EVALUATION OF DNA DAMAGE IN THE BUCCAL CELLS OF PATIENTS WITH ORAL SUBMUCOUS FIBROSIS USING COMET ASSAY

Dissertation submitted to

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY

In partial fulfillment for the Degree of

MASTER OF DENTAL SURGERY

BRANCH VI

ORAL PATHOLOGY & MICROBIOLOGY

2016 – 2019

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PLACE OF STUDY	Vivekanandha Dental College for Women, Elayampalayam, Tiruchengode, Namakkal District.
DURATION OF THE COURSE	3 Years (2016-2019).
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ACKNOWLEDGEMENT

First and foremost I humbly thank the **Almighty Creator** for His unmerited grace and mercy towards me in every phase of my life. Without Him this work would not have been possible.

I acknowledge my deep sense of gratitude to **Prof. Dr (Capt). S. Gokulanathan, B.Sc, M.D.S., Dean,** Vivekanandha Dental College for Women, for his constant support in my Post Graduate Curriculum.

I express my sincere thanks to **Prof. Dr. N. Balan, M.D.S., Principal,** Vivekanandha Dental College for Women, for his guidance and support in the successful completion of my work.

It gives me immense pleasure to express my profound gratitude to the ever supporting pillar of our department, respected Head of the Department of Oral Pathology and Microbiology, **Prof. Dr. N. Ganapathy**, **M.D.S.,** for providing me with all the facilities to carry out this research work.

I owe my utmost thanks and sincere gratitude to my post graduate guide and mentor, **Dr. T. Maheswaran, M.D.S, M.B.A.,** for his constant encouragement and wholehearted support at all stages of this research work. I am fortunate to have his inspiring guidance, pertinent criticism and pregmatic suggestions throughout my post graduate life. He has always been my source of inspiration in pursuing this work with sincerity and modesty. I am very thankful and honored to be called as his student.

I am grateful to my Co-Guide **Dr. J. Dinesh Shankar, M.D.S.,** associate professor, for his enthusiastic words and encouragement in every step of my post graduate curriculum.

I also extend my heartfelt thanks to my associate professors **Dr. V. Ilayaraja**, **M.D.S., Dr. T. R. Yoithap Prabhunath**, **M.D.S.,** and senior lecturers, **Dr. A. Yamuna Devi M.D.S., Dr. P. Tamil Thangam, M.D.S.,** and **Dr. M. Saranya, M.D.S.,** for their valuable suggestions, support and encouragement throughout my study period.

I extend my sincere thanks to the department of Biochemistry,

Prof. Dr. Sachu Philip, Prof. Dr. K. M. Tullanithi, Dr. S. Ayappan for their valuable suggestions and support in completion of my study.

I am very grateful to my senior, **Dr. S. Mohana Priya**. More than a senior, she was a good friend to me and without her priceless advice and support I would not have come this far.

I acknowledge my batch mates, **Dr. G. Jisha** and **Dr. K. Gayathri** for their moral support in pursuing my work.

Words cannot describe how I am thankful to my juniors, **Dr. J. Swathi Raman, Dr. S. Renuga Devi** and **Dr. J. Porkodi Sudha** for being there with me by my side in the ups and downs of my postgraduate life.

I am also thankful to my subjuniors **Dr. S. Nivethithaa, Dr. N. Preethi** and **Dr. V. Sathyarani** for their words of encouragement and support.

I am very grateful to my friends **Dr. Narendran, Dr. Kirthika** and **Dr.Ramyalakshmi** for their help and constant support in all my endeavors.

Last but not the least I cannot be what I am today without the loving care and motivation of my parents, **Mr. Krubakar Mithra** and **Mrs. Nallini Mithra** and siblings **Christina Mithra** and **Isaac Mithra.**

This work would not have been made possible without the guidance and support of each and everyone who have contributed their time and assistance in the successful completion of my dissertation.

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1	**OSF**	Oral Submucous Fibrosis
2	**DNA**	Deoxyribo Nucleic Acid
3	**ROS**	Reactive Oxygen Species
4	**HPLC**	High Performance Liquid Chromatography
5	**MN**	Micro Nuclei
6	**ISCN**	International System of Human Cytogenetic Nomenclature
7	**XRCC1**	X-Ray Repair Cross-Complementory protein 1
8	**PCR**	Polymerase Chain Reaction
9	**RFLP**	Restriction Fragment Length Polymorphism
10	**BQ**	Betel Quid
11	**TUSC**	Tumour Suppressor Candidate
12	**BPA1**	Binding Partner of ACD11 1
13	**EMT**	Epithelial Mesenchymal Transition
14	**TGF**	Transforming Growth Factor
15	**AEBN**	Aqueous Extract of Betel Nut
16	**HEBN**	HCl Extract of Betel Nut

17	**AAEBN**	Acetic Acid Extract of Betel Nut
18	**EEBN**	Ethanol Extract of Betel Nut
19	**UDS**	Unscheduled DNA Synthesis
20	**AgNOR**	Argyrophillic Nucleolar Organizing Regions
21	**CPD**	Cytobutane Pyrimidine Dimers
22	**SCG**	Single Cell Gel
23	**MTT**	3-(4,5-Dimethylthiazo-2-yl)-2,5-Diphenyltetrazolium bromide

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INTRODUCTION

INTRODUCTION

Oral submucous fibrosis is a chronic progressive disease that can affect any part of the oral mucosa and sometimes can also involve the pharynx.[1] The disease is characterized by chronic inflammation, epithelial atrophy, loss of rete ridges, subepithelial hyalinization and fibrosis of submucosal tissues that leads to stiffness of the oral mucosa and difficulty in mouthopening.[2]

The condition is considered to be a potentially malignant disorder with a higher chance of developing oral squamous cell carcinoma. The disease has been associated with the chewing of areca nut, a habit that is most prevalent in the south Asian population.[1] Several other factors such as chili consumption, genetic susceptibility, autoimmunity, nutritional deficiency states and collagen disorders have also been suggested to be involved in the pathogenesis of this condition. However areca nut quid has been accepted today as a chief etiological factor for oral submucous fibrosis.[3]

Areca nut (Areca catechu) is the fourth most commonly used psychoactive substance that is chewed to aid in digestion as well as to act as a stimulant. It is either chewed alone or in combination with different tobacco and non-tobacco substances. Areca nut is known to produce both mutagenic and carcinogenic effect. Because of its effect on the tissues of our body to cause various neoplastic and preneoplastic lesions, the International Agency for research on cancer in 2003 considered areca nut as a group I human carcinogen.[4]

The components of areca nut have been shown to have carcinogenic effects. The contents that have been proven to be carcinogens are tannins, some of the polyphenols: Safrole, hydroxychavicol, and catechins, and most of the alkaloids.[4] Normal oral epithelial cells are continuously subjected to the various genotoxic agents that are present in areca nut quid, tobacco, alchohols, reactive oxygen species (ROS) and nitrosamines. The antioxidants present in our body form conjugates with the reactive oxygen species and reactive intermediates, thereby degrading the reactive toxic species protecting the critical cellular macromolecules like DNA (Deoxyribo Nucleic Acid) against oral toxicants.[5]

An excessive amount of ROS, reactive metabolic intermediates and methylating agents from betel quid, tobacco, nitrosamines and other toxicants can attack the DNA of the cell and induce various kinds of genetic damage. If these DNA damaged cells are subsequently induced by proliferative agents to replicate, the genetic damage will remain permanently in the cells, leading to the formation of mutated initiated cells. The further promotion and progression of initiated cells can lead to the occurrence of oral precancerous lesions and cancer.[5] Thus the genetic damage caused by areca nut to the oral keratinocytes can lead to the malignant transformation of oral submucous fibrosis to oral squamous cell carcinoma.

DNA damage is of several forms like single strand break, double strand breaks and cyclobutane pyrimidine dimmers. These changes can be identified by various techniques like polymerase chain reaction, halo assay, comet assay, HPLC-electrospray tandem mass spectrometry, fluorescence in situ hybridization, terminal deoxyribonucleotidyltransferase-mediated deoxyuridine triphosphate nick end labelling assay, annexin V labelling, flow cytometry, immunological assays including

immunofluorescent, chemiluminescence thymine dimer detection, immunohistochemical assay, radio immunoassay, enzyme-linked immunosorbent assay, electrochemical methodisation and gas chromatography-mass spectrometry.[6]

Among the various techniques a technique called Comet assay or the single cell gel electrophoresis has gained popularity because of its use in quantifying DNA double strand breaks. The technique was first developed by Ostling and Johansson in the year 1984. This gel electrophoresis technique is known as single cell gel electrophoresis technique or the Comet assay. Later in the year 1988, Singh et al, modified the technique by using alkaline conditions.[7] This method not only identifies the double strand breaks but also the single strand breaks and alkali-labile sites expressed as frank strand breaks in the DNA.[8]

The assay has now become, a well established, simple, rapid, sensitive, versatile and extensively used tool to assess DNA damage and repair, both quantitatively as well qualitatively in individual cell populations.[7] Other types of DNA damage such as oxidative DNA damage and DNA cross linking (e.g. thymidine dimers) may also be assessed using lesion-specific antibodies or by using specific DNA repair enzymes in the comet assay technique. It has gained acceptance as a valuable tool in fundamental DNA damage and repair studies, human biomonitoring and genotoxicity studies.[9, 10]

Comet assay is not only a rapid technique but also a non - invasive technique. It is used to assess DNA damages caused by radiation, during carcinogenesis, to determine the genotoxicity and to analyse the irradiated food. The principle behind comet assay is to measure DNA strand breaks that cause relaxation of DNA supercoils. If the DNA contained breaks, the DNA supercoils get relaxed and the

broken ends migrate towards the anode during a brief electrophoresis because of the negative charge of DNA. If the DNA was undamaged, the lack of free ends and large size of the fragments will prevent the fragments from migration.[11]

The concept in comet assay is that the smaller the DNA fragment, farther it travels in electrophoresis and the greater is the damage. The undamaged DNA that appears to have a round intact shape and it is referred as the 'Head' of the comet. Whereas the DNA fragments that migrate toward the anode form the 'Tail'.[12] The head containing the high-molecular-weight DNA and the comet tail containing the leading ends of migrating fragments can be measured from digitized images using software.[11]

Epithelial cells, peripheral blood leukocytes, nasal cells, lens epithelial cells and corneal cells have all been used in comet assay. Lymphocytes are the most commonly used cells which are subjected to comet assay to identify the changes that occur due to the long-term exposure of carcinogens. Epithelial cells act as specialized components of many organs and have the potential to serve as biomatrices. These cells can be used to evaluate genotoxicity and furthermore, 80% of solid cancers are of epithelial origin.[13]

Many of the physical properties of epithelial cells are mainly dependent upon their attachment to one another, which is mediated by cell junctions. The specialized functions of epithelial cells are mediated by both structural modifications of their surfaces and the internal modifications, which helps these cells to fulfill their specific roles.[13]

Buccal epithelial cells have a direct contact with the carcinogens during tobacco usage. Hence the identification of DNA damage in these cells would be useful and appropriate. The buccal mucosal cells have also been used in micronuclei assay where it helps to identify the pyknotic nuclei and loss of nuclear material. Other nuclear features that can be used to assess nuclear abnormalities will include cells with two nuclei in the same cytoplasm, formation of nuclear bud or "broken egg" or small micronuclei (MN) near nuclei in the same cytoplasm. These biomarkers of genetic damage and cell death can be observed in both lymphocyte and buccal cell systems, and thus they help in the assessment of genetic damage.[14]

There is scarce literature about DNA damage in the buccal cells of Oral Submucous Fibrosis patients. There are no published reports regarding the evaluation of DNA damage in buccal cells of patients with OSF with associated tobacco habits using comet assay.

AIM AND OBJECTIVES

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Aim

To evaluate the DNA damage in the buccal cells of patients with oral submucous fibrosis using comet assay.

Objectives

- To assess the DNA damage in buccal cells of oral submucous fibrosis patients.
- To compare the DNA damage among subjects with different patterns of tobacco usage.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

DNA

Watson and Crick, 1953 published their proposed molecular structure of the DNA. The first model of the nucleic acid that was proposed by Pauling and Corey, consisted of three interwined chains with the phosphate groups near the fibre axis and the bases outside. However the authors found the model unsatisfactory due to several scientific reasons. Hence they gave their double stranded DNA helix model wherein the structure consisted of two helical chains each coiled around the same axis held to each other by hydrogen bonds. The nitrogenous bases are located inside and the sugars and phosphates are located outside of the double helical structure. This structure has paved way in understanding the remarkable genetic blueprint of life and its role in health and disease.[15]

Wiesmuller et al., 2002 discussed about the effects of environmental noxious agents on the DNA, the repair mechanisms and what happens in diseases where the repair mechanisms fail. The DNA undergoes various types of spontaneous modifications, and it can also react with many of the physical and chemical agents, of which some are endogenous products of cellular metabolism (eg, reactive oxygen species) while others, including ultraviolet light and ionizing radiation which are threats from the external environment. These alterations play a major role in the preservation and transmission of genetic information. Normally they are combated by the body's DNA repair systems. However deficiencies in DNA repair mechanisms have been linked as the major cause in many diseases including developmental conditions, autoimmune diseases and predisposition to cancer.[16]

Martinet et al., 2002 in their study found that there was an increased DNA damage in patient with atherosclerosis. The study was done to find whether the oxidative DNA damage and repair mechanisms lead to atherosclerotic plaques. The result of their study concluded that there was an increased level DNA damage with the severity of formation of the atherosclerotic plaques. They also described that comet assay has an advantage of identifying DNA damage both in carcinogenesis and in atherogenesis.[17]

Tulkia Bose 2003 found that the peripheral blood lymphocytes showed more chromosomal instability in various malignancies. The study was conducted among 250 patients with various types of malignancies. The chromosome plates in the study were classified using standard ISCN nomenclature. Karyotypes that showed acrocentric associations, premature centromeric division or aneuploidies were considered abnormal. It was concluded that many of the solid tumors showed cytogenetic changes in the leukocytes that had chromosomal instability. These cytogenetic changes may also be due to the effect of many environmental mutagens in multiple tissues. The inheritance of chromosomal instability through some of the genes involved in DNA synthesis and repair provided the proper introduction on which environmental mutagens cause the final damage. Sometimes these changes also indicate the effect of environmental mutagen in multiple tissues.[18]

Sellapa et al., 2009 mentioned that many polymorphisms affect the different DNA repair genes and modulate thereby modulate cancer. The study was conducted to correlate the relation between the risk of cancer development and polymorphisms in the DNA repair gene XRCC1399 and hOGG1326 genotypes using polymerase chain reaction-restriction fragment length polymorphism (PCR/RFLP). It was conducted

among 156 smokeless tobacco users and 70 controls without significant exposure to any mutagens. The occupational, smoking, and medical histories of the study subjects were collected using questionnaires. The relationship between DNA polymorphism and cancer development was analysed by using comet assay, micronuclei and chromosomal aberrations. A significant difference was found between smokeless tobacco users and the controls using the micronuclei assay. There exists a relationship between the XRCC1399 Gln/Gln and hOGG1326 Ser/Ser genotypes with DNA damage level in subjects who were exposed to smokeless tobacco. The study was done to evaluate the genotoxic effect of smokeless tobacco use and was recommended to identify the factor that promote carcinogenesis.[19]

Salmani et al., 2011 in an in-vivo study of peripheral blood mononuclear cells as a biomarker of oxidative stress to estimate the DNA damage. The base level DNA damage was examined in the blood samples collected by both venipuncture and lancet method using comet assay. It is a non invasive technique that only requires minimum amount of blood. The advantage is that the small amount of blood collected by the lancet can be used and stored without the addition of any cryopreservative without having any substantial changes in DNA. Thus the authors have described in detail about how the comet assay can be used for various bio-monitoring studies by simple sample collection, storage and analyses.[20]

ORAL SUBMUCOUS FIBROSIS

Pindborg JJ and Sirsat 1966 in their publication has given a detailed description of oral submucous fibrosis and also gave the definition of this progressive disease. According to Pindborg the disease may be defined as an insidious, chronic disease affecting any part of the oral cavity and sometimes the pharynx. Although

occasionally preceded by and/or associated with vesicle formation, it is always associated with a juxta-epithelial inflammatory reaction followed by a fibroelastic change of the lamina propria, with epithelial atrophy leading to stiffness of the oral mucosa and causing trismus and inability to eat. Although a lot of factors have been associated with the development of the disease, the habit of areca nut chewing has been found to be the major risk factor.[21]

Murti PR et al., 1984 did a longitudinal follow up study on the malignant transformation in oral submucous fibrosis over a period of 17 years in Ernakulam District, Kerala, India. Their analysis showed the oral submucous fibrosis to have a high degree of developing oral cancer with 7.6% malignant transformation rate. They found that oral cancer developed 3-16 years after the time of diagnosis of oral submucous fibrosis with the average age of occurrence to be 64.6 years. They also suggested that future follow up can show even greater increase in the malignant transformation in these patients.[22]

Sundqvist K et al., 1989 analysed the genotoxic and cytotoxic effects of areca nut related compounds in cultured human buccal epithelial cells. In their study they investigated the effects of areca nut extract, for areca nut alkaloids and four nitrosated derivatives on cultured human buccal epithelial cells. Their results showed that areca nut extract caused the formation of both DNA strand breaks and DNA protein cross-links. Taken together, the aqueous extract and, in particular, one yV-nitroso compound related to areca nut, i.e., 3-(V-nitrosomethylamino)propionaldehyde, are found to be highly genotoxic and cytotoxic to cultured human buccal epithelial cells and they are of potential importance in the induction of tumors in betel quid chewers.[23]

Merchant et al., 2000 has reviewed about how paan chewing without tobacco can be an independent risk factor for oral cancer. Betel quid (BQ) chewing produces reactive oxygen species (ROS) that is detrimental to the oral mucosa and can be directly involved in the tumor initiation process, by inducing mutation, or by making the oral mucosa susceptible to BQ ingredients and other environmental toxicants. BQ chewing produces many ROS, which can have multiple detrimental effects upon the oral mucosa. The production and release of these ROS will take place in the saliva of a BQ chewer under alkaline conditions during the autooxidation of areca nut (AN) polyphenols.[24]

Huang et al., **2006** studied the genes that were differentially expressed between normal epithelium and the epithelium in oral submucous fibrosis using cDNA microarray analysis. In their study they used laser capture microdissection to obtain the oral epithelium and subjected the tissues to cDNA microarray. The genes that were upregulated in OSF epithelium was 109 that included cyclins, tumor and suppressor candidate 3 (TUSC3) and 4, and BPA1. Around 169 genes were downregulated in OSF epithelium, including retinoic acid induced 3, apoptosis-related Harakiri and small GTPases. They validated the expression of TUSC3 in OSF epithelium by real-time reverse-transcriptase polymerase chain reaction. They also suggested that further investigations are to be needed to discover the roles of these candidate genes in the malignant transformation of oral submucous fibrosis epithelium into oral cancer.[25]

Mehrotra et al., 2006 reviewed the etiology, pathogenesis and value of genomic alterations in oral squamous cell carcinoma. Various forms of tobacco, betel quid, candida, viruses, alcohol etc have been found to be the most important etiological factors. Genomic instability, tumor suppressor genes, cytokine activity, apoptosis and

neovascularization were detailed as to have the most important role in the pathogenesis of cancer. Various proteomic array technologies, laser capture microdissection and lab-on - chip are the new recent advances in the diagnosis of premalignant lesions and cancer. The study of the carcinogenic process of head and neck, including the continued analysis of new genetic alterations, along with their temporal sequencing during initiation, promotion and progression, will allow us to identify new diagnostic and prognostic factors, which will provide a promising basis for the application of more effective treatments for these tumours.[26]

Jian et al., 2008 have analysed the expression profiles of 14,500 genes in human oral submucous fibrosis and normal control using Affymetrix U133A 2.0 Gene Chip arrays. Their results showed that in oral submucous fibrosis 716 genes were upregulated and 149 genes were downregulated. A list of significant differentially expressed genes involved in inflammatory response immune response and epithelial-mesenchymal transition (EMT) induced by TGF-ß signaling pathway were identified using gene oncology and relevant bioinformatics tools. These findings showed that the genetic abnormalities that were identified might play an important role in the pathogenesis and malignant transformation of oral submucous fibrosis.[27]

Ram et al., 2011 has reviewed the risk factors and the molecular pathogenesis of oral cancer. The literature search was carried out in NCBI Pubmed database using keywords like oral cancer, Risk factor, epidemiology and pathogenesis. The basic information was also obtained from the textbooks and medical university websites. Tobaccos, alcohol, Viruses, Candida, syphilis, nutritional factors are found to be the major risk factors. The genetic susceptibility and polymorphisms have been found to play the key role in the etio-pathogenesis of cancer. The development of oral cancer is a

multistep process that involves the accumulation of genetic and epigenetic alterations in the key regulatory genes. Experimental pathological studies of oral cancer in animal models and direct molecular genetic analysis of oral cancer subjects in recent times has revealed a substantial amount of knowledge on specific genetic alterations or other genetic mechanisms involved in the initiation and subsequent progression. Thus easier diagnosis, better prognostication and efficient therapeutic management should be followed for the prevention.[28]

Sharan et al., 2012 has reviewed the association of betel nut with carcinogenesis. Betel nut extract (BNE) was found to decrease the cell survival, membrane integrity and vital dye accumulation of cultured human buccal epithelial cells in a dose dependent manner. BNE also caused the formation of both DNA protein cross links and DNA single strand breaks. The different types of BNE, such as aqueous extract of betel nut (AEBN), HCl extract of betel nut (HEBN), acetic acid extract of betel nut (AAEBN), ethanol extract of betel nut (EEBN) and arecoline have shown different extents of cytostatic and cytotoxic effects, and they induced variable levels of dose dependent unscheduled DNA synthesis (UDS) in Hep2 cells in vitro.[29]

Ekanayaka et al., 2013 has reviewed the mechanism of pathogenesis and malignant transformation of oral submucous fibrosis and has stated that arecoline which is the major alkaloid in arecanut to have various genotoxic properties. The arecoline N–oxide, an active metabolite of arecoline, has been found to be the ultimate carcinogen in areca related oral carcinogenesis. Arecoline is found to downregulates p21 and p27 through reactive oxygen species/mTOR complex 1 (ROS/mTORC1) pathway. The reduced levels of p21 and p27 might facilitate G1/S transition of the cell cycle and subsequently may lead to error prone DNA replication.[30]

Anand et al., **2014** reviewed about the deleterious effects of betel nut on oral cavity. There is a historical evidence dating back nearly a century ago that suggests that the betel nut chewing may be involved in the development of oral squamous cell carcinoma. Although it is widely accepted that the presence of tobacco in BQ has an important role in the pathogenesis of oral squamous cell carcinoma, the carcinogenic potential of betel nut in the absence of other ingredients is less clear. However there are epidemiological data from several studies that confirms that the habit of BQ chewing increases the relative risk of developing oral squamous cell carcinoma.[31]

Shwetha H R et al., 2015 has reviewed in detail about the effects of areca nut in the oral epithelium. There are several studies done on animal models, in-vitro studies and about the pathobiological effects and genotoxic effects of areca nut on oral epithelial cells. Several membrane permeability studies have proven that arecoline and arecaidaine can diffuse across stratified epithelium. This research was further justified because genotoxic and mutagenic effects have not been proven to occur when mucous membrane was exposed to betel compound. Since there is lack of enough literature about the effect on areca nut on oral epithelium further studies are needed to completely understand the effect of areca nut on the oral epithelium.[32]

Ray JG et al., 2016 reviewed the histopathological aspects of the malignant transformation of oral submucous fibrosis. The oral submucous fibrosis being more prevalent in the Indian subcontinent has an estimated malignant transformation rate to be between 2-8%. They reviewed the histopathological aspects of the malignant transformation of oral submucous fibrosis. The changes in epithelial thickness, dysplasia, AgNOR counts and micronuclei in the epithelium, keratin protein alterations, p63 and E Cadherin expressions have a role in the malignant transformation of the

epithelium. There are also changes in the connective tissue and the epithelial connective tissue interactions that further lead to the malignant transformation. Epithelial hypoxia due to constriction of blood vessels because of fibrosis of the underlying connective tissue has also been given as an important mechanism.[33]

Das et al., 2017 studied the effect of areca nut and lime on rabbit oral mucosa. Their study showed progressive changes in the thickness of the epithelium from 3 months onwards from the exposure of areca nut. The protein expression pattern of these exposed tissues was analysed. Three major proteins, namely tropomyosin beta chain (in the skeletal muscle), actin and collagen alpha-1(I) chain, were identified in areca nut-treated rabbit tissues as compared to control. The genotoxic effect areca nut in the rabbit model was also evaluated using the comet assay in the blood of these animals. A significantly higher DNA damage was found in the areca nut treated rabbits when compared with the control group.[34]

COMET ASSAY

Martin et al., 1993 has reviewed the single cell gel electrophoresis procedure, the different protocols used, the principles determining the behaviour of DNA and the potential applications of the comet assay technique. Comet assay was first developed by Ostling and Johansson in 1984 and then later modified by Singh et al in the year of 1988. The comet assay technique particularly alkaline comet assay procedure has been found to be a rapid, simple, visual and sensitive technique for measuring DNA damage in individual mammalian cells. Comet assay has been continually evolving and as time passes the assay continues to find its application in diverse areas of scientific research and population monitoring.[35]

Olive et al., 2006 has presented the protocol for comet assay. The procedure for comet assay, a gel electrophoresis-based method can be a useful tool to measure DNA damage in individual eukaryotic cells. The materials, methodology and the troubleshooting errors that can occur during the application of comet assay were explained in detail. This technique can also be used to assess DNA damage and its repair in single-cell suspensions made from yeast, protozoa, plants, mammals and invertebrates. The applications of comet assay are wide ranging from human and sentinel animal biomonitoring to measurement of DNA damage in specific genomic sequences**.** The protocol is quick and can be completed in time fewer than 24 h.[11]

Kumaravel et al., 2009 described the methods, various advantages and disadvantages in using the comet assay procedure. The selection of the different parameters, staining methods along with inter-laboratory validation and methodologies was suggested in order to make the study more scientific and acceptable. The good analysis and measurements depends on the slide, staining and the parameter used for scoring. The Comet Assay has been found to be a reliable method for fundamental biological research, in addition to hazard and risk assessment in the field of genetic toxicology.[12]

Rojas et al., 2014 explained the importance of comet assay in epithelial cells of corneal, nasal, buccal and tear ductal cells. They have explained the various methodologies, sample preparations in various cells with proper guidelines and shortcomings of the procedures. The review also addresses the various variations in the comet assay procedure of different epithelial cells. The importance of various epithelial matrices as a biomonitoring tool in identifying the genotoxicity of carcinogens was also described. The comet assay technique can be used as an additional tool not only for the

genotoxic studies but also for the diagnosis, prognosis and treatment of various diseases.[13]

Collin et al., 2015 described the comet assay as an important tool in recent research along with many other developing technologies. The historical importance of the comet assay technique from the late 1970s was reviewed. It detailed about the past history, the present use of genotoxic studies in studying the nanoparticles and various other approaches. They also stated that inspite of various methods being followed, it was found to be a laboratory intensive procedure. They hoped that in the nearby future protocol standardization, inexpensive automated comet scoring, more human biomonitoring studies of DNA repair (accepting that phenotypic assays have an important place alongside genomics and transcriptomics), environmental monitoring using a variety of animal and plant species; and many more unpredictable developments and applications will come out for identifying the genotoxicity.[36]

Gunasekran V et al., 2015 reviewed about the analysis of DNA damage through the comet assay. They reviewed about the general harmful substances that can lead to various DNA damage. They have also explained the various methodologies of comet assay, its advantages and various applications with recent advances in comet assay. The various genetic methods with their advantages and their limitations were clearly distinguished. The comet assay was found to be a very useful tool in the assessment of DNA damage in diagnosing chronic diseases.[37]

Neri et al., 2015 confirmed the gradual increase in the number of publications that have discussed about the use of comet assay. The world wide use of this study in various fields like human population exposed to DNA damage and basic research on DNA damage and repair in cell cultures and animals was reviewed. The protocol for

comet assay needs much standardization and validation. The progressive use of high-throughput techniques and the clinical implication of the comet assay are research topics that will promote this assay in wide areas of genetic studies.[38]

Muthusamy et al., 2017 reported the Modified Comet Assays for the detection of Cyclobutane Pyrimidine Dimers and Oxidative Base Damages and mentioned that comet assay can be used for the detection of DNA strand breaks, base damages, and CPDs and to measure DNA damage during toxicity, ultraviolet radiation exposure, oxidative stress and human toxicological bio-monitoring scenarios. They also presented different versions of modified comet assay to detect the various DNA damages.[39]

APPLICATIONS OF COMET ASSAY

Valverde et al., 1999 analysed the DNA damage buccal epithelial cells, nasal epithelial cells and in leukocytes of the individuals who were exposed to air pollution in Mexico City using comet assay. The study group included 42 students in a medical school. The average age of the sample group was 19 years out of which 24 females and 18males. Significant difference in tail length was not found in the smokers who smoke less than 10 cigarettes per day. Among the epithelial cells significant difference was found in the nasal epithelial cells and the lymphocytes. However a mean difference in the buccal epithelial cells was not found. Since nasal cells are the first target for the environmental pollutants, they can be used for in biomarker studies, but several studies should be done in the future to identify the genotoxic effects due to pollutants.[10]

Albertini et al., 2000 proposed the guidelines for monitoring the group of individuals who were exposed to genotoxic agents and also the effect of these agents on humans as carcinogens. A concise guidance on the planning, performing and interpretation of studies to monitor groups or individuals who are exposed to genotoxic

agents was proposed. These serve as guidelines and as a check-list for evaluating the methodology and results of completed studies. The main emphasis is on circumstances of continuous exposures, such as those that occur in occupational environmental settings. However an effort is also made to give guidance in situations where unexpected short term exposures have occurred. The most important genotoxic endpoints and the numerical chromosomal aberrations can be assessed using classical chromosomal aberration analysis like micronuclei, DNA damage adducts, strand breaks, protein adducts and biochemical electrophoretic assays.[40]

Tice et al., 2000 stated the guidelines for both in vivo and in vitro genetic toxicology testing related to single cell gel electrophoresis. The various methodologies, analysis, interpretation of results for in vivo and invitro genetic toxicology testing were reviewed. The comet assay has been proven to have many advantages over the other geno-toxicity assays. The advantages were demonstrated sensitivity for detecting low levels of DNA damage, the requirement for small numbers of cells per sample, its ease of application, its flexibility, its low costs and the short amount of time needed to complete the study. The various other related methodologies included were the use of different pH conditions during electrophoreses to discriminate between DNA strand breaks and ALS, the use of a neutral diffusion assay to identify apoptotic/necrotic cells, the use of repair enzymes or antibodies to detect specific classes of DNA damage; and the use of the acellular SCG assay to evaluate the ability of the test substance to interact directly with the DNA. The expert panel mentioned that minimal experimental and methodological standards are needed to get the results of Comet studies, to be accepted as valid by knowledgeable scientists and by the regulatory agencies.[41]

Szeto et al., 2005 mentioned the comet assay as an important biomonitoring tool. However, they need an alternative tool for assessing the DNA damage. They described that epithelial cells are the most common sites of malignant transformation and that these cells would be easily accessible for a comet assay model. Buccal cells are found to be the better alternatives for lymphocytes in assessing the earlier invasion. The various intra- and inter-individual baseline DNA damage in buccal cells were investigated. The basic protocol for the comet assay was also summarized. Thus the framework for this evaluation will facilitate the use of the buccal cell comet assay model as an alternative test to the lymphocyte model for assessing genotoxicity and genoprotection in human trials.[42]

Moller et al., 2006 evaluated the effects of comet assay in analyzing the risks of individuals in the biomontoring studies. The various confounding factors and the occupational risk factors that lead to DNA damage were reviewed. The age, gender and smoking status were used as criteria in the selection of populations. The information on the level of exercise, infection, and diet on the day of the subjects was recorded. In the biomonitoring studies, comet assay was found to be a suitable technique in evaluating the buccal cells and nasal epithelial cells exposed to DNA damage. Both samples from exposed and unexposed populations should be taken at the same time to avoid seasonal variation. The comet assay has been described as a suitable and fast test for DNA-damaging potential in biomonitoring studies.[43]

McKenna et al., 2008 elaborated that comet assay has been widely used to detect the cellular responses to DNA damage and has found that it can be used in ecological testing genotoxicity studies, bio-monitoring and in the study of human disease. The applications of comet assay to study the DNA damage and repair

associated with cancer has been discussed. The advantages and the drawbacks of these studies involving comet assay in cancer were addressed. In addition comet assay was found to be the best tool in assessing the tumour hypoxia to surgical treatment and survival.[44]

Kaur et al., 2011 found the DNA damage in occupationally exposed farmers to pesticides in Punjab. They have assessed the level of DNA damage in 210 occupationally exposed Punjab farmers along with 50 matched control subjects. Results have shown a significant increase in the level of DNA damage in the exposed workers as compared to the control subjects and no variation was found to exist regarding age, drinking, smoking and dietary habits. There exists DNA damage in 35.7% of the workers and around 65.29% sprayers did not show any damage. This may be due to the protective mechanism that were by the workers against the toxic exposure or sometimes they could not be detected by this assay as it detect single strand breaks and alkali-labile sites.[45]

De Oliveira et al., 2011 has evaluated the DNA damage in paint industry workers. Blood samples were collected from these individuals. A significantly high level of DNA damage was found in the paint industry workers when compared to the control group. The hippuric acid levels in the urine samples of the individuals were analysed using high performance liquid chromatography (HPLC) with UV-VIS detector. It was found that chronic occupational exposure to paints causes more genetic damage. The study concluded that the mutagenicity of cells was low but the cytogenetic damage indicates the level of occupational health hazard of paint industry workers.[46]

Sharma et al., 2012 evaluated the precancerous genetic damage in minimally in professional sports players. The study was conducted in the players who were professionally active in their sport from 3 to 11 years on a daily training session of 4h/day. Both hockey and base-ball players had a significantly increased level of genetic damage. The mean tail length of the hockey players was found to be higher than the other teams. No difference in tail length exist between the genders.[47]

Powel et al., 2015 has described the various genotoxic effects that occur due to environmental and industrial factors. The new 3D DIP chip microarray based technique that is used to measure genotoxicity was explained. The measurements of both physical and chemical induced DNA damage spectra, integrating the analysis of these with the associated changes in histone acetylation induced in the epigenome was also detailed. The comet assay technique has the importance of using it as best predictive marker in identifying various genetic mutations.[48]

Arshad et al., 2015 evaluated the toxic effects of pesticides in the exposed individuals in Pakistan with an aim to prevent the harmful and anthropogenic effects. The study was conducted in the exposed workers from an industrial area in Multan, Pakistan, Malathion residues in blood samples were analysed by using gas chromatography. There existed a strong correlation of tail length in these samples in workers when compared to the controls. Thus adequate training and awareness programmes should be conducted for the safety practices and to change the industrial workers attitude to avoid the harmful environmental and anthropogenic effects.[49]

Sakhvidi et al., 2016 evaluated DNA damage in the healthcare providers who were working with anti-neoplastic drugs using comet assay. They systematically analysed various studies to determine the magnitude and the significance of DNA

damage in 15 studies and 14 studies were taken for the Meta analysis. An increased level of DNA damage was found and many studies should be carried out to find the intensity of these drugs in developing countries. The various protocols and strategies used should be standardized for the better generalization of the results.[50]

APPLICATIONS OF COMET ASSAY IN DENTISTRY

Saran et al., 2008 validated the biomarkers needed for the identification of risk of precancer. The risk assessment of oral cancer in precancerous patients were analysed using three assays, the micronuclei assay , the comet assay and the challenge assay. The study included 129 patients with cancer, 138 individuals with untreated precancerous stage and 176 control patients. Micronuclei and comet assay were done in the buccal mucosal cells and challenge assay was done in the blood lymphocytes. A stepwise increase in the level of DNA damage from control group to precancer and from precancer to cancerous patients by interobserver and intra-observer variability were noted.[51]

Mukherjee et al., 2011 evaluated the DNA damage in oral precancerous lesions patients and squamous cell carcinoma patients by using comet assay. They collected the peripheral blood samples by venepuncture method subjected the samples to comet assay. The study was conducted in 44 consecutive patients with oral cancer (n=26), leukoplakia (n=12) and OSF (n=6) and 10 healthy normal volunteers with normal oral epithelia (controls). The mean tail lengths between the diagnostic groups and between the people having different oral habits were compared. DNA damage was found to be greater in leukoplakia and squamous cell carcinoma patients and lesser in OSF patients. Greater DNA damage was found in patients with any form of oral habits when compared to the control with no habits.[52]

Baricevic et al., 2012 did an in vivo study in which they examined the genotoxic effect of casting alloys on oral epithelial cells in patients using removable and fixed appliances for more than five years. The study was done in 55years age matched subjects that included 30 subjects wearing prosthodontic appliances and 25 controls. The samples were collected from the buccal mucosa of the subjects and then processed for analysis. The cell viability of the samples were assessed using trypan blue exclusion test and genotoxicity was assessed using alkaline comet assay. It was found that comet assay parameters were significantly higher in the group wearing metal appliances when compared to the control group. In vivo evaluation of dental materials to analyze the genotoxicity will be helpful in enhancing the biocompatibility.[53]

Dodani et al., 2012 studied the periodontal findings in patients with oral submucous fibrosis and analysed the level of DNA damage in the affected gingival epithelial cells using comet assay. The study was conducted among 100 oral submucous fibrosis patients and 89 patients without OSMF were taken as the control group. After the gingival parameters were analysed, comet assay was done by scraping the gingival epithelial cells. The results showed an increase in tail length in the epithelial cells of gingiva in patients with oral submucous fibrosis when compared with the control group.[54]

Goncalves et al., 2014 analysed the genotoxic and cytotoxic effects of non-soldered and silver soldered orthodontic bands on HepG2 and HOK cell lines. The silver soldered bands were found to be genotoxic. The MTT reduction assay was performed for analysing the cytotoxicity and comet assay was used to identify the double strand breaks and micronuclei assay was used to verify the DNA damage.[55]

Udupa et al., 2014 assessed the DNA damage in the patients with oral submucous fibrosis and control patients using comet assay. 25 healthy individuals and 25 subjects with oral submucous fibrosis were included in the study. Buccal epithelial cells were collected from each individual and subjected to comet assay procedure. There was a statistically significant increase in tail length in OSF patients and also there was increase in tail length in these patients with respect to duration of the habit. Thus they concluded that OSF patients had a greater DNA damage when compared to the control group.[56]

Khan et al., 2014 compared the genetic damage that occurred in precancerous and cancerous patients by using micronuclei and comet assay. The study group consisted of 260 patients including leukoplakia, lichen planus, OSF, Oral squamous cell carcinoma and the normal control group. They mentioned that leukoplakia patients had more DNA damage than the oral squamous cell carcinoma patients. This finding suggests that oral leukoplakia has more tendency towards malignant transformation. The gender wise difference in DNA damage was not found in the study. The number of micronuclei was seen increasing from the normal group to precancerous and oral cancerous patients.[57]

Madhulika et al., 2015 studied the extent of DNA damage in patients with oral lichen planus and patients with lichenoid reactions. The study was conducted in 20 cases of lesional subjects and 20 normal individuals. The venipunctured blood was taken simultaneously after the incisional biopsy was done. Their results showed a significantly increased tail length and tail moment in the patients with lichen planus when compared to the control group. Thus comet assay can be used as a valid tool in

diagnosing potentially malignant disorders and thereby preventing cancer by early diagnosis.[58]

Vellappally et al., 2015 assessed the DNA damage in the leukoplakia patients having various grades of dysplasia. The study was conducted in 36 leukoplakia patients and 10 normal controls. The mean comet tail length of leukoplakia was found to be more than that of the controls. The mean tail length of the DNA tends to increase with different grades of dysplasia. The result of DNA damage helps us in understanding the biology of oral cancer and its prognosis.[59]

Shanmuga sundaram et al., 2017 evaluated the DNA damage in both oral submucous fibrosis and oral leukoplakia patients. The study subjects were divided in 3 groups. Group I consisted of 20 patients with oral submucous fibrosis, group II consisted of 20 leukoplakia patients and group 3 consisted of 20 control patients. Buccal exfoliated cells were collected from all the patients in each group and the level of DNA damage was evaluated by the method of Single cell gel electrophoresis technique/ comet assay technique. The mean tail length of DNA obtained from the buccal epithelial cells of leukoplakia was found to be significantly higher when compared to the epithelial samples obtained from the oral submucous fibrosis group and control group. The evaluation of DNA damage using comet assay in the potentially malignant disorder patients can be a useful tool in dentistry for early identification of the malignant transformation of the affected patients.[60]

MATERIALS AND METHODS

MATERIALS AND METHODS

Buccal epithelial cells not only form the first barrier for carcinogens entering via the ingestion route, they are also capable of converting the proximate carcinogens to reactive products. Therefore, the oral epithelial cells represent a preferred target site for early genotoxic events induced by carcinogenic agents entering the body via this route. DNA damage assessment in exfoliated buccal cells may be an innovative promising tool for genotoxic studies since sampling is easy and non invasive. Some results indicate that alkaline single-cell gel electrophoresis, using buccal epithelial cells, could be a good biomarker of early effects, and can be utilized for human monitoring.

With this background this study was aimed to assess the DNA damage in the buccal cells of oral submucous fibrosis patients using comet assay.

SUBJECT SELECTION

The study subjects were recruited from the patients attending Vivekanandha Dental College for Women. The study protocol was approved by the ethical committee of the institute with the reference number VDCW/IEC/30/2015. Patients who were clinically diagnosed with oral submucous fibrosis under standard diagnostic criteria were selected. The sampling method and the importance of the study were explained in detail to the study participants. The samples were collected from these subjects after obtaining their informed consent. The control group included age matched subjects without habits, other oral lesions and systemic illness.

The study group includes,

Group I – 30 individuals with oral submucous fibrosis

Group II – 30 individuals with clinically normal mucosa and without any systemic illness.

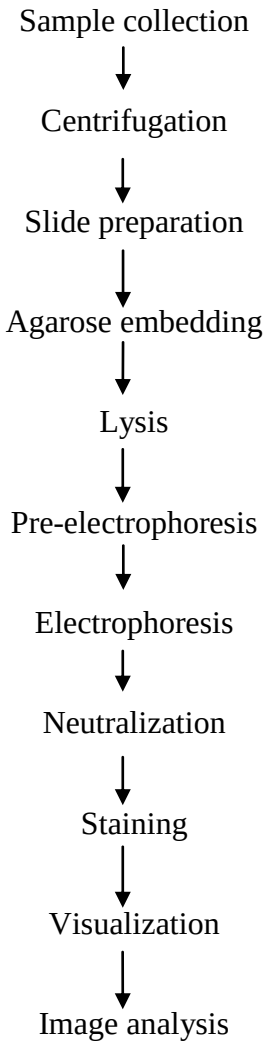
MATERIALS

1. Deionised water - LOBA 6483C5000
2. Normal melting agarose - SIGMA ALDRICH A9539-100G
3. Low melting agarose - HIMEDIA RM 861-25G
4. Proteinase-k - SISCO RESEARCH 49936
5. Sodium chloride - LOBA 0581900500
6. Ethidium bromide - LOBA 0371500005
7. Ethylene diamine Tetra acetic acid disodium salt - LOBA 0373000500
8. Triton X – 100 – LOBA LM03341605
9. Tris buffer- LOBA O039000500
10. Dimethyl sulphoxide – LOBA 0012300500
11. Sodium hydroxide pellets- LOBA 0581900500
12. Ethidium bromide – LOBA 0371500005
13. Sodium Dodecyl Sulpahate- MERCK 61841905001730
14. Sodium chloride – LOBA 0581900500
15. Frosted slides - ROHEM

EQUIPMENTS

1. Micro centrifuge tubes
2. Micropipette
3. Weighing machine
4. Horizontal electrophoresis system with power pack - BIORAD
5. Fluorescent microscope - OLYMPUS
6. Refrigerator.
7. Incubator-HERATHERM
8. Mechanical vibrator.
9. pH meter- EUTECH
10. Slide marker
11. Slide stand.
12. Slide tray
13. Pasteur pipette 3ml.
14. Dispenser
15. Centrifuge REMI-8C

PROCEDURE


Sample collection
Centrifugation
Slide preparation
Agarose embedding
Lysis
Pre-electrophoresis
Electrophoresis
Neutralization
Staining
Visualization
Image analysis

SAMPLE COLLECTION

The study subjects were selected from the patients attending the outpatient department of Oral Medicine & Radiology. The sample was collected from the patients with oral submucous fibrosis and the control subjects after obtaining their informed consent. In Group I and Group II, buccal smear sample was collected from one side of the oral mucosa. Samples were obtained by scrapping the cells from the buccal mucosa using an ice cream stick. Samples were then stirred in phosphate buffer saline contained in eppenderof tubes and placed in the refrigerator until processing.

CENTRIFUGATION

The samples were then centrifuged at 3500 rpm for 5 minutes to get a cell suspension. After centrifugation of the samples, the cells settled down at the bottom of the eppendorf tubes. The supernatant solution was then drained off.

SLIDE PREPARATION

Frosted slides were used and wiped with alcohol to get a dust- free surface. The slides were then placed in the incubator at 37 degree Celcius for 30minutes. The next procedure was pre-coating the slides with normal melting point agarose. Normal melting point agarose is the matrix that is used commonly for pre-coating of slides. It has a gelling temperature of 36 degree Celsius and melting temperature of 87 degree Celsius.

Normal melting point agarose for pre-coating was prepared by dissolving 0.5 gram of regular agarose in 100ml of distilled water. During the slide preparation, normal melting point agarose used for slide pre-coating have better adhesion when

compared to the low- melting point agarose. Low melting point agarose and the sample were used in the concentration of 2:1. The beaker containing agarose and distilled water was covered with the aluminium foil and then placed in boiling water till the solution became clear. The slides were then dipped in clear hot agarose solution and the undersides of the slides were wiped off. The slides were then placed in room temperature for 15-20 minutes until a clear homogenous layer was seen on top of the slides.

AGAROSE EMBEDDING

The samples were prepared with the low melting point agarose. Low melting point agarose was prepared by dissolving 0.5g of agarose in 12.5ml of Phosphate buffer saline. The mixture was placed in boiling water till a clear solution was obtained and then allowed to cool for 15-20 minutes till room temperature was reached. 85µl of the low melting point agarose was mixed with 10µl of the sample using a micropipette in a plastic tube. The suspension was then mixed using a mechanical vibrator. From this suspension, 10µl of the sample was placed on the slide and covered with a coverslip. Slides were then placed in the refrigerator for solidification process. After 3-4 minutes once the agar got solidified, the coverslip was gently removed from the slide.

LYSIS

Cell membranes are held together by phospholipids and polar interactions. Detergents and high salt concentration act as emulsifiers and help in the dissolution of proteins, fats and unwanted cellular substances. The most commonly used surfactants are sodium dodecyl sulphate and Triton-X.

In the preparation of lysis solution, the stock solution of 2.5N sodium chloride solution, 100mM EDTA solution and 10mM of Tris buffer, was prepared by dissolving 146.4 gm of sodium chloride, 37.2 gm of EDTA and 1.2 gm of Tris in 1000 ml of distilled water. NaOH was added and the pH was set to 10. Sodium lauryl sarcosinate, an anionic detergent, was excluded from the lysing solution, as identical results were obtained without it.

36 ml of the above prepared lysing solution was then taken and 0.4 ml of freshly prepared 1% Triton X – 100 and 4 ml of Dimethyl sulphoxide were added to give the final working lysing solution. The solution was stored in amber colored bottles at 4 degree Celcius. After the solidification of the sample, slides were then gently placed in the lysis buffer and kept for 1hr at 4 degree Celsius in a refrigerator.

PRE-ELECTROPHORESIS

After lysis, the next step is pre-electrophoresis to allow unwinding of the DNA. The slides were taken out from the lysis solution and placed in the alkaline electrophoresis solution for about 30 minutes.

ELECTROPHORESIS

The slides were gently removed from the lysing solution and placed in a horizontal electrophoresis system near the anode end, sliding them as closely as possible. The buffer reservoir was filled with freshly prepared electrophoresis buffer solution until the liquid level completely covered the slides without any bubbles over the agarose. Electrophoresis is one of the important steps of comet assay. During electrophoresis, broken DNA fragments tend to migrate towards the anode. The shorter the DNA fragment, the farther it travels.

For the electrophoresis, the electrophoresis buffer was prepared by dissolving 200 gm of NaOH in 500 ml of distilled water to get a 10N NaOH stock solution. The EDTA stock solution was prepared by dissolving 7.4 gm of EDTA, 1.2 gm NaOH in 100 ml distilled water and then pH was adjusted to get 200 ml EDTA stock solution. To make fresh electrophoresis before each run, 30 ml of NaOH solution and 5 ml EDTA solution were taken from the above stock solutions and made up to 1000 ml by adding distilled water. The solution is mixed well to get an electrolyte concentration of 500mM NaOH / 1mM EDTA.

After pre-electrophoresis, the electrodes were connected to the power-pack unit. The electrophoresis was run at constant current, voltage of 24volt and 300mA for 15minutes.

NEUTRALIZATION

To neutralize the alkaline pH to 7, slides were then kept in the neutralization buffer for 5minutes. Neutralization buffer was prepared by dissolving 48.5 gm of Tris in 1000 ml of distilled water to make a 0.4 M Tris buffer solution and the pH was adjusted to 7.5 using concentrated HCL and stored at room temperature.

After electrophoresis, the power unit was turned off. The slides were carefully lifted from the buffer and placed on a staining tray. The slides were coated drop wise with the neutralization buffer and left for 5 minutes. After neutralizing the pH, staining was done with a fluorescent dye to visualize the comets.

STAINING

A stock staining solution of ethidium bromide was prepared by dissolving 10 mg ethidium bromide in 50 ml distilled water and stored at room temperature. For the working solution, 1ml of the above prepared solution was mixed with 9 ml of distilled

water. The slides were drained and then 50 µl of working solution of ethidium bromide was added on top of the slides. The excess stain was blotted off.

VISUALIZATION OF SLIDES

The slides are visualized using Olympus CX 31 fluorescence microscope with Binocular 30 degree inclined and rotatable 360 degree. It has Plan C Achromat Infinity 4x, 10x, 40x, 100x Oil objectives. The fluorescent microscope has a blue halogen light with an excitation more than 510nm. The slides were visualized at 400X magnification and the images were captured using ProgRes® SpeedXT core 3 camera.

IMAGE ANALYSIS

The captured images were then measured using the ProgRes® Capture Pro 2.8.8 Jenoptik software**.** After capturing the image, a total of 15 cells with well stained nuclei were selected. After calibrating, the length and the diameter of each nucleus was measured using the line tool in the software and recorded in the Microsoft excel sheet**.** The tail length was obtained by subtracting the diameter from the total length of the nucleus. The mean tail length of each group was assessed from the mean value of the 30 individuals.

STATISTICAL ANALYSIS

The demographic data like age, sex, history and the duration of betel nut habit with average tail length were tabulated to assess the mean tail length. Statistical significance was assessed using Statistical Package for Social Science (SPSS) software version 17. The student t- test was used to compare the mean tail length between each groups. The average tail length was also compared with age, gender, type and duration of betel quid habit for any possible statistically significant association.

Figure 1: Sample collection

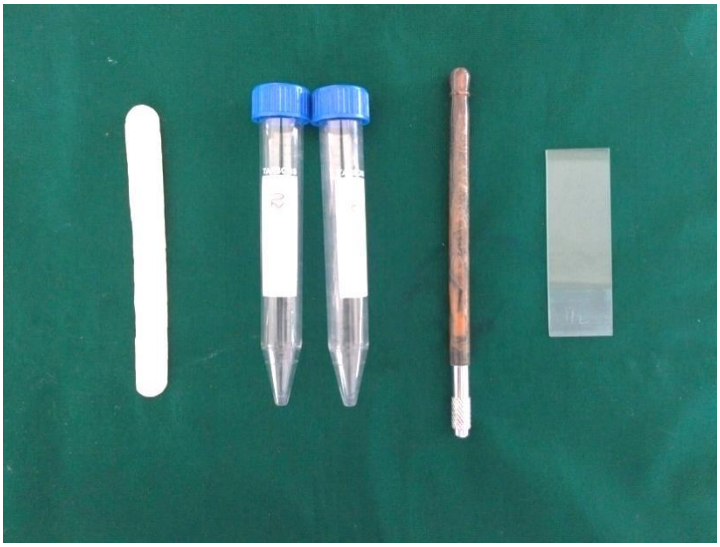
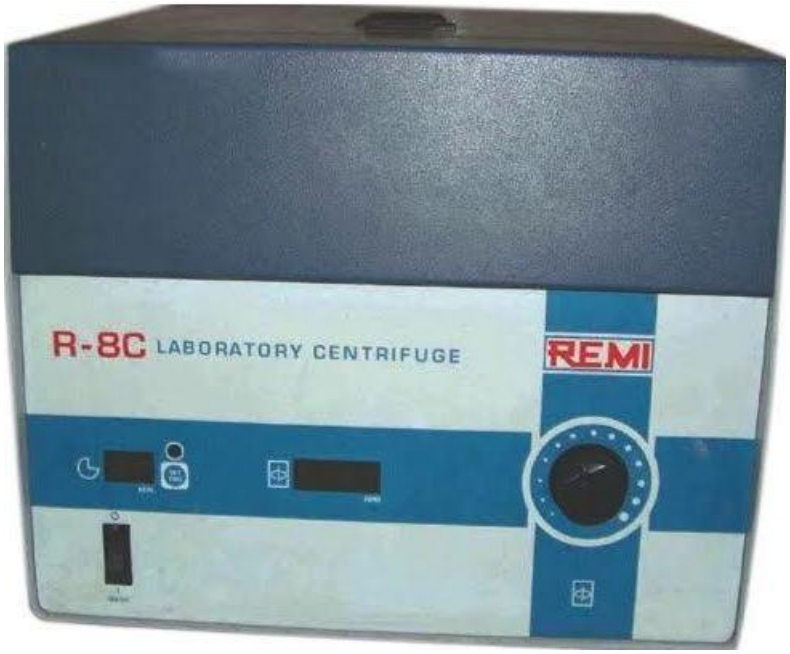


Figure 2: Centrifuge



AGAROSE EMBEDDING

Figure 3: Agarose

Figure 4: Micropipette

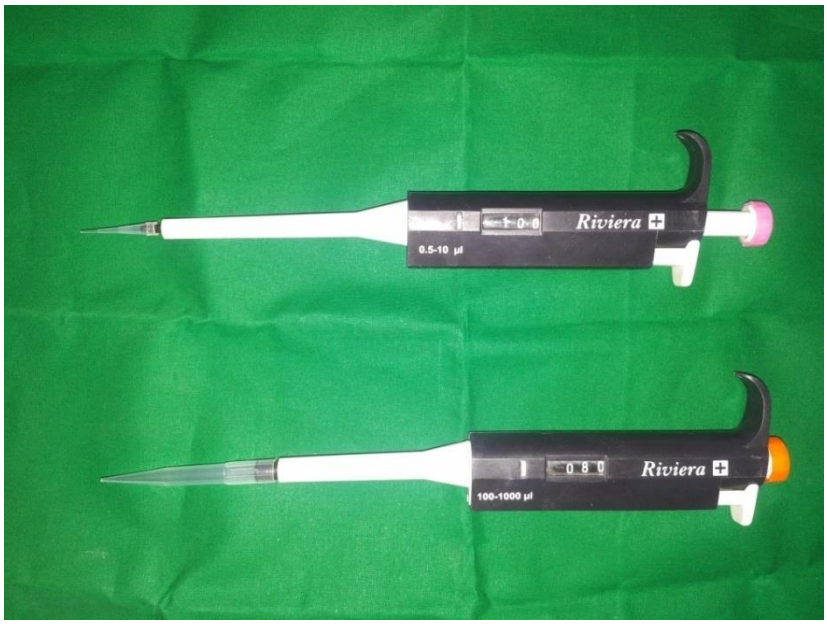


Figure 5: Microwave oven

Figure 6: Clear agarose solution

Figure 7: Slide preparation

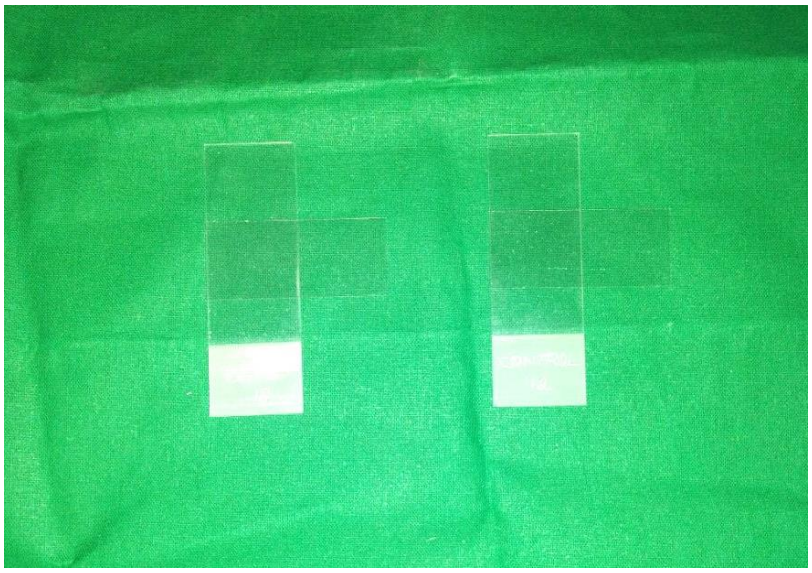


Figure 8: Stock solution

Figure 9: Lysis buffer

Figure 10: Lysis working solution

Figure 11: Electrophoresis Unit

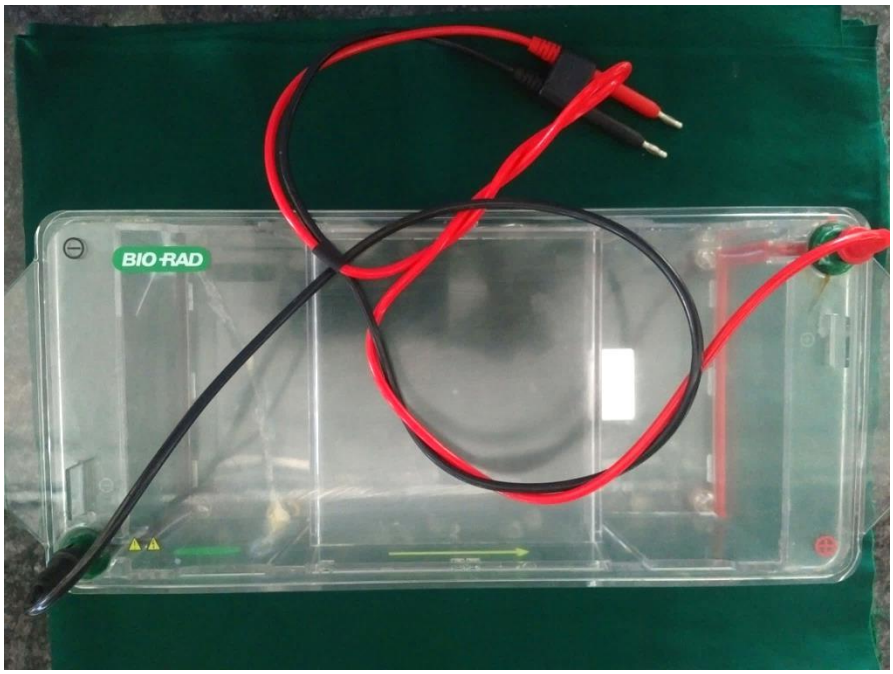


Figure 12: Power control unit

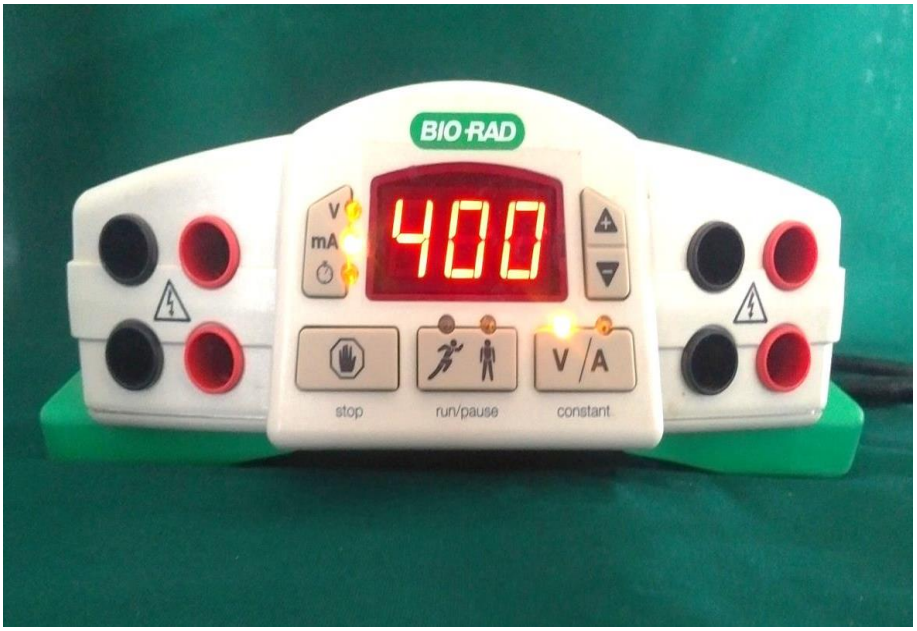


Figure 13: Neutralization buffer

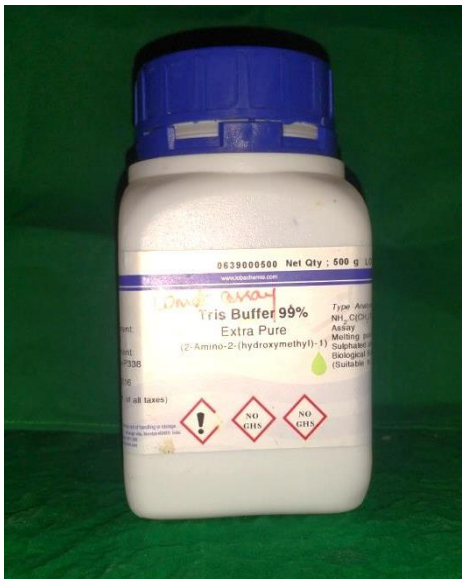


Figure 14: Ethidium Bromide Stain

Figure 15: Fluorescent microscope

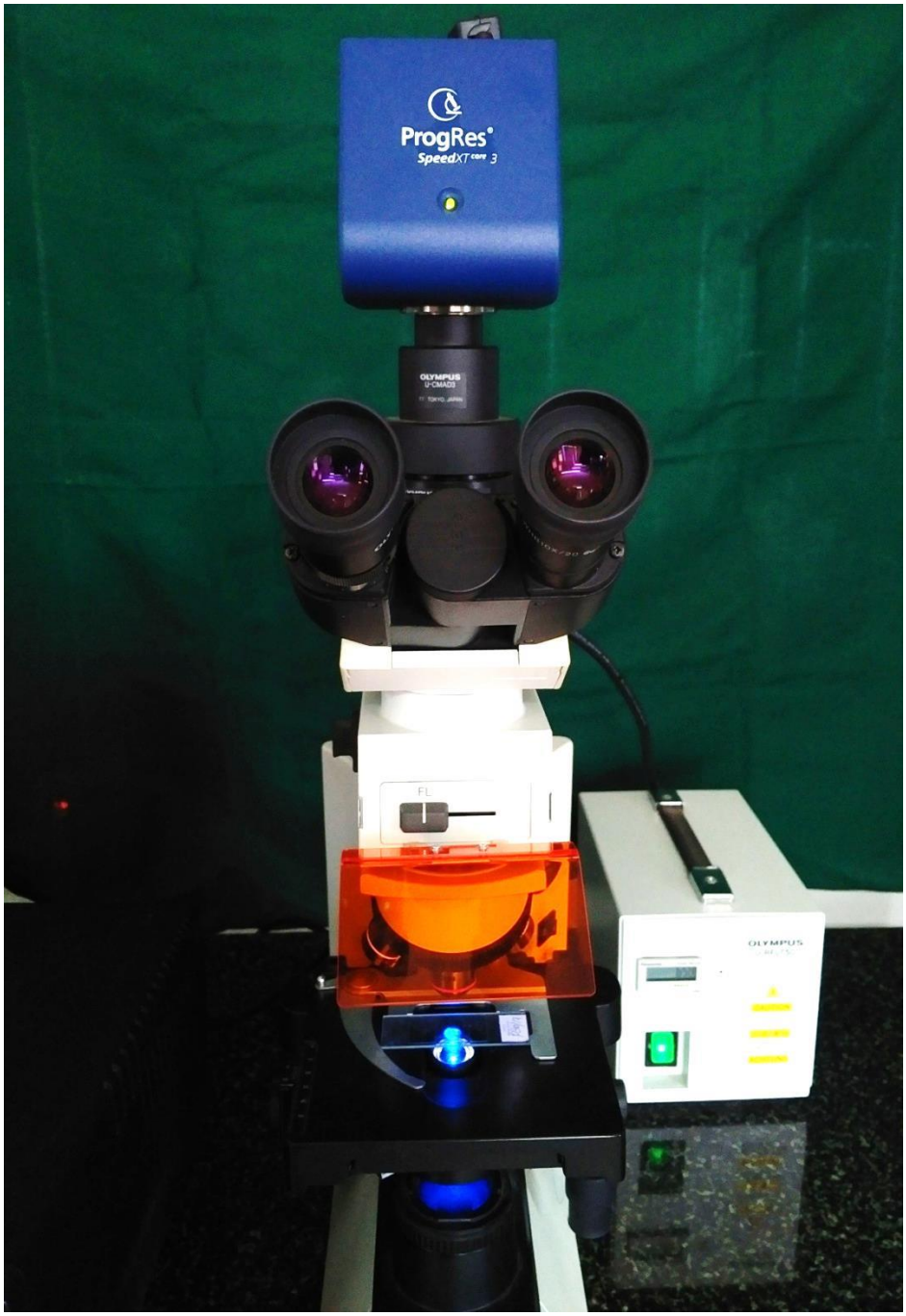
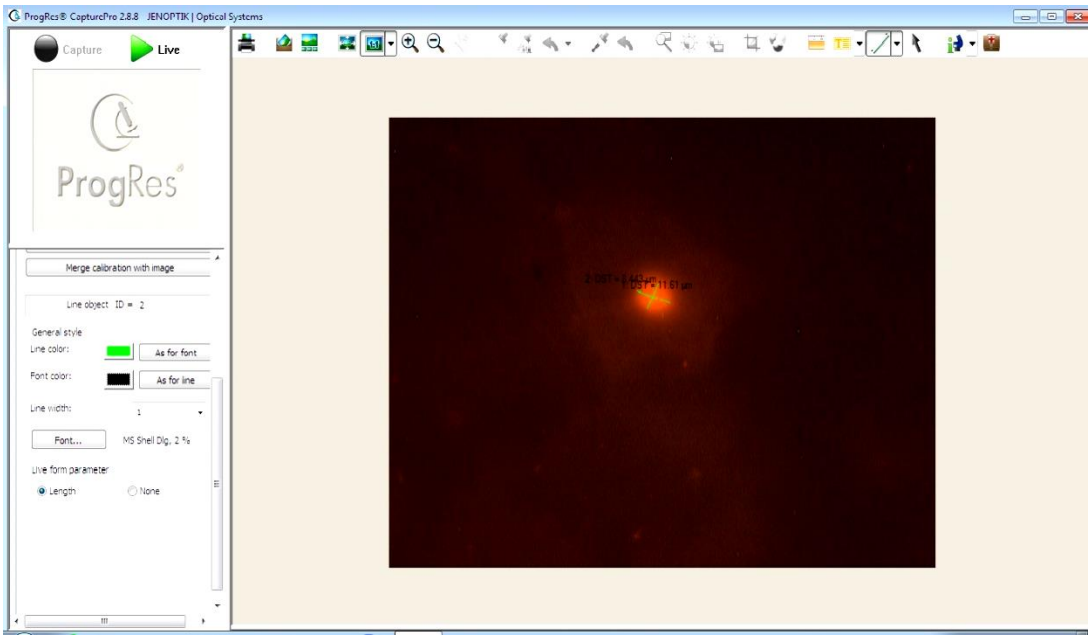


Figure 16: IMAGE ANALYSIS



RESULTS

RESULTS

The comet assay procedure was done to assess the DNA damage in the buccal cells of patients with oral submucous fibrosis patients. A total of 60 samples were taken for the study which included 30 submucous fibrosis patients (Group I) and 30 control patients (Group II). The distribution of cases is shown in Table 1.

Table 1: Distribution of cases between submucous fibrosis group and control group with their average age in years

Group	Number of study subjects	Average Age in years
Group I	30	45.4
Group II	30	45.5
Total	60	45.4

For every individual the average tail length was calculated and recorded. The Table 2 represents the average tail length of the buccal cell samples obtained from the study participants belonging to Group I i.e., patients with oral submucous fibrosis. The average tail length of the Group – II control patients is given in Table 3.

Table 2: Average tail length in patients with oral submucous fibrosis

S. No	Age (years)	Sex	Type of habit	Duration of the habit (years)	Average Tail length (µm)
01	35	Male	Betel quid	5	2.3
02	47	Male	Betel quid	9	2.3
03	43	Male	Betel quid + smoking	17	3.1
04	50	Male	Betel quid	8	2.4
05	39	Male	Betel quid	6	1.5
06	48	Female	Betel quid	2	1.6
07	41	Female	Betel quid	5	1.8
08	32	Male	Betel quid	3	1.7
09	44	Male	Betel quid + smoking	13	2.4
10	46	Male	Betel quid	9	2.2
11	41	Male	Betel quid + smoking	6	2
12	53	Male	Betel quid	8	2.7
13	50	Male	Betel quid	11	2.3
14	37	Male	Betel quid	7	2.1
15	50	Male	Betel quid	3	2.2

16	55	Male	Betel quid	10	2.5
17	48	Male	Betel quid	6	2.3
18	54	Male	Betel quid	20	2.7
19	41	Male	Betel quid	3	2.6
20	50	Male	Betel quid + smoking	9	2.6
21	48	Male	Betel quid	4	2.7
22	37	Female	Betel quid	7	1.8
23	60	Male	Betel quid	9	2.1
24	52	Male	Betel quid	2	1.6
25	51	Female	Betel quid	7	2.7
26	41	Male	Betel quid	5	1.8
27	48	Male	Betel quid	8	2.1
28	32	Male	Betel quid + smoking	6	1.9
29	46	Male	Betel quid	15	3.1
30	42	Male	Betel quid	3	2.4

Table 3: Average tail length in control group

S. No	Age (years)	Sex	Average Tail length (µm)
01	42	Male	1.2
02	46	Male	1.3
03	34	Male	1.1
04	44	Male	1.2
05	53	Male	1.6
06	37	Female	0.9
07	43	Male	1.4
08	41	Male	1.2
09	33	Male	1.2
10	38	Male	1.1
11	44	Male	1.2
12	48	Female	0.9
13	45	Male	1.3
14	41	Male	1.4
15	50	Male	1.5
16	43	Male	1.1
17	53	Male	1.5

18	44	Male	1.3
19	49	Male	1.6
20	55	Male	1.4
21	59	Male	1.9
22	62	Male	2.1
23	45	Male	1.2
24	48	Male	1.2
25	37	Female	1
26	44	Male	1.2
27	36	Male	1.4
28	48	Female	1.5
29	45	Male	1.7
30	57	Male	1.8

Student ‘t’ test was done to assess the average tail length between OSF patients and control patients and between different types of habits among the OSF patients. Pearson correlation was done to find the correlation of the tail length with the duration of habit and the age of the patients.

The average tail length in oral submucous fibrosis patients was comparatively higher than that of the control patients. The average tail length in OSF patients was found to be 2.25µm and that of the control patients was 1.35µm which is shown in Figure 17.

Figure 17: Average tail length in oral submucous fibrosis patients and the control group

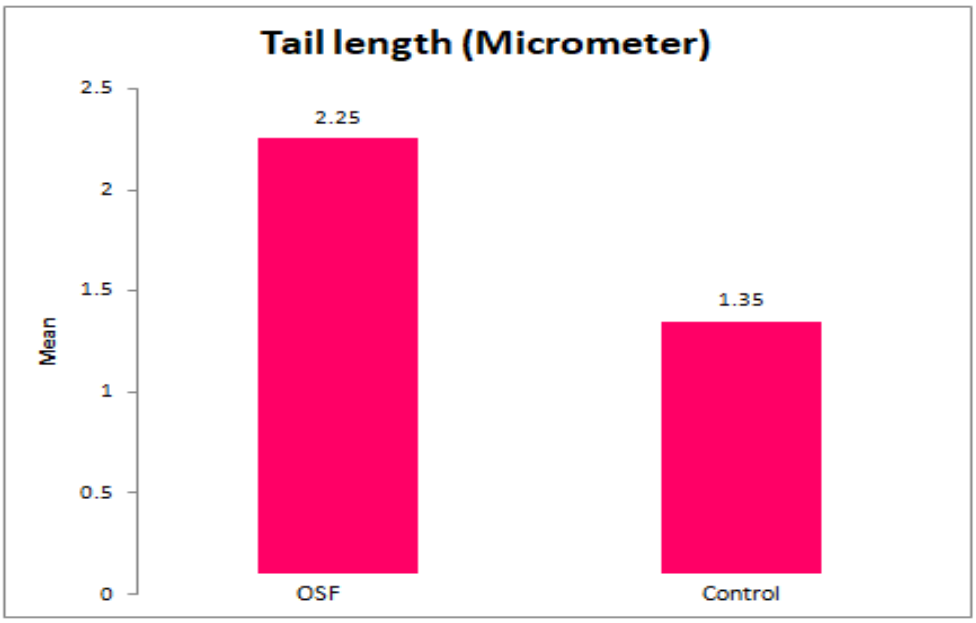
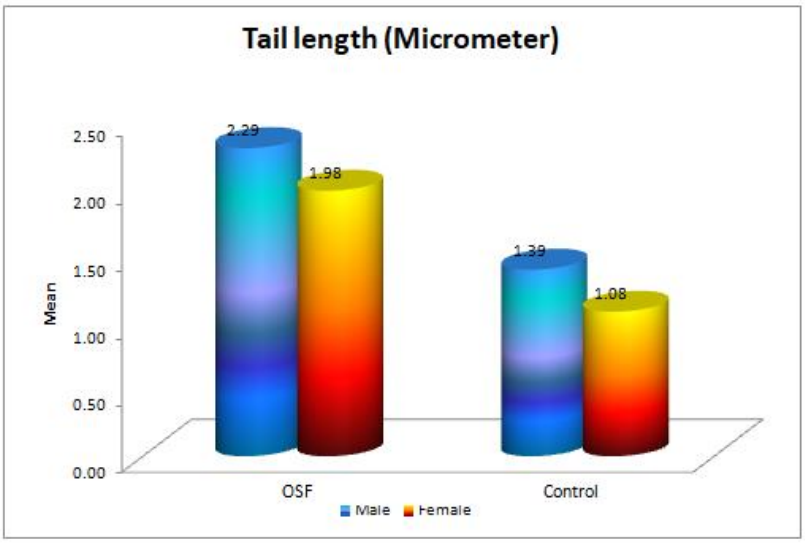


Table 4 and Figure 18 shows the variation in tail length between males and females in both the groups with males having a higher tail length than the females. However it is statistically significant only in the control group.

Table 4: Comparison of average tail length in oral submucous fibrosis patients and control group

			Tail length (Micrometer)			
Group	**Sex**	**N**	**Mean**	**SD**	**t**	**p**
OSF	Male	26	2.292	0.407	1.417	0.167
	Female	4	1.975	0.492		
Control	Male	26	1.388	0.261	2.209	0.036*
	Female	4	1.075	0.287		

Figure 18: Average tail length between males and females in both the groups

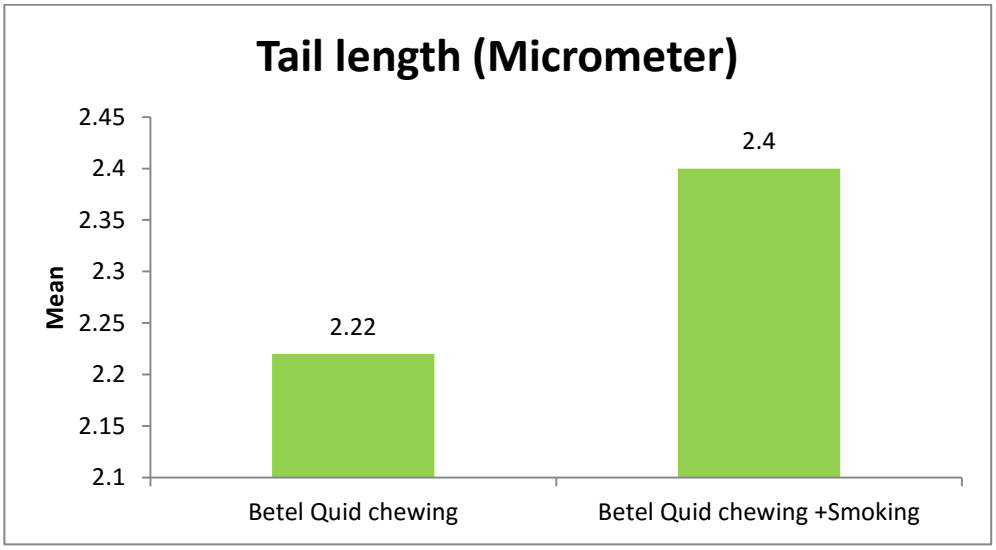


The tail length of patients with different types of habits that is patients with only betel quid usage and patients who had the additional habit of smoking tobacco was analysed using student 't' test. Patients with only betel quid usage has shown a higher tail length which is shown in Table 5 and Figure 19. However these results were statistically insignificant.

Table 5: Average tail length between different types of habits

	Type of Habit	N	Mean	SD	t	p
Tail length (Micrometer)	Betel Quid chewing	25	2.220	0.415	0.863	0.396
	Betel Quid chewing +Smoking	5	2.400	0.485		

Figure 19: Average tail length between different types of habits



Pearson correlation was done to correlate the average tail lengths with age and duration of habits of the subjects which is shown in Table 6. Positive correlation was found between age and tail length in OSF patients but was not statistically significant. However in the control group positive correlation with statistical significance was found between age and tail lengths implying that as age increased there was a significant increase in the tail length.

When correlating the duration of habits with the tail lengths in the OSF patients, as the duration of habit increased the tail length also increased. A positive correlation with statistical significance was obtained.

Table 6: Pearson Correlation done of average tail length with age and duration

		OSMF		**Control**	
		Age	**Duration of habit**	**Age**	**Duration of habit**
Tail length (Micrometer)	r	0.354	0.609	0.750	-
	p	0.055	0.001**	0.001**	-

The longest duration of habit was found in an OSF patient who had a high tail length of 2.7µm. Figure 20 is a scatter plot that shows the distribution of cases with respect to their average tail length and duration of habit.

Figure 20: Scatterplot showing average tail length in relation to duration of the habit in OSF patients.

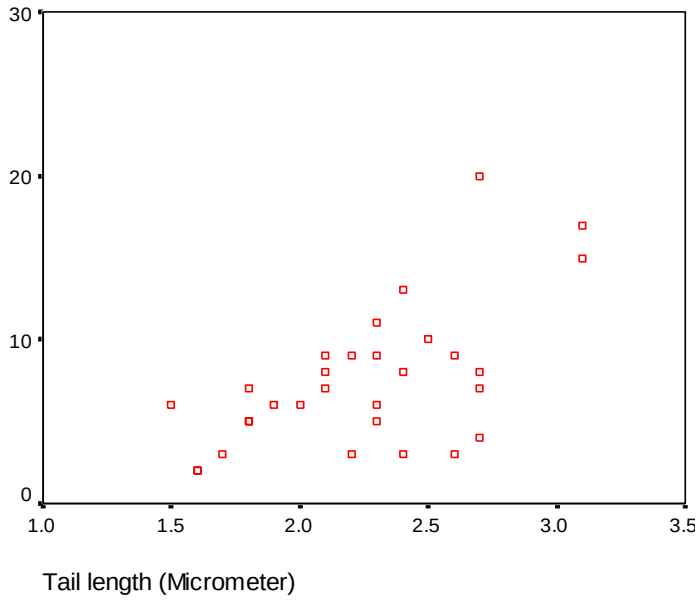
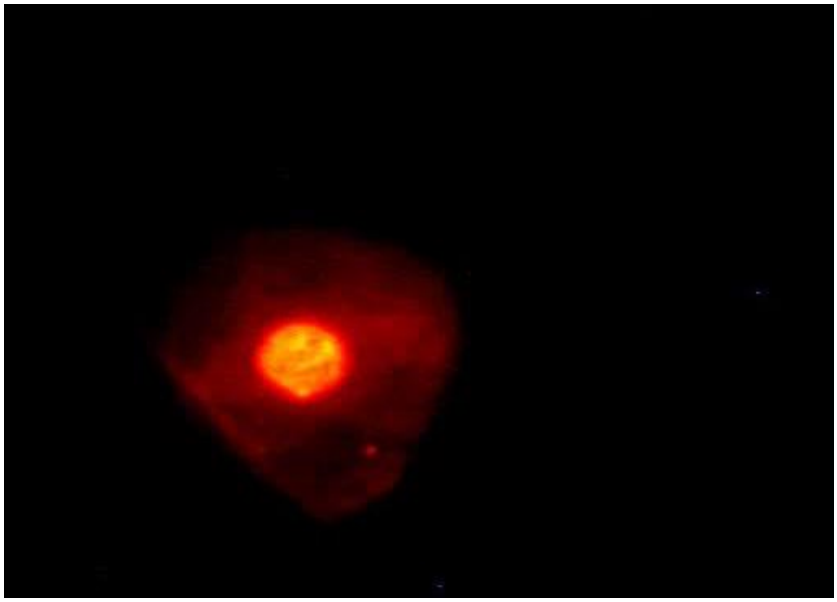
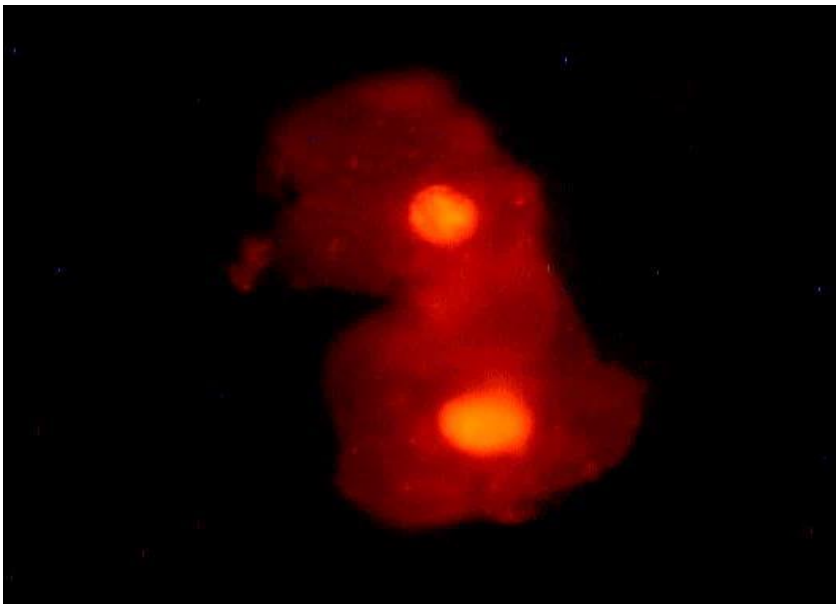
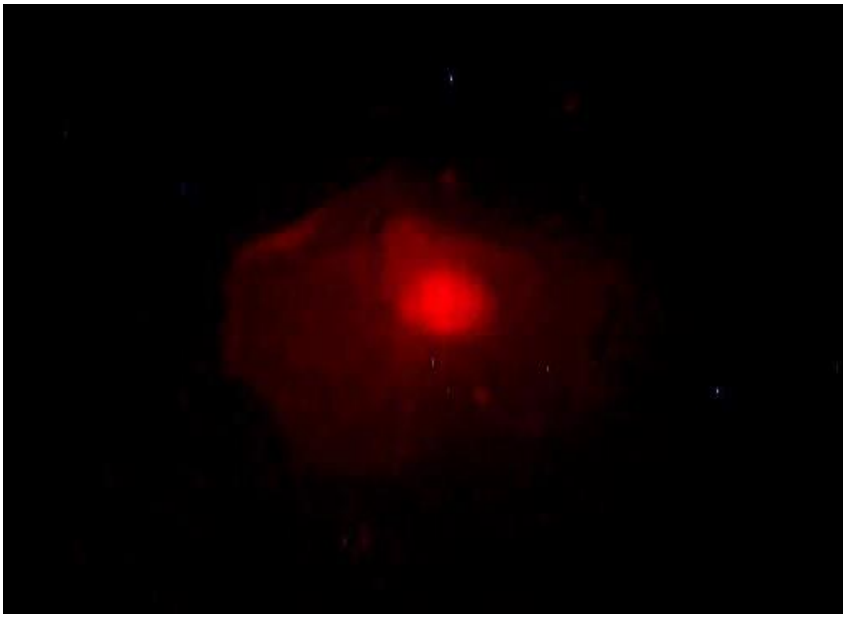
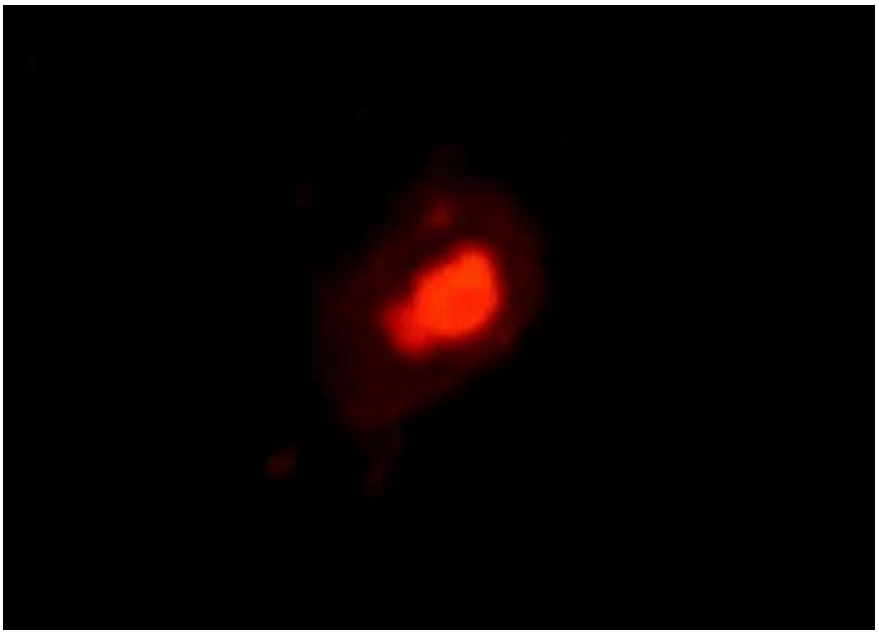


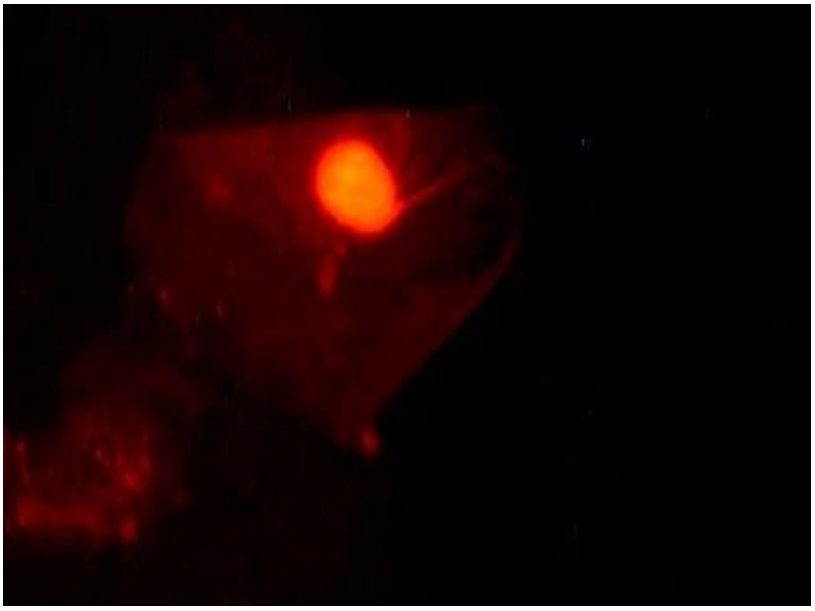
Figure 21: Microscopy of Patients with Oral Submucous Fibrosis (Group I)











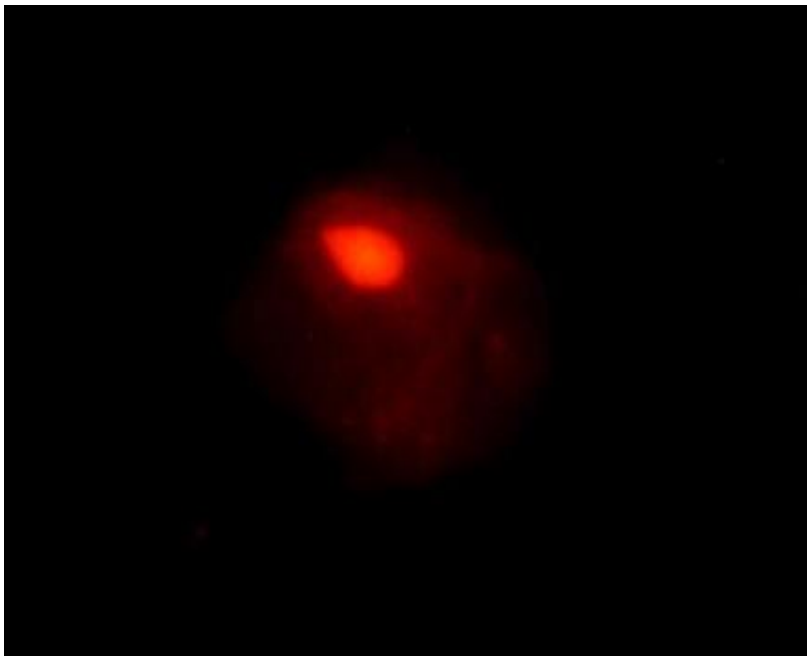
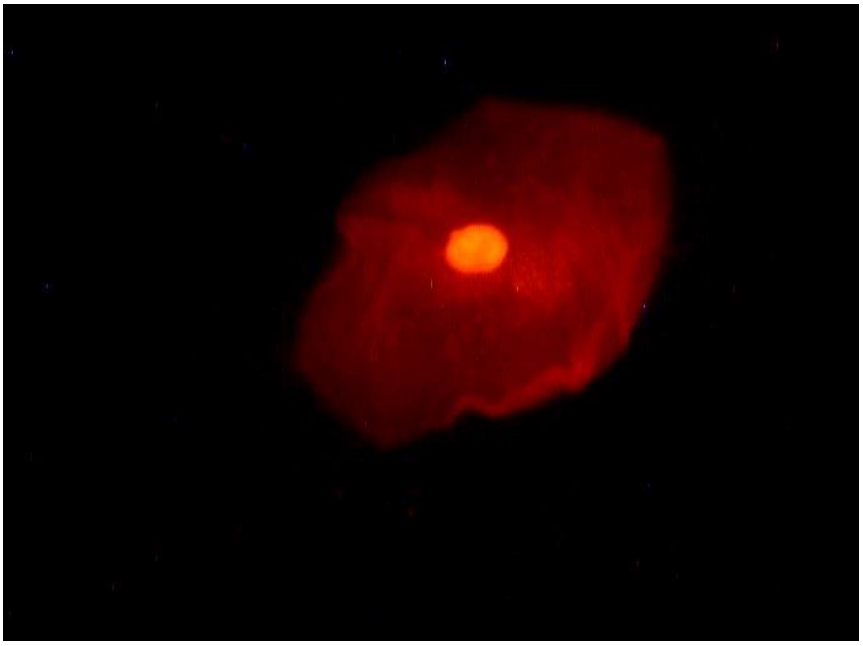
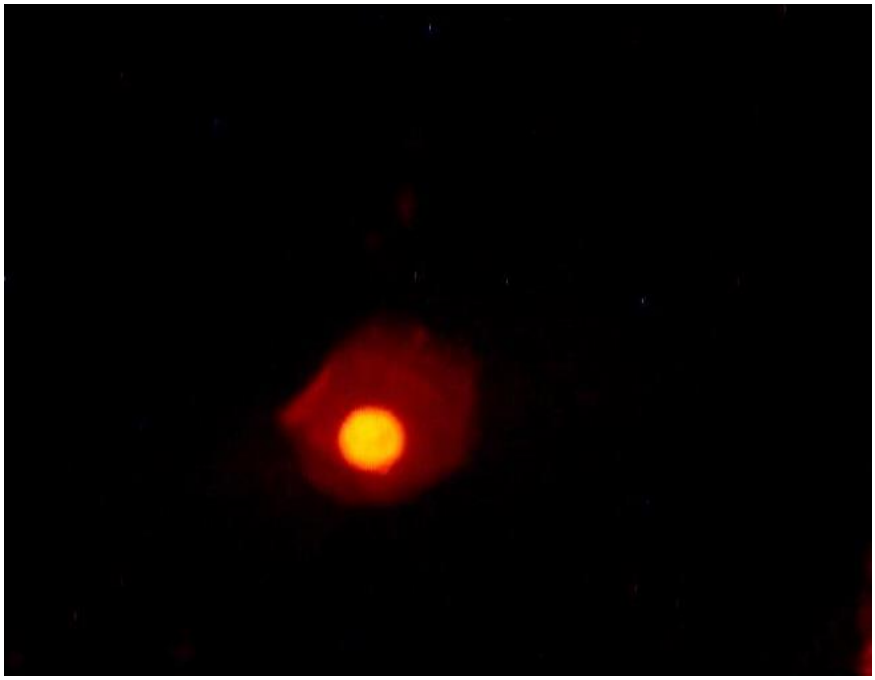
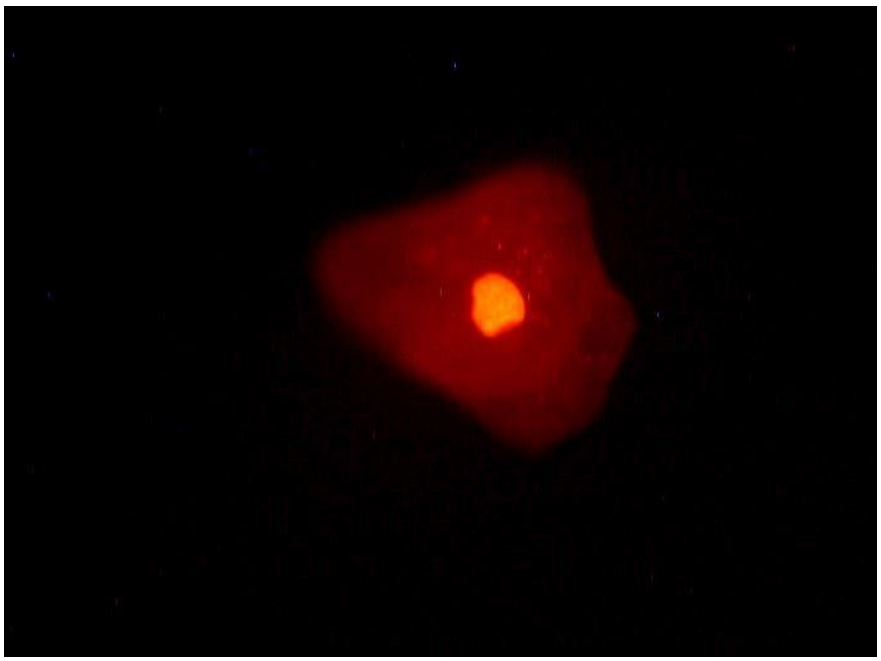
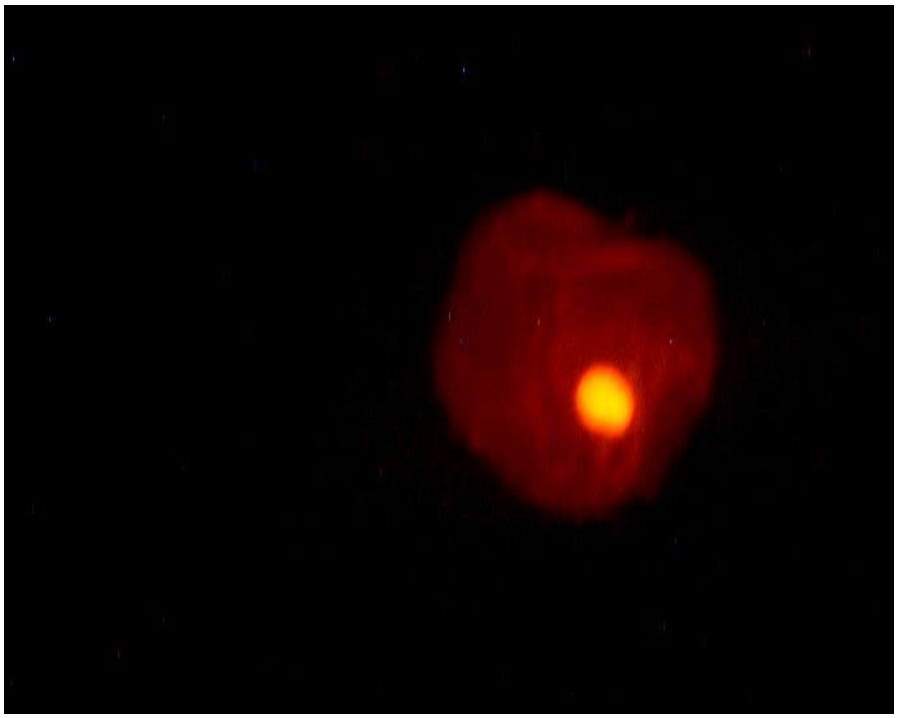


Figure 22: Microscopy of Control Patients (Group II)









DISCUSSION

DISCUSSION

Oral sub mucous fibrosis (OSF), is a potentially malignant disorder that is associated with the chewing of betel quid and areca nut. This disorder is more common in the population of Indian subcontinent and South East Asia. Malignant transformation of oral submucous fibrosis to oral squamous cell carcinoma has been estimated to be between 2-8%.[33] There is sufficient number of evidence to show that habitual gutkha and betel quid are associated with the occurrence of several oral mucosal disorders, including oral submucous fibrosis and oral cancer.[56]

The genotoxicity and mutagenicity of areca alkaloids has been detected by many assays. The reactive oxygen species produced during the auto-oxidation of areca nut polyphenols in the saliva of the chewers are crucial for the initiation and promotion of oral cancer. Several nitrosamines formed from areca nut have been demonstrated to be mutagenic and genotoxic and are capable of inducing tumours in the experimental animals.[5] Thus the constituents of areca nut are known to cause DNA damage and hence play a role in the malignant transformation of oral submucous fibrosis.

Comet assay has been known to be a versatile and simple method for the detection of DNA damage. It is a rapid, visual and sensitive method to assess DNA damage and repair.[7] On reviewing the literature few studies have been done in buccal cells and there are only few studies found to be reported that assessed the DNA damage using comet assay in the buccal cells of oral submucous fibrosis patients. Hence the aim of our study was to evaluate the DNA damage in the buccal cells of patients with oral submucous fibrosis using comet assay. The investigation was done

following sample collection, agarose embedding, lysis step, DNA unwinding, electrophoresis, neutralization, staining and analysis.

In the present study, patients who were clinically diagnosed with oral submucous fibrosis belonging to all clinical grades were included. The age matched control group of 30 individuals without any habit and without any oral lesion or systemic conditions was taken. Comet assay procedure was done and the mean tail length of patients with oral submucous fibrosis was found to be 2.25µm which was comparatively higher than the control group where the mean tail length was found to be 1.35 µm.

Our results correlated with a similar study (Udupa et al) that was done in the buccal epithelial cells of oral submucous fibrosis patients where the submucous fibrosis patients had a higher mean tail length when compared to the healthy control group. They also analysed the mean tail lengths between the different clinical grades and found no statistically significant difference between their tail lengths. They postulated that the insignificant results between the different clinical grades might be because of the uneven distribution of sample size and also once the disease develops there will be formation of tail length irrespective of the grading. [56]

The mean tail length of the control subjects with no habit, no oral lesion and no systemic conditions was found to be 1.35 µm in our study. This shows that even in the absence of any habit the cells undergo some sort of genetic damage that could be attributed to various environmental, occupational and age related factors. [43] Thus an antioxidant-rich environment may help in providing the optimal conditions needed for maintaining the integrity of DNA and thus prevent DNA damage.

The mean tail length of betel quid chewers and betel quid chewers with the habit of smoking tobacco was compared. The number of patients with only the habit of betel quid chewing was 25 and the rest had both smoking and betel quid chewing habit. Though we could not find any statistically significant difference between the two groups those individuals who had both the habits had a mean tail length of 2.4 µm which was slightly higher than those with only betel quid chewing habit who had a mean tail length of 2.2 µm. A case control study that was done to assess the DNA damage in the buccal cells of submucous fibrosis patients and leukoplakia patients found no significant change in tail length between chewers and smokers with chewers similar to our study.[60]

In relation to the duration of betel quid habit, there is statistically significant increase in the mean tail length of submucous fibrosis patients with the increase in duration of the habit. This significant increase is attributed to the fact that the longer the contact of the areca alkaloids with the oral mucosa, the greater will be the tail length and these results concur with the previous case control study done on the buccal epithelial cells of oral submucous fibrosis patients and leukoplakia patients.[60]

On comparing the mean tail length between males and females in both the study group and control group, a higher mean tail length was found in males compared to females. In the study group males had a average tail length of 2.29 µm whereas females had a mean tail length of 1.98 µm. On the other hand the mean tail length of males in the control group was 1.39 µm and females was 1.08 µm. This could be because of the skewed distribution of males and females subjects in our investigation.

The gingival and periodontal status of patients with oral submucous fibrosis and their genetic damage from gingival epithelial cells was assessed using comet assay. Their results showed that the periodontal status of the patients with oral submucous fibrosis was poor and the gingival epithelial cells of these patients showed a significantly higher tail length when compared to the control group. Just as in our study their results also showed a higher average tail length in the oral submucous fibrosis patients.[54]

Comet assay was done in the blood lymphocytes of patients with oral precancer and oral cancer in Kolkata. Four groups were taken which included 26 oral cancer patients, 12 leukoplakia partients, 6 oral submucous fibrosis patients and 10 healthy normal volunteers as the control group. It was found that the oral cancer patients had a higher mean tail length when compared to the rest of the groups. Among the patients with precancer, leukoplakia patients had a higher tail length when compared to oral submucous fibrosis and the latter had a higher mean tail length than the control group.[52] In our study also the oral submucous fibrosis patients had a higher mean tail length than the control group.

In assessing the DNA damage of the different stages of squamous cell carcinoma, the mean DNA damage of the control group was found to be 0.8616µm, where as in our study it was 1.35 µm. In their study the mean tail length of the different grades of squamous cells carcinoma was found to be 3.874µm and the mean tail length found in stage I was 2.3 µm which is similar to the results of our study where the mean tail length of oral submucous fibrosis patients is 2.25 µm.[61] This shows that the level of genetic damage in oral submucous fibrosis is equal to the genetic damage that is seen in the stage I cancer.

The IARC monograph 1998 on evaluation of carcinogenic risks to humans has identified areca nut to be a "group one carcinogen." The mutagenic potential of arecoline in areca nut is enhanced significantly by several other constituents of betel nut and the presence of other factors like the presence of alchohol or the extent of alkalinity/acidity. Areca nut is one of the major risk factors for the development of oral submucous fibrosis. Since OSF has the potential for malignant transformation, early detection at the cellular level will help to identify the individuals who are at high-risk of developing oral cancer.[56] Comet assay in the buccal cells of OSF patients will can aid in the identification of these changes at an earlier stage.

The limitation of this study is that there is a chance for subjective bias in measuring the tail length of the comet using the software. The tail lengths recorded were noted by a single observer which can lead to bias in the variation of mean tail length. The sample size was not sufficient enough to find an exact correlation between the DNA damage and other contributing factors like type of habit, duration of habit and the frequency of usage of the various. In addition to the above mentioned drawbacks, this research also suffers from the inherent drawbacks of cross sectional observational study.

The future research may be carried out in a prospective manner using comet assay in a larger sample of the study population with a well matched cases and control group, so as to get more clarity than the confusion to identify the base-level DNA damage in the initial stages of carcinogenesis. The comet assay is considered to be a fast and suitable test for assessing the DNA-damaging potential in biomonitoring studies. In addition to its applications in biomonitoring and genotoxicity, comet assay can be used as a potential tool in the assessment of DNA damage in the margins of

resections of cancer/precancer. They may also be helpful in the determination of success of intervention in these cases. The use of comet assay in other potential situations may lead to further advancement in our understanding of the biology of oral cancer and precancer for developing possible worthy early detection tests. [52]

SUMMARY &CONCLUSION

SUMMARY AND CONCLUSION

Potentially malignant disorders include a wide range of lesions that pave way to the development of oral cancer. Oral submucous fibrosis is one of the potentially malignant disorders that has a malignant transformation rate of 2%-8%. The occurrence of oral submucous fibrosis in developing countries like India has increased in the recent years because of the increase in the habit of using areca nut either alone or with tobacco. Once malignant transformation has occurred it leads to severe morbidity and mortality. Hence early detection of the genetic level changes in OSF that progresses to malignant transformation can help in preventing oral cancer.

The aim of our study was to evaluate the DNA damage in the buccal cells of patients with oral submucous fibrosis using the versatile technique comet assay. In our study a minimal level of tail length was seen in the control group without any habit or without any lesion. Our findings suggested that there was an increased level of DNA damage in the OSF group when compared to the control group. It also showed a significant increase in the tail length with respect to the duration of the habit implying that the longer the contact of the areca alkaloids in the patients mouth the greater is the DNA damage. The incidental findings of the present study imply that a much larger sample is needed to confirm the association between factors like age, gender and different types of habits. A prospective study should be carried out to find a better correlation among the above mentioned factors**.**

The lack of public awareness about the signs, symptoms and risk factors of these disorders is believed to be responsible for the diagnostic delay and treatment initiation of oral cancer. Hence investigations like comet assay can be used as powerful screening tools to create awareness among the public about the deleterious effects of harmful habits on the oral mucosa and thereby aid in the prevention of oral cancer.

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ANNEXURE

INSTITUTIONAL ETHICS COMMITTEE
VIVEKANANDHA DENTAL COLLEGE FOR WOMEN

SPONSORED BY : ANGAMMAL EDUCATIONAL TRUST

Ethics Committee Registration No. ECR/784/Inv/TN/2015 issued under Rule 122 DD of the Drugs & Cometics Rule 1945.

No:VDCW/IEC/ 30 /2015 Date: 05.11.2016

TO WHOMSOEVER IT MAY CONCERN

Principal Investigator: Dr. K. Rachel Sarah Vinodhini

Title: Evaluation of DNA damage in the buccal cells of patients with Oral Submucous Fibrosis using comet assay.

Institutional ethics committee thank you for your submission for approval of above proposal. It has been taken for discussion in the meeting held on 20.10.16. The committee approves the project and it has no objection on the study being carried out in Vivekanandha Dental College for Women.

You are requested to submit the final report on completion of project. Any case of adverse reaction should be informed to the institutional ethics committee and action will be taken thereafter.

CHAIRMAN
INSTITUTIONAL ETHICS COMMITTEE
VIVEKANANDHA
DENTAL COLLEGE FOR WOMEN
Elayampalayam-637 205.
Tiruchengode (Tk) Namakkal (Dt),
Tamilnadu.

SECRETARY
INSTITUTIONAL ETHICS COMMITTEE
VIVEKANANDHA
DENTAL COLLEGE FOR WOMEN
Elayampalayam-637 205.
Tiruchengode (Tk) Namakkal (Dt),
Tamilnadu.

VIVEKANANDHA DENTAL COLLEGE FOR WOMEN

DEPARTMENT OF ORAL PATHOLOGY AND MICROBIOLOGY

TIRUCHENGODE-637 205

INFORMED CONSENT FORM

Investigator: Dr. K. Rachel Sarah Vinodhini **Guide: Dr.T.Maheswaran**

TITLE: Evaluation of DNA damage in the buccal cells of patients with oral submucoys fibrosis using comet assay.

NAME: Mr/Ms/Mrs. SEX : Male / Female

Address: AGE : Yrs

I, _____, give my consent voluntarily to participate as a participant in this study. I agree to the following:

1. I have been informed to my satisfaction about the purpose of the study and study procedures.

2. I understand that the study involves questions which may sometimes be personal.

3. I agree to co-operate fully for complete examination.

4. I am told that the investigating doctor and the institution will keep my identity confidential.

5. I understand that I have rights to withdraw myself from the study and also that the investigator has the right to exclude me from the research at any point of time.

Name Signature/ Thumb impression of Participant/Parent/Guardian Date:

Investigator Signature of investigator Date:

விவேகானந்தா மகளிர் பல் மருத்துவ கல்லூரி

வாய்வழி நோயியல் மற்றும் நுண்ணுயிரியல் மருத்துவத்துறை

திருச்செங்கோடு - 637 205.

ஒப்புதல் படிவம்

ஆய்வாளர் : மரு. கி. ரேச்சல் சாரா வினோதினி **ஆய்வு நெறியாளர் :** மரு.த.மகேஸ்வரன்

தலைப்பு : OSMF கொண்ட நோயாளிகளின் வாய் செல்களில் டிஎன்ஏ சேதம் மதிப்பீடு.

பெயர் : திரு/திருமதி இனம் : ஆண்/பெண்

முகவரி : வயது :

நான் _____ என்னுடைய சுய நினைவுடனும் மற்றும் முழு சுதந்திரத்திடனும் நான் இந்த பல் மருத்துவ பரிசோதனையில் சேர்ந்து கொள்ள ஒப்புதல் அளிக்கிறேன்.

1. எனக்கு இந்த பரிசோதனை பற்றிய முழு தகவலும் அளிக்கப்பட்டுள்ளது.
2. இந்தப் பரிசோதனையில் கேட்கப்பட உள்ள கேள்விகள் என் சுயம் சார்ந்ததாக இருக்கலாம் என்பது எனக்கு முன் அறிவிக்கப்பட்டுள்ளது.
3. இந்த ஆய்வில் செய்யப்படும் பரிசோதனைகளுக்கு முழு ஒத்துழைப்பு அளிப்பேன்.
4. என்னைப் பற்றிய விவரங்களை பரிசோதனை செய்யும் மருத்துவரோ அல்லது மருத்துவமனையோ வெளியிடாது என்று உறுதியளிக்கப்பட்டுள்ளது.
5. இந்தப் பரிசோதனையிலிருந்து என்னை நானோ அல்லது மருத்துவரோ எப்பொழுது வேண்டுமானாலும் விடுவித்துக் கொள்ளலாம் என்பது தெரிவிக்கப்பட்டுள்ளது.

பெயர் கையொப்பம் / கைவிரல் ரேகை தேதி

பங்கேற்பாளர்

ஆய்வாளர் கையொப்பம் தேதி