

**EFFECT OF ARECOLINE AND TRANSFORMING GROWTH  
FACTOR  $\beta$  ON MYOFIBROBLAST TRANSDIFFERENTIATION  
BY ASSESSMENT OF MITOCHONDRIAL BIOENERGETICS:  
A CELL CULTURE STUDY**

**Thesis submitted to**

**THE KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH,  
BELAGAVI**

**(KLE DEEMED UNIVERSITY)**

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**For the award of the degree of**

*Doctor of Philosophy*

*In the Faculty of Dentistry*

*(Oral Pathology & Microbiology)*

by

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**(Registration No: KLEU/Ph.D/16-17/DO1216005)**



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## List of abbreviations

<b>Abbreviation</b>	<b>Full Form</b>
Ab	Antibody
Ag	Antigen
ALK5	Activin receptor-like kinase
ANOVA	Analysis of Variance
$\alpha$ SMA	Alpha Smooth Muscle Actin
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
Akt	Protein Kinase B
BSA	Bovine Serum Albumin
CAF	Cancer Associated Fibroblast
cDNA	complementary DNA
CTGF	Connective Tissue Growth Factor
CO <sub>2</sub>	Carbon dioxide
DAPI	4',6-diamidino-2-phenylindole
DDW	Double Distilled Water
DMSO	Dimethylsulfoxide
DMEM	Dulbecco's Modified Eagle's Media
DNA	Deoxyribonucleic acid
DPX	di-butyl phthalate xylene
EGR-1	Early Growth Response -1
EMT	Epithelial Mesenchymal Transition
Em	Emission
Ex	Excitation
FBS	Fetal Bovine Serum
FITC	Fluorescein isothiocyanate

HaCaT	Cultured Human Keratinocyte
HC	Heavy Chain
HGF	Human Gingival Fibroblasts
HK	Hexokinase
HRP/DAB	Horse radish peroxidase/3'3' diaminobenzidine
IFA	Immunofluorescence Assay
IF	Intermediate Filament
IHC	Immunohistochemistry
Ig	Immunoglobulin
LAPs	Latency-associated peptides
LC	Light Chain
LSD	Least significant difference
MAP	Mitogen-activated protein
M3	Muscarinic 3
mtDNA	Mitochondrial DNA
NaOH	Sodium hydroxide
NS	Non-significant
OSMF	Oral Sub Mucous Fibrosis
OXPHOS	Oxidative Phosphorylation
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PINK	Phosphatase and Tensin homolog-induced putative kinase
PI3K/AKT	Phospho-inositide-3-kinase/protein kinase B
qRT-PCR	Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction
RNA	Ribonucleic acid

ROS	Reactive Oxygen Species
SD	Standard Deviation
TGF $\beta$	Transforming Growth Factor Beta
TFAM	Transcription Factor of Mitochondria A
TBS	Tris buffered Saline
V+	Vimentin positive
VA+	Vimentin and Alpha Smooth Muscle Actin positive
VAD+	Vimentin , Alpha Smooth Muscle Actin and Desmin positive
VADM+	Vimentin , Alpha Smooth Muscle Actin, Desmin and Muscle Myosin positive
VFF	Vocal Fold Fibroblasts

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## ABSTRACT

### **Background:**

Oral sub mucous fibrosis (OSMF) is a condition wherein mesenchymal response to injury are mediated through changes in fibroblast structure and function. Fibroblasts differentiate to alpha smooth muscle actin positive ( $\alpha$ SMA+) myofibroblast, which is key feature underlying pathogenesis of OSMF. Studies highlight that myofibroblast phenotypes vary from physiological to pathological conditions. Recently, role of transforming growth factor  $\beta$  (TGF $\beta$ ) and mitochondrial reprogramming has been studied in organ fibrosis. In OSMF, the role of arecoline and TGF $\beta$  is well-recognized but there is a paucity in literature regarding their effect in myofibroblast differentiation and mitochondrial function. Thus, the present work aimed to evaluate the role of arecoline and TGF $\beta$  in myofibroblast differentiation by study of mitochondrial bioenergetics.

### **Objectives:**

**Primary Objective:** To evaluate role of arecoline and TGF $\beta$  on myofibroblast transdifferentiation using human oral fibroblast cell lines.

### **Secondary Objectives:**

1. To assess the effect of arecoline, TGF $\beta$  and combination of arecoline & TGF $\beta$  on myofibroblast transdifferentiation.
2. To assess the mitochondrial bioenergetics in myofibroblast transdifferentiation induced by arecoline, TGF $\beta$  and combination of arecoline and TGF  $\beta$ .
3. To compare the mitochondrial bioenergetics in myofibroblast transdifferentiation induced by arecoline, TGF $\beta$  and combination of arecoline and TGF $\beta$ .

**Methodology:** Primary human gingival fibroblasts (HGF) were used for studying effect of arecoline and TGF $\beta$  at different time points. After determining subtoxic concentrations and by literature survey, we designed six study groups (1 - untreated control, 2 -

arecoline 50µg/mL, 3 - arecoline (50ug/mL) with TGFβ receptor inhibitor SB431542 (10µM), 4 - TGFβ (10ng/mL), 5 - arecoline (50µg/mL) with TGFβ (10ng/mL) and 6 - SB431542 (10µM)). Myofibroblast phenotype was assessed using immunofluorescence assay (IFA) against vimentin, αSMA, desmin and muscle myosin. Mitochondrial changes were assessed by JC-10 assay, mitotracker red staining, QRT PCR for glycolysis (hexokinase II), mitophagy (phosphatase and tensin homolog-induced putative kinase 1), transcription factor of mitochondrial A (TFAM) and IFA for TFAM. Validation of TFAM and phenotype, immunohistochemistry was done using normal mucosa (5 cases) and OSMF tissues (60 cases).

**Results:** Myofibroblast differentiated after 48 hours of treatment and distinct cell phenotype are observed in study groups. Arecoline groups (2 and 3) show mitochondrial depolarization, fission, glycolysis and apoptosis while, TGFβ groups (4 and 5) show maintained membrane polarity, fusion and increased survival.

**Conclusion:** Distinct roles of arecoline and TGFβ are noted in myofibroblast differentiation and mitochondrial bioenergetics. TGFβ rescues arecoline mediated cytotoxicity and promotes survival of the differentiated myofibroblasts.

**Keywords:** Glycolysis, Myofibroblast, Mitochondria, Mitophagy, Oral Submucous Fibrosis.

# I. INTRODUCTION

## 1.1 Background

Oral submucous fibrosis (OSMF), from its erstwhile appellation as “Vidari” to the existing inclusion as a “potentially malignant disorder,” is described as a condition characterized by juxtaepithelial inflammation and progressive fibrosis of submucosa along with subsequent atrophy of the epithelium.<sup>1,2</sup> Clinically, OSMF presents with an array of signs and symptoms ranging from burning sensation, mucosal stiffness, blistering, blanching, reduction in the mouth opening, difficulty in swallowing to extended involvement of the middle ear and uvula. Palpable fibrous bands in the oral cavity, including sites like lips, buccal mucosa, and soft palate, constitute the hallmark of the OSMF.<sup>3-6</sup> Histologically, reduced vascularity with an increase in stromal fibrosis is considered a histological hallmark of OSMF. The condition is prevalent in South East Asian countries, predominantly India.<sup>3-6</sup>

Although multifactorial, research states that OSMF is primarily an abnormal healing process caused by unremitting chronic (chemical and mechanical) irritation to the oral mucosa led by areca nut chewing.<sup>6</sup> Bhosle RB et al reported a 34-100% frequency of areca nut chewing habit in the Indian subcontinent.<sup>7</sup> Besides the known psychoactive and parasympathomimetic effects of the areca nut and its components [Table 1], it also bears carcinogenic potential. The harmful effects of arecoline are not limited to the oral cavity but can involve many other body organs.<sup>8,9,10</sup>

**Table 1:** Key areca nut components and their chemical composition.<sup>10-12</sup>

<b>Constituent of areca nut</b>	<b>Chemical Composition</b>
Alkaloids [Active component]	Arecoline Arecaidine Guavacine Guavacoline
Polyphenols	Catechin Flavanoids, Hexahydroxyflavans, Flavan-3:4- diols Leukocyanidins Tannins
Trace elements	Sodium Magnesium Chloride calcium Vanadium Manganese Copper Bromide

Arecoline causes trauma and inflammation of the tissues primarily by the generation of reactive oxygen species (ROS) that causes upregulates pro-inflammatory and pro-fibrotic factors like transforming growth factor  $\beta$  (TGF  $\beta$ ), interleukins, fibroblast growth factor amongst many others.<sup>6</sup> In normal tissues, the balance between pro-fibrotic and anti-fi-

brotic factors regulates collagen synthesis and degradation.<sup>13</sup> The imbalance between these is responsible for stiffness and progressive fibrosis of the oral tissues. Hence, OSMF is also considered a collagen metabolic disorder.<sup>14</sup>

All human body organs can virtually undergo fibrosis when subjected to chronic trauma or inflammatory conditions. Intransigent tissue injury can override homeostasis and physiological repair leading to a mesenchymal response in the form of fibrosis, scarring, or a possible carcinomatous change.<sup>13</sup> In the process of wound healing, fibroblasts play a critical role in wound contraction and extracellular matrix formation. Research has brought to light that the regulated process of fibroblast activation or transdifferentiation to myofibroblasts is a cardinal event responsible for wound healing.<sup>13</sup>

Fibroblasts can differentiate into myofibroblasts which contain contractile protein alpha-smooth muscle actin ( $\alpha$ SMA).<sup>13</sup> Myofibroblasts produce enormous amounts of extracellular matrix components like collagen I. The actin stress fibers present in myofibroblast aid in production of isometric forces necessary for the process of wound contraction and closure.<sup>13</sup> Myofibroblasts are considered ubiquitous cells of mesenchymal origin seen in physiologic tissue repair, reactive or inflammatory conditions, and benign as well as malignant neoplasms.<sup>13,15,16,17</sup> The amassing and continual activation of myofibroblast leads to excessive fibrosis and scarring linked with numerous disorders, such as idiopathic pulmonary fibrosis, scleroderma, renal fibrosis, amongst many others.<sup>18,19,20,21</sup> There is snowballing in the number of  $\alpha$ SMA positive myofibroblasts with the advancing grade of tissue fibrosis. Along with proteins like  $\alpha$ SMA, cytoskeletal heterogeneity of myofibroblasts exists in these fibrotic conditions. In normal tissues, myofibroblasts can express:

vimentin positive (+), vimentin<sup>+</sup> with desmin<sup>+</sup> and vimentin<sup>+</sup> with  $\alpha$ SMA<sup>+</sup> phenotypes, while in pathological conditions: vimentin<sup>+</sup>,  $\alpha$ SMA<sup>+</sup> and desmin<sup>+</sup> or vimentin<sup>+</sup>,  $\alpha$ SMA<sup>+</sup>, desmin with smooth muscle myosin heavy chains<sup>+</sup> (heavy chains) may be expressed.<sup>21,22</sup>

In organ fibrosis, profibrotic cytokines like TGF $\beta$ , connective tissue growth factor (CTGF), endothelin, interleukins, amongst many others, regulate the activation of fibroblasts.<sup>23,24</sup> These activated fibroblasts have high energy demands for supporting their cellular changes and for the production of extracellular matrix components.<sup>24</sup> The role of mitochondria, specifically mitochondrial bioenergetics of muscle cells subjected to exercise or endurance training is well known.<sup>24</sup> Recently, there has been a focus on the cellular energy dynamics for fibroblast transdifferentiation in the healing of wounds. The role of TGF $\beta$  1 concerning fibroblast differentiation and energetic reprogramming of mitochondria has been brought to light in organ fibrosis and carcinomas.<sup>24,25,26</sup> TGF $\beta$  affects the mitochondria of human fetal lung fibroblast by activating the transcription factor of mitochondria (TFAM) and thereby leading to transcription and transduction of mitochondrial DNA necessary for biogenesis of the mitochondria.<sup>24</sup> Furthermore, the mitochondrial morphology, function, and clearance of damaged mitochondria by mitophagy are involved in the pathogenesis of numerous diseases, such as neurodegeneration, metabolic disorders, cardiovascular diseases, and organ fibrosis.<sup>27,28,29,30</sup> Phosphatase and Tensin homolog-induced putative kinase 1 (PINK1) has been studied in these conditions and is known to protect the cells against ROS-mediated oxidative stress. PINK1 gets activated

with depolarization of the mitochondrial membrane, and it helps to clear the damaged mitochondria of cells through mitophagy.<sup>30</sup>

Like in organ fibrosis, in OSMF, there is up-regulation of TGF $\beta$  by arecoline that also causes fibroblast differentiation and matrix remodeling of oral tissues.<sup>31,32</sup> Literature indicates high levels of TGF $\beta$  1 in the stroma of OSMF cases compared to normal tissues.<sup>32</sup>

The primary mechanism of action of TGF $\beta$  is by activation of transcriptional regulators, such as  $\beta$  catenin and smads, and mitogen-activated protein (MAP) kinase signaling.<sup>33,34</sup>

It thereby leads to myofibroblast differentiation that induces the production of  $\alpha$ SMA, collagen I, and fibronectin.<sup>33, 34</sup>

Like most other fibrotic diseases, OSMF draws clinical attention only after significant fibrosis has already taken place. Limited literature is available on arecoline and TGF $\beta$  induced mitochondrial bioenergetics in myofibroblast transdifferentiation in oral submucous fibrosis. Hence, research to explore the morphological, biological, and functional aspects of myofibroblast differentiation concerning OSMF is warranted. Thus, the present study aimed to evaluate the role of arecoline and TGF $\beta$  in myofibroblast differentiation by the study of mitochondrial bioenergetics of human oral fibroblast cell lines.

## **1.2 Objectives**

**Primary Objective:** To evaluate the role of arecoline and transforming growth factor  $\beta$  on myofibroblast transdifferentiation using human oral fibroblast cell lines.

### **Secondary Objectives:**

1. To assess the effect of arecoline, transforming growth factor  $\beta$  and combination of arecoline & transforming growth factor  $\beta$  on myofibroblast transdifferentiation.
2. To assess the mitochondrial bioenergetics in myofibroblast transdifferentiation induced by arecoline, transforming growth factor  $\beta$  and combination of arecoline and transforming growth factor  $\beta$ .
3. To compare the mitochondrial bioenergetics in myofibroblast transdifferentiation induced by arecoline, transforming growth factor  $\beta$  and combination of arecoline and transforming growth factor  $\beta$ .

### 1.3 Literature Review

The estimated prevalence rate for OSMF is 0.2 to 6.4% and although recognized initially as a condition that predominantly affects females, studies have observed predisposition towards the male population.<sup>35, 36, 37</sup> OSMF is a preventable condition that begins primarily due to factors that cause chronic injury to the oral tissues. OSMF continues as a persistent, inflammatory and scarring disorder that extensively involves the oral cavity and upper digestive system leading to limitations in oral functions and general debilitation.<sup>35-38</sup> The age groups affected are wide ranging from very young individuals to the elderly population.<sup>35-38</sup> With the advent of more processed products of areca nut the younger age groups are also being affected and the estimated cases have been snowballing.<sup>37</sup> The estimated cases in India are about 2.5 million.<sup>39</sup> Concern arises as this condition is irreversible, shows very little improvement to treatment and has the potential to undergo malignant change.<sup>2, 37, 38</sup> The progressive debilitation, the malignant change and its treatment can leave a huge impact on the patients physical and psychological health.<sup>2, 37, 38</sup> Clinically OSMF shows early and late features that include mucosal changes, fibrous bands, restricted tongue, involvement of uvula and associated sequelae or premalignant and malignant change.<sup>40</sup> Numerous researchers have tried to categorize the condition into stages so that the extent of involvement and communication for treatment can be made universal.<sup>41-50</sup> Most classification systems have categorized the condition based on mouth opening, mucosal changes and associated sequelae. Rao NR et al<sup>51</sup> have summarized these clinical features based on stages as described in Table 2.

**Table 2:** Clinical features of OSMF categorized into stages as per intra-oral and extra-oral manifestations. <sup>51</sup>

<b>Features</b>	<b>Early stage</b>	<b>Moderate stage</b>	<b>Advanced stage</b>
Intra-oral	(Mucosal changes) stomatitis, increased salivation, blanching, blistering, palpable bands, scant brown-black pigmentation of mucosa	Features of early stage with additional presentation like difficulty in whistling, restricted mouth opening, petechiae, inability to blow cheeks, blanching extending circumorally and involving soft palate, floor of mouth as well as faucial pillars. Shrunken/altered shape of uvula.	Features of moderate stage with loss of muscle suppleness, opaque/mottled/marbled white mucosa, de-papillated tongue, involvement of pharyngeal and esophageal mucosa.
Extra-oral	Not significant	Nasal twang, prominent masseter, sunken cheeks, inability to swallow, hoarse voice, impairment of hearing and weight loss.	Hypertrophy and stiffness of masseter, sunken cheeks, voice changes, folding of cheeks on opening of mouth, loss of nasio-labial fold, difficult deglutition, atrophy of facial muscles, and may extend to alterations in condylar forms and temporomandibular joint changes in severe cases.

OSMF histologically shows hyalinization, collagen deposition, reduced vascularity and secondary degeneration of muscles. <sup>6,38</sup> The condition of OSMF has been classified by numerous researchers based on its clinical presentation, histological features and combination of both.<sup>52-55</sup> The histopathological grading systems are based primarily on the pathological changes seen in the OSMF tissues and provide an insight based on progression and pathogenesis of the condition. Numerous histological classification systems have been put forth for the grading of OSMF, however, the early classification by Pindborg JJ and Sirsat SM (1966) incorporated all the essential histological features and classified the condition into four grades [Table 3]:

**Table 3:** Histological grading system of OSMF by Pindborg JJ & Sirsat SM.<sup>52</sup>

<b>Parameter</b>	<b>Grade I - Very Early OSMF</b>	<b>Grade II - Early OSMF</b>	<b>Grade III - Moderately advanced OSMF</b>	<b>Grade IV - Advanced OSMF</b>
Collagen & edema	Fine/Delicate fibrillar collagen, marked edema	Early juxtaepithelial hyalinization, thickened collagen bundles	Moderate hyalinization, thick bundles of collagen, changes observed juxtaepithelially with minimal residual edema	Abundant hyalinization of collagen fibers, bundles not discernible. Absence of edema.

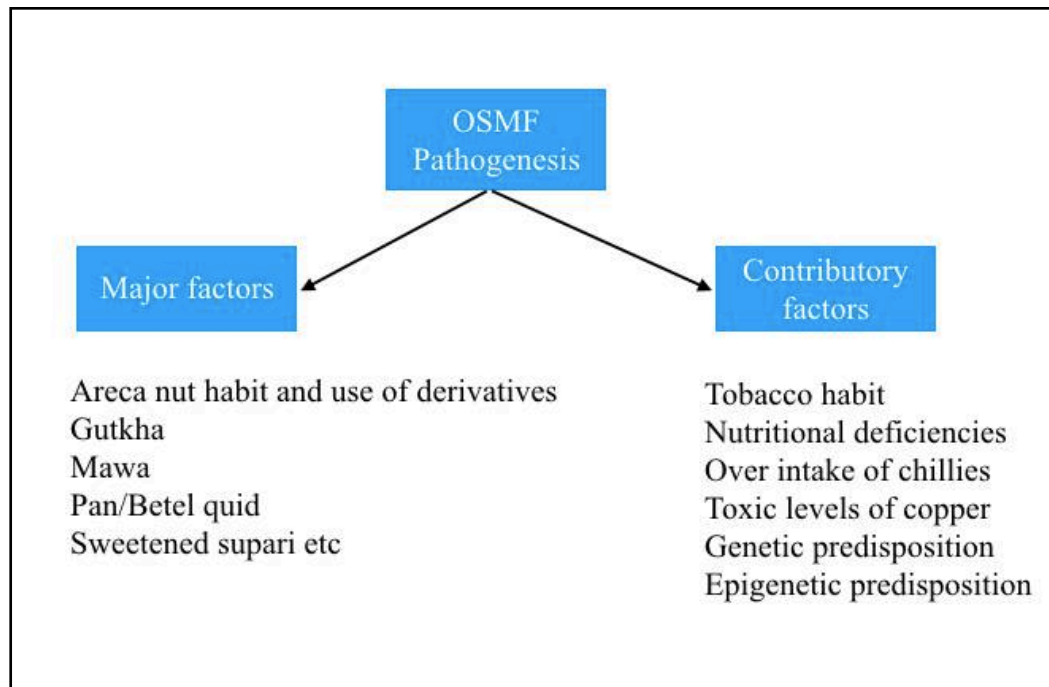
Fibroblasts response	Extensive response with plump and young fibroblasts with plentiful cytoplasm	Moderately dense occurrence of Plump fibroblasts	Less marked, fibrocytes with spindle nuclei and scant cytoplasm	Lack or scanty fibroblasts - thin and elongated
Vascularity	Normal vascularity with vascular dilation and congested	Vascular dilation and congestion prominent	Normal to constricted	Vasoconstriction/ obliteration of blood vessels
Inflammation	Occasional leukocytes and eosinophils	Abundant mononuclear inflammatory cells, eosinophils and plasma cells	Chronic inflammatory cells - plasma cells and lymphocytes observed	Chronic inflammatory cells

From the above classification we understand that the changes in the extracellular matrix and nature of fibroblasts are of key concern in OSMF progression. Utsunomiya H et al (2005) modified this criteria into three categories - early, intermediate and advanced OSMF [Table 4].<sup>54</sup>

**Table 4:** Histological grading system of OSMF by Utsunomiya et al.<sup>54</sup>

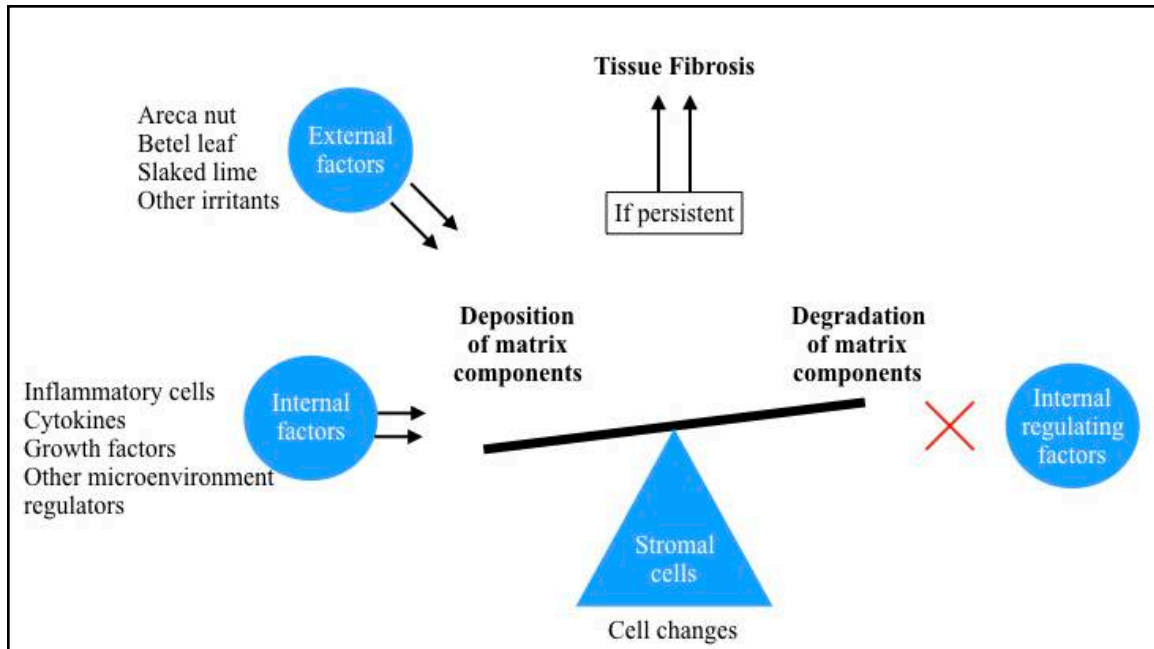
<b>Early OSMF</b>	<b>Intermediate OSMF</b>	<b>Advanced OSMF</b>
Lymphocytes - predominant in subepithelial tissue	Reduced inflammatory cells	Scanty inflammation
Zone of myxedematous change	Hyalinization appears subepithelially, granulation changes close to muscle	Marked fibrous tissue with hyalinisation extending from sub epithelial to superficial muscle layer, atrophy and degenerative changes in muscles
	Blood vessels compressed	Number of blood vessels reduced

The role of major factors like areca nut, slaked lime and betel leaf along with internal factors ie., downstream molecules like TGF $\beta$ , interleukins etc has been largely explored in pathogenesis of OSMF.<sup>6,38,55</sup> Other external contributing risk factors for the condition include tobacco, excessive chillies, vitamin deficiencies, low iron and zinc, malnutrition, amongst many others [Figure 1].<sup>6, 38, 51, 56</sup>



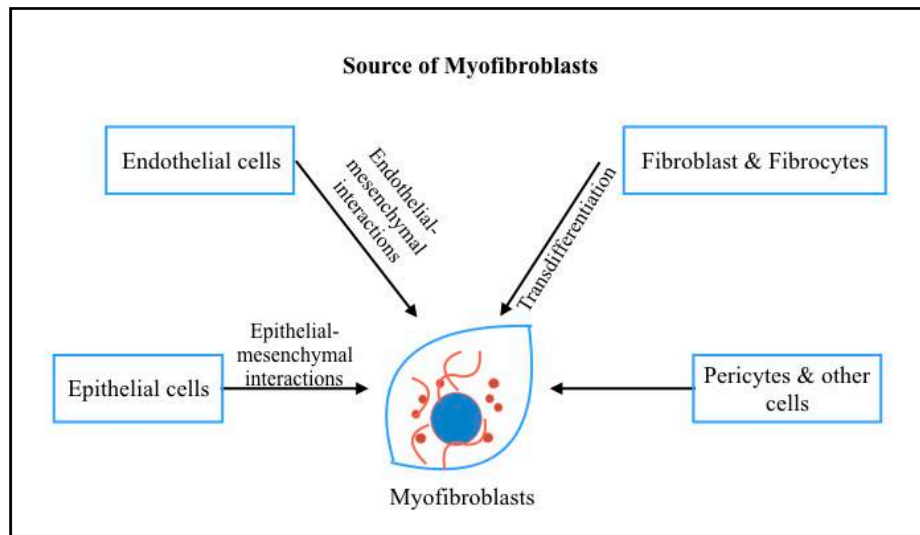
**Figure 1:** Major and contributory factors involved in pathogenesis of OSMF.

Understanding of the molecular mechanism that drives fibrosis primarily begins with identifying the internal and external factors responsible for initiation, regulation and persistence of the disorder.<sup>57</sup> Although organ fibrosis can begin with different etiological factors, it is common that these factors create an inflammatory milieu wherein secreted cytokines and growth factors cause cellular changes leading to excessive matrix and collagen production [Figure 2].



**Figure 2:** Basic mechanisms & factors involved in tissue fibrosis.

The cellular changes can occur in multiple cell types causing their transdifferentiation into an activated cell called as the myofibroblast.<sup>58</sup> Transdifferentiation is a well-regulated process of phenotypic change wherein reprogramming of genes causes switch between cell types.<sup>59</sup> Initially it was thought that mesenchymal cells namely fibroblasts are the only cells that get activated/transdifferentiated to myofibroblasts, however, research has indicated that mesenchymal cells like endothelial cells, pericytes and others, along epithelial cells can serve the need [Figure 3].<sup>13, 58, 59</sup> At molecular level, increase in  $\alpha$ sma, focal adhesion proteins paxillin, tensin and fibronectin, along with exuberant collagen synthesis in myofibroblasts as compared to fibroblasts are thought to be distinguishing features between these cells.<sup>60-63</sup>



**Figure 3:** Schematic diagram showing sources of myofibroblasts in tissues.

The process of fibroblast activation to a cell type with features capable of contraction similar to smooth muscle cells is the key event in OSMF initiation and progression.<sup>17, 32</sup> Numerous studies have highlighted that the count of differentiated  $\alpha$ smooth muscle actin positive myofibroblasts increases with the disease progression.<sup>17, 32</sup> In contrast to myofibroblast found in wound healing, it has been proposed that myofibroblasts with cytoskeletal heterogeneity have immune privilege and can elude apoptosis thereby contributing to sustained tissue fibrosis.<sup>13, 17, 64</sup> In physiological or pathological situations, cytoskeletal changes (structural and biochemical) in the fibroblast architecture aid in cellular adaptation. This suggests that fibroblast plasticity plays a role in the adaptation of cells to various stimuli.<sup>22, 65</sup> Cytoskeletal changes and differential localization of Vimentin, Desmin, muscle myosin and  $\alpha$ smooth muscle actin occur under these conditions.

### **Cytoskeletal proteins of interest:**

- 1) Vimentin - Vimentin derives its name from the word “vimentum” which in Latin stands for wickerwork. <sup>66</sup> This type III intermediate filament (IF) is one of the key modulators of the wound healing. It attributes plasticity to the mesenchymal cells, has a role in epithelial mesenchymal interaction and development of an invasive cell phenotype. Vimentin has distinct distribution and forms that determine the function. The commonly observed patterns of vimentin include highly organized networks, non filamentous, unit filaments and soluble forms. The organized forms that present as a network serve in resisting stress and maintain cellular shape, while the soluble forms largely function for cell signaling. <sup>67</sup> Recently, research has shown that extracellular vimentin can bind to the surface of the mesenchymal cells and promote wound closure. It is also involved in maintenance of architecture of the cytoskeleton of mesenchymal cells. Vimentin modulates cellular process involving interaction with immune cells, activation of MAP kinase pathways. In epithelial mesenchymal transition (EMT) and cell migration, vimentin interacts with microtubules, by limiting the flow of actin and modulating tractional forces and stiffening of cells. It closely works with the actin-myosin network, microtubules and other proteins to promote migration of cells. The role of vimentin is also observed in regulation of apoptosis of immune cells. <sup>67, 68</sup>
- 2)  $\alpha$ 5 $\beta$ 1 -  $\alpha$ 5 $\beta$ 1 belongs to the integrin family and similar to vimentin, it is essential for cellular motility, cytoskeletal stability and structure. It is the key protein that brings about myofibroblast contractility and plays a crucial role in wound repair. It regulates

the stress fiber assembly and microfilaments of the cell. It has been extensively used as a prognostic and indicative marker for myofibroblast differentiation in fibrosis and carcinomas. Besides fibroblasts, the other cells that express  $\alpha$  SMA include pericytes and smooth muscle cells. <sup>6, 17, 69-72</sup>

- 3) Desmin - Desmin, is a type III IF that is found in all types of muscles (cardiac, skeletal and smooth). Desmin functions similar to vimentin and maintains the cytoskeleton spatial organization by forming a scaffold around. It also functions to help in generation and transmission of forces. Pathology involving desmin gene manifests as desminopathies wherein intracellular aggregation of desmin causes apoptosis and subsequent replacement fibrosis. Desmin IF is also known to affect the mitochondria and mitochondrial respiration. <sup>73, 74</sup> Myofibroblasts in type II EMT and other fibrotic conditions have demonstrated expression of desmin. Varayoud J et al <sup>65</sup> studied pregnant rats and found that myofibroblasts that expressed desmin were thought to bring about rapid changes in shape and organelle distribution of cells consequent to collagen and elastin production in pregnancy.
- 4) Muscle Myosin - Myosin is a well known large protein that is found to play a central role in contraction of muscles. Myosin comprised of heavy chains (HC) and light chains(LC); differentiated myofibroblasts exhibit smooth muscle like features and typically express smooth muscle myosin HC and non muscle myosin. However, rare pathological conditions like Dupuytren's contracture show myofibroblast phenotype that exhibits positivity for vimentin, desmin,  $\alpha$  SMA and myosin. <sup>75</sup>

### **Myofibroblast biotype in physiological conditions, fibrosis and carcinomas:**

Research has highlighted that cytoskeletal proteins like vimentin and smooth muscle myosin HC are also found to be upregulated by TGF $\beta$  during the myofibroblast transdifferentiation.

In normal tissues, myofibroblasts can express: vimentin positive (V+) phenotype, vimentin and desmin positive (VD+) , and vimentin and  $\alpha$ SMA positive (VA); while in pathological conditions: VA+, Vimentin,  $\alpha$ SMA with Desmin (VAD)+ or Vimentin,  $\alpha$ SMA, Desmin with Muscle Myosin positive (VADM+) phenotype may be expressed [Table 5].<sup>21</sup>

**Table 5:** Immunophenotypes of myofibroblasts in various conditions.<sup>21</sup>

<b>Phenotype</b>	<b>Examples of associated conditions</b>
V+	Initial development of granulation tissue
VA+	Wound retraction
VAD+ or VADM+	Liver fibrosis, hypertrophic scars, Dupuytren's contracture and fibromatosis.

Besides, cytoskeletal phenotype, distinct functional and genotypic types of myofibroblasts are noted in various conditions. A study conducted by Branco et al, studied the effect of TGF $\beta$  on fibroblast of vocal folds (VFF) and elaborated  $\alpha$ SMA positive myofibroblast differentiation. Fibronectin,  $\alpha$  smooth actin, CTGF, and metalloproteinase inhibitor were the highest genes expression in TGF $\beta$ 1 treated vocal fold fibroblasts. They further con-

cluded that VFF treated with TGF  $\beta$  have similar phenotype but different genotypic behavior compared to scar VFF. <sup>65</sup>

Costa DE et al, studied cancer associated fibroblast (CAF) and found two distinct subtypes, one that had its profile (transcriptome and secretome) closer to normal or physiological fibroblasts and other with divergent expression pattern. They observed that both CAFs had different tumor-promoting capacity. <sup>26</sup>

Different myofibroblast biotypes and their functions in different pathologies are being largely explored to understand the nature and develop a targeted treatment plan for the same.

### **Role of arecoline and TGF $\beta$ in myofibroblast differentiation and pathogenesis of**

**OSMF:** The pleiotropic TGF $\beta$  family exists as 33 TGF $\beta$  associated polypeptides of which TGF $\beta$  has three isoforms namely TGF $\beta$ 1, TGF $\beta$ 2 and TGF $\beta$ 3. The TGF $\beta$  isoforms act along serine/threonine kinases receptors with the exception of TGF $\beta$ 3. These isoforms have pro segments called latency-associated peptides (LAPs). These LAPs form small latent complexes that restrict TGF $\beta$  from binding to the receptor. TGF $\beta$  needs activation from its latent form before activity. <sup>34, 76-81</sup> Thrombospondin 1, integrins and other macromolecules can activate the TGF $\beta$  latent complex and enable receptor binding. The signaling pathways for activity involve TGF $\beta$  1 receptor - activin receptor - like kinase (ALK5) with smad protein 2/3 i.e., canonical pathway and non-smad or non-canonical pathway. The canonical pathway is primarily involved in wound healing and fibrosis. <sup>34</sup>

In tissue fibrosis, TGF $\beta$  modulates different cellular and extracellular matrix responses. It induces  $\alpha$ SMA, collagens, fibronectins, and other components for excessive deposition of matrix and has a role in degradation of the same by repression of matrix metalloproteinases and up regulation of their inhibitors.<sup>18, 19, 82, 83</sup> Researchers found elevated TGF $\beta$ 1 levels in cases of OSMF as compared to normal mucosa.<sup>32,84,85</sup> Arecoline via its mitochondrial ROS production causes the activation of TGF $\beta$  and promotes further binding and signaling by the canonical pathway.<sup>6, 31, 83, 86</sup>

A study by Moutasim KA et al in 2011,<sup>32</sup> showed significant  $\alpha$ v $\beta$ 6-dependent activation of TGF $\beta$  1. They also found that the OSMF tissue stroma and the arecoline treated epithelial cells both showed high levels of  $\alpha$ v $\beta$ 6 integrin. They hypothesized that epithelial cells could induct the pathogenesis of OSMF, which further progresses by the arecoline/TGF $\beta$  1 associated fibroblast differentiation and matrix changes. TGF $\beta$  also increases CTGF and early growth response-1 (Egr-1) which aids in progression of the disorder.<sup>32,82,83</sup>

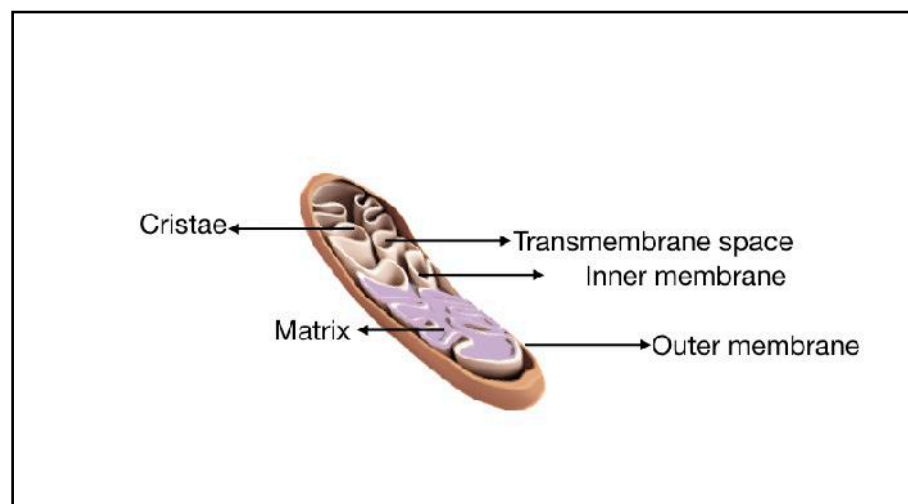
Besides the role of TGF $\beta$ 1 in OSMF, TGF $\beta$ 2 has also been studied in OSMF. Thangjam et al put forth that arecoline treated HaCaT cells showed Muscarinic 3 (M3) - muscarinic acid receptor and they induced TGF $\beta$ 2 through calcium and the protein kinase C pathway. Arecoline down-regulated collagens 1A1 and 3A1 in human primary gingival fibroblasts (HGF); but induced these when HGF were cultured along with human keratinocytes. On treatment with TGF $\beta$  blocker, reduction in collagen was noted, thus highlighting the role of TGF $\beta$  in collagen synthesis.<sup>83</sup>

Khan I et al<sup>85</sup> studied analyzed global gene expression of culture human keratinocyte (HaCaT) cells post treatment with areca water (extract water) and TGF $\beta$ . They found

64% differentially regulated genes by areca nut water extract when matched with TGF $\beta$  induced profile. On treating cells using TGF  $\beta$  receptor blocker they found that 57% of genes were compromised and 7% genes were separately induced by areca nut. They also found that human gingival fibroblast (HGF) did not show any smad or TGF  $\beta$  downstream targets while they were induced in HaCaT cells which suggested that epithelial cells can serve as a source of TGF $\beta$  in OSMF.

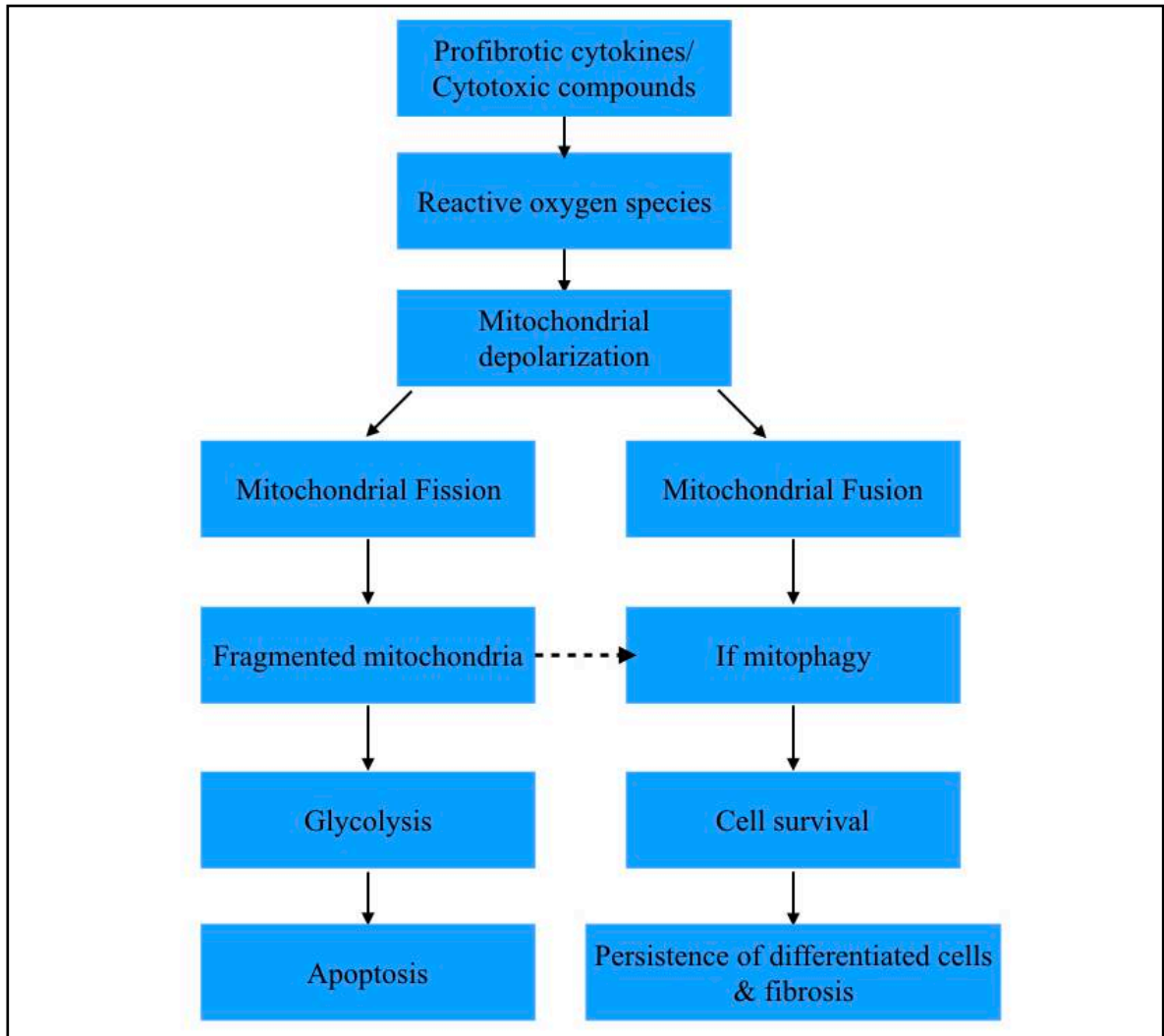
Further Pant I et al <sup>86</sup> studied the transcriptome profiling of HGF using areca nut as well as TGF  $\beta$ . They found 413 genes in HGF cells by combined treatment of areca nut and TGF $\beta$ . They also studied HaCaT epithelial cells and found that TGF $\beta$  is released by areca nut influenced activation of fibroblast and other genes implicated for fibrosis.

#### **Role of mitochondria and structural mitochondrial dynamics in mitophagy and apoptosis of myofibroblasts:**



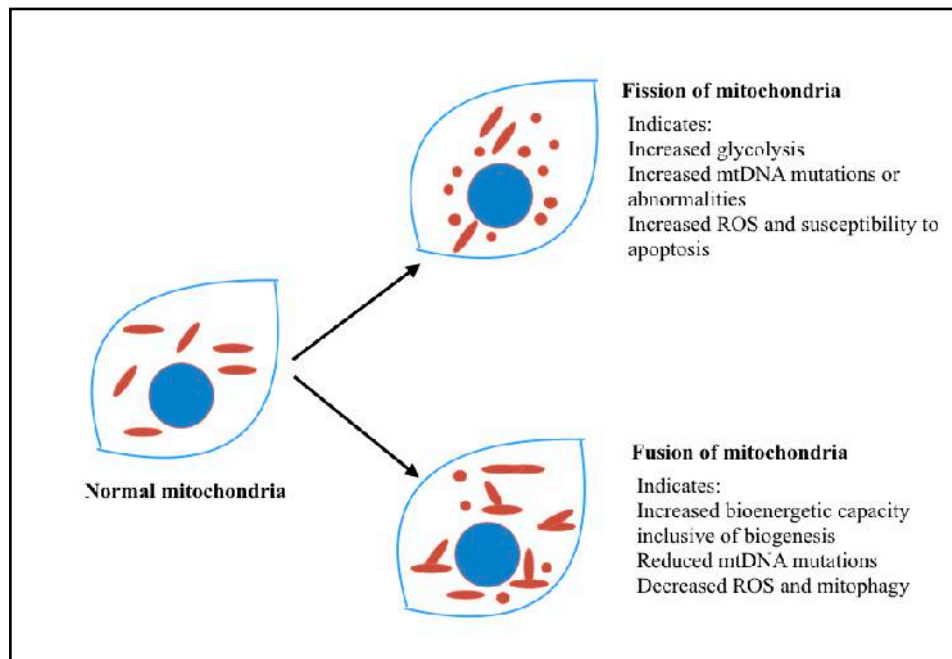
**Figure 4:** Schematic representation of mitochondrial structure.

Mitochondria are essential, double-membrane intracellular organelles [Figure 4] that can continuously divide by mitochondrial deoxyribonucleic acid (DNA) replication, undergo adaptive fusion and move within the cytoplasm of cell. Their key function include respiration for production of adenosine triphosphate (ATP) and regulating cell fate under ROS mediated cellular stress and metabolic alterations.<sup>27, 87</sup> Usually, in presence of oxygen, the tricarboxylic acid cycle occurs in the mitochondria to produce ATP by oxidative phosphorylation (OXPHOS) however, in condition with lack of oxygen or in special conditions like carcinomas, the shift to glycolytic pathway is observed.<sup>87, 88</sup> This pathway is an energy producing and is catalyzed by enzymes namely hexokinase (HK), phosphofructokinase and pyruvate kinase. HK is a rate-limiting and essential enzyme that is involved in regulating the first step of glycolysis. There are four isoenzymes of hexokinase - HKI, II, III and IV. HKII is of significance as it available in the cytosol and outer membrane of mitochondria. HK-II when bound to the membrane regulates glycolysis and is shown to be upregulated in pathological conditions like fibrosis and carcinomas.<sup>88</sup> Recently, it has been studied that glycolysis is essential for activation and maintenance of the profibrotic myofibroblast phenotypes.<sup>89</sup> However, the process of fibroblast activation is not limited to glycolysis but involves corresponding structural changes and regulated events like biogenesis, mitophagy and apoptosis [Figure 5].



**Figure 5:** Flow chart showing mitochondrial changes induced by profibrotic cytokines or irritants that guide the cell either to apoptosis or survival in tissue fibrosis.

Different cells contain mitochondria with distinct structural and biochemical characteristics.<sup>90</sup> Chen et al have put forth that in cardiac fibroblast activation, metabolic reprogramming plays a key role and that the cells are dependent on glycolysis for their energy demands.<sup>91</sup> Mitochondria are self regulatory and show structural adaptive changes like fission and fusion.<sup>28, 91</sup> Mitochondrial fusion i.e., interconnection, is said to maintain an energetic, active metabolic state, while fission i.e., fragmentation to smaller spherical mitochondria is associated with low or resting states [Figure 6].<sup>28, 91,92,93</sup> It is proposed that changes in these mitochondrial changes of fission and/or inadequate fusion regulate the process of glycolysis.<sup>92,93</sup>



**Figure 6:** Role of mitochondrial fission and fusion in myofibroblast differentiation.

Besides action of profibrotic molecules and cytotoxic drugs, the ROS generated by the mitochondria or its fission can mediated further mitochondrial damage. This damage could direct the cells to apoptosis. In order to maintain mitochondrial health the process of mitophagy is essential. <sup>93</sup> Mitophagy is essential for curbing the mitochondrial damage and for promoting survival of cells. It has been studied by authors that fission promotes fibrosis in renal, lung and liver. <sup>93, 94, 95, 96</sup> Wang Y et al found that the tissue biopsies also showed mitochondrial fission in case of renal fibrosis. <sup>94</sup>

Research suggests that this process of mitochondrial fission is regulated by PINK1. <sup>93, 97</sup> PINK1 is localized on the outer membrane of depolarized mitochondria which later functions by PINK1-Parkin pathway. PINK1 is largely studied in neurodegenerative diseases like Parkinson's disease. PINK1 regulated the mitochondrial quality by fission and ultimate mitophagy. <sup>97</sup>

Research by Lemos DR et al <sup>98</sup> showed that glycolytic reprogramming of renal cells occurs during fibrosis and that inhibition of glycolysis suppressed the activation of fibroblasts. Thus, it is essential to explore the role of mitochondrial bioenergetics mediated through glycolysis, biogenesis and other pathways for understanding the fibroblast differentiation in organ and tissue fibrosis. <sup>98</sup>

### **Myofibroblast mitochondrial bioenergetics in fibrosis and cancer:**

The human mitochondrial genome is a circular, double-stranded, compact molecule of DNA (16.5 kb). Depending on cell type, 2 to 10 copies of this DNA can be found in cells.

<sup>99</sup> This mitochondrial DNA (mtDNA) is attached to the inner mitochondrial membrane

within the mitochondrial matrix and packaged into protein–DNA complexes known as nucleoids. <sup>100</sup>

Functionally, increase in respiration by mitochondria can be supported by increase in mass of mitochondria. The increase in mitochondrial mass is associated with the increased transcription of mtDNA. TFAM regulates renewal of mitochondria by bringing about transcription and replication of mtDNA in mitochondrial biogenesis. TFAM is also known to regulate repair of damaged mtDNA. Synthesized in cytoplasm as a 29kD molecule, for activation by cytokines or regulators, TFAM gets cleaved to a 25 kD polypeptide when it enters the mitochondria. <sup>99,100</sup>

Bernard et al <sup>24</sup> studied TGF $\beta$ 1 induced differentiation and its association with metabolic or mitochondrial reprogramming of fibroblasts. TGF $\beta$  affects the mitochondrial metabolic reprogramming in human fetal lung fibroblast by activating mitochondrial transcription factor A (TFAM), replication and transduction of mtDNA and genes during biogenesis. They also observed that blocking the biogenesis or glycolysis results in reduced contraction and expression of  $\alpha$ sm $\alpha$ . <sup>24</sup>

A study conducted by Negmadjanov U et al <sup>25</sup> showed that TGF  $\beta$ 1 treated mouse fibroblasts had increased expression of  $\alpha$ sm $\alpha$ , higher mitochondrial function and an increased baseline oxygen consumption rate as compared to untreated cells.

Besides fibrosis, cancer associated fibroblasts also exhibit mitochondrial metabolic reprogramming which causes changes in biosynthesis and bioenergetics of cells. <sup>101</sup> Balliet et al showed fibroblasts with TFAM down regulation which is associated with dysregulation of Caveolin-1 and subsequent induction of ROS stress, dysfunction of mitochondria,

and glycolysis in the tumour microenvironment. They suggested that fibroblasts deficient in TFAM produce hydrogen peroxide and levo-lactate in excess, both of which promote development and progress of tumour. <sup>102</sup>

Thus, we understand that the process of fibroblast differentiation in pathological conditions involves interconnected, extensive morphological, biological and molecular events. Exact mechanism, profile of myofibroblast and the role of mitochondrial bioenergetics (if any) which contributes to oral submucous fibrosis is elusive. Like most other fibrotic diseases, OSMF draws clinical attention only after significant fibrosis has already taken place. Thus, there is a need to evaluate the role of arecoline and TGF  $\beta$  in relation to the differentiation dynamics of myofibroblasts in OSMF in order to develop appropriate therapy and curb the progression of this irreversible condition.

## 1.4 Justification

OSMF simulates an altered wound healing process that presents with amassing of activated fibroblasts and a progressive increase in fibrosis.<sup>54</sup> It is hypothesized that these myofibroblasts show adaptation by evading apoptosis and can produce excessive amounts of extracellular matrix.<sup>17, 103</sup> The irreversible nature of this condition warrants a detailed study of the behavior and pathogenesis of OSMF.

Arecoline is responsible for the hypersensitivity reaction and incessant juxtaepithelial inflammation in tissues of patients habituated to chewing areca nut. This chronic inflammation, increase in ROS, and the induced cytokines bring about changes and activation of fibroblasts in the tissues.<sup>17, 103</sup> Angadi et al through their immunohistochemical study using OSMF tissues showed that arecoline can bring about fibroblast differentiation in OSMF and that the frequency of these cells increases with progressive fibrosis of the tissues.<sup>17, 103</sup> Pant I et al have put forth that fibroblasts treated with areca nut components show differentiation to myofibroblasts and produce genetic alterations similar to those seen in OSMF cases.<sup>86</sup>

Furthermore, fibroblasts exhibit phenotypic plasticity wherein they undergo changes based on the physiologic needs and stimuli from the surrounding microenvironment. This plasticity and increased frequency of cancer-associated fibroblasts attribute to their ability to migrate and cause recurrence of the associated tumors. In several conditions, myofibroblasts with specific cytoskeletal phenotypes show colossal presence in the tissues depending upon the activating stimuli.<sup>22</sup> Interestingly, conditions like infantile myofibromatosis have shown spontaneous regression despite the amassing of myofibroblasts.<sup>22</sup> In

this regard, authors have postulated that a possible regulated activation of cytoskeletal proteins vimentin,  $\alpha$ SMA, desmin, smooth muscle myosin, and other proteins cause the development and progression of these lesions bearing quasi neoplastic characteristics. <sup>13,</sup>

22

Research has brought to light that the energetic process of fibroblast differentiation is essential for the contraction & healing of wounds. Mitochondria being the key source of energy meets the energy demands of the differentiating cells by functional changes including mitochondrial biogenesis. In lung fibrosis, it has been studied that the transformation of fibroblasts by TGF $\beta$ 1 is associated with increased mitochondrial biogenesis of the treated cells. <sup>25</sup> Further research in this area, propelled the understanding that mitochondrial bioenergetics inclusive of glycolysis and electron transport chain is essential for tissue contraction and the differentiation of fetal fibroblasts of the human lung. The dysfunction in mitochondrial bioenergetics and mitophagy leads to the subsistence of these myofibroblasts. <sup>24</sup> Research has shown an association between the role of growth factors like platelet-derived growth factor receptor- $\beta$ , evasion of autophagy, and Phospho-inositide 3 kinase/protein kinase B (PI3K/AKT) pathway in the regulation of cardiac myofibroblast differentiation in rats. <sup>29</sup>

However, it is not clear whether arecoline causes fibrosis by itself or by inducing secretion of cytokines like Transforming growth factor  $\beta$ . Also, the exact mechanism of fibroblast differentiation by TGF $\beta$  is not distinctly recognized, but TGF $\beta$  is known to be the most potent in modulating the mitochondrial bioenergetics and expression of  $\alpha$ SMA in fibroblasts of various organs undergoing fibrosis. Scarce literature is available on the role

of arecoline and TGF $\beta$  concerning mitochondrial energetics and cellular differentiation of fibroblast in OSMF. Thus, more studies are required to elaborate on the molecular pathogenesis occurring in the dynamic process of cell differentiation and progressive fibrosis.

## 2. MATERIALS AND METHODS

**Ethical Clearance certificate:** KLEU/EC/17-18/D-112 (Annexure I)

### **Cell lines, culture and study groups:**

After obtaining appropriate training (Annexure II) the experiments were conducted. Primary human gingival fibroblast (HGF)(PCS-201-018) cell lines obtained from American type culture collection (ATCC, USA) were passaged and used for all cell-culture experiments. Passages 2-8 were included for the time-based experiments. Cells were grown and maintained in Dulbecco's modified eagle's media (DMEM) (Hi-media, India) with antibiotics (Penicillin and Streptomycin, Hi-Media, India) and supplemented with 10% Fetal Bovine Serum (FBS)(Gibco, USA). The cells were maintained in 5% Carbon dioxide (CO<sub>2</sub>) incubator (Nuair, USA) at 37°C. The HGF cells (1x 10<sup>6</sup> cells/well) grown to achieve confluence in a 6-well plate. Prior to every experiment, serum starvation of the cells for 12 hours by means of 0.2% FBS-DMEM was done. Depending on objective the experiments were conducted at 12hours, 24 hours, 48hours and 72 hours.

Based on literature <sup>26, 86</sup> and cytotoxicity assay using Alamar Blue (Thermofisher scientific, USA) the study groups were designed and sub-toxic/ optimal concentrations were used for the treatment of cells.

The study groups were designed based on the research objectives as follows [Table 6]:

**Table 6:** Study groups and the optimal concentrations used for treatment of HGF cells.

No.	Study Group	Concentration
Group 1	Untreated Control	Untreated
Group 2	Arecoline treatment group [Arecoline hydrobromide (Sigma-Aldrich, USA)]	50 µg/ml
Group 3	TGF β treatment group [Recombinant human TGFβ 1 protein, R & D systems, USA]	10 ng/ml
Group 4	Arecoline + TGF β treatment group	50 µg/ml + 10 ng/ml
Group 5	Arecoline + TGF β1 ALK5 receptor blocker, SB431542	50 µg/ml + 10 µM
Group 6	TGF β1 ALK5 receptor blocker - SB431542 [Sigma-Aldrich (calbiochem)]	10 µM

**Rational for study group design:** Based on the literature review and understanding of OSMF pathogenesis, the following groups were considered - control group (Group1) comprised of untreated fibroblasts, cells treated with arecoline (Group 2) and cells treated with TGFβ (Group 3). Further, it is known that continuous arecoline consumption leads to increased production of TGFβ in the tissue microenvironment, thus to simulate a TGFβ rich environment we used a combination group wherein cells were treated with TGFβ an hour prior to the arecoline treatment (Group 4). To assess the differential roles of arecoline and TGFβ for bringing about myofibroblast differentiation, i.e., to evaluate the effect of arecoline alone we used the TGFβ1 receptor inhibitor SB431542 (Group 5) and treated the cells with 10 µM of SB431542 an hour prior to the arecoline treatment. An additional group was considered for understanding the effect of SB431542 (group 6) was considered

and treatments were performed after serum starvation and by using standard procedures and concentrations.

### **Standardization and determination of optimal concentration for arecoline and TGF $\beta$**

*Alamar Blue assay:* To study the effect of the treatment compounds, Alamar Blue assay was performed on serial dilutions of arecoline and TGF  $\beta$  for determining the optimal concentration.

Alamar Blue dye contains Resazurin that is cell culture media stable and nontoxic component. It is water-soluble and can pass through the membranes of living cells. As the dye adds no direct effect by itself, it can be used to study the effect of compounds for prolonged time periods. This compound is reduced to resorufin which is pink and fluoresces to higher wavelength than resazurin. This reduction allows easy quantification in the form of visible color change based on cell viability. The dye can indicate impaired cellular function or metabolism but not specific to changes in mitochondria.<sup>104</sup> Fluorescence is measured by fluorescence micro-plate reader (BioRad, USA) at two wavelengths - ex wavelength 530-560nm while em wavelength 590nm.

*Protocol and Experiment set up:*

**Table 7:** Alamar Blue assay reagents and preparation.

<b>Sr. No</b>	<b>Component</b>	<b>Criteria/Reagents</b>	<b>Requirement/Preparation</b>
1	Assay dye	10% Alamar Blue dye 0.2% FBS DMEM	1ml of dye stock in 9ml of 0.2% FBS DMEM media
2	Cells	HGF seeded at a density of 10,000 cells/well in 10% FBS-DMEM	Clear wells with flat bot- tom, 96-well plate
3	Fluorescence plate	For reading of fluorescence exhibited by cells after re- duction of dye	Black wall and clear flat bottom, 96-well plate
4	Incubation	To maintain stable environ- ment for the reaction	Incubate at 37°C in 5% CO <sub>2</sub> incubator for 1 hour in dark conditions
5	Replicates	Experiment to be performed in triplicates for reliability	Three wells per test group
6	Serum Starvation	To minimize effect of growth factors in the media and to synchronize cells be- fore the experiment	Incubating cells in 0.2% FBS-DMEM for 12 hours
7	Test solutions	Serial dilutions of Arecoline & TGF $\beta$	Stock solutions Plain DMEM

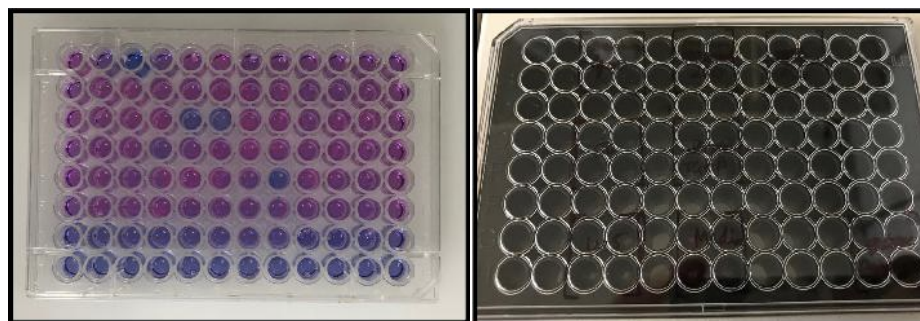
*Arecoline:* Arecoline hydrobromide dissolved in dimethylsulfoxide (DMSO) at 100mg/mL stock, which was further dissolved in plain DMEM to make serial dilutions from 0 to 200  $\mu$ g/mL.

*TGF  $\beta$ :* TGF  $\beta$  dissolved in 4mM hydrochloric acid in 1mg/mL Bovine serum albumin (BSA) at 20 $\mu$ g/mL, which was further dissolved in plain DMEM to make serial dilutions from 0 to 20 ng/ml.

The experiment was standardized using 10% Alamar Blue dye and using manufacturers instructions. Post standardization, the procedure was tested under different conditions. All the experiments were performed and results were tabulated for pertinent data analysis.

*Procedure:*

HGF cells grown in 10% FBS-DMEM were seeded 10,000 cells/ well in a 96-well plate. Triplicates were marked and used for control/untreated cells, arecoline serial dilutions and TGF  $\beta$  serial dilutions. After serum starvation, the 10% Alamar Blue dye was prepared and added to the cell groups. Post 1hour of incubation, the reaction solution was pipetted to a black wall, clear flat bottom 96-well plate [Figure 7] and fluorescence at excitation (Ex) wavelength 550nm and emission (Em) wavelength 580nm in a fluorescence plate reader (BioRad, USA). The readings were normalized with the blank wells (initially containing DMEM later replaced by alamar working solution) and divided for plotting the graph.



**Figure 7:** Alamar Blue assay a) Cell culture 96 well plate incubated with Alamar Blue solution for 1hour showing change to pink colored compound. b) Clear bottom, black wall 96 well plate used for obtained fluorescence reading of the reaction solution.

**Procedure to accomplish the first objective concerning myofibroblast transdifferentiation was as follows:**

**Experiment 1. Immunofluorescence assay (IFA):** For studying the myofibroblast cytoskeletal heterogeneity, all 6 groups of HGF cells were treated for 48 hours and stained with cytoskeletal markers for imaging using immunofluorescence assay. The procedure was standardized using known negative and positive controls for the antibodies. The antibody (Ab) dilutions were standardized and the standardized procedure was repeated before performing the experiment for study groups.

**Table 8:** Antibodies used for determination of cytoskeletal phenotype

<b>Sr no</b>	<b>Antibody</b>	<b>Clone and specificity</b>	<b>Company</b>
1	Vimentin	P o l y c l o n a l G o a t Immunoglobulin G (IgG)	R & D systems, USA
2	Anti-Actin, $\alpha$ SMA	Mouse Monoclonal IgG, Clone 1A4	Sigma Aldrich, USA
3	Desmin	Mouse Monoclonal IgG	Novus Biologicals, USA
4	Muscle Myosin	Mouse Monoclonal IgG, Clone MF20	R & D systems, USA
5	Secondary Ab (1:1000)	A n t i - r a b b i t I g G - Fluorescein isothiocyanate (FITC) conjugate Ab	Sigma Aldrich, USA
6	Secondary Ab (1:1000)	Anti-Goat IgG-FITC Ab	Sigma Aldrich, USA
7	Secondary Ab (1:1000)	Anti-Mouse IgG-FITC Ab	Sigma Aldrich, USA
8	4',6-diamidino-2-phenylindole (DAPI) (1:100)	Nuclear stain	Molecular Probes, UK

*Protocol and experiment set up:*

**Table 9:** Reagents and preparation for Immunofluorescence assay.

<b>Sr No</b>	<b>Process</b>	<b>Reagents</b>	<b>Preparation</b>
1	Coverslip wash solution	Sodium hydroxide (NaOH) (Sigma, USA) Ethanol (Sigma, USA) Round coverslips	Dissolve 25 g of NaOH in 500ml of ethanol solution in a glass bottle (highly caustic).
2	Fixation	3% Paraformaldehyde (PFA) in sterile phosphate buffered saline (PBS). (Sigma, USA)	1.5g PFA in 50ml of PBS. Vortex till reagents dissolve completely. Add 1 pellet of NaOH and adjust pH between 7.2-7.6.
3	Permeabilisation	Triton X buffer (Sigma, USA)	0.125 ml Triton x100 + PBS 50ml Shake till completely dissolved
4	Blocking buffer	1% BSA in sterile PBS (Sigma, USA)	1g BSA in 100ml PBS.
5	Antibody dilution	Primary cytoskeletal antibodies - Vimentin - 5µg/mL Myosin -10 µg/mL Desmin - 2µg/mL αSMA - 20µg/mL Blocking buffer Secondary Antibodies	Dissolve required antibody concentration in blocking buffer
6	DAPI staining	DAPI Sterile PBS	1:100 DAPI in PBS. Prepare fresh solution when staining.

*Procedure:*

*Preparation of coverslips:* For culturing the cells on coverslips and performing IFA we treated the coverslips using coverslip wash solution (labelled highly caustic) followed by washing with double distilled water (DDW) and 70% ethanol (3hours wash each on a shaker). After drying and autoclave the coverslips were used for seeding of cells.

*Immunofluorescence assay procedure:*

In laminar hood, after removal of the media and washing with sterile PBS, fixation of cells was done for 15minutes. This was followed by PBS washes (twice, 10minutes) on a shaker at room temperature. Permeabilization (10minutes) and blocking (1 hour) with appropriate PBS washes after each step was performed. The primary antibodies were used for incubation of coverslips in a humidifying chamber (1hour) and PBS was used as negative control. Following washing, 1:1000 dilution of secondary Ab was used in dark conditions. Final washing and incubation with 1:100 DAPI solution (10minutes) was done and coverslips were mounted on clean slides using fluoromount for visualization.

*IFA analysis:* IFA slides were analyzed for the positive or negative staining of cytoskeletal antibodies in the treatment groups. The myofibroblast differentiation was confirmed on evaluation of  $\alpha$ SMA expression. The expression of cytoskeletal proteins were tabulated for categorization into vimentin, vimentin with  $\alpha$ SMA , vimentin with  $\alpha$ SMA and desmin or Vimentin,  $\alpha$ SMA, desmin and myosin phenotype in each study group.

For validation of IFA results in relation to myofibroblast profile, we added an additional experiment and performed immunohistochemistry (IHC) for cytoskeletal proteins on a small sample of normal oral mucosa and different grades of OSMF.

### **Experiment 2. IHC for cytoskeletal proteins:**

Principle of antigen (Ag) - Ab binding was along with PolyExcel Horse radish peroxidase/3'3' diaminobenzidine (HRP/DAB) 2 step kit (Genepulse, Bangalore) was used for IHC against cytoskeletal proteins. All primary antibodies including vimentin, anti-actin, desmin and myosin heavy chain (Genepulse scientific, Bangalore) were used for IHC. DAB was used as chromogen for visual brown detection of the Ag-Ab complex.

OSMF tissues were categorized into three groups - early (20 cases), intermediate (20 cases) and advanced (20 cases) based on Utsunomiya et al classification. The grades were segregated based on evaluation of hematoxylin and eosin stained slides. Normal mucosa (5 cases) were taken as control. Six serial sections of 4um thickness were cut on to the gel coated slides using a semi-automatic microtome (Leica RM 2145). Routine de-paraffinization (10-15 minutes) and dehydration procedure (10 minutes) was done and microwave heat retrieval (3 rounds at 96°C for 6 minutes each) was used for retrieval of antigens. Buffers and blocking for endogenous peroxidases (15 minutes each) was performed followed by one hour of incubation of all primary Abs. Final treatment with HRP (30 minutes) and DAB enabled visualization of the positively stained areas. Hematoxylin applied as counter stain. Slides mounted after procedure using di-butyl phthalate xylene (DPX).

Standardization of IHC procedure was done using standard company provided positive and negative controls and these were included during IHC of study tissues. Standard IHC protocol was followed and the buffers used included citrate buffer (Genepulse, Bangalore) and tris buffer saline (TBS)(Genepulse, Bangalore).

*IHC analysis:*

For myofibroblast phenotype determination in the groups the slides were analyzed for cytoskeletal protein expression by tabulating the positive or negative expression and % of cells expressing the antibodies. Scoring of  $\alpha$ SMA was first done to determine myofibroblasts location in each group and corresponding areas were chosen for evaluation of positive staining of remaining cytoskeletal Abs. The scoring as per modified criteria<sup>16</sup> was done by two trained observers and variability (inter and intra observer) was evaluated statistically by use of kappa statistics (Agreement: 0.89 - Good to excellent).

**Procedures to accomplish the second objective concerning mitochondrial bioenergetics in myofibroblast transdifferentiation were as follows:**

Experiments:

- 1) Mitochondrial membrane permeability using JC-10 assay
- 2) Mitophagy, transcription and glycolysis using PINK-1, TFAM and HK-II using polymerase chain reaction; IFA and IHC for TFAM.
- 3) Mitochondrial morphology and intensity using Mitotracker Red assay
- 4) Cell survival using Alamar Blue assay

All experiments were standardized as per manufacturers instructions and repeated before the actual experiments were conducted.

### **Experiment 1. Mitochondrial membrane permeability using JC-10 assay**

JC-10 is a green color, lipophilic dye that is capable of entering into the mitochondria of cells. The principle of JC-10 dye is similar to JC-1, wherein it changes reversibly to orange-red color aggregates with increase of mitochondrial membrane potential in cells with intact mitochondria.<sup>105</sup> Fluorescence is measured at two wavelengths - Ex wavelength at 490/525 nm and Em wavelength at 540/590 nm using fluorescence micro-plate reader.

*Protocol and experiment set up:*

**Table 10:** Reagents and preparation for JC-10 assay.

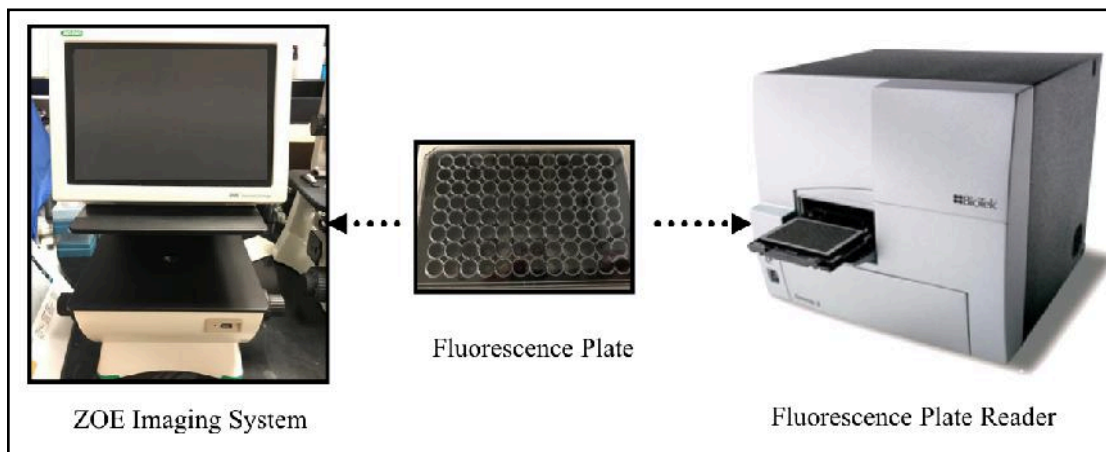
<b>Sr. No</b>	<b>Component</b>	<b>Criteria/Reagents</b>	<b>Preparation/Requirement</b>
1	Assay dye	JC-10 vial DMSO Hanks buffer with 20mM Hepes Pluronic F-127	2mg/mL (3mM) of JC-10 in DMSO. Kept at -20 degree Celsius. Working solution: 10 to 30 uM 1x solution in buffer, pH 7-8 with 0.02% Pluronic F-127
2	Cells	HGF culture of 10,000 cells/well in 10% FBS-DMEM	Cells seeded in a clear bottom, black wall 96-wells culture plate
3	Fluorescence plate	For reading of fluorescence due to JC-10 aggregates formed	Black wall and clear flat bottom, 96-well plate
4	Incubation	To maintain stable environment for the reaction	37 degree Celsius, 5% CO2 incubator for 30minutes in dark conditions
5	Replicates	Experiment in triplicates/group for reliability	Three wells/study group and three wells for blank (no cells seeded)
6	Serum Starvation	To minimize effect of growth factors in the media and to synchronize cells before the experiment	Incubating cells in 0.2% FBS-DMEM for 12 hours

7	Test solutions	0.2% FBS-DMEM for control group Arecoline - 50 µg/mL TGF β - 10 ng/ml Arecoline + TGF β - combination of above Arecoline + SB431542 - 50µg/mL arecoline + 10mM SB431542 SB431542 - 10mM SB431542	Post treatment, incubate cells with working solution of JC-10
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*Procedure:*

Cells were treated with reagents indicated in study groups for 24 hours and buffer was used for blank wells. JC-10 working solution (100 ul) was added in each well depending upon number of treatment groups. Incubation was done under controlled conditions (30 minutes) and fluorescence changes at Ex/Em = 490/525 (FITC channel) and 540/590nm (TRITC channel) was recorded using fluorescent plate reader for ratio analysis. Fluorescent images were captured using ZOE™ fluorescent cell imager [Figure 8](BioRad, USA).

*Analysis:* Normalization of the data using blank well was done before the ratio analysis and plotting of graph.



**Figure 8:** Components used in JC-10 assay - ZOE™ imaging system, fluorescence plate and fluorescence plate reader

## **Experiment 2. Mitochondrial morphology and intensity analysis using MitoTracker Red dye**

MitoTracker probes are membrane permeable and selective mitochondria dyes. They work on the principle that Mitotracker dye accumulate in the membrane component of mitochondria and are retained after cell fixation. <sup>106</sup>

*Protocol and experiment set up:*

*Reagents:*

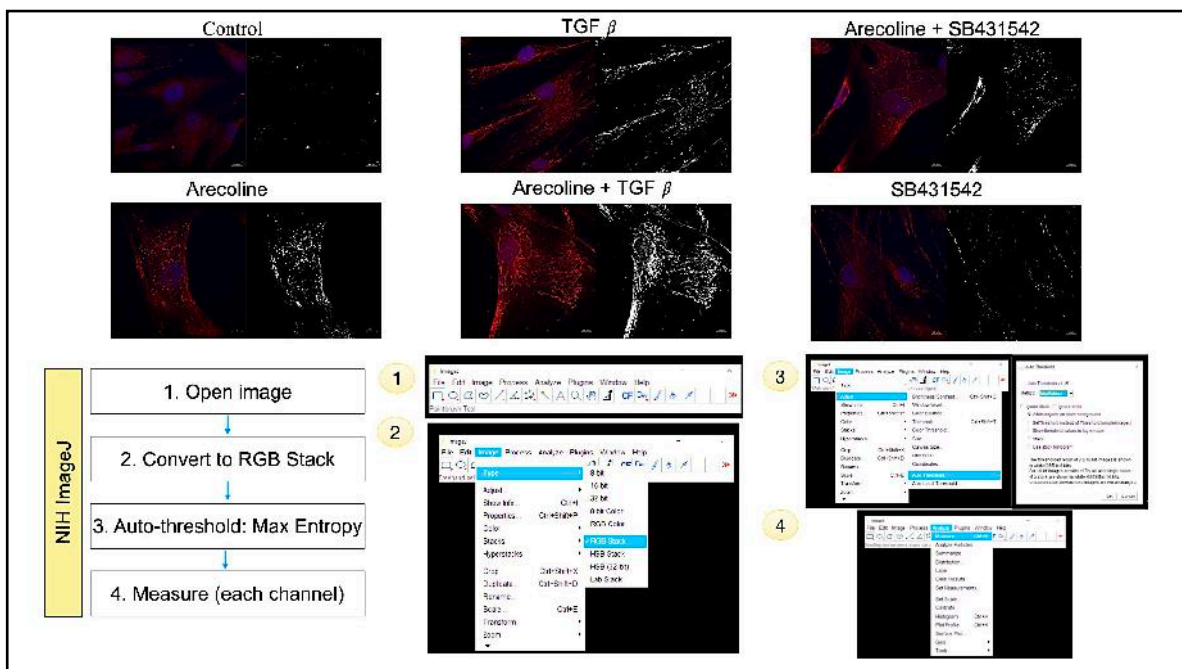
- Mitotracker Red
- DMEM plain media
- 3% PFA
- Permeabilisation buffer
- PBS 1x
- Fluoromount
- Treated coverslips for cell attachment
- Glass slides
- Cell culture plate

*Procedure:*

After dye optimization, 50nM Mitotracker Red aliquots were prepared. The cultured coverslips were washed with plain DMEM followed by three PBS washes (10minutes). Incubation was done using Mitotracker Red followed by PBS washes (10 minutes each). After this the 48 hour treated cells and control group were fixed using 3% PFA in PBS at room temperature (15minutes). Washes with PBS and permeabilization of cells (10min-

utes) was done. Post permeabilisation, incubation of coverslips with DAPI 1:100 and washes with PBS (10 minutes). The slides were labelled and coverslips were mounted using fluoromount for imaging under fluorescent microscope and for confocal microscopy. Images were captured at 40x magnification on Olympus BX-51 fluorescence microscope or on Zeiss LSM 510 Meta confocal microscope.

*Analysis:* Using Image J software [National institute of health (NIH)] the intensity and morphology of mitochondria in each of the treatment groups were analysed [Figure 9].



**Figure 9:** Process for measuring intensity and shape of mitochondria using MitoTracker Red and DAPI stained confocal images of the study groups using NIH Image J software.

**Experiment 3. Mitophagy, Transcription and Glycolysis using PINK-1, HK- II and transcription factor of mitochondria A (TFAM).**

**Quantitative Real-time Reverse Transcription-Polymerase Chain Reaction (qRT-PCR):** For evaluating the levels of PINK-1, HK-II and TFAM in the study groups, PCR was conducted using cells treated for 48 hours. The primers were prepared using primer blast and obtained from Integrated DNA Technologies, USA. Extraction of cellular ribonucleic acid (RNA) was performed using Ambion Purelink™ kit (Invitrogen, USA) as per instructions provided by the manufacturer. The extracted RNA was used with random hexamer primers, QuantiTect Reverse Transcription kit for preparation of complementary DNA (cDNA) as per protocol provided by the manufacturer. The detection of strands by PCR was done as per standard procedure using QuantiTect SYBR green PCR kit (Qiagen). The experiment was performed twice in duplicates.

*Primers:*

PINK1 Forward: 5' - TGCTGTGTATGAAGCCACCA-3'

PINK1 Reverse: 5'-AACCTGCCGAGATGTTCCAC-3'

Hk-II Forward: 5'-CCCTGCCACCAGACTAAACT-3'

HK-II Reverse: 5'-TGGACTTGAATCCCTTGGTC-3'

TFAM Forward: 5' - GAGTTGTGTATTGCCAGGAG-3'

TFAM Reverse: 5'-ACAGATGAAAACCCACCTCG-3'

*Analysis:* Relative expression of gene in each group was analyzed by use of comparative delta cycle threshold technique.

**IFA and IHC for TFAM:** For validation of PCR results in relation to TFAM expression, we performed IFA and IHC using Ab against TFAM (Rabbit monoclonal, Abcam, USA) using the same treatment groups of HGF cells and a small sample of normal oral mucosa and different grades of OSMF. The microwave retrieval system was standardized and set at 96°C for 18 minutes (3 cycle, each cycle - 6 minutes). The principle and procedure used for IFA and IHC of TFAM is same as that done for cytoskeletal proteins.

*Analysis:* IFA results for TFAM were analyzed similar to that of cytoskeletal proteins IFA. IHC analysis was done based on following criteria - localization of immunostaining (0 - Absent, 1-focal, 2- juxtaepithelial, 3- throughout the stroma), number of cells stained (0-10% cells, 10-25% cells, 25-50% cells and >50% cells), intensity of staining (negative, mild to moderate and dark brown) and frequency of positively stained fibroblasts (0- Absent, 1 to 3 - low expression and 4 to 6 - high or over-expression)

#### **Experiment 4. Cell survival assay**

*Protocol and experiment set up:* As mentioned in optimal concentration determination experiment using Alamar Blue assay.

*Procedure:* The study groups of treated HGF cells were examination at 12, 48 and 72 hours. At 12hours, the media was discarded and 10% Alamar Blue solution was added to the wells. Post 1hour of incubation, the fluorescence was measured using a black wall, clear flat bottom 96-well plate at 550nm & 580 nm. After reading the plate, the 0.2% DMEM FBS media was replenished and the experiment was continued for 48 and 72 hours for similarly evaluating the viability/survival of cells.

*Analysis:* Alamar Blue assay was analyzed by normalization of data with the blank wells reading and by dividing the values for plotting the graph.

### 3. DATA ANALYSIS PLAN

Cell culture experiments performed were tabulated and data was entered in excel software, which was further transferred to SPSS software (version 16.0) for applying appropriate tests for statistical analysis.

All the experiments were standardized, conducted post standardization and with replicates. Optimal concentrations of treatment reagents determined were used in the study.

Data normalization was done for the experiments where indicated.

**Table 11:** Statistical tests used for data analysis of experiments

<b>Experiments</b>	<b>Application</b>	<b>Statistical test used</b>
Alamar Blue assay	Optimal concentration determination	One way analysis of variance (ANOVA) test Post hoc tukey test
IFA and IHC	Determination of myofibroblast phenotype and TFAM expression	Distribution and frequency Chi square test
JC-10 assay	Mitochondrial membrane permeability	One way ANOVA test Post hoc tukey test
Mitotracker red staining	Mitochondrial localisation and intensity	One way ANOVA test Post hoc tukey test
Quantitative Real time Polymerase chain reaction	Mitophagy, transcription and glycolysis	One way ANOVA test Post hoc tukey test
Alamar Blue assay	Cell Survival	One way ANOVA Post hoc tukey test Repeated Measure ANOVA test Post hoc least significant difference (LSD) test

The differences were considered significant at 95% Confidence Interval and  $p \leq 0.05$ .

## 4. RESULTS

I. Results for optimal concentration determination of arecoline and TGF $\beta$  using human oral fibroblast cells and Alamar Blue assay.

**Table 12 (a):** Comparison of cell viability/relative fluorescence of human oral fibroblast cells treated with serial dilutions of arecoline (0 to 400  $\mu\text{g/mL}$ ) using one-way ANOVA test.

<b>Arecoline (A) groups (<math>\mu\text{g/mL}</math>)</b>	<b>Mean</b>	<b>Std. Deviation</b>	<b>F value</b>	<b>p value</b>
Ctrl	6018.58	351.66	90.347	0.001*
A6.25	6056.73	540.37		
A12.5	5378.02	144.99		
A25	5680.44	644.31		
A50	4772.10	246.17		
A100	3312.98	227.37		
A200	2557.57	11.54		
A400	1855.52	59.23		

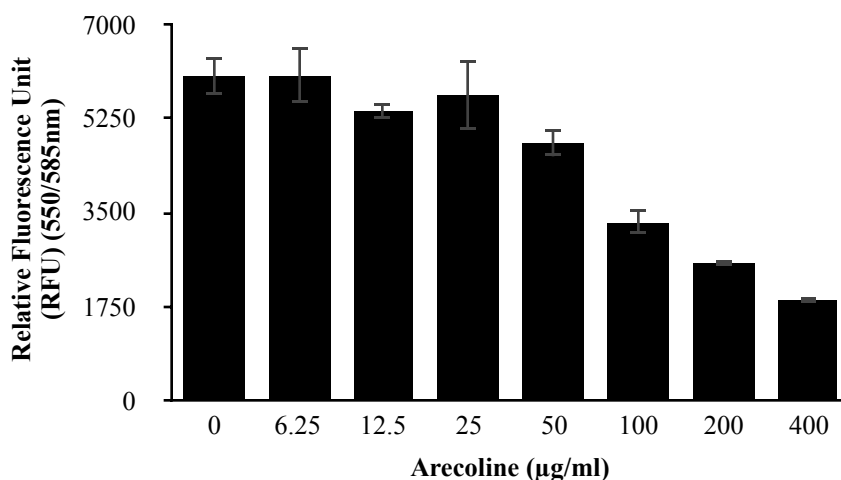
One-way ANOVA test; \* indicates significant difference at  $p \leq 0.05$ .

**Table 12 (b):** Pairwise analysis of serial dilutions of arecoline (0 to 400 µg/mL) using post-hoc Tukey’s test.

<b>Arecoline (A) Group Pair (µg/mL)</b>	<b>Difference</b>	<b>p value</b>
Ctrl VS A6.25	-38.16	1.000
Ctrl VS A12.5	640.56	0.203
Ctrl VS A25	338.13	0.860
Ctrl VS A50	1246.48	0.001*
Ctrl VS A100	2705.60	0.001*
Ctrl VS A200	3461.00	0.001*
Ctrl VS A400	4163.05	0.001*
A6.25 vs A12.5	678.72	0.153
A6.25 vs A25	376.29	0.784
A6.25 vs A50	1284.63	0.001*
A6.25 vs A100	2743.76	0.001*
A6.25 vs A200	3499.16	0.001*
A6.25 vs A400	4201.21	0.001*
A12.5 vs A25	-302.43	0.915
A12.5 vs A50	605.91	0.258
A12.5 vs A100	2065.039	0.001*
A12.5 vs A200	2820.44	0.001*
A12.5 vs A400	3522.49	0.001*
A25 vs A50	908.34	0.021
A25 vs A100	2367.47	0.001*
A25 vs A200	3122.87	0.001*
A25 vs A400	3824.92	0.001*

A50 vs A100	1459.13	0.001*
A50 vs A200	2214.53	0.001*
A50 vs A400	2916.58	0.001*
A100 vs A200	755.40	0.083
A100 vs A400	1457.45	0.001*
A200 vs A400	702.05	0.128

**Graph 1 :** Graph showing cell viability/relative fluorescence of human oral fibroblasts using serial dilutions of arecoline (0 to 400µg/mL) and Alamar Blue assay.



Error bars represent standard deviation. Post hoc tukey test; \* indicates significant difference at  $p \leq 0.05$

**Result:** As the concentration of arecoline increases from 6.25 µg/mL to 400 µg/mL gradual reduction in cell viability as indicated by relative fluorescence unit of treated cells was noted. The results of the assay showed significant difference between the groups. The optimal effect as seen from the graph was observed at arecoline 50 µg/mL.

**Table 13 (a):** Comparison of cell viability/relative fluorescence of human oral fibroblast cells treated with serial dilutions of TGFβ (0 to 20 ng/mL) using one-way ANOVA test.

<b>Groups TGFβ (T) groups ng/mL</b>	<b>Mean</b>	<b>Std. Deviation</b>	<b>F value</b>	<b>p value</b>
T ctrl	7884.64	149.085	13.878	0.001*
T 0.31	7915.93	169.805		
T 0.625	8051.26	172.142		
T 1.25	7794.57	218.935		
T 2.5	8592.03	254.114		
T 5	9187.22	337.933		
T 10	8315.28	215.498		
T 20	8565.35	190.673		

One-way ANOVA test; \* indicates significant difference at  $p \leq 0.05$

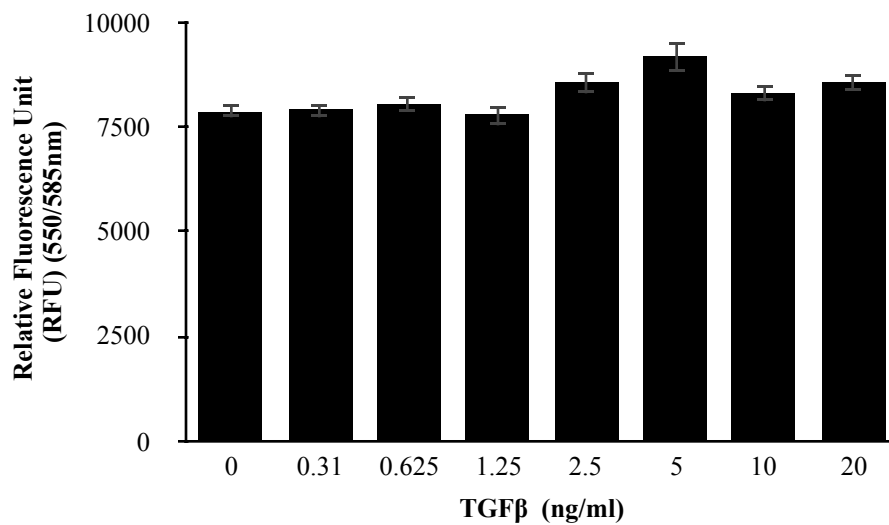
**Table 13 (b):** Pairwise analysis of serial dilutions of TGF $\beta$  (0 to 20 ng/mL) using post-hoc Tukey's test.

<b>TGF<math>\beta</math> group pair</b>	<b>Difference</b>	<b>p value</b>
T CTRL VS T 0.31	-31.29	1.000
T CTRL VS T 0.625	-166.62	0.979
T CTRL VS T 1.25	90.07	0.999
T CTRL VS T 2.5	-707.39	0.021*
T CTRL VS T 5	-1302.58	0.001*
T CTRL VS T 10	-430.65	0.309
T CTRL VS T 20	-680.71	0.028*
T 0.31 vs T 0.625	-135.33	0.994
T 0.31 vs T 1.25	121.36	0.997
T 0.31 vs T 2.5	-676.10	0.029
T 0.31 vs T 5	-1271.29	0.001*
T 0.31 vs T 10	-399.36	0.393
T 0.31 vs T 20	-649.42	0.038*
T 0.625 vs T 1.25	256.70	0.834
T 0.625 vs T 2.5	-540.77	0.116
T 0.625 vs T 5	-1135.96	0.001*
T 0.625 vs T 10	-264.02	0.815
T 0.625 vs T 20	-514.09	0.149
T 1.25 vs T 2.5	-797.47	0.008*
T 1.25 vs T 5	-1392.65	0.001*
T 1.25 vs T 10	-520.72	0.140
T 1.25 vs T 20	-770.78	0.010*
T 2.5 vs T 5	-595.18	0.067

T 2.5 vs T 10	276.75	0.779
T 2.5 vs T 20	26.68	1.000
T 5 vs T 10	871.93	0.004*
T 5 vs T 20	621.87	0.051
T 10 vs T 20	-250.07	0.851

Post hoc tukey test; \* indicates significant difference at  $p \leq 0.05$

**Graph 2 :** Graph showing cell viability/relative fluorescence of human oral fibroblasts using serial dilutions of TGF $\beta$  (0 to 20 ng/ml) and Alamar Blue assay.

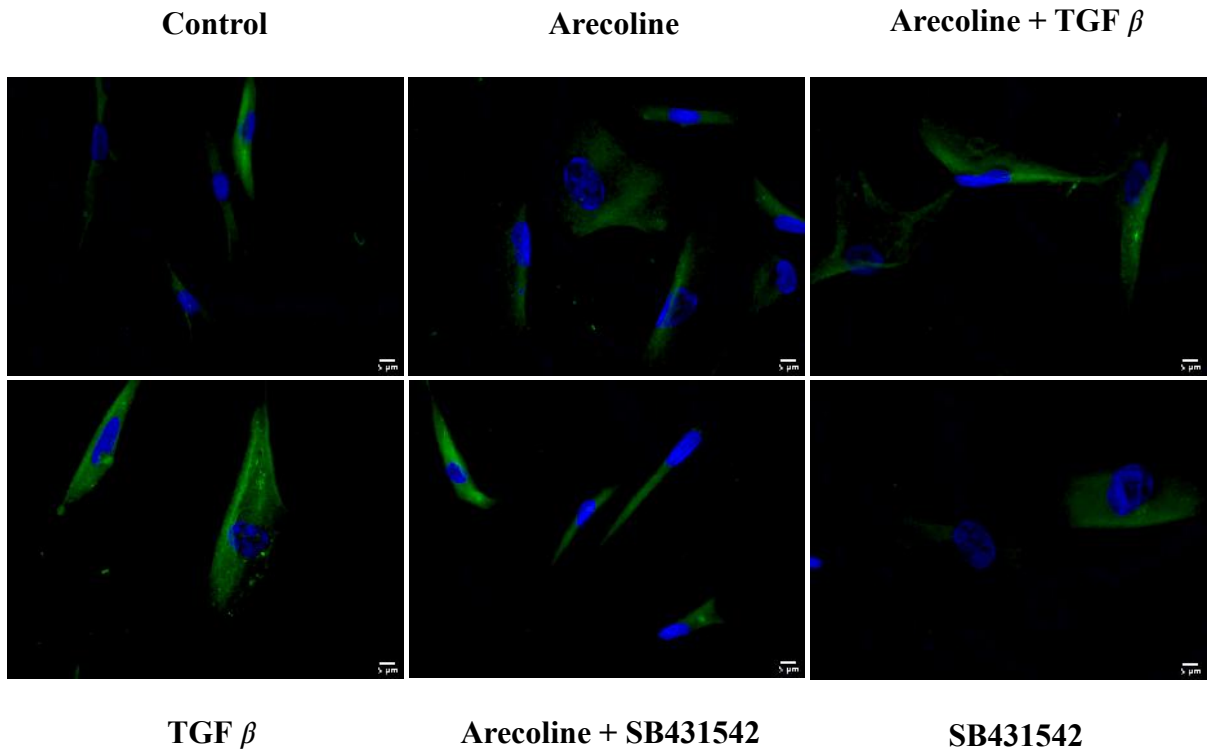


Error bars represent standard deviation. Post hoc tukey test; \* indicating significant difference at  $p \leq 0.05$ .

**Results:** The results of the assay showed significant difference between the TGF $\beta$  treatment groups. Upto 1.25 ng/mL results were similar to control group, increase was noted from 2.5 ng/mL to 5ng/mL while the optimal effect as seen from the graph and tables was observed at TGF $\beta$  10 ng/ml.

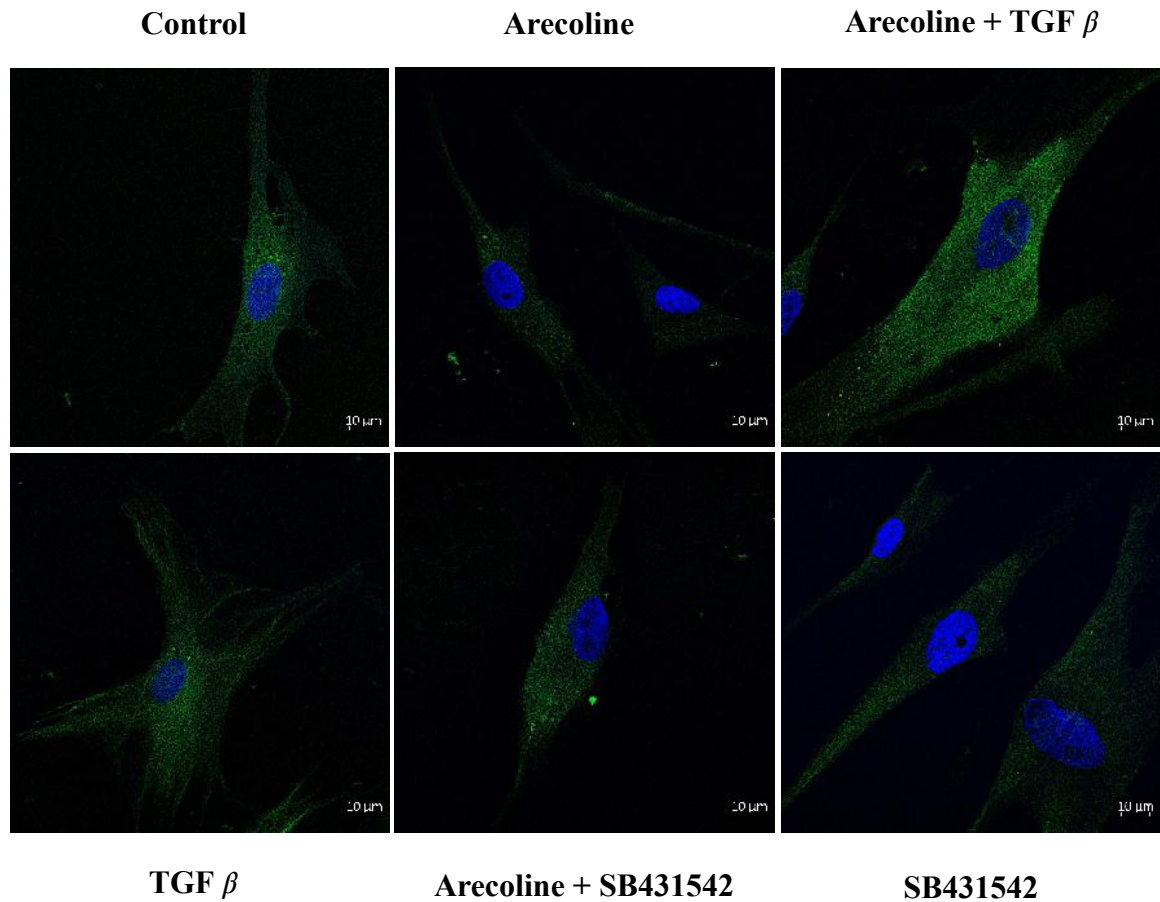
## II. Results for experiments of objective 1 - Myofibroblast cytoskeletal phenotype

**Figure 10 (a):** Vimentin immunofluorescence assay in study groups after 48 hours of treatment [40x]



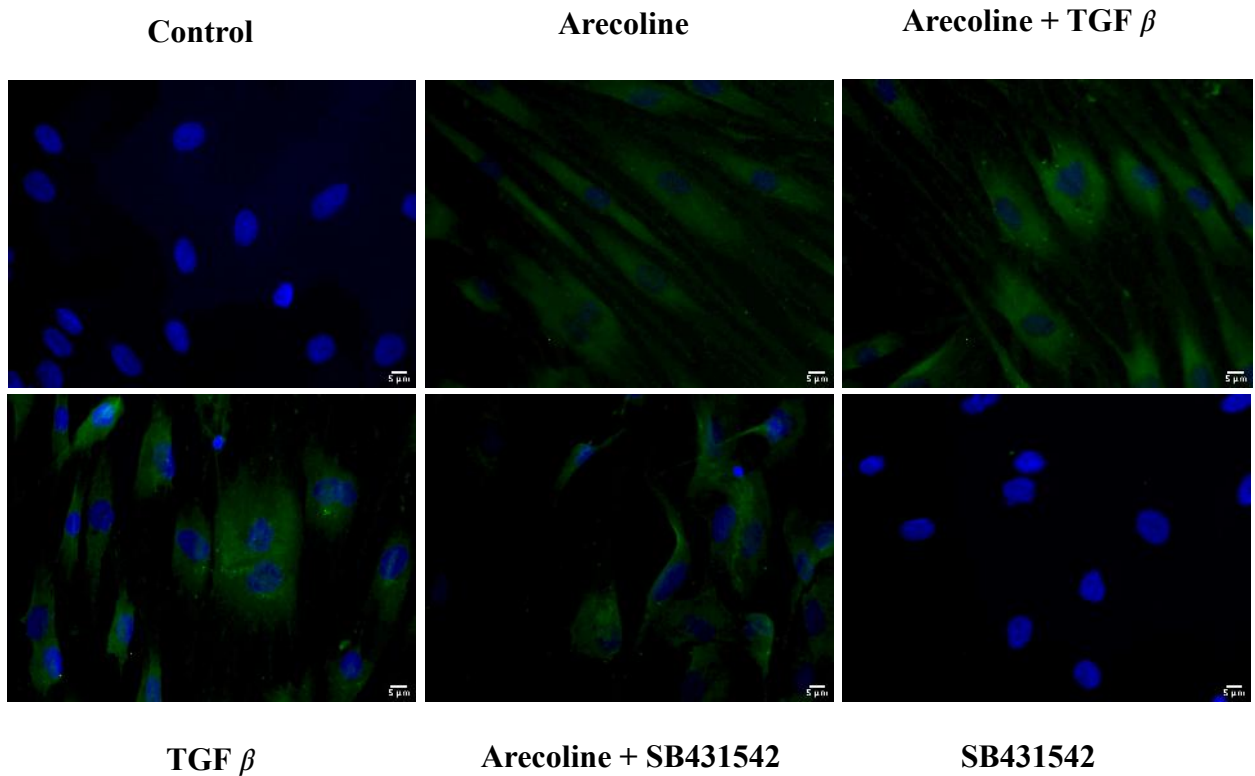
**Result:** Vimentin IFA shows positive expression of vimentin in all the study groups.

**Figure 10(b):** Vimentin immunofluorescence assay in study groups after 48 hours of treatment [Confocal microscopy images , 60x]



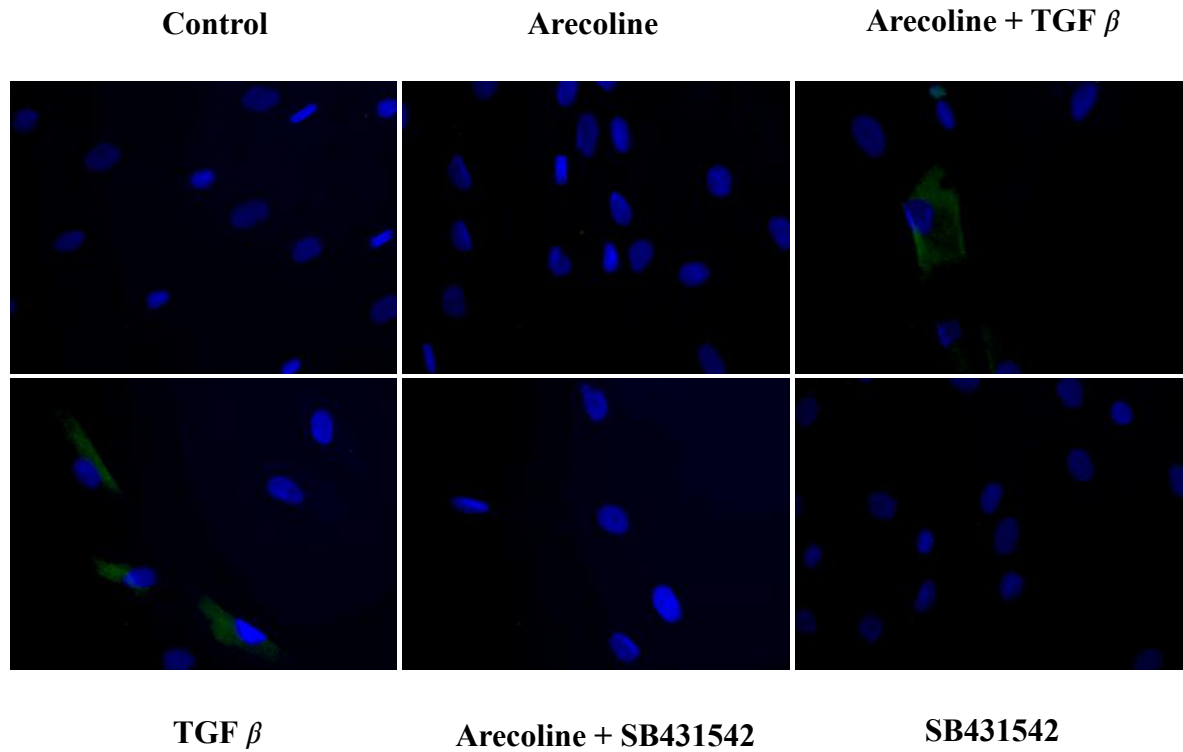
**Result:** Confocal images of vimentin IFA showing soluble form of vimentin in control, arecoline, arecoline with SB431542 and SB431542 groups while TGF $\beta$  shows predominantly fibrillar vimentin. Arecoline with TGF $\beta$  group shows peripheral filament like vimentin while perinuclear soluble vimentin.

**Figure 10(c):**  $\alpha$ SMA immunofluorescence assay in study groups after 48 hours of treatment [40x]



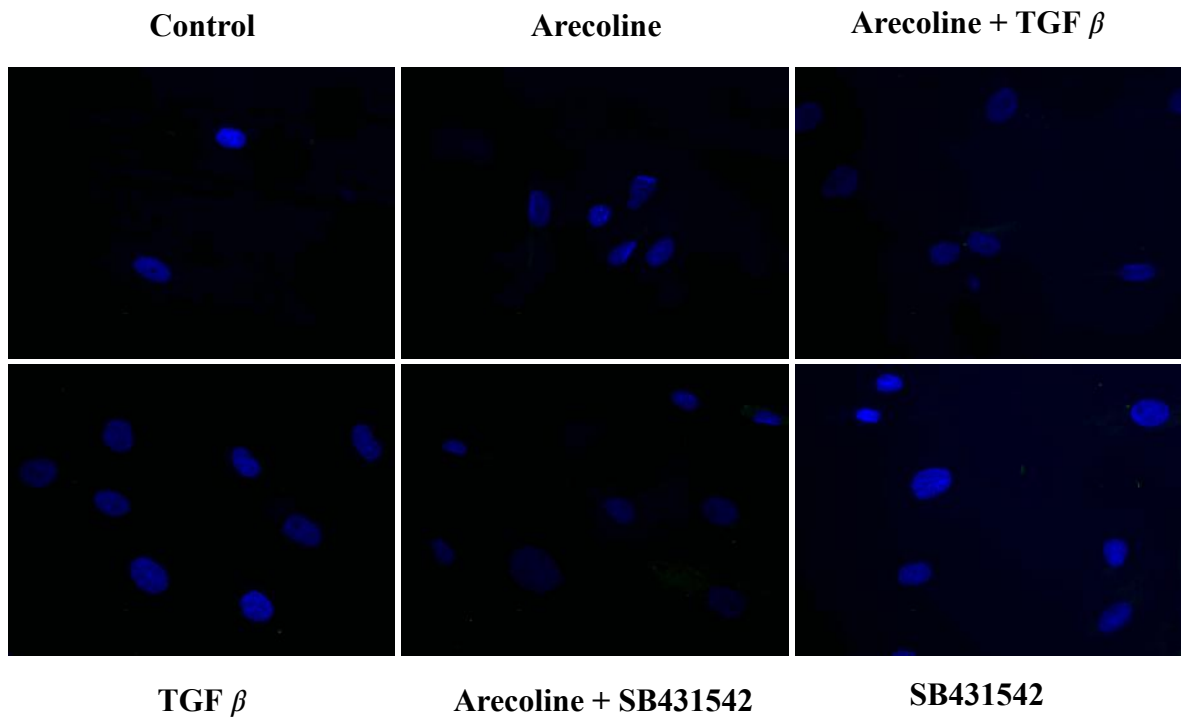
**Result:**  $\alpha$ SMA expression is noted in arecoline, arecoline with SB431542, TGF $\beta$  and the combination group of arecoline with TGF $\beta$ . This indicates myofibroblast differentiation after 48 hours of treatment. The control and SB431542 groups showed negative expression of  $\alpha$ SMA.

**Figure 10(d):** Desmin immunofluorescence assay in study groups after 48 hours of treatment [40x]



**Result:** Positive immunoexpression of desmin was noted in less than or equal to 25% cells in TGF $\beta$  and combination of arecoline with TGF $\beta$  groups. No expression was observed in control, SB431542, arecoline and arecoline with SB431542 groups.

**Figure 10(e):** Myosin immunofluorescence assay in study groups after 48 hours of treatment [40x]



**Result:** IFA for myosin showed negative expression in all the study groups.

**Table 14:** Immunofluorescence assay showing expression for vimentin,  $\alpha$ SMA, desmin and myosin in the different study groups.

<b>Group</b>	<b>Vimentin %</b>	<b><math>\alpha</math>SMA %</b>	<b>Desmin %</b>	<b>Myosin %</b>
1 - Untreated cells	>75% positive	Absent	Absent	Absent
2 - Arecoline treated cells	>75% positive	50 - 75% positive	Absent	Absent
3 - TGF $\beta$ treated cells	>75% positive	>75% positive	Less than or equal to 25% positive	Absent
4 - Arecoline and TGF $\beta$ treated cells	>75% positive	>75% positive	Less than or equal to 25% positive	Absent
5 - Arecoline and SB431542	>75% positive	50-75% positive	Absent	Absent
6 - SB431542	>75% positive	Absent	Absent	Absent

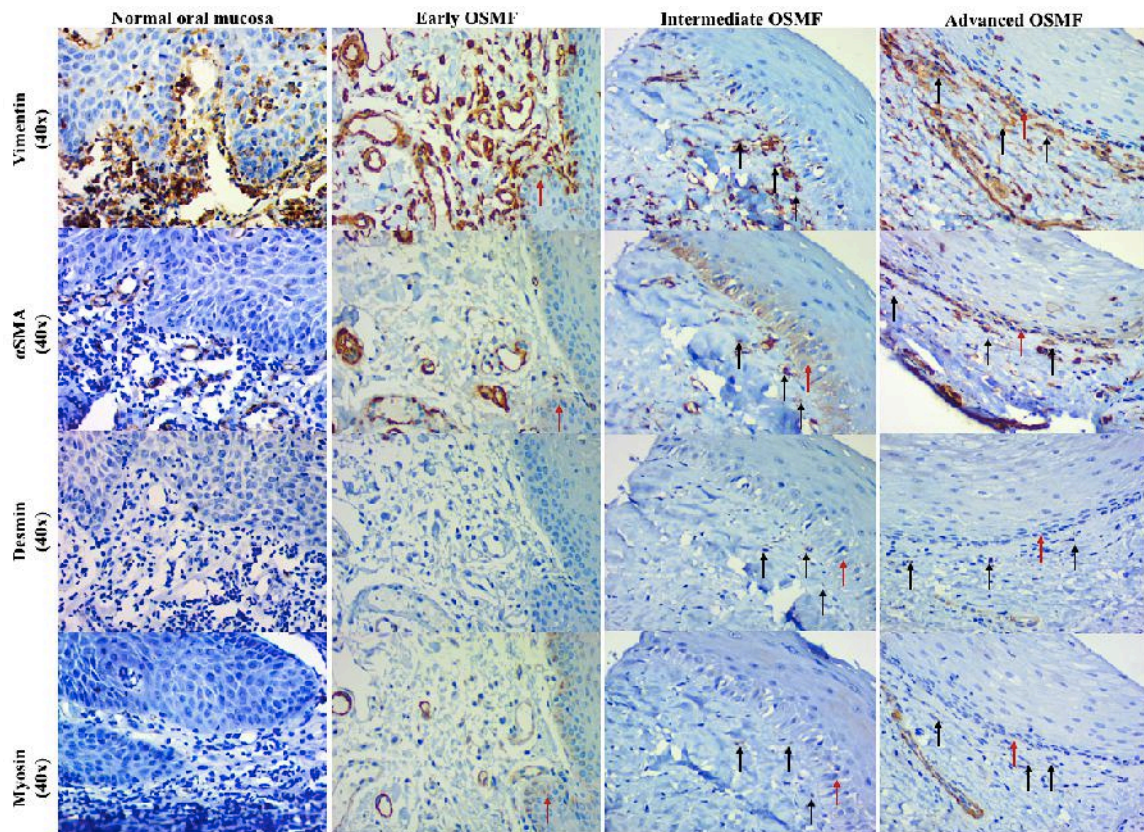
**Result:** Untreated and SB431542 cells exhibited a vimentin positive phenotype while other treatment group cells exhibited a vimentin with  $\alpha$ SMA to vimentin,  $\alpha$ SMA and desmin positive phenotype.

**Table 15:** Immunohistochemical expression of cytoskeletal proteins by fibroblasts in normal mucosa and different grades of OSMF.

Cytoskeletal protein	Normal mucosa (n = 5)	Early OSMF (n=20)	Intermediate (n=20)	Advanced OSMF (n=20)
Vimentin	5 cases positive	20 cases positive	20 cases positive	20 cases positive
$\alpha$ SMA	5 cases negative	20 cases positive	20 cases positive	20 cases positive
Desmin	5 cases negative	20 cases negative	2 cases positive 18 cases negative (less than 10% cells)	3 cases positive (10%-20%), 17 cases negative
Myosin	5 cases negative	20 cases negative	2 case positive (less than 10% cells) 18 cases negative	3 cases positive (less than 10% cells), 17 cases negative

**Result:** Normal mucosa exhibits a vimentin positivity (all 5 cases) in the stromal fibroblasts, while early OSMF exhibited vimentin and  $\alpha$ SMA positive fibroblasts (20 cases). The intermediate and advanced OSMF cases show a predominantly vimentin with  $\alpha$ SMA positive fibroblasts (20 cases) and only 4 cases of intermediate and 5 cases of advanced OSMF exhibited desmin and myosin positivity in less than 10% cells.

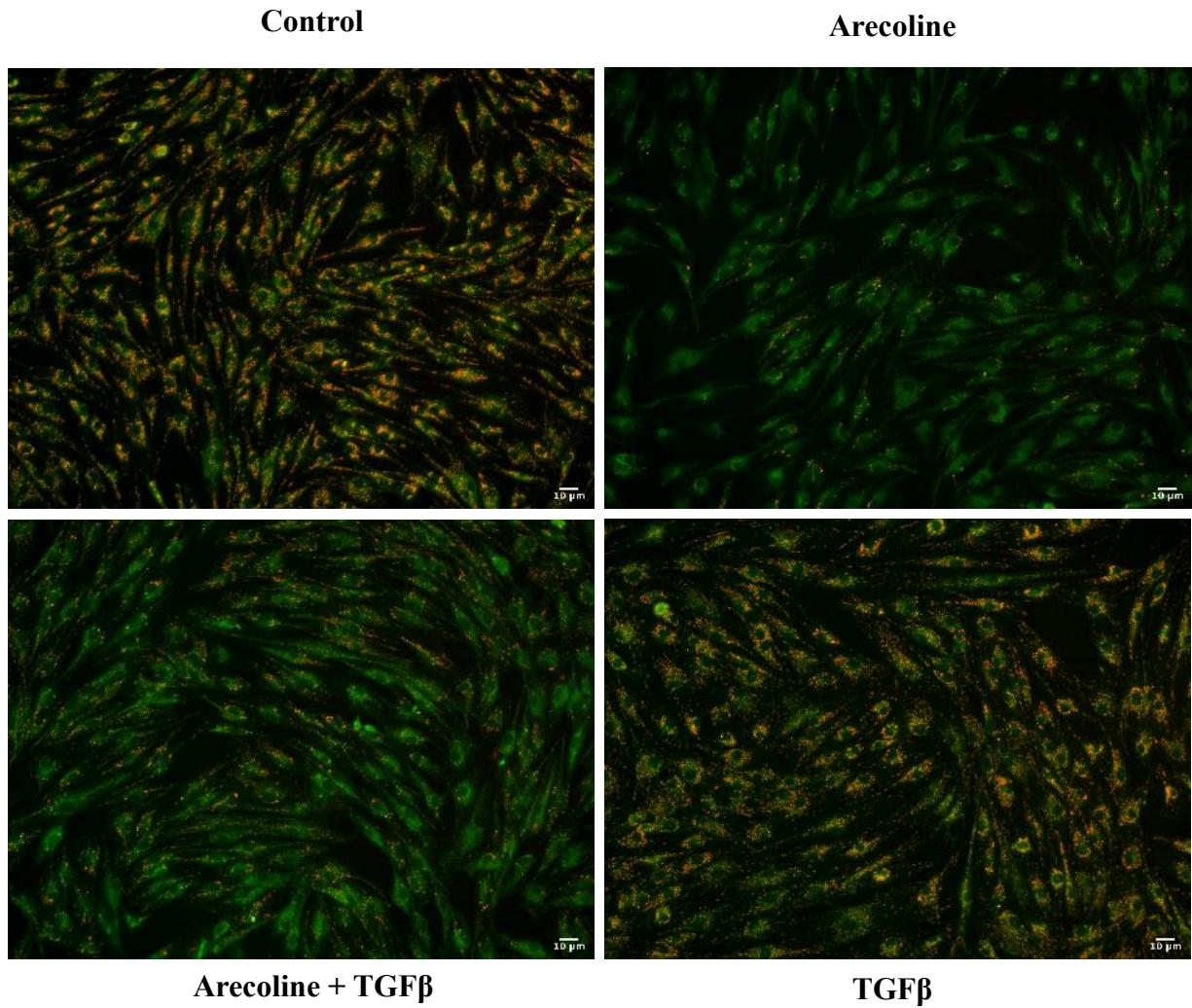
**Figure 11:** Photomicrographs of normal oral mucosa and grades of OSMF stained with antibodies against vimentin,  $\alpha$ SMA, desmin, and myosin (40x). Black arrows indicating positively stained myofibroblasts and red arrows indicating positivity in basal cells of epithelium.



**Result:** Vimentin positivity was noted in all the study groups.  $\alpha$ SMA expression noted in blood vessels (positive internal control), no expression noted in fibroblasts. Fibroblasts immunoexpression in stroma increased with increase in grade of OSMF. Desmin and myosin staining was absent in normal mucosa and early OSMF. The intermediate and advanced grades of OSMF showed focal (<25% cells) immunoexpression of desmin and myosin in  $\alpha$ SMA fibroblasts in few case as shown above. These cases also showed basal cells of epithelium stained focally positive (<25%) for  $\alpha$ SMA, desmin and myosin.

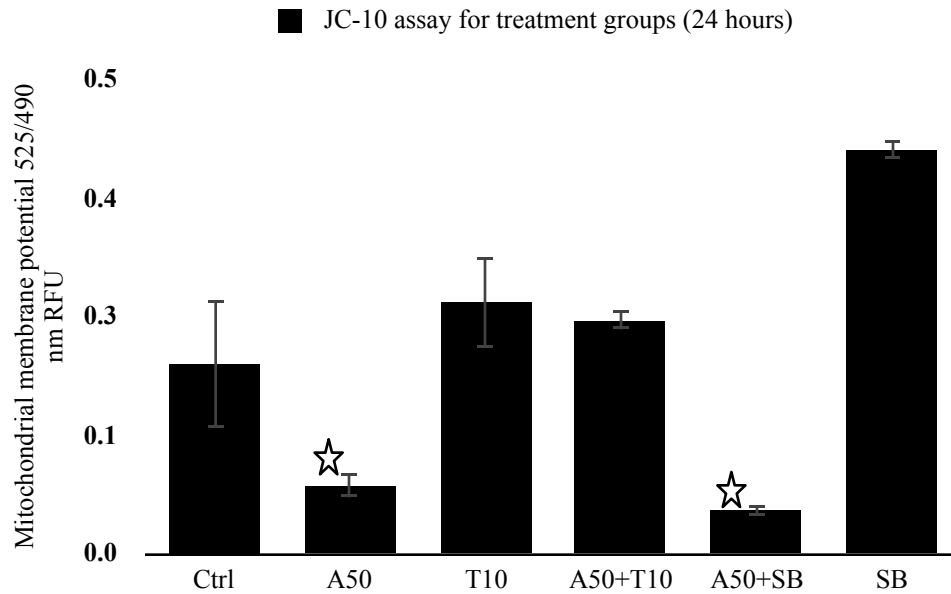
### III. Results for experiments of objective 2.

**Figure 12:** Fluorescent microscopy images showing JC-10 dye fluorescence in green and orange-red colour in the study groups at 24 hours (10x).



Predominantly green fluorescence - unhealthy mitochondria; Predominantly orange- red fluorescence - healthy mitochondria

**Graph 3:** JC-10 assay graph showing relative fluorescence of JC-10 in the study groups.



(Ctrl - control group, A50 - Arecoline 50  $\mu\text{g}/\text{mL}$ , T10 - TGF $\beta$  10  $\text{ng}/\text{mL}$ , SB - TGF $\beta$  ALK5 receptor inhibitor SB431542)

Error bars represent standard deviation. \*indicates significant difference at  $p \leq 0.05$ .

**Table 16 (a):** Intergroup comparison of JC 10 assay of study groups at 24hrs

<b>Groups</b>	<b>Mean</b>	<b>Standard De- viation (SD)</b>	<b>F value</b>	<b>p value</b>
Ctrl	0.200	0.067	50.369	0.001*
A50	0.071	0.007		
T10	0.264	0.047		
A50 + T10	0.246	0.009		
A 50 + SB	0.045	0.006		
SB	0.426	0.009		

One Way ANOVA test; \* indicates significant difference at  $p \leq 0.05$ .

(Ctrl - Control group, A50 - Arecoline 50  $\mu\text{g/mL}$ , T10 - TGF $\beta$  10  $\text{ng/mL}$ , SB - TGF $\beta$  ALK5 receptor inhibitor SB431542)

**Table 16 (b):** Pairwise comparison of JC10 assay of study groups at 24 hrs

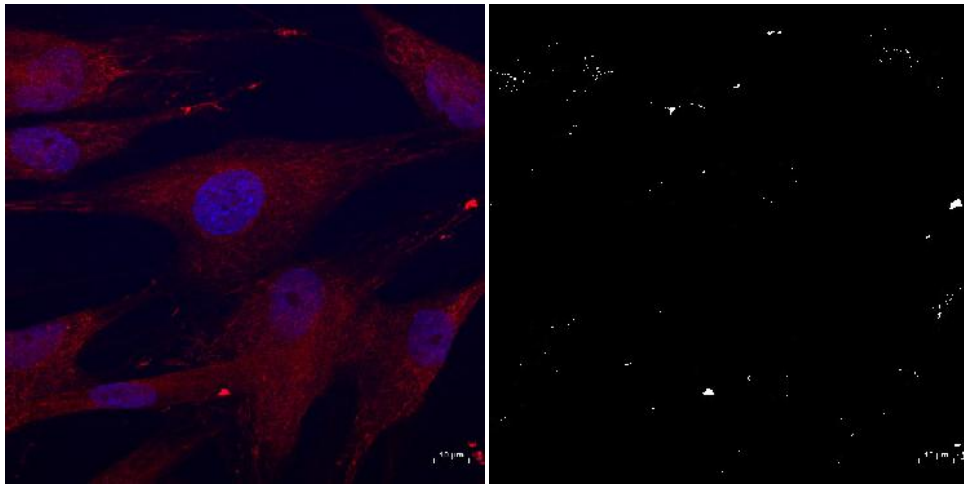
<b>Pair</b>	<b>Difference</b>	<b>p value</b>
Ctrl vs A50	0.129	0.006*
Ctrl vs T10	-0.064	0.270
Ctrl vs A50+T10	-0.045	0.597
Ctrl vs A50+SB	0.155	0.001*
Ctrl vs SB	-0.226	0.001*
A50 vs T10	-0.193	0.001*
A50 vs A50+T10	-0.175	0.001*
A50 vs A50+SB	0.026	0.930
A50 vs SB	-0.355	0.001*
T10 vs A50+T10	0.018	0.983
T10 vs A50+SB	0.219	0.001*
T10 vs SB	-0.162	0.001*
A50+T10 vs A+SB	0.201	0.001*
A50+T10 vs SB	-0.181	0.001*
A50+SB vs SB	-0.381	0.001*

Post hoc tukey test; \* indicates significant difference at  $p \leq 0.05$ . (Ctrl - control group, A50 - Arecoline 50  $\mu\text{g/mL}$ , T10 - TGF $\beta$  10  $\text{ng/mL}$ , SB - TGF $\beta$  ALK5 receptor inhibitor SB431542)

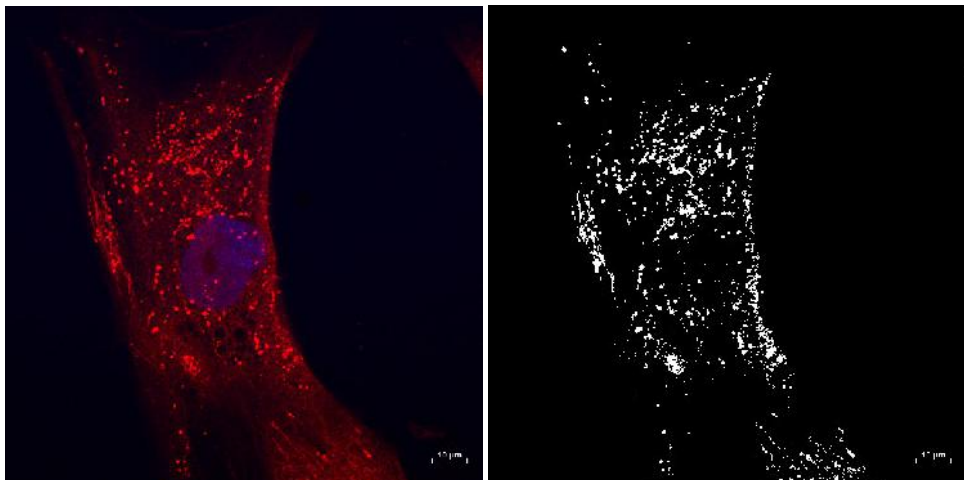
**Result:** Significant depolarization of mitochondria is noted in the arecoline and arecoline with SB431542 groups as shown in the graph. The arecoline group and arecoline with SB4431542 exhibits predominantly green fluorescence. TGF $\beta$  group exhibit least membrane depolarization followed by arecoline with TGF $\beta$  group as exhibit by orange-red fluorescence. While SB431542 and control groups has healthy mitochondria depicted by predominantly orange-red fluorescence.

**Figure 13 (a-f):** Confocal microscopy images of study groups [48 hours] showing mitochondrial morphology and localization using Mitotracker Red staining and conversion with NIH image J software.

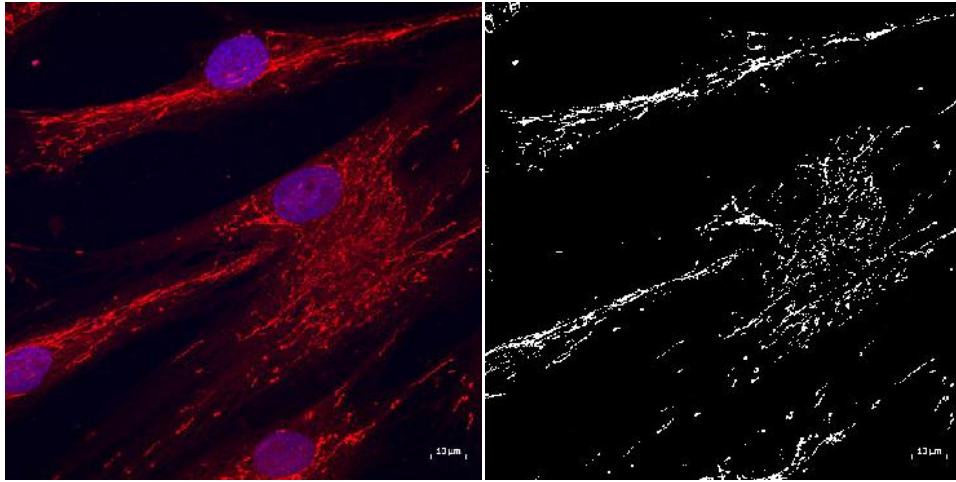
a. Mitotacker red and Image J converted confocal images of Untreated HuGF cells showing homogenous and delicate thread-like staining of mitochondria [60x].



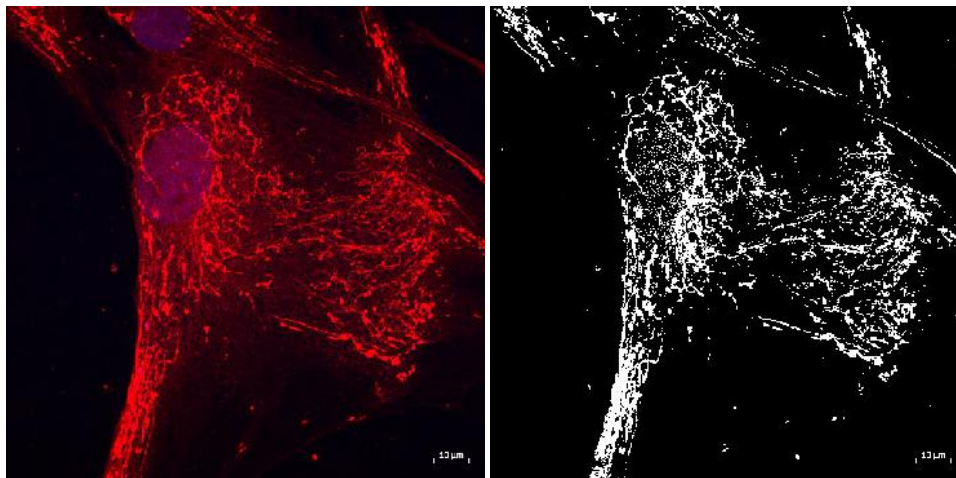
b. Mitotacker red and Image J converted confocal images of Arecoline treated cells showing round to punctate and dispersed staining of mitochondria [60x].



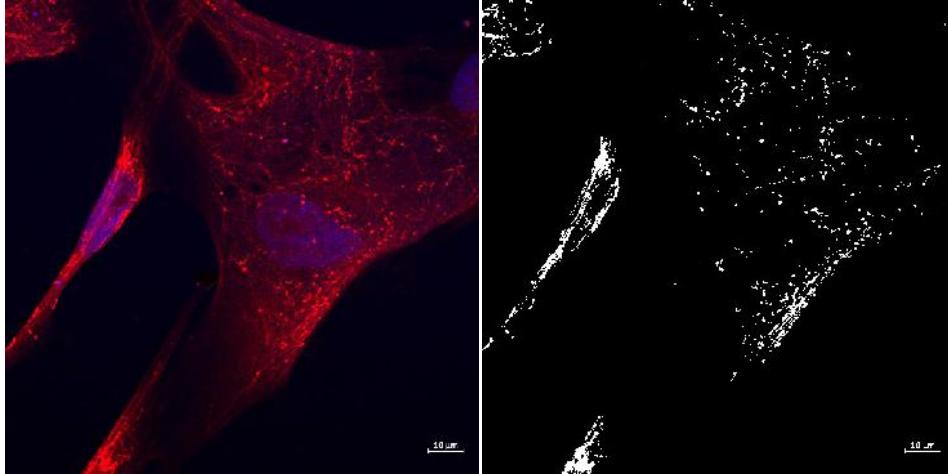
c. Mitotacker red and Image J converted confocal images of TGF $\beta$  treated cells showing dense, branched and elongated mitochondria [60x].



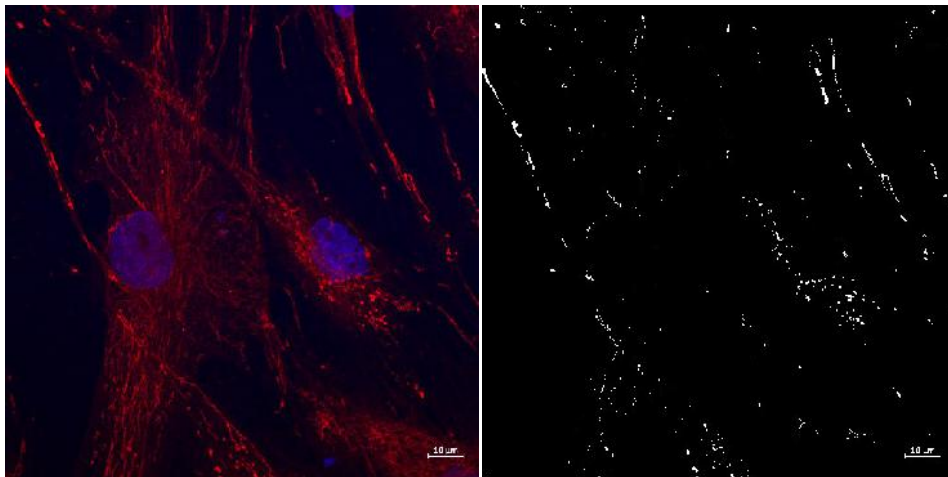
d. Mitotacker red and Image J converted confocal images of Arecoline with TGF $\beta$  treated cells showing predominantly dense, branched and elongated mitochondria with few punctate round mitochondria [60x].



e. Mitotacker red and Image J converted confocal images of Arecoline with SB431542 treated cells showing predominantly punctate round mitochondria [60x].



f. Mitotacker red and Image J converted confocal images of SB431542 treated cells showing predominantly fine fibrillar mitochondria [60x].



**Results:** Mitotracker Red staining demonstrates distinct mitochondrial morphology in all the study groups. Delicate thread like or fibrillar morphology - healthy mitochondria; punctate round morphology - fission; and elongated, branched swollen morphology - fusion of mitochondria.

**Table 17(a):** Intergroup comparison of normalized (Red/Blue) ratio of study groups by intensity analysis of Mitotracker Red dye using one-way ANOVA.

<b>Groups</b>	<b>Mean</b>	<b>SD</b>	<b>F value</b>	<b>p value</b>
Ctrl	0.05	0.03	18.772	0.001*
A50	0.54	0.11		
T10	0.79	0.20		
A50+T10	2.46	0.85		
A50+SB	0.34	0.06		
SB	0.10	0.03		

\* indicates significant difference at  $p \leq 0.05$ . (Ctrl - control group, A50 - Arecoline 50  $\mu\text{g}/\text{mL}$ , T10 - TGF $\beta$  10 ng/mL, SB - TGF $\beta$  ALK5 receptor inhibitor SB431542)

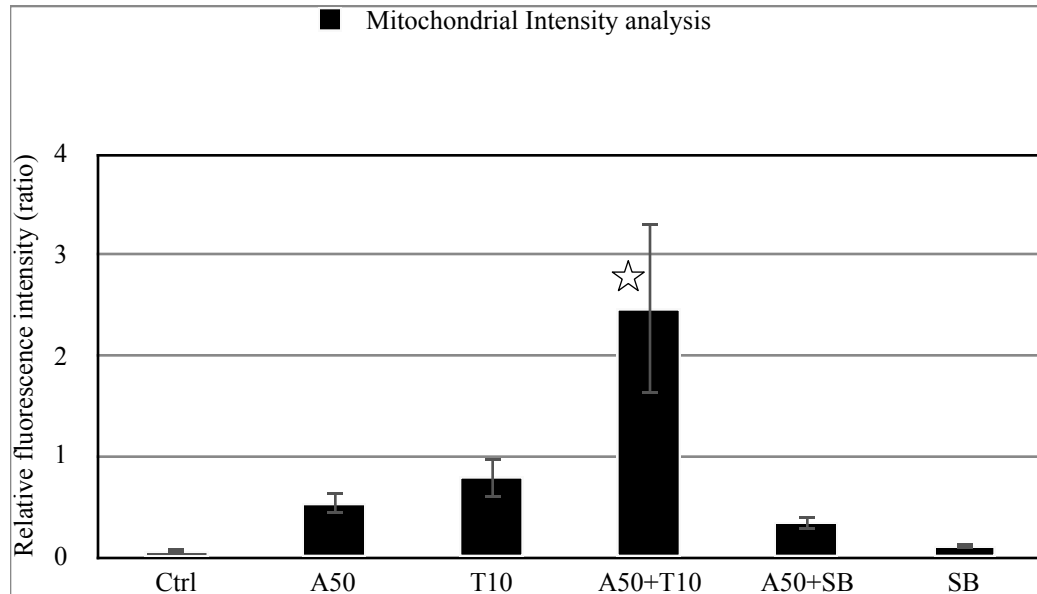
**Table 17(b):** Pairwise comparison of normalized ratio (Red/Blue) of study groups by intensity analysis of Mitotracker Red dye using post-hoc tukey test.

<b>Pair</b>	<b>Difference</b>	<b>p value</b>
Ctrl vs A50	-0.49	0.573
Ctrl vs T10	-0.73	0.199
Ctrl vs A50+T10	-2.41	0.001*
Ctrl vs A50+SB	-0.29	0.920
Ctrl vs SB	-0.05	1.000
A50 vs T10	-0.24	0.956
A50 vs A50+T10	-1.92	0.001*
A50 vs A50+SB	0.21	0.978
A50 vs SB	0.44	0.664
T10 vs A50+T10	-1.67	0.001*
T10 vs A50+SB	0.45	0.651
T10 vs SB	0.69	0.251
A50+T10 vs A50+SB	2.12	0.001*
A50+T10 vs SB	2.36	0.001*
A50+SB vs SB	0.24	0.961

\* indicates significant difference at  $p \leq 0.05$

(Ctrl - control group, A50 - Arecoline 50  $\mu\text{g/mL}$ , T10 - TGF $\beta$  10  $\text{ng/mL}$ , SB - TGF $\beta$  ALK5 receptor inhibitor SB431542)

**Graph 4:** Graph representing Mitotracker Red intensity analysis results of the study groups [48 hours].



Error bars represent standard deviation. One way ANOVA, post hoc-tukey test; \* indicates significant difference at  $p \leq 0.05$ .

(Ctrl - control group, A50 - Arecoline 50  $\mu\text{g}/\text{mL}$ , T10 - TGF $\beta$  10  $\text{ng}/\text{mL}$ , SB - TGF $\beta$  ALK5 receptor inhibitor SB431542)

**Results:** Significantly high intensity of fluorescence is noted in the arecoline with TGF $\beta$  group.

**Table 18 (a):** Intergroup comparison of HKII expression in the study groups at 24 hours using one-way ANOVA.

<b>Groups</b>	<b>Mean</b>	<b>SD</b>	<b>F value</b>	<b>p value</b>
Ctrl	0.00	0.04	23.350	0.001*
A50	2.280	0.08		
T10	0.555	0.20		
A50+T10	1.220	0.29		
A50+SB	1.965	0.16		
SB	1.785	0.50		

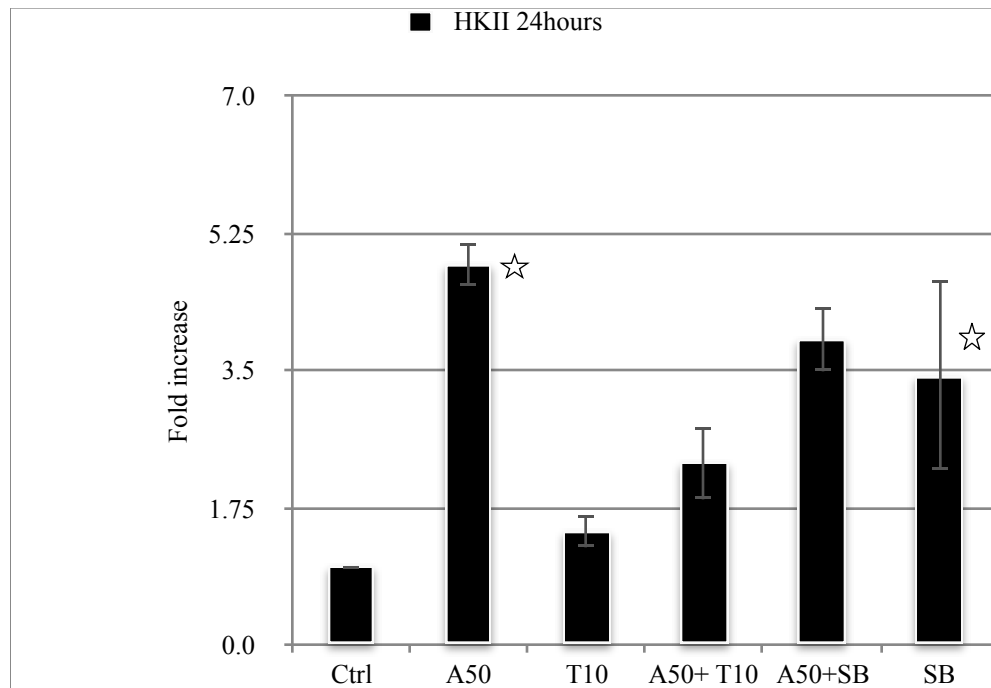
\* indicates significant difference at  $p \leq 0.05$ . (Ctrl - Control group, A50 - Arecoline 50  $\mu\text{g}/\text{mL}$ , T10 - TGF $\beta$  10 ng/mL, SB - TGF $\beta$  ALK5 receptor inhibitor SB431542)

**Table 18 (b):** Pairwise comparison of HKII expression in study groups at 24 hrs using post hoc-tukey test.

<b>Pair</b>	<b>Difference</b>	<b>p value</b>
Ctrl vs A50	-2.280	0.001*
Ctrl vs T10	-0.555	0.323
Ctrl vs A50+T10	-1.220	0.023*
Ctrl vs A50+SB	-1.965	0.002*
Ctrl vs SB	-1.785	0.003*
A50 vs T10	1.725	0.004*
A50 vs A50+T10	1.060	0.044*
A50 vs A50+SB	0.315	0.814
A50 vs SB	0.495	0.471
T10 vs A50+T10	-0.665	0.235
T10 vs A50+SB	-1.410	0.012*
T10 vs SB	-1.230	0.022*
A50+T10 vs A50+SB	-0.745	0.166
A50+T10 vs SB	-0.565	0.358
A50+SB vs SB	0.180	0.975

Post hoc tukey test; \* indicates significant difference at  $p \leq 0.05$ . (Ctrl - control group, A50 - Arecoline 50  $\mu\text{g/mL}$ , T10 - TGF $\beta$  10  $\text{ng/mL}$ , SB - TGF $\beta$  ALK5 receptor inhibitor SB431542)

**Graph 5:** Graph showing Hexokinase-II expression in study groups using qRT PCR at 24 hours.



One way ANOVA, post hoc-tukey test; \* indicates significant difference at  $p \leq 0.05$ . Error bars represent standard deviation. (Ctrl - control group, A50 - Arecoline 50  $\mu\text{g}/\text{mL}$ , T10 - TGF $\beta$  10 ng/mL, SB - TGF $\beta$  ALK5 receptor inhibitor SB431542)

**Result:** Cells treated with arecoline and arecoline with SB431542 exhibit significantly higher expression of HKII as compared to the controls. Increased HKII expression in cells is indicative of higher glycolytic switch in cells.

**Table 19(a):** Intergroup comparison of PINK1 expression in study groups at 48 hours using one way ANOVA test.

<b>Groups</b>	<b>Mean</b>	<b>SD</b>	<b>F value</b>	<b>p value</b>
Ctrl	0.000	0.099	99.224	0.001*
A50	-0.875	0.078		
T10	0.215	0.035		
A50+T10	0.530	0.085		
A50+SB	0.030	0.042		
SB	0.065	0.021		

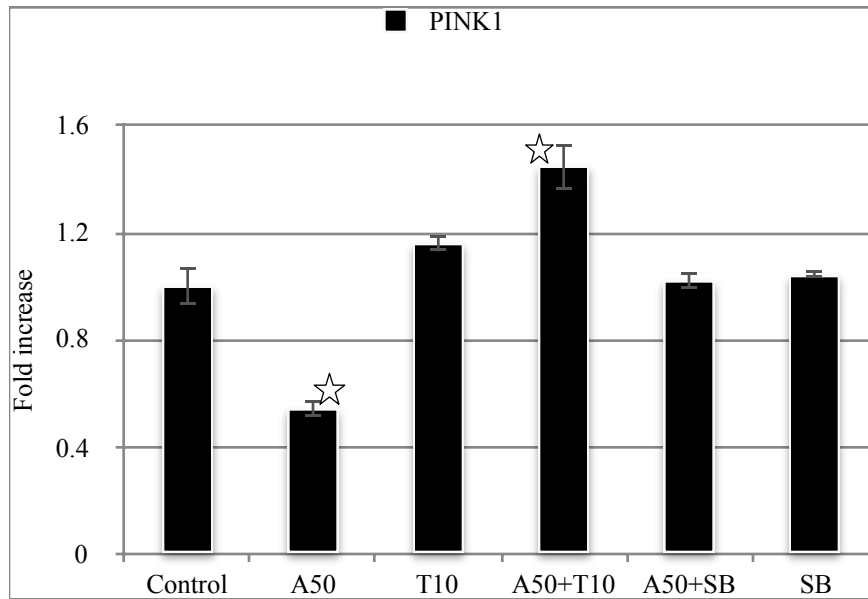
One Way ANOVA test; \* indicates significant difference at  $p \leq 0.05$ . (Ctrl - control group, A50 - Arecoline 50  $\mu\text{g/mL}$ , T10 - TGF $\beta$  10  $\text{ng/mL}$ , SB - TGF $\beta$  ALK5 receptor inhibitor SB431542)

**Table 19(b):** Pairwise comparison of PINK1 expression in study groups at 48 hrs using post hoc-tukey test.

<b>Pair</b>	<b>Difference</b>	<b>p value</b>
Ctrl vs A50	0.875	0.001*
Ctrl vs T10	-0.215	0.113
Ctrl vs A50+T10	-0.530	0.002*
Ctrl vs A50+SB	-0.030	0.996
Ctrl vs SB	-0.065	0.910
A50 vs T10	-1.090	0.001*
A50 vs A50+T10	-1.405	0.001*
A50 vs A50+SB	-0.905	0.001*
A50 vs SB	0.940	0.001*
T10 vs A50+T10	-0.315	0.023*
T10 vs A50+SB	0.185	0.187
T10 vs SB	0.150	0.334
A50+T10 vs A50+SB	0.500	0.002*
A50+T10 vs SB	0.465	0.003*
A50+SB vs SB	-0.035	0.993

Post hoc tukey test; \* indicates significant difference at  $p \leq 0.05$ . (Ctrl - control group, A50 - Arecoline 50  $\mu\text{g/mL}$ , T10 - TGF $\beta$  10  $\text{ng/mL}$ , SB - TGF $\beta$  ALK5 receptor inhibitor SB431542)

**Graph 6:** Graph showing PINK1 expression in study groups using qRT PCR at 48 hours.



One way ANOVA, post hoc-tukey test; \* indicates significant difference at  $p \leq 0.05$ . Error bars represent standard deviation. (Ctrl - control group, A50 - Arecoline 50  $\mu\text{g}/\text{mL}$ , T10 - TGF $\beta$  10 ng/mL, SB - TGF $\beta$  ALK5 receptor inhibitor SB431542)

**Result:** Significantly higher expression of PINK1 is found in the arecoline with TGF $\beta$  as compared to controls, while the arecoline group demonstrates down regulation as compared to the control group at 48 hours.

**Table 20(a):** Intergroup comparison of qRT PCR levels of TFAM in study groups at 48 hrs using one way ANOVA test.

<b>Groups</b>	<b>Mean</b>	<b>SD</b>	<b>F value</b>	<b>p value</b>
Ctrl	0.000	0.113	19.490	0.001*
A50	-0.320	0.028		
T10	0.345	0.219		
A50+T10	0.510	0.141		
A50+SB	-0.555	0.205		
SB	-0.535	0.049		

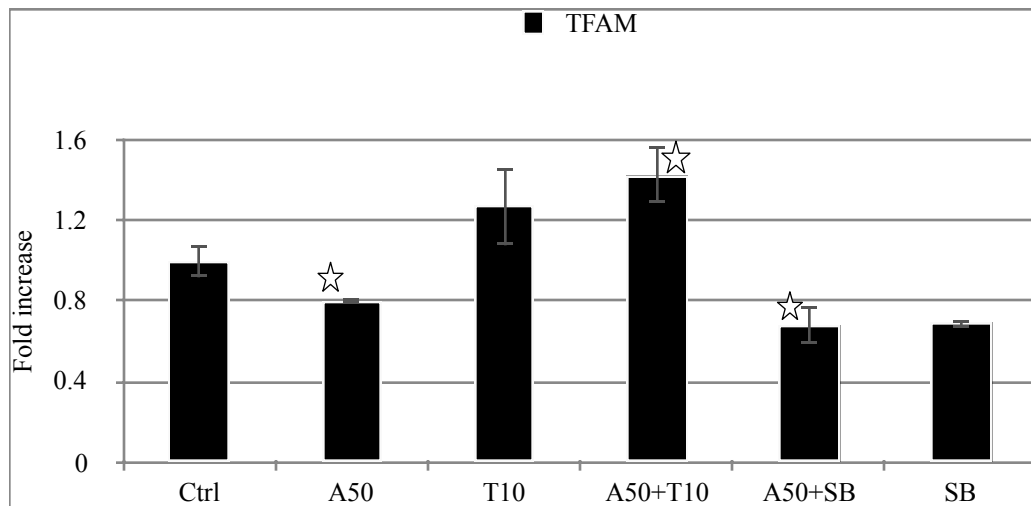
One Way ANOVA test; \* indicates significant difference at  $p \leq 0.05$ . (Ctrl - control group, A50 - Arecoline 50  $\mu\text{g/mL}$ , T10 - TGF $\beta$  10  $\text{ng/mL}$ , SB - TGF $\beta$  ALK5 receptor inhibitor SB431542)

**Table 20(b):** Pairwise comparison of qRT PCR levels of TFAM in study groups at 48 hours using post hoc-tukey test.

<b>Pair</b>	<b>Difference</b>	<b>p value</b>
Ctrl vs A50	0.320	0.351
Ctrl vs T10	-0.345	0.292
Ctrl vs A50+T10	-0.510	0.082
Ctrl vs A50+SB	0.555	0.059
Ctrl vs SB	0.535	0.068
A50 vs T10	-0.665	0.027*
A50 vs A50+T10	-0.830	0.009*
A50 vs A50+SB	0.235	0.615
A50 vs SB	0.215	0.686
T10 vs A50+T10	-0.165	0.850
T10 vs A50+SB	0.900	0.006*
T10 vs SB	0.880	0.007*
A50+T10 vs A50+SB	1.065	0.003*
A50+T10 vs SB	1.045	0.003*
A50+SB vs SB	-0.020	1.000

Post hoc tukey test; \* indicates significant difference at  $p \leq 0.05$ . (Ctrl - control group, A50 - Arecoline 50  $\mu\text{g/mL}$ , T10 - TGF $\beta$  10  $\text{ng/mL}$ , SB - TGF $\beta$  ALK5 receptor inhibitor SB431542)

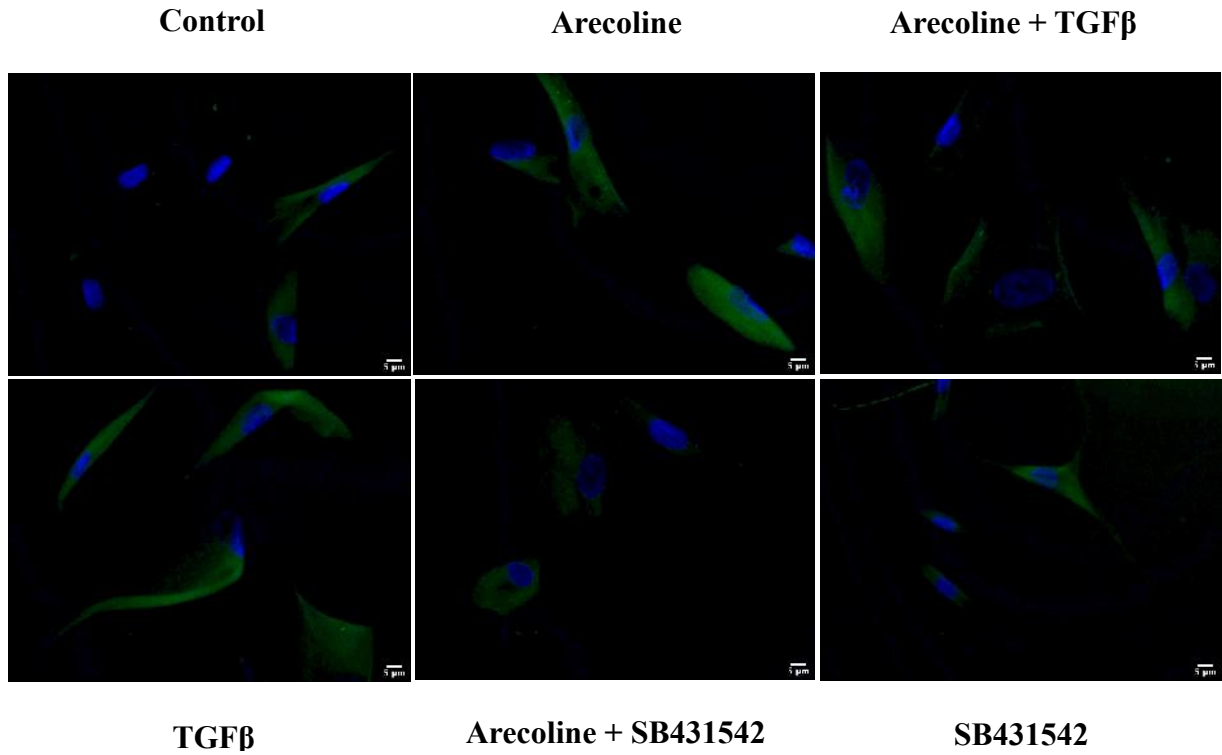
**Graph 7:** Graph showing TFAM expression in study groups using qRT PCR at 48 hours.



One way ANOVA, post hoc-tukey test; \* indicates significant difference at  $p \leq 0.05$ . Error bars represent standard deviation. (Ctrl - control group, A50 - Arecoline 50  $\mu\text{g}/\text{mL}$ , T10 - TGF $\beta$  10  $\text{ng}/\text{mL}$ , SB - TGF $\beta$  ALK5 receptor inhibitor SB431542)

**Results:** qRT PCR results demonstrate that TFAM levels of groups is similar to the control however, significant difference is observed between the arecoline and TGF $\beta$  and arecoline with TGF $\beta$  as compared to arecoline and arecoline with SB431542 groups.

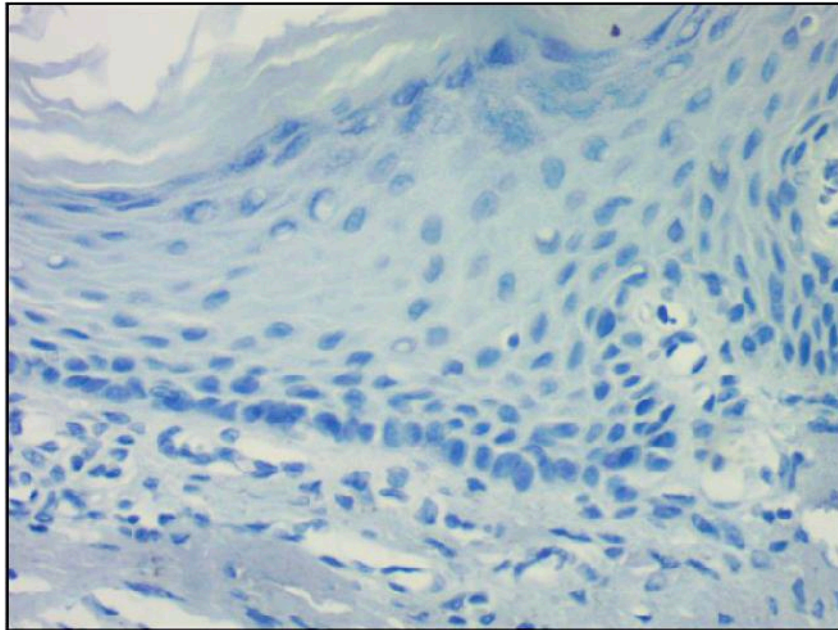
**Figure 14:** TFAM immunofluorescence assay in study groups after 48 hours of treatment [40x]



**Results:** IFA results indicate strong positive fluorescence in the TGFβ and arecoline with TGFβ group in >75% cells as compared to the other study groups (20 to 30% cells).

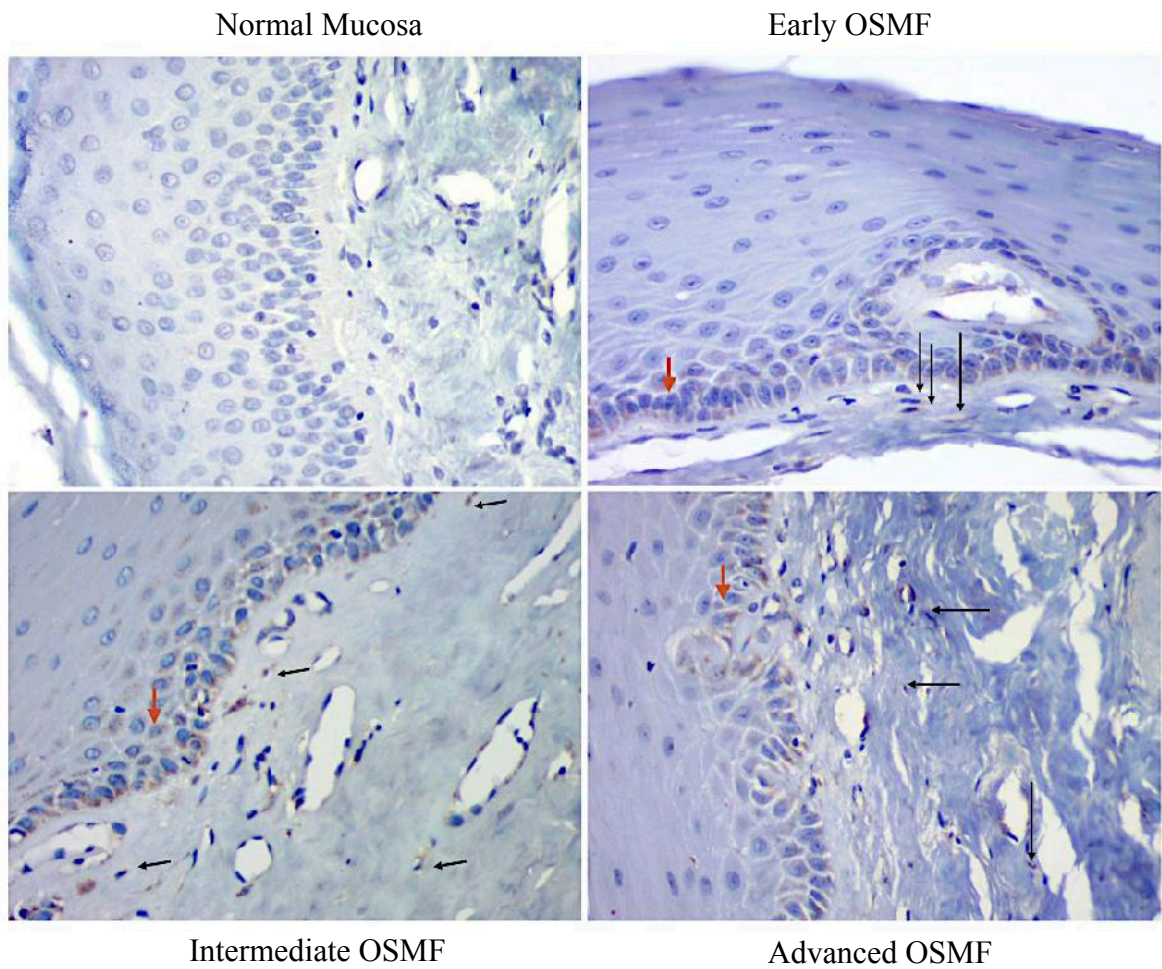
**Figure 15 (a,b):** Photomicrographs of normal oral mucosa and grades of OSMF stained with antibodies against TFAM [40x].

**a)** Absence of TFAM stained in normal oral mucosa [40x]



**Result:** Absence of TFAM staining in normal mucosa in both epithelium and connective tissue.

b) Immunoexpression of TFAM in OSMF tissues [40x]. Black arrows indicating positive expression in fibroblasts in grades of OSMF. Red arrows indicating staining in epithelium.



**Result:** Early OSMF show cytoplasmic TFAM immunoexpression in myofibroblasts located subepithelially. Moderate and advanced OSMF show myofibroblasts positive for TFAM even in the deeper stroma.

**Table 21 (a):** Distribution of positively stained fibroblasts localization in each study groups n (%)

Groups	Absent	Focal	Juxtaepithelial	Throughout
Normal mucosa	5 (100)	0	0	0
Early OSMF	9 (45)	9 (45)	2 (10)	0
Intermediate OSMF	0	1 (5)	13 (65)	6 (30)
Advanced OSMF	0	1 (5)	4 (20)	15 (75)

**Result:** No staining for TFAM was noted in normal mucosa and 45% early OSMF tissues. The remaining 55% cases of early OSMF showed TFAM positive fibroblasts focally or in the juxtaepithelial location. Intermediate OSMF showed 65% cases with juxtaepithelial localization, while 75% cases showed distribution throughout the stroma.

**Table 21(b):** Distribution of positively stained fibroblasts % in study groups [n (%)]

Groups	0-10	10-25	25-50	>50
Normal mucosa	5 (100)	0	0	0
Early OSMF	19 (95)	1 (5)	0	0
Intermediate OSMF	0	10 (50)	10 (50)	0
Advanced OSMF	0	1 (5)	10 (50)	9 (45)

**Result:** No or minimum staining for TFAM was noted in normal mucosa and 95% early OSMF tissues. The remaining 1 case of early OSMF showed less than 25% TFAM posi-

tive fibroblasts. Intermediate OSMF showed 50% cases with both less than 25% and 25 to 50% TFAM positive fibroblasts, while 95% cases of advanced OSMF showed more than 25% TFAM positive fibroblasts.

**Table 21(c):** Distribution of positively stained fibroblasts intensity in study groups [n (%)]

<b>Groups</b>	<b>Negative</b>	<b>Mild to moderately brown</b>	<b>Dark brown</b>
Normal mucosa	5 (100)	0	0
Early OSMF	9 (45)	11 (55)	0
Intermediate OSMF	0	2 (10)	18 (90)
Advanced OSMF	0	3 (15)	17 (85)

**Results:** All cases of normal mucosa and 45% of early OSMF showed a lack of TFAM staining in the stroma. Intensity of staining was mild to moderately brown in 55% cases of early OSMF. Dark brown intensity of staining was observed in 90% cases of intermediate and 85% cases of advanced OSMF.

**Table 21 (d):** Overall comparison of positively stained fibroblasts frequency among study groups [n (%)]

<b>Groups</b>	<b>Absent</b>	<b>Low</b>	<b>High</b>	<b><math>\chi^2</math> value</b>	<b>p value</b>
Normal mucosa	5 (100)	0	0	74.598	0.001*
Early OSMF	19 (95)	1 (5)	0		
Intermediate OSMF	0	12 (60)	8 (40)		
Advanced OSMF	0	3 (15)	17 (85)		

Chi-square test; \* indicates significant difference at  $p \leq 0.05$

**Result:** The frequency of TFAM positive fibroblasts increases with increase in the grade of OSMF. Highest TFAM expression was noted in the advanced OSMF cases (85%).

**Table 21 (e):** Pairwise intergroup comparison of TFAM positive fibroblasts frequency among study groups

<b>Pair</b>	<b><math>\chi^2</math> value</b>	<b>p value</b>
Normal mucosa vs Early OSMF	0.260	1.000 (NS)
Normal mucosa vs Intermediate OSMF	25.000	0.001*
Normal mucosa vs Advanced OSMF	25.000	0.001*
Early OSMF vs Intermediate OSMF	36.308	0.001*
Early OSMF vs Advanced OSMF	37.000	0.001*
Intermediate OSMF vs Advanced OSMF	8.640	0.008*

Chi-square test; \* indicates significant difference at  $p \leq 0.05$ ; NS: Non-significant

**Result:** The normal mucosa and early cases of OSMF showed similar results in terms of TFAM expression. Significant difference was noted amongst the other study groups.

#### IV. Cell survival assay for study groups using Alamar Blue at 12, 48 and 72 hours

**Table 22 (a)** Intergroup comparison of study groups for cell survival using Alamar Blue at 12 hours

<b>Groups</b>	<b>Mean</b>	<b>SD</b>	<b>F value</b>	<b>p value</b>
<b>Ctrl</b>	6734.726	306.4135	21.250	0.001*
<b>A50</b>	5991.76	128.0852		
<b>T10</b>	6893.803	160.734		
<b>A50+T10</b>	5832.723	234.610		
<b>A505+SB</b>	3960.906	863.590		
<b>SB</b>	5449.611	117.578		

One Way ANOVA test; \* indicates significant difference at  $p \leq 0.05$

(Ctrl - control group, A50 - Arecoline 50  $\mu\text{g}/\text{mL}$ , T10 - TGF $\beta$  10  $\text{ng}/\text{mL}$ , SB - TGF $\beta$  ALK5 receptor inhibitor SB431542)

**Table 22 (b)** Pairwise comparison of study groups for cell survival using Alamar Blue at 12 hours

<b>Pair</b>	<b>Difference</b>	<b>p value</b>
<b>Ctrl vs A50</b>	742.966	0.271
<b>Ctrl vs T10</b>	-159.076	0.996
<b>Ctrl vs A50+T10</b>	902.004	0.130
<b>Ctrl vs A50+SB</b>	2773.820	0.001*
<b>Ctrl vs SB</b>	1285.115	0.018*
<b>A50 vs T10</b>	-902.042	0.130
<b>A50 vs A50+T10</b>	159.038	0.996
<b>A50 vs A50+SB</b>	2030.854	0.001*
<b>A50 vs SB</b>	542.149	0.574
<b>T10 vs A50+T10</b>	1061.080	0.059
<b>T10 vs A50+SB</b>	2932.869	0.001*
<b>T10 vs SB</b>	1444.192	0.008*
<b>A50+T10 vs A50+SB</b>	1871.816	0.001*
<b>A50+T10 vs SB</b>	383.112	0.839
<b>A50+SB vs SB</b>	-1488.705	0.006*

Post hoc tukey test; \* indicates significant difference at  $p \leq 0.05$ . (Ctrl - control group, A50 - Arecoline 50  $\mu\text{g/mL}$ , T10 - TGF $\beta$  10  $\text{ng/mL}$ , SB - TGF $\beta$  ALK5 receptor inhibitor SB431542)

**Table 22 (c)** Intergroup comparison of study groups for cell survival using Alamar Blue at 48hours

<b>Groups</b>	<b>Mean</b>	<b>SD</b>	<b>F value</b>	<b>p value</b>
<b>Ctrl</b>	7911.885	688.639	14.909	0.001*
<b>A50</b>	7388.788	698.723		
<b>T10</b>	9099.850	815.362		
<b>A50+T10</b>	7889.326	128.509		
<b>A505+SB</b>	5639.970	226.426		
<b>SB</b>	6469.655	284.225		

One Way ANOVA test; \* indicates significant difference at  $p \leq 0.05$ . (Ctrl - control group, A50 - Arecoline 50  $\mu\text{g/mL}$ , T10 - TGF $\beta$  10  $\text{ng/mL}$ , SB - TGF $\beta$  ALK5 receptor inhibitor SB431542)

**Table 22 (d)** Pairwise comparison of study groups for cell survival using Alamar Blue at 48 hours

<b>Pair</b>	<b>Difference</b>	<b>p value</b>
<b>Ctrl vs A50</b>	503.098	0.839
<b>Ctrl vs T10</b>	-1187.965	0.152
<b>Ctrl vs A50+T10</b>	22.559	1.000
<b>Ctrl vs A50+SB</b>	2271.915	0.003*
<b>Ctrl vs SB</b>	1442.231	0.060
<b>A50 vs T10</b>	-1711.062	0.022*
<b>A50 vs A50+T10</b>	-500.538	0.861
<b>A50 vs A50+SB</b>	1748.818	0.019*
<b>A50 vs SB</b>	919.133	0.362
<b>T10 vs A50+T10</b>	1210.524	0.141
<b>T10 vs A50+SB</b>	3459.880	0.001*
<b>T10 vs SB</b>	2630.195	0.001*
<b>A50+T10 vs A50+SB</b>	2249.356	0.003*
<b>A50+T10 vs SB</b>	1419.671	0.066
<b>A50+SB vs SB</b>	-829.685	0.463

Post hoc tukey test; \* indicates significant difference at  $p \leq 0.05$ . (Ctrl - control group, A50 - Arecoline 50  $\mu\text{g/mL}$ , T10 - TGF $\beta$  10  $\text{ng/mL}$ , SB - TGF $\beta$  ALK5 receptor inhibitor SB431542)

**Table 22 (e)** Intergroup comparison of study groups for cell survival using Alamar Blue at 72 hours

<b>Groups</b>	<b>Mean</b>	<b>SD</b>	<b>F value</b>	<b>p value</b>
<b>Ctrl</b>	7892.489	52.742	92.788	0.001*
<b>A50</b>	8535.705	576.2208		
<b>T10</b>	9821.396	574.7894		
<b>A50+T10</b>	12811.94	456.0655		
<b>A505+SB</b>	7201.325	51.93396		
<b>SB</b>	6835.254	268.4913		

One Way ANOVA test; \* indicates significant difference at  $p \leq 0.05$ . (Ctrl - control group, A50 - Arecoline 50  $\mu\text{g/mL}$ , T10 - TGF $\beta$  10  $\text{ng/mL}$ , SB - TGF $\beta$  ALK5 receptor inhibitor SB431542)

**Table 22 (f)** Pairwise comparison of study groups for cell survival using Alamar Blue at 72 hours

<b>Pair</b>	<b>Difference</b>	<b>p value</b>
<b>Ctrl vs A50</b>	-643.216	0.404
<b>Ctrl vs T10</b>	-1928.907	0.001*
<b>Ctrl vs A50+T10</b>	-4919.450	0.001*
<b>Ctrl vs A50+SB</b>	691.164	0.335
<b>Ctrl vs SB</b>	1057.235	0.059
<b>A50 vs T10</b>	-1285.691	0.018*
<b>A50 vs A50+T10</b>	-4276.235	0.001*
<b>A50 vs A50+SB</b>	1334.380	0.014*
<b>A50 vs SB</b>	1700.451	0.002*
<b>T10 vs A50+T10</b>	-2990.544	0.001*
<b>T10 vs A50+SB</b>	2620.071	0.001*
<b>T10 vs SB</b>	2986.142	0.001*
<b>A50+T10 vs A50+SB</b>	5610.615	0.001*
<b>A50+T10 vs SB</b>	5976.686	0.001*
<b>A50+SB vs SB</b>	366.071	0.861

Post hoc tukey test; \* indicates significant difference at  $p \leq 0.05$ . (Ctrl - control group, A50 - Arecoline 50  $\mu\text{g/mL}$ , T10 - TGF $\beta$  10  $\text{ng/mL}$ , SB - TGF $\beta$  ALK5 receptor inhibitor SB431542)

**Table 22 (g):** Cell survival in study groups using Alamar Blue assay at 12 hours, 48 hours and 72 hours.

<b>Groups</b>		<b>12 hours</b>	<b>48 hours</b>	<b>72 hours</b>	<b>p value</b>
<b>Ctrl</b>	<b>Mean</b>	6734.726	7911.885	7892.489	0.077
	<b>SD</b>	306.413	688.639	52.742	
<b>A50</b>	<b>Mean</b>	5991.760	7388.788	8535.705	0.006*
	<b>SD</b>	128.085	698.723	576.221	
<b>T10</b>	<b>Mean</b>	6893.803	9099.850	9821.396	0.003*
	<b>SD</b>	160.734	815.362	574.789	
<b>A50+T10</b>	<b>Mean</b>	5832.723	7889.326	12811.940	0.001*
	<b>SD</b>	234.610	128.509	456.065	
<b>A50+SB</b>	<b>Mean</b>	3960.906	5639.970	7201.325	0.003*
	<b>SD</b>	863.590	226.426	51.933	
<b>SB</b>	<b>Mean</b>	5449.611	6469.655	6825.254	0.008*
	<b>SD</b>	117.578	284.225	268.491	

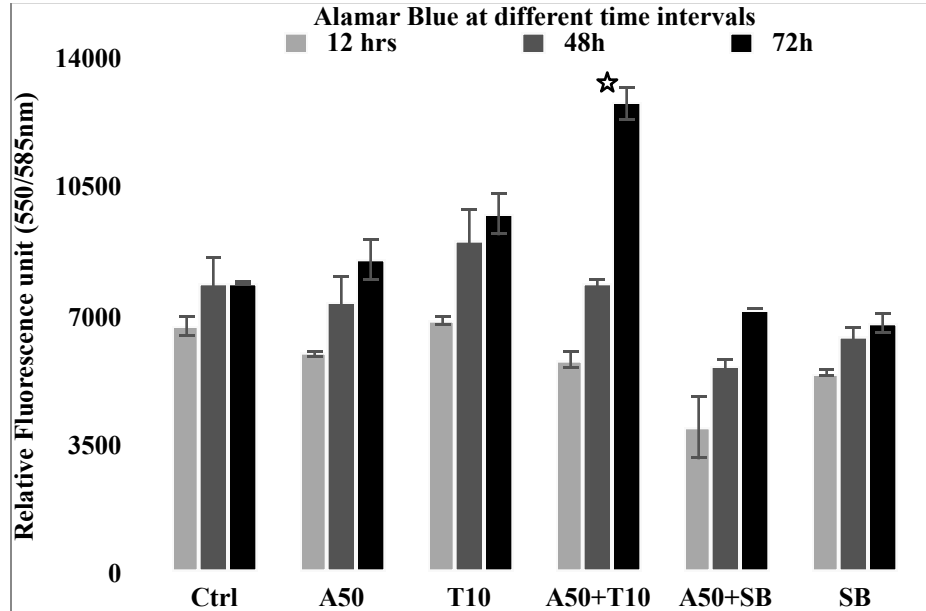
Repeated Measure ANOVA test; \* indicates significant difference at  $p \leq 0.05$ . (Ctrl - control group, A50 - Arecoline 50  $\mu\text{g/mL}$ , T10 - TGF $\beta$  10  $\text{ng/mL}$ , SB - TGF $\beta$  ALK5 receptor inhibitor SB431542)

**Table 22 (h):** Pairwise comparison of cell survival at 12 hours, 48 hours and 72 hours for cell survival using Alamar Blue assay (data represented using p-values).

<b>Groups</b>	<b>12 hours vs 48 hours</b>	<b>12 hours vs 72 hours</b>	<b>48hours vs 72 hours</b>
<b>Ctrl</b>	0.174	0.023*	0.966
<b>A50</b>	0.099	0.023*	0.028*
<b>T10</b>	0.044*	0.015*	0.045*
<b>A50 + T10</b>	0.008*	0.003*	0.003*
<b>A50 +SB</b>	0.054	0.025*	0.008*
<b>SB</b>	0.012*	0.025*	0.343

Post hoc LSD test; \* indicates significant difference at  $p \leq 0.05$ . (Ctrl - control group, A50 - Arecoline 50  $\mu\text{g/mL}$ , T10 - TGF $\beta$  10  $\text{ng/mL}$ , SB - TGF $\beta$  ALK5 receptor inhibitor SB431542)

**Graph 8:** Graph showing cell survival/relative fluorescence of human oral fibroblasts treatment groups at 12, 48 and 72 hours using Alamar Blue assay.



Error bars represent standard deviation. \* indicates significant difference

(Ctrl - control group, A50 - Arecoline 50  $\mu\text{g/mL}$ , T10 - TGF $\beta$  10  $\text{ng/mL}$ , SB - TGF $\beta$  ALK5 receptor inhibitor SB431542)

**Results:** On treating the cell groups and evaluating cell survival at 12hours, 48hours and 72 hours, significant cell survival is noted in the TGF $\beta$  and arecoline with TGF $\beta$  groups as compared to the control. Maximum survival is seen in the arecoline with TGF $\beta$  group. Significant cell death is noted in the arecoline with SB431542 group, while other groups showed survival similar to control group at 12, 48 and 72 hours.

## 5. DISCUSSION

OSMF presents with wide range of clinical and histological features that progresses irreversibly and affect the overall life of the affected patients. OSMF cases have been observed in patients from below 10 years to 80 years and above.<sup>35-38</sup> OSMF can cause significant debilitation and mortality considering its potential and estimated malignant change of 1.5-15% worldwide and 7.6% in India. <sup>35, 51, 107-113</sup>

The pathogenesis of OSMF is dynamic and involves spiraling of fibrotic changes due to interplay of various regulators. Extensive work in relation to OSMF has implicated primarily arecoline and TGF $\beta$  in the pathogenesis of OSMF. Histological findings accounted include cellular changes (inflammation and fibroblast differentiation), stromal changes (collagen and vascular changes) along with epithelial changes (atrophy and loss of rete pegs); these help in identifying the biological progression of OSMF. <sup>35, 51</sup> These tissue level changes primarily exhibit themselves as clinical signs and symptoms including mucosal alterations, fibrous bands, restriction of function and stiffening of involved tissues, etc.

At molecular level, arecoline can control the cell proliferation, release of ROS, DNA vitiation and cell death. <sup>6, 31, 55, 114, 115</sup> Arecoline also mediates lysyl oxidase based collagen cross-linking. <sup>6,55, 116</sup> Recently, synergistic actions of arecoline with tobacco use and alcohol have also been explored and found to be responsible in bringing about quick malignant change in the tissues. <sup>4, 6, 84, 117, 118, 119</sup>

To understand these dynamic cellular and molecular changes in OSMF, we aimed to evaluate the role of arecoline and TGF $\beta$  on mitochondrial bioenergetics during myofibroblast transdifferentiation.

Primary HGFs were used for our work and an environment essential for arecoline and TGF $\beta$  induced myofibroblast differentiation was created by treating the cells at 12 hours, 48 hours and 72 hours. Both arecoline and TGF $\beta$  have pleiotropic effects, hence evaluation of the optimal or sub-cytotoxic arecoline and TGF $\beta$  concentrations was needed. We first performed Alamar Blue assay for sub-toxic concentration determination. Arecoline at the concentration of 50 $\mu$ g/mL gave optimal results and TGF $\beta$  at 10ng/mL [Table 12(a, b), Table 13(a, b), Graph 1 & 2 ]. Literature suggests similar concentration of arecoline 50 $\mu$ g/mL to 100 $\mu$ g/mL were used for studying myofibroblast differentiation.<sup>26, 86, 115</sup> Chang YC et al <sup>115</sup> found that arecoline concentrations >50 $\mu$ g/mL could lead to cytotoxicity. Costea DE et al <sup>26</sup> and Pant I et al <sup>86</sup> also used concentrations of TGF $\beta$  ranging from 5ng/mL to 10ng/mL for studying fibroblast differentiation. As per literature, we used TGF $\beta$  ALK5 receptor inhibitor SB431542 at 10  $\mu$ M an hour prior to arecoline treatment. <sup>85, 86, 120, 121</sup>

It is known that in cultured fibroblasts can differentiate to functional biotypes of myofibroblasts that exhibit  $\alpha$ SMA between 48 to 72 hours after treatment with appropriate agent.<sup>24, 25, 85, 86</sup> It has been hypothesized that these cells show immune or cellular adaptations against apoptosis and thereby promote fibrosis.<sup>13, 17, 64</sup> The cell alters its cytoskeletal proteins form for modulating this adaptation and in response to matrix stress. Also, research has shown that with progression from granulation tissue like conditions to

scarring, the fibroblasts adapt from primarily expressing vimentin alone or vimentin with  $\alpha$ SMA to eventually showing additional transitions with positive expression of desmin and/or muscle myosin.<sup>21, 22, 65</sup> For confirmation of myofibroblast differentiation and to study their cytoskeletal heterogeneity, the study group cells were treated for 48 hours and evaluated for expression of  $\alpha$ SMA, vimentin, desmin and muscle myosin.

As fibroblasts belong to mesenchymal lineage, all the study groups demonstrated vimentin positivity in the cytoskeleton. The key role of vimentin was identified in healing and fibrosis and it was put forth that deficiency of vimentin leads to healing defects while excessive vimentin could bring about scarring. Vimentin is also known to foster development of an invasive property in apoptosis resistant fibroblasts.<sup>122</sup> Vimentin subsists in two main forms - soluble particles and filamentous/insoluble forms (short and long filaments) based on functional requirement. Soluble form enables migration while insoluble form aids in matrix invasion. Vimentin phosphorylation causes intermediate filament disassembly.<sup>123</sup> Vimentin also regulates the adhesion assembly necessary for adhesion and stabilization to matrix collagen. The expression of vimentin in epithelial cells is indicative of possible EMT. We found distinct arrangement of vimentin in the TGF $\beta$  and combination of arecoline with TGF $\beta$  group as compared to the control [Figure 10 (a, b)]. In groups with TGF $\beta$ , the vimentin showed a more filamentous arrangement while the other groups demonstrated predominantly homogenous-looking aggregates distributed diffusely in the cytoplasm [Figure 10 (b)]. This was similar to the findings of Surolia R et al<sup>122</sup> who explained that definite fibrillar arrangement of vimentin indicated its role invasion in IPF. Filamentous form of vimentin is commonly observed in cells that

are resistant to apoptosis. Thus indicating its possible role in cell survival. Chang YC et al<sup>124</sup> found that arecoline treatment could bring about vimentin expression in the fibroblasts cultured from the buccal mucosa. They indicated that this could probably be subsets of fibroblasts which react to internal genetic or other external modulation. Zheng L et al,<sup>125</sup> studied effect of miRNAs on arecoline induced EMT and put forth the role of vimentin in modulation of the same. Our results thus indicate that all cell groups showed a V phenotype (>75% cells ) [Table 14]. The HGF cells exhibited different patterns of vimentin arrangement in arecoline and TGF $\beta$  groups as compared to control, highlighting the possible role of vimentin in migration and survival, thereby in the pathogenesis of OSMF. This however, needs further assessment by silencing RNA treatments and knockout experiments.

$\alpha$ SMA is the prime indicator of myofibroblast transdifferentiation (contractile isoform), and is associated with the process of mechanosensing in healing/fibrosis. Mechanosensing is the process exhibited by myofibroblasts in response to matrix tension. In fibrosis and aging, the ROS mediated changes are said to eventually sulfonate actin which causes decreased new filament formation, thereby affecting the contractile function.<sup>126</sup> It has been observed in various models of fibrosis that  $\alpha$ SMA is upregulated on treatment with TGF $\beta$  and other profibrotic agents. In our work, we found that except the HGF cell control and SB431542 groups, all the remaining groups showed positive expression of  $\alpha$ SMA indicating myofibroblast differentiation and VA phenotype after 48 hours of treatment [Table 14, Figure 10(c)]. The arecoline treated cells showed 50-75% cells exhibiting  $\alpha$ SMA, while the remaining TGF $\beta$  and arecoline with TGF $\beta$  groups had

75%-100% cells exhibiting  $\alpha$ SMA expression [Table 14]. This indicated strong differentiation of fibroblasts in response to TGF $\beta$ . This was in accordance with the studies by Bernard K,<sup>24</sup> Negmadjanov et al <sup>25</sup> and Chang YC et al <sup>115</sup> who found up regulation of  $\alpha$ SMA in relation to myofibroblast differentiation.

Desmin, being a type III IF also plays a role in fibrosis and has been targeted for reversal strategies against desmin positive myofibroblasts in curtail or reverse fibrosis. In liver fibrosis and corneal injury-fibrosis, desmin along with vimentin is highly upregulated. <sup>127</sup> Chaurasia et al have shown a typical VA+ to VAD+ transition of myofibroblasts in corneal healing. <sup>128</sup> Similar to vimentin, the role of desmin has also been studied in EMT type II. To understand the expression of desmin in HGF cells, we used IFA for the treated cells and found that the TGF $\beta$  and their combination group showed expression of desmin in less than or equal to 25% cells [table 14, figure 10(d)]. No expression was observed in the untreated, arecoline, arecoline with SB431542, and SB431542 groups. Thus exhibiting a smaller subpopulation of treated cells showing VAD+ phenotype in response to TGF $\beta$  and arecoline with TGF $\beta$ . Another rare phenotype of myofibroblasts documented demonstrates the expression of muscle myosin heavy chains. However, expression of muscle myosin was negative in all the study groups [figure 10(e)]. Overall, except the control and SB431542 groups that demonstrate V+ phenotype, the other treated cells showed VA + to VAD+ phenotype.

To validate the findings of IFA and determine the phenotype of myofibroblasts in OSMF tissues, we immunostained the serial sections of tissues including grades of OSMF and normal mucosa against the cytoskeletal antibodies. We found that all cases of

normal oral mucosa and OSMF showed vimentin positive fibroblasts [Table 15, Figure 11]. This was in accordance with Sawant et al <sup>129</sup> who found vimentin expression in potentially malignant oral pathologies associated with areca nut chewing habit. They also observed that vimentin stained intense and was localized sub-epithelially as well as in the deeper tissues in advanced grades of OSMF. Nayak et al <sup>130</sup> have also demonstrated intense staining of vimentin in OSMF group than in normal mucosa.

Further, we observed that except normal mucosa, all cases of OSMF showed  $\alpha$ SMA positive myofibroblasts [Table 15, Figure 11]. In congruence with observations of Moutasim et al <sup>32</sup> and Angadi et al <sup>103</sup> we could demonstrate increased frequency of  $\alpha$ SMA myofibroblasts in progressive grades of OSMF as compared to the normal controls. These  $\alpha$ SMA positive cells were used as location guide for observing desmin and myosin immunoeexpression.

In relation to desmin and myosin, we found that the immunoeexpression was negative in normal mucosa group, except limited to muscle and blood vessels. In intermediate and advanced OSMF tissues, we found 2 out of 20 cases and 3 out of 20 cases that exhibited desmin positivity in less than 10% cells and 10% - 20% cells respectively [Table 15]. These myofibroblasts were typically located juxtaepithelially and also showed scanty/focal positive expression of desmin and myosin in the basal cells of epithelium. The same cases also demonstrated positive vimentin and  $\alpha$ SMA expression in epithelial basal cells [Figure 11]. Those cases showing VAD+ and VADM+ myofibroblast phenotype and epithelium with positive staining for the same may be indicating an intermediate stage of possible epithelial-mesenchymal transition and may need additional research with larger

sample for generalization of the results. This was also put forth by Chaw SY et al <sup>131</sup> who showed vimentin positivity in oral malignancies and EMT.

However, the IHC results support the IFA findings and in congruence with findings observed in pathological conditions, wherein the myofibroblast show a progressive change from V+ to VA+ or VAD+ and rarely VADM+ phenotype with specialized morphological arrangement of cytoskeleton.

To study the functional changes of cells undergoing the process of differentiation we studied the changes in the mitochondria. Mitochondria has been largely studied in cancer cells and cancer-associated fibroblasts in relation to their shift from OXPHOS to glycolysis. Recent literature reveals that mitochondrial changes in fibroblasts promote myofibroblast differentiation and have a role in their survival in tissue fibrosis. We assessed the functional changes by evaluating the mitochondrial membrane potential of the cells after 24 hours of treatment. At 24 hours, both arecoline with SB431542 and arecoline group (without SB431542) showed significant mitochondrial depolarization [Graph 3, Table 16(a, b)] as compared to the control and other treatment groups. This was also noted on imaging of cells used for the assay wherein maximum depolarization was depicted by predominantly green fluorescence of the cells was observed in the arecoline and arecoline with SB431542 groups [Figure 12]. In contrast, the TGF $\beta$ , arecoline with TGF $\beta$  and SB431542 groups demonstrated orange red fluorescence and results similar to the control group indicating healthy mitochondrial membrane potential. This confirms the understanding that arecoline via ROS generation can affect the mitochondria and lead to mitochondrial membrane depolarization. This is in contrast to the studies done on organ

fibrosis which showed depolarization of cells on treatment with TGF $\beta$ . This could be attributed to the differential cellular response and different concentrations used by researchers in models of organ fibroses.

Further, it is known that mitochondrial depolarization is associated with the morphological changes in mitochondria as mimicked through the underlying process of fission and fusion.<sup>93</sup> To study the mitochondrial morphology of 48 hours treated cells, we used Mitotracker Red staining and confocal microscopy images of the study groups. We found that the control and SB431542 groups behaved similar and showed a very delicate network of mitochondria not indicative of any evident changes [Figure 13 (a, f)]. Fission as indicated by round puncta-like mitochondria was observed in the arecoline and arecoline with SB431542 groups, while fusion indicated by elongated, branched and swollen mitochondria was found in the TGF $\beta$  and combination group of arecoline with TGF $\beta$  [Figure 13 (b-e)]. We further compared the intensity of Mitotracker Red staining in the study groups and found that arecoline with TGF $\beta$  showed significantly higher intensity as compared to the control and other groups [Graph 4, Table 17(a, b)]. Overall, mitochondria responds to ROS and stress by means of fission-fusion mechanism which further decides the fate of the mitochondria and cell. According to Gao QY et al,<sup>93</sup> cells showing adaptation by fission, show increased glycolysis and are more prone to further ROS mediated cellular apoptosis. We observed the same findings with cell treated using arecoline and arecoline with TGF $\beta$  blocker. TGF $\beta$  in this regards modulated the mitochondria to fusion and thereby probable increase in mitophagy and survival of cells. We could not find any studies indicating the changes in mitochondrial morphology in response to arecoline.

To check for glycolytic switch in arecoline treated cells showing fission, we evaluated the levels of the key enzyme involved in regulating glycolysis i.e., HK-II. We conducted qRT-PCR for cells treated for 24 hours and studied the levels of HK-II in the study groups. We found significant up regulation of HK-II levels in arecoline and arecoline with SB431542 group at 24 hours indicating that cells treated with arecoline undergo glycolysis and meet their energy needs by glycolytic metabolism [Graph 5, Table 18(a, b)]. Bernard K et al,<sup>24</sup> have put forth that protein synthesis and myofibroblast-associated matrix contraction both require energy by OXPHOS or glycolysis. However, they found a time-dependent (12hours to 48 hours) expression and up regulation of HK-II after TGF $\beta$  treatment of lung fibroblasts. Many researchers have found similar results wherein differentiating myofibroblasts demonstrated glycolytic reprogramming in various models of organ fibrosis. Xie N et al,<sup>89</sup> Chen ZT et al,<sup>91</sup> Wei Q et al,<sup>132</sup> and Ding H et al<sup>133</sup> have studied that suppressing or reducing TGF $\beta$  induced glycolysis can attenuate the differentiation of fibroblasts in fibrosis of heart, lung and kidneys. This glycolytic switch was also noted in cancer cells treated with arecoline.<sup>134</sup>

It is also proven that myofibroblasts undergo mitochondrial changes to adapt against ROS mediated injury by undergoing either biogenesis, mitophagy or cell apoptosis.<sup>24, 25, 134</sup> This probed us to evaluate the whether the arecoline treated cells undergo mitophagy for adaptation or undergo cell death. To evaluate possible mitophagy we used qRT PCR for PINK1, which is a well known marker for mitophagy and is being studied extensively in organ fibrosis and neurodegenerative diseases.<sup>30, 137, 138</sup> We evaluated the levels of PINK1 expression in the treatment groups in differentiated fibroblasts at 48

hours and found that the combination of arecoline with TGF $\beta$  showed up-regulation of PINK1 thereby indicating protection against action of ROS or oxidative stress. While, the arecoline group showed a significant reduction in PINK1 expression indicative that the cells would undergo apoptosis instead of survival [Graph 6, Table 19 (a, b)]. Bueno M et al<sup>137</sup> and Araya J et al<sup>139</sup> found that aggregated mitochondrial damage as well as elevated ROS were associated with reduced expression of PINK1 in alveolar epithelial cells (type II) in Idiopathic pulmonary fibrosis of lungs. They put forth that this correlated to increased cell apoptosis and senescence. However, arecoline with SB431542 showed results similar to control, which could be indicative of a possible effect of SB431542. The other treatment groups including TGF $\beta$  and SB431542 behaved similar to healthy controls.

As mentioned previously, biogenesis for maintaining of mitochondrial functions is essential and is modulated by TFAM. TFAM primarily maintains mtDNA, helps in migration, creates antioxidant environment and protects the cells. The protective function is largely explored in cardiac remodeling.<sup>140</sup> Research has shown that up regulation of TFAM is crucial for proliferation of vascular smooth muscles.<sup>141</sup> To determine cellular adaptation by biogenesis, we studied the levels of TFAM in treated cells by means of qRT PCR and Immunofluorescence assay. We found that the treatment groups showed significant differences but when compared to control cells they had marginal statistical difference in the TFAM levels. Arecoline treated cells (arecoline and arecoline with SB431542) showed significantly lower levels as compared to the TGF $\beta$  and arecoline with TGF $\beta$  groups (Table 20(a), Graph 7). This demonstrates a definite difference in transcriptional

activity of mitochondria of these treated cells (Table 20(b), Graph 7). The results of arecoline treatment groups (arecoline and arecoline with SB431542) are in accordance with studies wherein, differentiation of fibroblasts to cancer-associated active fibroblasts was associated with a decrease in TFAM expression and reduced OXPHOS.<sup>142</sup> In a research on endometrial carcinomas, TFAM knockout showed increased cell apoptosis.<sup>143</sup> Overall TFAM helps combat cellular apoptosis and tissue degeneration.<sup>142,143,144</sup>

The IFA results (Figure 14) appeared similar to those obtained through qRT PCR, wherein strong fluorescence was noted in the TGF $\beta$  and arecoline with TGF $\beta$  group (>75% cells). Bernard K et al <sup>24</sup> also who demonstrated increased TFAM activity in TGF $\beta$  treated lung fibroblasts. Minimum fluorescence observed in the other study groups wherein only 20 to 30% cells showed TFAM positivity indicating minimal transcriptional activity of the myofibroblast mitochondria.

We additionally performed IHC for TFAM using normal mucosa and OSMF tissues. The section used for TFAM staining was obtained by serial sectioning of tissue used for cytoskeletal markers IHC. This helped in confirmation that scoring was done for  $\alpha$ SMA positive myofibroblasts and not other stromal cells. We found significant difference in the cytoplasmic TFAM staining of myofibroblasts in the normal mucosa and OSMF tissues (Tables 21 (a-e)). Normal mucosa and 45% of early OSMF showed lack of TFAM expression while remaining 55% cases of early OSMF showed focal and juxtaepithelial localization of positive myofibroblasts. Intermediate and advanced OSMF cases predominantly showed juxta-epithelial and diffuse localization of TFAM positive myofibroblasts (Figure 15, Tables 21(a)). Percentage-wise, normal mucosa and early OSMF

showed less than 25% TFAM positive cells, while intermediate and advanced showed greater than 25% positively stained myofibroblasts (table 21(b)). The frequency and intensity of TFAM staining increased with advances in grade of OSMF. Pairwise comparison of study groups demonstrated significant results, except for normal mucosa and early OSMF which showed no difference in TFAM expression (Table 21 (c,d,e)). Our results indicate that as grade increased the myofibroblasts show marked transcription of mitochondria in myofibroblasts. Overall the TFAM expression results are similar to other studies which indicate that increased ROS causes mtDNA mutations and reduced TFAM expression with increased cell apoptosis. <sup>24, 145</sup> Adihetty PJ et al <sup>146</sup> have shown that increased mitochondrial regeneration is accompanied with increased TFAM and Peroxisome proliferator-activated receptor  $\gamma$  co-activator-1 $\alpha$  (PCG-1 $\alpha$ ) expression.

Interestingly, we also found that TFAM expression increased in muscle from normal mucosa to advancing grades of OSMF. Degenerating muscles in advanced OSMF showed lack of TFAM immunoexpression. This is in accordance with the research that muscles undergoing atrophy lack TFAM expression and that skeletal muscles with exercise show increased TFAM activity to combat against atrophy. <sup>144</sup>

It is known that imbalance between anabolic and catabolic process of mitochondria biogenesis and mitophagy can cause altered recovery and ultimate cell death. <sup>93, 147</sup> For validation of the mitochondrial changes in relation to cell apoptosis and survival, we performed the Alamar Blue over the key time points of 12, 48 and 72 hours [Graph 8, Tables 22(a-h)]. At 12 hours and 48 hours, arecoline with SB431542 showed maximum cell death as compared to all the other groups however at 72 hours it shows no major differ-

ence when compared to the untreated control. Significant cell death was noted in the arecoline and arecoline with SB431542 groups as compared to arecoline with TGF $\beta$  group and the TGF $\beta$  group at 72 hours. Researchers have also demonstrated that arecoline treatment can bring about cell death.<sup>114, 115</sup> Our results are also broadly comparable with research by Bras M et al,<sup>148</sup> Catanzaro MP et al,<sup>149</sup> and Zhou H et al<sup>150,151</sup> who observed that cells which exhibit excessive mitochondrial fission (as observed in arecoline and arecoline with SB431542 in mitochondrial morphology experiment) show apoptosis of the cells. We also found that arecoline with TGF $\beta$  group and the TGF $\beta$  group showed marked cell survival as compared to control and the other groups. This finding indicated that TGF $\beta$  rescues the arecoline treated cells from undergoing cell death and promotes myofibroblast survival and function.

Furthermore, like tumor cells that adapt to glycolysis, arecoline treated cells show glycolytic switch, however alone arecoline treated cells undergo cell death and need TGF $\beta$  for their survival. With the understanding of our present work, we can be theoretically hypothesize that it could be the chronicity and overrunning of this arecoline -TGF $\beta$  combined effect on myofibroblasts which directs them towards a possible malignant change. However this will need further research and validation for acceptance in regards to OSMF being a potentially malignant disorder.

We could possibly extrapolate the findings of the present work to the initial acute and chronic response of tissues to arecoline consumption and TGF $\beta$  as shown by the proposed model [Figure 16]. Our study, is the first study that explores the molecular and cellular changes in oral sub mucous fibrosis in terms of persistence and amassing of myofi-

broblasts, their (structural and functional) biotypes and mitochondrial as well as metabolic reprogramming of myofibroblasts.

## 6. SUMMARY

In our study, we treated HGF cells with the arecoline and TGF $\beta$  using different groups and optimal concentrations of treatment reagents. On evaluation of myofibroblast phenotype and mitochondrial changes, we found that distinct mechanisms are orchestrated at different time points from early mitochondrial membrane changes, mitochondrial morphology changes like fission and fusion to energetic reprogramming and transcription between 24hours to 48 hours. The persistent effect of treatment with arecoline and TGF $\beta$  were further demonstrated by studying the cell survival at 72 hours. We found that the transdifferentiation of myofibroblasts and phenotypic changes were induced by arecoline and TGF $\beta$  treatments at 48 hours. The control and SB431542 groups did not show differentiation (V+ phenotype), neither marked mitochondrial changes.

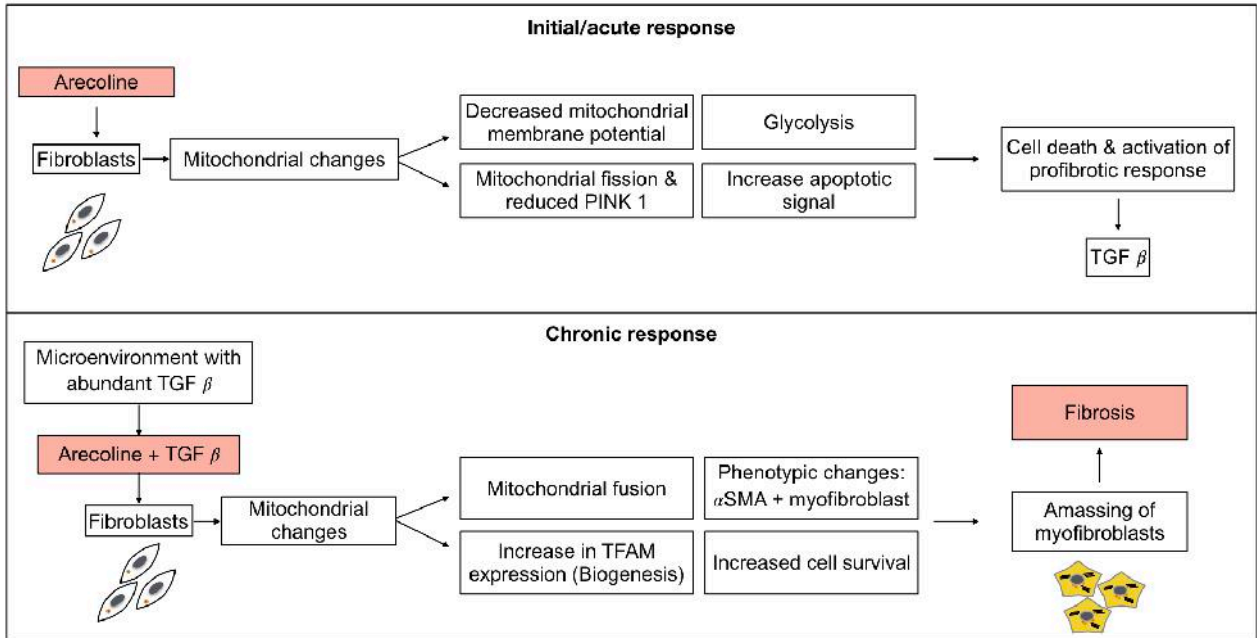
Cytoskeletal phenotypes of treated cells (arecoline and TGF $\beta$ ) ranged VA+ to VAD+ myofibroblasts. Arecoline with blocking of TGF $\beta$  receptor and arecoline treated cells demonstrated a VA+ phenotype (50 to 75% cells). While a subpopulation of TGF $\beta$  and arecoline with TGF $\beta$  treated cells (less than or equal to 25% cells) showed VAD+ expression. No myosin expression was noted in any study groups. The results were validated using of OSMF tissues. In OSMF tissues, a very small subset demonstrated presence of desmin and myosin, possibly indicating epithelial mesenchymal interactions.

The differences in metabolism and cellular function based on the stimuli were also observed in the treatment group. To describe briefly **arecoline** by itself leads to early increased mitochondrial membrane depolarization and altered mitochondrial morphology indicative of fission in myofibroblasts. Further, arecoline treated cells show glycolytic

reprogramming at 24 hours i.e, prior to myofibroblast differentiation. Indicating that these cells need glycolysis for differentiation and adaptation against mitochondrial damage. Post differentiation, these cells undergo mitophagy as observed by down-regulation of PINK1 at 48hours and eventual cell death at 72 hours.

**TGF $\beta$  and combination of arecoline with TGF $\beta$**  showed mitochondrial membrane potential similar to untreated control and expressed increased mtDNA transcription (upregulated TFAM) with an elongated, branched mitochondrial morphology of mitochondria indicative of fusion. We also found myofibroblasts in TGF $\beta$  group demonstrated long filamentous form of Vimentin that is postulated to be possibly involved in of cell structure stabilization and protection against apoptosis. TGF $\beta$  in the combination group with arecoline, is shown to rescue the arecoline-induced mitochondrial damage in myofibroblasts and promoting survival of these cells that lead to tissue fibroses.

Thus **TGF $\beta$ -rich environment** can protect the differentiated myofibroblasts from undergoing cell death, thereby assisting in the accumulation of myofibroblasts that can sustain themselves and bring about continuous collagen secretion. Although cytoskeletal phenotypes in the treated cells did not vary much in arecoline and TGF $\beta$  groups but the functional differences indicate that there are subsets of differentiated cells. Based on the present findings of our research work we propose a model of acute and chronic response of myofibroblast differentiation in OSMF [Figure 16].



**Figure 16:** Proposed model of acute and chronic response to arecoline and TGFβ in OSMF fibrosis based on findings of research work.

## 7. CONCLUSION

OSMF as well as organ fibrosis cause generalized debilitation and affect the quality of life of the individual. Both of which, have limited treatment methods and demand stringent protocols for management of the condition. However, understanding of the pathogenesis and development of targeted therapy for the same appears to be the most reliable approach for management of fibrosis. Our study enables understanding of the possible response of cells to arecoline consumption in relation to early response and persistent or chronic response. It highlights that the role of  $TGF\beta$  in OSMF is significant and shows “rescue” of cells from cytotoxicity caused by arecoline treatment thereby advances fibrosis.  $TGF\beta$  favors differentiation of fibroblasts to a pro-survival phenotype that functions primarily by activation of mitochondrial bioenergetics of the cells. The study largely elucidates the role of mitochondria and cell survival in the pathogenesis of OSMF. The research highlights the potential for development of targeted therapy for mitochondria and functional fibroblast biotype for management of the irreversible condition of OSMF.

### **Limitations:**

Although our work extensively elaborates cell survival and model of OSMF tissue response, the limitation of lack of study of collagen production and contraction function of the differentiated myofibroblasts exists. Collagen contraction assay could possibly aid confirmation of the functional role of the differentiated myofibroblasts that undergo cell survival. The results of the present study can further be supported if cellular dynamics in

terms of ultrastructural findings could be elicited. Performing seahorse respirometry for the treatment groups can aid in validation of the findings of the present research. Performing immunohistochemical analysis with larger sample size of human tissues would guide for clinical application of the study results and for development of targeted therapy against cytoskeletal phenotype or mitochondria.

**Recommendation:**

- 1) The role of electron transport chain (collagen contraction assay) and cellular respiration (seahorse respirometry experiment) can be evaluated for exploring and validating the metabolic reprogramming of myofibroblasts.
- 2) Further validation of mitochondrial biogenesis and mitophagy by performing loss of function experiments for TFAM & overexpression studies for PINK1.
- 3) Co-culture of epithelial cells and fibroblasts to study epithelial cell dynamics and interactions with fibroblasts in response to Arecoline and TGF  $\beta$  therapy.

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## 9. ANNEXURE

### Annexure - I: Ethical Clearance

**KLE UNIVERSITY**  
(Formerly known as KLE Academy of Higher Education & Research, Belagavi)  
[Declared as Deemed-to-be-University act 3 of the U.C. Act, 1956 vide Government of India Notification No F-9-19-2004 U-3(A)]  
Accredited 'A' Grade by NAAC (2<sup>nd</sup> Cycle) Placed in Category 'A' by MHRD (GoI)

**Office of the Director, Academic Affairs**  
JNMC Campus, Nolew Nagar, Belagavi-590 010, Karnataka State, India  
☎: 0831-2444444/2493779 FAX: 0831-2493777 Web: <http://www.kleuniversity.edu.in> E-mail: [diracademic@kleuniversity.edu.in](mailto:diracademic@kleuniversity.edu.in)

Ref. No. KLEU/EC/17-18/D-1) 2 \_\_\_\_\_ 16<sup>th</sup> May 2017

To,  
**Dr. Karishma Desai**  
Full Time Research Scholar,  
2016-17 batch, Faculty of Dentistry  
KLE VK IDS, Belagavi

Dear Research Scholar,


Sub:- Regarding Ethical Clearance.

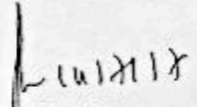
The KLE University **Ethics Committee on Human Subjects** for Ph. D Research Project met on **22<sup>nd</sup> March 2017** to consider your application for approval of the research project **"Effect of Arecoline and Transforming Growth Factor - $\beta$  on Myofibroblast Trans Differentiation by Assessment of Mitochondrial Bioenergetics: A Cell Culture Study."**

After review of the documents submitted by you and satisfactory explanations provided to the members, the committee has approved this research project as no Ethical issues are involved in the proposed research project.

You are requested to report to Ethical Committee in case of the following:

1. Any deviation from or change of the protocol.
2. Any changes in study documents.

  
**(Dr. Anita Dalal)**  
Member Secretary,  
Ph.D. Ethical Committee(Human),  
K.L.E. University,  
Belagavi.

  
**(Dr. Anil Hogade)**  
Chairman  
Ph.D. Ethical Committee(Human),  
K.L.E. University,  
Belagavi.

CC to: - The Director Academic Affairs, KLE University, Belagavi.  
- The Director Research Foundation, KLE University, Belagavi.  
- The Registrar, KLE University, Belagavi

Annexure - II Training certificates





Sinhgad Institutes



# Sinhgad Dental College & Hospital & Sinhgad College Of Engineering

## Certificate of Attendance

Presented to

.....*Dr. Karishma Desai*.....

for participating in

***“Workshop on Cell Culture”***

Organized by

Department of Oral Pathology & Microbiology  
and Department of Biotechnology

on 17<sup>th</sup> - 19<sup>th</sup> January 2017.

Dr. Sameer Patil  
Principal  
SDCH, Pune

Dr. S. D. Lokhande  
Principal  
SCOE, Pune

Dr. Kalpana Joshi  
Professor & HOD  
Dept. of Biotechnology

Dr. Sangeeta Palaskar  
Professor & HOD  
Dept. of Oral Pathology & Microbiology



Completion Date 11-Feb-2018  
Expiration Date 10-Feb-2021  
Record ID 25356585

This is to certify that:

**Karishma Desai**

Has completed the following CITI Program course:

**Human Research** (Curriculum Group)  
**Biomedical Research Investigators** (Course Learner Group)  
**1 - Basic Course** (Stage)

Under requirements set by:

**SUNY - Buffalo (University at Buffalo)**



Verify at [www.citiprogram.org/verify/?w35d00aea-97e6-4e0e-aaa7-90cd90b0a2c7-25356585](http://www.citiprogram.org/verify/?w35d00aea-97e6-4e0e-aaa7-90cd90b0a2c7-25356585)



Completion Date 13-Feb-2018  
Expiration Date 12-Feb-2021  
Record ID 25356590

This is to certify that:

**Karishma Desai**

Has completed the following CITI Program course:

**Human Research** (Curriculum Group)  
**Stem Cell Oversight** (Course Learner Group)  
**1 - Basic Course** (Stage)

Under requirements set by:

**SUNY - Buffalo (University at Buffalo)**



Verify at [www.citiprogram.org/verify/?wf93df3d3-b6d3-49a0-9672-8248599c093d-25356590](http://www.citiprogram.org/verify/?wf93df3d3-b6d3-49a0-9672-8248599c093d-25356590)

Original Study

## CLINICOPATHOLOGICAL EVALUATION OF ORAL SUBMUCOUS FIBROSIS - A RETROSPECTIVE, SINGLE INSTITUTE STUDY

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### ABSTRACT

Oral submucous fibrosis (OSMF) is a fibrotic, potentially malignant disorder of the oral cavity. The prevalence of OSMF in the south-east Asian population and the role of the areca nut in its pathogenesis are well documented. However, to understand the pathophysiology of the condition, it is necessary to study the risk factors and population characteristics of individuals affected by the condition. Thus, the present retrospective study aimed to evaluate the clinicopathological features of OSMF. Clinically and histologically diagnosed cases of OSMF reported from 1998 to 2018 were included in the present study. Clinicopathological parameters of gender, age, stage, grade, and habit history were included. To test the association, the chi-square test was used.

The OSMF cases showed a male predominance with the male to female ratio being 10.7:1. A wide age range from 14 years to 84 years was noted. Histological grade and clinical stage of OSMF showed significant association ( $p < 0.05$ ). The majority of cases (63 out of 141) showed advanced grades of OSMF. An association between gender and age ( $p < 0.05$ ) as well as between gender, habit history, and grade of OSMF ( $p < 0.05$ ) was observed. Betel nut and tobacco consumption in the younger age group and their significant association with advanced grade and stage of OSMF was observed in the present study. The study highlights a need for education of patients regarding the use of the substances and their deleterious effect on the disease progression and quality of life of the patient.

**Key words:** Oral Submucous Fibrosis, Carcinoma, Areca nut, Tobacco.

### Introduction

Oral Submucous Fibrosis (OSMF) is a fibrotic condition of the oral cavity that occurs predominantly in the South-East Asian population. [1] It is also known as a potentially malignant disorder and causes debilitation due to the progressive fibrosis of the affected areas. [2] OSMF presents an array of clinical and histopathological features. [1, 2] Although multifactorial, the primary factor responsible for bringing about these changes is considered to be the persistent consumption of areca or betel nut. [2, 3] Commercially available forms of areca nut like gutkha, are implicated with the early presentation and progress of OSMF. [2, 4, 5] Consumption of Pan and gutkha are reported to be commonly associated with OSMF in the northern parts of India. [6] Although gutkha has been banned, the rising incidence of OSMF, its irreversible nature, and its malignant transformation deems essential the study of clinicopathological factors like patient age, gender, habits, stage, and grades of OSMF. Hence, the present retrospective study aimed to evaluate the clinical and histological features of reported cases of OSMF in a single institute.

### Materials and Methods

After obtaining Institutional ethical clearance, the present retrospective study was undertaken. A total of 141 clinically and histologically diagnosed cases of OSMF reported from 1998 to 2018 were included in the study. Clinical data consisting of age, gender, clinical signs and symptoms, stage, and habits were retrieved. Habits documented were grouped as 0 - no documentation of history on patient record; 1- Betel leaf with or without lime; 2- Betel nut alone; 3- Gutkha; 4- Tobacco; 5- The combination of two or more and 6- No habit of addictive substance consumption. Clinical signs & symptoms as available from patient records were grouped into 0 - presenting with a sharp tooth, denture trauma, and other; 1- Burning sensation and blanching of mucosa with or without blisters; 2- Palpable bands in the buccal mucosa and/or circumoral mucosa with or without blistering and burning sensation, difficulty in mouth opening (26-35mm); 3- Palpable bands extending to raphe, uvula, tongue and difficult mouth opening (15-25mm) or one finger opening, erythematous areas; 4- Difficulty in mouth opening (less than 15mm) with ulcers/white lesions; 5- Difficulty in

mouth opening and associated with carcinomatous growth. For grading of OSMF, we used modified Pindborg and Sirsat classification and added categories of OSMF with dysplasia and OSMF with carcinoma. [1] The available clinical stage as mentioned on patient record as per Khanna JN and Andrade criteria was also included for comparison. [7] All the cases included were histologically evaluated by two trained pathologists of similar experience in diagnosing head and neck pathologies for confirmation of the histological grade of OSMF. The data was tabulated and Chi-Square analysis was performed to test the difference between the clinical and histopathological parameters ( $P < 0.05$ ).

## Results and Discussion

### Gender and Age

The gender distribution showed the male to female ratio of 10.7:1 indicative of a male predisposition. Males were predominantly in the younger age group of 40 years and less (97%), while the majority of females belonged to the more than 40 years age group (78.6%). The age range for the cases was from 14 years to 84 years. 70% of cases (99 out of a total of 141 cases) were noted in the less than 40 years age group (Table 1).

**Table 1.** Distribution of OSMF cases according to Gender & Age groups

Gender	40 and fewer years of age (%)	More than 40 years of age (%)	Total no. of participants n (%)
Male	96 (97)	33 (78.6)	129 (91.5)
Female	3 (3)	9 (21.4)	12 (8.5)
Total	99 (100)	42 (100)	141 (100)

### Habits and Grade

The majority of cases (63 out of 141 cases) presented with moderately advanced OSMF (Table 2). A total of 116 cases gave the history of habits, 5 cases had no habit history given by the patient, amongst which one was associated with denture trauma. No habit history was documented for 20 cases. Maximum cases had a history of Gutkha chewing or a combination of two or more habits (Table 3).

**Table 2.** Distribution of OSMF cases according to Histopathological grade

Histopathological Grade	No of participants n (%)
Very early OSMF	10 (7.1)
Early OSMF	34 (24.1)
Moderately advanced OSMF	63 (44.7)
Advanced OSMF	22 (15.6)
OSMF with dysplasia	6 (4.3)
OSMF with Carcinoma	6 (4.3)

**Table 3.** Distribution of OSMF cases according to habits

Habits (Score assigned)	No of participants n (%)
No history documented (0)	20 (14.2)

Betal leaf with or without lime (1)	2 (1.4)
Betal nut (2)	26 (18.4)
Gutkha (3)	32 (22.7)
Tobacco (4)	24 (17.0)
Combination of any two or more (5)	32 (22.7)
No habit of addictive substance consumption (6)	5 (3.5)

### Gender and grade

More than 50% of cases with moderately advanced OSMF were noted in males, while females showed the majority of cases that were in the early grade. Out of the total 12 cases showing OSMF with dysplasia (6 cases) and OSMF with carcinoma (6 cases), the majority of them were noted in male patients (11 out of 12 cases).

### Association between gender, habits, grade, and age

All gutkha chewers were males and about 50% of females showed a habit history of betel nut consumption. A significant association was noted between gender and habit ( $p=0.046$ ) (Table 4). Amongst Gutkha chewers, 50% of cases showed moderately advanced OSMF followed by advanced OSMF (28.1%). A significant association was noted between the habit history and grades of OSMF ( $p = 0.048$ ) (Table 5). Similarly, a significant association was noted between the histological grade and age of patients ( $p=0.001$ ) (Table 6).

**Table 4.** Comparison between Gender and Habit

Gender	Habit	Total
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		0	1	2	3	4	5	6		P-value
Female	Count	0	0	6	0	3	2	1	12	0.046*
	% within gender	0.0%	0.0%	50.0%	0.0%	25.0%	16.7%	8.3%	100.0%	
Male	Count	20	2	20	32	21	30	4	129	
	% within gender	15.5%	1.6%	15.5%	24.8%	16.3%	23.3%	3%	100.0%	
Total	Count	20	2	26	32	24	32	5	141	
	% within gender	14.2%	1.4%	18.4%	22.7%	17.0%	22.7%	3.6%	100.0%	

Chi-square test: \* indicates a significant difference at  $p \leq 0.05$ .

Habits: 0 -No history documented; 1- betel leaf with or without lime; 2- betel nut; 3- Gutkha; 4- Tobacco; 5- the combination of two or more; 6 - no habit of addictive substance consumption.

**Table 5.** Association of Histopathological grades with habits

Histopathological Grade	Habit							Total	P-value
	0	1	2	3	4	5	6		
Very early OSMF	1 (5)	0	4 (15.4)	3 (9.4)	1 (4.2)	1 (3.1)	0	10 (7.1)	0.048*
Early OSMF	6 (30)	0	10 (38.5)	3 (9.4)	9 (37.5)	4 (12.5)	2 (40)	34 (24.1)	
Moderately advanced OSMF	9 (45)	2 (100)	11 (42.3)	16 (50)	11 (45.8)	12 (37.5)	2 (40)	63 (44.7)	
Advanced OSMF	2 (10)	0	1 (3.8)	9 (28.1)	2 (8.3)	7 (21.9)	1 (20)	22 (15.6)	
OSMF with dysplasia	2 (10)	0	0	0	1 (4.2)	3 (9.4)	0	6 (4.3)	
OSMF with Carcinoma	0	0	0	1 (3.1)	0	5 (15.6)	0	6 (4.3)	
Total	20 (100)	2 (100)	26 (100)	32 (100)	24 (100)	32 (100)	5 (100)	141 (100)	

Chi-square test: \* indicates a significant difference at  $p \leq 0.05$ .

Habits: 0 -No history documented; 1- betel leaf with or without lime; 2- betel nut; 3- Gutkha; 4- Tobacco; 5- the combination of two or more; 6 - no habit of addictive substance consumption.

**Table 6.** Association of Histopathological grade of OSMF with age groups

Histopathological grade	Age group		Total	p-value
	40 or less	More than 40 yrs		
Very early OSMF	6 (5.9)	4 (10)	10 (7.1)	0.001*
Early OSMF	19 (18.8)	15 (37.5)	34 (24.1)	
Moderately advanced OSMF	56 (55.4)	7 (17.5)	63 (44.7)	

Advanced OSMF	15 (14.9)	7 (17.5)	22 (15.6)
OSMF with dysplasia	1 (1)	5 (12.5)	6 (4.3)
OSMF with Carcinoma	4 (4)	2 (5)	6 (4.3)
Total	101 (100)	40 (100)	141 (100)

Chi-square test: \* indicates a significant difference at  $p \leq 0.05$

#### Stage of OSMF, Clinical presentation, and grade

According to the staging criteria used, 79 out of 141 total participants presented with Stage 2 OSMF (56%), while 11 out of 141 cases presented with Stage 4 OSMF (7.8%) (Table 7). Clinically, 53 out of 141 cases presented with reduced mouth opening, few erythematous areas, and palpable bands extending to involve the raphe, uvula, and tongue (Table 8).

**Table 7.** Distribution of OSMF cases according to the clinical stage

Stage	No of participants n (%)
Stage 1	30 (21.3)
Stage 2	79 (56.0)
Stage 3	21 (14.9)
Stage 4	11 (7.8)

**Table 8.** Distribution of OSMF cases according to clinical presentation

Clinical signs and symptoms (Score)	No of participants (%)
Not relevant to OSMF/other symptoms like a sharp tooth, denture irritation, etc (0)	6 (4.3)

Burning sensation, blanching of the mucosa, and blistering (1)	14 (9.9)
Palpable bands in BM and/or circumoral, difficulty in mouth opening [26-35mm], blistering, burning sensation (2)	47 (33.3)
Palpable bands extending to raphe, uvula, tongue, and difficult mouth opening [15-25mm] or one finger opening, erythematous areas (3)	53 (37.6)
Restricted mouth opening [ $<15\text{mm}$ ] with Ulcers/white lesions (4)	15 (10.6)
Restricted mouth opening and associated cancer [5]	6 (4.3)

#### Association between Grade, stage, and Clinical signs - symptoms

Clinical signs and symptoms, as well as clinical stage, showed significant association with the histopathological grade of OSMF ( $p=0.001$ ) (Tables 9 and 10). The majority of patients presented with stage 2 OSMF and amongst these 70% of cases had a histological grade of moderately advanced OSMF. 50% of cases with stage 1 OSMF showed histological features of early OSMF whereas stage 3 & 4 OSMF cases showed either advanced OSMF or dysplasia and carcinoma with OSMF (Table 10).

**Table 9.** Association of Histopathological grades with clinical signs and symptoms

Histopathological Grade	Clinical Signs & symptoms						Total	P-value
	0	1	2	3	4	5		
Very early OSMF	1 (16.7)	4 (28.6)	5 (10.6)	0	0	0	10 (7.1)	0.001*
Early OSMF	5 (83.3)	4 (28.6)	18 (38.3)	3 (5.7)	4 (26.7)	0	34 (24.1)	
Moderately advanced OSMF	0	6 (42.9)	23 (48.9)	28 (52.8)	6 (40)	0	63 (44.7)	
Advanced OSMF	0	0	1 (2.1)	21 (39.6)	0	0	22 (15.6)	
OSMF with dysplasia	0	0	0	1 (1.9)	5 (33.5)	0	6 (4.3)	

OSMF with Carcinoma	0	0	0	0	0	6 (100)	6 (4.3)
Total	6 (100)	14 (100)	47 (100)	53 (100)	15 (100)	6 (100)	141 (100)

Chi-square test; \* indicates a significant difference at  $p \leq 0.05$ .

Clinical Signs & symptoms: 0 - Sharp tooth, denture trauma and other; 1- Burning sensation and blanching of mucosa with or without blisters; 2- Palpable bands in the buccal mucosa and/or circumoral mucosa with or without blistering and burning sensation, difficulty in mouth opening (26-

35mm); 3- Palpable bands extending to raphe, uvula, tongue and difficult mouth opening (15-25mm) or one finger opening, erythematous areas; 4- difficulty in mouth opening (less than 15mm) with ulcers/white lesions; 5- difficulty in mouth opening and associated with carcinomatous growth.

**Table 10.** Association of Histopathological grades with stages of OSMF

Histopathological Grade	Stage				Total	p-value
	Stage 1	Stage 2	Stage 3	Stage 4		
Very early OSMF	8 (26.7)	2 (2.5)	0	0	10 (7.1)	
Early OSMF	15 (50)	16 (20.3)	3 (14.3)	0	34 (24.1)	
Moderately advanced OSMF	7 (23.3)	56 (70.9)	0	0	63 (44.7)	
Advanced OSMF	0	5 (6.3)	16 (76.2)	1 (9.1)	22 (15.6)	0.001*
OSMF with dysplasia	0	0	2 (9.5)	4 (36.4)	6 (4.3)	
OSMF with Carcinoma	0	0	0	6 (54.5)	6 (4.3)	
Total	30 (100)	79 (100)	21 (100)	11 (100)	141 (100)	

Chi-square test; \* indicates a significant difference at  $p \leq 0.05$

The pathogenesis of OSMF is considered to be multifactorial. However, the areca nut is implicated as the key etiological agent for OSMF. [2, 3, 5] In India, the habit of betel leaf and betel or areca nut consumption is common. Recently, an upsurge has been observed concerning the consumption of various commercially available products of Areca nut alone or in association with other agents like lime and tobacco. [6, 8] With the change in available forms and accessibility of these substances, there is a need to evaluate the association between the consumption of these Areca nut products along with the study of characteristics of the population affected by OSMF.

Previous studies showed a female preponderance for OSMF, however, recently more number of OSMF cases are reported in male patients. [6] Hazarey VK et al, suggested this could be attributed to easy access to the commercially available areca nut products. [6] Our cases showed a male predisposition, with the male to female ratio being 10.7:1. This was following the findings of More CB et al [4], Biradar S et al [9], Srivastava R et al [10], Angadi PV et al [11], Cai X et al [12], and Yang SF et al [13] who also found a male predominance in OSMF cases.

In our present study, the OSMF cases showed an age distribution ranging from 14 years to 84 years. Recently, studies have shown that OSMF commonly affects

individuals in the younger age group. [6, 14] A few Indian and Chinese studies have reported OSMF in children and young patients with ages ranging from 9 to 16 years. [12, 14, 15,16] Our study showed similar results wherein maximum cases were seen in the less than 40 years age group. Further, the age-wise distribution showed that the younger age group was predominantly comprised of male patients and showed moderate to advanced clinical grade and stage of OSMF. This was in concordance with the results of Angadi PV et al [11] and Cai X et al [12], who noted a similar association between age, gender, and clinicopathological features. Furthermore, it has been reported that OSMF with carcinoma is more common in the younger age groups. [15]

In our study, the most common clinical presentation noted was restricted mouth opening (between 15 to 25mm) with palpable bands and burning sensation. Angadi PV et al [11] also found mouth opening to be most affected in OSMF patients.

In the present study, we found that the female patients majorly presented with an early grade of OSMF, and only half of the female patients had a history of areca nut consumption. Contrary to this, all gutkha chewers were male and showed an advanced grade of OSMF. More CB et al [5] and Hazarey VK et al [6] also found a similar habit-gender association in their studies. The consumption of commercial

areca nut products like gutkha has been linked to the presentation of advanced grades of OSMF. [5, 17] OSMF being a potentially malignant disorder, can present with dysplasia or transformation of OSMF to carcinoma. In our study, we found 4.2% cases in both, OSMF with dysplasia and OSMF with carcinoma groups. Interestingly, all cases of OSMF with carcinoma had a history of consumption of two or more substances like betel nut, tobacco, and/or gutkha for longer than 10 years. While, amongst the 32 gutkha chewers, 78.1% of cases developed moderately advanced to advanced OSMF and 75% of cases of these cases reported less than 10 years of gutkha consumption history. Our study supports the findings of Hazarey VK et al [6], Angadi PV et al [11] and Avinash Tejasvi ML et al. [18] who found similar results. It can be hypothesized that consumption of commercial products with high levels of arecoline and tobacco or the consumption of two or more such agents could lead to persistent chemical and mechanical irritation, early progression, and a possible malignant transformation of OSMF.

Even with the ban of Gutkha [19], the easy availability of commercial as well as natural areca nut products, tobacco, and other addictive substances highlights the need for regular awareness programs for the youth regarding the association between these habits and potentially malignant disorders like OSMF [6, 15]. The impact on the quality of life and the economical burden of treatment needs as well as the possible malignant transformation of OSMF needs to be addressed. [20-23] A strict scrutiny to regulate the availability of these natural and commercial betel nut/tobacco products needs to be implemented all over the country.

As the present study is a retrospective study, factors like nutritional status, general health, frequency of habit for all patients, and their socioeconomic status were not available. Being a single institute study, a fewer number of cases with the dysplastic and malignant change in OSMF were observed. To study the associations and enable research to discern the pathogenesis of OSMF, further multicentric studies should be undertaken.

### Conclusion

The present retrospective study analyses the various clinicopathological features of OSMF and highlights the key associations observed in these patients. Clinically, restricted mouth opening was found to be the most common presentation of OSMF while, histologically, advanced grades of OSMF were observed in male patients of less than 40 years of age. History of consumption of gutkha or more than two substances like betel nut and tobacco was observed in patients with advanced, dysplasia and carcinoma-associated OSMF cases.

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**Conflict of interest:** None

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**Ethics statement:** As the study is a retrospective research study, the ethics review board approved the waiver of written informed consent.

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## Original Article

# Role of programmed cell death 4 in myofibroblast differentiation in oral submucous fibrosis

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## Abstract

**Background:** Fibrosis is an uncontrolled healing process, led by persistent differentiation of fibroblast to alpha-smooth muscle actin ( $\alpha$ SMA) positive activated fibroblast or myofibroblast. Oral submucous fibrosis (OSMF) is one such condition that is associated with areca nut use. Recently, Programmed Cell Death 4 (PDCD4), a pro-apoptotic marker, has been shown to modulate fibroblast differentiation in various organ fibrosis. The present study aimed to evaluate the role of PDCD4 in the regulation of fibroblast differentiation in OSMF.

**Materials and Methods:** Paraffin-embedded tissue sections from 45 cases of the normal oral mucosa, early OSMF and advanced OSMF were examined for PDCD4 and  $\alpha$ SMA expression by immunostaining. Co-expression of PDCD4 and  $\alpha$ SMA in fibroblasts was examined using Spearman's correlation test.

**Results:** The stromal fibroblasts showed minimal expression of  $\alpha$ SMA in the normal mucosa and early OSMF, while advanced OSMF groups demonstrated a higher frequency of  $\alpha$ SMA myofibroblasts. The PDCD4 expression was noted in the normal stromal fibroblasts. However, this expression appeared to progressively reduce with an increasing grade of OSMF. Thus, a negative correlation was noted between stromal PDCD4 and  $\alpha$ SMA expression with progressive OSMF.

**Conclusion:** This study demonstrated a putative role for PDCD4 in oral fibrosis consistent with its role in other tissues. The lack of PDCD4 expression with increasing myofibroblast expression in OSMF suggests that targeting its dysregulation may be an attractive translational therapeutic target.

**Keywords:** Areca, betel nut, myofibroblast, oral submucous fibrosis, programmed cell death

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## INTRODUCTION

Oral submucous fibrosis (OSMF) constitutes an oral premalignant condition induced by areca nut ingestion and is considered an uncontrolled tissue healing response

resulting in fibrosis.<sup>[1]</sup> Clinically, OSMF presents with a burning sensation in the mouth, stiffening of the mucosa, and reduced mouth opening, among other changes that lead to difficulty in food consumption and generalized

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debilitation. Histologically, it is associated with progressive changes in the connective tissue such as fibrosis and hyalinization, along with atrophy of the epithelium in advanced cases. The connective tissue response to arecoline, a major alkaloid of areca nut, is known to up-regulate pro-inflammatory and pro-fibrotic cytokines, such as Transforming Growth Factor beta (TGF  $\beta$ ). TGF- $\beta$  leads to the differentiation of stromal fibroblasts to alpha smooth muscle actin positive ( $\alpha$ SMA) myofibroblasts that are well known to promote matrix synthesis and tissue contraction.<sup>[2,3]</sup> Recently, alkaloids of arecanut, namely arecaidine and guvacine, along with the polyphenols such as catechin and tannins, have also been shown to induce TGF  $\beta$  signaling in epithelial cells contributing to the complex pathogenesis of OSMF.<sup>[4,5]</sup>

Programmed cell death 4 (PDCD4) is a well-known pro-apoptotic protein recognized for its roles in transcription and translation pathways in tumor growth and metastasis. PDCD4 has been observed to promote fibroblasts differentiation in renal and liver fibrosis through TGF- $\beta$  and other regulatory pathways.<sup>[6-8]</sup> In a recent study, we observed reduced PDCD-4 expression in oral dysplastic and oral carcinomas compared to normal epithelial mucosa.<sup>[9]</sup> The decrease in PDCD4 expression in transforming epithelia appeared to correlate well with reduced cell death observed in these lesions. A key reason for the persistence of myofibroblast phenotypes has been their ability to evade apoptosis.<sup>[10]</sup> Thus, the premise for this study was to examine if the changes in PDCD4 expression would correlate with increased differentiation of oral stromal fibroblasts into  $\alpha$ SMA positive myofibroblasts. We investigated  $\alpha$ SMA and PDCD4 expression in the normal and progressive stage of OSMF oral mucosa to assess its putative role in tissue fibrosis.

## MATERIALS AND METHODS

### Human tissue samples

Following ethical clearance from the Institute, archival formalin-fixed, paraffin-embedded tissues of normal mucosa ( $n = 10$ ), and OSMF ( $n = 30$ ) were retrieved. Clinical data were used to evaluate the history with areca nut consumption and for the absence of other local and systemic factors or illnesses.

### Histological analyses

Three continuous sections of 4  $\mu$ m were cut, and one section was stained using hematoxylin and eosin. Histological grading from I through IV was based on criteria elaborated by Pindborg and Sirsat.<sup>[1]</sup> Cases were then categorized into two groups as either early OSMF for

histological Grades I and II cases (Group 2) or advanced OSMF for histological Grade III and IV cases (Group 3) and compared to normal mucosa (Group 1).

### Immunostaining

Two sections were used for immunohistochemistry with PDCD4 (Clone EPR3432, BioGenex Lab) and  $\alpha$ SMA (clone EP188, PathnSitu). The primary antibody was detected using a one-step polymer HRP kit (PCH2, PathnSitu) by the Avidin-Biotin complex and counterstained with Harris's hematoxylin.<sup>[11]</sup>

### Immunostaining analyses

Sections were analyzed for the staining intensity, localization and pattern by two independent examiners. The two examiners had similar levels of experience with training in immunohistochemical and pathological evaluation of oral lesions. The inter observer agreement using a training set was 0.89 (Kappa statistic). For study groups, any discrepancy in scoring was resolved by the consensus on a multi head microscope.

A modified scoring criterion was adopted due to the compression and amassing of the cells noted in histological sections in OSMF. To compare the expression of PDCD4 and  $\alpha$ SMA in fibroblasts, consecutive sections were stained and analyzed. Subjective errors on identifying cell types such as endothelial cells, inflammatory cells and fibroblast were reduced by confirmed at high magnification prior to scoring. For  $\alpha$ SMA expression, the intensity of positive cells was scored as 0 for no staining, 1 for cells visible at high power and 2 for cells visible at medium power. For the  $\alpha$ SMA expression pattern, scoring criteria by Etemad-Moghadam *et al.* were modified as follows: 0-absent to 1, 2 or 3 for low expression and 4, 5 or 6 for high expression of  $\alpha$ SMA<sup>+</sup> cells.<sup>[12]</sup> The intensity for PDCD4 expression was scored as 0 for no staining, 1 for mild to moderate brown, 2 for dark brown as per modified Reis and Tomenson criteria.<sup>[13]</sup> To assess percentage of expression for both  $\alpha$ SMA and PDCD4, percentage of positively stained fibroblast were scored as 0 = 0%–10% positive cells, 1 = 11%–30% positive cells, 2 = 31%–60% positive cells and 3 = 60%–100% positive cells per field. A cumulative total score representing the intensity and percentage was expressed as 0, 1 and 2 indicated low/normal expression pattern while 3, 4 and 5 indicated overexpression pattern.

### Statistical analyses

Expression data for PDCD4 and  $\alpha$ SMA were tabulated in Excel and analyzed in SPSS (version 16.0, IBM, Seattle, Washington, USA) for statistical significance. To test the association between the two, the Chi-square and Fisher

exact tests were performed, and their correlation was determined using the Spearman's correlation test.

## RESULTS

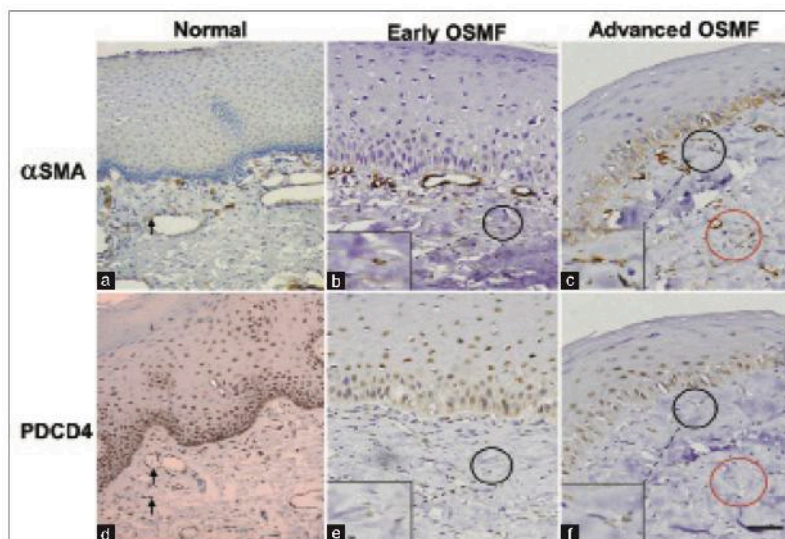
### *$\alpha$ SMA expression and localization within connective tissue cells*

All the cases of normal mucosa and 10 of 15 (66.7%) of early OSMF cases demonstrated a low frequency of fibroblasts expressing  $\alpha$ SMA [Figure 1a]. Amongst these, eight cases of normal mucosa presented with an absence of staining for  $\alpha$ SMA fibroblasts. Total expression of  $\alpha$ SMA showed similar results in normal mucosa and early OSMF ( $P = 0.061$ ) [Table 1]. In the advanced OSMF group, 13 out of 15 cases (86.7%) presented with a high frequency of  $\alpha$ SMA myofibroblasts. The frequency of  $\alpha$ SMA myofibroblast between normal mucosa and early OSMF versus advanced OSMF groups was statistically significant ( $P = 0.001$  and  $P = 0.0008$ , respectively) [Table 1]. On examining their localization,  $\alpha$ SMA<sup>+</sup> myofibroblasts were present within superficial connective tissue and epithelial-connective tissue junction in early OSMF cases [Figure 1b]. In advanced OSMF cases, the localization varied and could be appreciated even in the

deeper stroma [Figure 1c]. Taken together, normal mucosa and early OSMF cases presented with a low frequency of  $\alpha$ SMA fibroblasts, while advanced OSMF exhibited high frequency ( $P = 0.001$ ) [Table 2]. These results clearly established the preponderance of  $\alpha$ SMA<sup>+</sup> myofibroblasts with progressive OSMF stages.

### *Programmed cell death 4 expression and localization within connective tissue cells*

Next, we examined PDCD4 intensity and pattern in these tissues. PDCD4 expression is predominantly limited to the nucleus, with low cytoplasmic expression in normal cells. We observed positive staining for fibroblasts in all three groups [Figure 1d-f]. Nuclear PDCD4 staining in the epithelium in normal and early OSMF was evident, while it appears to be predominantly cytoplasmic in advanced OSMF. A significant reduction in total PDCD4 expression in early OSMF ( $P = 0.05$ ) and advanced OSMF ( $P = 0.001$ ) was observed compared to normal mucosa [Table 3]. PDCD4 expression among the OSMF groups appeared to be similar. Next, we examined the pattern of PDCD4 expression in these tissues and noted lower percentage of positive cells with increasing grade of fibrosis. Overall, the



**Figure 1:** Consecutive histological sections of normal oral mucosa, early and advanced Oral submucous fibrosis stained with  $\alpha$ SMA, and programmed cell death 4. (a) Photomicrograph of normal oral mucosa stained with  $\alpha$ SMA with positive cytoplasmic staining of  $\alpha$ SMA (arrows), predominantly in a perivascular location; (b) Photomicrograph of early Oral submucous fibrosis showing  $\alpha$ SMA positive fibroblasts in the connective tissue stroma with a high-power magnification (black circle and inset image); (c) Photomicrograph of advanced Oral submucous fibrosis showing  $\alpha$ SMA positive fibroblasts in the juxtaepithelial (black circle) and deeper connective tissue stroma (red circle), inset shows high-power image with prominent  $\alpha$ SMA expression; (d) Photomicrograph of normal oral mucosa stained with Programmed Cell Death 4 showing epithelium, stromal fibroblasts (marked by arrow) and a nuclear and peri-vascular localization; (e) Photomicrograph of early oral submucous fibrosis showing mild programmed cell death 4 expression in stromal fibroblasts (black circle and inset image); (f) Photomicrograph of advanced oral submucous fibrosis showing few programmed cell death 4 positive fibroblasts throughout superficial (black circle) and deep connective tissue (red circle), inset shows high-power image noting lack of prominent staining

differences in PDCCD4 staining among the three groups were strikingly significant ( $P = 0.001$ ) [Table 4]. These observations established a reduced expression of PDCCD4 were consistent with prior observations noting PDCCD4 in tissue fibrosis.

#### Correlating $\alpha$ SMA and programmed cell death 4 expression in fibroblasts

Finally, to examine the  $\alpha$ SMA and PDCCD4 expression in fibroblasts, we compared immunostaining in the consequent section in each case. Normal oral mucosa and advanced OSMF demonstrated an inverse relationship between  $\alpha$ SMA and PDCCD4 staining using Spearman's rank correlation (correlation coefficient =  $-0.71$ ) [Table 5].

## DISCUSSION

Oral tissues are well known to be minimally fibrotic, leading

to comparisons with fetal-like nonscarring wounds.<sup>[14,16]</sup> The significant immune-active surveillance, including the role of saliva in the oral cavity, has been correlated with a mitigated inflammatory response that appears to shift the tissue healing response to a nonscarring resolution. Several studies have examined the role of matrix molecules (small leucine proteoglycans), adhesion molecules (integrins  $\alpha$ V $\beta$ 6, Connexin 43, and CD 44), and growth factor isoforms (IGF- $\beta$ 3 versus  $\beta$ 1) in mediating this phenotype.<sup>[17-23]</sup> It appears to be a critical teleological adaptation that enables normal oral functions due to injury and rapid healing necessary for normal oral physiological functions. In this context, an oral disease with prominent clinical manifestation of fibrosis, as evident in OSMF is a striking dichotomy from the normal oral pathophysiological responses. Normal oral healing has transient expression of myofibroblasts, analogous to cutaneous wounds but has been demonstrated to involve minimal matrix synthesis and contraction.<sup>[14]</sup> Myofibroblasts play key, permissive roles in

**Table 1: Comparison of the alpha-smooth muscle actin expression of fibroblasts in normal oral mucosa, early oral submucous fibrosis, and advanced oral submucous fibrosis cases using the Fischer's exact test**

Groups	$\alpha$ SMA expression (intensity + percentage)			P		
	Low	High	Total	1 versus 2	2 versus 3	1 versus 3
Normal mucosa (1)	10 (100)	0	10 (100)	-	-	1 versus 3
Early OSMF (2)	10 (66.7)	5 (33.3)	15 (100)	0.061 (NS)	2 versus 3	0.008*
Advanced OSMF (3)	2 (13.3)	13 (86.7)	15 (100)	-	0.001*	

Fisher's exact test, \* $P \leq 0.05$ , PDCCD4 expression scores: 0-2 low, 3-5 high. NS: Not significant,  $\alpha$ SMA: Alpha-smooth actin, PDCCD4: Programmed cell death 4, OSMF: Oral submucous fibrosis

**Table 2: Comparison of the alpha-smooth muscle actin expression pattern of fibroblasts in normal oral mucosa, early oral submucous fibrosis, and advanced oral submucous fibrosis cases using Chi-square test**

Groups	$\alpha$ SMA pattern (intensity + percentage)			P
	Low/normal expression	Over expression	Total	
Normal mucosa (1)	10 (100)	0	10 (100)	0.001*
Early OSMF (2)	10 (66.7)	5 (33.3)	15 (100)	
Advanced OSMF (3)	2 (13.3)	13 (86.7)	15 (100)	

Chi square test, \* $P \leq 0.05$ , PDCCD4 expression scores: 0-2 low, 3-5 high.  $\alpha$ SMA: Alpha smooth actin, PDCCD4: Programmed cell death 4, OSMF: Oral submucous fibrosis

**Table 3: Comparison of Programmed Cell Death 4 expression of fibroblasts in normal oral mucosa, early oral submucous fibrosis, and advanced oral submucous fibrosis cases using the Fischer's exact test**

Groups	PDCCD4 expression (intensity + percentage)			P		
	Low	High	Total	1 versus 2	2 versus 3	1 versus 3
Normal mucosa (1)	0	10 (100)	10 (100)	-	-	1 versus 3
Early OSMF (2)	6 (40)	9 (60)	15 (100)	0.050*	2 versus 3	0.001*
Advanced OSMF (3)	11 (73.3)	4 (26.7)	15 (100)	-	0.139 (NS)	

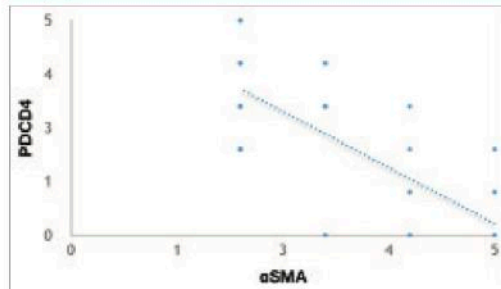
Fisher's exact test, \* $P \leq 0.05$ , PDCCD4 expression scores: 0-2 low, 3-5 high. NS: Not significant, PDCCD4: Programmed cell death 4, OSMF: Oral submucous fibrosis

**Table 4: Comparison of Programmed Cell Death 4 expression pattern of fibroblasts in normal oral mucosa, early oral submucous fibrosis, and advanced oral submucous fibrosis cases using Chi-square test**

Groups	PDCCD4 pattern (intensity + percentage)			P
	Low/normal expression	Over expression	Total	
Normal mucosa (1)	0 (0)	10 (100)	10 (100)	0.001*
Early OSMF (2)	6 (40)	9 (60)	15 (100)	
Advanced OSMF (3)	11 (73.3)	4 (26.7)	15 (100)	

\* $P \leq 0.05$ , Chi-square test, PDCCD4 expression scores: 0-2 low, 3-5 high. PDCCD4: Programmed cell death 4, OSMF: Oral submucous fibrosis

**Table 5: Correlation between Programmed Cell Death 4 and alpha-smooth muscle actin expression of fibroblasts in the study groups using Spearman Rank Correlation Test**



Group	Correlation coefficient	Interpretation
Overall	-0.71	High negative correlation

PDCD4: Programmed cell death 4, αSMA: Alpha-smooth actin

the progression of malignant tissues.<sup>125,241</sup> Given the reported premalignant potential for OSMF, the role of myofibroblasts in determining its biological behavior has important clinical implications.

Recent advances in matrix biology have unraveled a broad heterogeneity in fibroblasts within connective tissue.<sup>125</sup> While the precise origin of the myofibroblasts remains unclear, phenotypic similarities have suggested that they arise from peri-vascular (pericyte) and stromal fibroblasts.<sup>241</sup> A key signaling pathway, TGF-β1, has been well established as a primary inducer of the αSMA differentiated myofibroblast phenotype.<sup>151</sup> Further, several downstream signaling intermediates such as H1C5, miR21, and PDCD4 have been implicated in the TGF-β driven process leading to organ fibrosis and tumor stroma modulation.<sup>16-8,27-271</sup> Among them, PDCD4 is known to regulate cell apoptosis and has roles in the activated fibroblast phenotype. The presence of nuclear (active) versus cytoplasmic localization of PDCD4 reflects its functional state in various cell types.<sup>125</sup> Loss of PDCD4 expression is associated with increased cell survival and changes in cellular functions. However, differences in apoptosis sensitivity and rate cell turnover of oral versus cutaneous cells have been well reported.<sup>121</sup>

This study was aimed at examining the role of PDCD4 and myofibroblasts in OSMF. As anticipated, there was a distinct increase in the number of αSMA myofibroblasts in progressive OSMF stages, as reported previously.<sup>122-241</sup> Moreover, we noted PDCD4 expression in normal mucosa and early OSMF was predominantly nuclear in the stromal fibroblasts. Interestingly, the advanced cases of OSMF showed either low or absence of nuclear PDCD4 expression

in the stromal fibroblasts. Further, consecutive sections stained with PDCD4 and αSMA demonstrated a high negative correlation in advanced OSMF cases. These results are consistent with recent reports by Zang *et al.* and Sun *et al.*, who reported αSMA myofibroblasts lacked PDCD4 expression in hepatic and renal fibrosis, respectively.<sup>17,8</sup> They implicate the lack of PDCD4 expression to a TGF β driven miR 21 and activation protein 1 feedback loop during myofibroblast transformation. Thus, our findings are in congruence and support the hypothesis that PDCD4 plays a central role in myofibroblast differentiation leading to tissue fibrosis.

OSMF represents both features of a fibrotic stroma and an atrophic epithelial component with discrete histological and clinical courses in stark contrast to oral squamous cell carcinomas.<sup>125,241</sup> Recent reports have emphasized that the OSCC arising in preexisting OSMF has a more verrucous and ulcero hyperproliferative nature with an indolent clinical course.<sup>125-381</sup> These together suggest that while OSMF is a degenerative (epithelial atrophy) and aberrant healing (stromal fibrosis) response, a secondary crucial transformative event is necessary for malignancy. The chronic, sustained exposures of epithelial components to the injurious agent (areca nut, tobacco, or combinations) along with permissive changes in the underlying stroma such as fibrosis and hypoxia may contribute to the low but consistent, premalignant potential of OSMF. While the key molecular event mediating this transformation remains to be fully investigated, a potential candidate could be PDCD4 with its low, cytoplasmic expression evident in the OSMF epithelium as well as other dysplastic, premalignant oral lesions.<sup>121,3</sup> These observations are supported by the well-documented tumor suppressor role of PDCD4 in carcinomas from multiple anatomical sites such as cervical, colorectal, endometrial, breast, pancreatic, prostate, gastric, brain, esophagus, lung and ovarian cancers, among others.<sup>125-127</sup> Thus, PDCD4 expression in oral premalignant lesions, including OSMF in their epithelial compartment, could potentially useful for prognostication of clinical risk, protracted surveillance, and monitoring.

## CONCLUSION

This study noted a negative correlation of PDCD4 expression with increased myofibroblast differentiation in OSMF. Future investigations can focus on therapeutically targeting the TGF-β regulated miR21-PDCD4-mediated stromal signaling for OSMF management.

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Nil.

**Conflicts of interest**

There are no conflicts of interest.

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