"ASSESSMENT OF EFFECT OF CURCUMIN ON ARECOLINE INDUCED HUMAN BUCCAL MUCOSAL FIBROBLASTS: A CELL CULTURE STUDY"

Thesis Submitted to

THE KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH, BELAGAVI

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In the Faculty of

Dentistry

By

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(Registration No: KLEU/Ph.D./2015 -16 /DO1215006)

Under the Guidance of

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KLE V. K. Institute of Dental Sciences KAHER

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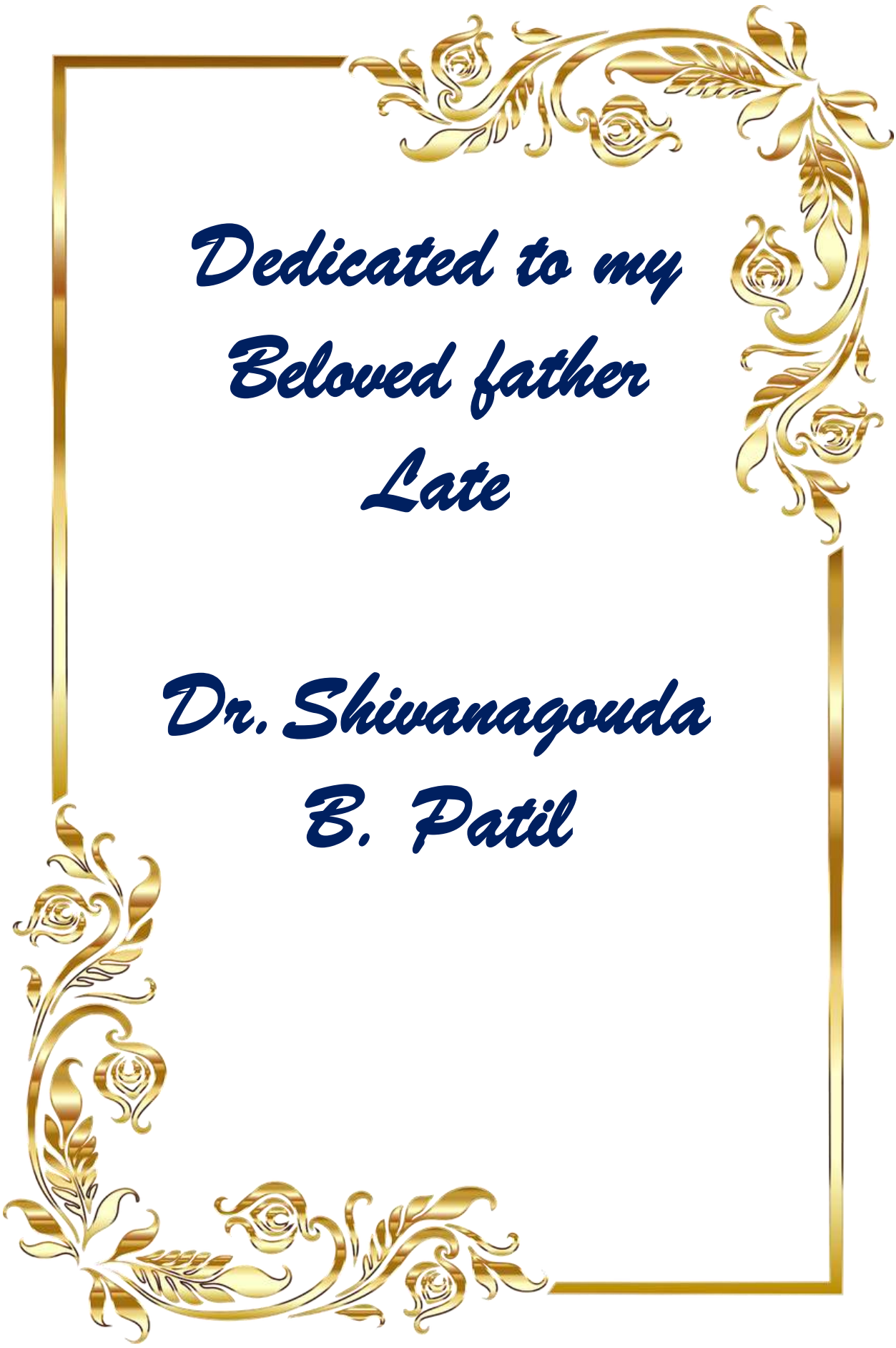
Dedicated to my
Beloved father
Late
Dr. Shivanagouda
B. Patil

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Dr Ritiha S Patil…

LIST OF ABBREVIATIONS

AH	ArecolineHydrobromide
ANE	Arecanut Extract
ANOVA	Analysis of Variance
BMF	Buccal Mucosal Fibroblasts
BMP's	Bone morphogenetic proteins
BSL	Biosafety cabinet laminar.
BQ	Betel quid
CAF	Cancer associated fibroblast.
CD	Cluster differentiation
CTGF	Connective Tissue Growth Factor
DAPI	Diamidino-phenylindole.
DMEM	Dulbeccos Modified eagles Mediam
DMSO	Dimethyl Sulfoxide.
DNA	Deoxyribose Nucleic Acid.
EDTA	EthylediamineTetraacetic acid
EGF	Endothelial Growth factor
FBS	Fetal bovine Serum
FCS	Fetal Calf Serum
FGF	Fibroblast growth factor
FN	Fibronectin
FSP	Fibroblast Specific protein
GH	Growth Hormone.
GAG	Glycosaminoglycans
HA	Hyaluronidase

IARC	International Agency for Research and cancer.
IHC	Immunohistochemistry
IF	Immunofluroscence
LDH	Lactate Dehydrogenase
LOH	Loss of Heterozygosity
MF	Myofibroblast
MMP's	Matrix metalloproteinases
MSC	Mesenchymal Stem cells
MTT	Thyzolyl blue tetrazoliumromide
NK	Natural Killer
NR	Neutral Red
OC	Oral Cancer
OPL	Oral premalignant Lesion
OSMF	Oral submucous Fibrosis
OSCC	Oral Squamous Cell Carcinoma
PBS	Phosphate buffer Saline
PMD	Potentially Malignant disorders.
TGF	Transforming Growth Factor
STR	Short Tandem Repeat
XTT	Methoxy nitro Sulfophenyl.

ABSTRACT

Introduction: Oral cavity is lined by the oral mucous membrane that is made up of an overlying epithelial layer and an underlying connective tissue. Fibroblasts are the principle cells of the oral mucosa and buccal mucosa which in normal condition secrete collagen and elastin fibres. In pathological condition fibroblasts actively take part in wound healing. An abnormal increased secretion of collagen (Fibrosis) by the cells is noted in both systemic and oral diseases. One of the common conditions associated with fibroblast pathologies is Oral sub mucous fibrosis (OSMF). OSMF is a multi-factorial pathological disorder in which arecanut plays a major role for the pathogenesis of the precancerous condition affecting the fibroblasts at both cellular and molecular level. Despite many treatment modalities like cessation of habit surgical excision, diathermy, the disease still remains irreversible .Recent clinical studies have proved that the use of herbal products like curcumin, neem, tulsietc. have improved the signs and symptoms of OSMF. As curcumin is considered a safe natural product by Food and Drug Administration (FDA), it becomes a choice of herbal product in ourstudy.

Aims of the study: Our study aims to isolate the buccal mucosal fibroblasts from healthy individuals and assess the effect of arecoline, curcumin and curcumin on arecoline induced cells for morphological and cytotoxic alterations.

Materials and methods: Isolation and primary culture was done using a combination technique explant enzamytic technique. All the chemicals were procured from Gibco, Himedia and sigma Aldrich. The assessment of each compound was done by morphological assay and cytotoxic assessment using MTT, Neutral red uptake and LDH Assay (kit based method).

Results and observation : The results were statistically analyzed using and spread on excel sheet using SPSS software version 24 .The morphological assay showed that arecoline at higher concentration altered the morphology of cells from spindle (F1) to more stellate (F3) and epitheloid shape (F2) .Whereas curcumin maintained the spindle F1 shape in all the concentrations.(Higher and lower).The cytotoxic assay of MTT, Neutral Red and LDH assay were similar where arecoline proved to be cytotoxic at higher concentration (500-125µg/ml).Whereas curcumin was nontoxic maintaining the cell viability in almost all the concentration promoting the proliferation of cells.

Conclusion: Our results of morphological and cytotoxic assays suggests that curcumin proves to be a safe nontoxic compound maintaining both morphology and cell viability of cells. Hence when our results correlated with the clinical trials of Curcumin in OSMF, curcumin is safe herbal compound in the management of Oral submucousfibrosis.

Keywords: Oral sub mucous fibrosis, Buccal mucosal fibroblasts, Arecoline, Curcumin, Lactate dehydrogenase.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF GRAPHS

INTRODUCTION

Oral cavity is lined by the oral mucous membrane protecting the underlying tissue from the stress either due to external forces from the tissue or due to mastication. The mucous membrane has an outer epithelium and underlying connective tissue known as lamina propria comprising of cells. The major cells of lamina propria include fibroblasts, macrophages, mast cells, and inflammatory cells and adipocytes. Fibroblasts are the principle cells responsible for the synthesis of collagen fibres, regulation of deposition of extracellular matrix and ground substance. Fibroblasts originate from primitive mesenchyme and hence express protein vimentin, a marker of tissue of mesodermal origin.[1]

Fibroblasts form extracellular fibers of the connective tissue proper and mainly produce Collagen and Elastin. Cytoskeleton of Fibroblast consists of microtubules of 240 nm, tubulin which maintains cell shape and intracellular structure. Filaments are made of microfilaments with intracellular "Muscles" are of <80 nm in thickness that help in contractile, cell motility. [2]

Fibroblasts of connective tissue are the major cells responsible for the secretion of components of extracellular matrix, thus providing a supportive framework which maintains the integrity of the cells, tissue and organ. Fibroblasts also play vital role in wound healing by depositing the extracellular matrix and restoring the structure of injured tissue. Fibroblasts secrete many chemo tactic molecules like fibrin, fibronectin and proteins like "platelet Derived Growth Factor, platelet factor 4 and β-thromboglobulin" which function as key factors at the site of

wound. Fibroblasts along with integrin receptors migrate and accelerate the process of wound healing by secreting the matrix.[3]

Fibroblasts usually divide at a slower rate in resting tissues, but highly proliferative in tissue subjected to tissue injury. The proliferation is generally driven by growth factors like fibronectin, fibroblast Growth factor (FGF).Whenever there is an injury the fibroblasts migrate to the site of injury and proliferate to produce large amounts of collagenous matrix, which helps to isolate and repair the damaged tissue. In tissue remodeling the fibroblasts secrete enzymes like Matrix Metalloproteinase's (MMPs) that degrade the extracellular matrix and further facilitate secretion of one of the major growth factor like Fibroblast growth factors (FGFs) that stimulate or inhibit cell proliferation, differentiation, inflammation, or angiogenesis.[2]

Fibroblasts when subjected to pathological stress either due to physical or chemical injury result in proliferation and increased synthesis of collagen leading to fibrosis. Increased proliferation of fibroblasts is seen in conditions like atrial fibrosis, cirrhosis, myelofibrosis, scleroderma, Oral Submucous fibrosis, gingival enlargement, fibromas, etc.[2] the ability of fibroblasts to proliferate during injury and also due to injury caused by chemicals makes these cells a unique nature to grow in in-vitro conditions. This unique feature has made fibroblasts a topic of interest for the cell biological researchers.[3]

One of the common generalized states of disease in oral cavity where altered fibroblasts are involved is Oral submucous fibrosis. "It is a premalignant condition where there is fibrosis in the connective tissue of buccal mucosa leading to stiffness of buccal mucosa, restricted mouth opening and burning sensation". The patients present with clinical signs and symptoms of intolerance to spicy food, rigidity in

buccalmucosa and other parts of mouth eventually leading to restricted mouth opening with paleness of the oral mucosa, burning sensation, presence of fibrous bands, and oral ulceration . The etiological factors that have reported to date are areca nut, chilies, deficiency of micronutrient like iron, zinc, vitamins. Recent studies and research have revealed that demonstration of antibodies and HLA antigens thus proving autoimmune basis for the pathogenesis of the disease.[4]

Among all the etiological factors, betel nut or arecanut is considered to be most important factors for OSMF. Arecanut has four major alkaloids like arecoline, arecadine, guvacoline, guvacine. Arecoline a major alkaloid of arecanut is reported to cause the toxicity at cellular and genetic levels of human cells leading to cancer.[3,4] International Agency of Research Cancer by World Health Organization has grouped arecanut a Group I carcinogen.[5]

Harvey et al conducted a study where they proved that Arecoline stimulates the fibroblast proliferation. An exposures of 0.1-10μg/ml stimulates fibroblasts proliferation and a concentrations of 25 μg/ ml, inhibits fibroblast growth and collagen synthesis. Jeng et al in their study reported that arecoline induced genetic defects in the cells even at lowest concentrations.[6]

The treatment of oral sub mucous fibrosis involves foremost the cessation of habbit followed by surgical excision, steroid therapy, diathermy, hyaluronidase and physiotherapy. Despite of so many treatment modalities available, the disease relapses and is still found to be irreversible. In the past two decade, a lot of studies are being carried out in treating Oral sub mucous fibrosis using herbal products like Curcumin, Propolis, Aloe Vera, Tulsi. [8]

For thousands of years, the use of plants and herbs as therapeutic approach in many diseases has renewed an interest and hope alternative therapies of natural substances with minimal toxicity related to health.8 Ancient studies have revealed the extensive use of Turmeric in India as a spice and also as a medicament in many disease. Literature and research in the past century has proved the antioxidant and anti-cancerous properties of turmeric and indicated it as an effective phyto-therapeutic measure for the treatment of cancerous lesions. Curcumin is derived from a perineal plant that belongs to ginger family and is named as Curcuma longa. Curcuma extract contains three curcuminoids namely, "curcumin, demethoxycurcumin and bis demethoxycurcumin".[9]

The scientific literature shows a large evidence that the contents of curcumin has a wide range of biological and pharmacological actions like "anti-oxidant, anti-inflammatory, anti- bacterial, anti-fungal, anti-parasitic, ant mutagen, anti-cancer and detox properties."[10, 11]

Curcumin with its chemo sensitizing and radio-sensitizing properties inhibits the growth and metastasis. Curcumin has the ability to inhibit tumour growth when given orally by inhibiting the proliferation and migration of fibroblasts. It also inhibits the growth of new vessels thus inducing apoptosis in cancer cells.[11,12,13]

Since ages, curcumin has a wide range of applicability like in inflammatory conditions, cancer, wound healing and ulcers etc. In recent years its molecular potency has been extensively studied. It's reported nontoxic capacity and good tolerability in humans in clinical trials has urged the researchers to use it as a preventive and modifying compound compound in the treatment of OSMF.[12]

Several clinical studies are being carried out to establish the potency of curcumin in the treatment of OSMF. Shrivastav et al in the year 2013, found that curcumin when used in the form of paste and applied to the OSF patients showed effective results with increased the opening of mouth and reduced irritation and burning sensation.[114]

Agarwal et al in the year 2014 studied the effect of curcumin OSMF, the diagnosed patients were given curcumin in the form of tablets and were kept under observation at regular intervals. The results showed an increase in the mouth opening of 10.4 mm in these cases.[111]

In the year 2015, Hazare et al conducted a study on a group of patients with OSF where curcumin was applied in the form of lozenges for 3 months duration and found that curcumin showed an increase in the mouth opening of 5.4 mm.[112]

In a study done by Yie Deing Tang et al in the year 2009 , the molecular effects of arecoline and curcumin on tissue growth factors was observed and they found that arecoline increased the production of Connective Tissue Growth Factor(CTGF) in buccal fibroblasts. Further the immunohisto-chemical staining proved the presence of CTGF in the cells. CTGF expression indicates the fibrotic activity of the cells in the OSMF condition.[81]

Based on the above literature background, the role of curcumin found to be effective and proven to be one of the natural therapeutic targets in several diseases. However limited literature supports the molecular mechanism of curcumin on arecoline induced oral mucosal fibroblasts. Thus the aim of our study is to develop primary cell lines of BMF form the healthy individuals and further subject to arecoline and curcumin to study the morphological and cytotoxic changes, so as to propose curcumin as treatment option in Oral Submucous Fibrosis.

REVIEW OF LITERATURE:

Human body tissues are usually lined by the epithelium and supported by fibrous connective tissue. Epithelium layer comprises of cells that are well adapted to the connective tissue, thus providing support and nutrition. The tissues that are lined by epithelium or mucosa are gastrointestinal tract, oral cavity, skin, the small and large intestine esophagus. The epithelium of any tissue reflects the functional adaptability of the organs. The stomach and the small intestine is lined by a layer of simple epithelium which helps in absorption. Whereas the skin, oral cavity and esophagus are covered by multiple layers of cells called the stratified epithelium that further differentiates into other cells.[1]

The oral and esophageal mucosa has a moist surface, absence of appendages unlike the epithelium of skin and other parts of body. The skin possesses hair follicles, sebaceous and sweat glands. The oral cavity and esophagus usually consists the major and minor salivary glands which secrete the saliva and are responsible for maintaining the moisture of oral cavity.[2]

The lining the mouth is the oral mucous membrane with oral epithelium and the connective tissue that is lamina propria. Its structure and function of oral mucosa has been extensively discussed and studied both at the cellular and molecular level. The oral cavity is considered as complex biological network of individual it becomes a subject of interest to study the normal physiological and pathological process of diseases. The Tissues, the cells, their embrylogical relationship with other mucosa and as a gateway for a huge influx of microbial have made oral mucosa the gatekeeper / housekeeper functions of the mouth.[3]

The layers of oral epithelium have been divided into four that is the basal layer stratum basale, stratum spinosum, stratum granulosum and stratum keratinosum or keratin layer.[3]

Depending on the region of mouth the epithelium is either keratinized or non-keratinized. The non-keratinized squamous epithelium covers the soft palate the inner lips, buccal mucosa and the floor of mouth whereas stratified squamous keratinized epithelium lines gingival, hard palate and the dorsal surface of tongue, lamina propria is thick and fibrous layer consisting collagen and elastin fibers and cells like fibroblasts mainly with mast cells, plasma cells, red blood cells and lymphocytes. The fibroblasts form the major part of connective tissue with many functions in both physiological and pathological condition. The lamina propria , has a papillary layer which is the superficial layer and is made up of loose connective tissue with some blood vessels and nerve tissue. The other layer of lamina propria has two layers the papillary and deep layer with blood vessels and dense fibers. The chief cells of the connective tissue of oral mucosa are the Fibroblast cells that produce and maintain the extracellular matrix. The physiological fibroblast is termed as fibrocyte which is less active and stable when compared to fibroblasts that are highly active. The mature fibroblasts which are termed as fibrocyte the current studies and research have emphasized mainly on the regenerative capacity of the fibroblasts used in tissue culture. In 2006, the mouse fibroblasts were transformed into cells with combined features of embryo and stem cells with the use of transcription factors. This led to a major success in the field of Tissue Culture and regenerative research and the cells were further named as IPSc (induced pluripotent stem cells). In 2007, IPSc were obtained from human fibroblasts. Thus these studies opened a new path for the tissue and cellular culturing which is different from Cloning of cells.[4]

Development and Origin of Fibroblasts.

Primary fibroblasts (PF) basically originate from the primitive mesenchyme and undergo epithelial mesenchymal transition. The primitive mesenchyme forms mesoderm and Subsequently to other germ layers. The mesoderm further leads to the formation of more mature fibroblasts with connective tissue. Fibroblast, the principal active cell of connective tissue that is flat and elongated spindle shape cells with flat oval nucleus and elongated processes extending from both the ends. Fibroblast produce collagen, gel like ground substance filling up the spaces of cells and tissue.[4]

Fibroblasts also play an important role in healing of wound. When there is tissue injury the fibroblast has the ability to migrate to the site of injury and deposit new collagen and thus help the tissue undergo healing process.[4]

Fibroblasts are one of the main components of connective tissue that are basically spindle shaped with an oval nucleus. They are the primary source of extracellular matrix and connective tissue stroma which provide support for the tissue. Fibroblasts are responsible for the synthesis and regulation of extracellular matrix and collagen.[5]

Structure:

Fibroblasts originate from the mesenchymal cells which are elongated, spindle shaped with extensions from cytoplasm. The cytoplasm consists of rough endoplasmic reticulum (rER) and a large Golgi apparatus. Fibroblast is the term used to describe these cells when they are in an activated state whereas fibrocyte refers to the less active state, when the cell is involved in tissue maintenance and metabolism.[5] The basic spindle shapes of fibroblasts gets altered in both physiological state either

getting transformed into another cells like myofibroblasts or in pathological condition like fibrosis, cancers etc.[6]

Origin

The cells take their origin from the primitive meshyme and therefore express the vimentin as their main protein which is the marker of cells that are derived from mesoderm. The epithelial cells may also give rise to fibroblasts due to epithelial mesenchymal transition and this process can go vice versa leading to the development of growth of the tumour.[7]

Function :

Wound Healing

Wound healing is the response of the tissue to any injury that begins immediately after injury. It an organized process that involves cellular hormonal and molecular mechanism of the body. The most important cells involved in wound healing are the fibroblasts. Fibroblasts mainly participate in wound healing by proliferation that helps in tissue regeneration by secreting the extracellular matrix and collagen. Further Fibroblasts help in contraction of the matrix to close the wound of the tissue. Apart from proliferation and synthesis of matrix the cells take part in clotting mechanism by the synthesis of urokinase plasminogen activators.[8]

Inflammation

Inflammation is a complex process that reveals the hosts needs to deal effectively with injury and the injurious agents to maintain a balance in the tissues and organ. Fibroblasts produce the extracellular matrix proteins that mediate the process

of inflammation. The cells produce inflammatory cytokines like Tumour Growth Factor β1, Interleukin -1β, interleukin-6 (IL-6), IL- 13, IL-33, prostaglandins and leukotrines. The factors secreted by fibroblast activate the cells and help to migrate the immune cells. The fibroblast cells also produce proteins that activate the nonresident immune cells and help in maintenance of fibroblast function in acute and chronic inflammation as well as in chemical and cell mediated immunity.[8]

Angiogenesis.

Angiogenesis is the formation of new blood vessels seen in physiological condition like embryonic development, wound healing and pathological condition like tumour growth. Fibroblasts have been mainly involved in the synthesis and maintenance of extracellular matrix and connective tissue, apart from that the cells play a key role in angiogenesis. A number studies have revealed that the cells secrete angiogenic growth factors like "vascular endothelial growth factor (VEGF), transforming growth factor β (TGF-β), platelet derived growth factor (PDGF)." Fibroblast interacts closely with endothelial cells and carries the process of angiogenesis in the existing blood vessels. Many studies have reported the over expression of VEGF in squamous cell carcinoma patients in response to anticrine TGFβ signaling. The VEGF actively takes part in the vascular damage which further activates the fibroblasts to produce collagen leading to fibrosis.[7]

Fibroblasts are the most common cell type found in connective tissue which produce collagen (type I, III, IV)"proteoglycans, fibronectin, laminins, glycosaminoglycans, metalloproteinases, and prostaglandins". The cells synthesize the extracellular matrix and reorganize by degredation and crosslinking of enzymes. The cytokines and growth factors like transcription growth factor- alpha and beta

(TGF-A and TGF-B), platelet-derived growth factor (PDGF), colony-stimulating factor, epidermal growth factor (EGF), and tumor necrosis factor (TNF) regulate the enzymes of fibroblasts. The function of fibroblasts varies with their origin and location in the body. The fibroblasts located superficially function in the formation of hair follicle and re-epithelization during wound healing. The fibroblasts of deeper tissue deposit the extracellular matrix and the extracellular matrix. Hence the formation of cells like adipocytes, pericytes endothelial cells that further differentiate into many other cells.[9]

The most common and classic transformation of fibroblast is myofibroblast. The myofibroblast cells are very much similar to the fibroblasts which function both as fibroblasts and smooth muscle cells. The number of fibroblast is usually maintained by the proliferation of resident fibroblast. The cells are also derived from the resident stem cells and mesenchymal tissue cells. Due to dysregulation of the cells leads to the pathogenesis of many diseases resulting in morbidity and mortality.[9]

The role of oral fibroblasts in the inflammatory response in acute wounds: As discussed earlier fibroblasts majorly primarily synthesize the ECM for the repair of the damages tissue during wound healing. The fibroblasts at resting stage are known as sleeper cells which do not produce the extracellular matrix whereas the cells need to be activated either by external or internal stimuli for further synthesis of collagen and fibers that forms the extracellular matrix. The fibroblasts which are at risk take part in homeostasis and are the first cells to respond any damage or tissue injury or any infection .In acute inflammation when the cytokines are released by the platelets, the resident cells are also activated to release added chemokines mostly IL6 and IL-8. They also form a direct cell to cell contact on immune cells.[10]

Pathologies associated with Fibroblasts.

The fibroblast under pathological condition gets transformed into different cells either morphologically or genetically with altered function. In many diseases that have been reported, fibroblasts either secrete excess of collagen leading to increased formation of extracellular matrix leadin Fibrosis is usually observed in many tissue like heart, lung, kidney, liver and Gastro Intestinal tract. Literature has many studies and research where the normal function and morphology are altered in pathogenesis leading to mortality and morbidity of an organ. Hence the different behavior of the fibroblasts is mainly to their origin of sites, the intrinsic and the extrinsic factors like mechanical stress or any toxic compounds acting on the fibroblasts. The mechanism of pathogenesis of fibrosis involves interaction of cell types and cytokines like type 2 CD4 CD40 receptor and ligand interaction, and "cytokines like IL-4, transforming growth factor B(TGFβ), platelet derived growth factor (PDGF)". Some of the common pathologies reported due to fibrosis include skin scarring, hypertrophic scar, cirrhosis of liver, pulmonary fibrosis, cardiac fibrosis, kidney bone marrow fibrosis and scleroderma. The fibrotic tissues possess myofibroblasts that are spindle shaped cells resembling to fibroblasts and smooth muscle cell which express alpha smooth muscle actin (α-SMA) immuno histochemically along with myofilaments observed ultra structurally. These are also called as juxta-epithelial cells as they are commonly seen residing in tissue like skin, pulmonary septa, periodontal ligament. The main function of these cells is their role in wound healing.[11]

Some common fibrotic disorders of oral cavity include Oral submucous fibrosis, Ehlers–Danlos syndrome (EDS), Osteogenesis imperfecta., Stickler syndrome. , Alport syndrome., Marfan syndrome., Epidermolysis bullosa., Systemic

sclerosis, Oral lichen planus, giant cell granuloma, giant cell fibroma and phenytoin-induced gingival hyperplasia.[11]

The Oral submucous fibrosis is the most common and high risk "precancerous condition characterized by changes in the connective tissue fibers of the lamina propria and deeper parts leading to stiffness of the buccal mucosa and restricted mouth opening." OSMF was first described by Shwartz from East Africa in 1908, where it is more commonly found among the g to the stiffness of connective tissue. This condition is called as fibrosis.[12]

The Oral submucous fibrosis (OSF) has been traditionally described as chronic scarring disease of oral cavity involving the pharynx and upper esophagus leading to tightness or rigidness of buccal mucosa, restricted mouth opening, causing trismus. The disease has more prevalence with South and South East Asian countries associated with betel nut or arecanut chewing.[12]

History:

In ancient medicine, Shushrutha has mentioned a condition and named it as "vidari" under mouth and neck diseases and reported the limited opening of mouth, depigmentation of oral mucosa, and irritation in the mouth with intake of food. For the first time a case of "atrophica idiopathica tropica mucosae oris was observed by Schwartz in 1952 in Indians and East Africa. In 1953, the condition was first described by Lal and coined the term "oral submucous fibrosis". Sirsat SM and Pindborg JJ in 1966 described OSMF with four histological stages. Seedat HA, Van Wyk CW in 1988 mentioned the irreversible nature of the precancerous condition even after cessation of betal nut che Prevelance of the disease:[13]

Oral submucous fibrosis (OSMF) is commonly reorted in India and south east Asia. From past 4 decades the prevalence of the disease has increased from 0.03% to 6.42%. Reports and studies have published around 5 million diagnosed cases of OSMF in India. The age of onset of the condition is not specific where even the reports of as early as 14 year old children to about 60 year old patients have developed the precancerous condition with a range of 20-55 years. The onset is insidious with duration of 2-5 years , the clinical complications of the disease is directly proportional to the intensity and form of consumption of tobacco like gutkha, pan masala, flavoured supari etc.The concern of the condition is that the symtoms are irreversible and the malignant transformation rate of oral has been reported to 7.6%.[13]

Etiology:

Oral submucous fibrosis is a multifactorial precancerous condition. Articles and studies reveal various factors for the pathogenesis of disease. The epidemiological data suggests that chewing of tobacco as areca nut is the main etiological factor for OSMF. Other etiological factors include consumption of chilies, diet with nutritional deficiencies such as iron and zinc, autoimmune disorders collagen diseases. .

1. **Tobacco Consumption**

The consumption of tobacco in the form of gutkha ,pan, supari is the one of factor for the pathogenesis of Oral Submucous Fibrosis. These mainly consists of Arecanut derived from betelnut seeds.The arecanut consists of alkaloids that stimulate the fibroblasts in the synthesis of collagen by 150%.The main alkaloids include arecoline, arecaidine, guvacoline, and guvacine . Of all these alkaloids arecoline is the most potential and active ingredient of arecanut in the pathogenesis of OSMF.[17]

1. Immune system: Tobacco or the ebtel nut chewing affects the immune system. The levels of TGF and the interferon's interfere with the pathogenesis of the disease. In patients diagnosed with OSMF the levels of TGF beta and interferons are decreased. Consumption of Chillies and spicy food: The consumption of chillies in the regular diet leads to the progression of pathogenesis of OSMF. The active ingredient of chillies i.e Capsaicin-vanillylamide of 8- methyl-6-nonenicacid play an etiological role in oral submucous fibrosis.[15]
2. Nutritional deficiency:-

   The OSMF cases have been correlated with nutritional defeciencies with decreased values of vit B complex, leading to ulceration and vesiculations of Oral cavity and buccal mucous membrane. Cases have also been reported with Iron deficiency anaemia that interferes with healing and scarring of tissues.[17]

3. Genetic and Immunologic Processes: Any defect in the genetic configuration is poroposed to be an etiological factor for the disease process. OSMF diagnosed patients express HLA-A10, HLA-B7, and HLA-DR3 with or without the habbit of tobacco chewing.
4. Immunological disorders:

   An increase in the ESR levels and globulins indicate the immunological disturbance. OSMF diagnosed patients show an increased levels of Serum antibodies (IgA, IgG and IgM) which have also been reported in OSMF.[15,17,19]

Role of Arecanut in Oral submucous Fibrosis.

The past literature one of the major etiological factors for the pathogenesis of Oral submucous fibrosis various epidemiological studies and histopathological effects on fibroblast and keratinocytes support the chewing of areca nut as one of the most important risk factors for OSMF. Indian Culture has the tradition of chewing arecanut with betel leaves since ancient times. Betel nut is the fourth most commonly used psychoactive substance in the world Betel which is present in mawa, paan, gutkha, khaini, and paan masala. The world health organization has considered International Agency for Research on Cancer (IARC) classified betel nut as a group 1 human carcinogens.[20]

Areca catechu L. is a palm family areca plants, perennial evergreen trees and is widely cultivated in tropical areas.Arecanut is derived from the plant areca catechu L, with husk which is green in colour and the nut is haed in consistency. The seed or endosperm is consumed as fresh or boiled or after sun drying.[21]

Chemical composition:

Shen reported that the main components of the fruits of Areca catechu L. are phenols (31.1%), polysaccharides (18.7%), fat (14.0%), fiber (10.8%) and alkaloids (0.5%). While the main ingredients of its flower are alkaloids and polyphenols.

Polyphenols: The polyphenols in the fruits of Areca catechu L. are phenolic acids, flavonoids and xylophenolic acids. They are mainly present in the roots, stems, leaves and fruits of Areca catechu L., and the amounts of them are related to the growth cycle and maturity.

Flavonoids: Flavonoids are mainly contained in the seed and fruit of Areca catechu L. Zhang et al. obtained four flavonoids from the fruits of Areca catechu.

Tannins: The tannins in Areca catechu L. are tannins (proanthocyanidins), also named as flavanol derivatives, which exist after combining with arecoline and the content in Areca catechu L. is about 15%. Some tannins have been isolated and confirmed as arecatannin, arecatannin , arecatannin , arecatannin , arecatannin arecatannin.[23]

Fatty acids: The fatty acids were mainly contained in the seeds of Areca catechu L., and the total content of fatty acid is about 14%. The lauric acid, myristic acid, palmitic acid, stearic acid , oleic acid , linoleic acid , α-lipoic acid, decanoic acid, decanoic acid.[23]

Amino acids: It is reported that the content of proline and tyrosine in Areca catechu L. The phenylalanine, arginine, a small amount of tryptophan and methionine were also confirmed in Areca catechu. [24]

The constant contact between the betel quid and the oral mucosa, the alkaloids and the flavonoidsfrom the quid are absorbed and undergoes metabolism. These constituents along with their metabolites serve as a constant source of irritation to the oral mucosa.25 Along with this chemical irritation, the mechanical irritation of the oral mucosa also occurs due to the presence of the coarse fibers in the betel quid. The microtrauma resulting from this continues friction of coarse fibers of areca nut facilitates the diffusion of betel quid alkaloids and flavonoids into the subepithelial connective tissue, resulting in juxtaepithelial inflammatory cell infiltration. Due to the persistent habit of areca nut chewing, chronic inflammation sets in at the site. Inflammation is characterized by the presence of activated T cells, macrophages, etc[26]

Cytokines like interleukin 6 (IL 6), tumor necrosis factor (TNF), interferon α and growth factors like Transforming growth factor – beta (TGF-β) are synthesized at the site of inflammation. Initial irritation leads to the further atrophy and ulceration of the mucosa. Persistent inflammation is essential for the occurrence of tissue fibrosis and cancer.[28,29,30]

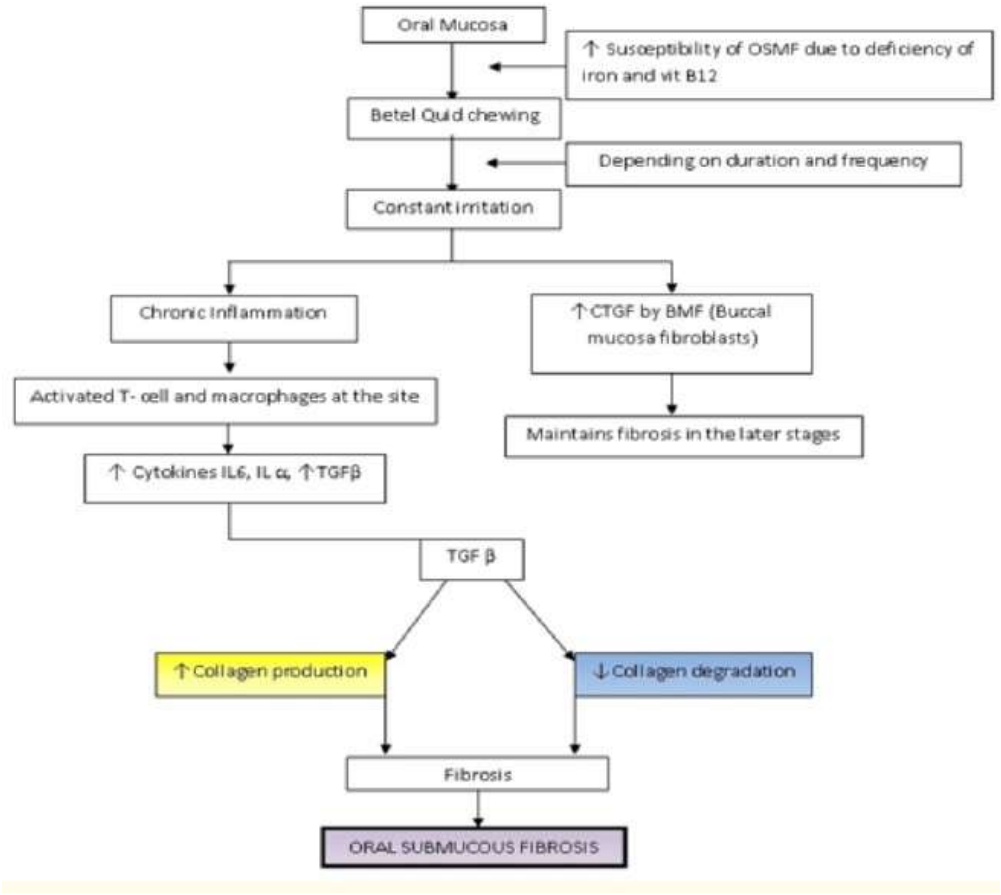

Fig 1. : Role of arecanut in OSMF ***(Rachana V. Prabhu et al J Oral Medicine and Pathology)***

Increased collagen synthesis.

TGF- β is a key regulator of Extra- cellular matrix (ECM) assembly and remodelling. It helps in activation of procollagen genes and elevation of pro-collagen proteinase levels (PCP – Procollagen C-proteinase, BMP 1- bone morphogenetic protein1 PNP – Procollagen N-proteinase).[29]

Procollagen genes are transcribed and translated to form pro-collagen monomeric chains (procollagen precursor). Three of these monomers assemble into a trimer triple helix which is aided by disulphide bridge formation. These trimeric procollagen chains are acted upon by N and C – terminal proteases to cleave the terminal domains and form the fibrils. The newly formed fibrils are then covalently stabilized through cross-linking to form a stable mature structure of collagen.[31.32]

The genes COL1A2, COL3A1, COL6A1, COL6A3, and COL7A1 have been identified as definite TGF- β targets. The transcriptional activation of types I and type VII collagen gene expression by TGF- β has been demonstrated. Due to this it results in increased expression of pro-collagen genes and thus contributes to the increased collagen levels in OSF.[35]

Many invitro studies have been conducted and proved that arecoline is cytotoxic to the normal fibroblasts in a dose dependent manner. Cheing yah yeh in their study ANE exhibited cytotoxicity to C3H10T/12 cells at concentrations higher than 320 mg/ mL as shown by a decrease in colony numbers. ANE-induced inflammatory reactions are important for human response to infection, irritation, and injury, as well as to oral cancer. AN not only induces oral epithelial hyperplasia and

carcinogenesis, but also has an impact on subcutaneous connective tissue resulting in OSF. This can be due to genotoxicity and of BQ components.[37,38]

Clinical presentation depends on the stage of the disease.10 Initially, most patients present with a burning sensation or intolerance to spicy food, and they may have vesicles, particularly on the palate. Ulceration and dryness of the mouth is later followed by fibrosis of the oral mucosa, which leads to rigidity of the lips, tongue, and palate, and trismus The most obvious clinical signs include blanched, opaque oral mucosa with palpable fibrous bands .Furthermore, the overlying epithelium may become dysplastic and malignant. Restricted mouth opening interferes with examination of the oral mucosa, and makes early diagnosis of cancer a daunting task.

Treatment options include iron and multivitamin supplements including lycopene, an extract of tomato, and a range of medicines (e.g. intralesional injection of steroids, hyaluronidase, human placenta extracts, chemotrypsin, pentoxifylline and collagenase). Surgery, including cutting of the fibrous bands and jaw muscles and joint, has been used for more extreme cases.

Apart from the routine standardized protocol for the management of OSMF, there have been many studies on the natural method of treatment of precancerous condition like use of tulsi aloe vera, neem, curcumin etc. Curcumin is an old Indian spice with wide pharmacological actions, has been documented with various clinical trials in treating oral diseases.

CURCUMIN

Curcumin is an Indian spice commonly known as haldi or turmeric. It originates from the rhizomes or the roots of Curcuma longa. The Curcuma longa belongs to a member of the ginger family (Zingiberaceae). It is a yellow colored pigment derived from fat soluble, polyphenolic pigments the main component of curcumin are the curcuminoids namely Curcumin, demethoxycurcumin and bisdemethoxycurcumin. From many centuries in Asia and eastern parts of the world curcumin was introduced and mainly used as a common spice. Further research lead to the exploration of its application with respect to medicinal values.[40]

Historical background of Curcumin

Fig 2: Curcumin root – Curcuma Longa

Kocaadam B,et al . Curcumin, an active component of turmeric (Curcuma longa), and its effects on health.

The Vedic literature and the vedic age has described the use of curcumin nearly 4000 years back in India. The most common application or its use was as a common kitchen or with some religious significance. This Indian spice was expanded to many countries like China during 700 AD, and by 800 -1200 in Africa, and by the end eighteenth century in Jamaica in the. Turmeric or curcumin has been reported with its wide application in medicinal use in Asia as mentioned in Sanskrit literature of Ayurveda and Unani for example in Susruta's branch of Ayurveda. Curcumin has been used as ointment to relieve the pain and side effects of food poisoning.[42]

An epidermiological study reported that curcumin was consumed by as large as complete population of Nepal and no side effects were reported. Marco Polo in 13th century introduced turmeric to Europe and western parts of world. Further studies and research have revealed its wide applications like anti-inflammatory, anticancer, detoxifying agent etc.[44] This could be due the curcuminoids and the polyphenols responsible for the yellow coloured pigment Chemically curcumin exists as two tautomeric forms that is ketol and enol form. The ketol form is less stable when compared with enol form form which is stable and exists in solid form. The "Food and Drug administration (FDA)" have recommended curcumin as a safe agent and a food additive with an E number 100.Recent advances and research had lead to the scientific attention of its medical and therauptic actions in Western world. The "United States Food and Drug Administration has approved Curcumin as "Generally Recognized As Safe (GRAS)", thus proving that curcumin safe food additive and tolerable and does not require a premarket review and approval by any food agency.[45]

Thus with its wide applicability and many properties curcumin in worldwide can be found not just as a spice in the kitchen but also a medicinal alteratative in the form of capsules, tablets ,ointments, soaps, cosmetics in treating many diseases.[46]

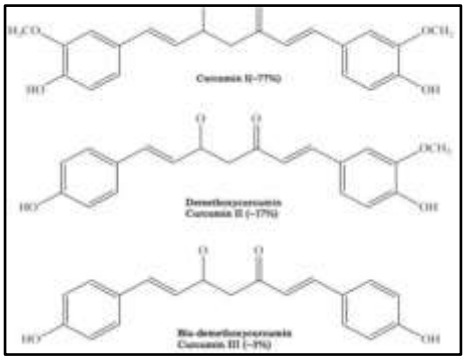

Fig 3: Structure of Curcumin [48]

Structure of Curcumin

The keto enol tautomerism in curcumin is because of the presence of carbonyl groups on the carbon number 3 and 5 in heptadiene ring. The stabilization of enol tautomer with respect to the keto tautomer is due to the conjugation of the carbonyl double bond with the enol double bond and a pi orbital system, i.e., phenyl group in conjugation with the conjugated C=C double bonds. The enol tautomer is characterized by the formation of strong intramolecular hydrogen bonding compared to intermolecular hydrogen bonding which exists in the keto form.The enolisation of curcumin brings about a fundamental change, i.e., the polar keto tautomer is converted to the non-polar enol tautomer. The dependency of the structure of curcumin on solvent has already been proved as it exhibits different λmax in different solvents .Depending upon the polarity of the medium, curcumin exists in different proportion as a tautomeric mixture of keto and enol forms in the medium. Both the tautomers get solubilized in the medium through different forces. Polar-polar

solubilization takes place by means of dipole-dipole forces, while nonpolar–non-polar and polar–non-polar solubilization takes place by means of dispersion forces.

Properties of Curcumin.

Curcumin as mentioned earlier is the most common Indian spice used as a an ingredient in curries and routine meals. Indian ancient literature highlights the use of plants and herbs in medicine since ayurvedic and vedic age.[48] This extraordinary property of herb which can be used both as a food ingredient and also a medicinal component has attained a spotlight in the west in recent decade. Literature has many studies of curcumin with its wide properties like "antioxidant, anti-inflammatory, antiviral and antifungal actions, anticancer etc". Recent advances and studies have proved that curcumin as a non toxic and can be used as an alternative to medicine in treating the diseases. In general to name a few, action of curcumin in inflammation is because of inhibition of inflammatory molecules of Curcumin by lowering the synthesis of inflammatory enzymes like histamine thus prolonging the action of the body's natural hormonal and immune levels leading to improved circulation by toxins.[49] This property of curcumin helps in postsurgical healing. Curcumin acts in atherosclerosis and other cardiac diseases by preventing the blood clot formation. Curcumin is a potent inhitor of apoptotic and inflammatory pathways that is "cyclooxygenase pathway, lipoxygenase and glutathione S-transferase pathway." Its extended actions also include in reduction of reactive oxygen species in process of oxidation. Each properties of curcumin will be discussed in detail in further review.[50,51,55]

MECHANISMS OF ACTION

1. Antioxidant Effects. The antioxidant property of curcumin is mainly due to the water and fat soluble components when compared to Vitamin C and E. Studies have demonstrated that in pathological condition like ischemia of a heart when treated with curcumin reduced the ischemic changes in the heart. Oxidative stress is an imbalance due to reactive oxygen species and ability of these ROS and neutralizes the damage caused at the tissue injury.[57] At normal physiological conditions the cells maintain low levels of ROS, whereas in oxidative stress condition as there are high levels of ROS .modify the mitochondrial function leading to cell death. Research suggests that curcumin is an effective antioxidant agent that reduces the reactive oxygen levels and minimizes the oxidative stress of the cells and tissue by chelating heavy metals and regulate various enzymes. In an vitro study done by Roberto morteni et al where they assessed the effect of curcumin on endothelial heme - oxygenase enzyme which is an inducible stress protein of aorta cells. An incubation of these cells with curcumin for 18 hours resulted in increased resistance of cells.[58,59,60]
2. Anti-inflammatory Effects. Curcumin exhibit potent anti-inflammatory effects during inflammation and is proved by many research and in-vitro studies. Curcumin in acute inflammation was found to be as effective as cortisone or phenylbutazone, and one-half as effective in cases of chronic inflammation. In rats with Freund's adjuvant-induced arthritis, oral administration of Curcuma longa significantly reduced inflammatory swelling compared to controls. [66,69]

   In monkeys, curcumin inhibited neutrophil aggregation associated with inflammation. Curcuma longa's anti-inflammatory properties may be

attributed to its ability to inhibit both biosynthesis of inflammatory prostaglandins from arachidonic acid, and neutrophil function during inflammatory states. Curcumin may also be applied topically to counteract inflammation and irritation associated with inflammatory skin conditions and allergies, although care must be used to prevent staining of clothing from the yellow pigment.[71]

3. Anticarcinogenic Effects. Several animal studies and research involving rats and mice which were proved using in-vitro studies on cell lines proved that curcumin has a potent anticarcinogenic property. The anticancer activity of curcumin can be discussed in three stages namely tumour promotion, angiogenesis and tumour growth. Studies on colon and prostate cancer have proved that administration of curcumin inhibited the tumour growth by suppressing the activity of mutagens and carcinogens. Curcumin is due to its antioxidant and scavenging property further helps in suppression of growth of tumour by indirectly increasing the glutathione levels and thus helping in detoxification of carcinogens.[74,74]
4. Antimicrobial Effects.

   As Turmeric extract consists of many essential oil which has an antibacterial action on bacteria, parasites, and other pathogenic organisms. A study was conducted where the chicks were infected with ceacal parasite Eimera maxima and when supplemented with 1% curcumin resulted in the reduction of bacterial scores in small intestine with weight gain. Curcumin as an antimicrobial was also tested on guinea pigs, where they were infected with pathogenic fungi. Further when the pigs were topically applied with turmeric oil inhibited growth of pathogenic fungi. An improvement in the healing of

lesion was also noted when the turmeric was continued for 8 days with disappearance of the lesions. With its wide range of action on different organisms curcumin can be called as antibacterial,antifungal and antiviral.[74]

5. Cardiovascular Effects: Turmeric has been reported to have protective effect on cardiovascular system. Its actions include decreasing the cholesterol and triglycerine levels, lower the susceptibility of LDL proteins, and inhibit the platelet aggregation. The above effects have been reported to even low levels of curcumin that is 20M. These effects have been noted even with low doses of turmeric. A study was conducted on rabbits where athererosclerosed rabbits were supplemeted with 1.6–3.2 mg/kg body weight of turmeric daily and observed. They found a decreased levels of LDL to lipid peroxidation plasma cholesterol and triglyceride levels. The action of curcumin on cholesterol levels may be due to decreased cholesterol uptake in the intestines and increased conversion of cholesterol to bile acids in the liver. This shows that turmeric had a healing effect on the atherosclerotic and ischemic heart diseases.[77]
6. Effect on Gastrointestinal Tract.

   Constituents of Curcuma longa extract is founf to be protective on the gastrointestinal tract and digestive sytem. The sodium curcuminate of curcumin inhibits the intestinal spasm and increases the secretion of enzymes like gastrin, secretin and pancreatic enzymes. Turmeric also has a healing property in the treatment of gastric ulcers that are caused due to stress, alcohol consumption, indomethacin effects, etc.

7. Curcumin enhances immunity.

   Curcumin along with different other properties has also been reported to play a vital role in enhancing the immunity. It helps in the body to fight off cancer and allow some cells to escape apoptosis. Studies have revealed that the curcumin when ingested enhances the production of antigens and antibodies like in a study done by Gupta et al, where they found that after the ingestion of curcumin the lining of intestine showed a large number of CD4 T helper cells and B type of immune cells. The immune-modulatory property of curcumin is due its interaction with immunomodulators like dendritic cells, macrophages, B and T cells cytokines, and other transcription factors in the signaling pathways. The activity of curcumin to enhance the immune system has also been reported on brain models during brain injury and colitis. Thus curcumin interferes with pathways involved in the disease process and helps in increasing the immune factors and cells.[78,78]

8. Antifibrotic activity of curcumin: Curcumin is a natural safe herbal compound that has been used in the management of various pathological disease and cancerous conditions. To name a few atherosclerosis, acute infections, skin scarring, lung fibrosis, cardiac fibrosis, brain tumour, breast cancer, and in oral conditions like "Oral sub mucous fibrosis, mouth ulcers, squamous cell carcinoma of tongue etc". Besides from the wide action of curcumin , its also plays and important role in fibrotic diseases.[81,80]

   The fibrotic action of curcumin has been reported in many literature and its mechanism of action has been studied. The mechanism is due to decreased synthesis of collagen and its accumulation in the tissues by its antioxidant and anti-inflammatory action. It inhibits the fibroblast proliferation by blocking the

TGFβ1 signaling cascade and up regulating the heme oxygenase -1.It further increases the expression of cathepsin that induces the apoptosis in fibrotic tissue and cells. Thus curcumin is a potent agent used to reduce the fibrosis caused by the excessive proliferation of fibroblasts.[80]

9. Curcumin enhances immunity. Curcumin along with different other properties has also been reported to play a vital role in enhancing the immunity. It helps in the body to fight off cancer and allow some cells to escape apoptosis.[83,84]

Curcumin in Oral diseases

Curcumin is used in many oral pathologies , oral cancer and diseases with the data providing its action on targeted oral diseases. Curcumin is proved to be a potent agent in treating Oral ulcers, oral precancerous condition like "oral submucous fibrosis, and even in squamous cell carcinoma leukoplakia etc".[90]

In a clinical trial done by Wagmore et al curcumin was administered as mouth was in individuals with gingivitis and periodontitis. The results were as effective as the standard chlorhexidine in reducing the symptoms of gingivitis. Behel et in their study selected the periodontitis patients with deep pockets, where the controls were treated with scaling and root planning whereas the experimental group were administered the curcumin gel (2%). They found that both the groups statically demonstrated a reduction in the plaque index, gingival index, sulcus bleeding.[91] Similarly Suhag et al used curcumin as a local irrigants and they also reported a reduction in the symptoms associated with periodontal and gingival inflammation. Curcumin as previously discussed is a potent anticancer agent due to its mechanism of action at various level of cell cycle regulation, induce apoptosis, regulate mutagenesis

and arrest of cycle at G2/M phase, becomes a choice of natural herb in treating the precancerous condition like OSMF and oral cancers.[92]

As in our study we have selected Oral submucous fibrosis due to its common prevalanve in the Indian population and the frequency of occurrence, there are several clinical trials of curcumin on Oral submucous fibrosis and evaluate its effect.[93]

Several clinical studies are being carried out to establish the effect of curcumin in the treatment of OSMF. Shrivastav et al 2013, showed that curcumin when used in the form of paste and applied to the OSF patients showed effective results thereby increasing the mouth opening and reducing the burning sensation.[95]

Agarwal et al in the year 2014 assessed curcumin in the treatment of OSMF in diagnosed patients, where the patients were administered curcumin tablets and were kept under observation at regular intervals. The results showed an increase in the mouth opening of 10.4 mm in these cases.[96]

In the year 2015, Hazare et al conducted a study on a group of patients with OSF where curcumin was applied in the form of lozenges for 3 months duration and found that curcumin showed an increase in the mouth opening of 5.4 mm.[99]

In a study done by Yie Deing Tang et al in the year 2009, the molecular effects of arecoline and curcumin on tissue growth factors was observed and they found that arecoline has stimulated "Connective Tissue Growth Factor(CTGF)" production in buccal Mucosal Fibroblasts, which was confirmed with CTGF expression by immunohisto-chemical staining in OSMF fixed tissues.

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Thus they concluded that arecoline induced the production of CTGF which lead to fibrosis in tissue and cells and their experiment also included the counter action of curcumin on arecoline induced cells. The CTGF levels were decreased when the arecoline treated cells were acted by curcumin.[100]

Despite these clinical trials proving curcumin an effective therauptic for treating the OSMF, an insight to the cellular changes of action of curcumin on fibroblast is yet to be explored. Literature lacks the articles with in-vitro studies of action of curcumin in Oral submucous fibrosis. Thus in our research curcumin was the choice of treatment for oral submucous fibrosis to assess the morphological and cytotoxic changes that are caused by arecoline.[101]

Hence our study aims in the isolation and primary culture of Human buccal mucosal fibroblasts from healthy individuals and the assessment of arecoline and curcumin on the morphological and cytological aspects.

JUSTIFICATION

Fibroblast is the primary cells and form extracellular fibers of the connective tissue .They produce Collagen and Elastin. Cytoskeleton of Fibroblast consists of microtubules of 240 nm, Tubulin which maintains cell shape and intracellular structure. Filaments are made of Microfilaments with intracellular "Muscles" <80 nm in thickness , contractile, cell motility Actin & Myosin Intermediate filament 80-100 nm, Vimentin, maintains cell shape and contact between adjacent cells . Fibroblasts synthesize size and secrete: Collagen, Elastin, Proteoglycans, Glycoproteins.

The oral mucosal fibroblasts are affected in one of the oral disease - OSF because of the effect of alkaloids present in arecanut. The alkaloids cause increased formation of collagen leading to stabilization and cross linking of collagen fibres which further causes accumulation of collagen resulting in disease – OSF.9 Evidence from the literature suggest that oral mucosal fibroblasts undergo morphological and biological changes due to alkaloids present in arecanut.

Oral Submucous Fibrosis is now globally accepted as an Indian disease with highest rates of 8%-14% malignant transformation . Recent studies indicates that the number of cases of OSF has risen rapidly in India, ranking as highest with 2 million cases reported in 2011.Despite of various treatment modalities, the disease still remains irreversible.

Currently, in India studies using natural products such as curcumin, aloe vera and oxitard are ongoing for various diseases. In the last decade role of curcumin in treatment of oral diseases has been tried. Clinically curcumin is proven to be effective

in OSF with clinical data suggesting increase in mouth opening. There is no evidence of the mechanism of action of curcumin at molecular level to explain its efficacy.[16]

Therefore due to lack of literature of effect of curcumin on human buccal fibroblasts in OFS, this study intends to evaluate the effect of curcumin in the molecular and biological events occurring in altered human buccal mucosal fibroblasts cell lines.

METHODOLOGY

I. Isolation and Development of Primary cultures of "Human Buccal Mucosal Fibroblasts (HBMF)"

a) Ethical Consideration and Sample collection :

An ethical clearance was taken from the ethics committee of "KLE Academy of Higher Education and Research with the number "KLEU/Ethic/2016-2017/D-227". An informed consent and a detailed case history was recorded from each patients and confidentiality of the patient was maintained. Tissue samples from the healthy individuals who came for the routine impacted third molar extraction to the Department of Oral and maxillofacial surgery were collected under aseptic conditions. The samples were then transferred to DMEM media and further subjected to processing of Tissue for isolation of cell lines in tissue culture lab.

b) Processing of the tissue sample :

The processing was done in a bio-safety cabinet under sterilized condition (Fig 1-b) using a combination of explants - enzymatic technique. The tissue specimens obtained from the surgical department were washed with Phosphate Buffer Saline as per the protocol 2-3 times and then washed with DMEM media. Samples were then minced into 1mm x1mm pieces with sterilized BP blade (21) in a glass petri plate (Fig 1-c, d). During mincing/processing of tissue trypsin enzyme was added which contains ("0.02% Ethylenediaminetetra-acetic acid (EDTA- HiMedia)" (Fig 1–e) to separate the cells and isolation from parent tissue. The minced tissue was subjected to centrifugation for 4000 rpm which resulted in the formation of pellet of cells.

Supernatant solution was discarded; fresh DMEM media was added and transferred to culture plates. In addition the minced tissue was flooded with complete media containing were added to culture to prevent contamination and growth of microorganisms. The culture were kept for incubation for

24hours in 5% CO2 incubator (Fig 1-f)

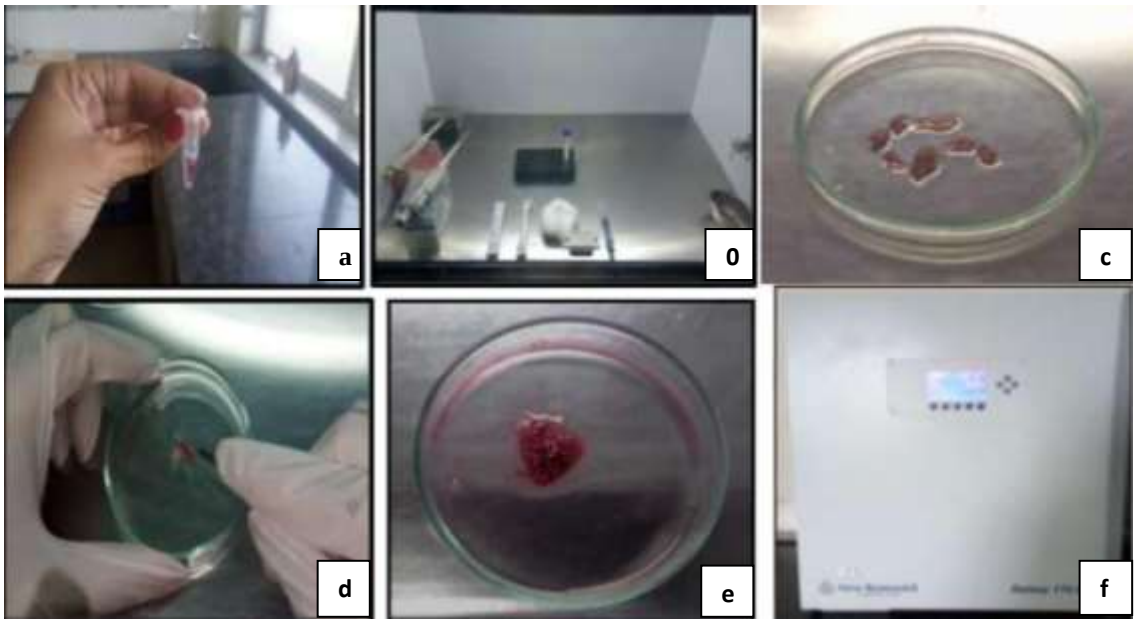


Fig 4 : a) Sample collection . b) Laminar air flow chamber with all the equipments for the tissue processing. c-d-e) Mincing of the tissue sample in petri plate. f) Incubation in CO2 Incubator.

c) Monitoring of the cultures:

The cells were monitored every 24 hours with constant change of DMEM media, FCS that is fetal calf serum, antibiotics as and when required during the depletion of media and to prevent the contamination. The culture were constantly observed under inverted microscope for any further development of the processed tissue i.e explants dislodgment, radial migration of the cells, or contamination.

The old media was replaced with fresh complete DMEM. Any change in the color of media, fungal or bacterial contamination if observed, the cultures were immediately discarded .In our study a total of 11 samples were collected and two samples were discarded due to contamination either during sample collection or during the processing of the tissue samples.

The cells were cultured and maintained constantly by changing the media and observed over a period of time under inverted microscope for morphological development and presence of any contamination and the observation were recorded. Following were the day wise changes observation of buccal mucosal fibroblast cells observed:

Day 1: The Primary culture when observed had a mixed variety of cells from round to spherical clumps of cells still attached to explants tissue sample (Fig 4–a). The cultures predominantly showed RBC and WBC. No contamination was noted in culture plates.

Day 2: The cultures on second day showed round to ovoid cells detaching from the main explants tissue indicating the outgrowth of primary culture (Fig 4-b). The culture wells were further maintained in incubators with addition of more DMEM (Dulbecco's Modified Eagles Media), fetal calf serum and antibiotics to avoid any contamination.

Day 3-4: The fibroblasts when observed were attached to the culture wells, at the periphery small spindle - shaped cells were observed covering complete wells. (Fig 4-c, d). The cultures also showed a mixture of cells from round to ovoid with small extension acquiring the spindle form of fibroblasts.

Day 6-14: During these particular days of culture, the cells showed drastic changes in their morphology where spindle-shaped cells were observed covering majority of wells of culture plates with stellate-shaped cells with small fibroblastic extensions, and round dividing cells . Cells reached around 60%–70% confluency on day 8-10 (Fig 4 –e). On day 10-14 (Fig 4-f), the cell lines were completely adherent to the wells and further processed for sub culturing and passaging in t flask.

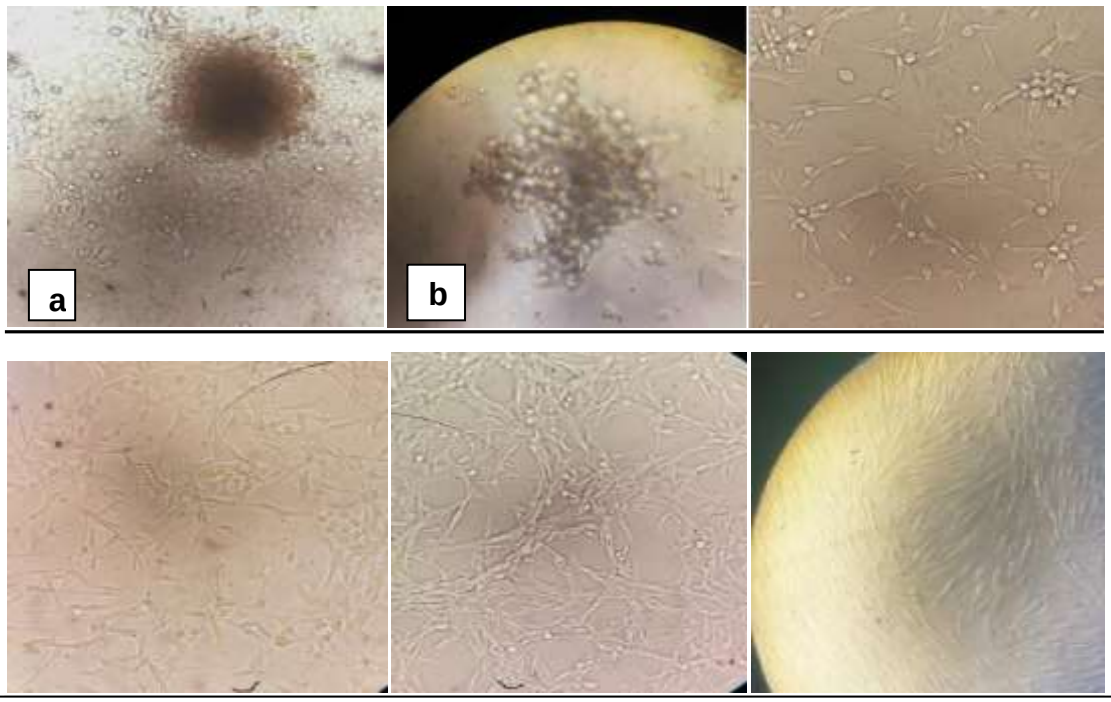


Fig 5: Isolation and primary culture of buccal mucosal fibroblast cell lines

a) Day 0 .b) Day 3-4.c) Day 5. D) Day 6-8. e) Day 8-10 f) Day 10-14.

Growth curve assay:

The growth curve assay was conducted to assess and study the growth characteristics of the KLED-BF18 cell lines. The assay helps in the evalualtion of cellular growth over a period of time which reflects the rate of growth of each cell population and their pattern of growth cycle that is log, lag and stationary phase.

Procedure:

The cells of 3-4 passage were selected for the growth curve assay. The cells were trypsninized to detach from the culture t flasks followed by centrifugation resulting to form a cell pellet. Cell counting was done using tryphan blue dye exclusion method. Once the counting was done, the cells were seeded in culture plates.

The cells were regularly counted for 6 days and the number of cells were plotted on a graph against the number of days for understand the growth characteristics of cell lines

Table 1: Growth Curve Assay: Table showing an increase in the number of cells from Day 0 to Day 5

Number of days	Number of cells per well
Day 0	7000
Day 1	22000
Day 2	156000
Day 3	171000
Day 4	172000
Day 5	182000

c) Subculturing and Passaging :

Once the confluency is reached where the cells surrounding the explant tissue has started growing and expanding, the medium was poured out and the cell surfaces washed gently. The fibroblast cells were detached by trypsinization. On action of trypsin the cells attained round shape and get detached from the culture plates. The cell suspension was pippeted in appendorf tube and centrifuged. Fresh complete media was and cultured fin new wells and t25 culture flask. Cultures were incubated in 5% CO2 incubators with 37 degree temperatures. The culture medium was changed once in every 48 hour and the cells were further processed for morphological characterization and authentication by STR Profiling.

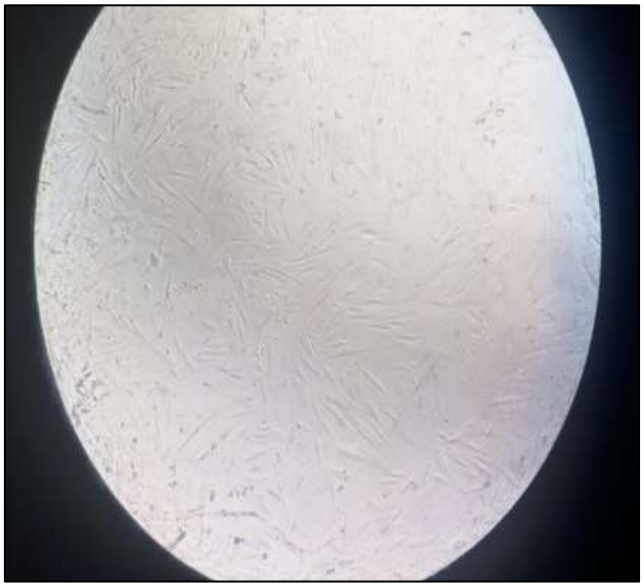


Fig 6: Confluent growth of the cells in the flasks.

Morphological Characterization and Authentication of cell lines:

a) Morphological characterization :

The primary cultures were monitored regularly for the changes in morphology and growth and observed. The isolated buccal mucosal fibroblasts were observed regularly over a constant period of time for the morphological variants under inverted microscope. The initial cultures showed round to ovoid cells which were in the form of large clusters and clumps. Gradually, the round cells appeared more as plump shaped cells with abundant cytoplasm and round nucleus. On further observation the cells appeared spindle shape with round nucleus and clear abundant cytoplasm. Assessment of morphological phenotypes of buccal fibroblasts was based on de Waal J et al and thus the following three main morphological variants of fibroblasts were noted in our cultures (Fig 3).

1. F1: Spindle shaped cells that are highly proliferative which secrete low levels of type I Type III collagen**.**
2. F2 – These are the epitheloid, less proliferative that secrete more Type I collagen.
3. F3 - Stellate shaped cell, least proliferative, produces large quantities of collagen type 1 and Predominate in FIBROSIS.

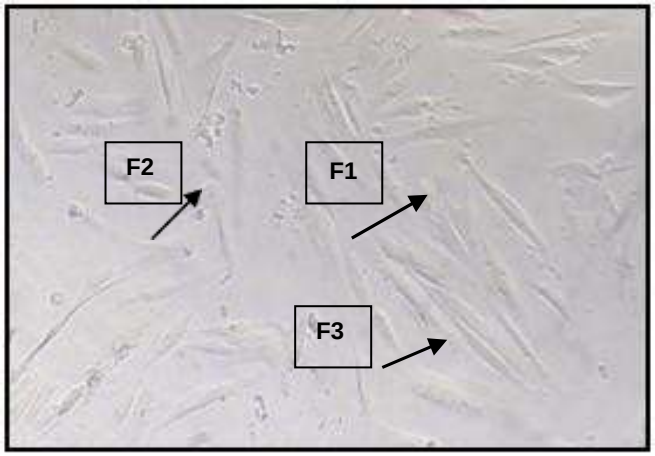


Fig 7: Morphological phenotypes of cultured human buccal mucosal fibroblasts.

a) "Short Tandem Repeat Profiling/STR or DNA profiling of the cells".

The cell lines were further processed for authentication by Short tandem Repeat (STR) profiling from DNA forensics lab, New Delhi India**.** The cells from the passage 3-4 of the confluent flask were selected for authentication .The pellet was prepared and sent to the lab as per the standard protocol recommended by the lab. The cells were also named and coded as KLED-BF18 for future use. The cell pellet was then properly sealed and transferred to the DNA Forensics lab, New Delhi. Once the authentication procedures are completed, the cells were processed for the assessment of arecoline and curcumin effects on morphology and cytotoxicity.

DNA Forensics Laboratory Pvt. Ltd.
44F/9, Rajendra Complex, Kishangarh, Vasant Kunj,
New Delhi- 110070, India

Cell Line Authentication STR Profile Report

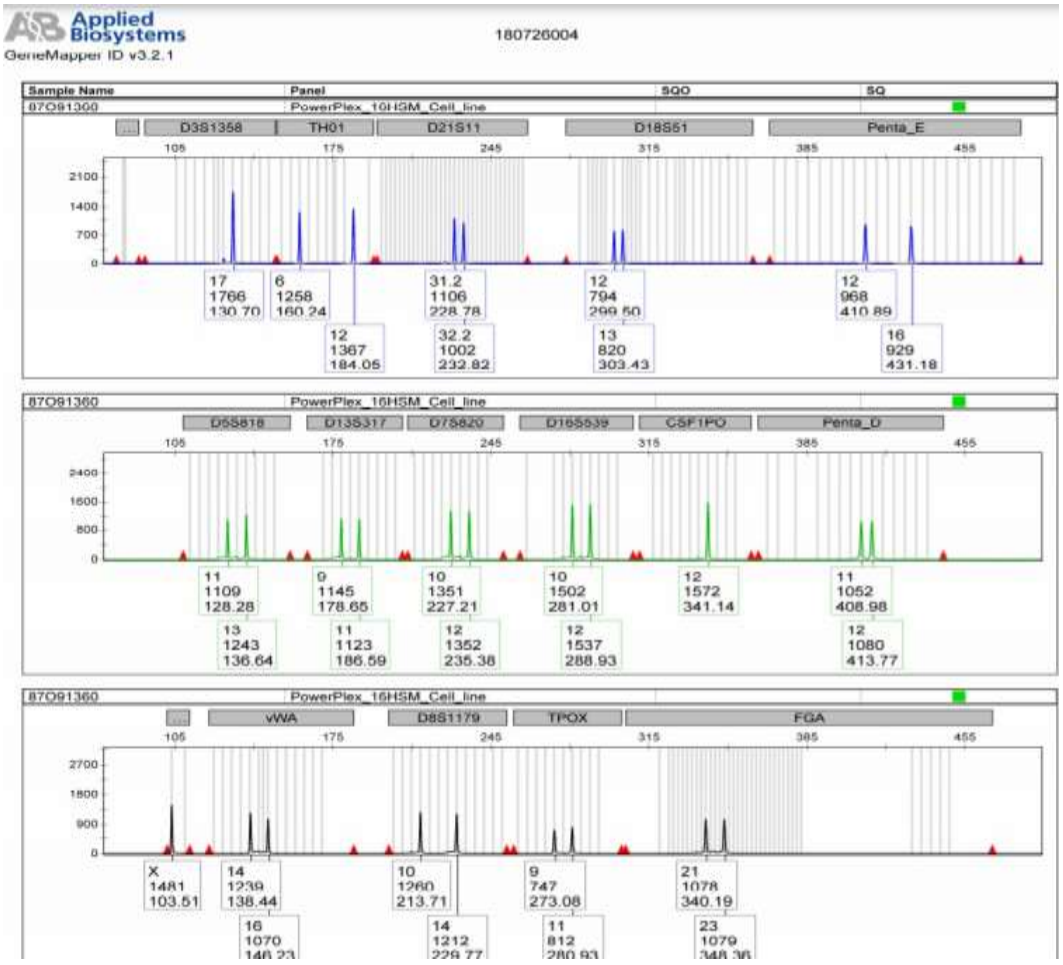
Sample Submitted By: Dr. Ritiha Cuppin
Email Address: ritihapatil111@gmail.com
Cell line Designation: KLED-BF18
Date Sample Received: 16-07-18
Report Date: 03-08-18

Test Result for submitted Sample			**Reference Database Profile**	
Loci	**Query profile (KLED-BF18)**		**Database Profile (No any significant Match)**	
D3S1358	17			
TH01	6	12		
D21S11	31.2	32.2		
D18S51	12	13		
Penta E	12	16		
D5S818	11	13		
D13S317	9	11		
D7S820	10	12		
D16S539	10	12		
CSF1PO	12			
Penta D	11	12		
vWA	14	16		
D8S1179	10	14		
TPOX	9	11		
FGA	21	23		
Amelogein	X	X		
No of shared allele between query sample and database profile- NA				
Total no of allele in database profile- NA				
Percentage Match Between the submitted sample and database profile- NA				

Note: *Loci highlighted in grey can be made public to verify cell identity. In order to protect the identity of the donor* ***Please do not publish*** *the allele calls from all the STR loci tested.*

Comparative output from Database:

EV	Cell No.	Cell name	Locus names								Figures
			D5S818	D13S317	D7S820	D16S539	VWA	TH01	AM	TPOX	CSF1PO	
	Query (Your Cell)		11,12	9,11	9,12	11,14	15,18	7,10	X,Y	8,8	12,12	
1.00(36/36)	397	HT-1376	11,12	9,11	9,12	11,14	15,18	7,10	X,Y	8,8	12,12	-
1.00(36/36)	CRL-1472	HT-1376	11,12	9,11	9,12	11,14	15,18	7,10	X,Y	8,8	12,12	-
1.00(36/36)	CRL-7927	HT-1376	11,12	9,11	9,12	11,14	15,18	7,10	X,Y	8,8	12,12	-
0.72(26/36)	246	Y-79	11,12	11,12	8,9	13,14	15,18	6,10	X,Y	8,8	11,12	-



The results of the STR profiling of our primary cell lines sample say that the cells that are isolated and established are of human in origin which have unique features of and genetic characteristics that resemble the fibroblasts of humans.

Once the cell lines were characterized and authenticated as KLED-BF18, the sub culturing and cryopreservation were carried out for further morphological and cytotoxic assessment of Arecoline, Curcumin on Human buccal mucosal fibroblasts i.e. KLED-BF18.

Cryopreservation of fibroblasts.

Cryopreservation was carried out using the standard protocol with "Dimethy l-sulfoxide (DMSO)," that stores the cells for a longer duration in liquid nitrogen. Cells on reaching the confluency, 4^{th} to 5^{th} passage subcultures were selected subjected to trypsinization . Cells were subjected for centrifugation at 4000 rpm for 3 minutes. The cell pellet was re-suspended in freezing medium (90% FBS and 10% DMSO).Cell suspensions were aliquot into cryogenic storage vials and frozen subsequently at 2-4°C for 1 hour, further in -20 °C for 1 hour, -80°C in a deep freezer overnight and finally kept in liquid nitrogen till the further usage of cell lines.

1 PREPERATION OF COMPOUNDS:

a. **Preperation of dilutions of Arecoline hydro bromide:** Arecoline Hydrobromide was procured from Sigma Aldrich (Fig 3) and stored at room temperature. The dilution of the arecoline hydrobromide was prepared by dissolving 2mg in 2ml of DMEM media and found to be completely soluble in media. The stock solution i.e. 2mg in 2ml was further subjected to serial dilution to get the following dilutions in table 2 of Arecoline Hydrobromide in DMEM media.

Table 2: Preparation of dilutions of Arecoline

Arecoline Hydrobromide .
500 µg/ml
250 µg/ml
125 µg/ml
62.5 µg/ml
31.25 µg/ml
15.6 µg/ml
7.8 µg/ml
3.9 µg/ml
1.9 µg/ml

Fig 5 Arecoline Hydrobromide Compound

a. Preparation of dilutions of Curcumin :

Curcumin C3 complex was procured from Sami labs Bangalore (Fig 4). Curcumin on solubility was found to be insoluble in water and hence the dilutions were prepared by first dissolving the stock solution in 1% DMSO as shown in table and further diluting in Phosphate buffered saline. Following similar dilution were prepared by serial dilution method in PBS.

Table 2: Preparation of dilutions of Curcumin

Curcumin
500µg/ml
250 µg/ml
125 µg/ml
62.5 µg/ ml
31.25 µg/ml
15.6 µg/ml
7.8 µg/ml
3.9 µg/ml
1.9 µg/ml

Fig 6: Curcuma Longa (Sami Labs)

I. **Morphological Assay :**

The cell lines KLED-BF18 were assessed for the morphological changes or effect caused by Arecoline, Curcumin and Curcumin on Arecoline treated cells. The cells were observed for 48 hours and all the cytological and nuclear changes were noted.

Day 1:

The cells of passage number 3 and 4 on reaching the confluency were detached from the flasks and seeded in 12 well culture plates and labeled respectively. The cells are transferred and allowed to and get attached to the new culture flasks with constant monitoring and observation under inverted microscope.

Day 2:

The cells were treated with different dilutions of Arecoline, Curcumin and Curcumin on arecoline induced buccal fibroblast cell lines. The cell lines were kept for incubation period of 48 hours to assess the morphological alteration of the respective compounds. The morphological changes were noted and recorded.

I. Assessment of Cytotoxic assay on Buccal mucosal fibroblasts.

The cellular mechanism and metabolism is determined by various factors. The cells are affected by the temperature, chemical composition of the surrounding, the pH and the interaction of the surrounding chemicals or compounds. There are many in-vitro assays to determine the normal functioning of the cells like viability assays, proliferation assays and cytotoxic assays. The cytotoxicity analysis of compounds are one of the most important and critical analysis of the effect of drugs, compounds or chemicals and their screening on the cells. The agents may cause cellular destruction, prevention of protein synthesis, inhibition of receptor binding, enzyme interaction etc. Thus in-vitro assessment of these chemicals acting on the cell lines has become a major development in the field of oncology, drug development and drug screening.

In our study we analyzed the effect of compounds, Arecoline hydrobromide Curcumin and Curcumin on arecoline induced fibroblasts using "MTT assay, Neutral Red Uptake assay and Lactate Dehydrogenase Assay".

1. MTT ASSAY:

"MTT assay is a colorimetric assay to assess the cytotoxicity compounds on the cells. The yellow tetrazolium MTT (3- (4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) gets reduced by active cells the metabolically active dehydrogenase enzyme, that results in the formation of NADH and NADPH with the formation of purple formazan which can be solubilized. The optical density readings are and quantified using a spectrophotometer of particular wavelength.

Principle:

MTT assay is a colorimetric assay where there is reduction of a yellow tetrazolium salt (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, or MTT). Viable cells contain NAD (P) H-dependent oxido-reductase enzymes which reduce the MTT reagent to formazan, an insoluble crystalline product with a deep purple color. These Formazan crystals are then dissolved using a solubilizing solution and absorbance is measured at 500-600 nanometers using an ELISA reader. The darker the solution, the greater the number of viable indicating metabolically active cells"

Equipment and Materials Required: 96 well plate, Dulbeccos Modified Eagles Media (DMEM), MTT reagent, Dimethyl Sulfoxide (DMSO), ELISA Reader, Inverted microscope, 37°C incubator and Laminar air flow chamber.

Protocol :

Day 1 : Seeding of the cells.

The cells of 3 or 4^{th} passage were selected for the assay and the cell counting was done using Tryphan Blue dye exclusion method .The cells were first detached from the main flask trypsinisation where 1ml of Trypsin was used followed by centrifugation, resulted to form the pellet. The supernatant media was discarded and the cells were counted by Trypan Blue dye exclusion test. Three different plates were seeded so as to record the cytotoxic changes for 24, 48 and 72 hours.

Principle of tryphan blue dye exclusion method : The living cells with intact cell membranes do not allow the dye to penetrate the cell whereas the cells on toxicity of compounds eventually die and dead cells take up the dye to appear as dark blue in color. The cell suspension is further treated with the dye and examined visually, the

viable cell showed a clear cytoplasm and are translucent whereas the dead cells appeared dark in colour.

Equipments required: Pipette and tips**,** Trypan Blue**.** Hemocytometer, coverslip Inverted Microscope, 96 well culture plates and ELISA reader.

Procedure

Cell counting: Tryphan blue dye method:

Cell suspension of 10 µl of was taken in an eppendorf tube and about equal parts (10 µl) of trypan blue dye (Fig 8) was added to the cell suspension. With the cover slip already in place on hemacytometer counter was filled with 10µl of cell suspension was mixed with tryphan blue and the cells were focused and counted under inverted microscope.

Counting

The counting of the cells is carried out using a hemocytometer under inverted microscope with 10X objective. The living cells and the dead cells were counted accordingly in all 4 squares. The total cell count was done by using the following formula. After the cell Counting, fibroblast cells of about 10^6 cells were added to each well with 100µl of fresh DMEM media for the assessment of Arecoline and Curcumin and combination of both.

Day 2 : Addition of compound .

The compound Arecoline Hydrobromide, curcumin were measured as stock solution and around 100µl of the prepared dilutions were added to the cell lines in triplicates (Fig 9). The cell was incubated for the next 24 hours in CO2 incubators.

$$\text{Cell counting} = \frac{\text{Total number of cells (I+II+III+IV)} \times 2 \times 10{,}000}{4}$$

Fig 10: 96 well microtitre plate with compounds added to the fibroblast cell lines.

Day 3 : MTT assay analysis .

20µl of MTT Compound was added on day 3 and cells were incubated for 3 hours in CO2 incubator. After three hour of incubation, 100µl of DMSO was added which dissolves the formazon crystals. The optical density reading were recorded by running the plate in ELISA reader at 490 nm and the cell viability was calculated with the given formula.

$$\text{Cell Viability (\%)} = \frac{\text{Mean OD of test compound}}{\text{Mean OD at control (untreated cells}} \times 100$$

The same procedure was carried for 3 days.(24, 48 and 72 hours) the readings were recorded to calculate the % cell viability of human buccal mucosal fibroblast cell lines.

NEUTRAL RED UPTAKE ASSAY:

"The neutral Red assay allows the assessment and quantification of the cytotoxicity of compounds on cells and estimation of total viable cells. It is called as "toluydene red, Basic Red 5, or C.I. 50040" dye used for staining the cells. The principle relies of the assay is that the living cells allow the permeation of the red dye and bind to it in the lysosomes of healthy living cell lines."

Equipments required:

Micropipettes, 96 well culture plates, desorbing solution, laminar air flow chamber, DMEM media, heamocytometer, Elisa Reader

Procedure:

Day 1: The cells from the 3-4th passage were detached from the t flasks and counted using tryphan blue dye exclusion method. Around 1x10^5 cells were seeded in the 96 well and kept for incubation in 5% CO2 incubators. Three plates of the cell lines were seeded to record subsequently for three days i.e. 24, 48 and 72 hours.

Day 2: The cells were exposed to the compounds i.e. Arecoline, Curcumin and curcumin on arecoline induced cells seeded in 96 well plates. The plates were again incubated for the next 24 hours in 5% CO2 chambers.

Day 3: 100µl of Neutral red was added (Fig 10 a, b) to each well and incubated for 3 hours. The procedure was followed by discarding of supernatant dye solution along with media with the addition of desorbing solution (DMSO). The optical density readings were recorded at 630 nm in the ELISA reader.

$$\text{Cell Viability (\%)} = \frac{\text{Mean OD of test compound}}{\text{Mean OD at control (untreated cells}} \times 100$$

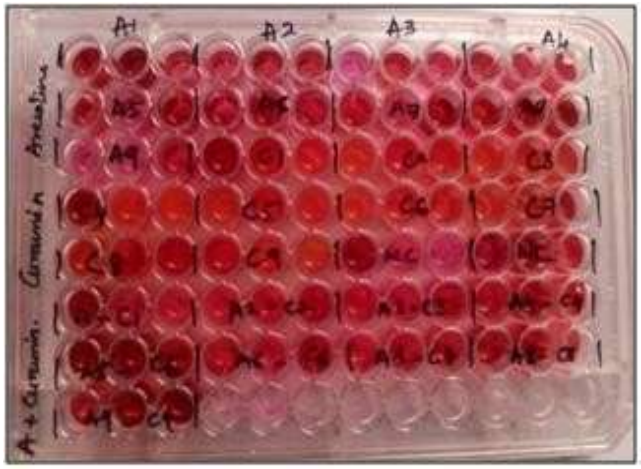


Fig 11: 96 well Microtitre plate with Neutral red Dye added to the fibroblast cell lines

LACTATE DEHYDROGENASE ASSAY:

Lactate Dehydrogenase enzyme is a stable enzyme and is present in all cell types. The damage of the cell by any toxic compound breaks the cell membrane and releases of the lactate dehydrogenase enzyme which is a marker for cytotoxicity. It reversibly converts lactate into pyruvate, with the concomitant inter-conversion of NADH and NAD+. It is a sensitive, quick, and easy way for detection of LDH released from damaged cells, where LDH enzyme converts lactate to pyruvate and NADH, at a particular fluorescence at two wavelengths. (Ex/Em = 535/587 nm).The fluroscence is measured and the number of cell viability is calculated. In this assay only the arecoline and curcumin groups were assessed as the cytotoxicity caused by arecoline were irreversible. The assay was performed using LDH Assay kit based method procured from Biovision Company.

Sample Preparation:

The adherent cells were collected and washed once with fresh regular culture medium. An appropriate number i.e 10x5 cells were seeded in 100 µl culture medium

per well of 96-well tissue culture plate for Low Control, High Control and to evaluate test compounds. The cells were incubated in incubator overnight. After 24 hours of incubation the test compounds were added test wells and incubated for an appropriate time based on the compound (~8-24 hrs).

The plates were shaken for 1 min. to mix them properly. 10 µl Cell Lysis Solution was added and plates were shaken well for 1 min. to mix, and incubated for 30 min. 5µl of supernatant from each well into a new 96-well white plate with flat bottom. During analysis, the background value should be subtracted from all readings.

Contents of the kit:,

- LDH Assay Buffer
- LDH Substrate mix
- Pico Probe
- Cell Lysis solution
- LDH Positive Control

95µl of Reaction mix is added to each well. Gently the plates are shaken well and the flouroscence readings are recorded with the wavelength, Ex/Em = 535/587 nm and thus the percentage cell viability was calculated and quantified with standard formulas.

$$\text{Cell Viability (\%)} = \frac{\text{Mean OD of test compound}}{\text{Mean OD at control (untreated cells}} \times 100$$

Wound scratch- Healing Assay:

The wound scratch assay is an invitro assay that involves steps creating a "scratch" in a cell monolayer, capturing the images at the beginning and at regular intervals during cell migration to close the scratch, and comparing the images to quantify the migration rate of the cells.

Procedure:

The cultured buccal mucosal fibroblasts cells were counted approximately 10x3 cells/ml of DMEM media and seeded in a six well culture well plate. The cells lines were allowed to grow to a confluent growth covering all the surfaces of well in CO2 incubator. After 24 hours of cell growth in the wells curcumin of higher concentration was added and, a scrath or a wound was created using 100µl micro tip and the microphotographs were taken at 0^{th} hour, 12^{th} hour, 24 hour and 48 hour to assess the closure and approximation of the wound.

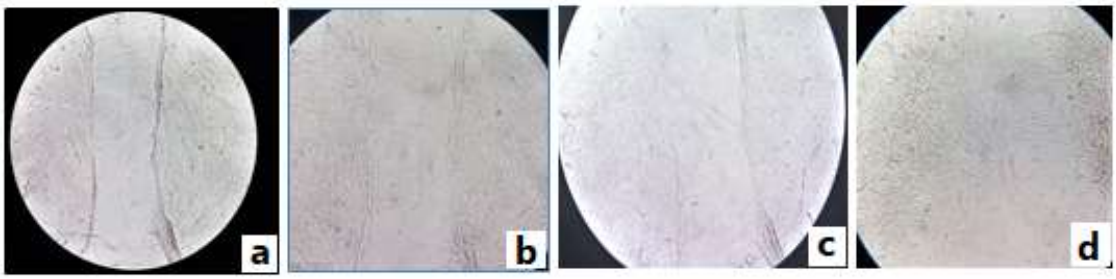


Fig 11: a) 0^{th} Hour b) 12^{th} hour c) 24^{th} hour d) 48^{th} hour. of untreated buccal mucosal fibroblasts.

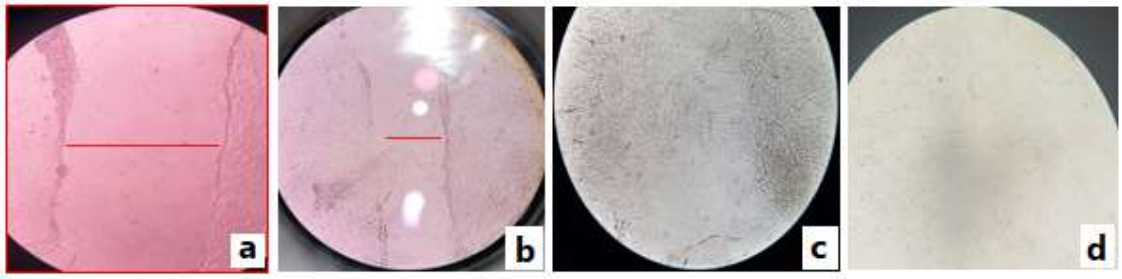


Fig 12: a) 0th Hour b) 12th hour c) 24th Hour d) 48th hour. of buccal mucosal fibroblasts treated with high concentration of curcumin.

Fluorescent Staining of the cells using DAPI stain.:

A simple-to-use fluorescent stain, 4',6-diamidino-2-phenylindole (DAPI), visualizes nuclear DNA in both living and fixed cells. DAPI staining was used to determine the number of nuclei and to assess gross cell morphology. Following light microscopic analyses, the stained cells were processed for electron microscopy. Cells stained with DAPI show ultra structural changes compared to the appearance of cells not stained with DAPI.

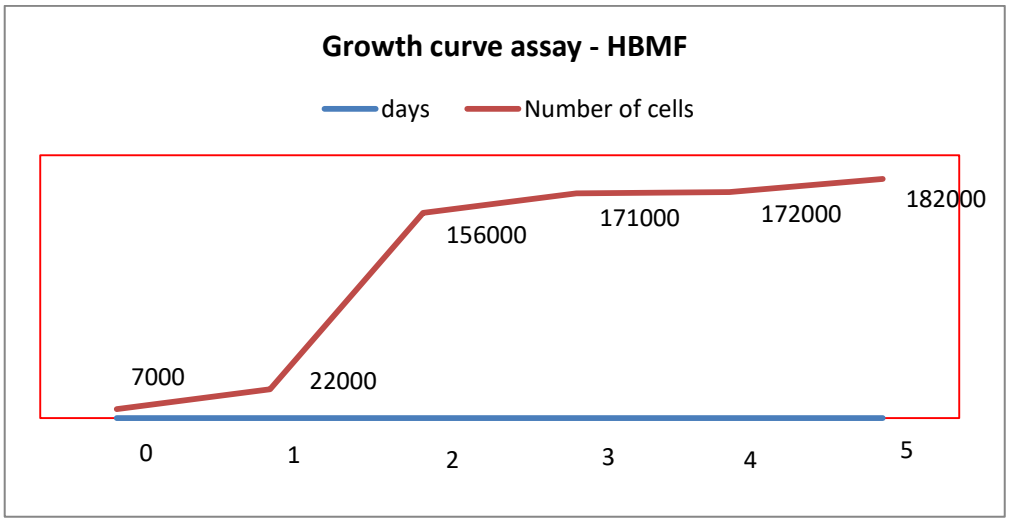
Procedure: The BMF were selected from the subcultures and Trypsinized to detach them from flasks attached with slide .The cells were incubated for 24 hours till they reach the confluency. After 24 hours of incubation, a desired concentration of compound was added. The media was aspirated from the flasks. The cells were fixed by 4% paraformaldehyde followed by 20 mins of incubation. The cells were washed with PBS and DAPI stain was added with observation under fluorescent microscope.

DATA ANALYSIS PLAN:

All the data was entered in an excel spreadsheet, and further data was imported into SPSS version 24 software for further statistical analysis. Confidence interval was set at 95% and the propability value of ≤ 0.05 was usually considered to be statistically significant in the study. The morphological assessment data was interpreted based on only observation whereas cell viability of all the assays (MTT, Neutral Red, and LDH) was calculated based on cell viability percentage formula and the same was graphically represented for the respective groups. The data regarding all the assays(MTT, Neutral Red, LDH) which had two or more mean values of different concentration of compounds, hence the Analysis of variance (ANOVA) test for all the groups at different points of time was carried out. An inter-group and intra-group comparison of all the assays (MTT, Neutral Red, LDH) was analyzed using dependent t test and one way ANOVA for all the group of cells treated with Arecoline, Curcumin and curcumin on arecoline treated or induced cell lines.

RESULTS

Growth Curve Assay:



I MORPHOLOGICAL ASSESSMENT:

The morphological assay of curcumin, arecoline and curcumin and arecoline was assessed on the cultured primary buccal mucosal fibroblasts cells (KLEBF-18) and the cells were observed under inverted microscope for 48 hours by the effect of the selected compounds. The morphological changes were assessed based on the parameters like shape of the cell, outline of the cells (spindle, plump, round), type of nucleus (open/ closed), and type of cytoplasm (clear/ granular).

Morphological Assessment of Cell Lines with Arecoline Compound:

Arecoline showed a dose dependent effect on the cultured fibroblasts, where the higher concentration had irreversible morphological alteration with degenerative changes, however with lower concentration showing no alteration of morphology of cells depicting normal fibroblasts. The morphological alteration of buccal fibroblast cells was noted only at specific concentration and they are as follows.

At 500 µg/ml- 250µg/ml of Arecoline Compound:

At these range of concentration the morphology of fibroblasts showed predominantly F2 (epitheloid cells) and round cells, with granular cytoplasm and open nucleus indicating cells undergoing degenerative changes with granulation and death **(Figure 11 a ,b)**

At 125µg/ml of Arecoline Compound:

At this concentration the morphology of fibroblasts showed all the three shaped fibroblasts with predominant F1 (Spindle shape) along with F2 (epitheloid) and F3 (Stellate) shape fibroblasts. All the three types of fibroblasts observed at this concentration showed cell outline as either spindle, plump or round. The nucleus of this concentration found to be closed type and the cytoplasm was granular and sparse. **(Figure 11 b)**

At 62.5µg/ml of Arecoline Compound:

At this concentration the morphology of fibroblasts showed only F1 (Spindle shape) and F3 (Stellate) shape fibroblasts. Here at this concentration we did not observe F2 (epitheloid) shaped Fibroblasts. Though some of the cells were round immature type of fibroblasts but majority of cells showed spindle outline, open nucleus with abundant clear cytoplasm.

At 31.25–1.9 µg/ml of Arecoline Compound:

After 31.25 µg/ml till 1.9µg/ml the fibroblasts did not show any morphological changes suggestive of normal fibroblasts with no effect of arecoline hydrobromide.

Majority of the cells were F1 (spindle-shaped), the cell outline being spindle and plump along with few immature type of cells possessing abundant clear cytoplasm and alteration of fibroblasts was observed only at 125 µg/ml of arecoline.

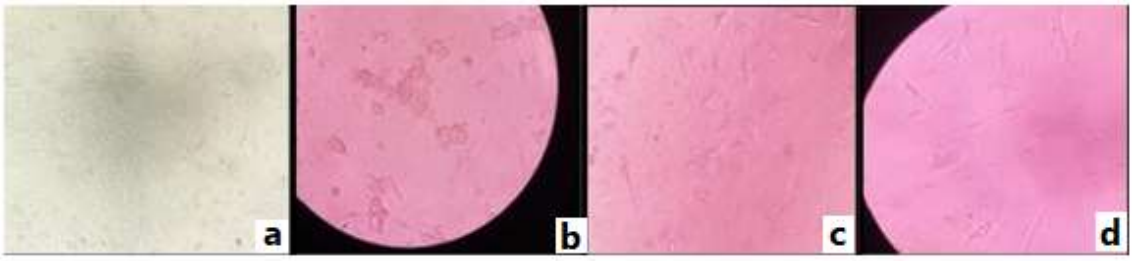


Fig 11: Morphological Assessment of Arecoline on Human Buccal Mucosal Fibroblasts a) Untreated cell. b) 500µg/ml. c) 250µg/ml d) 125µg/ml.

Overall Observation:

On assessment of morphological changes observed with the effect of arecoline compound, we found that at lower concentration from 31.25 µg/ml to 1.9 µg/ml had no effect on shape of fibroblasts. Only at 125 µg/ml we could observe all the three shape (Spindle, epitheloid and stellate) of fibroblasts, whereas at higher concentration that is 500 µg/ml to 250 µg/ml the cells showed degenerative changes suggestive of toxicity with effect of arecoline. This suggests that lower concentration had no effect on cells but higher concentration was found to be toxic to cells.

Table 4: Morphological Assessment of Arecoline on HBMF

CONCENTRATION of ARECOLINE (µg/ml)	F1	F2	F3	OUTLINE			NUCLEUS		CYTOPLASM	
				Spindle	Plump	Round	Open	Closed	Abundant	sparse
500	-	-	-	-	-	R**	-	-	Sparse	-
250	-	-	F3	-	-	R	-	Closed	Granular	Sparse
125	F1	F2	F3	S **	P	R	-	Closed	Granular	Sparse
62.5	F1		F3	S*	-	-	Open	-	C lear	-
31.9	F1	-	-	S*	P	-	Open	-	Clear	-
15.62	F1	-	-	S*	P	-	Open	-	Clear	-
7.8	F1			S**	P	-	Open		Clear	-
3.9	F1			S**			Open		Clear	-
1.9	F1			S**			Open		Clear	-

Morphological Assessment of Cell Lines with Curcumin Compound:

At 500µg/ml -250µg/ml of Curcumin compound:

At these range of concentration of curcumin, we found predominantly F1 and F3 shape fibroblasts. Moreover we did not observe F2 shape fibroblasts at these concentrations. The outline of fibroblast was spindle and round with closed nucleus possessing abundant clear cytoplasm. **(Fig 12–a,b,c.)**

At 125µg/ml of Curcumin compound:

At this concentration of curcumin, we found all the three shaped fibroblasts i.e F1 (spindle), F2 (epitheloid) and F3 (stellate) shapes. The outline of fibroblasts found to be either spindle, plump or round. The nucleus of fibroblasts possessed closed type with abundant clear cytoplasm.

62.5 µg/ml of Curcumin compound:

At this concentration of curcumin, we found F1 (spindle) and F3 (stellate) shaped fibroblasts. But we did not find F2 shaped fibroblasts. The cells had spindle and plump outline with open nucleus and abundant clear cytoplasm.

At 31.25 µg/ml of Curcumin compound:

Similar to 125 µg/ml of curcumin even at this concentration showed all the three shape fibroblasts i.e. F1, F2 and F3. The outline of the fibroblasts showed spindle and plump with open nucleus and abundant clear cytoplasm.

15.62 µg/ml to 1.9 µg/ml of Curcumin compound:

The lower concentration of this range showed only F1 (spindle) shape of fibroblasts with open nucleus and abundant clear cytoplasm

Overall Observation:

On observing the morphological changes of fibroblasts with the effect of Curcumin compound, we found that at most of the concentration the compound did not posses toxicity to the cells. Only at 125µg/ml and 31.9 µg/ml we could able appreciate all the three shapes of fibroblasts, whereas the other concentrations of curcumin had maintained throughout spindle shaped fibroblasts.

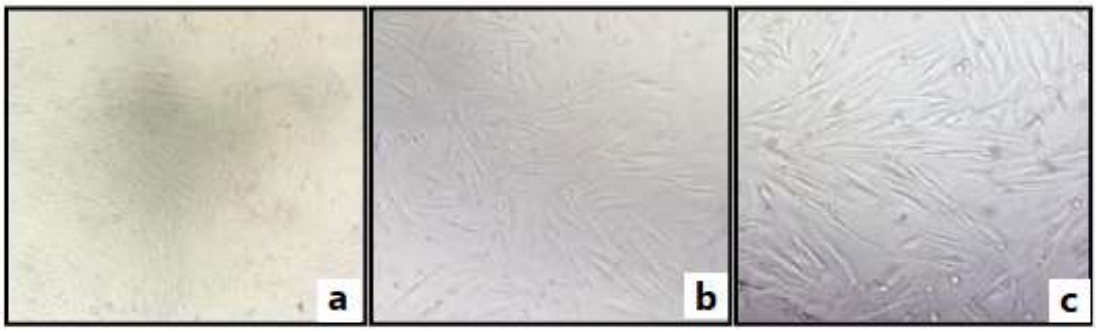


Fig 12: Morphological assessment of curcumin on Human Buccal Mucosal Fibroblasts a) Untreated cell, b) 500µg/ml, c) 250µg/ml

Table 5: Morphological assessment of Curcumin on Human buccal mucosal fibroblasts.

Concentration (µg/ml)	**F1 F2 F3**			**OUTLINE**			**NUCLEUS**	**Cytoplassm**
				Spindle	Plump	Round	Open Closed	Aliundant Sparse
500	F1**			**S**		R		
250	F1	F2	F3	S			Closed	Clear
125	F1	F2	F3	S **	P	R	Open	Clear
62.5	Fl		F3				Open	Clear
31.9	F1	F2	F3	S	P		Open	Clear
15.62	Fl			S	P		Open	Clear
7.8	Fl			s			Open	Clear
3.9	Fl			S			Open	Clear
1.9	Fl			S			Open	Clear

Morphological Assessment of Cell Lines with Curcumin on arecoline induced fibroblasts:

At 500µg/ml -250µg/ml of Curcumin compound on arecoline induced fibroblasts :

At 500 concentration of Curcumin on arecoline induced fibroblasts, the types of fibroblasts was not able to distinguish, but with serial dilution of upto 250 we found predominantly F2 (epitheloid) shape fibroblasts. The outline of fibroblast was round with open nucleus possessing spare granular cytoplasm indicating degeneration of cells. (Figure, table)

At 125µg/ml of Curcumin compound on arecoline induced fibroblasts:

At this concentration, we found all the three types of fibroblasts i.e. F1 (spindle), F2 (epitheloid) and F3 (stellate). The outline of fibroblasts found to be either spindle, plump or round. The nucleus of fibroblasts possessed closed type with abundant clear cytoplasm.

At 62.5µg/ml Curcumin compound on arecoline induced fibroblasts:

At this concentration, we found only F1 (spindle) and F3 (stellate) shaped fibroblasts. The cells had spindle and plump outline with closed nucleus and abundant clear cytoplasm.

At 31.25 µg/ml to 1.9 µg/ml of Curcumin compound

The lower concentration of this range showed only F1 (spindle) shape of fibroblasts with closed nucleus and abundant clear cytoplasm.

Overall Observation:

On observing the morphological changes of fibroblasts with the effect of Curcumin on arecoline induced fibroblasts, we found that at higher concentration that is at 500 µg/ml we could not to distinguish shape of cells due to degenerative changes suggestive of toxicity that was already caused by the effect of arecoline. However at 125µg/ml we found all the three shapes of fibroblasts and at 62.5 µg/ml we did not find F2 shape of fibroblasts but F1 and F3 shapes of fibroblasts were evident. Whereas at lower concentration from 31.25 µg/ml we found only F1 shape fibroblasts. This suggests that the higher concentration of curcumin on arecoline induced cell lines showed the morphological alteration caused by the toxicity of arecoline whereas at lower concentration the normal spindle shape of fibroblasts were observed.

Table 6: Morphological assessment of Curcumin on Arecoline induced Human buccal mucosal fibroblasts.

Concentration (µg/ml)	**FI**	**F2**	**F3**	**Outline Spindle/ Plump/Round**			**Nucleus Open / Close**	**Cytoplasm A Bilndant / Sparse**
500						R	Open	Sparse
250		F2				R	Open	Sparse
123	Fl		FS	S	P	R	Closed	Clear’
62.3	Fl	M	F3	S			Closed	Clear
31.25	FI	F2	F3	S	P		Closed	Clear
1 3.62	Fl			S	P		Closed	Clear
7.8	F1			S			Closed	Clear
3.9	F1			S			Closed	Clear
1.9	Fl			S			Closed	Clear

CYTOTOXIC ASSAY

MTT ASSAY:

The MTT Assay was carried out for 24, 48 and 72 hours on Human Buccal Mucosal Fibroblats exposed to arecoline, curcumin and curcumin on arecoline induced cells.

Arecoline:

1. **24 hours:**

The results of MTT assay showed that the higher concentration i.e 500 µg/ml showed a cell viability of 60% and 250µg/ml showed a slight increase in the cell viability of 72%. Thus the first two concentrations were considered as cytotoxic as the number of viable cells was reduced to half. Whereas the concentration 125µg/ml and 62.5µg/ml showed the cell viability of 73% and 83% indicating it to be less toxic compared to the first two concentration as tabulated in the table and graph respectively. Further the lower concentration concentration of Arecoline hydrobromide ranging from 31.25-1.9 µg/ml maintained a cell viability of 80-100% of viable cells thus indicating it to be nontoxic at lower concentration of arecoline hydrobromide.

2. **48 hours :**

When the cell lines were exposed to Arecoline for 48 hours, the cell count was decreased to about 50%. The higher concentration i.e 500µg/ml, 250µg/ml and 125µg/ml showed a marked reduction in the cell number to 58. %, 69% and 78% respectively as shown in the table and graph. The viability count of cells of 48 hours when compared to 24 hours was reduced to 50%. Thus the above concentrations are

cytotoxic, decreasing the cell viability of fibroblasts. The lower concentration ranging from 62.5-1.9µg/ml had a cell viability ranging between 90%-100% of viable fibroblasts suggesting that these concentration are less toxic compared to the higher concentration of arecoline.

3. 72 hours :

On exposure of the cells for 72 hours to arecoline hydrobromide, there was a decrease in the cell count in almost all the concentration from higher to lower except the concentration from 15.62-1.9µg/ml. Arecoline at 500, 250, 125 and 62.5µg/ml showed a cell viability ranging from 40-65% as tabulated in the table and graph respectively.

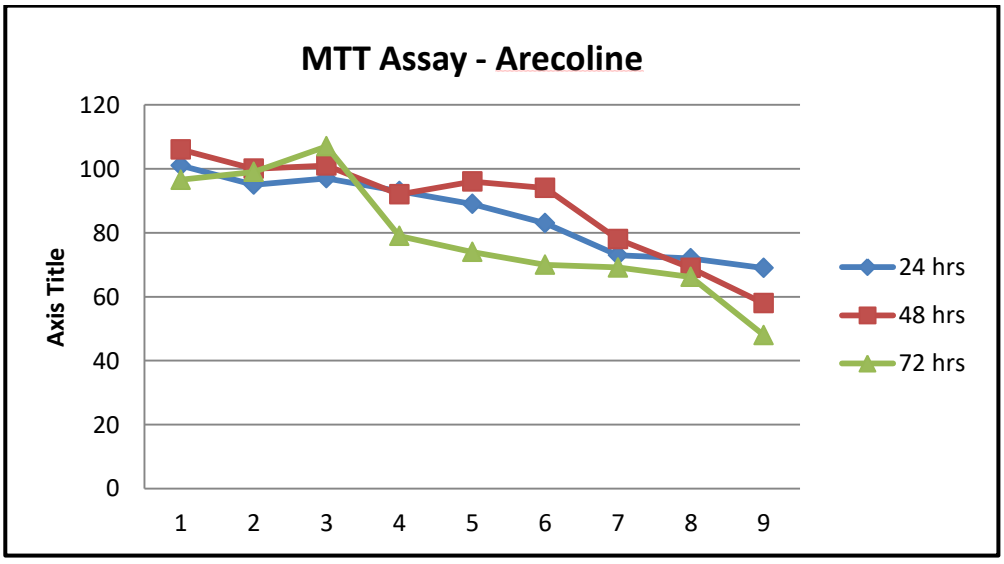
Overall observation:

The cells when exposed to arecoline for 24, 48 and 72 hours, the higher concentration showed decrease in percentage viability of cells and found to be cytotoxic to the cells than compared to the lower concentration of arecoline.

Table 7: MTT Assay of effect of Arecoline on BMF

Concentration (µg/ml)	%Viability 24 hours	%Viability of cells 48 hours	% Viability of cells 72 hours
1.9	101	106	98.55
3.9	95	100	99
7.8	97	101	107
15.6	93	92	99
31.25	89	96	84
62.5	83	94	85
125	**73**	**78**	**69.13**
250	**72**	**69**	**66.2**
500	**60**	**58**	**48**

Graphical representation of effect of arecoline on BMF



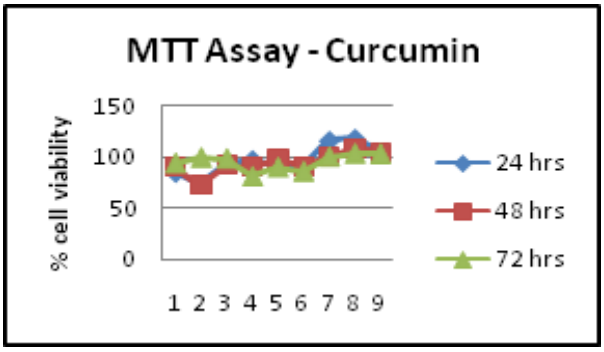
Curcumin

The cytotoxic assessment of different concentration of curcumin using MTT assay on fibroblasts for 24, 48 and 72 hours suggested that the majority of the concentration maintained a good number of viable cells. The higher concentration from 500-125µg/ml showed the viability ranging from 105-95% indicating the proliferation of fibroblasts cells. The lower concentration had no effect thus maintaining the cell viability of about 75-95% of viable cells. Thus curcumin promoted the cell proliferation maintaining the cell number and vitality with minimum cellular death indicating it to be nontoxic to the human buccal mucosal fibroblasts.

Table 8: MTT Assay of effect of curcumin on HBMF

Curcumin (µg/ml)	%viability of cells 24 hrs	%viability of cells 48 hour	%viability of cells 72 hours
1.9	84.5	89.5	94
3.9	73.1	85.2	99
7.8	93	91.9	98
15.6	97	89.5	81.3
31.6	91	97.7	90.9
62.5	90	91	86
125	116	112	107
250	118	108.7	105
500	104	105	103.7

Graphical representation of effect of Curcumin on BMF



Curcumin on Arecoline induced human buccal mucosal fibroblasts:

The arecoline induced fibroblasts were treated with different concentration of curcumin and assessed for the cytotoxicity for 24, 48 and 72 hours respectively. The 24 hours observation revealed that the cell viability for the concentration of 500,250,125 and 62.5µg/ml was found to be 89%, 101%, 98.5% and 107% respectively. The lower concentration less than 62.5 µg/ml showed a good number of viable cells indicating the proliferation of fibroblasts of about 95- 115% of cell viability as shown in table and tabulated on graph. Thus the curcumin when added to the previously arecoline treated cells increased the viability of the cells at lower concentration.

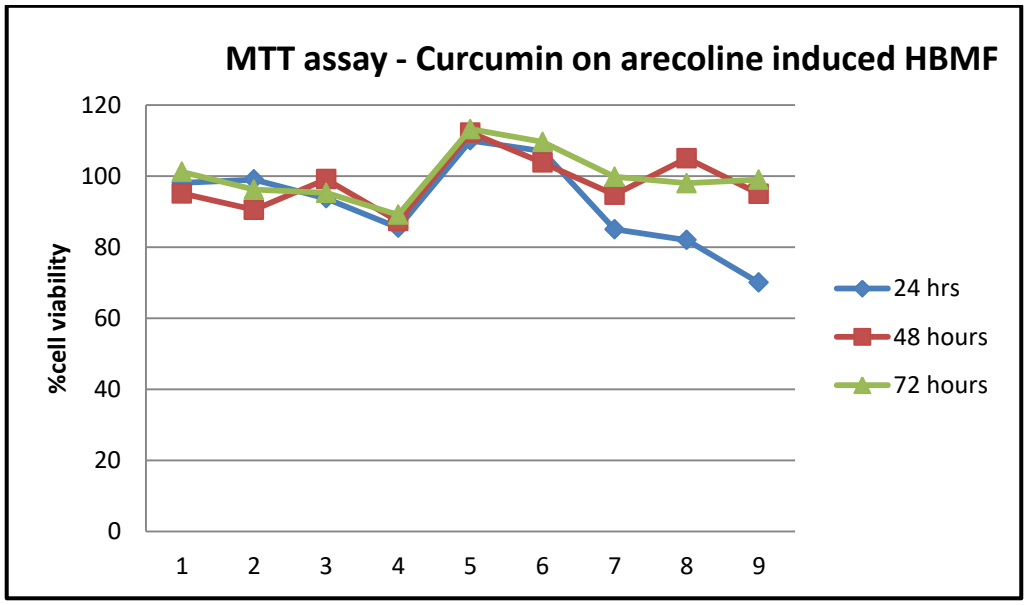
On further exposure of the arecoline treated cells to curcumin for 48 hours, the results were more marked with respect to % cell viability. The higher concentration 500-125µg/ml when calculated for the viability showed viable cells ranging from 85-90 %. The lower concentration had a much higher viable cells ranging between 90-110% of fibroblasts.

Thus the MTT assay assessment for the cytotoxicity of the compounds on HBMF suggested that arecoline hydrobromide was found to be cytotoxic at higher concentration reducing the cell viability to 50% when exposed for 48 and 72 hours. The curcumin was found to be nontoxic in almost all the concentration when exposed for longer duration for 72 hours promoting cell proliferation and maintaining the cell viability. The curcumin when assessed on arecoline induced HBMF increased the viability of the cells by 20-30% thus promoting the proliferation of the fibroblast cells.

Table 9: MTT Assay of effect of Curcumin on Arecoline induced BMF cells

Concentration(µg/ml)	% Cell viability 24 hours	% Cell viability 48 hours	% Cell viability 72 hours
1.9	98.13	95.1	101.2
3.9	99.02	90.5	96.2
7.8	93.7	99.1	95.3
15.6	85.48	87.3	89.12
31.6	110	112.24	113.22
62.5	107	103.8	109.6
125	**98.7**	**94.77**	**99.8**
250	**101**	**105**	**98**
500	**89**	**95**	**99**

Graphical representation of effect of Curcumin on Arecoline induced BMF



Statistical Analysis

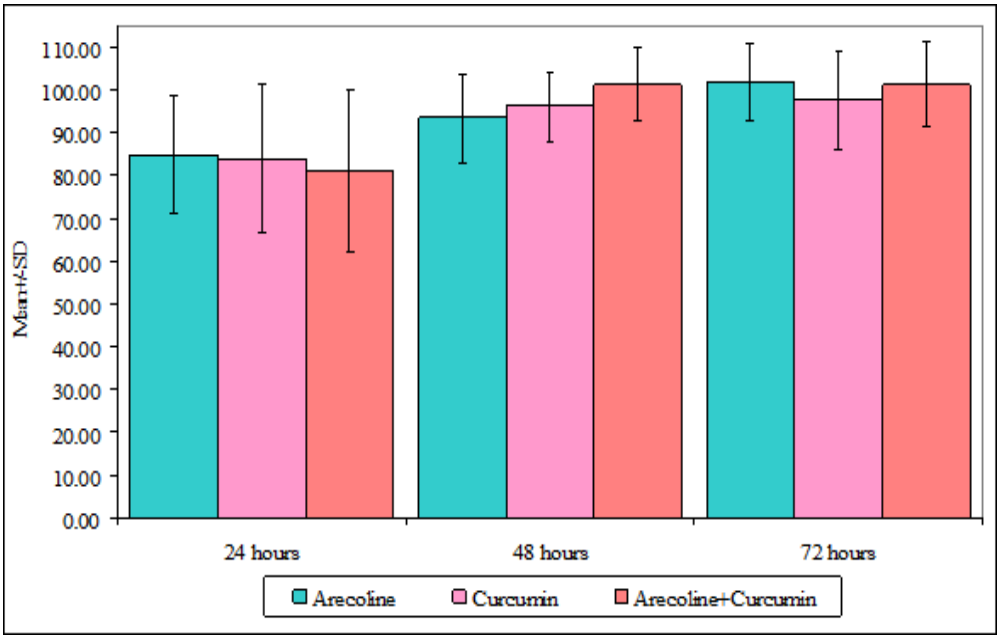
The percentage cell viability scores of all the three groups were compared at different time points by **one way ANOVA**

Time points	Sources of variation	Sum of squares	df	Mean square	F-value	P-value
24 hours	Between Groups	1307.369	2	653.684	5.1080	**0.0140***
	Within Groups	3071.491	24	127.979		
	Total	4378.860	26			
48 hours	Between Groups	1006.716	2	503.358	3.0060	0.0680
	Within Groups	4018.482	24	167.437		
	Total	5025.199	26			
72 hours	Between Groups	2452.349	2	1226.174	6.8160	**0.0050***
	Within Groups	4317.511	24	179.896		
	Total	6769.860	26			
24hrs to 48hrs	Between Groups	216.339	2	108.169	1.9350	0.1660
	Within Groups	1341.547	24	55.898		
	Total	1557.885	26			
24hrs to 72hrs	Between Groups	638.072	2	319.036	5.3540	**0.0120***
	Within Groups	1430.184	24	59.591		
	Total	2068.256	26			
48hrs to 72hrs	Between Groups	331.561	2	165.780	8.5950	**0.0020***
	Within Groups	462.936	24	19.289		
	Total	794.496	26			

$p < 0.05$

Interpretation of the table:

The above table represents the percentage viability scores of all the three groups' i.e between and within the groups at different point times using ANOVA. Statistical significance was observed 24 hours and 72 hours when compared to the viablility percentage scores of 48 hours within and between the groups.



Interpretation of graph:

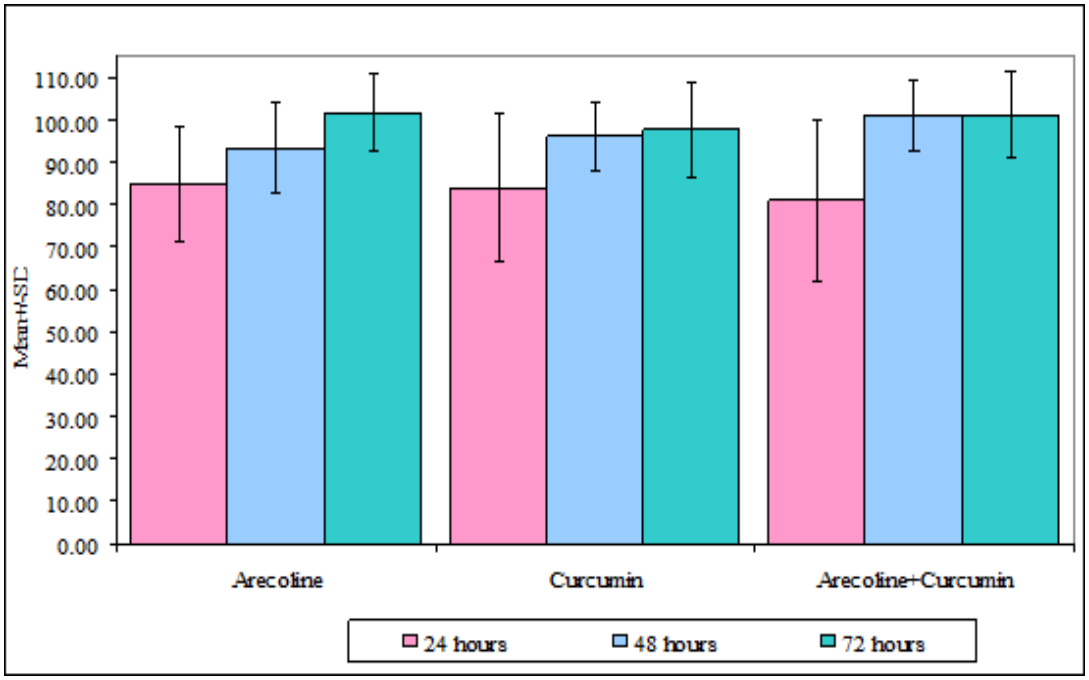
The bar graph shows the percentage viability scores of all the three groups for 24, 48 and 72 hours. The cell viability scores was not variable in the arecoline group at different point times. Whereas in the curcumin and curcumin on arecoline induced group there was an increase in viability of the cells from 24 to 72 hours.

Further the scores of MTT assay of each individual group i.e intra group comparison at different point times were analyzed by dependent t test.

Groups	Time points	Mean	Std.Dv.	Mean Diff.	SD Diff.	% of change	t-value	p-value
Arecoline	24 hours	84.78	13.79					
	48 hours	84.00	17.41	0.78	3.96	0.92	0.5890	0.5721
	24 hours	84.78	13.79					
	72 hours	81.08	19.08	3.69	5.92	4.36	1.8719	0.0981
	48 hours	84.00	17.41					
	72 hours	**81.08**	**19.08**	**2.92**	**3.97**	**3.47**	**2.2062**	**0.0500***
Curcumin	24 hours	93.40	10.47					
	48 hours	96.14	8.10	-2.74	4.92	-2.94	-1.6731	0.1328
	24 hours	93.40	10.47					
	72 hours	**101.24**	**8.48**	**-7.84**	**8.56**	**-8.40**	**-2.7490**	**0.0251***
	48 hours	**96.14**	**8.10**					
	72 hours	**101.24**	**8.48**	**-5.10**	**5.14**	**-5.30**	**-2.9787**	**0.0176***
Arecoline+Curcumin	24 hours	101.83	9.17					
	48 hours	97.63	11.57	4.19	11.31	4.12	1.1118	0.2985
	24 hours	101.83	9.17					
	72 hours	101.37	10.16	0.45	8.39	0.45	0.1620	0.8753
	48 hours	97.63	11.57					
	72 hours	**101.37**	**10.16**	**-3.74**	**3.97**	**-3.83**	**-2.8276**	**0.0222***

Interpretation of the table:

The above table represents the viability scores of MTT assay of each individual group i.e intra group comparison at different point times analyzed by dependent t test. Arecoline group showed decrease in the viability of the cells at 72 hours which was statistical significance (p-0.05). Similarly in the second group curcumin we found an increase in viability percentage of cells at 48 and 72 hours and was significant (p value 0.02) .Whereas, the third group had an increased percentage viability of cells only at 72 hours and found to be statistical significance (p-0.02).The above scores were again analyzed by plotting a bar graph representing viability percentage of all the three groups as shown below.



Interpretation of graph:

The above graph represents the viability scores analysed using dependent t test at different time intervals. We observed that all the three compounds showed increase in proliferation of cells from 24 hrs to 72 hrs

1. Neutral Red Assay (NR)

The Neutral Red Assay was conducted further to assess cytotoxicity of Arecoline, curcumin and effect of Curcumin on arecoline induced buccal fibroblasts.

Arecoline:

Arecoline was assessed for the cytotoxicity on buccal mucosal fibroblasts for 24, 48 and 72 hours.

1. **24 hours** : The concentration 500 µg/ml showed a cell viability of 70% that is about 30% of the cell death was observed, whereas 250µg/ml and 125 showed a slight increase in the cell viability of around 80% with 20 % of cell death. Thus the first

three concentrations are considered as cytotoxic based on the number of viable cells. The further concentration of Arecoline hydrobromide did not alter the viability of the cells with cells maintaining the cell number above 90 %(Table, graph).

2. **48 hours:** There was a steep decrease in the cell count when exposed for 48 hours to about 50% of the count observed for 24 hours. The concentration 500µg.ml, 250µg/ml and 125µg/ml showed a marked reduction in the cell number to 50. % , 61% and 69% respectively. Thus the above concentrations are cytotoxic to fibroblasts decreasing the cell viability. The lower concentration ranging from 62.5- 1.9µg/ml had a cell viability ranging between 70 – 90% of viable fibroblasts suggesting less toxic compared to the higher concentration of arecoline.

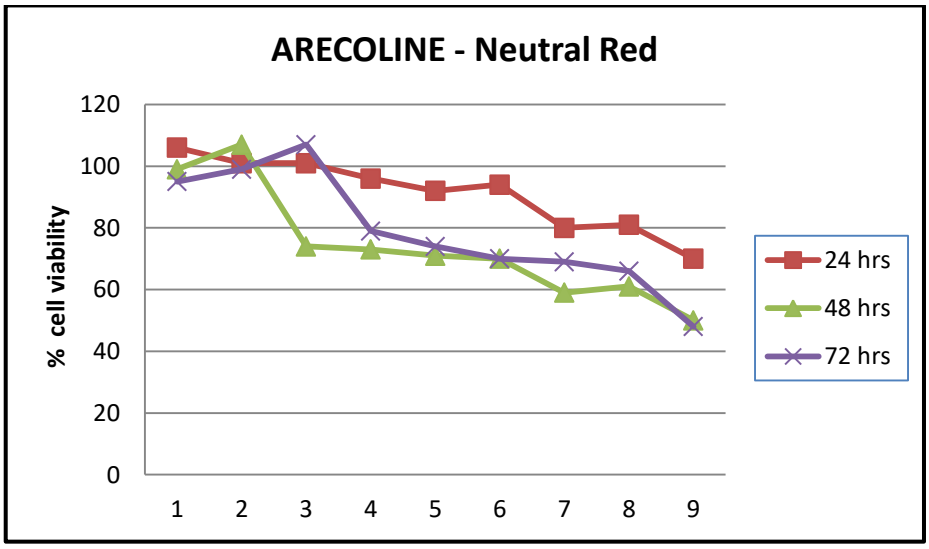
72 hours:

On exposure of the cells for 72 hours to arecoline hydrobromide, there was a decrease in the cell count in almost all the concentration except the concentration from 7.8 -1.9µg/ml. arecoline at 500,250, 125 and 62.5µg/ml showed a cell viability ranging from 40-65% .Thus when cells exposed for longer duration, their viability also decreased in a dose dependent manner.

Table 12: NR Assay - effect of Arecoline BMF cells

Concentration (µg/ml)	%Cell Viability 24 hrs	48 hrs	72 hrs
1.9	106	99	95
3.9	101	107	99
7.8	101	74	107
15.6	96	73	79
31.6	92	71	74
62.5	94	70	70
125	**80**	**59**	**69**
250	**81**	**61**	**66**
500	**70**	**50**	**48**

Graphical representation of effect of Arecolineon BMF

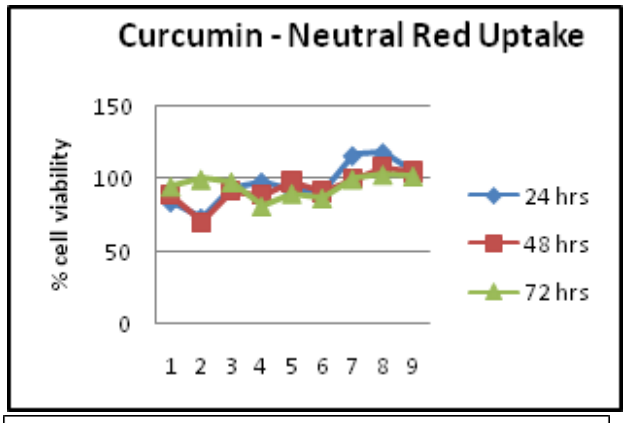


CURCUMIN

The neutral red assessment of different concentration of curcumin on fibroblasts for 24, 48 and 72 hours suggested that the majority of the concentration had a good number of viable cells. The higher concentration 500-125µg/ml showed the viability ranging from 105-95% (table and graph). The lower concentration did not affect viability of the cells maintaining the 85-95% of viable cells and represented graphically. Thus curcumin promoted the cell proliferation maintaining the cell number and viability with minimum cellular death though it was exposed to 72 hours, indicating it to be nontoxic to the human buccal mucosal fibroblasts.

Concentration (µg/ml)	% cell viability 24 hrs	48 hrs	72 hrs
1.9	84	89	94.7
3.9	73	70	99.7
7.8	93	91.7	98
15.6	97.8	89	81
31.6	91.7	97.8	89.9
62.5	90.6	91.1	86.7
125	**116**	**100**	**100**
250	**118**	**108**	**103**
500	**104**	**105**	**102**

Table: NR **Assay - effect of Curcumin on BMF cells**



Graphical representation of effect Curcuminon BMF

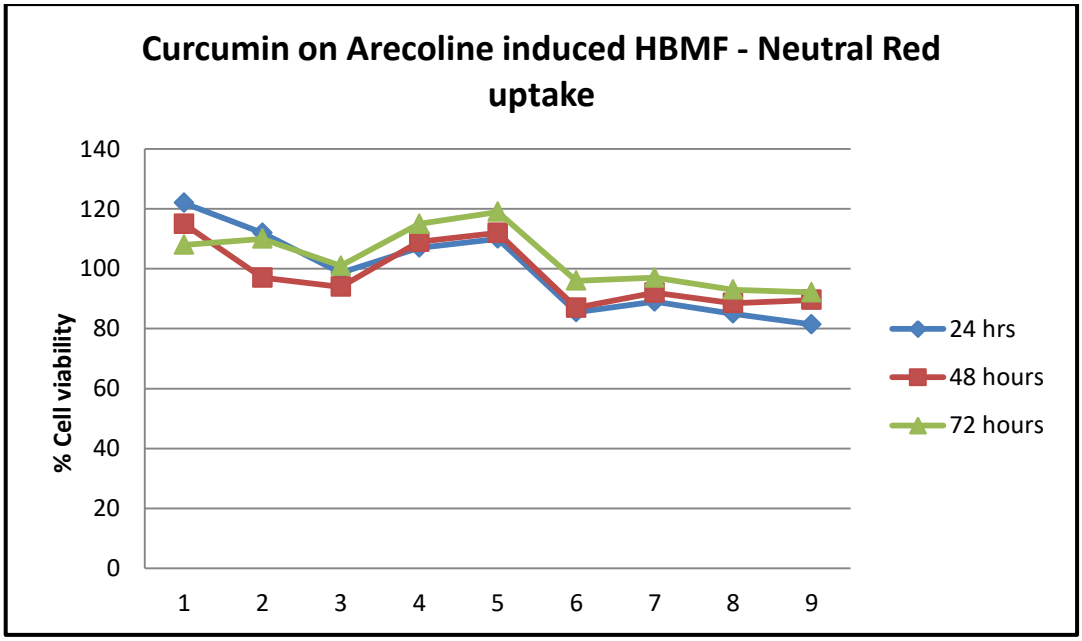
CURCUMIN ON ARECOLINE INDUCED HBMF:

The arecoline induced fibroblasts when treated with different concentration of curcumin for 24,48 and 72 hours revealed that the percentage viability of the cells for higher concentration 500,250,125 and 62.5µg/ml was found to be 80-90 % of cell viability indicating the proliferation of fibroblasts. Whereas lower concentration of curcumin on arecoline treated cells showed a much higher viable cells ranging between 90-120% of fibroblasts maintaining the cell viability in almost all the concentration of curcumin compound..

Table: NR **Assay - effect of Curcumin on Arecolne induced BMF cells**

Concentration (µg/ml)	% cell viability 24 hrs	% cell viability 48 hrs	% Cell viability 72 hrs
1.9	122	115	108
3.9	112	97	110
7.8	98.7	94	101
15.6	107	109	115
31.6	110	112	119
62.5	85.5	87	96
125	89	92	97
250	85	88.5	93
500	81.4	89.6	92.13

Graphical representation of effect Curcumin on Arecolineinduced BMF



Statistal analysis of Neutral red assay:

Comparison of three groups with cell viability scores at different time points by one way ANOVA

Table 15: Cell viability scores of three groups by one way ANOVA.

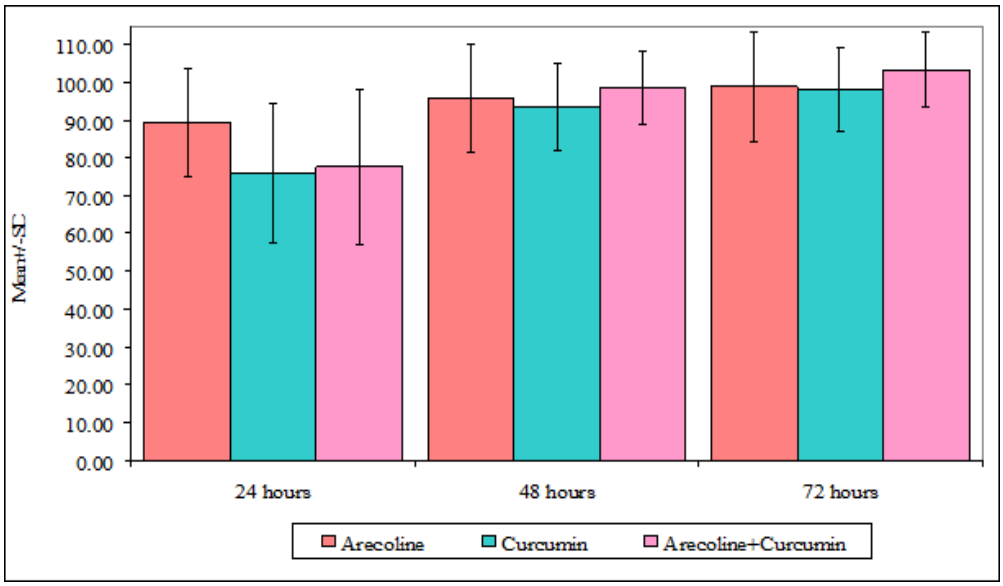
Time points	Sources of variation	Sum of squares	df	Mean square	F-value	P-value
24 hours	Between Groups	425.334	2	212.667	1.0120	0.3790
	Within Groups	5044.767	24	210.199		
	Total	5470.101	26			
48 hours	**Between Groups**	**2477.512**	**2**	**1238.756**	**6.3690**	**0.0060***
	Within Groups	**4668.109**	**24**	**194.505**		
	Total	7145.621	26			
72 hours	**Between Groups**	**3352.570**	**2**	**1676.285**	**8.2480**	**0.0020***
	Within Groups	**4877.822**	**24**	**203.243**		
	Total	8230.392	26			
24hrs to 48hrs	**Between Groups**	**863.722**	**2**	**431.861**	**6.1350**	**0.0070***
	Within Groups	**1689.558**	**24**	**70.398**		
	Total	2553.280	26			
24hrs to 72hrs	**Between Groups**	**1411.701**	**2**	**705.850**	**6.9070**	**0.0040***
	Within Groups	**2452.698**	**24**	**102.196**		
	Total	3864.399	26			
48hrs to 72hrs	Between Groups	67.134	2	33.567	0.2830	0.7560
	Within Groups	2849.664	24	118.736		
	Total	2916.799	26			

Interpretation of the table:

The above table represents the percentage viability scores of all the three groups' i.e. between and within the groups at different point times using ANOVA where a statistical significance was observed in 48 hours and 72 hours when compared

to the scores of 24 hours within and between the groups. When the time points were compared with each other a significant increase in the viability of the scores was noted between 24-48 hours (p-0.007) and 24-72 hours (p- 0.004).Further the scores were plotted on a bar graph for all the three groups.

Graph 9: Graphical representation of effect Curcumin on Arecoline induced BMF.



Interpretation of graph:

The above graph represents the viability scores of all the three groups compared at different point times. At 48 and 72 hour percentage cell viability scores were markedly increased when compared to the scores of 24 hours of all groups. Thus indicating the cell viability scores increased in curcumin and combination group when exposed to 48 and 72 hours.

Table 16: Comparison of different time points with cell viability scores in three groups by dependent t test

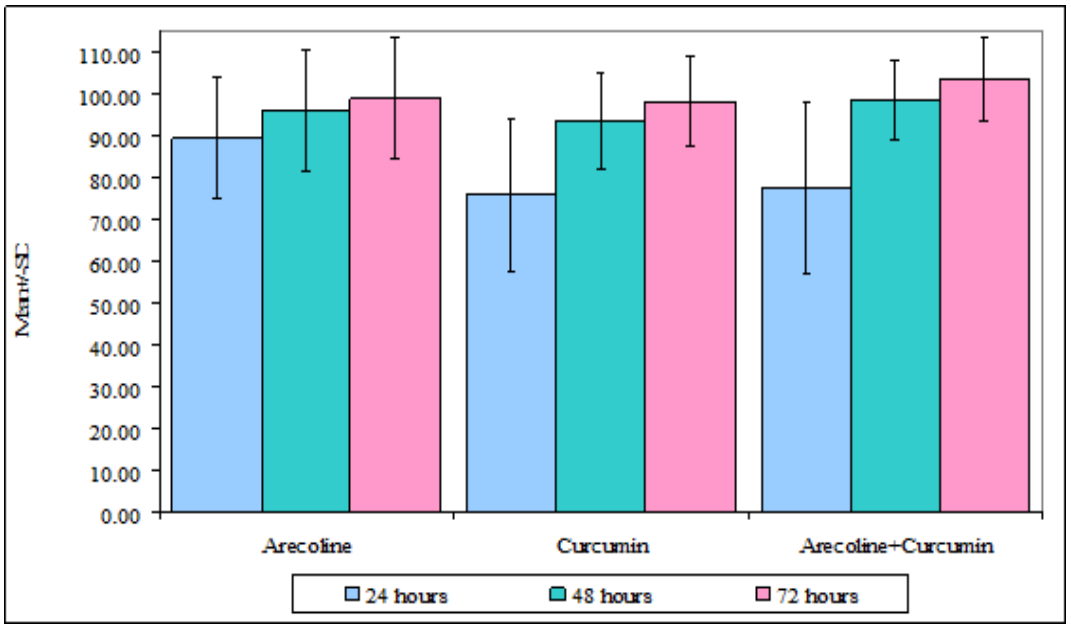
Groups	Time points	Mean	Std.Dv.	Mean Diff.	SD Diff.	% of change	t-value	p-value
Arecoline	**24 hours**	**89.44**	**14.53**					
	48 hours	**76.00**	**18.28**	**13.44**	**10.61**	**15.03**	**3.8022**	**0.0052***
	24 hours	89.44	14.53					
	72 hours	**77.77**	**20.50**	**11.68**	**9.55**	**13.06**	**3.6683**	**0.0063***
	48 hours	76.00	18.28					
	72 hours	77.77	20.50	-1.77	14.01	-2.32	-0.3782	0.7151
Curcumin	24 hours	95.94	14.51					
	48 hours	93.61	11.48	2.33	7.06	2.43	0.9909	0.3507
	24 hours	95.94	14.51					
	72 hours	98.60	9.60	-2.66	12.26	-2.77	-0.6499	0.5340
	48 hours	93.61	11.48					
	72 hours	98.60	9.60	-4.99	11.40	-5.33	-1.3129	0.2256
Arecoline+Curcumin	24 hours	98.96	14.45					
	48 hours	98.23	10.84	0.72	6.98	0.73	0.3103	0.7643
	24 hours	98.96	14.45					
	72 hours	103.46	9.87	-4.50	8.07	-4.55	-1.6739	0.1327
	48 hours	**98.23**	**10.84**					
	72 hours	**103.46**	**9.87**	**-5.23**	**5.46**	**-5.32**	**-2.8688**	**0.0209***

Interpretation of the table:

The above table represents the comparison (intra-group) of percentage viability scores of all the three groups at different point times using dependent t test .A statistical significance was observed in arecoline group for 24 ,48 and 72 hours with decrease in the cell viability. We did not find any significant changes in the curcumin treated group. A significance of p value 0.029 was noted in the curcumin and

arecoline combination group for 48 and 72 hours indicating an increase in the viability of cells when treated with curcumin on arecoline induced fibroblasts.

Graph 10: Graphical representation of all the three groups' comparisons at different point times.



Interpretation of graph:

The above bar graph represents the percentage viability scores of cells compared within the groups at different point times. The 48 and 72 hour scores were markedly increased in all the three groups when compared to the percentage cell viability scores of 24 hours in curcumin and combination of curcumin and arecoline group.

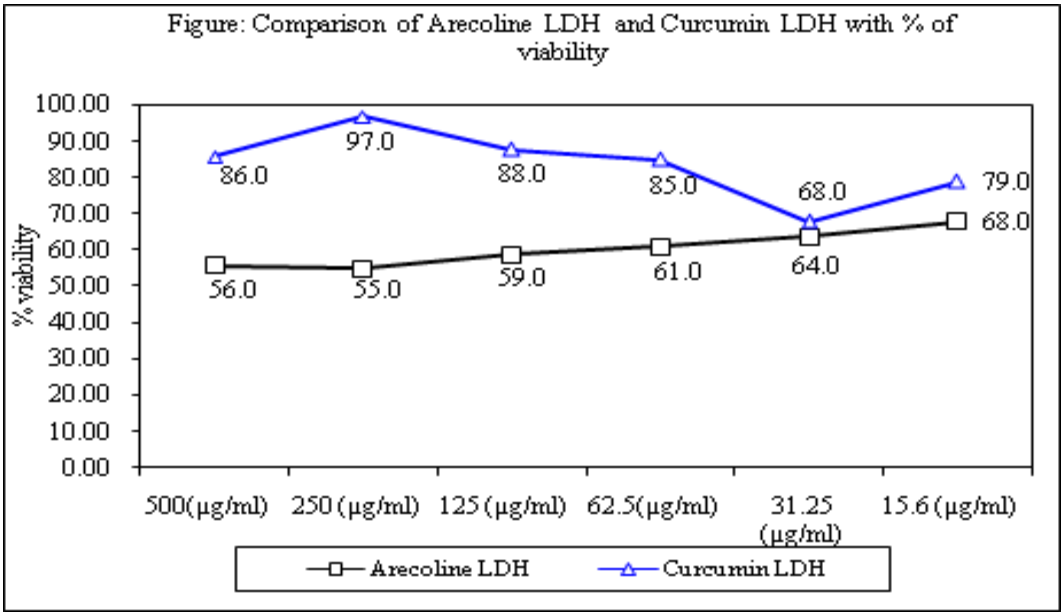
Latate Dehydrogenase Assay:

The lactate dehydrogenase assay was carried out to assess the cytotoxicity of compounds Arecoline and curcumin on human buccal mucosal fibroblasts The assay showed that when the cells treated with higher concentration of arecoline i.e 500 - 125

µg/ml, the % viability was reduced to 50% indicating the cell death and cytoxicity(table) indicating the arecoline cytotoxic to the cells with the release of lactate dehydrogenase enzyme. Whereas the similar concentration of curcumin maintained the cell viability more than 85%. Proving it to be nontoxic and promoting the proliferation of the buccal fibroblast cell lines.

Table 17: Lactate Dehydrogenase assay

Concentration(µg/ml)	Arecoline LDH Ex/em	% viability	Curcumin LDH ex/em	% viability	Negative control	Positive Control
500	0.651	56	0.100	86%	0.078	0.115
250	0.642	55	0.112	97%		
125	0.683	59	0.102	88%		
62.5	0.707	61	0.098	85		
31.25	0.741	64	0.091	68		
15.6	0.787	68%	0.088	79		



Florescent staining –DAPI Technique:

ARECOLINE:

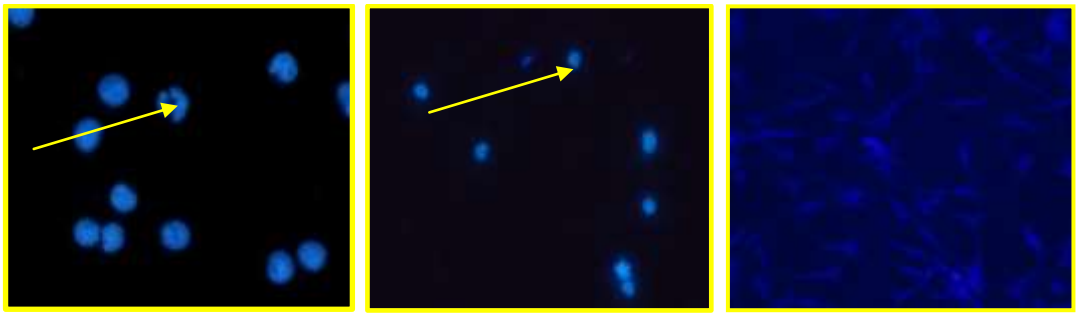


Fig: 15 Florescent images a) 500 µg.ml b) 250 µg/ml c)125µg.ml

CURCUMIN

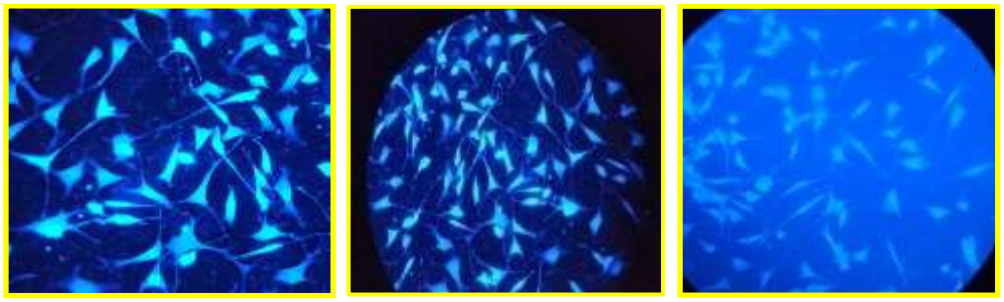


Fig: 16 Florescent images a) 500 µg.ml b)250 µg/ml c)125µg.ml

The flouroscent images of cellls treated with higher concentrations of arecoline showed pyknotic and fragmented nuclei when compared to the cells treated with curcumin which was able to maintain the spindle shape of the cells in almost all the concnetrations that is 500,250 and 125µg.ml of curcumin. This insdicates arecoline is cytotoxic at higher concentrations altering the morphology and nuclear changes leading to cell death. Whereas curcumin maintained the cell morphology without causing the nuclear changes and hence proves to be nontoxic to cells.

DISCUSSION

The oral cavity is a complex structure lined by oral mucous membrane consisting of oral epithelium and the connective tissue. The majority of cells of the connective tissue are formed by the fibroblasts that play an important role in wound healing in oral cavity, mainly synthesizing the collagen fibers that support the adjacent structures and anchor the cells to each other thus providing strength and elasticity. There are pathological conditions where the fibroblasts have altered morphology and function like cardiac fibrosis, lung fibrosis, scleroderma, liver fibrosis, "Oral submucous fibrosis (OSMF), etc. Oral Submucous Fibrosis is a premalignant condition caused by consumption of betel nut or arecanut and nutritional deficiencies leading to burning sensation, blanching of oral mucosa and restricted mouth opening" .The morphological and functional aspects of fibroblasts are affected by arecoline from arecanut.[7]

Our study includes the isolation and culture of primary cell lines of buccal fibroblasts from healthy patients and to assess the effect of arecoline on the cultured fibroblasts. The cells will be further assessed by the effect of curcumin on arecoline treated or induced primary fibroblast cell lines. The tissues for the processing were procured from healthy individuals undergoing routine impacted third molar extraction and processed in tissue culture lab for isolation of buccal mucosal fibroblasts cells. Primary culture of the cells was performed using a combination technique that is explants - enzamytic technique. The isolated cells were authenticated and characterized using STR profiling from DNA forensics lab, New Delhi and named as KLEBF 18. After authentication the buccal mucosal fibroblasts cells were assessed using the compounds arecoline, curcumin and combination of both for morphological

analysis and cytotoxic analysis using MTT assay, Neutral Red Uptake assay and LDH assay".

The culturing and the research on primary cells is since decades using commercial cell lines. The cells provide great information and an insight on the biological processes of human cells with wide applications like drug testing, its metabolism and toxicity, genetic research, in the synthesis of various vaccines, antibodies and other compounds related to biological process.[15] A lot of studies have been conducted to culture the primary cells of human origin like skin fibroblasts, epithelial cells, cardiac fibroblasts etc. Whereas a very few studies have been reported on culture of oral fibroblasts.[16]

The studies have revealed that there are two basic techniques for the primary culture and their isolation from human tissue samples. Bolls and Lee suggested enzymatic and explant method. Daniels et al was the first to describe the enzamytic technique of isolation and culture of cells. Billingham and Reynolds in their study proposed a method to isolate the epithelial cells using enzyme that is trypsin, later they called this technique as enzymatic method.[17] The other method is the tissue dissociation or enzymatic technique with the use of proteolytic enzymes to isolate the cells. The common enzyme used in many tissue culture techniques for primary culture is trypsin a proteolytic enzyme which breaks down the protein and inactivate the adhesive and intracellular molecules so as to detach the cells from the plate and float. The main advantage of the enzamytic technique is that the cells easily get disassociated from the main explant tissue and the undesired cells are removed. The optimum concentration of trypsin used is 0.25 to 1% along with EDTA a chelating

agent that weakens the cell matrix. In our study we have used 0.25 % Trypsin with EDTA as this concentration did not alter the cell morphology and behavior.[17]

Carrel and Burrows, in their study isolated the epithelial cells using explant method where the cells isolation was done without using the enzymes. However, they concluded that the cells did not dissociate easily from the parent tissue and it was time consuming for the cells to dissociatefrom the explants tissue. The main disadvantage of the explant method is that the cells do not get separated from the tissue sample and isolation becomes difficult resulting in the failure of development of monolayer.[19]

The study by Orazizadeh et al. suggest explants method to isolate the keratinocytes, there were successful in detaching the cells from main tissue within 24 h whereas the fibroblasts were isolated after 4 days of culture where no enzyme was used for isolation of cells.[17]

Thus considering the advantages and disadvantages of both enzamytic and explants technique we used a combination of the technique that is explant – enzamytic technique where 0.5 % of Trypsin with EDTA was used. The use of trypsin with this particular concentration showed no alteration in the biological behavior of the cells, also helped in the removal of other contaminating cells, such as epithelial cells, RBCs and other inflammatory cells for the isolation of cells from tissue samples. By using this technique we were successful in isolation of cells on 3rd day of our culture and a confluent culture was obtained around 14th day. Once the primary culture of buccal fibroblasts reached confluency, the cells were morphologically identified as buccal fibroblasts and three different shapes were noted that is spindle shape (F1), epitheloid shape (F2) and stellate shape (F3) that were also noted in the study by Oliver et al.[19]

The cell lines were further processed for characterization and authenticated using DNA/STR profiling from DNA Forensic Laboratory, New Delhi India. The STR Profiling results confirmed that the cells resemble the fibroblasts genetically and are of human origin possessing a unique loci on genes tandem repeats. The cells were the subcultured and further stored for further assays to assess the effect of Arecoline and Curcumin on the Human buccal mucosal fibroblast cell lines.

The International Agency for Research and Cancer (IARC) a sponsored group of World Health Organization declared arecanut as Group I carcinogen in development of oral premalignant and malignant cancers.[29]

Arecoline is one of the potent constituent for the causation of premalignant conditions like Oral Submucous Fibrosis. The alkaloids of aecanut have a deleterious effect on both morphology and molecular changes on the fibroblasts of oral cavity. Previous animal studies have established different shapes of fibroblasts that are F1-spindle shaped which produces low levels of collagen and is highly proliferative, F2-Epitheloid which produces high collagen than F1 and F3- stellate shaped which is least proliferative with maximum level of type 1 and type 3 collagen production.[45]

After the authentication of buccal mucosal fibroblasts, the cells were subjected for the Morphological and cytotoxic assessment of the compounds. In our study when the buccal fibroblasts were treated with different concentration of arecoline, we found that the morphological alteration were in a dose dependent manner. The higher concentration of arecoline from 500-250µg/ml altered the spindle shape (F1) of the cultured fibroblasts resulting in the degenerative changes when exposed for 48 hours. Whereas the lower concentration of arecoline from 15 -1.9 µg/ml maintained the spindle shape (F1) of fibroblast. The arecoline concentration of 125 µg/ml showed all

the three i.e F1, F2 and F3 variants of fibroblasts. The results of our study were very similar to the study done by Deepu George Mathew et al where they compared the effect of arecoline on fibroblasts of tobacco chewers with or without Oral Submucous Fibrosis. They concluded that in their primary culture of fibroblasts the three basic variants of fibroblasts were noted i.e F1, F2, F3. The chronic tobacco chewers for long duration of 3 months showed more of stellate (F3) cells and epitheloid (F2) than spindle shaped.[27]

(F1) cells. Thus when fibroblasts are subjected to betel nut chewing the morphological variations are noted. In another study by Abhishek Banerjee et al in 2017 reported various morphological alterations after treatment of isolated buccal fibroblasts with different concentrations of arecoline. They reported that F2 (epitheloid) cell lines predominated in most of the cultures which was due to F1 cells transforming to F2 cells when continuously exposed to arecoline for 8 days. The F3:F1 ration was found to progressively increase at higher concentration of>100µg/ml.[60]

Our observation was found to be consistent, where F3 and F2 population of cells predominated than F1 fibroblast with the effect of arecoline in dose dependent manner. In our experiment results it was found that higher concentration of arcoline was toxic to cells showing degenerative changes but noted that some of the cells were round and stellate shape suggestive of transformation with the effect of arecoline. However with the concentration of 125, all the three shapes of fibroblasts were observed suggesting that all these fibroblasts helps in secretion of abnormal collagen (seen in moderate and advanced stage of Oral submucous fibrosis). Whereas the lower concentration maintained the spindle shape (F1) throughout the culture which secretes

low levels of collagen and are highly proliferative. Our experiment is in consistent with the clinical findings of OSF with the effect of arecoline.

When we observed the morphological changes with the effect of curcumin, it was surprising to find that the higher concentration of arecoline showed degenerating changes but curcumin at all concentration did not show any degenerative changes suggesting that curcumin is non toxic to BMF. At all the concentration of curcumin, consistently F1 type fibroblasts were present.

A very limited data is available on the effect of curcumin on the morphology of normal cells. However some studies found that curcumin was non toxic for normal cells at concentration of 100µg/ml and maintained the morphology. In a study done by Sangeetha et al in the year 2020 where they compared the effect of curcumin and chlorhexidine on human fibroblasts .They found that an increase in concentration of chlorhexidine altered the morphology of fibroblast with nuclear fragmentation, cytoplasmic blebbing and echinoid spikes at cellular level. Whereas curcumin did not alter the shape of fibroblasts maintaining F1 shape fibroblasts throughout the culture. The observations were very similar to our study.[84]

The cultured cells were further assessed for the effect of curcumin on arecoline induced fibroblasts and observed for 48 hours. In our study we found that at higher concentration i.e 500- 250µg/ml the curcumin did not reverse back the altered morphology caused due to arecoline i.e loss of F1 shape. However, curcumin maintained the spindle (F1) and stellate shape (F3) at concentration from 62. 5- 1.9µg/ml. The results were similar a study done by Praveen kumar et al in 2017 where they assessed the effect of curcumin on cultured pulpal fibroblasts. According

to their study curcumin maintained the morphology and viability of normal pulp fibroblasts at as high as 174%, 310%, and 317%, respectively.[97]

Thus with above data and discussion curcumin proves to be nontoxic when compared with arecoline with respect to the morphology of cultured buccal fibroblasts.

Our study also includes the assessment of cytotoxic effect of all the three compounds using MTT, Neutral red assay and LDH assay on cultured buccal mucosal fibroblasts. The first cytotoxic assay we perfrmed was MTT assay. The cytotoxicity of arecoline has been reported in literature proving it to be a toxic compound to cells in a dose dependent mannerIn a study done Sundqvist et al in year 1992, the effect of aqueous extract of arecanut on cultured human buccal epithelial cells was assessed. The MTT assay of arecoline on the cells showed a decreased cell viability and morphological alteration in dose dependent manner, that is the acute exposure of the cells for 3 hour to higher concentration of arecoline>300µg/ml that there were alteration in the cell morphology, loss of colony formation of the normal cells, and a decreased cell viability was noted of less than 50 percentage of cells.

Another study by Chang et al in 2001 where they discussed the adverse and cytotoxic effects of arecoline on PDL that is periodontal fibroblasts and found that arecoline at concentration 100µg/ml inhibited the DNA synthesis and reduced the percentage cell viability to 50% for 6 hour of incubation. Chiang et al cultured the primary gingival fibroblasts from healthy individuals and subjected them with different concentrations of arecoline that is 20,40,100 and 200µg/ml. They found that arecoline inhibited the growth of cells with 40% of the cell viability observed at 100µg/ml of arecoline. They also reported that at concentration>200µg/ml the

arecoline completely inhibited the DNA synthesis resulting in cell death. Abhishek et al in the year 2017 cultured the fibroblasts from both normal and Oral submucous fibrosis diagnosed tissue and assessed the concentration (50/100/150/300/500 ug/ml) of arecoline on the cell viability and cell count for a period of 8 days. They reported that arecoline at concentration 50µg/ml had a stimulatory effect whereas at 150µg/ml had an inhibitory effect.[68]

When our results were compared with the previous experiments we also found that the concentration of arecoline above 125-500µg/ml were cytotoxic to the fibroblasts with 50-60% reduction of cell viability for 72 hours. Whereas the lower concentration from 62.5-1.9µg/ml maintained the cell viability of 80-90%, considering as nontoxic to the cells. This could be probably due to the reason that higher concentration of arecoline induces oxidative stress andgenotoxic effects. Arecoline was found to decrease the percentage of cells in G1 and S phase, resulting in the depletion of cell membrane and the mitochondrial dysfunction that fails in the action of NADPH oxido- reductase cell death. Thus our study results were in accordance with above study where curcumin promoted cell proliferation in normal cells and maintained the viability in all the concentration.

The cytotoxic assay of curcumin on arecoline induced fibroblast also had similar results like curcumin except in the higher concentration from 500-250µg/ml due to the toxic effect of arecoline that already damaged the fibroblasts leading to cell death. Whereas the lower concentration maintained the cell number of arecoline induced fibroblasts. Thus curcumin is said to be nontoxic and promoted the proliferation of fibroblasts and maintained the cell viability in all the concentration when compared to arecoline which is cytotoxic to the cells.

Enzyme to reduce the MTT formazon crystals in to purple coloured crystals. Whereas the probable hypo thesis for The Neutral red Assay for further assesing the lysosomal damage of the cultured fibroblasts on cells treatement with Arecoline, curcumin and curcumin on arecoline induced cells was performed in our study. The results of our study showed that at higher concentration from 500- 125 µg/ml the cell viability reduced to 50% than at lower concentration i.e 62.5-1.9µg/ml where the cell viability was 80-95 %. The results of effect of arecoline on fibroblasts were very similar to the previous (ref and quote the studies) cytotoxic studies of arecoline on gingival, periodontal and pulp fibroblasts where the toxicity of arecoline at higher concentration is due to the lysosomal damage and this may due to inhibition of Activated Metabolic Protein kinases by ROS and resulting in increased ROS levels in the cells with attenuation of N-acetyl cysteine and glutathione resulting in apoptosis and cell death. Thus the arecoline is cytotoxic and leading to lysosomal damage causing progressive cell death of fibroblasts at higher concentration when compared to lower concentration which maintained the cell viability of 70-95%.[65]

Literature shows few studies on the assessment of effect of curcumin using neutral red assay on normal fibroblasts. In our study curcumin at higher concentration from 500-125µg/ml showed the viability ranging from 105-95% whereas at lower concentration maintained the cell viability 85-95% of viable cells for 72 hours. Similar results were also noted in our study when curcumin was assessed on arecoline induced fibroblasts where all the concentration maintained the cell viability to 80-100% .This is due the antioxidant property of curcumin inhibiting the ROS activation and attenuating the proliferative factors like CDKs enhancing the S phase and M phase of cell cycles Further our study also included the cytotoxic assessment of effect arecoline and curcumin on fibroblasts using Lactate dehydrogenase assay

(LDH) .[70] The higher concentration of Arecoline was cytotoxic to the fibroblasts with increased levels of lactate dehydrogenase enzyme expressed reducing the viability of cells to 50% whereas curcumin with respective concentration maintained the viability to 85%. This proves arecoline as a toxic compound when compared to curcumin causing the cellular damage with the release of lactate dehydrogenase enzyme and leading to cell death. Thus curcumin is nontoxic compound maintaining the cell number without damaging the cellular components of fibroblasts.

Hence with the above results and discussion arecoline is considered as a cytotoxic compound altering the morphology and inhibiting the cell growth and proliferation of normal fibroblasts resulting in reduced remodeling of collagen fibres and progression of fibrosis condition as observed in Oral submucous fibrosis. Whereas Curcumin on the other hand was nontoxic to the buccal fibroblasts without altering the morphology, maintaining the cell viability, inhibiting the cells to undergo apoptosis and promoting the proliferation of cells in all the concentrations thus proving it to be a safe natural product in the management of Oral submucous fibrosis.

SUMMARY

"Oral Submucous fibrosis is a potentially premalignant condition characterized with submucosal fibrosis, burning sensation ,blanching of oral mucosa and eventually leading to restricted mouth opening .which is most commonly seen in Indian population." Arecanut is considered to be the most potent factor for the pathogenesis of this precancerous condition. The arecanut consists of alkaloids of which arecoline plays a major role in Oral submucous fibrosis. Arecoline has been reported to cause deleterious effect both on morphology and cytotoxicity on fibroblasts leading to abnormal collagen production. Though there are many treatment modalities for the management of Oral submucous fibrosis like cessation of habbit followed by corticosteroid therapy, surgical excision of fibrous bands, diathermy etc but the disease still remained irreversible. Other than this conventional method of approach, the natural mode of management of Oral submucous fibrosis has also been reported using Curcumin ,tulsi ,neem etc. Studies and the clinical trials of crcumin in OSMF have proved that use of curcumin has minimized the clinical signs and symptoms. Hence with this background of literature we designed our study to assess the effect of arecoloine and curcumin on human buccal fibroblasts.

To assess the effect of these compounds we first developed Human Buccal Mucosal Fibroblasts (HBMF) and authenticated the primary cell lines using STR/DNA profiling and named as KLEBF-18. The STR profiling proved that the primary cultured cells were unique in nature and resembles fibroblasts of human origin. Once the primary cultures of fibroblasts were established the cell lines were assessed for the morphological changes with the addition of compound arecoline, curcumin and curcumin on arecoline induced fibroblasts. The serial dilutions of the

compounds were standardized and the experiments were carried from 500 µg/ml to 1.9 µg/ml .

We observed:

- At highest concentration of arecoline the fibroblasts showed degenerative changes indicating cell death, with the same concentration curcumin maintained the spindle shaped fibroblasts.
- Only at particular concentration of arecoline and curcumin (125 µg/ml) all the types of fibroblasts (F1, F2, F3) were observed.
- Curcumin had maintained consistently spindle shaped fibroblasts at all concentrations.

The addition of curcumin compound on arecoline induced fibroblasts revealed that curcumin could not reverse the degenerative changes caused by arecoline at higher concentrations (500 µg/ml to 250 µg/ml) but could maintain spindle shape at other concentrations. It proved that curcumin maintained the normal spindle shaped morphology of fibroblasts at all the concentrations.

- The cytotoxic assays with MTT, Neutral Red and LDH on developed primary cell lines of HBMF (KLEBF-18). Arecoline at higher concentration was found to be cytotoxic with 50% cell viability as compared to the lower concentrations.
- Whereas in contrast to arecoline, curcumin was nontoxic to the fibroblasts in all the concentrations and maintained the cell viability more than 80% and promoted proliferation. Thus proving curcumin to be a non toxic compound on HBMF.

CONCLUSION

"Oral submuocus fibrosis is one of the most prevalent premalignant conditions of oral cavity in India with irreversible changes noted due to the effect of arecoline." India is known to be the hub of innumerous herbal products and curcumin being one of the potent herbal drug for wide clinical application. Hence we focused our research on curcumin. We would like to propose that curcumin was found to be non toxic to oral fibroblasts, as it showed minimal morphological alteration and maintained the cell viability in all our cytotoxic assays. Our country is the maximum producer of curcumin and is considered to be an important Indian spice that is routinely used. Our bench data proposes that curcumin is a safe choice of natural product in the clinical management of Oral sub mucous fibrosis.

Limitations and future scope of our study

Our study could prove the effect of arecoline and curcumin only at cellular level but we did not prove the genetic alteration related to these cellular changes and other signaling pathways causing the cytotoxic effect of arecoline and the same with curcumin. Thus the future scope of our research is to study the genetic alteration related to respective compounds which will help the biological researchers and clinicians for the targeted therapy of Oral submucous fibrosis. Furthermore, we would like to implement curcumin in clinical trials with the help of safe drug delivery formulations for the management of Oral submucous Fibrosis.

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ANNEXURES

KLE UNIVERSITY

(Formerly Known as K.L.E. Academy of Higher Education & Research, Belagavi)

'Accredited 'A' Grade by NAAC' Placed in Category 'A' by MHRD (GoI)

Director, Academic Affairs

Ref.No.KLEU/Ethic/2016-17/D- 227 27th August 2016

To,
Dr. Ritiha Patil
Ph.D. Part-Time Research Scholar,
2015-16 Batch,
KLE University,
Belagavi

Dear Research Scholar,

Sub:- Regarding Ethical Clearance.

The KLE University **Ethics Committee on Human Subjects** for Ph. D Research Project met on **Wednesday, 29th June 2016** to consider your application for approval of the research project **"Assessment of the effect of curcumin on arecoline induced human buccal mucosal fibroblasts : A cell culture study."**.

As there are no ethical issues involved in your proposed research project, the Committee has provided approval for this 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. All serious adverse events.
3. 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

Research and Ethics Committee
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Sl. No.: 1064

CERTIFICATE

This is to Certify that the synopsis titled

Assessment of effect of curcumin on arecoline induced human buccal mucosal fibroblasts : A cell culture study

Submitted by

Dr. Ritika Patil (PhD Research Scholar) *P. G. Student / Staff, Guided by* Dr. Alka Kale *from Department of* Oral Pathology & Microbiology *has been critically evaluated by committee members and granted ethical clearance to conduct the above mentioned study*

Date : 21/04/2016

Member Secretary
Research and Ethical Committee
KLEVK Institute of Dental Sciences
Belagavi

Chairman
Research and Ethical Committee
KLE VK Institute of Dental Sciences
Belagavi

Original Article

Isolation, culture and characterization of primary cell lines of human buccal mucosal fibroblasts: A combination of explant enzamytic technique

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Abstract

Background: The cell culture technique has become a routine and a popular method for its wide applications in the field of cell biology and biotechnology and in medical research. Isolation of primary cells over the cancer cells is an essential component of cell culture technology as they are the reliable source to understand normal physiological, morphological and molecular process of human cells. As fibroblasts are the prominent cells of the connective tissue of oral mucosa, many disease entities and histogenesis are linked to fibroblasts. Culture of oral fibroblast cells helps the oral biologists and researchers to study the morphological and molecular process in the oral diseases.

Aim: The aim of our experiment is to isolate and culture the human buccal mucosal fibroblast cells from healthy individuals using a combination of explant–enzymatic method and characterization of the cells by short tandem repeat (STR) profiling.

Materials and Methods: The tissue samples were collected from healthy individuals undergoing routine impacted third molar extraction. A combination of explant–enzymatic technique was used for the isolation from the tissue samples. The cells were further subcultured, maintained and stored as per the standard protocols. Thus, to confirm the oral fibroblasts of human origin and its uniqueness, they were characterized using STR profiling.

Results and Conclusion: Using the combination technique, we were successful in isolating the cells at a faster rate by detachment of cells on day 3 and confluency on day 10. The morphological assessment and STR profiling further confirmed that the isolated cell lines resemble human fibroblast cells.

Keywords: Cell lines primary culture, enzymatic technique, explant culture, human buccal mucosal fibroblast, short tandem repeats

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INTRODUCTION

In recent years, the tissue culture technology has a great influence in human society. Cell culture technique has become popular and a widely used method in various fields like in understanding cell biology, medical research,

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in the field of pharmacology to test efficacy and toxicity of new drugs, manufacture of vaccines and also in assisted reproductive technology. Isolation of the primary cell lines and their primary cultures allows researchers to study the morphological, cellular and functional behavior of the cells.[1]

Developed and established cell lines have disadvantage because there are chances to carry several copies of mutations. This could be due to cross contamination or inappropriate exchange of cell lines from laboratories against ethical guidelines. Although primary cell line has finite life span, it is always preferred to study the process of cell cycle, apoptosis and cell repair in a controlled condition to understand the original characteristics and functions of the cells for further clinical translation. Thus, it is the need of the hour to establish standardized protocol for primary culture in the field of cell biology.[2]

There are many established standard protocols for the primary culture of systemic normal and neoplastic cells. Availability of standard protocols for the isolation of normal primary cell lines of human cells such as keratinocytes, skin fibroblasts, epithelial cells and embryonic cells has been well documented. Surprisingly, there is very sparse literature regarding the primary culture of human buccal mucosal fibroblasts.[3]

Fibroblasts are the important cells in the connective tissue of the oral mucosa. They play an important role in the synthesis of structural proteins and extracellular matrix, help in the differentiation of the adjacent epithelium by production of epithelial growth factors and regulate the inflammatory process by secreting the chemokines and cytokines. Hence, to further study the mechanisms of cell functioning and the morphology, a need for the primary culture of human oral fibroblast has been a concern. Primary cultures of fibroblasts allow researchers to obtain cells that can be grown in controlled *in vitro* conditions by retaining their original characteristics and functions.[4]

Establishing a primary cell lines of oral fibroblast can help the oral biologists in understanding the proper structural characteristics, growth rate and morphological alteration to check the toxicity level with carcinogens and also to assess the response of specific novel treatment.[5]

One of the best examples of alteration of fibroblast leading to irreversible disease is oral submucous fibrosis. There are several *in vitro* studies on fibroblasts to assess the effect of arecoline toxicity. A study done by Jheng *et al*. in 1999 found that there were morphological alterations when treated with different concentrations of arecoline.[6] A similar study was carried out by Abhishek *et al*., where they concluded that different concentrations of arecoline showed a decrease in the cell count of the gingival fibroblasts. Hence, developing accurate primary cell line of oral mucosal fibroblasts can further help in validating and performing several *in vitro* studies to link with novel therapeutic targets.[7,8]

Over the past decade, two tissue culture techniques widely used are enzymatic and direct explant techniques described by Bernice in 1994 and Kedjarune *et al*. in 2001. Daniels *et al*. in 1996 described a detailed protocol for enzymatic technique where they used an enzyme for the isolation of the cells from tissue sample using trypsin or diaspase enzyme. Whereas in the direct explant technique, the tissue samples are processed without using enzyme which involves fewer steps compared with the enzymatic technique.[9,10] Moreover, it has been observed that there are advantages and disadvantages mentioned about both the techniques. To overcome these, we decided to develop primary cell lines by a combination of both the techniques.[11,12]

Hence, the aim of our study was to establish a detailed protocol in developing a standard primary cell line of human buccal mucosal fibroblasts (HBMFs) by a combination of explant–enzymatic technique, study their morphological and growth characteristics and further characterize the cell lines by DNA/short tandem repeat (STR) profiling.[13]

MATERIALS AND METHODS

Ethical consideration

Ethical clearance for the study was obtained from the Ethics Committee of KLE Academy of Higher Education and Research (KLEU/Ethic/2016-2017/D-227). Informed consent was also obtained from all the patients involved in our study. Confidentiality of the documentation of the detailed case history, clinical details and personnel information of the patients was maintained and has not been revealed even in this paper.

Tissue sample collection

Tissue samples for primary HBMFs were obtained from 10 healthy human subjects (age ranging from 18 to 55 years), undergoing routine extraction of impacted third molar at the Department of Oral and Maxillofacial Surgery, KLE V K Institute of Dental Sciences, Belagavi. The tissues were carried from the oral surgery department to the cell culture laboratory in an Eppendorf tube containing the cell culture media [Figure 1a] which also acted as the transport media (Dulbecco's Modified Eagle's Medium, Gibco [pH 7.2]) and were stored at 4°C before processing.

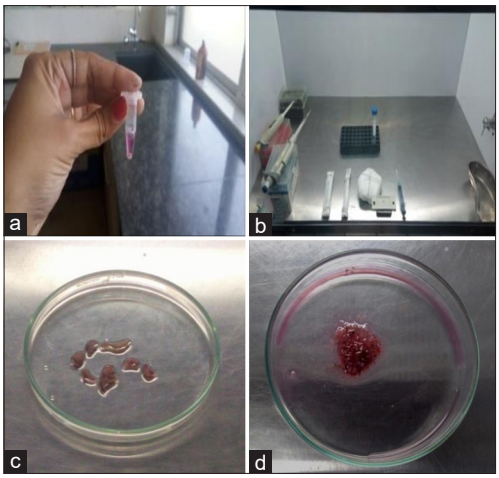

Figure 1: Photomicrograph of images (a-d) depicting steps involved in the tissue processing like collection of sample in sterilized Eppendorf tube containing dish containing the culture media (a), process of mincing of tissue sample in the laminar air flow chamber with proper arrangement of sterilized instruments (b), tissue samples are minced or processed in a glass Petri dish using a BP blade No. 22 into small pieces of 1 mm × 1 mm in size (c and d)

The cell culture technique

The tissue processing was carried out in a biosafety cabinet and all the sterilization protocols were maintained before the experiments as mentioned in Table 1. The tissue specimens obtained were first washed and disinfected in phosphate-buffered saline (pH 7.3–7.4) solution for 2–3 min and then washed in culture media. Tissues were then minced into 1 mm × 1 mm pieces with the help of sterile BP blade No. 22 in a sterilized Petri dish containing the culture media (DMEM) [Figure 1b-e]. Trypsin (0.25%) containing 0.02% ethylenediaminetetraacetic acid (EDTA) (HiMedia) was added for the separation of the cells, which further helps in the passaging of the cells. The minced tissue was then centrifuged at 4000 rpm for 3 min (Eppendorf 5418R) resulting in the formation of pellet to which the fresh media was added and the sediment was seeded in a 6-well culture plate. The culture plate with the minced tissue was flooded with culture media containing DMEM media pH 7.2 supplemented with fetal calf serum (FCS). To prevent the growth of microorganisms, 100 U/ml penicillin, 100 μg/ml streptomycin and 1% amphotericin B (Gibco-BRL, New York, USA) were added to culture media. The culture plate was then incubated at 37°C in a humidified chamber of 95% air and 5% CO_2 incubator New Brunswick - Eppendorf company, German (Bangalore distributers).

Monitoring of primary cell cultures and contamination

Daily observation was done to check for any contamination and explant dislodgment and the overall radial migration of primary cells from the explants. Constant monitoring for any microbial or fungal contamination was done under an inverted microscope. Replacement of old medium by new complete media was also done based on the color change of media. If microbial contamination was detected in any dish or flask, the entire content was immediately discarded. In our experiment, no contamination was found with six tissue samples; however, four samples were detected with contamination. On day 3 of culture, the migration of primary cells was seen, and a monolayer of cells was observed. The primary cell lines were moved from the culture plate to a culture flask. Tissue fragments were covered with culture medium containing fetal calf serum and antibiotics, and the process of culture was continued until the primary outgrowing cells reached around 70%–80% confluency in the culture flasks.

Establishing secondary cultures/passaging

During the subculturing or passaging, the cells morphologically spindle in shape were identified as fibroblasts. On reaching confluency, the cells surrounding the explant tissue further expanded. The medium was poured out of the culture flasks and the cell surfaces washed gently with phosphate-buffered saline (PBS) three times. The cells were removed from the cultures by trypsinization with 0.25% trypsin and 0.05% EDTA. After 2–3 min, the cells attained round shape and began to detach from the plastic surface of the culture flask. The cell suspension along with media were then transferred to a 1.5-ml Eppendorf tube and subjected for centrifugation. Fresh complete media was added for culture flasks to expand cell numbers in a new T25 culture flask. Cultures were grown at 37°C in a humidified atmosphere containing 5% CO_2. The culture medium was changed once in every 48 h. The 2nd–4th passages of cultures of these cells were frozen for further STR profiling, as the cells were in lag phase of growth.

Cryopreservation of fibroblasts

Cryopreservation of the buccal mucosal fibroblasts was performed according to the standard protocol of cryopreservation of the cell lines using dimethyl sulfoxide (DMSO), which permits long-term storage of cells in liquid nitrogen. Once the cells reached the confluency, the cells of the 4th–5th passage are selected and routine trypsinization of cells is done with 0.25% of trypsin. The cells were subjected for centrifugation at 4000 rpm for 3 min. The cell pellet was resuspended in freezing medium consisting of 90% fetal bovine serum (FBS) and 10% DMSO. Cell suspensions were aliquoted into cryogenic storage vials and frozen subsequently at 2°C–4°C for 1 h, −20°C for 1 h and −80°C in a deep freezer overnight.

Table 1: Sterilization protocol to prevent contamination

Procedure	Measures taken to prevent contamination
Sterilization and maintenance of culture laboratory	Before any procedure, the entire cell culture laboratory was properly cleaned with disinfectants The walls, floors, outer surface of culture hood, refrigerators, CO_2 incubators and other equipment were thoroughly cleaned with disinfectant After cleaning the laboratory, UV light for the entire laboratory was kept on for 1 h Fumigation of laboratory was done at the every 10 days
Sterilization of the laminar air flow hood	UV light was switched on 15-30 min before and after the experiment The laminar chamber was cleaned with 70% ethanol as a disinfectant prior and after the experiment The chemicals and instruments were constantly wiped with 70% ethanol before placing them in the laminar air flow chamber
Sterilization of CO_2 chamber	Fresh distilled water was regularly replaced every 15 days with a pinch of cupric sulfate dissolved in it The inner surface of incubator was cleaned with 70% ethanol every alternate day to prevent contamination of the flasks and culture plates All the flasks and culture plates were thoroughly wiped with 70% ethanol before placing them in incubator
Sterilization of accessory instruments	Instruments such as BP blade holder, kidney trays, tissue holding forceps and the Petri plates were sterilized using autoclave before the tissue processing at a temperature of 121°C for 30 min at 15 psi pressure
Procurement and transfer of the tissue sample to culture laboratory	The tissue samples were procured from patients undergoing impaction of third molars under the guidance of oral surgeon with aseptic precautions taken while surgery The samples were collected and transferred in the sterilized vials containing DMEM media to the culture laboratories
Tissue processing	Sterilization of the laminar air flow chamber as mentioned above The optimum operating area should be maintained The instruments before the tissue processing should be neatly organized Use of laboratory coats, gloves, masks and head caps was used to prevent contamination The tissue is first washed with PBS 3-4 times to remove the blood and any other contamination Mincing of the tissue sample was done using preautoclaved and sterilized instruments Cleansing of the operator hands was done with 70% ethanol often during the tissue processing helps avoid contamination from external factors
Subculturing	Once the primary cell lines are established, the cultures were maintained with complete DMEM media containing 1%-2% of antibiotics to avoid contamination The tissue culture plates were observed frequently for any contamination, if any contamination was found the plates were discarded immediately
Maintaining and preservation of cell lines	Sterilized Eppendorf tubes were used for the preservation of cell lines The cell lines to be preserved were transferred to Eppendorf tubes containing 90% FBS and 10% DMSO under sterilized aseptic condition The Eppendorf tubes with the cell lines and preservation media were thoroughly sealed with paraffin wax with cell name and coding

UV: Ultraviolet, DMEM: Dulbecco's Modified Eagle's Medium, PBS: Phosphate-buffered saline, FBS: Fetal bovine serum, DMSO: Dimethyl sulfoxide, BP: Bard-Parker Company

Characterization of the primary cell lines of human buccal mucosal fibroblast

Morphological characterization of the primary cell lines

Once the primary cell lines were established, the cells were observed under an inverted microscope on day 1–day 8 and the morphological changes were noted. The morphological variation of the cells was recorded as F1 which are spindle-shaped cells, F2 which are epithelioid-shaped cells and F3 as stellate-shaped cells.[3]

Characterization of cell lines by short tandem repeat profiling

The cells were characterized for STR/DNA profiling from DNA Forensics Laboratory, New Delhi. The cells from the confluent flask with passage 3rd or 4th were selected for the DNA profiling. The cell pellet was prepared according to the standard protocol provided by the laboratory and transferred to the laboratory. Before sending the cell lines to the laboratory, the cells were named and coded for the convenience and future use as KLED-BF18. The cell pellet was then properly sealed and transferred to the DNA Forensics Laboratory for further processing. Once the primary cell lines were established, the cells were observed under an inverted microscope on day 1–day 8 and the morphological changes were noted. The morphological variation of the cells was recorded as F1 which are spindle-shaped cells, F2 which are epithelioid-shaped cells and F3 as stellate-shaped cells.[3]

RESULTS AND OBSERVATION

The results showed that six out of ten primary cultures of oral fibroblast cells done by a combination of explant–enzymatic technique were successful. In our experiment, we were able to isolate the fibroblast cells within 3–5 days and 70%–80% of confluency reached around 8–10 days of culture. The remaining tissue samples or the cells were discarded due to contamination.

First day: Primary culture

The culture showed mixture of cells which include round/spherical clumps of cells, red blood cell (RBC) and white blood cell (WBC). The primary culture of the tissue sample also showed some areas of tissue degeneration with fibrin and round cells [Figure 2a]. On observation, the culture

plates showed the absence of contamination. The culture was then kept for incubation for 24 h overnight.

Second day: Primary culture

The culture plates showed the same round cells with a slight decrease in the number of RBC and WBC. The spherical cells were seen detaching and an outgrowth of the primary tissue was observed [Figure 2b]. Primary cultures were maintained with the addition of fresh media, FBS and antibiotics to avoid and control the contamination.

Third–fifth day

The cultures showed a series of changes. The fibroblasts were attached to the base of the culture wells and small spindle-shaped cells (F1 type) were observed on the periphery of the culture plates [Figure 2c]. A mixture of cells were observed showing from round to spherical cells; small extension of the cells was observed, resembling that spindleshaped cells (F1) were noted.[3] There was absence of contamination observation of the culture.

Sixth–tenth day: Secondary culture/passaging

The cultures showed majority of long spindle-shaped cells (F1) extending and covering all the surfaces of the wells. The cell population also showed stellate-shaped cells (F3) with fibroblastic extensions, and small round dividing primary cells were noted [Figure 2d]. The buccal fibroblast cell lines reached around 60%–70% confluency on day 8. On day 10, the cell lines were subcultured and passaging was done and labeled as T1 and T2 flask. The cells were then maintained and incubated with constant monitoring for any contamination.

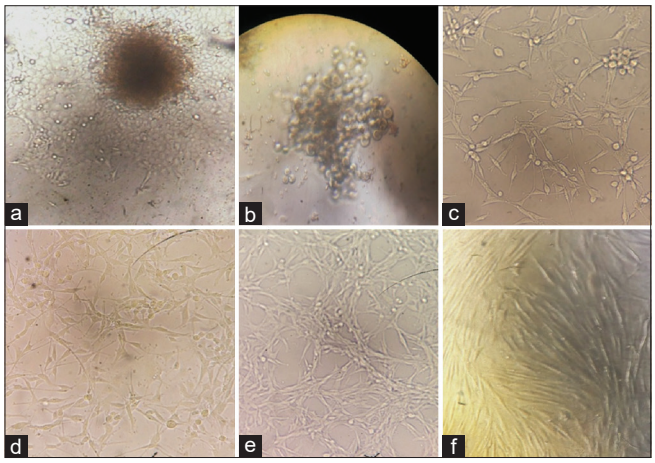

Figure 2: Photomicrograph of images showing the isolation and primary outgrowth of the cells from the explant tissue. Cells migrating and outgrowing from the explant tissue on day 2 (a). Round clump of cells seen indicating the outgrowth of the explant tissue and multiplication of cell lines on day 3 (b). Small spindle-shaped fibroblast cells (F1) with mixture of spherical dividing cells (c). Cultures showing majority of F1-shaped fibroblasts with few F2- and F3-shaped cells (d). The fibroblasts reach the confluency around day 10 with mostly F1-shaped fibroblast cells (e and f)

Eleventh–fourteenth day: Secondary culture

On observation under an inverted microscope, subculture T1 flask showed a monolayer of long spindle-shaped cells (F1 type), and T2 flask showed the cells with confluency of 70% with some areas showing small dividing spherical cells [Figure 2e and f]. The subcultured flasks were then incubated with appropriate media, FCS and antibiotics for the further growth and storage of the cell lines.

Growth curve assay

The growth curve assay was carried to assess the cell growth pattern and proliferation of the fibroblast cell lines. The results of the growth curve assay are mentioned in Table 2, and the graph was plotted [Figure 3]. Our experiment started by seeding around 7000 cells per well in a 12-well culture plate; the cells were allowed to grow and observed using an inverted microscope under ×40 magnification and counted daily for 5 days. The cell count was done every day using trypan blue count assay and an average was recorded at the end of the week. The cells in the last well were recorded as 182,000 cells per well. The experiment shows an increase in the number of cells and the growth pattern of cell lines from day 0 to day 5.

Results of characterization of cell lines

Morphological assessment of primary cell lines of human buccal mucosal fibroblast

The cells were monitored for the morphological changes from day 1 to 14. There were no cell changes observed on day 1 and 2 as they retained round to spherical shape. On day 3–5, the cells showed cellular extensions, resembling spindle-shaped morphology of fibroblast cells. The cultures showed the cells resembling mature spindle-shaped fibroblast (F1) [Figure 4a] on day 7–10. The epithelioid (F2)-shaped cells were observed

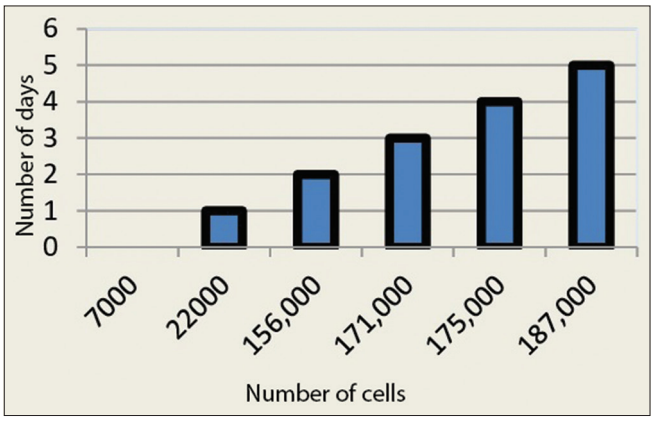

Figure 3: Linear bar graph describing the growth characteristics of primary cell lines of human buccal mucosal fibroblast where the number of cells dividing (X) was plotted against the number of days (Y)

at around day 10 with mixture of very less number of stellate (F3)-shaped cells [Figure 4b].

Results of short tandem repeat/DNA profiling for cell line authentication report

In our study, we observed that the STR profiles of DNA isolated from the cell pellet of primary cell lines of HBMF had some unique features as compared to other standard cell lines considered. Table 3 describes the loci of the DNA of our sample of the cell line KLED-BF18 comparing it with standard fibroblast cell lines. Table 4 shows the locus point that are matching with the standard fibroblast cell lines such as CRL 7201, CRL-7226, CRL7553 and CRL 7554, whereas the cells are showing less resemblance with loci of cells of epithelial origin such as JCRB0178.0, JCRB0178.1 and JCRB0234.0. Thus, the overall results of the STR profiling of our sample of the cell lines state that the established cell line is of human origin, with unique features not resembling to any previous human genes and showing the characteristics of cell of fibroblastic origin.

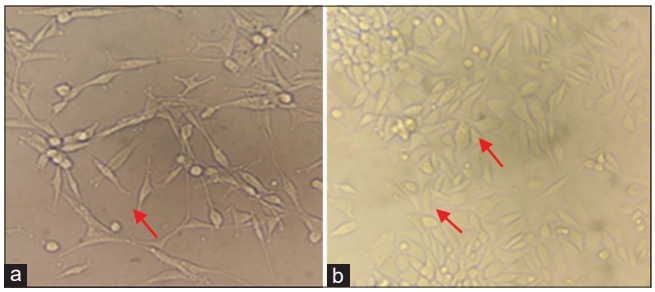

Figure 4: Morphological changes of fibroblasts noted during culture. Predominant spindle-shaped fibroblasts (F1) (a). Mixture of cells with few number of both stellate-shaped (F3) and epithelioid-shaped fibroblasts (F2) (b)

Table 2: Growth curve assay

Number of days	Number of cells/well
Day 0	7000
Day 1	22,000
Day 2	156,000
Day 3	171,000
Day 4	172,000
Day 5	182,000

Table 3: STR profile report table

Test result for submitted sample		
Loci	Query profile (KLED-BF18)	
TH01	6	12
D5S818	11	13
D13S317	9	11
D7S820	10	12
D16S539	10	12
CSF1PO	12	
VWA	14	16
TPOX	9	11
Amelogein	X	X

DISCUSSION

Animal cell is a fundamental unit of life and form an incredible tool in biological research. It acts as a model system for understanding physiological processes and screening of toxic or therapeutic compounds for use in medical treatments. The cells play a major role in the production of functional enzyme, growth factor and vaccines. Cell culturing is a process of maintaining cells of multicellular organisms outside their original body under precise conditions. Over the last three decades, there have been advances in the field of tissue culture and tissue engineering technology. The isolation of primary cells from whole tissues for biological studies is becoming widely recognized in studying and understanding the mechanism of cell cycle, apoptosis and DNA repair. To isolate and establish a cell line with standardized protocols has now become an essential tool in biomedical research and tissue culture technology.[1,2]

Primary cells have the capacity to undergo replicative senescence or aneuploidization which makes their isolation and infinite growth difficult. Therefore, new cultures need to be established regularly with proper monitoring and maintenance. The concerns of Primary cell cultivation, the need for a high quantity of primary buccal fibroblasts in obtaining the greatest number of clonogenic cells, cell performance, and the best culture lifespan cells has been in demand since decades has lead to development of standard

Table 4: Comparative table of STR profile data

EV	CELL NUMBER	CELL NAME	D5S818	D13S317	D7S820	LOCUS NAMES		TH01	AM	TPOX	CSF1PO
						D16S539	VWA				
	Query (Your Cell)		11,13	9,11	10,12	10,12	14,16	6,8	X, X	9,11	12,12
0.67 (24/36)	675	LS	12,13	9,11	8,10	10,11	14,16	6,7	X, X	8,8	12,12
0.67 (24/36)	CRL-7201	HS235.SK	11,12	11,13	10,13	10,12	14,16	6,9	X, X	9,11	11,14
0.67 (24/36)	CRL-7226	HS280.T	11,13	9,11	11,12	10,11	16,17	7,9.3	X, X	8,11	10,12
0.67 (24/36)	CRL-7553	HS821.SK	12,13	9,11	10,12	10,12	14,15	7,9.3	X, X	8,11	12,13
0.67 (24/36)	CRL-7554	HS821.T	12,13	9,11	10,12	10,12	14,15	6,9.3	X, X	8,11	12,13
0.67 (24/36)	JCRB0178.0	KP-3	13,13	9,11	10,12	9,13	17,18	6,9.3	X, X	9,11	12,12
0.67 (24/36)	JCRB0178.1	KP-3L	13,13	9,11	10,12	9,13	17,18	6,9.3	X, X	9,11	12,12
0.67 (24/36)	JCRB0234.0	TYK-NU	12,13	10,11	10,10	9,10	14,16	9,9	X, X	9,11	12,12

protocols for primary cultures of cell lines. Literature and standardized protocols are available for the isolation of rodent embryonic fibroblasts, gingival fibroblasts and oral keratinocytes, but there is minimum literature available on the isolation and culturing of HBMFs.[3]

For obtaining the primary culture of cells, Bolls and Lee suggested two basic techniques involved and they are enzymatic and explant method. Billingham and Reynolds proposed a technique for the separation of epithelial cells using an enzyme (trypsin), thus called the enzymatic method. The tissue dissociation or enzymatic method involves proteolytic enzymes to obtain a single cell suspension or subsequent subcultures. Trypsin which is a proteolytic enzyme breaks down the protein by inactivating the adhesion molecules and integrins to detach the cells from the plate and float.[7,8]

Trypsin along with EDTA is used in cell culture where EDTA acts as a chelating agent that weakens the cell matrix interaction. Trypsin is the most widely used enzyme for dissociation of cells from tissues, adhesive cells from the flasks and for rapid passaging of large cell suspension. However, in a study done by Stems *et al*. and Plaskach *et al*. in 1993, their results indicated that trypsinization or enzymatic method can cause the modification in the adhesive properties of the cells, dysregulation of the cell functions, loss of cell activity, alteration of the cell membrane permeability and disruption of the cell resulting in the cell deformity. A study done by Mexican *et al*. concluded that exposure to high concentration of trypsin can disrupt the cell morphology and their adhesive properties, which is a common problem in subculture.[9,10] P K Nanda *et al*. conducted a study to assess the enzymatic disintegration of the tissue samples for establishing the primary culture; different concentrations of trypsin (0.12% and 0.25%) with regard to time were used. The effect of different concentrations of trypsin showed that the tissue samples partly dissociated with 0.12% and completely dissociated with 0.25% of trypsin. Further, the cells showed morphological alteration and failure of attachment of cells to flasks in the subsequent subculture.[11,12]

According to literature, a minimum concentration of 0.25%–0.5% of trypsin is considered safe for tissue culture studies. In our experiment, trypsin of minimum concentration of 0.25% with EDTA was used for isolation and subculture of the cell lines. Its concentrations did not alter the biological behavior of the cells, and allowed the removal of other contaminating cells, such as epithelial cells, RBCs and other inflammatory cells. We would like to propose that the combination of explant–enzymatic technique showed that 0.25% of trypsin was appropriate for the detachment and developing a primary cell line faster and accurate.[14]

In 1910, Carrel and Burrows described a method for the extraction of epithelial cells called direct explant method where the cells are isolated directly from the tissue samples without using dissociating enzymes such as trypsin. However, they concluded that the cells were not able to dissociate easily from the explant tissue and it took long duration for the first cells to come out from the parent explant tissue. The main disadvantage of the explant technique is that the cells remain in clusters, which becomes difficult for the disassociation of the tissue and fails in the development of monolayer. The explant technique also results in the appearance of undesired cells.

In a study done by Orazizadeh *et al*. in 2015, the keratinocytes were able to get detached from the explant tissue within 24 h whereas the fibroblasts were isolated after 4 days of culture.[12,14]

Similarly, another study done by Punita *et al*. in year 2018 found that the success rate of outgrowth of fibroblast cell from skin biopsy was 4–12 days of processing of the tissue samples. Comparing with these studies, we were successful in isolating the buccal mucosal fibroblast cell lines using a combination of explant–enzymatic method, which helped the cells to detach and migrate from the main explant tissue sample on day 3 of our experiment. The combination method of tissue culture in our study showed that cells were easily separated from the main tissue and were able to grow at faster rate without any morphological alteration.[14]

The major concern in our study was the prevention and maintenance of primary cultures from contamination. The tissue samples obtained from the oral cavity are always associated with bacterial contamination, which hinders the rate of growth of primary cell lines. O W Merten described about bacterial and viral contamination and incorporated some measure to prevent the contamination like washing the tissue samples before the processing with PBS, using sterilized and autoclaved instruments and checking the raw materials for any contamination. They were successful in establishing the primary cell lines with absence of bacterial and viral growth in the cell lines.

In our experiment, measures were taken to prevent any bacterial or fungal contamination right from the procurement of the tissue samples to maintaining and subculturing of the cell lines. Based on our experiments, we would like to propose the list of measure to be taken in developing a primary cell line of BMF cell line [Table 1].

Of ten tissue samples, we were successful in isolating the fibroblast cell line from four tissue samples. The failure of other samples was due to contamination while tissue processing or from the source of sample.

On observation of the morphology of fibroblasts, we observed that F1 type of fibroblasts was seen on 3rd–5th day, whereas a combination of F1 and F2 type of fibroblasts was observed on 6–10th day. However, subsequently, we found predominantly spindle-shaped fibroblasts in all our cultures. Hence, appearance of F3-shaped cells during culture would suggest terminal stage of fibroblasts, synthesizing high amounts of collagen. Surprisingly, we found less number of F2- and F3-shaped fibroblasts during our culture, as these fibroblasts are predominantly seen with the effect of exogenous addition of arecoline.[15] The above findings further prove that our developed fibroblast cell lines are pure primary cell lines developed with no toxic effect during culture.

The cell lines were further subjected for authentication and characterization through DNA or STR profiling from DNA Forensics Laboratory, New Delhi, according to the standard protocol provided by the laboratory. STR profiling or DNA profiling is the most commonly and routinely used method for identification and authentication of human cell lines, which generates a specific molecular and genomic identity code of cell lines and stem cells. This can establish an identity to the specific individual cell lines and helps in further research and reproducibility of the cells for any clinical intervention. STR profiling is cost-effective and can be considered as a standard tool for cell line authentication. As the cell lines from the surgical specimens are difficult to isolate and generate subcultures, we thought to characterize and authenticate our primary cell lines using STR or DNA profiling. Hence, in our study, by STR analysis, we confirmed that cell lines are of human origin and resemble the fibroblast cells with unique loci of tandem repeats.[13]

Pure cultures of authenticated primary HBMF cell lines with subsequent subcultures were incubated, maintained and stored for the future application in the research and clinical aspects of dentistry.

CONCLUSION

The experiment was carried out to standardize the protocols for isolating and establishing the primary cell lines of HBMFs in large numbers from healthy individuals by a combination of explant–enzymatic technique. Obtaining primary cell lines of HBMF from the buccal tissues was very challenging as they were susceptible to source of contaminations. The above-mentioned standardized protocol helps in authentication and establishment of primary cell lines of HBMFs with morphological and genetic profiling.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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Determination of phenotypic alteration of arecoline-induced buccal mucosal fibroblasts: An *in-vitro* cell culture study

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Abstract:

INTRODUCTION: Oral cancer is one of the worldwide health problems accounting as the 6th common of all malignancies. Majority of the oral cancer develop from premalignant conditions of the oral cavity due to the chronic habit of tobacco chewing and smoking. The prominent cells of the oral mucosa are fibroblasts playing a major role in synthesis of extracellular matrix, wound healing, and wound repair. Arecoline, one of main ingredients of tobacco is considered as a risk factor for the development of oral premalignant lesions and cancer. The arecoline is reported to have both genotoxic and morphological alteration of oral fibroblasts leading to Oral submucous fibrosis. Thus in our study a dose dependent effect of arecoline was assessed on the morphology of cultured Human Buccal Mucosal Fibroblasts.

AIM: The aim of our experiment is to assess the effect of different concentration of arecoline on the morphological variation of primary cell lines of human buccal mucosal fibroblasts to develop a model of altered fibroblasts.

MATERIALS AND METHODS: The primary cell lines of human buccal mucosa were established in BSRC KAHER Belagavi, and authenticated by STR profiling from DNA forensics Lab New Delhi. The cells were further cultured and assessed after treating with different concentration of arecoline hydrobromide. The treated cells were then observed for the phenotypic changes and recorded. The morphological alterations were compared to the untreated fibroblasts.

RESULTS AND CONCLUSION: In our study, a dose-dependent effect of arecoline was assessed on phenotypic or morphological alteration of buccal mucosal fibroblasts. The results justified that concentration of arecoline lower than 125 μg/ml did not show change in the morphology of buccal mucosal fibroblasts, whereas the concentration of arecoline >250 μg/ml showed altered fibroblasts. Hence, it can be concluded that the levels of arecoline in arecanut chewers if it is >250 μg/ml, the mucosal fibroblasts may undergo changes to cause fibrosis of collagen. The future scope of our study is to determine the genotoxic effects of arecoline on buccal mucosal fibroblasts and also to develop the therapeutic effects.

Keywords:

Arecoline hydrobromide, cytotoxicity, human buccal mucosal fibroblasts, oral submucous fibrosis

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Introduction

Oral cancer is one of the worldwide health problems accounting as the 6th common of all malignancies. The most common oral malignancy is oral squamous cell carcinomas (SCCs). The oral cancer develop from premalignant conditions of the oral cavity such as leukoplakia, erythroplakia, palatal lesion cigar smoking, oral lichen planus, oral submucous fibrosis, discoid lupus erythematosus, and epidermolysis bullosa. Although there are many advancements in the treatment modalities of oral cancer and precancerous

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conditions, there is no significant improvement in over the past several decades.[1,2]

Oral mucosa is the lining of oral cavity, located anatomically between the skin and gastrointestinal tract. It protects the deeper tissues from mechanical forces resulting from mastication and from abrasive nature of foodstuffs. The cells of the oral mucosa comprise the epithelial cells, keratinocytes, and connective tissue cells mainly the fibroblasts. Fibroblasts play an important role in the synthesis of extracellular matrix in connective tissue and in wound healing. Human oral fibroblasts located in the oral cavity contrast to skin fibroblasts, have the ability to rapidly repair defects in the oral cavity more quickly, reorganize the extracellular matrix and migrate for wound repair. Abnormal proliferation or any morphological or genetic alteration of human oral fibroblasts can lead to the development of oral SCC, oral submucous fibrosis. Human oral fibroblasts are a useful model for elucidating the mechanisms of fibrosis and developing treatments for oral cancers. One of the common conditions of oral cavity where altered fibroblasts are involved is oral submucous fibrosis. It is a potentially malignant condition characterized by changes in the connective tissue fibers of the lamina propria and deeper parts, leading to stiffness of the mucosa and restricted mouth opening seen predominantly in people of Asian descent. Oral submucous fibrosis predominantly affects the buccal mucosa and other parts of oral cavity, pharynx, and upper third of the esophagus.[3,4]

In 2003, the International Agency for Research and Cancer (IARC), a World Health Organization sponsored group, found sufficient evidence that the habit of chewing betel quid, with or without tobacco, causes cancer and has considered as an important environmental risk factor for the development of oral premalignant lesions and cancer. Arecanut is now considered to be Group I carcinogen by IARC, International Agency of Research Cancer by the World Health Organization.[5]

Arecoline consists of alkaloids such as arecoline, arecadine, guvacoline, and guvacine. Arecoline is the major alkaloid of areca nut, causes cytotoxicity and genotoxicity in mammalian cells causing carcinogenicity.[6,7]

Arecoline has been documented as the main agent responsible for fibroblast proliferation, in presence of slaked lime (Ca (OH) 2), arecoline get hydrolyzed to arecadine, thus affecting the fibroblast proliferation. In a study done by Harvey *et al*. they found that showed that exposures to 0.1–10 μg/ml arecoline stimulates fibroblasts and concentrations >25 μg/ml, inhibits fibroblast growth and collagen synthesis. Jeng *et al*. in their study found that depletion of cellular glutathione levels by arecoline predisposes the oral mucosal fibroblasts to various genotoxic and cytotoxic stimulation. Hence, based on the literature survey, the effect of arecoline on the oral fibroblasts is concentration dependent, frequency of consuming the arecanut and geographical variation.[8,9]

In OSMF, the oral mucosal fibroblasts are affected because of the effect of alkaloids present in arecanut. The alkaloids cause increased formation of collagen, leading to stabilization and cross linking of collagen fibers, which further causes accumulation of collagen resulting in disease – OSF.[10,11] Evidence from the literature suggest that oral mucosal fibroblasts undergo morphological and biological changes due to alkaloids present in arecanut based on the consuming habit of arecanut. Thus, the present study aims at assessing the effect of different concentration of arecoline on the morphological variation of oral fibroblasts using HBMF cell lines.[10,11]

Materials and Methods

The study comprised the primary cell lines of human buccal mucosal fibroblasts derived from the tissue samples of healthy individuals undergoing extraction of impacted third molars with the informed consent of the patients from Department of Oral surgery of KLE V K Institute Dental Sciences. The culture of primary cell lines was carried out using standard protocols in Tissue Culture Laborotary of Dr. Prabahakar Kore Basic Science Research Centre KAHER, Belagavi. The cell lines was characterized and authenticated from DNA Forensics Lab, New Delhi.[2]

Ethical clearance for the study was obtained from the Ethics Committee of KLE Academy of Higher Education and Research with the reference number /ethical number: KLEU/Ethic/2016-2017/D-227. Informed consent was also obtained from all the patients involved in our study.

Cell growth and culture

The authenticated cell lines were cultured in T-25 flask with complete Dulbeccos Modified Eagles Media (DMEM) media and on reaching a confluency of 80%, the cell lines were sub-cultured and passage number was given as P1-2-3 and P4. During the initial days of culture, fibroblasts showed spindle (F1) epithelioid (F2) and stellate shaped (F3) fibroblasts and mixture of round-to-spherical-shaped cells.[2,12]

Arecoline hydrobromide was procured from Sigma Aldrich in the powder form and different concentration was prepared by dissolving in DMEM Media. The stock solution was prepared as 1 mg/2 ml of DMEM and further concentration were prepared using serial dilution method.

Morphological assessment

Day 1: The cell counting and seeding

The cells were cultured till they reached the confluency and the cells of the 4th passage were selected for the study which showed all the shapes of fibroblast cells (F1, F2, and F3). The cells were incubated for 24 h in CO2 incubator supplemented with DMEM Media, fetal bovine serum, and antibiotics. The adherent cells were detached from the flask by trypsinization and centrifuged to form a cell pellet. The pellet is then mixed with fresh media and cell counting is done. Approximately 6000 cells were seeded in a 12 well culture plate. The cells were allowed for attachment in a CO2 incubator for 24 h.

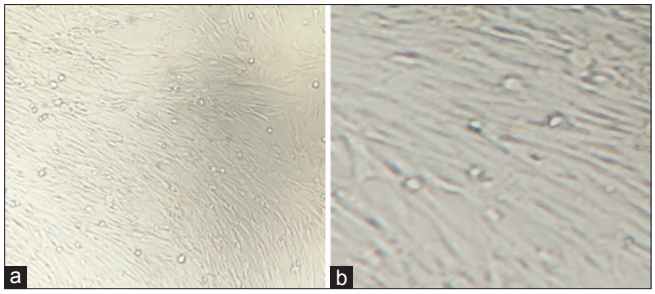

Figure 1: (a and b) Primary culture of human buccal mucosal fibroblasts

Day 2: Addition of arecoline hydrobromide on the cell lines

Arecoline hydrobromide was incorporated on the cell lines with different concentrations by using serial dilution method [Table 1]. DMEM media was used as a dissolving media and around 200 µl of the compound was added in each well. The cells were incubated for 24 h in CO2 incubator and were assessed for the morphological changes.

Assessment of morphology of fibroblast cell lines

The morphological changes were categorized as shape of the cell, outline of the cells, nucleus, and cytoplasm for the assessment of the effect of AH on cell lines as seen in Table 2.

1. 500 µg/ml: The cells treated with this concentration of AH were morphologically altered showing mainly

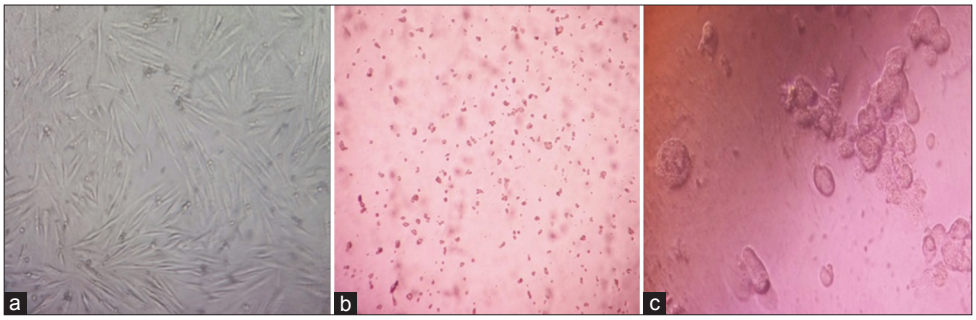

Figure 2: (a-c) :Photomicrogrph of fibroblast cell lines treated with 500 µg/ml arecoline hydrobromide

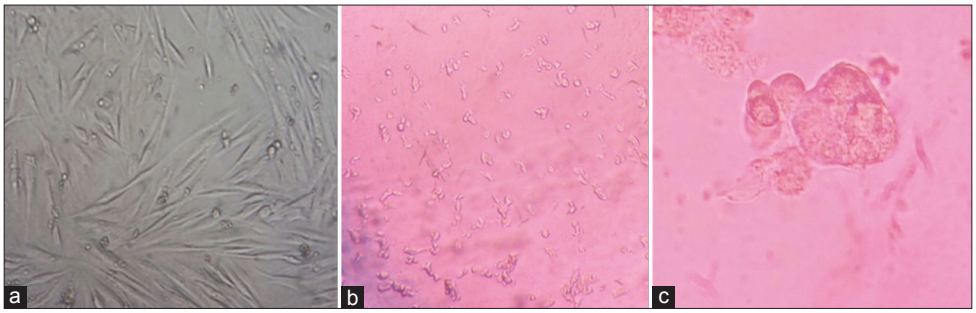

Figure 3: (a-c) Photomicrograph of fibroblast cell lines treated with 250 µg/ml of arecoline hydrobromide (a: ×10, b: ×20, c: ×40)

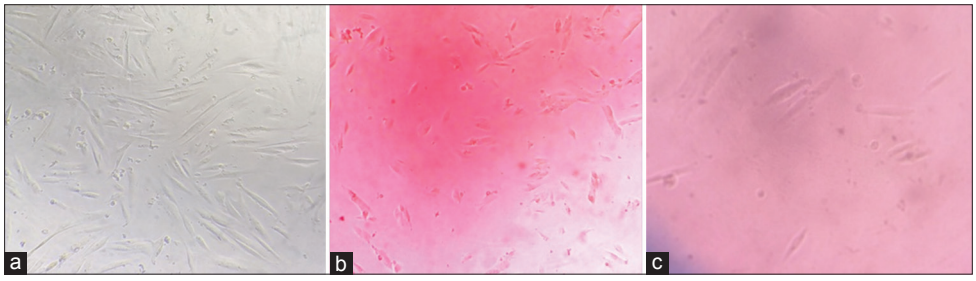

Figure 4: (a-c) Fibroblast cell lines treated with 125 µg/ml of arecoline hydrobromide. (a: ×10, b: ×20, c: ×40)

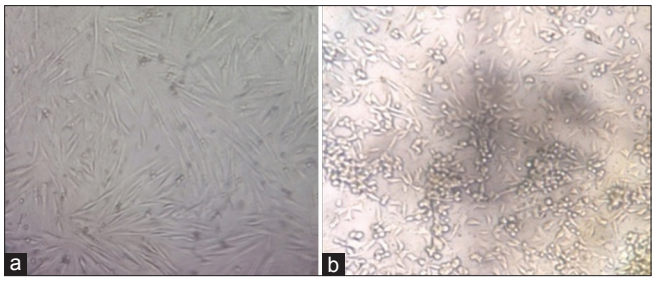

Figure 5: (a and b) Fibroblast cell lines treated with 62.5 µg/ml of arecoline hydrobromide. (a: ×10, b: ×20)

F2 (epithelioid)-shaped cell lines. When observed under inverted microscope the outline of the cells were dead cells with majority of them being round cells, closed nucleus-containing dense granular scanty cytoplasm [Figure 2b and c]

2. 250 µg/ml: The culture wells treated with 250 µg/ml showed epithelioid (F2)-shaped cells. The fibroblasts had a plump-shaped outline with the absence of spindle-shaped fibroblasts [Figure 3b and c]. The cells possessed closed nucleus with scanty dense granular cytoplasm indicating granular cells [Figure 3c]
3. 125 µg/ml: The culture wells showed cells of mixed population with F1, F2, and F3-shaped fibroblasts. The majority of the cells were spindle shaped and few plump cells. The nucleus was open with scanty clear cytoplasm which is feature of granular cells
4. 62.5 µg/ml: The cells treated with this concentration of AH were not much morphologically altered as they showed majority of the cell lines possessing F1 cells and remaining of the well with both F2 and F3 cells. The cells were spindle shaped, round and plump fibroblasts with open nucleus showing abundant clear cytoplasm
5. 31.25–3.90 µg/ml: There were no much morphological changes seen with the cells treated with 31.25–3.90 µg/ml of arecoline hydrobromide. Majority of the cells were spindle-shaped, and stellate-shaped cells. The culture wells also showed cells with open nucleus and abundant clear cytoplasm.

Results and Observation

The primary cell lines were established in BSRC KAHER Belagavi, and authenticated by STR profiling from DNA forensics Lab New Delhi and published.[12] The cultures had long spindle shaped cells with round nucleus and abundant cytoplasm as seen in Figure 1a and b. The cells were further cultured and assessed after treating with different concentration of Arecoline Hydrobromide. The results showed that the concentration of 500 µg/ml [Figure 2a-c] was cytotoxic to the cell lines where the majority of the cells were round with granular nucleus and scanty cytoplasm indicating dead cells. Table 3 shows the effect of different concentration of Arecoline Hydrobromide on the morphology of buccal mucosal fibroblasts. The concentration from 250 to 125 µg/ml [Figure 3a-c] showed mixture of cells with majority of them being granular cells indicating the cells attaining death and very few being spindle/plump-shaped cells. The concentration from 125 to 7.81 µg/ml [Figure 4a-c] and [Figure 5a-c] had no much cytotoxic effect with F1, F2, and F3 types [Table 3] with clear abundant cytoplasm. The lower concentration of the Arecoline hydrobromide showed less toxicity to the cells and could not exhibit altered shapes of fibroblasts with closed nucleus and abundant cytoplasm.

Table 1: Concentration of arecoline hydrobromide

Arecoline hydrobromide concentration (µg/ml)
500
250
125
62.5
31.25
15.6
7.81

Discussion

Oral cavity is lined by oral mucosa consisting of epithelium and connective tissue. The major cells of connective tissue in oral cavity are fibroblasts. The fibroblasts play an important role in the synthesis of collagen fibers maintaining the architecture of tissue microenvironments by depositing and remodeling of extracellular matrix components. The cells actively play role in the production of collagen and the maintenance of extracellular matrix, thus regulating the wound healing.[1,2] Hence, any damage or alteration in the shape or function of fibroblasts may lead to pathologies of oral mucous membrane such as oral submucous fibrosis and oral SCC. The etiology of the precancerous condition in India is mainly the consumption of tobacco either in smoke and smokeless form. Tobacco is the leading cause for the precancerous condition in India. The main ingredient of tobacco is arecanut which consists of Arecoline and Arecadine. The increased concentration of Arecoline and the constant habit of chewing tobacco results in the fibrosis of oral mucous membrane mostly resulting in oral submucous fibrosis. The arecoline will be absorbed by buccal mucosa harming the connective tissue and hence is considered as the initial risk factor of OSF in betel chewers. The increased gingivitis, periodontitis and the changes in the buccal mucosa in betel chewers might implicate that there exist some possible morphological and molecular damage.[2,3]

In previous studies done on fibroblasts cell culture, three basic morphological forms of fibroblasts were appreciated. Based on the animal model and cell

Table 2: Morphological assessment of arecoline induced primary human buccal mucosal fibroblasts

AH (µg/ml)	Type			Outline			Nucleus		Cytoplasm	
	F1	F2	F3	Spindle	Plump	Round	Open	Closed	Abundant	Scanty
7.81	√	√	√	√	√	√		√	√	
15.62	√	√	√	√	√	√		√	√	
31.25	√		√	√	√	√		√	√	
62.5	√	√	√	√	√	√		√	√	
125	√		√	√	√	√	√	√	√	√
250			√			√	√			√
500										√

AH: Arecoline hydrobromide

Table 3: Shapes of fibroblast

Type of fibroblast	Shape of the fibroblast	Function
F1	Spindle shape	Type I collagen - Highly proliferative
F2	Epetheloid shape	Type II collagen - Proliferative
F3	Stellate shape	Type I and III collagen - Less proliferative

culture experiments, they were classified as F1 (spindle shaped), F2 (epithelioid shaped), and F3 (stellate shaped). F1 produces very low levels of collagen (type 1 and 3), are more proliferative in nature. F2 is less proliferative and secretes more collagen (type 1 and 2). F3 cells produces high levels of collagen (type 1 and 3) with proliferative activity indicating the terminal fibroblasts [Table 3].[1]

The isolation and culture of primary cells from the explant tissue have been documented since decades. The primary culture is an essential component of animal tissue and cell culture technology.[2] The advantage of culturing the primary cells over the cancer cells is that as they are the reliable source in understanding the normal physiological, morphological, and molecular process of the human cells. Our study mainly aims to study the morphological variation of the cultured primary cell lines of human buccal mucosal fibroblasts taken from healthy individuals. The alteration in the morphology, genetic, and their function help in understanding the pathogenesis of many pathological conditions. Culture of oral fibroblast cells helps the oral biologists and researchers to study the morphological and molecular process in the oral diseases.[11,12]

In continuation to our previous research of developing primary cell line, we conducted this research to determine the dose-dependent effect of Arecoline on morphology of primary buccal mucosal fibroblasts. The primary cell lines of buccal mucosal fibroblasts were cultured, treated with different concentration of Arecoline for 24 h and the morphological changes were observed under inverted microscope. On observation, we found that the concentration of 500 µg/ml was cytotoxic as the majority of the cells showed the features of granular cells or dead cells. The remaining cells which (10-15) showed alteration from spindle shape to round or spherical with scanty granular cytoplasm thus attaining cell death. At concentration of 250 µg/ml to 150 µg/ml in our study, we had a mixture of cells from spindle cells to plump-shaped fibroblast. Along with plump-shaped cells, round dead cells were also present. Hence in our study, we observed that the concentration of 500 µg/ml was completely cytotoxic to the cells, whereas the concentration ranging from 250 to 150 µm/ml was partially cytotoxic showing mixture of dead and viable cells.

A study conducted by Chang Y C *et al*. in the year 2001 assessed the cytotoxic effects of Arecoline on the PDLF in Taiwan in patients with the habit of smoking. Their study showed that the concentration >200 µg/ml arecoline inhibits cell growth, proliferation, and protein synthesis on human PDLF indicating that betel nut chewing might be another risk factor in the pathogenesis of periodontal diseases. The above observations are similar to our findings.[2,3]

Shang Lu Chiang in the year 2007 conducted a study to assess the dose-dependent effect of Arecoline on Human Gingival Fibroblasts (HGF) of normal individuals. The cells were treated with 100 µg/ml (or >100 µg/ml) of arecoline for 24 h, the cells appeared less dense than control and cell retraction was observed. Whereas the concentration >400 µg/ml resulted in granular and dead cells indicating cyto-toxicity of Arecoline to normal fibroblasts. This was in accordance to our observation.[4]

A similar experiment was conducted by Deepu George Mathew *et al*. in 2011 to compare the arecoline effect on fibroblasts of chewers with or without OSMF. They divided their study groups as Arecanut chewers with normal mucosa, arecanut chewers with OSMF and a control group with no chewing habit and normal mucosa. Their study concluded that the primary culture of fibroblasts revealed not only the basic F1, F2, F3 morphological variants, but in addition, they also found f1, f2, f3 (mitotic) and f4, f5, f6 (postmitotic) shapes

of fibroblasts in arecanut chewers with OSMF. This showed that there are morphological variations when fibroblasts in the buccal mucosa are subjected to betel nut chewing. However, this study did not highlight the dose-dependent effect of arecoline on the morphology of buccal fibroblasts.[5]

A recent study was carried out by Abhishek Banerjee *et al*. in the year 2017 to identify the various morphological forms of fibroblasts and to understand and assess the response of the fibroblast cell lines to different concentrations of Arecoline. The primary cell lines were cultured and treated with different concentration ranging from 50/100/150/300/500 ug/ml of Arecoline. The cell lines were then observed for 8 days to assess the morphological variation and cell counts. Our results were concomitant to the above findings when less concentration of arecoline was stimulative and showed toxic effect at higher concentration.[10]

Conclusion

The adverse effects of arecanut chewing and its constituents on buccal mucosa have been studied from the past decades. The arecoline and arecadine being the main constituent of arecanut have both phenotypic and genotoxic effect on the cells of buccal mucosa that are predominantly fibroblasts. Hence in our study, the dose-dependent effect of Arecoline was assessed on phenotypic or morphological alteration of buccal mucosal fibroblasts. The results justified that concentration of arecoline lower than 125 µg/ml did not show change in the morphology of buccal mucosal fibroblasts in Indian healthy individuals. Whereas the concentration of arecoline >250 µg/ml showed altered fibroblasts. Hence, it can be concluded that the levels of arecoline in arecanut chewers if it is >250 µg/ml, the mucosal fibroblasts may undergo changes to cause fibrosis of collagen. The future scope of our study is to determine the genotoxic effects of arecoline on buccal mucosal fibroblasts and also to develop the therapeutic effects.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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