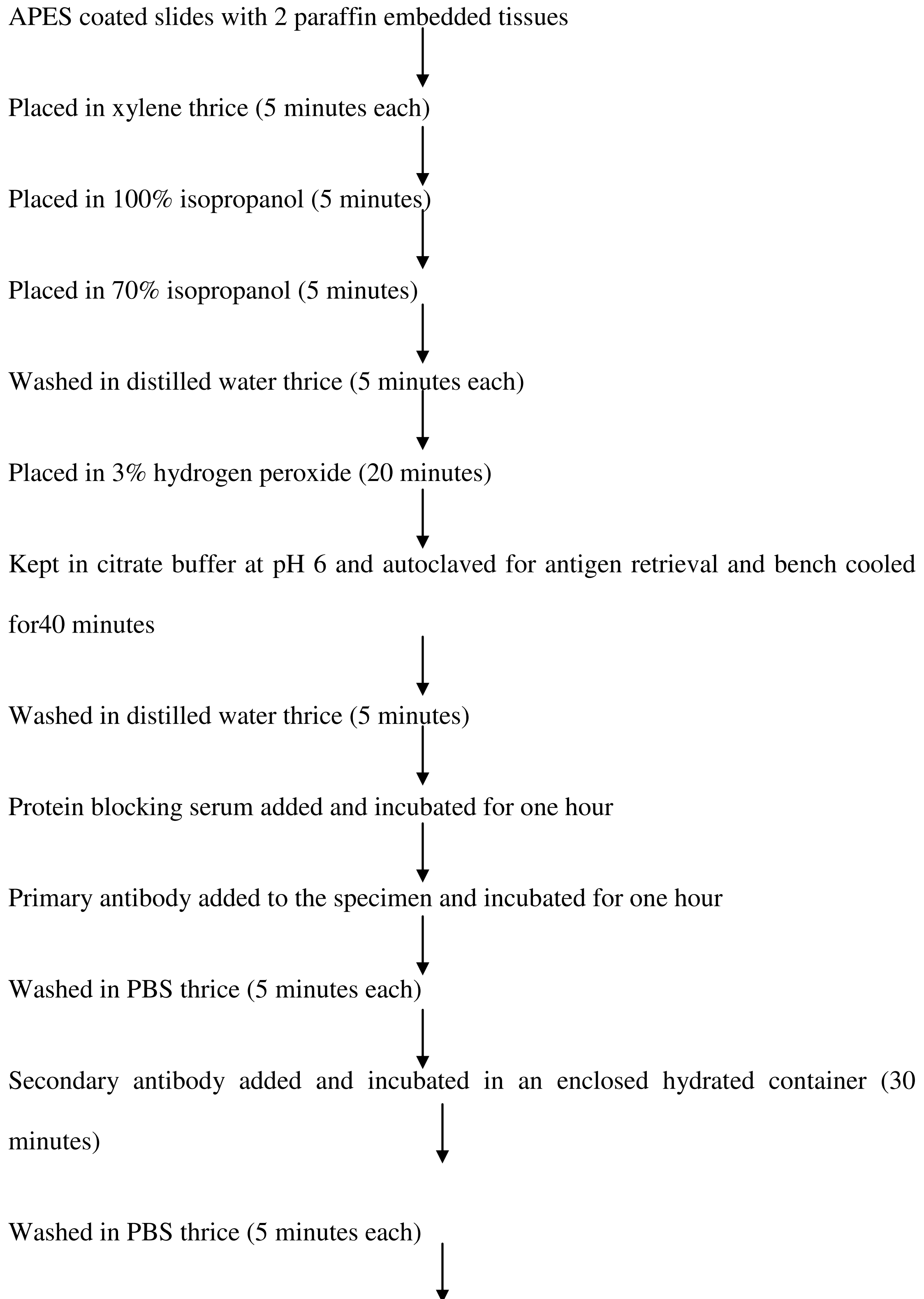
After the slides were dry, tissue section of 5 micron thickness were made in a rotary manual microtome. The ribbons of tissue section were transferred onto the APES coated slide from the tissue float bath such that two tissue bits come on to the slide with a gap in between. One of the tissue sections was labeled positive (P) and the other negative (N).

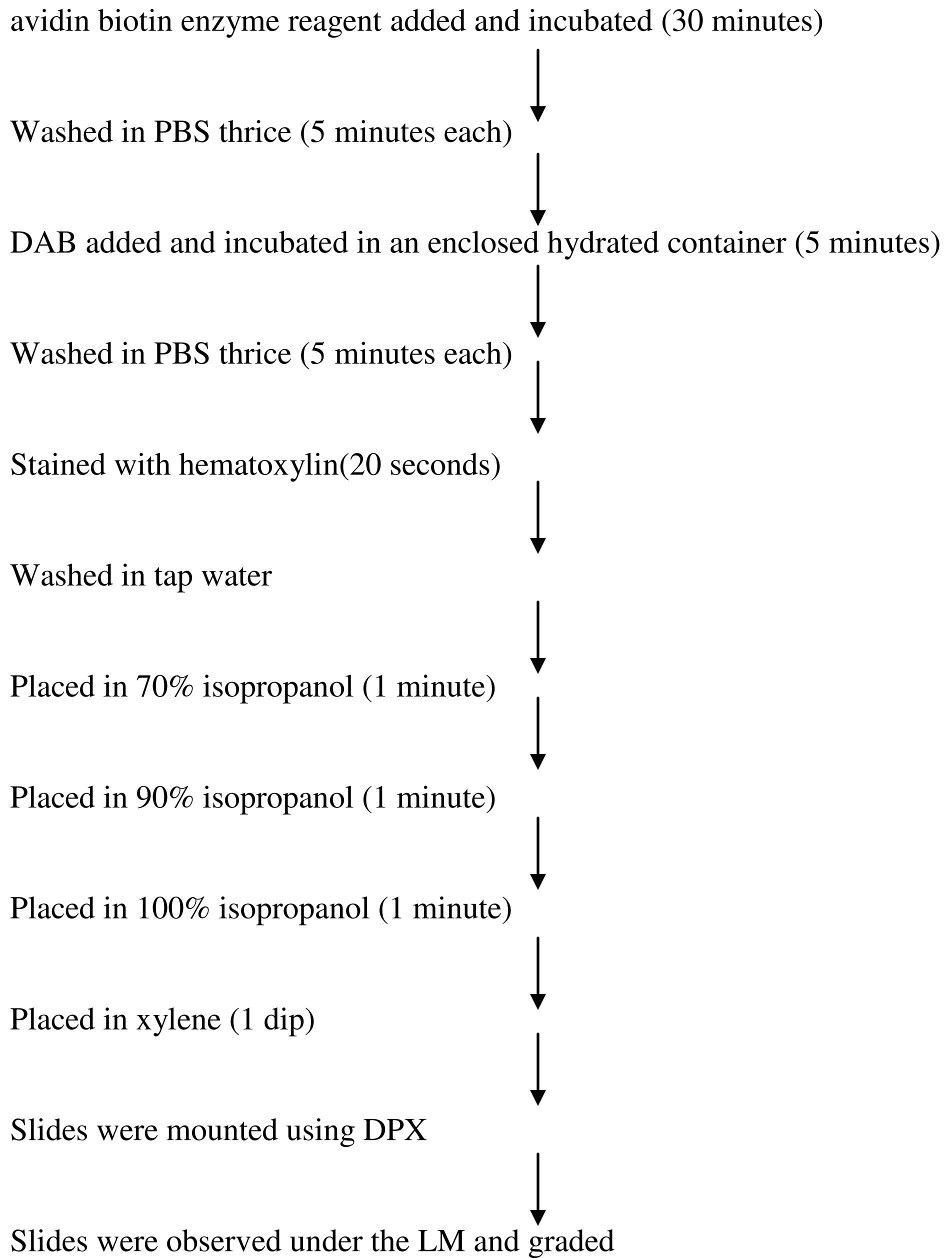
PROCEDURE

The slides with tissue sections were treated with three changes of xylene to remove paraffin wax. They were put in descending grades of alcohol and then rehydrated with water. Slides were then treated with 3% hydrogen peroxide for 30 minutes to quench

endogenous peroxidase activity of cells that would otherwise result in non – specific staining. The slides were then transferred to citrate buffer and autoclaved for antigen retrieval at 15 lbs pressure for 30 minutes. The slides were dipped in 3 changes of distilled water for 5 minutes each. Circles were drawn around the tissues, so that the antibodies added later on do not spread and are restricted to the circle. The tissues were incubated in protein blocking serum for one hour in an enclosed hydrated container. Then the slides were wiped carefully without touching the tissue section to remove excess of blocking serum. The primary antibody, pre-diluted α-smooth muscle actin was added to P tissue on the slide and then to the N, PBS was added. The slides were incubated for half an hour . Then the slides were wiped carefully without touching the tissue section to remove excess of antibody and washed with three changes of cold PBS for 5 minutes. Then a drop of ENVISION horseradish peroxidase was added on both the sections and the slides were incubated for 30 minutes. Later slides were washed in three changes of cold PBS for 5 minutes in each. The slides were wiped carefully without touching the tissue section to remove excess PBS. The sections were washed in 3 changes of cold PBS for 5 minutes in each. Then the slides were wiped carefully without touching the tissue section to remove excess PBS. Then a drop of DAB was added to the sections. Slides were then washed in distilled water to remove excess chromogen and counter stained with hematoxylin. Then the slides were transferred to 70% alcohol, 100% alcohol and one change of xylene. The tissue sections were mounted with DPX. The slides were then observed under the microscope. Throughout the procedure care was taken not to dry the tissues.

IHC PROCEDURE FLOW CHART




avidin biotin enzyme reagent added and incubated (30 minutes)
Washed in PBS thrice (5 minutes each)
DAB added and incubated in an enclosed hydrated container (5 minutes)
Washed in PBS thrice (5 minutes each)
Stained with hematoxylin(20 seconds)
Washed in tap water
Placed in 70% isopropanol (1 minute)
Placed in 90% isopropanol (1 minute)
Placed in 100% isopropanol (1 minute)
Placed in xylene (1 dip)
Slides were mounted using DPX
Slides were observed under the LM and graded

Statistical analysis was done using SPSS ™ software (version 11.5). $p \leq 0.05$ was considered to be statistically significant.

- Chi-square test analysis was done to compare the proportion of myofibroblasts expression among the three study groups.
- The inter-observer variability for the intensity of stain and percentage of cells stained was assessed using kappa statistics.

STAINING CRITERIA AND STAINING INDEX CALCULATION METHODS

Calculation of staining intensity (SI)

No staining scored as **0** (SI 0)

Staining visible only at 40X scored as **1** (SI 1)

Staining visible at only at 10X not in 4X scored as **2** (SI 2)

Staining visible even at 4X scored as **3** (SI 3)

Calculation of percentage of cells (Labeling Index-LI)

0 positive cells scored as **0** (LI 0)

1-25% of positive cells scored as **1** (LI 1)

25-50% of positive cells scored as **2** (LI 2)

50-100% of positive cells scored as **3** (LI 3)

Calculation of staining index

It is derived from multiplication of staining intensity and percentage of cells. (SI xLI)

The final score is grouped into no stain, mild, moderate and intense categories.

1. If score is 0 then it is grouped to **no stain** category

2. If the score is 1, 2 then it is grouped to **mild** category

3. If the score is 3, 4 then it is grouped to **moderate** category

4. If the score is 6 to 9 then it is grouped to **intense** category

Schwartz in 1952 described five Indian women from Kenya with a condition of the oral mucosa including the palate and the pillars of the fauces which he called "atrophica idiopathica mucosa oris". Later on it was termed Oral Submucous Fibrosis by Joshi in 1953.

Paymaster JC in 1956[6] conducted a clinical study with 650 patients in India and concluded that OSF is a potential precancerous lesion and also he described the development of slow-growing squamous cell carcinoma in one third of the cases with submucous fibrosis.

Pindborg JJ and Sirsat SM in 1966 [1] described OSF (Oral Submucous fibrosis) is an insidious, chronic disease affecting any part of the oral cavity and sometimes the pharynx. Although occasionally preceded by and/or associated with vesicle formation, it is always associated with a juxta-epithelial inflammatory reaction followed by a fibroelastic change of the lamina propria, with epithelial atrophy leading to stiffness of the oral mucosa and causing trismus and inability to eat. There are connective tissue changes present in oral submucous fibrosis. The early stage is characterized by a finely fibrillar collagen, dispersed with marked edema. The fibroblastic response is produced by fibroblasts which are plump of young cells with abundant cytoplasm. In later stages the collagen present in connective tissue is moderately hyalinized, the amorphous change starting from the juste-epithelial basement membrane. Occasionally, thickened collagen bundles are still seen separated by slight residual edema. The fibroblastic response is less marked and the fibroblasts are mostly having elongated spindle-shaped nuclei and scanty cytoplasm.

Oral epithelium in the affected areas is markedly atrophic as compared with the thickness of normal oral epithelium. The rete pegs are completely lost. The buccal mucosa, normally non-keratinised are showing. The atrophy of the oral epithelium is probably secondary to the connective tissue changes.

Ranganathan K, Devi MU, Joshua E et al in 2004[7] conducted a case control study in Chennai. They concluded that (OSF) is a precancerous condition strongly associated with the use of areca nut. OSF is significantly associated with use of pan masala and pan masala plus areca nut, with or without concurrent alcohol use. Tobacco smoking and use of alcohol without use of areca nut containing products is not associated with OSF. The areca nut and areca nut-containing agents, with or without alcohol or tobacco, are of likely aetiological significance in the development of OSF. In particular, individuals having the habit of using pan masala plus alcohol are particularly at risk of developing OSF. Tobacco or alcohol were not risk factors for OSF to occur, whether taken alone or together.

Ahmad MS, Ali.S.A., Ali. A.S. et al in 2006[8] studied about the etiological factors associated with OSF. It was found that average betel quid consists of areca nut, lime, catechu, and tobacco wrapped in betel leaf approximately 3.5 grams to 4 grams in weight. The moisture content of betel quid is 70% and dry weight of tobacco and areca nut is about 1.14 grams whereas in pan and gutkha the weighing around 3.5 grams approximately has 7% of moisture and 3.26 grams of dry areca nut. Hence habitual chewer of pan or gutkha is having more chance of developing OSF than

those who are chewing only betel quid. Due to the established malignant potential and the complications of trismus, it is recommended that the patients diagnosed as having OSF are followed up regularly. In India, the prevalence of substance use with or without other associated habits is increasing as the socioeconomic status of the population is increasing. The impact of OSF has thus become an imperative public health concern**.**

Clinical and histopathological classifications of Oral Submucous Fibrosis

Rajendran R in 2003[9] studied about the clinical and histopathological features. He described that the onset of OSF is insidious over a 2 to 5-year period. The prodromal symptoms start with a burning sensation in the mouth while taking spicy food, appearance of blisters usually seen in the palate, ulcerations or recurrent generalized inflammation of the oral mucosa, increased salivation, defective gustatory sensation, and dryness of the mouth. There are appearance of small vesicles in the cheek and palate. Petechiae are also seen along the vascular dilatations in early stages of the disease. External irritant like chilli or areca nut may induce vascular response due to hypersensitivity of the mucosa and formation of petechiae mostly on the tongue followed by the labial and buccal mucosa with no sign of blood dyscrasias or systemic disorders. Histologically slightly atrophic epithelium with numerous dilated and blood-filled capillaries juxta-epithelially will be present. As the disease progresses, the oral mucosa becomes blanched and slightly opaque, and white fibrous bands appear. The buccal mucosa and lips may be affected at an early stage although

it was thought that the palate and the faucial pillars are the areas involved first. The oral mucosa is involved symmetrically and the fibrous bands in the buccal mucosa run in a vertical direction. The density of the fibrous deposit varies from a slight whitish area on the soft palate causing no symptoms to a dense fibrosis causing fixation and shortening or even deviation of the uvula and soft palate. The fibrous tissue in the faucial pillars varies from a slight submucosal accumulation in both pillars to a dense fibrosis extending deep into the pillars with strangulation of the tonsils. It is this dense fibrosis involving the tissues around the pterygomandibular raphe that causes varying degrees of trismus .The exact site and extent of the fibrosis and its role in the causation of trismus are determined by several factors. For example, the anatomical and physiological integrity of the underlying musculature is vital for the degree of mouth opening. Based on electron microscopical observations. Muscle degeneration which is present in OSMF, significantly affect the already existing trismus in these patients. Equally important is the involvement of the pterygomandibular raphe, a site commonly reported to accentuate the extent of trismus. Another factor is the duration of the disease in the affected individuals, which depends on the subjective evaluation of signs and symptoms. Current views of a protracted and insidious onset of the disease and its very slow progression make any sort of objective diagnostic criterion difficult, at least in the earlier stages. A factor which seems to be overlooked by many investigators while recording the extent of mouth opening is the acuteness of oral symptoms (persistent/ recurrent stomatitis and glossitis) at the time of recording. Most investigators agree that in OSMF the patient experiences a protracted period of stomatitis and/or glossitis with remissions and

exacerbations , which must be taken into consideration, together with the age of the patient and the extent and site of fibrosis, when recording the extent of trismus. Sometimes the fibrosis spreads to the pharynx and down to the pyriform fossae. Upon palpation, a circular band can be felt around the entire rima oris, and these changes are quite marked in the lower lip . All observers have noted impairment of tongue movement in patients with advanced OSMF, but only some have registered an atrophy of the tongue papillae. With progressing fibrosis, patients complain of stiffening of certain areas of the mucosa leading to difficulty in opening the mouth, inability to whistle or blow out a candle and difficulty in swallowing. When the fibrosis involves the pharynx, the patient may experience referred pain in the ear. The clinical and histological features of OSF are salient and various investigators have correlated the same.

Oral Submucous Fibrosis – Etiopathogenesis

Ranganathan K and Gauri Mishra in 2006[10] described about different schemes in OSF classifications. Classification of oral submucous fibrosis done on the following basis

1. **Classification systems based on clinical features.**

    Pindborg JJ (1989)

    Lai DR et al (1995)

    Ranganathan K et al (2001)

    Rajendran R (2003)

2. **Classification systems based on histopathological features.(6)**

Pindborg JJ et al (1966)

Utsunomia H et al (2005)

3. **Classification systems based on clinical features and histopathological features.**

    Khanna JN et al (2005)

1. Classification based on clinical features.

Pindborg JJ (1989) divided OSMF based on the physical findings into 3 stages

Stage 1: Stomatitis includes erythematous mucosa, vesicles, mucosal ulcers, melanotic mucosa pigmentation and mucosal petechiae.

Stage 2: Fibrosis occurs in healing vesicles and ulcers, which is the hallmark of this stage.Early lesions demonstrate blanching of the oral mucosa. Older lesions include vertical or circular palpable fibrous bands in the buccal mucosa and around the mouth opening or lips. This results in a 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 the mouth opening stiff and small tongue, blanched and leathery floor of the mouth, fibrotic and depigmented gingiva, rubbery soft palate with decreased mobility, blanched and atrophic tonsils, shrunken bud like uvula, and sunken cheeks, not commensurate with age or nutritional status.

Stage 3: Leukoplakia is found in more than 25% of individuals **.**Speech and hearing deficits may occur .

Lai DR (1995) conducted a study and divided the OSMF based on their inter incisal distances

Group A: Mouth opening greater than 35mm

Group B: Mouth opening between 30-35mm

Group C: Mouth opening between 20-30mm

Group D: Mouth opening less than 20mm.

Ranganathan K et al (2001) used baseline study on the mouth opening parameters of normal patients and divided OSMF patients as

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

Group II: Limited mouth opening 20mm and above

Group III: Mouth opening less than 20 mm

Group IV: OSMF with advanced with limited mouth opening(>7mm and <20mm). Precancerous or cancerous changes seen throughout mucosa.

This classification includes the common clinical presentations of fibrosis and burning sensation along with range of mouth opening would be more convenient and appropriate for the clinicians in diagnosis and follow up.

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 the mouth.

Advanced OSMF: Blanched and slight opaque mucosa,fibrous bands in buccal mucosa running in vertical direction. Palate and faucial pillars are the areas first involved. Gradual impairment or tongue movement and difficulty in mouth opening.

2. Classification based on histopathological features

Pindborg JJ and Sirsat SM (1996) were the first to divide OSMF depending on only histopathological features alone

Very early stage: Fine fibrillar collagen dispersed with marked oedema. Plump young fibroblasts containing abundant cytoplasm. Blood vessels are dilated and congested. Inflammatory cells mainly polymorphonuclear leukocytes with occasional eosinophils are formed.

Early stage: Juxtaepithelial area shows early hyalinization. Collagen present are still in separate thick bundles. Moderate number of plump young fibroblasts is present. Dilated and congested blood vessels. Inflammatory cells are primary lymphocytes, eosinophils and plasma cells.

Moderately advanced stage: Collagen is moderately hyalinized. Thickened collagen bundles are separated by slight residual oedema. 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 hyalanized. Smooth sheets with no separate bundles of collagen are seen. Edema is absent. Hyalinized area is devoid of fibroblasts. Blood vessels are completely obliterated or narrowed. Inflammatory cells found are lymphocytes and plasma cells.

Utsunomiya H, Tilakratne WM, Oshiro K et al (2005)

Early stage: Large number of lymphocytes in subepithelial, connective tissue, zone along with myxodematous changes.

Intermediate stage: Granulation change close to the muscle layer and hylanization appears in subepithelial zone where blood vessels are cmpressed by fibrous bundles.

Advanced stage: Inflammatory cell infiltrate hardly seen. Number of blood vessels dramatically small in subepithelial zone. Marked fibrosis areas with hylaine changes extending from subepithelial to superficial muscle layers. Atropic degenerative changes start in muscle fibres.

3.Classification system based on clinical features and histopathological features

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

Group I: Very early cases

Group II: Early cases

Group III: Moderately advanced cases

Group IV A: Advanced cases;

Group IV B: Advanced cases with premalignant and malignant changes.

Maher R, Lee AJ, Warnakulasuriya KA et al in 1994[11] conducted a case control study in OSF. They described the direct role of areca nut in the etiopathogenesis of OSF. Experimentally, an alkaloid component of the areca nut, "Arecoline" can induce fibroblast proliferation and collagen synthesis and may penetrate the oral mucosa to cause progressive cross linking of collagen fibres .

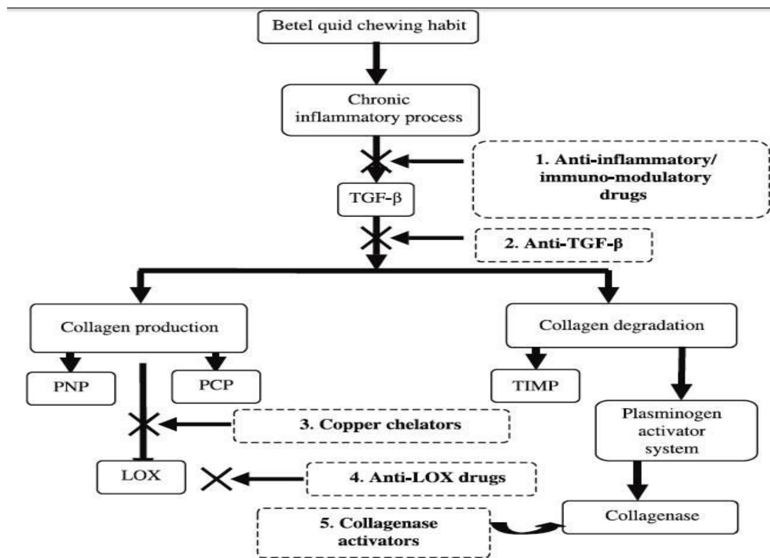
In IARC Monographs (2004)[12] They found that the aetiology of OSF is largely unknown, there is a relationship between the use of areca nut extract and development of OSF prompting the World Health Organization to classify areca nut as a group I carcinogen. Areca nut is consumed in various forms with and without tobacco such as betel quid (areca nut + slaked lime + betel leaf), pan masala (powdered areca nut with additives + flavouring agents + tobacco) and raw areca nut (seeval flakes and kotta paaku granules). In India, there are regional variations in the type of areca nut product used. However, OSF pathogenesis is multifactorial.

P. Rajalalitha and S. Vali in 2005[13] studied about the various molecular events that occur in OSF. They highlighted the role of locally acting irritants on the oral mucosa. The local irritants include areca nut, capsaicin, tobacco, pungent and spicy foods and alcohol. Another equally important causative aspect suggested to contribute to OSF is the prolonged, chronic deficiency of Iron/Vitamin B complex. Since such nutritional deficiencies are more common in females than males, it explains the reason why OSF

is more prevalent in the female population. This evidence implies that OSF may be considered a collagen-metabolic disorder resulting from exposure to areca nuts. Collagen is the major structural component of the connective tissues and its composition within each tissue needs to be maintained for proper tissue integrity. The synthesis of collagens is influenced by a variety of mediators, including cytokines, growth factors, lymphokines and hormones. Transforming growth factor-beta (TGF-b); TGF-b1, in particular plays a major role in wound repair and fibrosis. This growth factor has also been involved in the development of many fibrotic diseases. It induces the deposition of extra cellular matrix (ECM) by increasing the synthesis of matrix proteins like collagen and decreasing its degradation of collagen by stimulating various inhibitory mechanisms. Although TGF-b is essential for healing, overproduction leads to scar tissue, and fibrosis. As in other fibrotic diseases, TGF-b signaling pathway might be critical for pathogenesis of OSF. There are many reasons for chewing betel. It causes euphoria, increases salivation, satisfies hunger, relieves tooth pain, etc. The areca nut containing major alkaloids like arecoline, arecaidine, arecolidine, guyacoline, and guacine will induce various molecular events via TGF-b and eventually results in fibrosis. The important flavonoid components of areca nut are tannins and catechins. Arecoline is the most abundant alkaloid. These alkaloids undergo nitrosation and give rise to N-nitrosamines, which might have a cytotoxic effect on cells. Oral keratinocytes secrete prostaglandins (PGs) in response to areca nut extract. This causes inflammatory mediators like activated T cells, macrophage, etc emergence and will lead to fibrosis. Persistent tissue inflammation is crucial factor for cancer and tissue fibrosis to occur. Thus, it can be considered that induction of

oral mucosal inflammation by BQ (Betel Quid) ingredients to be a critical event in the pathogenesis of OSF. Cytokines like interleukin 6, tumor necrosis factor (TNF), interferon A, etc. and growth factors like TGF-b are synthesized at the site of inflammation. Increased susceptibility among individuals who are anemic due to iron or vitamin B12 deficiencies has been demonstrated. This could be due to increased fragility of the mucosa by which there is more BQ absorption. TGF-b1 is a key regulator of ECM (Extra Cellular Matrix) assembly and remodeling. The action of TGF-b on the genes implicated in the formation and degradation of the ECM is mostly exerted at the transcriptional level through ill defined Intra-cellular pathways.

An overview of pathogenesis of Oral submucous fibrosis



Tilakaratne, M.F. Klinikowski, Takashi Saku et al in 2006[14] reviewed the etiology and pathogenesis of Oral Submucous Fibrosis from data taken from recent epidemiological studies. The studies provided evidence that areca nut is the main aetiological factor for OSF. A dose-dependent relationship was observed for both

frequency and duration of chewing areca nut (without tobacco) in the development of OSF. Commercially freeze dried products such as pan masala, Guthka and mawa have high concentrates of areca nut per chew and appear to cause OSF more rapidly than by self prepared conventional betel quid that contain smaller amounts of areca nut. The authors hypothesized that the increased collagen synthesis or reduced collagen degradation as possible mechanisms in the development of the disease. These chemicals appear to interfere with the molecular processes of deposition and/or degradation of extracellular matrix molecules such as collagen.In vitro studies on human fibroblasts using areca extracts or chemically purified arecoline support the theory of fibroblastic proliferation and increased collagen formation that is also demonstrable histologically in human OSF tissues.

Oral Submucous Fibrosis – Laboratory Findings

Rajendran R in 2003[9] studied about the clinical and histopathological features. He described about various haematological abnormalities including anemia, increased erythrocyte sedimentation rate, eosinophilia, increased gamma globulin, a decrease in serum iron and an increase in total iron binding capacity. The percentage saturation of transferrin also decreased and a significant reduction in total serum iron and in albumin was found. It is doubtful regarding the role of iron deficiency anaemia as the cause or the effect of the disorder. A rise in serum mucoproteins, mucopolysaccharides and antistreptolysin titre '0' (measured in Todd's unit) has also

been reported. A significant depression of the lactate dehydrogenase isoenzyme ratio (LDH IV / LUH 11) is reported at the tissue level in OSF.

Rooban, George A et al in 2004[15] conducted a cytological study of copper in OSF. They found that high copper content present habitual areca nut chewers plays a vital role in pathogenesis of OSF. This study evaluates the copper-staining pattern of buccal epithelial cells in oral cytological smears of non-chewers, chewers, and OSF. Copper appeared as shades of pale red within the cytoplasm of chewers and did not show any stain in non-chewers. Intense red stain was seen in OSF smears as dark granules within the cytoplasm. They concluded that intense staining of copper in OSF buccal smears in areca nut chewers support the role of copper in the pathogenesis of OSF.

Malignant transformation of oral submucous fibrosis

Murti PR, **Bhonsle RB, Pindborg JJ et al in 1985**[2] conducted in OSF affected individuals where 66 patients with oral submucous fibrosis were followed-up for a period of 17 yr (median observation 10 yr) in Ernakulam District, Kerala, India. Oral cancer developed in five (7.6%) patients. The malignant transformation rate in the same sample was 4.5% over a 15-yr observation period (median 8 yr). These findings impart a high degree of malignant potential of oral submucous fibrosis.

Radhakrishna Pillai, Prabha Balaram, PhD and Kannan Sankara Reddiar, MS in 1992[16] suggested that OSF is a condition with a high risk of malignant transformation. Till date, no conclusive etiologic agent has been identified, although plenty of data have been generated on various aspects of the disease. These include genetic, carcinogenic, immunologic, viral, nutritional, and autoimmune possibilities, all of which also have been implicated in the development of oral cancer

J.H. Jeng, **M.C. Chang and L.J. Hahn in 2000**[17] reviewed about the association between areca nut chewing and carcinogenesis. Areca nut products induce mutagenic and genotoxic effects, in addition to inducing preneoplastic as well as neoplastic lesions in experimental animals. Areca nut should, thus, be highly suspected as a human carcinogen. Toxicity studies relating to areca nut contained polyphenols and tannins are not conclusive, with both carcinogenic and anti-carcinogenic effects being reported. The mutagenicity and genotoxicity of areca alkaloids has been detected by many short-term assays. It would thus appear that areca nut toxicity is not completely due to its polyphenol, tannin and alkaloid content. Reactive oxygen species produced during auto-oxidation of areca nut polyphenols in the BQ-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.

N. Afroz, S.A. Hasan and S. Naseem in 2006[18] studied about the association between areca nut and malignant transformation of OSF. They concluded that arecoline in areca nut can induce fibroblast proliferation and collagen synthesis and may penetrate the oral mucosa to cause progressive cross linking of collagen. Tobacco chewing and smoking are not considered to play a role in the development of this disease.

Siddharth Pundir, Susmita Saxena and Pooja Aggrawal in 2010[19] reported development of cancer from OSF in 2 cases.

In patients with submucous fibrosis, the oral epithelium becomes atrophic and thereby becomes more vulnerable to carcinogens. It is now accepted that chewing areca is the most important aetiological factor for developing OSF. The atrophic epithelium shows first an intercellular edema and later epithelial atypia associated with moderate epithelial hyperplasia. From then on, carcinoma may develop any time. It is suggested that submucous fibrosis should be regarded as a condition that causes predisposition to the development of oral cancer. Here we are presenting two cases of oral submucous fibrosis showing malignant potential and development of oral squamous cell carcinoma.

Myofibroblasts in fibrosis and wound healing

Desmouliere.A in 1995[20] reviewed the factors influencing myofibroblast differentiation during wound healing and fibrosis. The author reviewed that the granulation tissue fibroblasts develop several ultrastrucutural and biochemical

features of smooth muscle cells, including the presence of microfilament bundles and expression of alpha smooth muscle actin , the actin isoform typical of contractile vascular smooth muscle cells. They suggested that when granulation tissue evolves into a scar, myofibroblasts containing alpha smooth muscle actin disappear, as a result of apoptosis. In contrast myofibroblasts expressing alpha smooth muscle actin persist in excessive scarring and in fibrotic conditions. The author concluded by stressing on the need for further studies and the factors regulating the phenotype of myofibroblasts and understanding their behavior.

Mutsaers and Bishop in 1997[21] reviewed mechanisms of tissue repair. The overview briefly described the process of wound healing and highlighted some of the key recent advances in this field of research. It emphasizes the importance of cell-cell and cell-matrix interactions, particularly relating to the role of cell surface adhesion molecules, and describes developments that have led to a better understanding of the dynamic nature of matrix turnover with reference to negative and positive mediators that regulate procollagen gene expression and protein production. An important component of this was concerned with the development of tissue fibrosis, which accompanies a number of disease states and demonstrates remarkable parallels with the normal wound healing process; excessive amounts of matrix are laid down but the resolution of scarring, which would be anticipated in wound healing, is impaired. Since cytokines play an important role in regulating cell function such as proliferation, migration and matrix synthesis, it is the balance of these mediators which is likely to play a key role in regulating the initiation, progression and

resolution of wounds. Finally, this review highlights areas of tissue repair research in which recent developments have important clinical implications that may lead to novel therapeutic strategies.

Jyoji Yamate, Akiko Okado, Mitsuru Kuwamura et al in 1998[22] reviewed the localization of myofibroblasts during rat renal interstitial fibrosis following long-term unilateral ureteral obstruction by immunohistochemical analysis. They observed thatb fibrogenic changes were seen in the medulla and papilla but not in the cortex of the obstructed kidneys. They also added that the myofibroblasts are major cells producing extra cellular matrix such as collagen fibers in fibrogenic lesions and they are highly immunopositive for α-smooth muscle actin(SMA). The myofibroblasts are characterized by the presence of cytoplasmic myofilaments which are immunopositive for SMA and also confirmed this through electron microscopy for the presence of myofibroblasts with cytoplasmic actinlike microfilaments in rat fibrotic lesions. In their study , they found that as the medullary fibrosis increases the number of SMA-positive cells also sequentially increases

D. W. Powell, R. C. Mifflin, J. D. Valentich et al in 1999[23] the morphological and immunohistochemical properties of myofibroblasts. The myofibroblasts are a unique group of smooth-muscle-like fibroblasts that have a similar appearance and function regardless of their tissue of residence. The myofibroblasts secrete cytokines(IL1, IL6, TNFα, IL10), growth factors (TGF-b ,CSF-1,GM-CSF , PDGF-AA and PDGF-BB) , chemokines IL-8 and inflammatory mediators Phospholipase A2 activating protein ,PGE, ,Prostacyclin. Through these secretions they play an important role in

organogenesis and oncogenesis, inflammation, repair, and fibrosis in most organs and tissues.Platelet-derived growth factor (PDGF) and stem cell factor are two secreted proteins responsible for differentiating myofibroblasts from embryological stem cells. These and other growth factors cause proliferation of myofibroblasts and myofibroblast secretion of extracellular matrix (ECM) molecules and various cytokines and growth factors causes mobility, proliferation, and differentiation of epithelial or parenchymal cells. Repeated cycles of injury and repair lead to organ or tissue fibrosis The myofibroblasts possess several distinguishing morphological characteristics, some of which are present in fibroblasts or smooth muscle cells. They display prominent cytoplasmic actin microfilaments (stress fibers), and they are connected to each other by adherens and gap junctions . These cells are also in contact with the ECM by focal contacts once known as the fibronexus, a transmembrane complex made up of intracellular contractile microfilaments and the ECM protein fibronectin . Often, an incomplete basal lamina surrounds the myofibroblasts. Gap junctions couple some myofibroblasts to the tissue smooth muscle, and the cells are commonly in close apposition to varicosities nerve fibers. Immunohistochemical characterization of myofibroblasts is based on antibody reactions to two of the three filament systems of eukaryotic cells. These three systems are composed of *1*) actin, a component of the microfilaments; *2*) vimentin, desmin, lamin, or glial fibrillary acidic protein (GFAP), members of the intermediate filament system; and *3*) the tubulins of the microtubules

Veronique Moulin, Francois A. Auger, Dominique Garrel et al in 2000[24] investigated the role of wound healing myofibroblasts on re-epithelialization of human skin.In human skin, large burned surfaces heal using two concomitant phenomena: re-epithelialization and dermal neoformation. Numerous studies report the role of interactions between keratinocytes and fibroblasts, but the relationship between wound healing myofibroblasts and keratinocytes is not clear, even though these two cell types coexist during healing. The authors investigated the influence of myofibroblasts on keratinocyte growth and differentiation using an *invitro* skin model. A histological study was performed to determine the speed and quality of epithelialization. When the dermis was populated with myofibroblasts, a continuous epidermis was formed in 7±10 days. In contrast, with wound healing myofibroblasts or without cell in dermis, the complete re- epithelialization never occurred over the 10-day period studied. After 7 further days of epidermal differentiation, histology showed an epidermis more disorganized and expression of basement membrane constituents was reduced when wound healing myofibroblasts or no cells were added in the dermis instead of fibbroblasts. These results suggest that wound healing myofibroblasts are not efficient to stimulate keratinocyte growth and differentiation. Treatment of fibroblasts with TGFb1 induced an increase of epidermal cell differentiation as seen when myofibroblasts were present. However, this cytokine did not change re-epithelialization rate and induced an increase of basement membrane matrix deposition in opposition to myofibroblasts. Thus, TGFb1 action is not suffcient to explain all the different keratinocyte reactions towards fibroblasts and wound healing myofibroblasts. They concluded that myofibroblasts seem to have a

limited role in the re-epithelialization process and might be more associated with the increased extracellular matrix secretion.

Sem H. Phan in 2002[25] reviewed about the association between pulmonary fibrosis and myofibroblasts. In active fibrosis activated fibroblasts phenotypically converted into myofibroblasts which secrete increased amounts of extracellular matrix. The extra cellular matrix secreted by myofibroblasts are type I collagen. They also found that transforming growth factor- β provides protection against apoptosis and disappearance of myofibroblasts. They conclude that the appearance and disappearance of the myofibroblasts appears to correlate with the initiation of active fibrosis and its resolution, respectively. In addition, the myofibroblasts have many phenotypic features, which induce much of the pathologic alterations in fibrotic lung tissue.Furthermore, the persistence of the myofibroblast may lead to progressive disease and, conversely, its disappearance may be an indicator of resolution. By testing the characteristics of myofibroblasts will yield new therapeutic aspects of fibrosis and subsequent treatment of the fibrosis.

Marilena Vered, Izhar Shohat, Amos Buchner et al in 2002[26] investigated if stromal myofibroblasts can contribute to variations in the biological behavior of lesions like odontogenic cysts and tumors. Myofibroblasts have the potential to facilitate progression of neoplastic epithelial lesions that could contribute to their biological behavior. The study was assessed immunohistochemically and the frequency of stromal myofibroblasts in different odontogenic cysts and tumors were

found and it was correlated to their aggressive biological behavior. The study included cases of dentigerous cyst (DC, n = 7), odontogenic keratocyst—parakeratinized type (OKC-P, n = 8), orthokeratinized type (OKC-O, n = 9), ameloblastic fibroma/fibro-odontoma (AMF/O, n = 11), unicystic ameloblastoma (UAM, n = 6), and solid ameloblastoma (SAM, n = 7). Cases of oral squamous cell carcinoma (SCC, n = 5) served as control. Myofibroblast frequency was assessed as the number of alpha smooth muscle actin (aSMA)-positive stromal cells in 10 high-power fields, presented as the mean number of positive cells per field. Counts showed that mean number of positive cells in OKC-P was significantly higher than in DC and SAM. It was significantly higher than in UAM and AMF/O. Counts in OKC-P and SAM were not significantly different from SCC. The high frequency of stromal MF in known aggressive odontogenic lesions, such as OKC-P and SAM, implies that MF can contribute to the biological behavior of these odontogenic lesions

Alexis Desmouliere, Ian A. Darby and Giulio Gabbiani in 2003[27] reviewed the retractile and remodeling phenomena accompanying and, in pathologic situations, following the healing of an open wound. According to their review mechanical stress was crucial for the fibroblast-myofibroblast modulation. Another important point clarified is the direct participation of _-SM actin in force generation by the myofibroblast. They also stated that during wound healing of a full-thickness rat skin wound, cellular retinol-binding protein-1 (CRBP-1) is transiently expressed by a significant proportion of fibroblastic cells including myofibroblasts, suggesting that it plays a role in the evolution of the granulation tissue. The activity of myofibroblast depends on the combined action of cytokines and growth factors, extracellular matrix

components, and, importantly, on the development of mechanical tension. Recent studies have shown that externally applied mechanical load can lead to the rapid and sequential induction of distinct extracellular matrix components in (myo)fibroblasts, indicating that extracellular matrix composition is adapted specifically to changes in load .The generation of force by the myofibroblast is starting to be elucidated, and further work on the mechanisms of stress fiber isometric contraction will furnish tools leading to the understanding not only of granulation tissue contraction mechanisms but also of the evolution of granulation tissue into a more permanent pathologic fibrotic tissue. As discussed above, the transmission of the isometric force produced by the myofibroblast is regulated by both stress fiber contractile activity and deposition and quality of extracellular matrix. They concluded that understanding the interplay between these processes will be important to improve knowledge of several physiologic and pathologic situations.

Gabbiani in 2004[28] reviewed the evolution of the myofibroblast which is considered a key cell for wound healing and fibrotic diseases. Wound healing and fibrocontractive diseases are characterized by the presence of a cell called myofibroblast that is responsible for pathological tissue remodeling. TGF-beta is the main stimulus for the fibroblast/myofibroblast modulation. Alpha-smooth muscle (SM) actin, the actin isoform typical of vascular smooth muscle cells, is the main marker of the myofibroblastic differentiation and in addition is responsible for the high retractile activity of this cell. He concluded that the N terminal sequence of alpha-SM actin inhibits myofibroblast contraction *invitro* and *invivo* and could represent a therapeutic tool in fibrotic diseases.

Witold W Kilarski, Natalia Jura and Par Gerwins in 2005[29] studied the migration, proliferation and invasive growth activity of myofibroblasts during formation of granulation tissue in situations of wound healing, arteriosclerosis and tumor growth. They studied the invasive phenotype of myofibroblasts, establishing an assay where arterial tissue from chicken embryos was embedded in fibrin gels and stimulated with growth factors. Addition of serum, PDGF-BB and FGF-2, but not VEGF-A, resulted in an outgrowth of cellular sprouts with a pattern that was similar to the organization of cells invading a provisional matrix in an *invivo* model of wound healing using the chicken chorioallantoic membrane. Sprouting cells were defined as myofibroblasts based on being a-smooth muscle actin-positive but desmin-negative. Invasive growth and sprouting of vascular smooth muscle cells was not limited to chicken cells since a similar response was seen when spheroids composed of purified primary human aortic smooth muscle cells were embedded in fibrin. This ex vivo model allows quantitative analysis of invasive growth and differentiation of vascular smooth muscle cells and fibroblasts into myofibroblasts. The method described in this report allows studies of myofibroblast differentiation and matrix invasiveness and can be complementary to known *invitro* models design to study myofibroblast dependent force generation in three-dimensional matrices.

H.E. van Beurden, J.W. Von den Hoff, R. Torensma, J.C. Maltha et al in 2005[30] discussed the role of myofibroblasts in palatal wound healing. Wound contraction and subsequent scar tissue formation, during healing of these surgical wounds, contribute largely to these growth disturbances. Fibroblasts initiate wound contraction, but proto-myofibroblasts and mature myofibroblasts are by far the most important cells in

this process. Myofibroblasts are characterized by their cytoskeleton, which contains alpha-smooth-muscle actin. Additionally, their contractile apparatus contains bundles of actin microfilaments and associated contractile proteins, such as non-muscle myosin. This contractile apparatus is thought to be the major force-generating element involved in wound contraction. After closure of the wound, the myofibroblasts disappear by apoptosis, and a less cellular scar is formed. A reduction of contraction and scarring might be obtained by inhibition of myofibroblast differentiation, stimulation of their de-differentiation, stimulation of myofibroblast apoptosis, or impairment of myofibroblast function and also reviewed the role of myofibroblasts in palatal wound healing. The authors postulated that the surgical closure of orofacial clefts is considered to impair maxillary growth and dento-alveolar development. Wound contraction and subsequent scar tissue formation, during healing of these surgical wounds, contribute largely to these growth disturbances. The potential to minimize wound contraction and subsequent scarring by clinical interventions depends on the surgeon's knowledge of the events responsible for these phenomena. Fibroblasts initiate wound contraction, but proto-myofibroblasts and mature myofibroblasts are by far the most important cells in this process. Myofibroblasts are characterized by their cytoskeleton, which contains alpha-smooth-muscle actin. Additionally, their contractile apparatus contains bundles of actin microfilaments and associated contractile proteins, such as non-muscle myosin. This contractile apparatus is thought to be the major force-generating element involved in wound contraction. After closure of the wound, the myofibroblasts disappear by apoptosis, and a less cellular scar is formed. A reduction of contraction and scarring

might be obtained by inhibition of myofibroblast differentiation, stimulation of their de-differentiation, stimulation of myofibroblast apoptosis, or impairment of myofibroblast function.

Brigham C. Willis, Roland M. duBois, and Zea Borok in 2006[31] reviewed about the origin and postulated that myofibroblasts are of epithelial origin during fibrosis in the lung. They believed that inflammation and chronic fibrosis, especially in diseases such as idiopathic pulmonary fibrosis/usual interstitial pneumonia, are often dissociated and that inflammation is neither necessary nor sufficient to induce fibrosis. The origin of the primary effector cell of fibrosis in the lung, the myofibroblast, is not clearly established. Although conversion of resident fibroblasts and differentiation of circulating bone marrow–derived progenitors likely play a role, the possible contribution of alveolar epithelial cells (AECs), through a process termed epithelial–mesenchymal transition" (EMT), has only recently received consideration. A process by which epithelial cells lose cell–cell attachment, polarity and epithelial specific markers, undergo cytoskeletal remodeling, and gain a mesenchymal phenotype; EMT plays a prominent role in fibrogenesis in adult tissues such as the kidney. The authors summarized the evidence supporting a central role for EMT in the pathogenesis of lung fibrosis, the potential for EMT in AECs *in vitro* and *in vivo* and role of transforming growth factor-1 in this process, and the implications of epithelium-driven fibrosis on future research and treatment.

Christophe Helary, Ludmila Ovtracht, Bernard Coulomb et al 2006[32] studied the dense fibrillate collagen matrices and the behavior of myofibroblast during wound healing. Human dermal fibroblasts were seeded onto three-dimensional fibrillar collagen matrices and then migrated into the collagen network and differentiated into myofibroblasts. In order to evaluate the use of collagen matrices as model systems for studying myofibroblast phenotype during wound healing, myofibroblast behaviour migrating into dense or loose matrices was compared. The effect of collagen concentration on cell morphology, remodelling, proliferation and apoptosis of human myofibroblasts was evaluated. Myofibroblasts within dense collagen matrices (40 mg/ml) were spindle shaped, similar to cells observed during tissue repair. In contrast, cells within loose matrices (5 mg/ml) were more rounded. Matrix hydrolysis activities (MT1-MMP and MMP2) did not differ between the two collagen concentrations. The myofibroblast proliferation rate was measured after 24 h bromodeoxyuridine incorporation (BrdU). Cells in dense collagen matrices proliferated at a higher rate than cells in loose matrices at each culture time point tested. Apoptotic cells were only detected in dense matrices from day 21 onwards when cells had already migrated into the collagen network. These results showed that a high collagen concentration has a stimulatory effect on myofibroblast proliferation and apoptosis, two important events in wound healing. Thus, dense matrices can be used to create controlled conditions to study myofibroblast phenotype.

Boris Hinz in 2007[33] reviewed the formation and function of the myofibroblast during tissue repair. The authors said that it is generally accepted that fibroblast-to-

myofibroblast differentiation represents a key event during wound healing and tissue repair. The high contractile force generated by myofibroblasts is beneficial for physiological tissue remodeling but detrimental for tissue function when it becomes excessive such as in hypertrophic scars, in virtually all fibrotic diseases and during stroma reaction to tumors. Specific molecular features as well as factors that control myofibroblast differentiation are potential targets to counteract its development, function, and survival. Such targets include smooth muscle actin and more recently discovered markers of the myofibroblast cytoskeleton, membrane surface proteins, and the extracellular matrix. Moreover, intervening with myofibroblast stress perception and transmission offers novel strategies to reduce tissue contracture. Stress release leads to the instant loss of contraction and promotes apoptosis.

Mytien T. Goldberg, Yuan-Ping Han, Chunli Yan in et al 2007[34] suggested that the cause for abnormal wound healing could be due to TNF-α which suppresses α-smooth muscle actin expression in human dermal fibroblasts. Abnormal wound healing encompasses a wide spectrum, from chronic wounds to hypertrophic scars. Both the conditions are associated with an abnormal cytokine profile in the wound bed. In this study, the authors sought to understand the dynamic relationships between myofibroblast differentiation and mechanical performance of the collagen matrix under tissue growth factor–β (TGF-β) and tumor necrosis factor–α (TNF-α) stimulation. We found TGF-β increased α-smooth muscle actin (α-SMA) and TNF-α alone decreased the basal α-SMA expression. When TGF-β1 and TNF-α were both added, the α-SMA expression was suppressed below the baseline. Real-time PCR

showed that TNF-α suppresses TGF-β1-induced myofibroblast (fibroproliferative) phenotypic genes. The results further showed that TNF-α inhibits TGF-β1-induced Smad-3 phosphorylation via Jun N-terminal kinase signaling. Mechanical testing showed that TNF-α decreases the stiffness and contraction of the lattices after 5 days in culture. The authors finally concluded that changes in α-SMA, collagen, and fibronectin expression result in decreased contraction and stiffness of collagen matrices.

Qiong Gan, Tadashi Yoshida, Jian Li et al in 2007[35] studied about the difference in the transcriptional mechanism for expression of α smooth muscle actin by smooth muscle cells and myofibroblasts. They studied that myofibroblasts are induced in multiple pathological state such as the granulation tissue of contracting wounds, fibroproliferative diseases and play a major role in the inflammatory response. In addition, both smooth muscle cells and myofibroblasts contribute to a wide range of human diseases including vein graft remodeling, tumor metastasis and myocardial remodeling accompanying renovascular hypertension. They found that both smooth muscle cells and myofibroblasts express α smooth muscle actin and expression, it can be distinguished by different transcriptional mechanisms involving MCAT (a gene sequence) elements

Bin Li a, James H.-C. Wang in 2009[36] reviewed the role fibroblasts and myofibroblasts in wound healing and fibrosis and they measured the contractile force exerted by fibroblasts and myofibroblasts . Fibroblasts are one of the most

abundant cell types in connective tissues. These cells are responsible for tissue homeostasis under normal physiological conditions. When tissues are injured, fibroblasts become activated and differentiated into myofibroblasts, which generate large contractions and actively produce extracellular matrix (ECM) proteins to facilitate wound closure. Both fibroblasts and myofibroblasts are involved in wound healing by generating traction and contractile forces, respectively, to enhance wound contraction. Myofibroblasts are an intermediate cell type between fibroblasts and smooth muscle cells (SMCs). Myofibroblasts start to appear in the early phase of granulation tissue formation, become abundant in the proliferation phase of wound healing, and progressively disappear in the later stage of healing, possibly by an apoptotic mechanism. Myofibroblasts have ultra-structures distinctive from those of fibroblasts, including extensive cell-matrix adhesions, abundant intercellular adherens and gap junctions, and bundles of contractile cytoplasmic microfilaments Multiple sources of myofibroblasts exist in order to meet the temporarily high demand for contractile cells in wound repair. Myofibroblasts in skin wounds are derived from fibroblasts in the dermis, subcutaneous tissues surrounding the wound, pericytes and vascular smooth muscle cells which play important role in vascular wound healing . There has been evidence that myofibroblasts may be derived from tubular epithelial cells through epithelial mesenchymal transition (EMT). The main function of myofibroblasts is to synthesize ECM proteins, collagen types I-VI and XVIII, glycoproteins, and proteoglycans for normal growth, differentiation, andn repair. Myofibroblastsn secrete many other matrix molecules including laminin and thrombospondin, glycosaminoglycans (GAGs), hyaluronic acid (HA), and heparan

sulfuate (HS), as well as matrix-modifying proteins such as matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs). Myofibroblasts also generate contractile forces which bring together the edges of an open wound and therefore facilitate wound closure. However, excessive myofibroblast activities, including excessive contraction and over-production of ECM are also the major causes of tissue fibrosis and scar formation.

Marcello Guarino MD, Antonella Tosoni BSc, Manuela Nebuloni MD in 2009[37] reviewed the contribution of epithelium to organ fibrosis. Fibrosis is a complex response initiated to protect the host from an injurious event. It involves massive deposition of matrix by an expanded pool of fibrogenic cells, disruption of the normal tissue architecture, and parenchymal destruction. Fibroblasts, the effector cells of matrix production, when engaged in fibrogenesis, display the highly activated phenotype characteristic of myofibroblasts. Although proliferation of pre existing stromal fibroblasts, emerging evidence seems to indicate that an important number of matrix-producing fibroblasts/myofibroblasts arises through a mechanism of epithelial-mesenchymal transition. Through this process, epithelial cells would lose intercellular cohesion and would translocate from the epithelial compartment into the interstitium where, gaining a full mesenchymal phenotype, they could participate in the synthesis of the fibrotic matrix. Epithelial mesenchymal transition is induced by the integrated actions of many stimuli including transforming growth factor-β and matrix-generated signals that are also known to be implicated in inflammation, repair responses and fibrosis. The authors suggested markers of epithelium undergoing

epithelial mesenchymal transition include loss of E-cadherin and cytokeratin; de novo expression of fibroblast specific protein 1/S100A4, vimentin, and α-smooth muscle actin; basement membrane component loss; and production of interstitial-type matrix molecules such as fibronectin and type I/III collagen.

Kevin K. Kim, Ying Wei, Charles Szekeres et al in 2009[38] investigated the role of epithelial cell integrin links catenin and Smad signaling to promote myofibroblast formation and pulmonary fibrosis. The authors explored the role of the prominent epithelial integrin in experimental fibrosis by generating mice with lung epithelial cell–specific loss of integrin expression. These mice had a normal acute response to bleomycin injury, but they exhibited markedly decreased accumulation of lung myofibroblasts and type I collagen and did not progress to fibrosis. Signaling through catenin has been implicated in EMT. Integrin was required for catenin phosphorylation at tyrosine residue 654 (Y654), formation of the pY654 catenin/pSmad2 complex, and initiation of EMT. Finally, analysis of lung tissue from IPF patients revealed the presence of pY654-catenin/pSmad2 complexes and showed accumulation of pY654-catenin in myofibroblasts. These findings demonstrated that epithelial integrin–dependent profibrotic crosstalk between catenin and Smad signaling and support the hypothesis that EMT is an important contributor to pathologic fibrosis.

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Oral Submucous Fibrosis and the role of myofibroblasts

Li X, Ling TY and Gao YJ 2007[39] studied the effects of arecoline on the differentiation of myofibroblasts of oral mucosa. They isolated and cultured oral keratinocytes and fibroblasts. The expression of the alpha-smooth muscle actin in the fibroblasts was examined by immunohistochemistry and reverse transcriptase polymerase chain reaction (RT-PCR). No difference was found in the expression of alpha-smooth muscle actin between the fibroblasts that were directly stimulated by arecoline and the control. The expression of alpha-smooth muscle actin in the keratinocyte and fibroblast-cocultured group was higher than in the control group, and higher in fibroblasts cocultured with keratinocytes preprocessed by arecoline than in fibroblasts cocultured with keratinocytes without preprocessing with by arecoline. They concluded that differentiation of myofibroblasts from fibroblasts in oral submucous fibrosis might be induced by the interaction of arecoline and keratinocyte.

Kundendu Arya Bishen, Raghu Radhakrishnan and Kapaettu Satyamoorthy in 2008[40] studied and analyzed the role of basic fibroblast growth factor in oral submucous fibrosis pathogenesis. They studied a series analysis of 30 cases of OSF and carried out bFGF expression using immunohistochemistry. Connective tissue changes in these cases were corroborated using aldehyde fuchsin and Verhoeff's hematoxylin special stains. They found that bFGF immunoreactivity was found to be increased in fibroblasts and in endothelial cells in early OSF cases, while the expression of bFGF in stroma increased notably in advanced fibrosis. On staining the tissues with Verhoeff's hematoxylin, the fibers were seen as uniformly distributed black-colored fine fibers in the lamina propria of normal mucosa. There was an

evident change in the nature of fibers in the OSF cases,as there was an increase in the coarse fibers in the deeper tissues in the early OSF graded by H&E. Increased bFGF expression in early stages of the disease was explainable to an initial injury phase because of areca consumption, followed by cellular activation by chemotactic cytokines and other growth factors with eventual fibrosis occurring as a result of molecular alteration at the cellular level.

K.A. Moutasim, D. Mirza, D. Marsh et al in 2009[41] studied and investigated the role of αvβ6 integrin in the pathogenesis of OSF. The cytokine TGF-β1 is considered to have a central role in inducing this myofibroblastic phenotype, and its expression is increased in numerous fibrotic conditions. The epithelial-specific integrin αvβ6 is not detectable on normal oral keratinocytes but is upregulated during tissue remodelling. αvβ6 is regarded as a key activator of TGF-β1 through its interaction with the latency associated peptide (LAP) of the cytokine. Immunochemistry was used to examine αvβ6 expression in 41 cases of OSF compared with 14 cases of fibroepithelial hyperplasia. TGF-β1 activation assays were carried out using a keratinocyte cell line genetically modified to express high levels of αvβ6 (VB6). To determine whether VB6 cells could induce myofibroblast differentiation, co-culture experiments with HFFF2(Human Fetal Foreskin Fibroblasts 2) fibroblasts were performed. Immunochemistry revealed high expression of αvβ6 in 54% of OSF cases. This was significantly higher than expression in fibroepithelial hyperplasia ($p=0.009$). Co-culture experiments revealed markedly increased SMA expression by HFFF2 cells, indicating myofibroblast transdifferentiation. Upregulation of SMA was suppressed

by inhibiting αvβ6. Myofibroblast transdifferentiation resulted in increased synthesis of collagen I. These *in vitro* findings were confirmed by immunochemistry, which demonstrated SMA-positive myofibroblasts in the collagen-rich connective tissue of OSF cases. They concluded that αvβ6 integrin is upregulated by keratinocytes in OSF, and that αvβ6-dependent TGF-β1 activation promotes myofibroblast transdifferentiation.

Oral squamous cell carcinoma and myofibroblasts

Hu Yanjia and Jian Xinchun in 2007[42] investigated the role of epithelial–mesenchymal transition in oral squamous cell carcinoma and oral submucous fibrosis. Epithelial–mesenchymal transition (EMT) is an indispensable mechanism during morphogenesis. Interest and research in EMT are currently at a high level due to its important role in cancer and fibrosis. Emerging evidence suggests that EMT is also a crucial event in oral squamous cell carcinoma (OSCC). The keratinocytes secrete a variety of profibrotic cytokines, participating in a bi-directional communication network with neighboring fibroblasts whereby each cell type influences the proliferation/survival of the other. Keratinocyte plays a major role in the pathogenesis of OSF, with the capacity to produce TGF-β1, regulate the function and differentiation of fibroblasts, and modify cell morphology and gene expression in response to injury, all independent of the degree of inflammation. The authors highlighted the signaling pathways involved in EMT and the recent advances in the study of EMT in OSCC.

Moghadam, M. Khalili, F. Tirgary et al in 2009[4] evaluated and studied myofibroblasts in oral epithelial dysplasia and squamous cell carcinoma. They studied a sample consisting of three groups, including 40 oral squamous cell carcinomas, 15 dysplasias, and 15 sections of normal oral epithelium. Vimentin, desmin, and alpha-smooth muscle actin were used to identify myofibroblasts. The percentage and intensity of alpha-smooth muscle actin were examined, and positive immunostaining was observed in the myofibroblasts of all squamous cell carcinomas; however these cells did not stain in the dysplasias or normal epithelium specimens. The presence of myofibroblasts was significantly higher in oral squamous cell carcinomas compared to both, dysplasias and normal mucosa cases ($P < 0.001$). A significant difference was not observed between the different grades of oral squamous cell carcinoma ($P = 0.2$). These findings show the presence of myofibroblasts in the stroma of oral squamous cell carcinoma but not dysplasia and normal mucosa, suggesting further investigation to clarify the role of myofibroblasts in the carcinogenesis of this tumor.

PATIENT CHARACTERISTICS

Twenty cases of OSF (Group I), 20 cases of OSCC (Group II) and 10 cases of clinically appearing normal mucosa (Group III) were analyzed for immunoreactivity of myofibroblasts. All the samples in group I, II and III were taken from the buccal mucosa.

DISTRIBUTION OF GENDER AMONG GROUPS

In Group I 19 (95%) were men and 1(5%) was women. In Group II 15(75%) were men and 5(25%) were women. In group III 8 were men (80%) and 2 (20%) were women. **(Table 1, Graph 1).**

DISTRIBUTION OF GENDER AMONG GROUPS

The age groups are divided into 20to 40 years, 41 to 60 years and 61+ years. In Group I out of 20 cases 13(65%) belonged to age Group 20-40 years and 7(35%) belonged to age Group 41-60 years. In Group II out of 20 cases 2(10%) belonged to age Group 20-40 years, 5 cases (25%) belonged to age Group 41-60 years and 13 cases (65%) belonged to age Group 61+years. . In Group III out of 10 cases 9(90%) belonged to age Group 20-40 years and 1(10%) belonged to age Group 41-60 years. (**Table 2, Graph 2**)

DISTRIBUTION OF HABITS AMONG GROUP I (OSF) AND GROUP II (OSCC)

In total of 40 cases in group I & II, 31 had habits and details of 9 cases not available. Within those who had habits 9.7 %(n=3) had no stain, 51.6 %(n=16) had mild staining, 22.6 %(n=7) had moderate staining and 16.1% (n=5) had intense staining. Out of 9 who had no habits, 33.3% (n=3) had mild staining, 55.6% (n=5) had mild staining and 11.1% (n=1) had moderate staining. **(Table 3, Graph 3)**

STAINING INTENSITY

DISTRIBUTION OF STAINING INTENSITY (SI) OF α-SMA (ALPHA SMOOTH MUSCLE ACTIN) AMONG 3 GROUPS:

α-SMA revealed positivity in group I, II and III. In Group I and Group II cases showed 85% staining for **α-SMA**, whereas in Group III, positive staining was observed 30%.

In Group I out of 20 OSF cases 30 %(n=6) cases have a score of 3 (SI3), 20 %(n=4) cases have a score of 2 (SI2), 35 %(n=7) cases have a score of 1 (SI1), and 15% (n=3) cases have a score of 0 (SI0). Group II out of 20 cases 20 %(n=4) cases have a score of 3 (SI3), 30 %(n=6) cases have a score of 2 (SI2), 35 %(n=7) cases have a score of 1 (SI1), and 15% (n=3) cases have a score of 0 (SI0). In Group III out of 10 normal cases 30 %(n=3) cases have a score of 0 (SI0) and 70 %(n=7) cases have a score of 1 (SI1). (**Table 4, Graph 4)**

DISTRIBUTION OF STAINING INTENSITY AMONG DIFFERENT OSF GRADINGS

Total 20 cases of OSF, 6 cases belonged to Grade I, 10 cases belonged to Grade II and 4 cases belonged to Grade III. In Grade I 16.7% (n=1) have a score of 0 (SI0), 50% (n=3) have a score of 1 (SI1), 16.7 % (n=1) have a score of 2 (SI2) and 16.7 %(n=1) have a score of 3 (SI3). In Grade II 40 %(n=4) have a score of1 (SI1), 30 %(n=3) have a score of 2 (SI2), and 30% (n=3) have a score of 3 (SI3), In Grade III 50

%(n=2) have a score of 0 (SI0) and 50 % (n=2) have a score of 3 (SI3) (**Table 5, Graph 5**)

DISTRIBUTION OF STAINING INTENSITY AMONG DIFFERENT OSCC GRADINGS

Total 20 cases of OSCC, 13 cases belonged to well differentiated group, 3 cases belonged to moderately differentiated and 4 cases belonged to poorly differentiated. In well differentiated group, 23.1% (n=3) have a score of 0 (SI0), 23.1% (n=3) have a score of 1 (SI1), 23.1 % (n=3) have a score of 2 (SI2) and 30.8 %(n=4) have a score of 3 (SI3). In moderately differentiated group II 66.7 %(n=2) have a score of1 (SI1) and 33.3 %(n=1) have a score of 2 (SI2) In poorly differentiated group 50 %(n=2) have a score of 1 (SI1) and 50 % (n=2) have a score of 2 (SI2) (**Table 6, Graph 6)**

KAPPA STASTISTIC VALUE

The inter-observer agreement for the staining intensity of stain for all the 3 groups was arrived using kappa statistics and kappa value is **0.689**.

PERCENTAGE OF IMMUNOPOSITIVE CELLS- LABELING INDEX (LI)

DISTRIBUTION OF LABELING INDEX (LI) OF α-SMA (ALPHA SMOOTH MUSCLE ACTIN) AMONG 3 GROUPS:

α-SMA revealed positivity in group I, II and III. In Group I and Group II cases showed 85% staining for **α-SMA**, whereas in Group III, positive staining was observed 40%.

In Group I out of 20 OSF cases 5 %(n=1) cases have a score of 3 (LI3), 40 %(n=8) cases have a score of 2 (LI2), 40 %(n=8) cases have a score of 1 (LI1), and 15% (n=3) cases have a score of 0 (LI0). Group II out of 20 cases 15 %(n=3) cases have a score of 0 (LI0) and 85 %(n=17) cases have a score of 1 (LI1). In Group III out of 10 normal cases 60 %(n=6) cases have a score of 0 (LI0) and 40 %(n=4) cases have a score of 1 (LI1). (**Table 7, Graph 7)**

DISTRIBUTION OF LABELING INDEX (LI) AMONG DIFFERENT OSF GRADINGS

Total 20 cases of OSF, 6 cases belonged to Grade I, 10 cases belonged to Grade II and 4 cases belonged to Grade III. In Grade I 16.7% (n=1) have a score of 0 (LI0), 50% (n=3) have a score of 1 (LI1) and 33.3 % (n=2) have a score of 2 (LI2). In Grade II 50 %(n=5) have a score of1 (LI1), 40 %(n=4) have a score of 2 (LI2), and 10% (n=1) have a score of 3 (SI3). In Grade III 50 %(n=2) have a score of 0 (LI0) and 50 % (n=2) have a score of 2 (LI2) (**Table 8, Graph 8)**

DISTRIBUTION OF STAINING INTENSITY AMONG DIFFERENT OSCC GRADINGS

Total 20 cases of OSCC, 13 cases belonged to well differentiated group, 3 cases belonged to moderately differentiated and 4 cases belonged to poorly differentiated. In well differentiated group, 23.1% (n=3) have a score of 0 (LI0) and 76.9 % (n=10) have a score of 1 (LI1). In moderately differentiated group 100 %(n=3) have a score of1 (LI1). In poorly differentiated group 100 %(n=4) have a score of 1 (LI1) (**Table 9, Graph 9)**

STAINING INDEX

DISTRIBUTION OF STAINING INDEX AMONG THE GROUPS.

Using Kruskal-Wallis test the comparison among groups derived, there is a significant difference in the expression of myofibroblasts ($p=0.004 < 0.05$) (**Table 10, Graph 10)**

COMPARISON OF STAINING INDEX BETWEEN GROUP I AND GROUP III

In OSF, 15% of the cases showed no staining while 40% mild staining, 20% moderate staining and 25% of the cases had intense staining in connective tissue. In normal mucosa, 40% exhibited mild staining and no staining was seen in 60% of the cases. The result is statistically significant ($p = 0.005 < 0.05$) **(Table 11, Graph 11)**

COMPARISON OF STAINING INDEX BETWEEN GROUP II AND GROUP III

In OSCC, 15% of the cases showed no staining while 65% mild staining and 20% moderate staining in connective tissue. In normal mucosa, 40% exhibited mild staining and no staining was seen in 60% of the cases. The result is statistically significant ($p = 0.019 < 0.05$) **(Table 12, Graph 12)**

COMPARISON OF STAINING INTENSITY BETWEEN GROUP I AND GROUP II

In OSF group, 15% of the cases showed no staining while 40% mild staining, 20% moderate staining, and 25% of the cases had intense staining in connective

tissue. In OSCC group, 15% of the cases showed no staining while 65% mild staining and 20% moderate staining in connective tissue. As both the lesions expressing myofibroblasts, the result is statistically not significant (p = 0.157) (**Table 13, Graph 13)**

COMPARISON OF STAINING IN DIFFERENT OSF GRADING

Total 20 cases of OSF, 6 cases belonged to Grade I, 10 cases belonged to Grade II and 4 cases belonged to Grade III. In Grade I 16.7% (n=1) showed no stain, 50% (n=3) showed mild stain 16.7% (n=1) moderate and 16.7 %(n=1) showed intense staining. In Grade II 50 %(n=5) showed mild staining 20 %(n=2) showed moderate and 30% (n=3) showed intense staining. In Grade III 50 %(n=2) showed no stain, 25% (n=1) showed moderate and 25 %(n=1) showed intense staining. (**Table 14, Graph 14)**

COMPARISON OF STAINING IN DIFFERENT OSCC GRADING

In total 20 cases of OSCC, 13 belonged to well differentiated, 3 belonged to moderately differentiated and 4 belonged to poorly differentiated. In well differentiated group 23.1% (n=3) showed no stain, 46.2% showed mild stain and 30.8% showed moderate staining. In OSCC moderately differentiated 100% (n=3) showed mild staining. In OSCC poorly differentiated 100% (n=4) showed mild staining **(Table 15, Graph 15)**

Myofibroblasts may be morphologically and immunologically identified through the expressed cytoskeletal proteins. The myofibroblasts are contractile cells expressing α SMA(smooth muscle actin). They are smooth muscle like cells and are capable of augmenting and down-regulating the inflammatory response by secreting soluble mediators of inflammation.[23] They are induced by TGFβ,[41, 23] a potent pro-inflammatory and pro-fibrotic cytokine that regulates the balance between collagen deposition and degradation in oral submucous fibrosis. Owing to the various functions of the myofibroblasts and its active role in formation and repair of extracellular matrix healing, this study was undertaken to identify the relationship of myofibroblasts to the various grades of oral submucous fibrosis, oral squamous cell carcinoma compared to normal mucosa using α SMA which is expressed by myofibroblasts.

Twenty cases each of oral submucous fibrosis and oral squamous cell carcinoma archival tissue and ten normal mucosa obtained during impaction removal procedures were utilized for the study. The distribution of genders in the study groups was 4:1 (Males: Females). In the oral submucous fibrosis group the gender distribution is 19:1(Males: Females). The male preponderance among oral submucous fibrosis has been documented in several studies from South India.[7]

The age distribution of oral submucous fibrosis as observed was between 20-40 years. Oral submucous fibrosis commonly occurs in the age group of 20-40 years[7]. Similarly the occurrence of oral squamous cell carcinoma commonly occurs among older age groups as seen in this study.[12]

Staining intensity did not show any difference among the three study groups. It was further observed that the staining intensity 3 (30%) expression was seen more in oral submucous fibrosis than in oral squamous cell carcinoma(20%). This finding could be probably explained by the fact that altered wound healing process of the oral mucosa could have occured after chronic sustained injury and thus resulting in fibrosis. This could also be due to hypersensitivity response caused by arecoline and the resultant persistent inflammatory response in oral submucous fibrosis, which acts as an initiator in the activation of fibroblasts culminating in fibrosis.[13] On analyzing the staining intensity among grades of oral submucous fibrosis it was observed that staining intensity progressively increased from early oral submucous fibrosis to advanced oral submucous fibrosis. This finding is consistent with the findings of Angadi et al[3], though the classification employed in that study is different[1], we employed a more clinically relevant classification.[10]

Higher staining intensity was observed among well differentiated oral squamous cell carcinoma group while the moderate and poorly differentiated groups of oral squamous cell carcinoma show mild staining intensity. This suggests that myofibroblasts are stimulated during the repair of extracellular matrix and are capable of augmenting and down-regulating the inflammatory response by secretion of these soluble mediators of inflammation like prostaglandins, hydroxyeicosatetraenoic acids (HETEs), growth factors TGF-β and cytokines interleukin 1, interleukin 6, TNF-α, inteleukin 10.[23]

The percentage of positively stained cells as indicated by the labeling index showed the labeling index grades of LI 2 and 3 were observed in oral submucous

fibrosis and not in oral squamous cell carcinoma or normal mucosa. The chronic inflammation and continuous tissue remodeling in oral submucous fibrosis as well as chronic sustaining injury to factors such as arecoline and micro trauma could cause the transient or continuous differentiation of fibroblast from undifferentiated stem cell or a differentiated fibroblast pool. Our study confers with findings of Angadi et al[3]

The labeling index among the various grades of oral submucous fibrosis were studied. In Grade II and Grade III oral submucous fibrosis 50% of the cases show labeling index score of 2 and 3 while in Grade I oral submucous fibrosis 33.3% of cases show labeling index score 2. This indicates that with the increase of stimulus and the severe grade of the disease, more amount of myofibroblasts differentiation occurs.[23, 3] However the difference between the grades was not statistically significant. When comparing the labeling index (LI) within the grades of oral squamous cell carcinoma there was no significant difference. Thus the expression pattern of α SMA in our study indicates that differentiation of oral squamous cell carcinoma does not correlate with the differentiation of myofibroblasts.

Staining index among the study group was studied. There was statistically significant difference in the pattern of staining index among the study groups. Intense staining of α SMA was observed in oral submucous fibrosis and moderate staining was seen in both oral submucous fibrosis and oral squamous cell carcinoma. Normal mucosa had mild staining index. This could be the effect of mild inflammation in normal mucosa. The intense staining in oral submucous fibrosis were similar to the findings of Angadi et al[3]. Though 65% cases of oral squamous cell carcinoma showed a mild Staining Index and 20% cases had moderate Staining Index, none had intense

Staining Index. The absence of intense Staining Index in oral squamous cell carcinoma needs to be studied further.

On comparing the Staining Index between oral submucous fibrosis and normal mucosa, it is observed that 85% of oral submucous fibrosis cases showing positivity while 40% of cases in normal mucosa show positivity of α SMA. The uniform mild expression in both the groups, probably could be a result of the mild chronic inflammation in both these conditions.

On comparing the staining index between oral squamous cell carcinoma and normal mucosa, 65% of oral squamous cell carcinoma cases showed mild staining index and 20% of cases showed moderate staining index while in normal mucosa 40% cases showed mild staining index. However it was statistically significant. The uniformity in expression probably relates to the chronic inflammation that exists in both the conditions.

There is no significant difference of staining index between oral submucous fibrosis and squamous cell carcinoma. However 24% of cases exhibited intense staining in oral submucous fibrosis while no cases of oral squamous cell carcinoma had intense staining index. This indicates that oral submucous fibrosis had more myofibroblasts expression than oral squamous cell carcinoma probably due to failure of healing and continuous fibrosis[3].The staining index did not considerably vary within the grades of the oral submucous fibrosis.

Our results suggest that persistent, chronic inflammation leads to formation of a subset of fibroblastic population-myofibroblasts. The chronic inflammatory

mediators such as TGFβ and αvβ6 integrin could cause more numbers as well as increased intensity or wide functional ability of myofibroblasts. Though our study failed to demonstrate a statistically significant difference between grades of oral submucous fibrosis, a trend that is suggestive of such a phenomenon was seen. Further studies will shed more light on the pathogenesis of oral submucous fibrosis with reference to myofibroblasts.

Summary and conclusion

- The aim of the study was to evaluate expression of myofibroblasts in oral submucous fibrosis, oral squamous cell carcinoma and normal mucosa.
- In this study the immunohistochemical analysis was done with α-SMA (smooth muscle actin). Total of 50 blocks from archives were taken.
- 20 cases of oral submucous fibrosis(Group I), 20 cases of oral squamous cell carcinoma(Group II) and 10 cases of normal mucosa.
- It was observed that Staining Intensity score (S.I- 3) was seen more in oral submucous fibrosis than in oral squamous cell carcinoma.
- Staining intensity among grades of oral submucous fibrosis progressively increased from early oral submucous fibrosis to advanced oral submucous fibrosis, but it was not statistically significant.
- Among the grades of oral squamous cell carcinoma, higher staining intensity was observed among well differentiated oral squamous cell carcinoma while others show mild staining intensity.

- As determined by labeling index, more percentage of cells expressing α SMA in oral submucous fibrosis than in oral squamous cell carcinoma and Normal mucosa
- When comparing the Staining Index between oral submucous fibrosis and normal mucosa, there is a significant difference.

Our results suggest that oral submucous fibrosis actually represents an abnormal healing process in response to chronic mechanical and chemical irritation because of areca nut chewing as demonstrated by the increased incidence of myofibroblasts in this disease. Additionally, as myofibroblasts are responsible for producing a variety of factors that are involved in the fibrotic processes, they could be the key link in the pathogenesis of oral submucous fibrosis. Interruption of development, recruitment or activation could provide a unique therapeutic approach to combat fibrosis.

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Clinica Chimica Acta 383 (20

From,

Institutional Review Board,

Ragas Dental College and Hospital,

Uthandi,Chennai

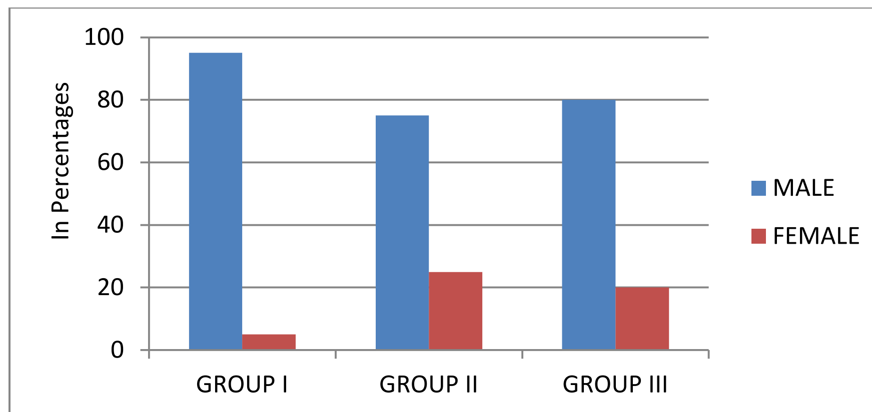
The dissertation topic titled 'Immunohistochemical study of the expression of myofibroblasts in Oral Submucous fibrosis , Oral squamous cell Carcinoma and Normal mucosa' submitted by Dr.A.Sivachandran has been approved by the Institutional Review Board of Ragas Dental College and Hospital on 14th March 2011.

Dr.K.Ranganathan	**Dr.S.Ramachandran**
Secretary,	**Chairman,**
Ragas , IRB	**Ragas , IRB**

TABLE 1 : DISTRIBUTION OF GENDER IN THE STUDY GROUPS (n=50)

Groups		Gender		p VALUE
		Male	Female	
Group I	n=20	19	1	0.210
	%	95	5	
Group II	n=20	15	5	
	%	75	25	
Group III	n=10	8	2	
	%	80	20	

GRAPH 1 : DISTRIBUTION OF GENDER IN THE STUDY GROUPS



GROUP I-OSF

GROUP II-OSCC

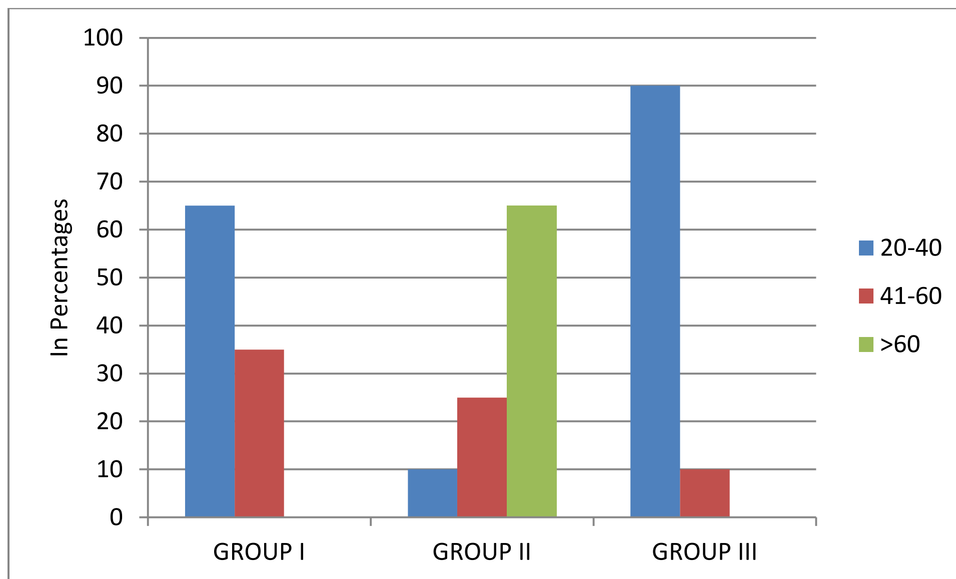
GROUP III-NORMAL

TABLE 2 : DISTRIBUTION OF AGE IN THE STUDY GROUPS (n=50)

AGE (in years)	GROUP I		GROUP II		GROUP III		p VALUE
	n=20	%	n=20	%	n=10	%	
20-40	13	65	2	10	9	90	0.000*
41-60	7	35	5	25	1	10	
>61	0	0	13	65	0	0	

*p value < 0.05 was considered to be statistically significant

GRAPH 2 : DISTRIBUTION OF AGE IN THE STUDY GROUPS (n=50)



GROUP I-OSF

GROUP II-OSCC

GROUP III-NORMAL

TABLE 3: DISTRIBUTION OF HABITS AMONG STAINING GROUPS (n=31)

STAIN. INDEX	SMK		CHW		ALC		CHW+ SMK		SMK+ ALC		CHW+ALC		ALL		pVALUE
	n=5	%	n=6	%	n=2	%	n = 3	%	n= 1	%	n=10	%	n=4	%	
NO STAIN	0	0	0	0	1	50	0	0	0	0	2	20	0	0	0.597
MILD	4	80	3	50	0	0	3	100	1	100	3	30	2	50	
MODERATE	1	20	2	33.3	1	50	0	0	0	0	2	20	1	25	
HIGH	0	0	1	16.7	0	0	0	0	0	0	3	30	1	25	

(Details of 9 cases not available)

GRAPH 3 : DISTRIBUTION OF HABITS AMONG STAINING GROUPS

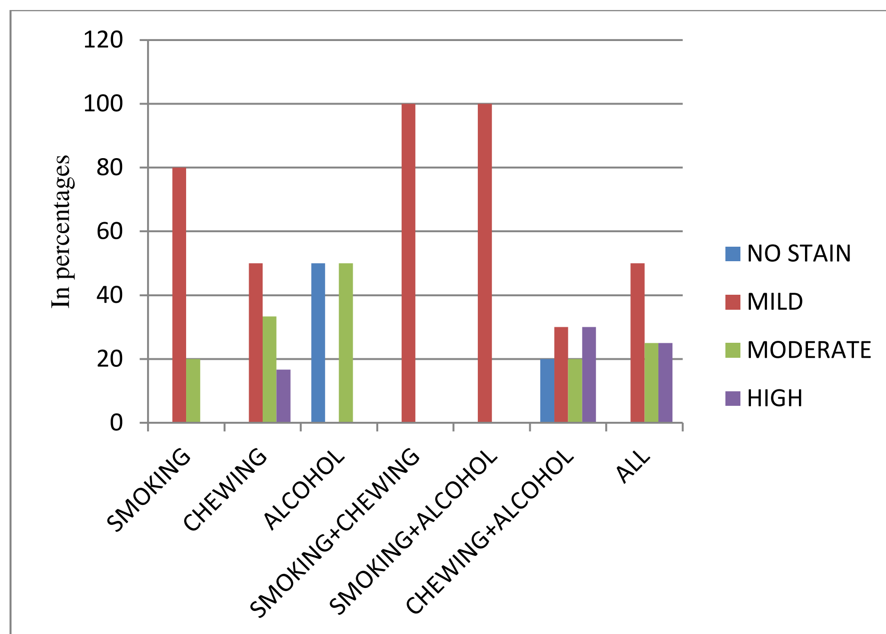
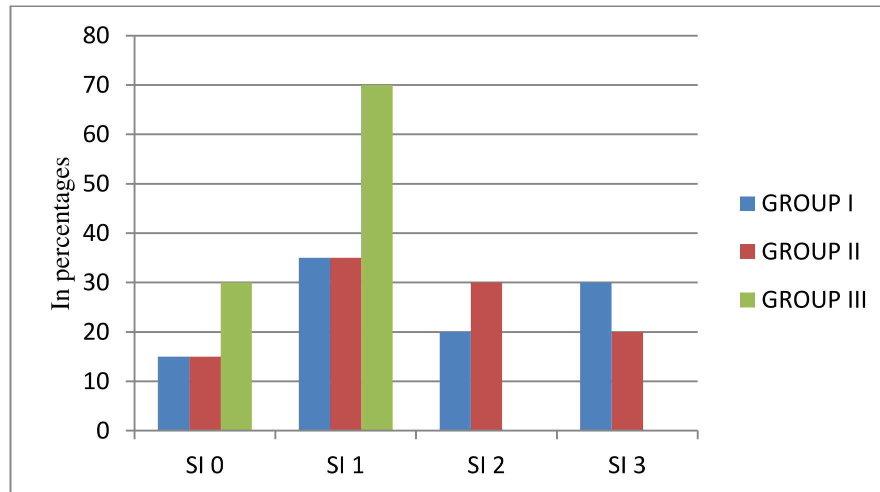


TABLE 4 : DISTRIBUTION OF STAINING INTENSITY AMONG THE GROUPS (n=50)

STAINING INTENSITY		SI 0	SI 1	SI 2	SI 3	p VALUE
GROUP I	n=20	3	7	4	6	0.156
	%	15	35	20	30	
GROUP II	n=20	3	7	6	4	
	%	15	35	30	20	
GROUP III	n=10	3	7	0	0	
	%	30	70	0	0	

GRAPH 4 : DISTRIBUTION OF STAINING INTENSITY AMONG THE GROUPS (n=50)



No staining scored as **0** (SI 0)

Staining visible only at 40X scored as **1** (SI 1)

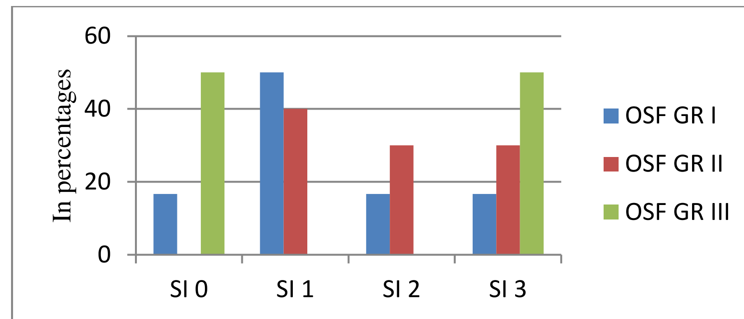
Staining visible at only at 10X not in 4X scored as **2** (SI 2)

Staining visible even at 4X scored as **3** (SI 3)

TABLE 5: DISTRIBUTION OF STAINING INTENSITY AMONG DIFFERENT OSF GRADINGS (n=20)

STAINING INTENSITY		SI 0	SI 1	SI 2	SI 3	p VALUE
OSF GRADE I	n=6	1	3	1	1	0.182
	%	16.7	50	16.7	16.7	
OSF GRADE II	n=10	0	4	3	3	
	%	0	40	30	30	
OSF GRADE III	n=4	2	0	0	2	
	%	50	0	0	50	

GRAPH 5: DISTRIBUTION OF STAINING INTENSITY AMONG DIFFERENT OSF GRADINGS (n=20)



No staining scored as **0** (SI 0)

Staining visible only at 40X scored as **1** (SI 1)

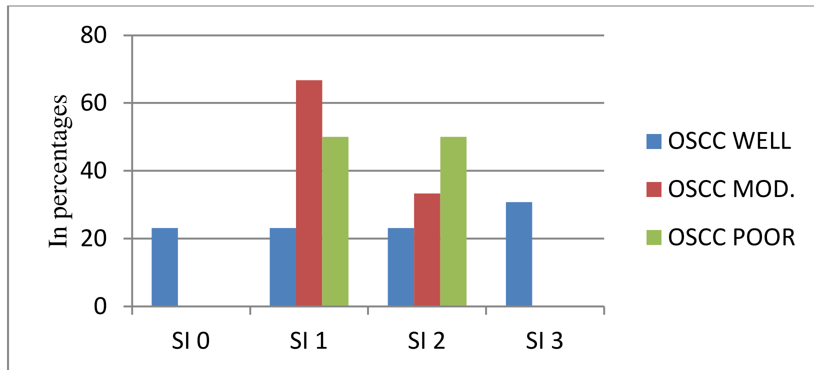
Staining visible at only at 10X not in 4X scored as **2** (SI 2)

Staining visible even at 4X scored as **3** (SI 3)

TABLE 6: DISTRIBUTION OF STAINING INTENSITY AMONG DIFFERENT OSCC GRADINGS (n=20)

<table>
<tr><th colspan="2">STAINING INTENSITY</th><th>SI 0</th><th>SI 1</th><th>SI 2</th><th>SI 3</th><th>p VALUE</th></tr>
<tr><td rowspan="2">OSCC-WELL DIFFERENTIATED</td><td>n=13</td><td>3</td><td>3</td><td>3</td><td>4</td><td rowspan="6">0.405</td></tr>
<tr><td>%</td><td>23.1</td><td>23.1</td><td>23.1</td><td>30.8</td></tr>
<tr><td rowspan="2">OSCC MODERATELY DIFFERENTIATED</td><td>n=3</td><td>0</td><td>2</td><td>1</td><td>0</td></tr>
<tr><td>%</td><td>0</td><td>66.7</td><td>33.3</td><td>0</td></tr>
<tr><td rowspan="2">OSCC POORLY DIFFERENTIATED</td><td>n=4</td><td>0</td><td>2</td><td>2</td><td>0</td></tr>
<tr><td>%</td><td>0</td><td>50</td><td>50</td><td>0</td></tr>
</table>

GRAPH 6: DISTRIBUTION OF STAINING INTENSITY AMONG DIFFERENT OSCC GRADINGS (n=20)



No staining scored as **0** (SI 0)

Staining visible only at 40X scored as **1** (SI 1)

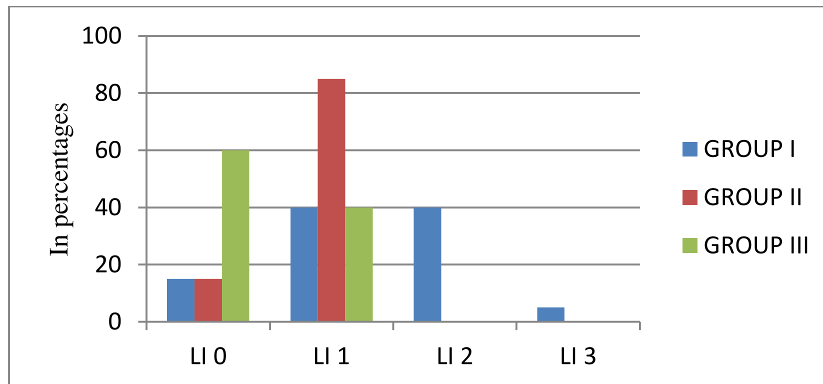
Staining visible at only at 10X not in 4X scored as **2** (SI 2)

Staining visible even at 4X scored as **3** (SI 3)

TABLE 7 : DISTRIBUTION OF PERCENTAGE OF CELLS AMONG THE GROUPS (n=50)

PERCENTAGE OF CELLS		LI 0	LI 1	LI 2	LI 3	p VALUE
GROUP I	n=20	3	8	8	1	0.000*
	%	15	40	40	5	
GROUP II	n=20	3	17	0	0	
	%	15	85	0	0	
GROUP III	n=10	6	4	0	0	
	%	60	40	0	0	

GRAPH 7 : DISTRIBUTION OF PERCENTAGE OF CELLS AMONG THE GROUPS (n=50)



0 positive cells scored as **0** (LI 0)

1-25% of positive cells scored as **1** (LI 1)

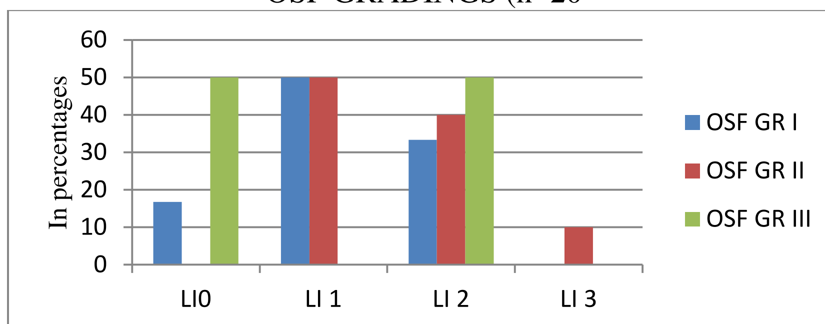
25-50% of positive cells scored as **2** (LI 2)

50-100% of positive cells scored as **3** (LI 3)

TABLE 8: DISTRIBUTION OF PERCENTAGE OF CELLS AMONG DIFFERENT OSF GRADINGS (n=20)

PERCENTAGE OF CELLS		LI 0	LI 1	LI 2	LI 3	p VALUE
OSF GRADE I	n=6	1	3	2	0	0.242
	%	16.7	50	33.3	0	
OSF GRADE II	n=10	0	5	4	1	
	%	0	50	40	10	
OSF GRADE III	n=4	2	0	2	0	
	%	50	0	50	0	

GRAPH 8: DISTRIBUTION OF PERCENTAGE OF CELLS AMONG DIFFERENT OSF GRADINGS (n=20



0 positive cells scored as **0** (LI 0)

1-25% of positive cells scored as **1** (LI 1)

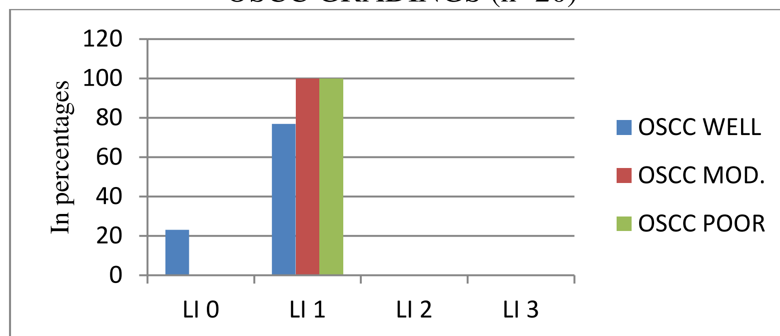
25-50% of positive cells scored as **2** (LI 2)

50-100% of positive cells scored as **3** (LI 3)

TABLE 9: DISTRIBUTION OF PERCENTAGE OF CELLS AMONG DIFFERENT OSCC GRADINGS (n=20)

PERCENTAGE OF CELLS		LI 0	LI 1	LI 2	LI 3	p VALUE
OSCC-WELL DIFFERENTIATED	n=13	3	10	0	0	0.387
	%	23.1	76.9	0	0	
OSCC MODERATELY DIFFERENTIATED	n=3	0	3	0	0	
	%	0	100	0	0	
OSCC POORLY DIFFERENTIATED	n=4	0	4	0	0	
	%	0	100	0	0	

TABLE 9: DISTRIBUTION OF PERCENTAGE OF CELLS AMONG DIFFERENT OSCC GRADINGS (n=20)



0 positive cells scored as **0** (LI 0)

1-25% of positive cells scored as **1** (LI 1)

25-50% of positive cells scored as **2** (LI 2)

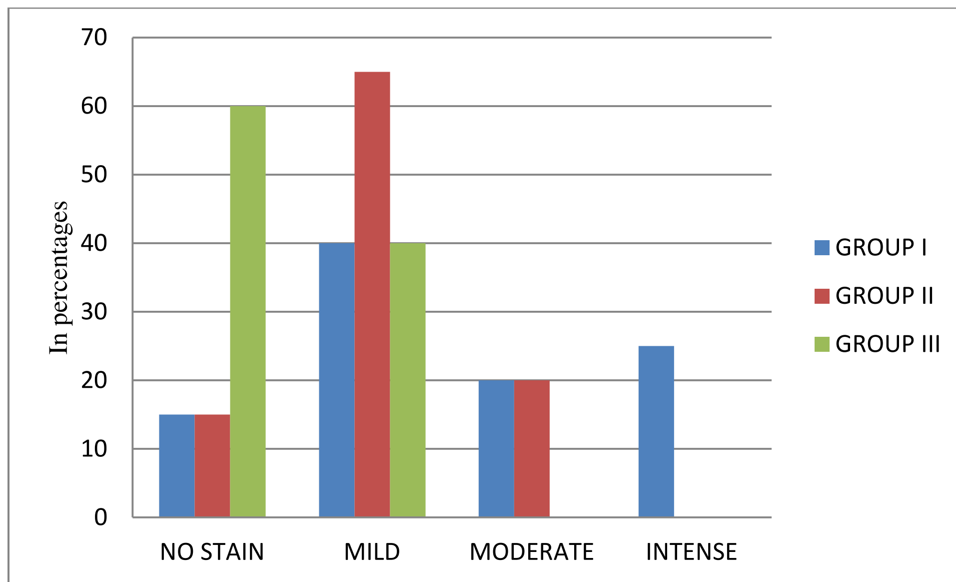
50-100% of positive cells scored as **3** (LI 3)

TABLE 10 : DISTRIBUTION OF STAINING INDEX AMONG THE GROUPS (n=50)

STAINING INDEX		NO STAIN	MILD	MODERATE	INTENSE	p VALUE
GROUP I	n=20	3	8	4	5	0.004*
	%	15	40	20	25	
GROUP II	n=20	3	13	4	0	
	%	15	65	20	0	
GROUP III	n=10	6	4	0	0	
	%	60	40	0	0	

*p value < 0.05 was considered to be statistically significant

GRAPH 10 : DISTRIBUTION OF STAINING INDEX AMONG THE GROUPS



STAINING INDEX

GROUP I-OSF

GROUP II-OSCC

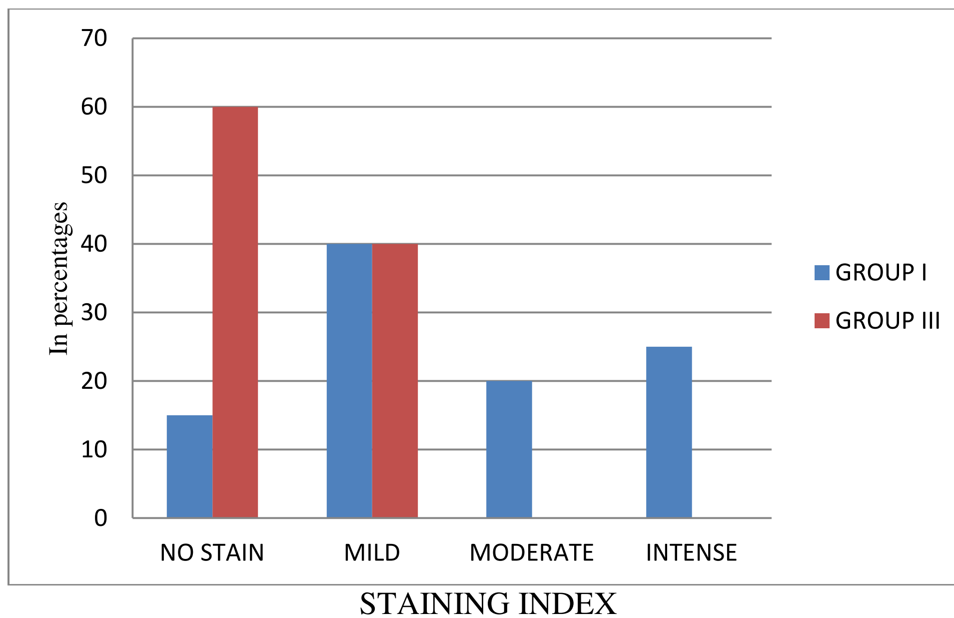
GROUP III-NORMAL

TABLE 11: COMPARISON OF STAINING INDEX BETWEEN GROUP I AND GROUP III

STAINING INDEX	NO STAIN (%)	MILD (%)	MODERATE (%)	INTENSE (%)	P VALUE
Group I n=20	15	40	20	25	0.005*
Group III n=10	60	40	0	0	

*p value < 0.05 was considered to be statistically significant

GRAPH 11: COMPARISON OF STAINING INDEX BETWEEN GROUP I AND GROUP III



GROUP I-OSF

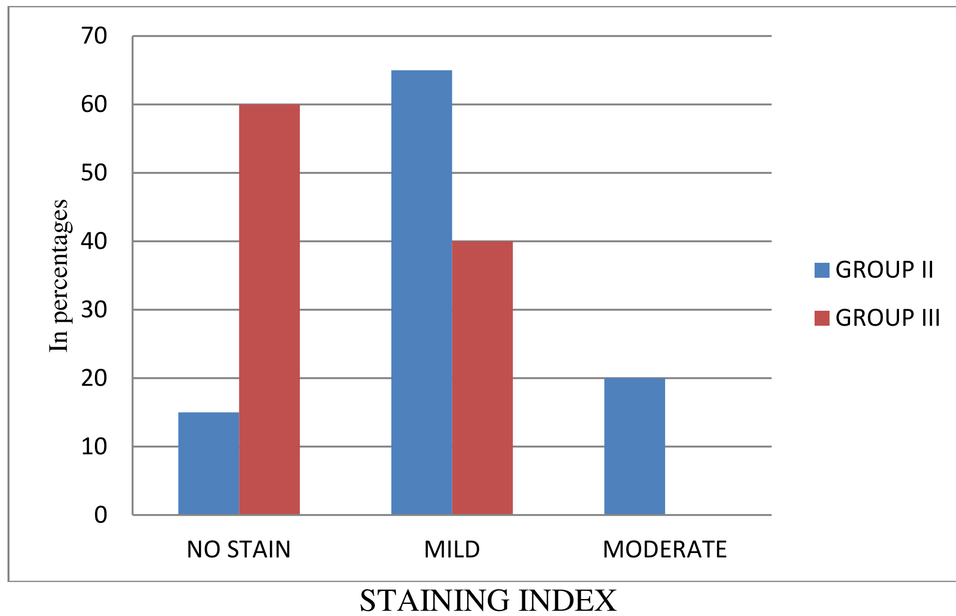
GROUP III-NORMAL

TABLE 12: COMPARISON OF STAINING INDEX BETWEEN GROUP II AND GROUP III

STAINING INDEX	NO STAIN (%)	MILD (%)	MODERATE (%)	P VALUE
Group II n=20	15	65	20	0.019*
Group III n=10	60	40	0	

*p value < 0.05 was considered to be statistically significant

GRAPH 12: COMPARISON OF STAINING INDEX IN BETWEEN GROUP II AND GROUP III



STAINING INDEX

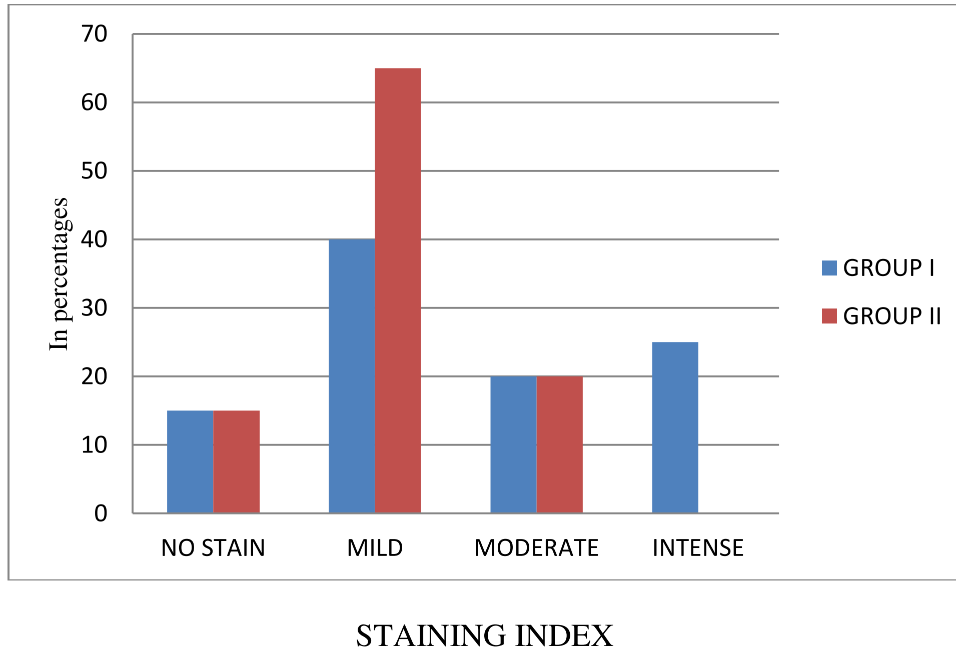
GROUP II-OSCC

GROUP III-NORMAL

TABLE 13: COMPARISON OF STAINING INDEX BETWEEN GROUP I AND GROUP II

STAINING INDEX	NO STAIN (%)	MILD (%)	MODERATE (%)	INTENSE (%)	pVALUE
Group I n=20	15	40	20	25	0.157
Group II n=20	15	65	20	0	

GRAPH 13: COMPARISON OF STAINING INDEX BETWEEN GROUP I AND GROUP II



STAINING INDEX

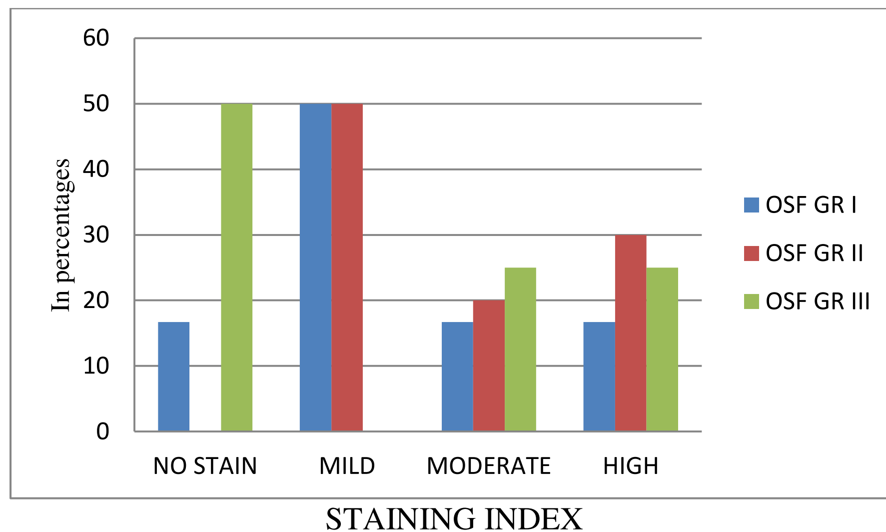
GROUP I-OSF

GROUP II-OSCC

TABLE 14: DISTRIBUTION OF STAINING INDEX AMONG DIFFERENT OSF GRADINGS (n=20)

STAINING INDEX		NO STAIN	MILD	MODERATE	INTENSE	p VALUE
OSF GRADE I	n=6	1	3	1	1	0.309
	%	16.7	50	16.7	16.7	
OSF GRADE II	n=10	0	5	2	3	
	%	0	50	20	30	
OSF GRADE III	n=4	2	0	1	1	
	%	50	0	25	25	

GRAPH14: DISTRIBUTION OF STAINING INDEX AMONG DIFFERENT OSF GRADINGS



STAINING INDEX

TABLE 15: DISTRIBUTION OF STAINING INDEX AMONG DIFFERENT OSCC GRADINGS (n=20)

STAINING INDEX		NO STAIN	MILD	MODERATE	p VALUE
OSCC-WELL DIFFERENTIATED	n=13	3	6	4	0.215
	%	23.1	46.2	30.8	
OSCC MODERATELY DIFFERENTIATED	n=3	0	3	0	
	%	0	100	0	
OSCC POORLY DIFFERENTIATED	n=4	0	4	0	
	%	0	100	0	

GRAPH 15: DISTRIBUTION OF STAINING INDEX AMONG DIFFERENT OSCC GRADINGS

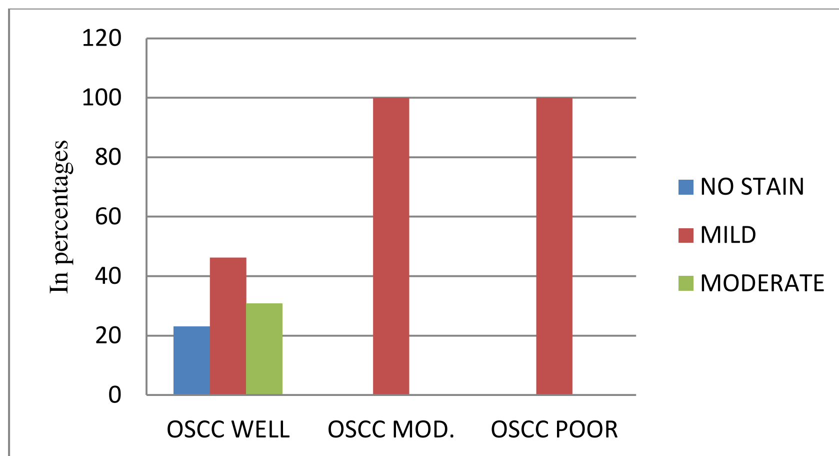


Figure 1

Armamentarium

Figure2

Armamentarium

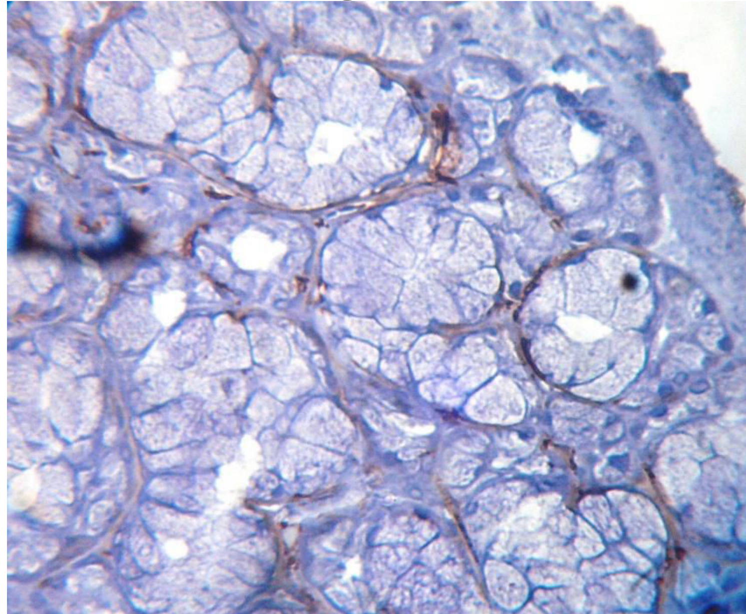
Figure 3

Primary Antibody

Figure 4

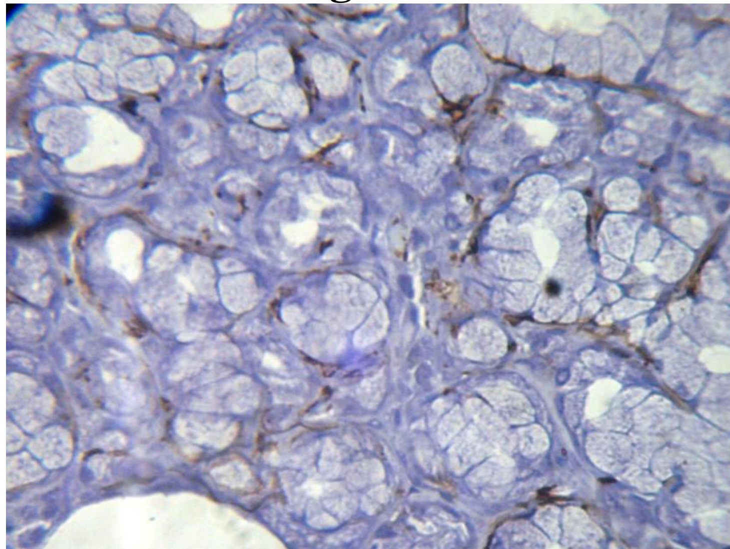
Secondary Antibody

Figure 5



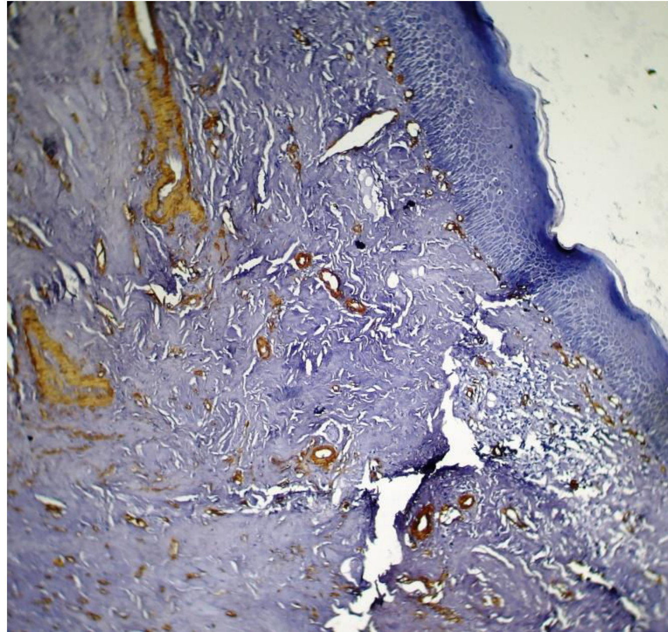
α-SMA Positive staining of normal salivary gland (control-40X)

Figure 6



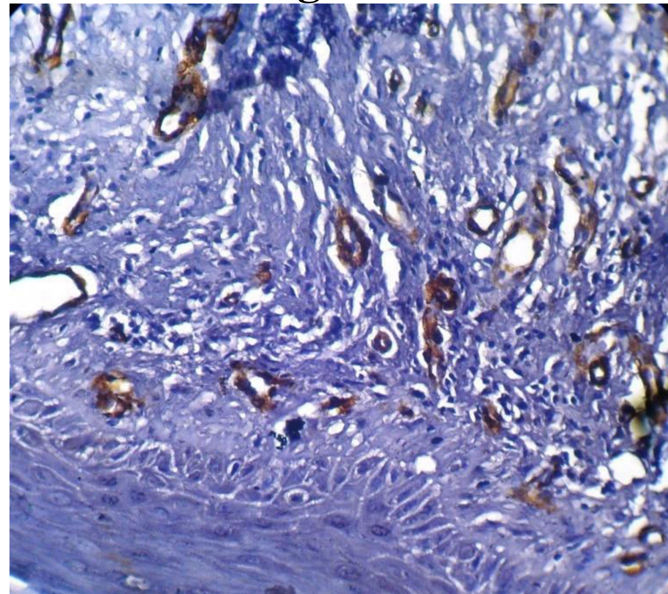
α-SMA Positive staining of normal salivary gland control-40X

Figure 7



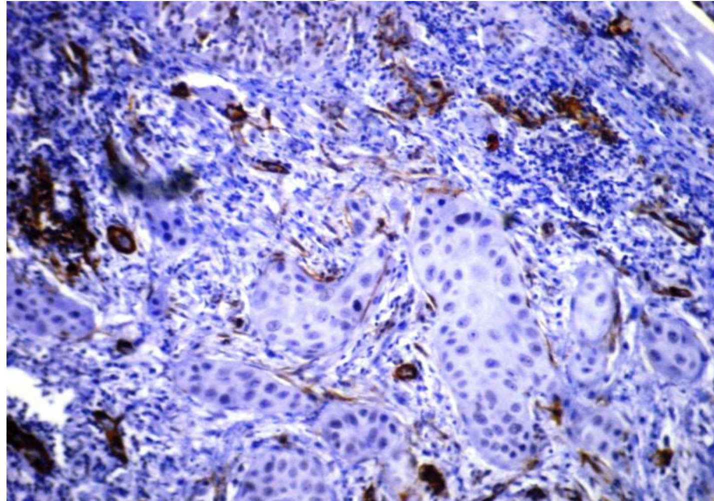
α-SMA Positive staining of OSF-10X

Figure 7



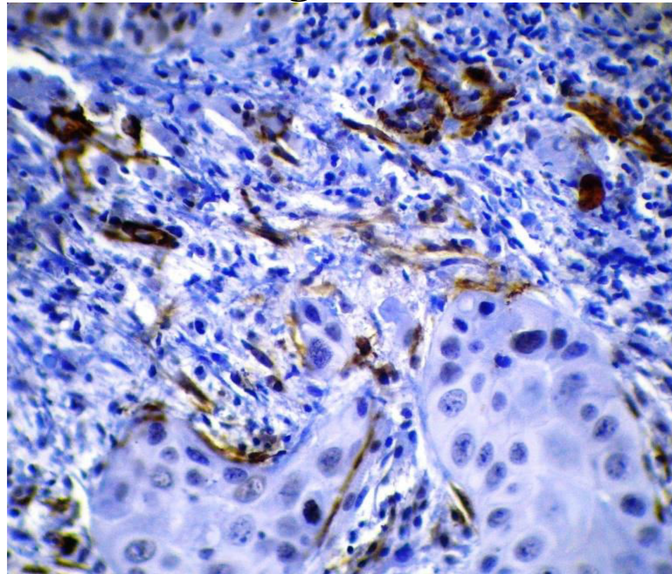
α-SMA Positive staining of OSF-40X

Figure 9



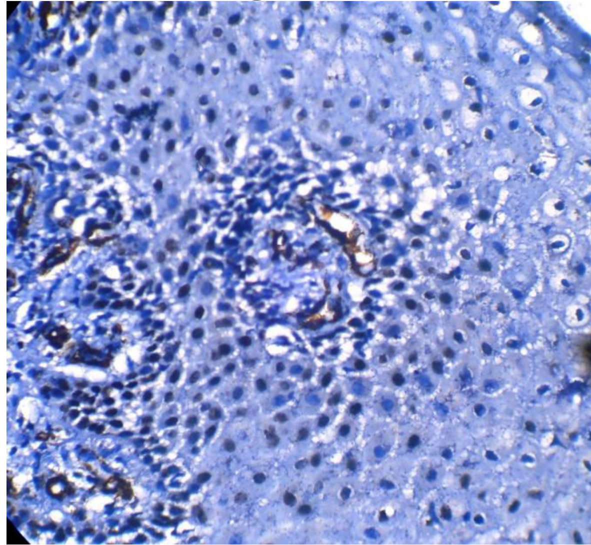
α-SMA Positive staining of OSCC-10X

Figure 10



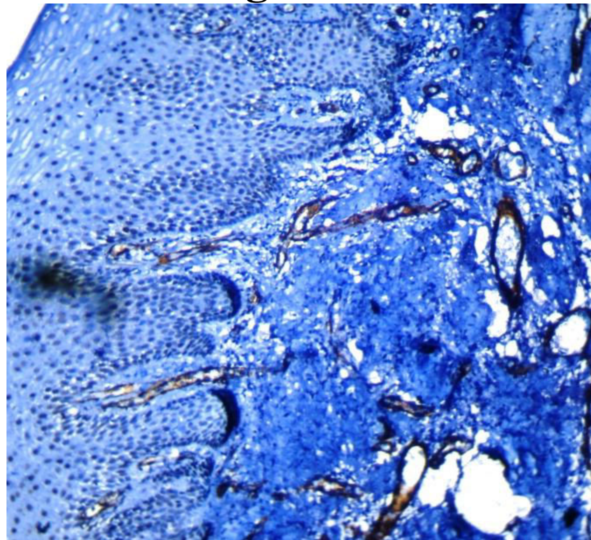
α-SMA Positive staining of OSCC-40X

Figure 11



α-SMA Positive staining of Normal mucosa-10X

Figure 12



α-SMA Positive staining of Normal mucosa-40X