CYTOLOGICAL CHANGES AND SPECIFIC SALIVARY BACTERIAL ASSESSMENT IN ORAL SQUAMOUS CELL CARCINOMA PATIENTS UNDERGOING RADIATION THERAPY

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INTRODUCTION

Oral cancer is one of the most common cancers in the world and a major cause of morbidity and mortality. The incidence of oral cancer is said to be greatest in the developing countries, with squamous cell carcinoma being the most common histological type.

Radiotherapy is frequently used as a standard treatment for carcinoma of the oral cavity either alone or in combination with surgery.**(Zheng JW et al 2008)** The reaction of tumor tissue, as well as the surrounding oral tissues, to radiotherapy depends on their growth rate and inherent susceptibility to radiation, which often shows a marked inter-individual variability**(V Raj et 2011)**. Hence, assessing and predicting the outcome of treatment for individual patients becomes difficult. Estimation of radiosensitivity will be useful in planning the optimum radiation schedule for each patient.

Tests to predict the response of tumor to radiotherapy in an individual patient have been made in past and are still being pursued. Biopsies from the lesional site in order to predict response to radiotherapy were used initially. Later Graham found exfoliative cytology to be useful in assessing radiation related changes and to predict the response to treatment in cervical cancer patients **(Graham RM 1947).** Graham's radiation response (RR) test, though reproduced by some later studies, was not universally accepted as an indicator for treatment outcome.**(Silverman S Jr, 1967)**

Nucleoprotein patterns of irradiated malignant exfoliated cells as well as persistence of malignant cells in smears after completion of therapy were also proposed to correlate with clinical outcome.**(Silverman S Jr et al, 1967)** Also, radiation induced acute nuclear changes as an assay to assess radiosensitivity.**(Bhattathiri NV et al, 1998)**

Exfoliative cytology is a simple and noninvasive technique and the smear obtained can be analyzed quantitatively and qualitatively.Qualitative assessment of the exfoliated cells has lost its reputation as a result of a large number of false negative results arising due to the ill-defined diagnostic criteria.**(Einstein TB, 2005)** In the past exfoliative cytology has not gained wide acceptance due to problems such as inadequate samples, technical errors and the incorrect interpretation of findings. Presently with advanced imaging techniques, computerized systems and the use of quantitative techniques to verify the reliability of cytomorphometric analysis, this method is achieving credit once again.**(Goregen M et al, 2011)**.

In exfoliative cytology, the quantitative parameters are objective and reproducible. One such quantitative parameter is morphometry.**(Hande AH et al, 2010)** Cytomorphological parameters such as cellular diameter (CD), nuclear diameter (ND), nuclear area (NA), cytoplasmic area, NA/CA ratio, nuclear shape, nuclear membrane continuity, optical density and nuclear texture have shown meaningful results in the diagnosis of oral lesions,especially NA and NA/CA ratio. **(Goregen M et al, 2011)**. The variations obtained in these parameters have been attributed to exposure to carcinogenic agents like tobacco through their end-products.**(Hande AH, 2010)** There is little mention in the literature about quantitative cytologic assessment of the effects of radiation therapy upon normal oral mucosa.

The morphology of the exfoliated cells depends on the nature of alterations taking place in the epithelial layer. Alterations in cytologic pattern may be attributed to changes occurring in the epithelial layer. **(Einstein TB et al, 2005)**

. The present study utilized exfoliative cytology and image analysis for scrutinizing the exfoliated keratinocytes for the cellular changes induced by radiation therapy in oral squamous cell carcinoma patients to estimate the NA, CA, ND and CD and to quantify the changes in the above variables and to explore the possibility of utilizing them as an assay to predict tumor response to radiotherapy.

Several discoveries in microbiological literature since 19th century have led its way to suggest that bacteria were implicated in all studies, and hence, the theory of bacterial infection leading tooral cancer was born. Various epidemiological and laboratory based studies have shown number of bacterial species to be associated with different cancers. Few such propositions that gained widespread interest were the associations seen between Salmonella typhi and gall bladder cancer, Chlamydophila pneumoniae and lung cancer, Streptococcus bovis and colorectal cancer, E. coli, crohn's disease and colon cancer **(Chocolatewala N et al, 2007).**

The pathogenesis of carcinogenesis due to bacteria can be attributed to chronic inflammatory mechanisms and bacterial toxins.

There are various proposed mechanisms for bacterial role in carcinogenesis. Chronic infections by several bacteria or production of toxins disturb the cell cycle and lead to alteration in cell growth**. (Koyi H et al, 2001).**

Chronic infections can trigger increased rate of genetic mutation which induce cell proliferation and DNA replication through activation of mitogen activated kinase (MAPK) pathways and cyclin D1 thereby increasing the rate of tumor development **(Parsonnet J, 1995)**. Suppression of apoptosis by modulated expression of Bcl-2 family proteins or by inactivation of retinoblastoma protein, pRb.**(Lara-Tejero M,2000).**

Host cell signaling pathways may be altered by intracellular organism thereby enhancing its survival inside the cell**.(Lax AJ,2005).**

Another possible mechanism is the metabolism of potentially carcinogenic substances by the bacteria. Conversion of ethanol to its carcinogenic derivative acetaldehyde can facilitate tumorigenesis by increased DNA damage **(Salaspuro MP, 2003).** This is supported by increased levels of microbial acetaldehyde production in heavy drinkers and smokers. Nitroso compounds formed by bacteria can cause carcinoma of oesophagus and other mucosal areas like oral cavity.**(Lijinsky W, 1982)**

Studies have shown that DNA counts of Capnocytophaga gingivalis, Prevotella melaninogenica, and Streptococcus mitis were significantly higher in the saliva of oral cancer patients compared to normal healthy individuals..

In the other part of this study, these organisms were cultured from the saliva of oral cancer patients before receiving radiotherapy, and during radiotherapy and the counts compared to analyse the effect of radiotherapy on the count of these organisms in their saliva. The counts at various stages were also compared to those in normal healthy individuals to analyse the response to therapy.

AIM AND OBJECTIVES

AIM OF THE STUDY

To assess the cytological changes and specific salivary bacteria in histopathologically proven oral squamous cell carcinoma patients undergoing radiation therapy.

OBJECTIVES OF STUDY

- To assess if there are significant variations in the cytological measurements like nuclear area (NA), cytoplasmic area(CA) , nuclear diameter(ND), cell diameter(CD) and nuclear cytoplasmic ratio(N:C), in the exfoliated oral mucosal cells at various stages of radiotherapy in oral squamous cell carcinoma patients
- To assess if there are significant variations at various stages of radiotherapy in oral squamous cell carcinoma patients compared to cells from normal healthy individuals.
- To compare specific bacterial species - Capnocytophaga gingivalis, Prevotella melaninogenica, Streptococcus mitis in the saliva of oral squamous cell carcinoma patients before and during various stages of radiation therapy
- To compare the counts of these organisms at various stages of radiotherapy with those of normal healthy individuals

REVIEW OF LITERATURE

EXFOLIATIVE CYTOLOGY

Exfoliative cytology is the study of superficial cells shed from mucous membranes and the continuous exfoliation is a part of physiological turnover. There are two types of exfoliation from which sampling of cells can be done. They are (i) Natural spontaneous exfoliation in which cells from sites containing naturally exfoliated cells like saliva, urine specimen and vaginal pool secretions can be studied for normal changes of aging and pathological changes and (ii) Artificial or abrasive cytology in which cells are forcibly removed from mucosal surfaces before their natural time of shedding. These cells are usually smaller and less mature than the naturally desquamated cells. (**Naib Z.M & Willis D, 1985**)

HISTORY OF EXFOLIATIVE CYTOLOGY

Lionel Beale in **1860** identified and described cancer cells in sputum and published an article on Cyotological Examination of Sputum in a Case of pharyngeal carcinoma.

George N. Papanicolaou a Greek anatomist and pioneer in cytopathology introduced PAP staining procedures in **1942** for cyotological smears and along with Hebert Traut in 1943, used cytological smears in diagnosis in a case of uterine cancer.

In **1949, Morrison LF** described cytomorphology of malignant cells in a case of oropharygeal carcinoma.

ORAL EXFOLIATIVE CYTOLOGY

Ziskin (1940) first used exfoliative cytology in the oral cavity.

In **1951, Paul W Montgomery** used exfoliative cytology to study the normal features of the oral epithelial mucosa.

Raymond et al in **1961** studied exfoliated cells of the tongue in cases of severe iron deficiency anemia and concluded that markedly less cornified and keratinized population of epithelial cells in those cases.

Green Berg et al in **1981** obtained cells from the saliva and also smears from buccal mucosa in patients with pernicious anemia.

In **1986, Barret et al** showed that exfoliative cytology was 95% reliable and can be used to confirm herpes simplex infections in immunocompromised patients and also graded the cytopathological features.

A review of oral exfoliative cytology done by **Sugarman P et al in 1996** concluded that exfoliative cytology is a useful adjunct to conventional biopsy to assess dysplastic changes within the oral epithelium.

Ogden GR et al (1997) reviewed methods of assessment by oral cytology and indicated exfoliative cytology to be an important adjunct in assessing patients with potentially cancerous lesions.

D. Maraki et al (2004) evaluated the diagnostic accuracy of exfoliative cytology and DNA image cytometry and concluded that both together constituted sensitive, specific and non invasive method to diagnose oral epithelial neoplasia.

Ravi Mehrothra et al in 2006 reviewed the recent advances in cytological techniques and stated that oral cancers can be diagnosed much earlier using diagnostic techniques such as cytomorphometry, DNA cytometry and molecular analysis.

CYTOMORPHOMETRY

The term computerized nuclear morphometry was introduced by **Bjorn Stenkvist et al in 1978**. He showed that the multivariate numerical score that correlate well with nuclear atypia and had greater reproducibility when compared to conventional histological preparation.

Cowpe JG et al in 1985 measured nuclear and cell size in normal smears taken from five different sites(soft palate, buccal mucosa, lower lip, tongue and gingival) in the oral cavity in an attempt to make a baseline which might help in comparison of similar measurements from pathological smears. They showed that significant variations in nuclear and cytoplasmic area were displayed between different sites.

Ogden GR et al in 1991 studied the effect of smoking on oral mucosa by quantitative cytomorphometric analysis of buccal smears. He measured nuclear and cytoplasmic areas of cells using a Vids V semi-automatic image analysis system and found that there was significant elevation in nuclear area in smokers compared to normal group.

Ramaesh et al in 1999 studied the effect of betel chewing with tobacco on buccal mucosal cells using cytomorphometry and studied cell diameter and nuclear diameter of cells from dysplastic lesions and normal mucosa of healthy subjects.

They found that there was increase in nuclear diameter and decrease in cell diameter in cells from dysplastic lesions compared to normal. They concluded that tobacco influenced the cells and the changes in nuclear diameter was marked.

Anuradha et al in 2007 studied exfoliated gingival cells to evaluate nuclear diameter, cytoplasmic diameter and nuclear cytoplasmic ratio in normal healthy individuals of different age and sex. They showed that there were age related and sex related alterations in gingival smears.

CYTOMORPHOMETRY AND ORAL CANCER

Jonathan G Cowpe, in 1984 carried out DNA cytophotometry and cytomorphology of normal and abnormal oral mucosal squames. He indicated that the use of these techniques may improve the diagnostic reliability of exfoliative cytology in the management of oral malignancy.

JG Cowpe, RB Longmore and MW Green in 1988 studied smears collected from suspicious lesions in buccal mucosa and floor of the mouth and showed significant variation in nuclear area and cytoplasmic area compared to smears taken from healthy sites in the same patients. Thus they attempted at improving the diagnostic sensitivity of cytology in the detection of early oral malignancy and they concluded that quantitative cytology could be of great value for monitoring and follow-up of suspicious lesions and can be an excellent additional diagnostic test to detect early oral malignancy.

T. Ramaesh et al in 1999 found that cytomorphometric analysis of smears from buccal lesions (leukoplakia and squamous cell carcinoma) was useful in differentiating dysplastic and malignant squamous cells from normal squames using discriminant analysis based on ND and CD values. A sensitivity of 89% and a specificity of 89.7% were reported by them.

Khandelwal et al in 2010 analysed the cytomorphological features of keratinocytes in smears obtained from the oral mucosa of tobacco users and from oral squamous cell carcinoma lesions. They found significant reduction in the cellular area of keratinocytes from oral squamous cell carcinoma lesions when compared with those from oral smears of tobacco users and concluded that cytomorphometric analysis of keratinocytes can serve as a useful adjunct in the early diagnosis of oral squamous cell carcinomas.

Veda Hegde in 2011 measured the cell and nuclear diameter from the squames of cases with oral leukoplakia, oral submucous fibrosis, oral lichen planus which showed no dysplastic changes histologically and oral squamous cell carcinoma. The nuclear to cytoplasmic ratio in each of these were deduced from the values obtained and compared with the controls which were age, sex and site matched. It was concluded from the findings of the study that a decrease in the mean cytoplasmic diameter of exfoliated buccal mucosal cells could serve as an early indicator of dysplastic change especially in lesions which appear histologically benign.

In 2011, Smitha T, Sharada P and Girish HC studied and compared the changes in nuclear and cellular size, shape and nuclear–cytoplasmic ratio of the cells in the basal layer of oral leukoplakia and well-differentiated oral squamous cell carcinoma (SCC) with normal buccal mucosa, using computer-aided image analysis in tissue sections. From the results obtained, they concluded that, of the morphometric parameters studied, size was useful to differentiate between normal, potentially malignant leukoplakia and SCC.

HG Ahmed et al in 2011 conducted a study to find a better way for predicting the cellular proliferative activity of apparently healthy oral epithelium exposed to certain carcinogens. The nuclear area (NA) and nucleolar organizer regions (NORs) counts were compared with that of cytological atypia in 100 cases of epithelia exposed to toombak (carcinogen), 100 controls (nonexposed) and two cases of squamous cell carcinoma (SCC), as internal controls. Significant differences in AgNOR mean count and NA mean values were identified between cases and controls. Significant differences were also noted in AgNOR mean count and NA mean values between cases and two cases of SCC and hence it was concluded that AgNOR mean count and NA are useful markers for prediction of cytologically nonevident proliferative activity of oral mucosa exposed to carcinogens.

Joshi et al in 2013 carried out a study to analyze the cytomorphometric features of cells obtained by cytobrush and stained with Feulgen stain from oral premalignant and malignant lesions. They found that there was increase in nuclear diameter, nuclear area, nuclear cytoplasmic ratio and decrease in cell diameter, cytoplasmic area in lesions

compared to normal healthy individuals. Hence they concluded that morphometry could be used to detect dysplasia and malignancy in their early stages.

K.Kuyama et al (2013) conducted a study to compare among dysplasia of oral, uterine cervix and bronchus. It was a retrospective review of smears of cases diagnosed with dysplasia of oral, bronchial and uterine cervix, from 2002 to 2010. The results displayed a significant variation in cytomorphometrical values among the 3 regions. N/C values for uterine cervix and bronchus were well distinguished in comparison with oral dysplasias.

A.Nadaf et al in 2013 conducted a phase contrast cytomorphometric study of wet and unstained smears containing squames from normal oral mucosa and oral leukoplakia. They found significant increase in mean nuclear diameter and decrease in mean cellular diameter between the two groups. They concluded that cytomorphometry using phase contrast microscopy can be a better diagnostic tool as it was quicker and cost-effective.

Shaila et al in 2014, published an original study in which they analyzed the cytomorphological features of keratinocytes in smears obtained from the oral mucosa of oral squamous cell carcinoma (OSCC) lesions and normal controls using oral rub and rinse technique. Quantitative assessment of nuclear diameter (ND), cytoplasmic diameter (CD), cellular area (CA), nuclear area (NA), and nuclear cytoplasmic ratio(N:C) were carried out.

They found that there was increase in the mean ND, NA, and N: C; and decrease in CA and CD of cancer subjects when compared to that of normal controls.

Jagannathan et al in 2014 reviewed exfoliative cytology as a predictive diagnostic tool. Though conventional cytology had limited sensitivity and specificity, the authors noted that improved accuracy was obtained by combining cytology with computer -assisted morphometry. This method is also suggested as a regular chair monitoring tool in patients who need a routine follow up to diagnose any changes in the early stages of development of malignancy.

CYTOMORPHOLOGY AND RADIOTHERAPY

S Silverman Jr. et al in 1967 compared the cytologic radiation changes and clinical tumor responses in 84 patients with oral squamous carcinoma treated with ionizing radiation. These patients were observed during therapy and for 1 to 7 years following therapy. Cellular changes noted in response to radiation were cytoplasmic vacuolization, cell enlargement, multinucleation and nuclear alterations. The percentage of each specific cellular morphologic change was also calculated. It was concluded that cell enlargement and multinucleation, indicative of radiation response occurred within two to three weeks from the beginning of treatment. But the changes were found to be static after 3 weeks. The radiation response indices after radiotherapy were, however, found to be highly increased and also persistent for long periods.

G R Ogden, J G Cowpe and M W Green in 1988 investigated the effect of radiotherapy on normal buccal mucosa were using the quantitative techniques of cytomorphology (measurement of nuclear and cytoplasmic area) and DNA cytophotometry. These techniques were applied to smears obtained before, during, and after irradiation.

They found that Nuclear area and cytoplasmic area increased and DNA values were abnormal in most cases as a result of radiotherapy, returning to within normal limits one month after treatment. This was said to contrast strongly with the changes seen in smears from previously irradiated uterine cervices, where changes in cytomorphology were seen to persist for several years.

In 1998, NV Bhattathiri et al tried to identify the relationship between the radiosensitivity of oral cancers and the induction of micronucleation, nuclear budding and multinucleation (polynucleation) by serial cytological evaluation during fractionated radiotherapy. Serial scrape smears were taken from the tumor site (44 patients with oral epidermoid carcinoma) before and during radiotherapy and stained by Giemsa and the frequency of micronucleated cells, nuclear budded cells and multinucleated cells were evaluated by light microscopy. After a minimum follow-up period of 30 months the patients were classified as having resistant or sensitive tumours, depending on whether the primary tumour had recurred or not within that time. It was concluded from their observations that serial cytological assay of nuclear changes (SCANCing) during radiotherapy is a potentially useful test to predict radiosensitivity. Also, as multinucleation showed the greatest relation with radiosensitivity, injury to the cytokinetic apparatus was considered important in determining tumour radiosensitivity.

In 2005, P Sharma et al did a a quantitative analysis of radiation-associated cellular changes in oral cancer and their correlations with histologic grade and clinical stage. According to their findings, cellular changes did not show a relationship with clinical stage either before or after irradiation. However, they demonstrated a partial pretherapy correlation with histological findings.

This relationship was observed to diminish progressively with increasing radiation dose. Micronucleation and nuclear budding remained significantly different between histologic grades even after 24 Gy of irradiation this was considered clinically practicable alternatives to biopsy at this stage and useful in further studies on cytologic prognostication of irradiated oral cancer.

R Kumari et al in 2005 evaluate the dose-dependent relationship of nuclear abnormalities by serial cytology during fractionated radiotherapy in head and neck cancer patients. Serial scrape smears were taken from the tumor before and during radiotherapy (0 to 24 Gy), and stained with Giemsa and May Grunwald's stain. The frequency of micronucleated, binucleated and multinucleated cells was evaluated with the help of light microscope and the counts were expressed per 1000 uninucleated cells. Their study results revealed that micronucleus assay is a very useful tool in the assessment of biological damage that predicts tumor radiosensitivity.

HG Ahmed et al in 2008 assessed the oral cytological changes associated with exposure to chemotherapy and/or radiotherapy in 100 patients with cancers of different regions(head and neck cancers -27, breast cancer -30, hematolymphoid malignancies-13, cervical cancer -10, prostatic cancer-10, and the remaining 10 patients had tumors with unknown primary). The aim of this study was to investigate the feasibility of using cytological evaluation to detect oral epithelial atypia amongst cancer patients. Pap stained oral smears were assessed for cellular atypia. When examining the association between the number of radiotherapy cycles and the degree of epithelial atypia, it was found that high proportions of severe epithelial atypia were observed among those exposed to a higher number of radiotherapy cycles.

BM Biswal et al in 2010 conducted a preliminary study on 26 head and neck squamous cell carcinoma patients to predict radiation response before completion of the radiation therapy schedule. Intra-radiotherapy nuclear morphometry combined with AgNOR were done on cells obtained by fine needle aspiration. From their observations the authors concluded that morphometry and AgNOR were simple and useful tools for the prediction of radiation response in head and neck cancers.

V Raj and H Mahajan in 2010, performed a study in order to establish the relationship between various nuclear changes and radiation dose and to explore the possibility of utilizing them as an assay to predict tumor response to radiotherapy. Acridine orange fluorescent dye and May-Grunwald Giemsa stain were used to assess nuclear abnormalities like micronucleation, nuclear budding, binucleation, and multinucleation and these were then correlated with tumor response. It was concluded that a direct dose-response relationship existed between the frequencies of various nuclear abnormalities and radiation in oral squamous cell carcinoma patients undergoing fractionated radiotherapy.

D.Agarwal et al in 2011 carried out a prospective, non-randomized study to assess the role of serial cytological assay in predicting radiosensitivity of squamous cell cancer of oral cavity in patients on fractionated radiotherapy (RT) and to evaluate the relationship of radiosensitivity with the histological grade of oral cancer. They observed changes such as multinucleation, micronucleation, karyorrhexis and cytoplasmic vacuolation occurring in irradiated cancer cells, with a statistically significant dose related increase in these changes.

Less differentiated tumors were seen to be less radiosensitive and exhibited increased rate of persistence of dysplastic cells and a higher rate of recurrence (33%) after completion of radiotherapy as compared to well differentiated tumors. The authors also recommended regular use of serial cytological assay as it was shown to provide valuable evidence of radiosensitivity and persistence of tumor/dysplastic cells at 8 weeks post-radiotherapy.

MICROORGANISMS AND MALIGNANCIES

The hypothesis that certain bacteria are capable of causing cancer is supported by studies of animal-specific pathogens that promote tumor formation like Helicobacter hepaticus which was discovered in 1992 as a cause of chronic active hepatitis that progressed to hepatocellular carcinoma in mice(**Ward, et al., 1994**).

D'Costa J et al in **1998** and later **Tran N et al** in 2007 showed with well documented evidences that viruses like Human Papilloma Virus(HPV) and Epstein Barr virus play a role in carcinogenesis by abrogation of p53 and pRb tumor suppressor genes and other cellular proteins with subsequent alteration in host genome function.

Dutta U et al and **Shukla VK** in **2000**, showed that typhoid carriers were at an increased risk of developing carcinoma of gall bladder thus suggesting Salmonella typhi in its etiology.

Newman, et al in 2001 proved that chronic infection with *Citrobacter rodentium*, a mouse pathogen that is genetically similar to enteropathogenic *Escherichia coli*, can result in colon cancer.

Chlamydia pneumoniae infection has been shown to be associated with increased risk lung cancer as shown in studies done by et **Littman AJ et al** and **Koyi H et al** in 2004 and 2001 respectively.

According to **Crowe SE(2005)** and **Montalban C et al(2001)**, Helicobacter pylori infection, known to cause stomach ulcers, has been proven to subsequently lead to gastric carcinomas and Mucosa Associated Lymphoid Tissue (MALT) Lymphoma.

Biarc J et al and **Gold JS et al** in 2004 proved that Streptococcus bovis mediated bacteremia linked with colonic and extracolonic malignancies.

Dehio in 2005 also observed a link between infection with Bartonella species and vascular tumor formation.

Also, **Rao, et al** in 2006 and 2007 showed H. hepaticus to promote cancer formation in the mammary gland of mice by innate immune inflammatory response.

Wagenlehner et al in 2007 showed a link between infection of the prostate and prostatic carcinoma as PCR analysis of prostate tissue of prostate carcinoma patients showed sequences of the organisms Mycoplasma geniculatum and Chlamydia trachomatis.

Microbes induce an estimated 20% of all the fatal cancers in human beings, as suggested by **Blaser in 2008.**

It has been estimated by **Martin D** and **Gutkind GH** in 2008 that at least six human viruses, EBV, hepatitis B virus (HBV), hepatitis C virus (HCV), HPV, human T-cell lymphotropic virus (HTLV-1) and Kaposi's associated sarcoma virus (KSHV) contribute to 10–15% of the cancers worldwide.

Rosa DD et al in 2008 have presented cases where chronic mucocutaneous Candida infection was associated with esophageal cancer.

BACTERIA AND ORAL SQUAMOUS CELL CARCINOMA

Nagy KN et al.(1998), conducted a study on biofilms present on the surface of the oral SCC in patients suggested an increase of the Veillonella, Fusobacterium, Prevotella, Porphyromonas, Actinomyces and Clostridium, Hemophilus, Enterobacteriaceae and Streptococcus spp. compared to healthy mucosal surface of the same individuals.

Lara- Tejero et al (2000) showed that several infections cause intracellular accumulation of pathogens, Campylobacter jejuni in case of their study, thus leading to suppression of apoptosis primarily through modulation of the expression of Bcl-2 family proteins or by inactivation of retinoblastoma protein, pRb.

Several bacteria can cause chronic infections or produce toxins that disturb the cell cycle thus leading to altered cell growth(**Koyi H et al., 2001,Anttila T et al., 2003Littman AJ et al., 2004**).

According to **Lax AJ and Thomas W (2002),** the rich oral microbiota and the frequent episodes of transient bacteremia in normal life, maintain a low-grade chronic inflammation triggering a variety of systemic reactions. These in turn may lead to carcinogenic mechanisms which are unclear but irrespective of the focus of infection, bacteria may induce cellular proliferation, inhibit apoptosis, interfere with cellular signaling mechanisms, and act as tumor promoters.

According to **Salaspuro MP (2003)**, another possible mechanism is the metabolism of potentially carcinogenic substances by the bacteria. In the oral cavity, where the pre-existing local microflora facilitate tumourogenesis by converting ethanol into its carcinogenic derivative, acetaldehyde to levels capable of inducing DNA damage, mutagenesis and secondary hyperproliferation of the epithelium. This is evidentially proven by increased levels of microbial acetaldehyde production in heavy drinkers and smokers.

Sheu BC in 2008 showed that cytokine reactions to play a role in the immune-related mechanisms of cancer development. Up-regulation of cytokines and other inflammatory mediators are shown to affect complex metabolic pathways thus creating a link between chronic infections and sugar metabolism, the products of which are shown to induce carcinogenesis.

RAGE (receptor for advanced glycation end products), a member of immunoglobin protein family, expressed as a multi-ligand receptor expressed on various cell membranes, has also been suggested to play a role in carcinogenesis by **Katz J et al** in 2010. The protein has been shown to trigger multiple intracellular signaling molecules in several inflammation associated clinical diseases like diabetes and cancer.

Smruti Pushalkar et al (2012) compared the oral microbiota in tumor and non-tumor tissues of patients with oral squamous cell carcinoma and found that Streptococcus sp. oral taxon 058, Peptostreptococcus stomatis, Streptococcus salivarius, Streptococcus gordonii, Gemella haemolysans, Gemella morbillorum, Johnsonella ignava and Streptococcus parasanguinis were highly associated with tumor site where as Granulicatella adiacens was prevalent at non-tumor site.

EFFECT OF RADIOTHERAPY ON ORAL MICROFLORA

K.A.Abu Shara et al in **1992** studied the radiotherapeutic effect on oropharyngeal flora in 80 head and neck cancer patients so that it will help in controlling post-operative infections in patients who were already irradiated. Swabs were taken before and at the end of radiation therapy. They found significant increase in Staphylococcus aureus, beta hemolytic streptococci, Candida, Proteus and Pseudomonas aeruginosa. It was concluded from this study that irradiation has a significant effect on oropharyngeal flora.

M.Panduranga Kamath et al in **2002** studied the radiotherapeutic effect on oropharyngeal flora in head and neck cancer by culturing swabs from the tonsillar fossae, prior to commencement of radiotherapy and soon after completion of the full course, for aerobic, anaerobic bacteria and fungi. Commonest organisms isolated in the preirradiation group and controls were Streptococcus pneumoniae and Klebsiella species. Commonest organisms isolated from post irradiation group were Streptococcus viridans, Klebsiella speciesand Bacteroides species. Streptococcus pneumonia was found to be significantly decreased with irradiation compared to pre irradiation period. Moraxella species and Klebsiella also decreased with irradiation but organisms like Staphylococcus aureus, Pseudomonas species, Bacteroides species and Candida species showed marginal increase with irradiation.

L.Kun et al in **2005** investigated the impact of radiotherapy on microbial contents of the biofilms on the oral mucosal surfaces by culturing swabs from the centre of the radiotherapy region.

They found that after radiotherapy, the colonies of Streptococci, Candida albicans, Staphylococcus aureus were found significantly increased while Neisseria were significantly decreased.

Mager et al (2005) conducted a study on 40 common salivary microbiota of oral squamous cell carcinoma patients and showed that six common bacteria - P. melaninogenica, Capnocytophaga gingivalis, Capnocytophaga ochracea, Eubacterium saburreum, Leptotrichia buccalis and Streptococcus mitis were found at significantly higher salivary levels in OSCC patients compared with the controls. Also, three of the 40 species tested namely, Capnocytophaga gingivalis, Prevotella melaninogenica and Streptococcus mitis were found to be elevated in the saliva of oral squamous cell carcinoma patients. Hence these three organisms have been suggested as salivary diagnostic markers for oral squamous cell carcinoma with 80% sensitivity.

Elerson Gaetti et al(2011) evaluated the occurrence of yeasts, pseudomonas and enteric bacteria in the oral cavity of patients undergoing radiotherapy for treatment of head and neck cancer. Saliva, mucosa and biofilm samples were collected from fifty patients before receiving RT, during therapy and 30 days after RT and microorganisms were detected by culture and polymerase chain reaction. Pseudomonas aeruginosa, Proteus, Klebsiella were found to be more prevalent organisms before onset of radiotherapy. They found that modifications in the oral environment due to RT treatment seemed to facilitate the colonization of oral cavity by members of family Enterobacteriaceae, genera Enterococcus and Candida.

Panghal et al in **2012** analysed oral squamous cell carcinoma patients undergoing radiotherapy for bacterial pathogens in oral swab by culture and biochemical procedures.

Prevalent bacterial pathogens isolated were Staphylococcus aureus, Escherichia coli, Staphylococcus epidermidis, Pseudomonas aeruginosa, Klebsiella pneumonia, Proteus mirabilis, Proteus vulgaris and the fungal pathogens were Candida albicans and Aspergillus fumigatus.

Yue- Jian Hu et al in **2013** investigated the compositional profiles and microbial shifts of oral microbiota during head and neck radiotherapy to identify the imbalance of the oral microecosystem caused during therapy, so that effective preventive oral care can be given to such patients. Plaque samples were taken before and during radiotherapy and subjected to high-throughput sequencing which is a recent technology to sequence DNA and RNA in a quicker manner compared to other sequencing methods.. The top three genera observed pretreatment were Neisseria, Streptococcus and Capnocytophaga. These three genera were observed to vary significantly across different time points during therapy. Also, 11 genera namely, Streptococcus, Actinomyces, Veillonella, Capnocytophaga, Derxia, Neisseria, Rothia, Prevotella, Granulicatella, Luteococcus and Gemella were found in relative abundance during the course of radiotherapy.

The present study was carried out in 30 oral cancer patients with histopathologically proven oral squamous cell carcinoma of alveolus, buccal mucosa or tongue who are planned to receive radiotherapy. 30 normal healthy individuals without any habits formed the control group. Informed consent (Annexure I) was obtained from all the patients before collection of samples.

The study was performed after obtaining approval from the institutional ethical committee.

The patients were categorized into the following groups.

Study group:

30 oral cancer patients with histopathologically proven oral squamous cell carcinoma of alveolus, buccal mucosa or tongue and are planned to receive radiotherapy of dosage 60 gy in 30 fractionated doses, at 2 Gy/day. The salivary samples were collected before the onset of radiotherapy and at completion of 30 Gy and 60 Gy.

Control group:

This group comprised of 30 normal healthy individuals with clinically normal oral mucosa.

Part I: **Cytomorphometric analysis**

i. Armamentarium required for smear preparation

a. Container for collection of saliva

b. Ethanol

c. Micropipette with tips

d. Test tubes

e. Centrifuge

f. Glass slides

g. Spray fixative

ii. Collection of specimen:

Unstimulated whole saliva was collected from the patient. The patient was asked to rinse mouth with isotonic saline and was asked to spit into a sterile container.

0.1 – 0.2 ml of the collected saliva was transferred to a test tube and 0.5 ml of 95% ethanol was added to it. Then the test tube was centrifuged at 2000 rpm for 10 minutes. The supernatant was decanted, the cell pellet was collected with a micropipette, placed on a marked glass slide, smeared and fixed with spray fixative (Biolab diagnostics).

iii. Staining of smears

Rapid pap staining kit (Biolab diagnostics) was used to stain the smears.

Technique:

The kit consists of one solution for nuclear staining and two solutions (A and B) to be mixed in equal proportions for cytoplasmic staining (cytoplasmic working stain).

The marked slide was first hydrated by dipping slide in tap water for 1-3 minutes.

Nuclear staining:

Excess water was blotted out from the slide and the slide was dipped in nuclear stain for 60 seconds. Then the slide was dipped in tap water for 45 seconds.

Excess water was blotted out and the slide was dipped in dehydrant (100% ethyl alcohol) with 2 changes for 30 seconds each.

Cytoplasmic staining:

Then the slide was dipped in cytoplasm working stain for 45 seconds. Then the slide was dipped in tap water for 1 minute.

Excess water was blotted out, the slide was dipped in alcohol (2 changes), then in xylene and mounted.

Image analysis

20 cells per slide, with clear outline were selected for analysis. Quantitative parameters such as cytoplasmic diameter, cytoplasmic area (CD, CA) and nuclear diameter and nuclear area (ND, NA) and nuclear cytoplasmic ratio were assessed for each cell using image analysis software (ProgRes CapturePro version 2.8).

The nuclear and cell diameters were obtained by drawing two perpendicular lines along two planes in the nucleus and cell respectively and then obtaining the mean value of the two measurements. The data obtained were copied to excel sheet and means generated.

The means of all parameters(Annexure II) were then statistically analysed using Wilcoxon signed rank test for comparison between three stages in study group and using Mann Whitney U test for comparison between study and control group.

Part II: Bacterial Culture

i. Armamentarium required for culture
    a. Agar bases
        i. Streptococcus mitis selective medium(Hi media) – Streptococcus mitis
        ii. Wilkins chalgren medium (Hi media) – Prevotella melaninogenica
        iii. Tryptone soy agar (for TBBP medium) – Capnocytophaga gingivalis
    b. Distilled water
    c. Conical flasks
    d. Autoclave
    e. Petri plates
    f. L- rod
    g. Test tubes
    h. Anaerobic gas pack (Hi media – LE 002A)

ii. Collection of salivary sample

The patient was asked to rinse mouth with isotonic saline and then was asked not to swallow for 10 minutes. It was made sure that the patient had not eaten anything for 1 hour before collection of sample and not used any mouthwash for 24 hours prior to sample collection. After 10 minutes, the patient was asked to spit into a sterile container. It was repeated until 2 – 3 ml saliva was collected.

iii. Preparation of culture media

a. Streptococcus mitis

90.07 grams of agar was suspended in 1000 ml distilled water. It was heated to boiling to dissolve the medium completely. The medium was then sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. It was then cooled to 50-55°C and 1 ml of sterile 1% Potassium Tellurite Solution was added. It was mixed well and poured into sterile Petri plates.

b. Prevotella melaninogenica

43 grams of Wilkins Chelgren agar was suspended in 1000 ml distilled water. It was heated to boiling to dissolve the medium completely. The medium was then sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. It was then cooled to 50°C mixed gently and poured into sterile Petri plates.

c. Capnocytophaga gingivalis

50 grams of Tryptone soy agar was suspended in 1000 ml distilled water. It was heated to boiling to dissolve the medium completely.

The medium was then sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. After cooling to 50°C, Bacitracin 50 μg, sheep's blood 2 ml and Polymyxin 100μg were added. Then the medium was poured into sterile Petri plates.

Culture method:

The collected sample of saliva was serially diluted with distilled water.

Serial dilution :

0.5 ml of the salivary sample was added to 9.5 ml distilled water and mixed well. 1 ml of this solution is added to 9 ml distilled water to obtain10^2 dilution. Similarly, upto 10^6 dilution was obtained.

Spread plate method of culture was done. 0.1ml of 10^6 diluted salivary samples was poured into the petriplates with the selective media. Then the petriplates were placed in airtight jar along with anaerobic gas pack and left for 48 hours. After 48 hours, the plates were taken out and the number of colonies were counted.

Bergey's Microbiology manual and biochemical tests were used to confirm the respective organisms.

BIOCHEMICAL TESTS

Streptococcus mitis	**Prevotella melaninogenica**	**Capnocytophage gingivalis**
VP – negative Urease – negative Catalase – negative Mannitol – negative Lactose – positive Esculin hydrolysis - positive	Indole – negative Oxidase – negative Catalase – negative Mannitol – negative Maltose – positive Nitrate reduction – positive Alpha heamolysis	Indole – negative Glucose – positive Lactose – positive Sucrose – positive Esculin – negative

The appearance of the colonies was as follows:

Streptococcus mitis – Blue colonies

Prevotella melaninogenica – Pale yellow colonies

Capnocytophaga gingivalis – Yellow to brown colonies

The numbers of colony forming units were counted manually after dividing the plate into four quadrants.

The above said procedures were done for the control group and in the study group before radiotherapy, at 30 Gy radiation and after completion of 60 Gy. The counts from control and study group were tabulated (Annexure III) and statistically analysed using Wilcoxon signed rank test for comparison between three stages in study group and using Mann Whitney U test for comparison between study and control group.

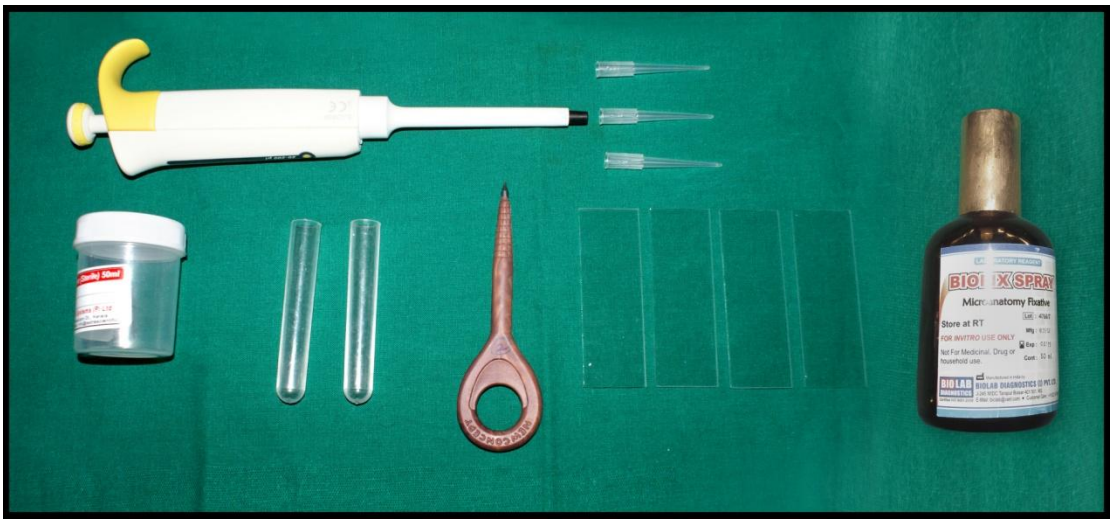


Figure 1- Micropipette, Slides, Glass Marking Pencil, Test Tube and Biofix Spray

Figure 2- Sterile Container Containing Sample

Figure 3- Placement of Test Tube Containing Sample into Centrifuge

Figure 4- Rapid Papanicalaou Smear Kit (Biolab Diagnostics, India)

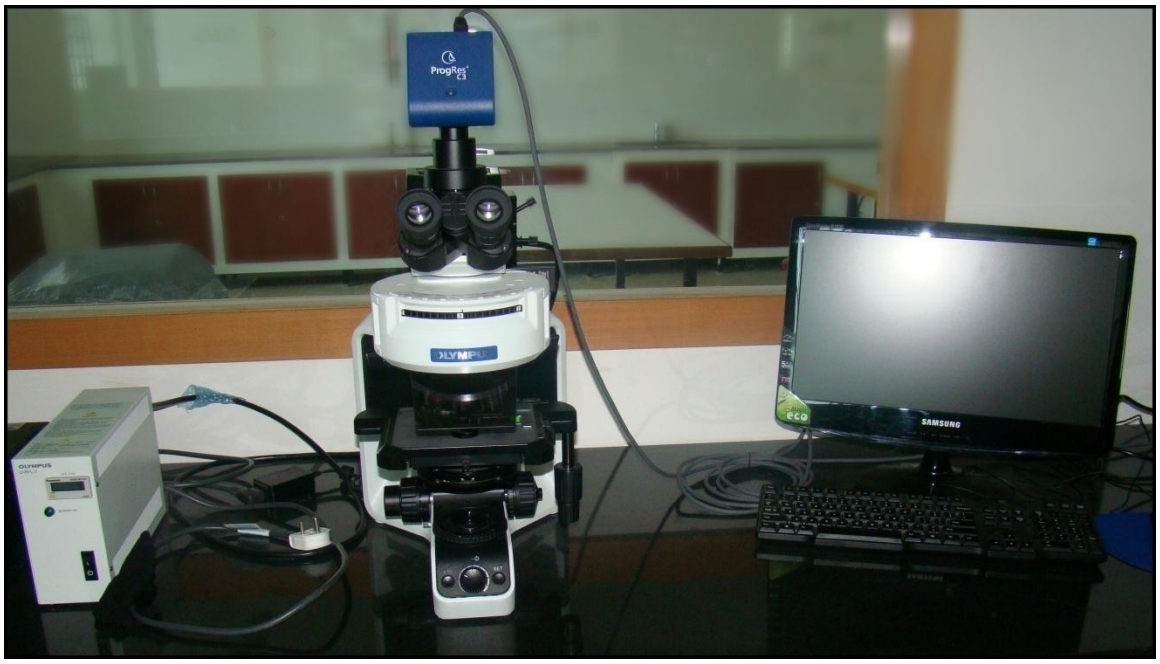


Figure 5- Research Microscope with ProgRes Capture Pro 2.8 Software (Olympus Opto Systems India Pvt. Ltd,)

Figure 6- Thermocol Box with Sample

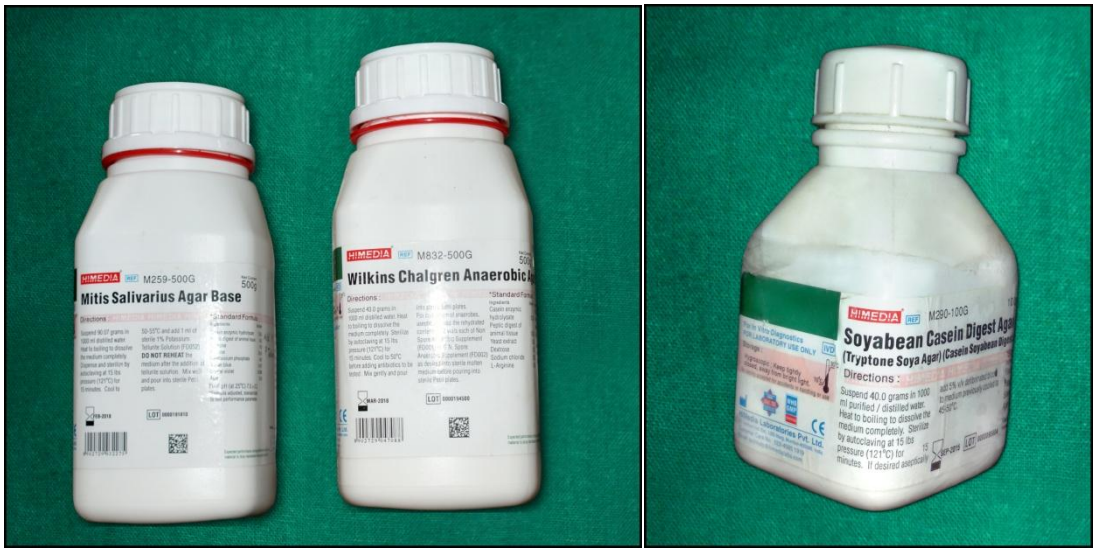


Figure 7- Agars used for Media Preparation

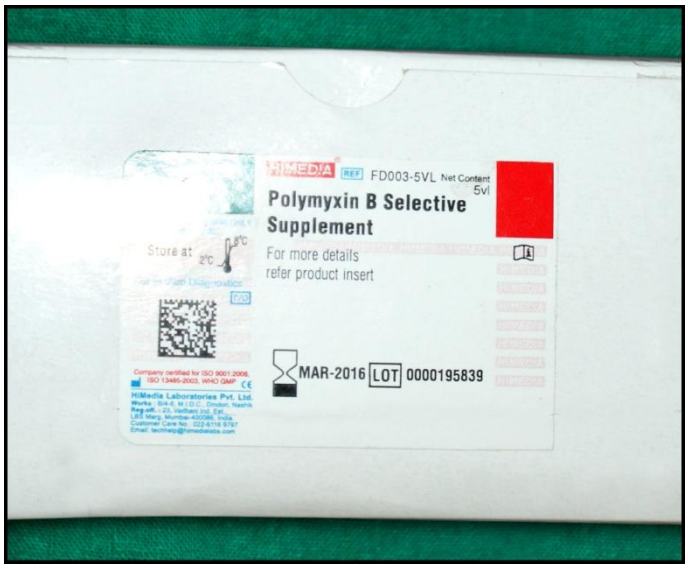


Figure 8- Polymyxin B for selective medium of Capnocytophaga gingivalis

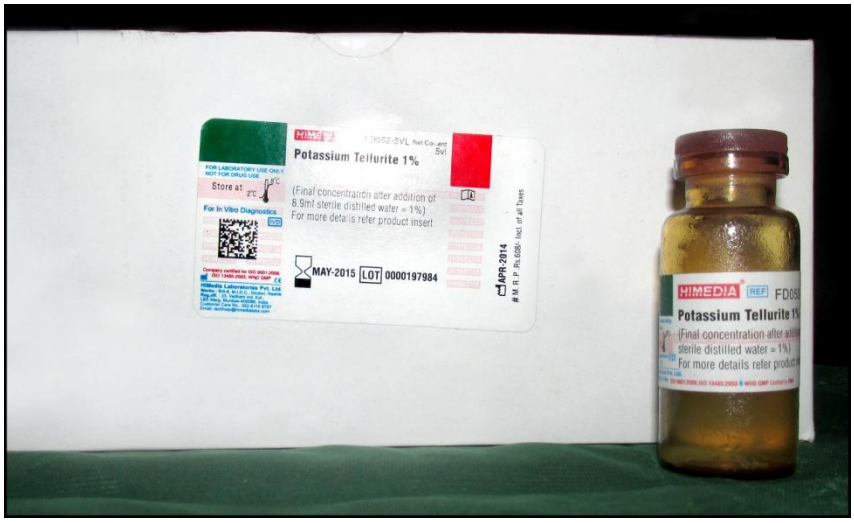


Figure 9- Potassium Tellurite for selective medium of Streptococcus mitis

Figure 10- Jar used for Culture and Anaerobic Gas Pack

Figure 11- Pouring Selective Medium into the Plate

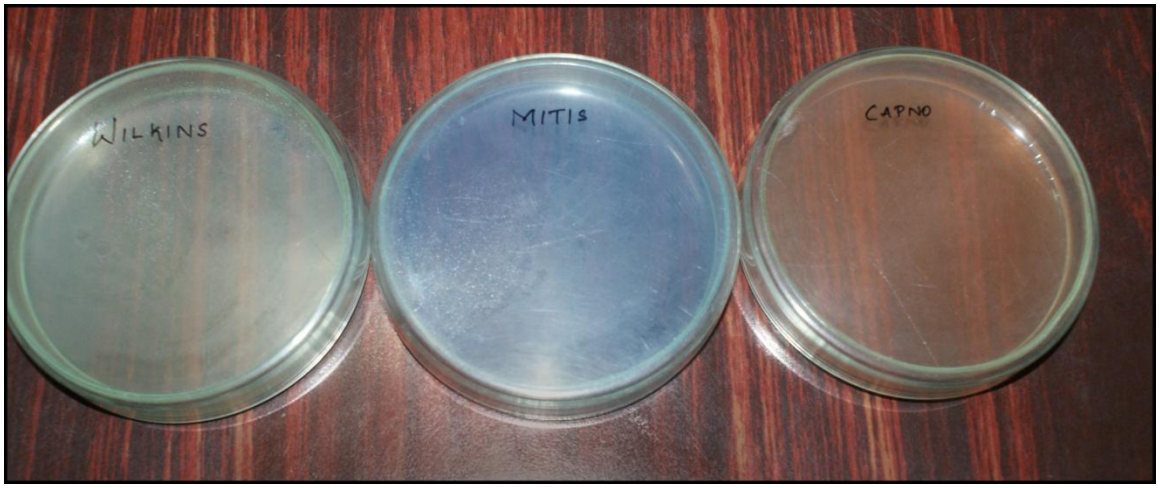


Figure 12- Petri Dishes with Poured Selective Media

Figure 13- Poured Plate Placed on Rotating Table

Figure 14- Serial Dilution of Saliva

Figure 15- Diluted Saliva into Petridish containing Selective Medium

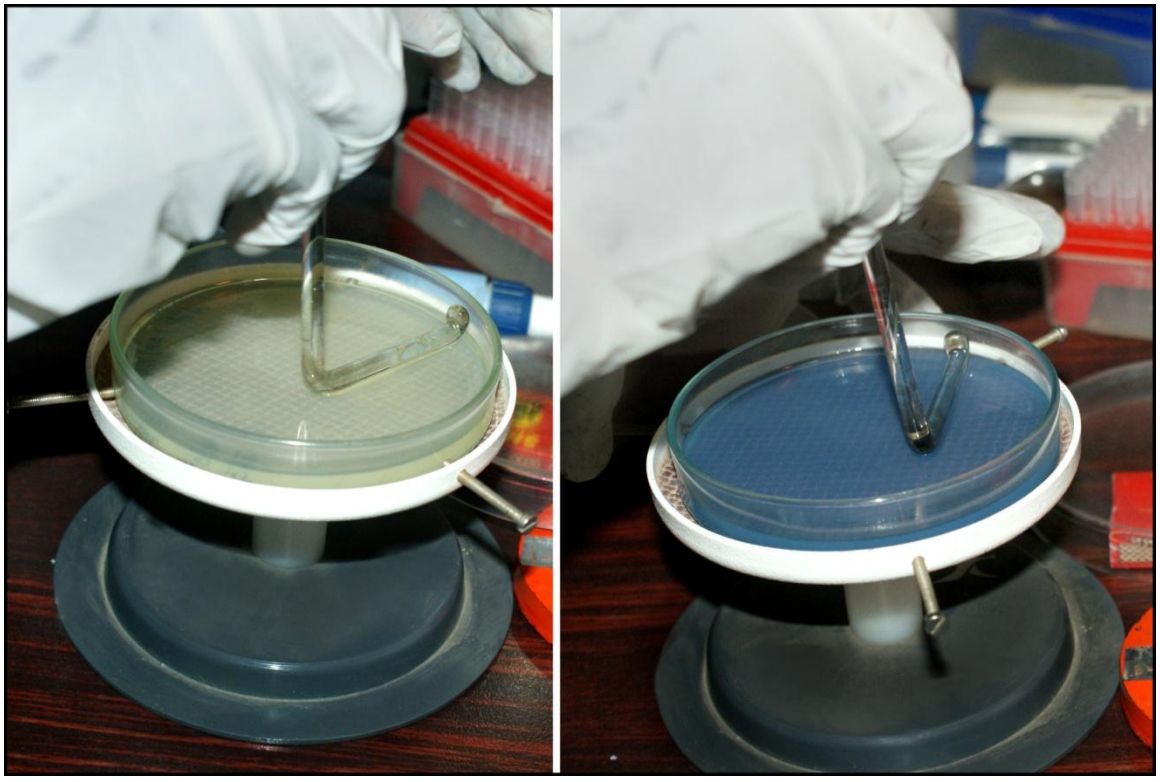


Figure 16- Spreading Plate with L- Rod

Figure 17- Anaerobic Gas Jar with Poured and Spread Plates

Figure 18- Incubator

Figure 19- Oxidase Discs for biochemical test

RESULTS

NUCLEAR AREA

Table I a

Descriptive Statistics for Nuclear Area

GROUPS	N	MEAN (μm^2)	STANDARD DEVIATION	MINIMUM (μm^2)	MAXIMUM (μm^2)
CONTROL	30	62.97	7.54	51.06	90.85
STAGE I	30	69.05	11.29	55.5	104.23
STAGE II	30	75.79	15.49	48.35	106.31
STAGE III	30	71.62	17.09	44.67	128.33

The mean value of nuclear area in normal group was 62.97 μm^2**,** while it was 69.05 μm^2**,** 75.79 μm^2 and 71.62 μm^2 in stage I, II and III of study group subjects respectively.

Table I b

Comparison of Nuclear Area of study group at the three Stages

COMPARISON GROUPS	Z VALUE	P VALUE
STAGES I & II	-1.800	0.072
STAGES II & III	-1.594	0.111
STAGES I & III	-0.422	0.673

Wilcoxon signed rank test

The study group data were assessed for significance using Wilcoxon signed ranks test and the p value was not found to be significant between various stages.

Stage I and II – 0.072

Stages II and III – 0.111

Stages I and III – 0.673

Table I c

Comparison of Nuclear Area of study group at each stage with control group

COMPARISON GROUPS	Z VALUE	P VALUE
CONTROL AND STAGE I	-2.380	0.017*
CONTROL AND STAGE II	-3.696	0.000***
CONTROL AND STAGE III	-2.779	0.004***

Mann Whitney U test * Significant *** Highly significant

Mann Whitney U test was used to compare the nuclear area of the controls with those of the study group at various stages. The results indicated that p value was found to be statistically significant between the groups.

Control vs Stage I – 0.017(significant)

Control vs Stage II – 0.000(significant)

Control vs Stage III – 0.004(significant)

CYTOPLASMIC AREA

Table II a

Descriptive Statistics for Cytoplasmic Area

GROUPS	N	MEAN (μm^2)	STANDARD DEVIATION	MINIMUM (μm^2)	MAXIMUM (μm^2)
CONTROL	30	2233.48	238.42	1716.0489	2794.0286
STAGE I	30	2069.39	551.65	1413.84	3833.03
STAGE II	30	2137.99	830.55	1187.61	5621.64
STAGE III	30	1924.03	541.51	919.11	3140.55

The mean value of cytoplasmic area in control group was 2233.48 μm^2, while it was 2069.39 μm^2, 2137.99 μm^2 and 1924.03 μm^2 in stage I, II and III of study group subjects respectively.

Table II b

Comparison of Cytoplasmic Area of study group at the three Stages

COMPARISON GROUPS	Z VALUE	P VALUE
STAGES I & II	-0.586	0.558
STAGES II & III	-1.368	0.171
STAGES I & III	-1.429	0.153

Wilcoxon signed rank test

The study group data were assessed for significance using Wilcoxon signed ranks test and the p value was not found to be significant between various stages.

Stage I and II – 0.558

Stages II and III – 0.171

Stages I and III – 0.153

Table II c

Comparison of Cytoplasmic Area of study group at each stage with control group

COMPARISON GROUPS	Z VALUE	P VALUE
CONTROL AND STAGE I	-2.5558	0.011**
CONTROL AND STAGE II	-1.922	0.05*
CONTROL AND STAGE III	-2.691	0.007***

Mann Whitney U test * Significant ** *** Highly significant

Mann Whitney U test was used to compare the cytoplasmic area of the controls with those of the study group at various stages. The results indicated that p value was found to be statistically significant between the groups.

Control vs Stage I – 0.011

Controll vs Stage II – 0.055

Control vs Stage III – 0.007

NUCLEAR DIAMETER

Table III a

Descriptive Statistics for Nuclear Diameter

GROUPS	N	MEAN (µm)	STANDARD DEVIATION	MINIMUM (µm)	MAXIMUM (µm)
CONTROL	30	9.101	0.53	8.23	10.8369
STAGE I	30	9.46	0.63	8.59	11.17
STAGE II	30	9.85	1.01	7.96	11.82
STAGE III	30	9.56	1.01	7.55	12.43

The mean value of nuclear diameter in normal group was 9.101 µm, while it was 9.46µm , 9.85µm and 9.56 µm in stage I, II and III of study group subjects respectively.

Table III b

Comparison of Nuclear Diameter of study group at the three Stages

COMPARISON GROUPS	Z VALUE	P VALUE
STAGES I & II	-1.779	0.075
STAGES II & III	-1.820	0.069
STAGES I & III	-0.381	0.704

Wilcoxon signed rank test

The study group data were assessed for significance using Wilcoxon signed ranks test and the p value was not found to be significant between various stages.

Stage I and II – 0.075

Stages II and III – 0.069

Stages I and III – 0.704

Table III c

Comparison of Nuclear Diameter of study group at each stage with control group

COMPARISON GROUPS	Z VALUE	P VALUE
CONTROL AND STAGE I	-2.3006	0.021*
CONTROL AND STAGE II	-3.460	0.001***
CONTROL AND STAGE III	-2.646	0.008***

Mann Whitney U test * Significant *** Highly significant

Mann Whitney U test was used to compare the nuclear diameter of the controls with those of the study group at various stages. The results indicated that p value was found to be statistically significant between the groups.

Control vs Stage I – 0.021(Significant)

Control vs Stage II – 0.001 (Significant)

Control vs Stage III – 0.008 (Significant)

CELL DIAMETER

Table IV a

Descriptive Statistics for Cell Diameter

GROUPS	N	MEAN (µm)	STANDARD DEVIATION	MINIMUM (µm)	MAXIMUM (µm)
CONTROL	30	54.37	2.58	47.67	60.99
STAGE I	30	51.92	6.20	43.28	67.86
STAGE II	30	52.42	9.09	40.47	85.94
STAGE III	30	50.07	7.07	34.98	63.58

The mean value of cell diameter in control group was 54.37 µm , while it was 51.92 µm, 52.42µm and 50.07 µm in stage I, II and III of study group subjects respectively.

Table IV b

Comparison of Cell Diameter of study group at the three Stages

COMPARISON GROUPS	Z VALUE	P VALUE
STAGES I & II	-0.216	0.829
STAGES II & III	-1.306	0.192
STAGES I & III	-1.347	0.178

Wilcoxon signed rank test

The study group data were assessed for significance using Wilcoxon signed ranks test and the p value was not found to be significant between various stages.

Stage I and II – 0.829

Stages II and III – 0.192

Stages I and III – 0.178

Table IV c

Comparison of Cell Diameter of study group at each stage with control group

COMPARISON GROUPS	Z VALUE	P VALUE
CONTROL AND STAGE I	-2.587	0.010**
CONTROL AND STAGE II	-2.025	0.043*
CONTROL AND STAGE III	-2.469	0.014**

Mann Whitney U test * Significant ** *** Highly significant

Mann Whitney U test was used to compare the cell diameter of the controls with those of the study group at various stages. The results indicated that p value was found to be statistically significant between the groups.

Control vs Stage I – 0.010 (Significant)

Control vs Stage II – 0.043 (Significant)

Control vs Stage III – 0.014 (Significant)

NUCLEAR CYTOPLASMIC RATIO

Table V a

Descriptive Statistics for Nuclear Cytoplasmic Ratio

GROUPS	N	MEAN	STANDARD DEVIATION	MINIMUM	MAXIMUM
CONTROL	30	0.03	0.002	0.026	0.035
STAGE I	30	0.036	0.006	0.026	0.05
STAGE II	30	0.039	0.007	0.016	0.054
STAGE III	30	0.04	0.009	0.025	0.058

The mean value of nuclear cytoplasmic ratio in normal group was 0.03, while it was 0.036**,** 0.039 and 0.04 in stage I, II and III of study group subjects respectively.

Table V b

Comparison of Nuclear Cytoplasmic Ratio of study group at the three Stages

COMPARISON GROUPS	Z VALUE	P VALUE
STAGES I & II	-2.129	0.033*
STAGES II & III	-0.977	0.329
STAGES I & III	-2.335	0.020*

Wilcoxon signed rank test * Significant

The study group data were assessed for significance using Wilcoxon signed ranks test and the p value was found to be significant between Stage I & II and between stage I & III.

Stage I and II – 0.033 (Significant)

Stages II and III – 0.329

Stages I and III – 0.020 (Significant)

Table V c

Comparison of Nuclear Cytoplasmic Ratio of study group at each stage with control group

COMPARISON GROUPS	Z VALUE	P VALUE
CONTROL AND STAGE I	-4.317	0.000***
CONTROL AND STAGE II	-5.441	0.000***
CONTROL AND STAGE III	-5.544	0.000***

Mann Whitney U test *** Highly significant

Mann Whitney U test was used to compare the nuclear cytoplasmic ratio of the controls with those of the study group at various stages. The results indicated that p value was found to be statistically significant between the groups.

Control vs Stage I – 0.000 (Significant)

Control vs Stage II – 0.000 (Significant)

Control vs Stage III – 0.000 (Significant)

Streptococcus mitis

Table VI a

Descriptive Statistics for Streptococcus mitis count in study and control groups

GROUPS	N	MEAN (X 10^6 CFU /ml)	STANDARD DEVIATION	MINIMUM (X 10^6 CFU /ml	MAXIMUM (X 10^6 CFU /ml)
CONTROL	30	161.43	74.564	28	289
STAGE I	30	247.07	59.101	168	386
STAGE II	30	208.87	55.944	103	294
STAGE III	30	167.03	43.090	93	260

The mean value of Streptococcus mitis in control group was 161.43 x 10^6 CFU/ml saliva, while it was 247.07 x 10^6 CFU /ml , 208.87 x 10^6 CFU /ml and 167.03 x 10^6 CFU /ml in stage I, II and III of study group subjects respectively.

Table VI b

Comparison of Streptococcus mitis in study group at the three Stages

COMPARISON GROUPS	Z VALUE	P VALUE
STAGES I & II	-4.721	0.000***
STAGES II & III	-4.721	0.000***
STAGES I & III	-4.782	0.000***

Wilcoxon signed rank test *** Highly significant

The study group data were assessed for significance using Wilcoxon signed ranks test and the p value was not found to be significant between various stages.

Stage I and II – 0.000 (Significant)

Stages II and III – 0.000 (Significant)

Stages I and III – 0.000 (Significant)

Table VI c

Comparison of Streptococcus mitis in study group at each stage with control group

COMPARISON GROUPS	Z VALUE	P VALUE
CONTROL AND STAGE I	-3.940	0.000***
CONTROL AND STAGE II	-2.388	0.017**
CONTROL AND STAGE III	-0.251	0.802

Mann Whitney U test ** *** Highly significant

Mann Whitney U test was used to compare the Streptococcus mitis of the controls with those of the study group at various stages. The results indicated that p value was found to be statistically significant between Control & stage I and between control & stage II.

Control vs Stage I – 0.000 (Significant)

Control vs Stage II – 0.017 (Significant)

Control vs Stage III – 0.802

Prevotella melaninogenica

Table VII a

Descriptive Statistics for Prevotella melaninogenica count in study group and control group

GROUPS	N	MEAN (X 10^6 CFU /ml)	STANDARD DEVIATION	MINIMUM (X 10^6 CFU /ml)	MAXIMUM (X 10^6 CFU /ml)
CONTROL	30	22.97	10.67	9	58
STAGE I	30	49.33	18.417	21	94
STAGE II	30	33.93	12.020	16	69
STAGE III	30	26.30	9.966	11	48

The mean value of Prevotella melaninogenica in control group was 22.97, while it was 49.33 x 10^6 CFU /ml , 33.93 x 10^6 CFU /ml and 26.30 x 10^6 CFU /ml in stage I, II and III of study group subjects respectively.

Table VII b

Comparison of Prevotella melaninogenica in study group at the three Stages

COMPARISON GROUPS	Z VALUE	P VALUE
STAGES I & II	-4.701	0.000***
STAGES II & III	-3.408	0.001***
STAGES I & III	-4.784	0.000***

Wilcoxon signed rank test *** Highly significant

The study group data were assessed for significance using Wilcoxon signed ranks test and the p value was found to be significant between various stages.

Stage I and II – 0.000 (Significant)

Stages II and III – 0.001(Significant)

Stages I and III – 0.000 (Significant)

Table VII c

Comparison of Prevotella melaninogenica in study group at each stage with control group

COMPARISON GROUPS	Z VALUE	P VALUE
CONTROL AND STAGE I	-5.398	0.000***
CONTROL AND STAGE II	-3.536	0.000***
CONTROL AND STAGEIII	-1.443	0.149

Mann Whitney U test *** Highly significant

Mann Whitney U test was used to compare the Prevotella melaninogenica of the controls with those of the study group at various stages. The results indicated that p value was found to be statistically significant between the groups.

Control vs Stage I – 0.000 (Significant)

Control vs Stage II – 0.000 (Significant)

Control vs Stage III – 0.149

Capnocytophaga gingivalis

Table VIII a

Descriptive Statistics for Capnocytophaga gingivalis count in study group and control group

GROUPS	N	MEAN (X 10^6 CFU /ml)	STANDARD DEVIATION	MINIMUM (X 10^6 CFU /ml)	MAXIMUM (X 10^6 CFU /ml)
NORMAL	30	4	1.03	1	5
STAGE I	30	7.90	2.975	4	17
STAGE II	30	5.90	2.734	3	16
STAGE III	30	4.40	1.773	2	9

The mean value of Capnocytophaga gingivalis in control group was 4x 10^6 CFU/ml saliva while it was 7.90 x 10^6 CFU /ml**,** 5.90 x 10^6 CFU /ml and 4.40 x 10^6 CFU /ml in stage I, II and III of study group subjects respectively.

Table VIII b

Comparison of Capnocytophaga gingivalis in study group at the three Stages

COMPARISON GROUPS	Z VALUE	P VALUE
STAGES I & II	-4.136	0.000***
STAGES II & III	-3.202	0.001***
STAGES I & III	-4.623	0.000***

Wilcoxon signed ranks test *** Highly significant

The study group data were assessed for significance using Wilcoxon signed ranks test and the p value was found to be significant between various stages.

Stage I and II – 0.000 (Significant)

Stages II and III – 0.001 (Significant)

Stages I and III – 0.000 (Significant)

Table VIII c

Comparison of Capnocytophaga gingivalis in study group at each stage with control group

COMPARISON GROUPS	Z VALUE	P VALUE
CONTROL AND STAGE I	-6.580	0.000***
CONTROL AND STAGE II	-5.919	0.000***
CONTROL AND STAGE III	-3.806	0.000***

Mann Whitney U test *** Highly significant

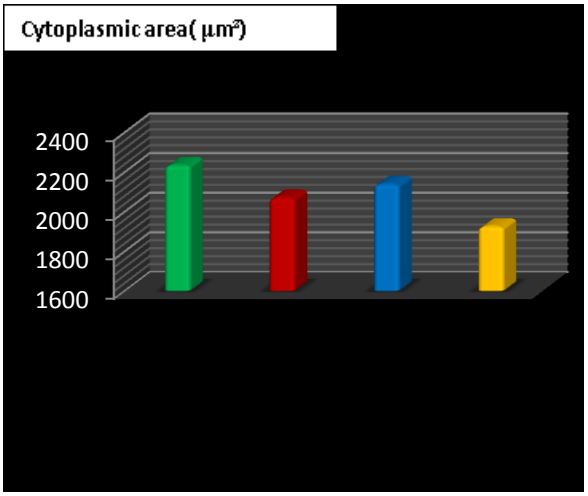
Mann Whitney U test was used to compare the Capnocytophaga gingivalis of the controls with those of the study group at various stages. The results indicated that p value was found to be statistically significant between the groups.

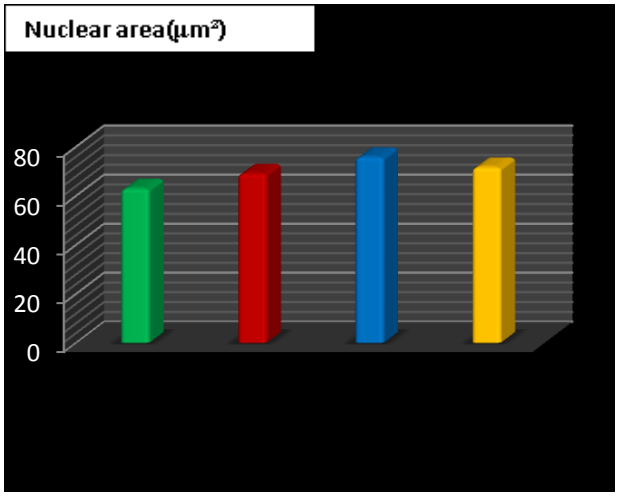
Control vs Stage I – 0.000 (Significant)

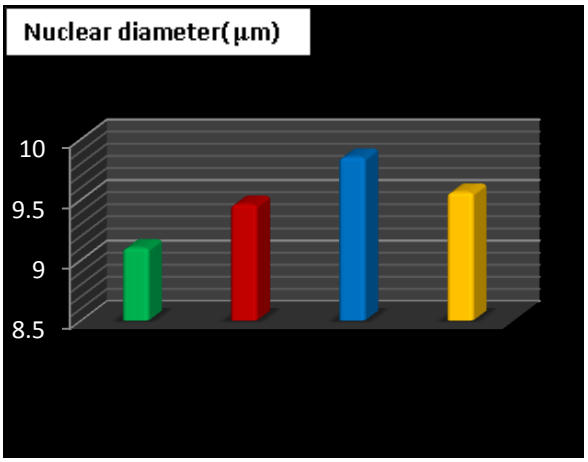
Control vs Stage II – 0.000 (Significant)

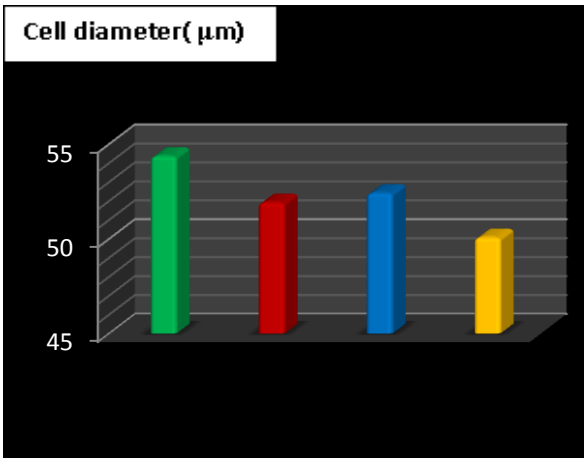
Control vs Stage III – 0.000 (Significant)

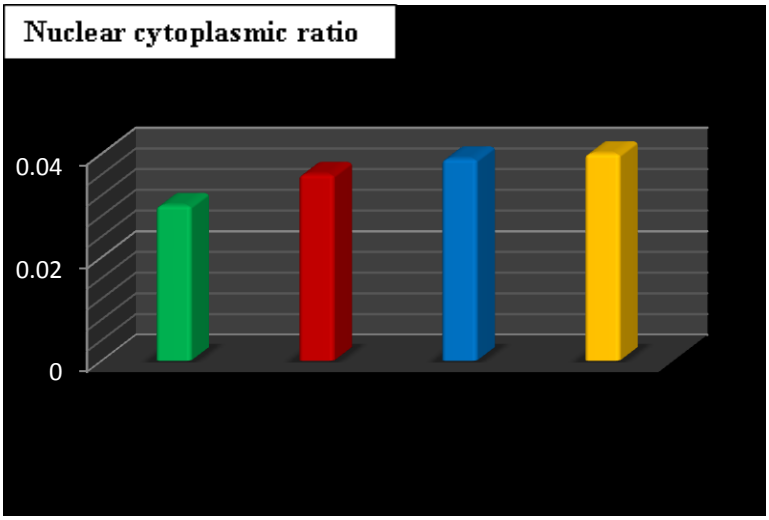
CHARTS SHOWING CYTOLOGICAL PARAMETERS











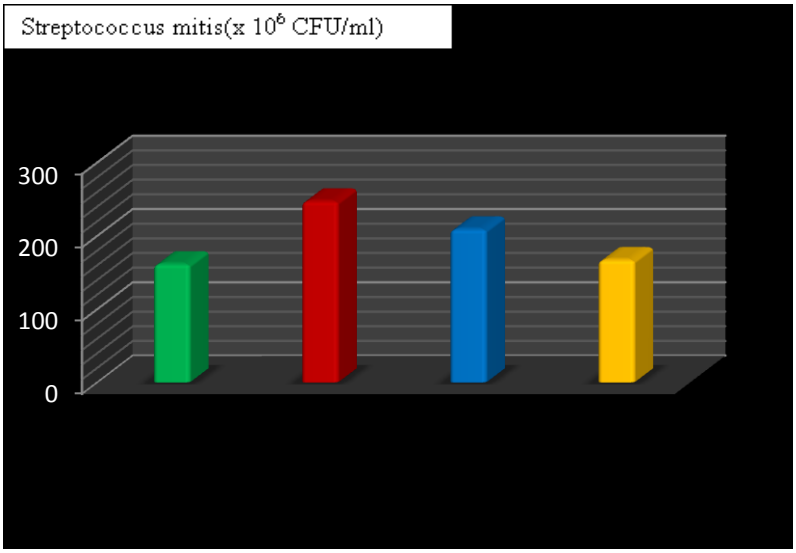
Control

Stage I

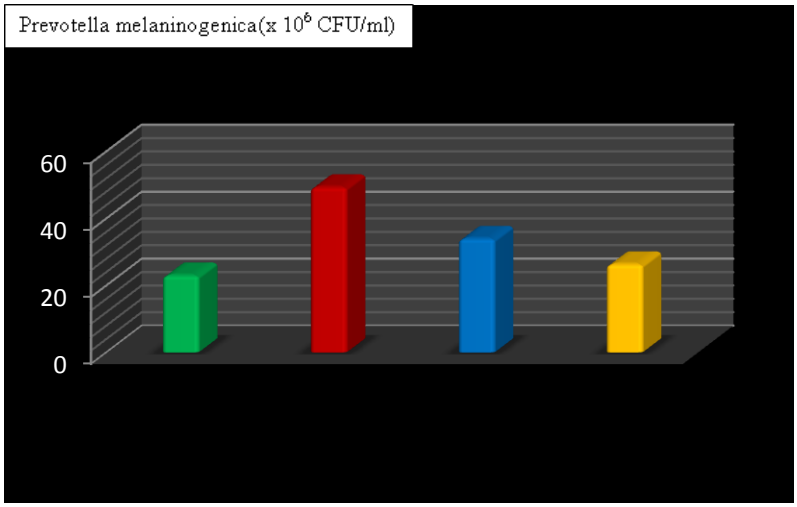
Stage II

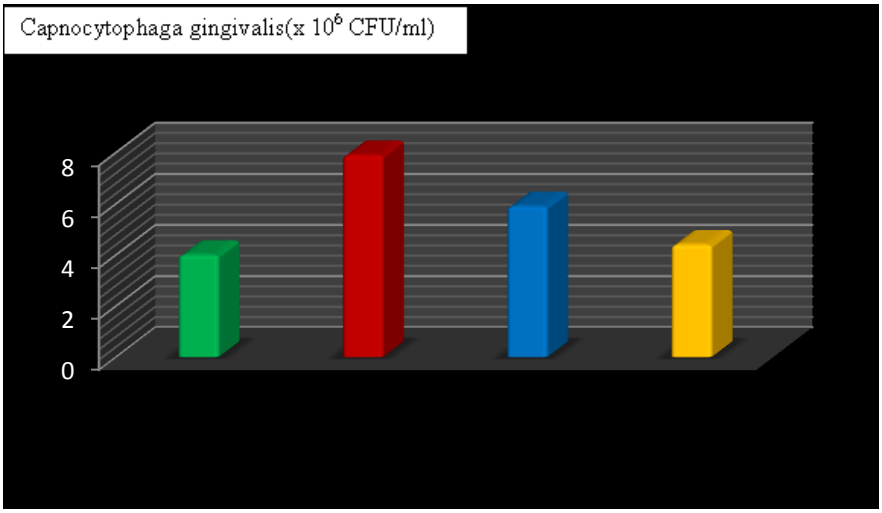
Stage III

CHARTS SHOWING BACTERIAL COUNTS



- Control
- Stage I
- Stage II
- Stage III





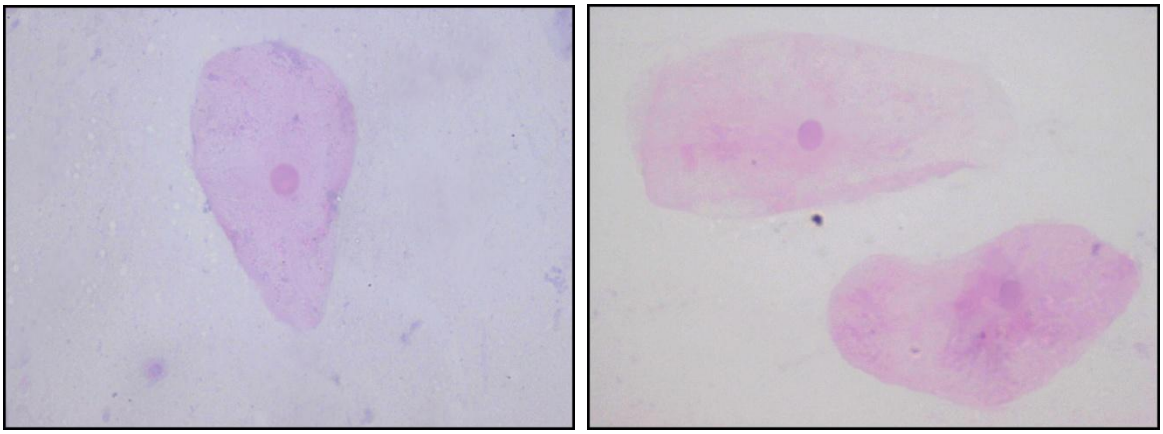


Figure 20: Photomicrographs (40X) Showing Exfoliated Cells in Control Groups – PAP

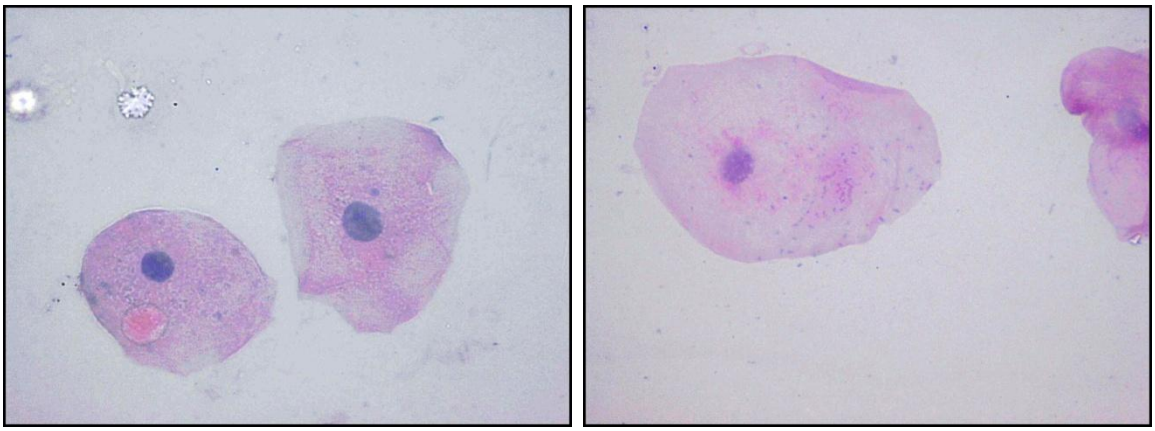


Figure 21: Photomicrographs (40X) Showing Exfoliated Cells in Stage I – PAP

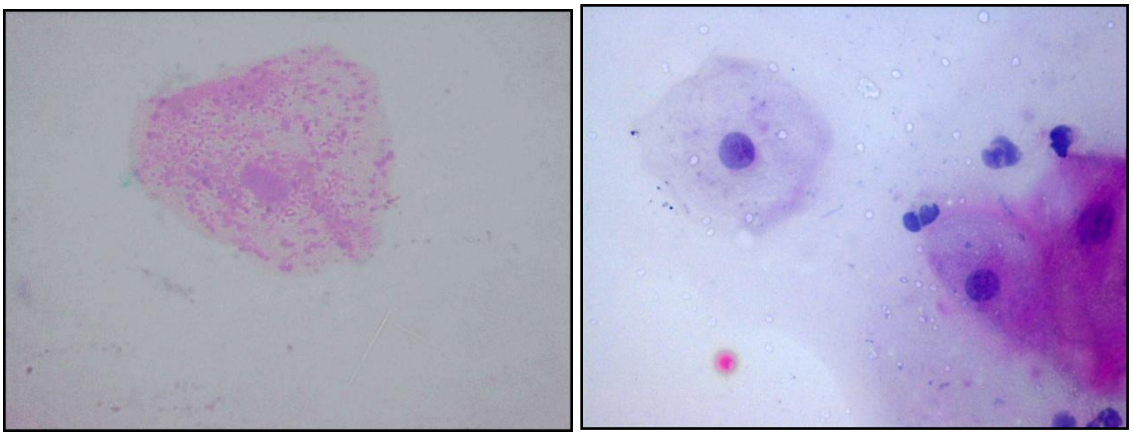


Figure 22: Photomicrographs (40X) Showing Exfoliated Cells in Stage II – PAP

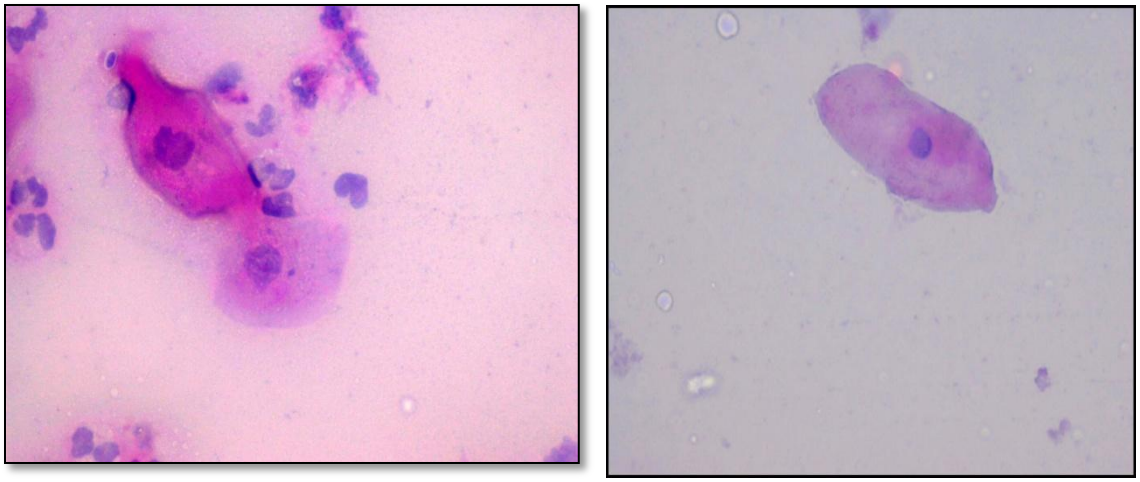


Figure 23: Photomicrographs (40X) Showing Exfoliated Cells in Stage III – PAP

CYTOMORPHOMETRIC ANALYSIS

Nuclear Area & Cytoplasmic Area

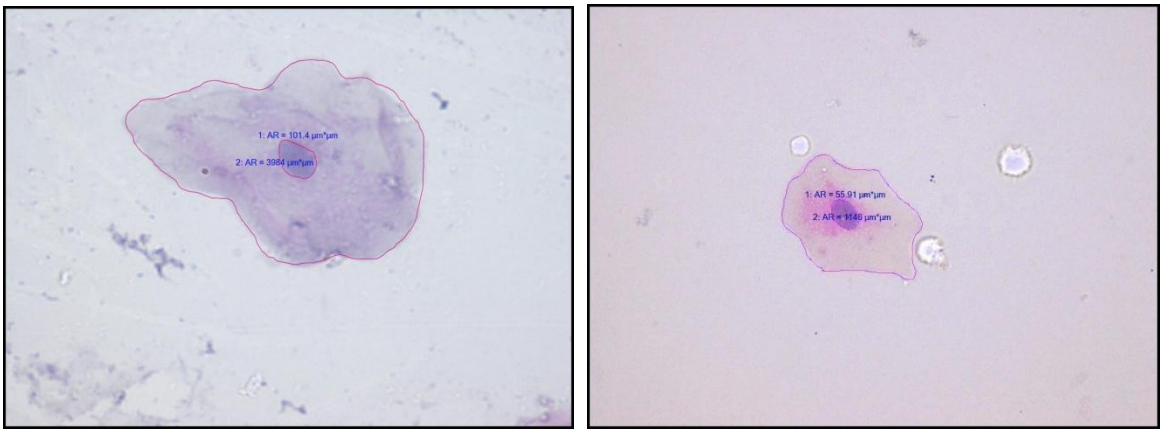


Figure 24: Photomicrographs (40X) Showing Exfoliated Cell Cytomorphometry in control and study groups – PAP

Nuclear & Cell Diameter

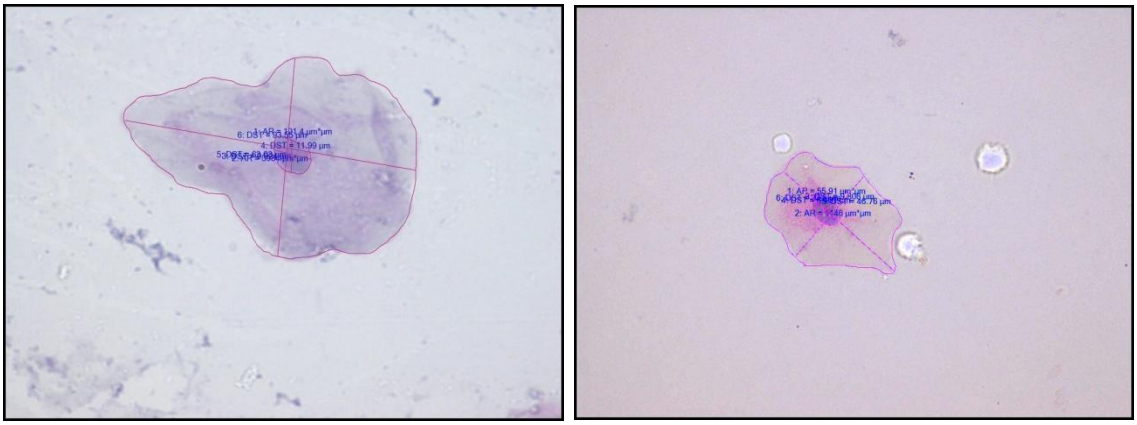


Figure 25: Photomicrographs (40X) Showing Exfoliated Cell Cytomorphometry in control and study groups – PAP

BACTERIAL CULTURE

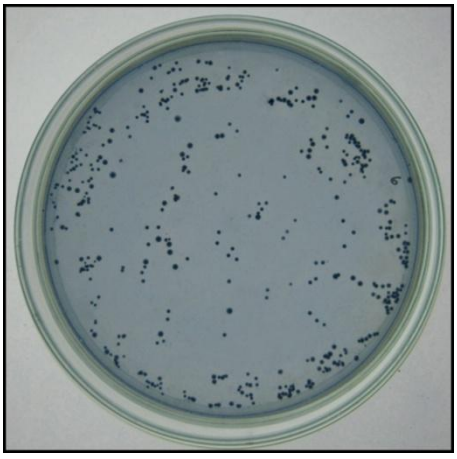


Figure 26: Colonies Of Streptococcus Mitis (Blue)

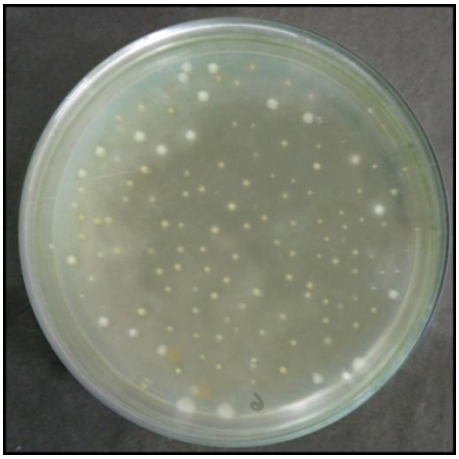


Figure 27: Colonies of Prevotella melaninogenica (Yellow)

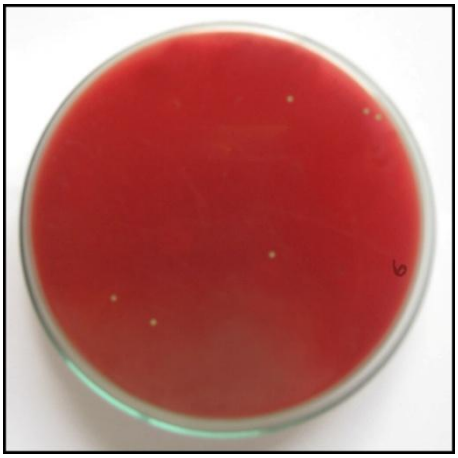


Figure 28: Colonies of Capnocytophaga gingivalis(α-hemolytic)

BIOCHEMICAL TESTS

Figure 29: Tests to confirm Streptococcus mitis

Figure 30: Tests to confirm Prevotella melaninogenica

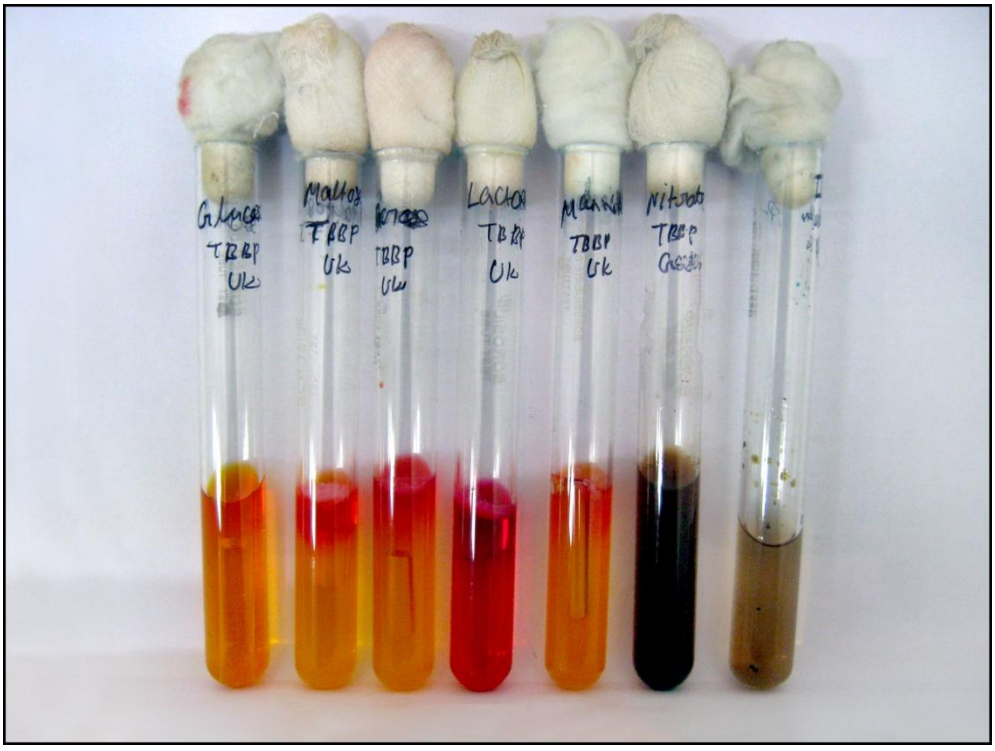


Figure 31: Tests to confirm Capnocytophaga gingivalis

DISCUSSION

Radiation Therapy (RT) is an important treatment modality in Oral Squamous Cell Carcinoma. It is used alone or combined with chemotherapy. The reaction of the tumor tissue to RT has been shown to vary between individuals hence making it difficult to assess and predict the treatment outcome for patients. **(Vineet Raj et al., 2010)**

Exfoliative cytology, a simple, non-invasive and economical tool for diagnosis had its use limited in oral squamous cell carcinoma because of the subjective nature of its interpretations and false negative results **(Sivapathasundaram et al,2004)**. But image analysis technique enables quantification of nuclear and cellular changes from normal on an objective basis and this has been used in various studies to establish relationship between tumor response and morphological changes in the cells **(Smitha T et al, 2011)**.

An association between oral cancer and bacteria has been studied since many years as various discoveries suggested the role of bacteria in many cancers. Three bacterial species, namely, Streptococcus mitis, Prevotella melaninogenica and Capnocytophaga gingivalis have been shown to be increased in oral squamous cell carcinoma patients **(DL Mager et al, 2005)**.

Altered immunological system changes the profile of microbiologic flora both in normal people and in head and neck cancer patients. The oropharyngeal flora in these patients can be altered and can consist of abnormal organisms with significantly associated decrease in normal commensals. RT has been shown to influence the oropharyngeal flora, with growth of many abnormal pathogens **(MP Kamath et al,2002)**. It has been proved that about half of cancer deaths are due to infection.

Most common causes of sepsis are pneumonia and septicemia caused by gram negative bacilli **(Inagarki et al 1974)**.

The present study was carried out in 30 patients who were histopathologically diagnosed with oral squamous cell carcinoma and planned RT with 60Gy radiation in 30 fractionated doses at 2 Gy/day for 5 days a week and 30 normal healthy individuals. Saliva samples were collected from all subjects belonging to study group; before RT, at 2 weeks (30Gy) and 4 weeks (60Gy) and also from the control group.

The samples were also centrifuged, smeared, PAP stained and analyzed cytomorphometrically for the following parameters:

- Nuclear Diameter
- Nuclear Area
- Cell diameter
- Cytoplasmic area
- Nuclear cytoplasmic ratio

The samples were cultured for the 3 organisms namely, Streptococcus mitis, Prevotella melaninogenica and Capnocytophaga gingivalis to analyse the effect of radiotherapy on these organisms.

Cytomorphometric analysis

This study showed an increased mean value of nuclear area in oral squamous cell carcinoma subjects compared to controls. The mean value of nuclear area in the control group was 62.97μm^2 while it was 69.05μm^2, 75.79 μm^2 and 71.62 μm^2 at the three stages in oral squamous cell carcinoma subjects.

These differences between the control and study group (in all the 3 stages), were found to be statistically significant, the p values being 0.017, 0.000 and 0.004 respectively.

The mean value of nuclear areas in case of the study group subject was seen to be increased at stage II compared to stage I, with decrease in the value again at stage III. However, these values were not found to show any statistical significance. (Stage I and II: p value – 0.072; Stage II and II: p value – 0.111; Stage I and III: p value – 0.673)

The increase in nuclear area in cases of squamous cell carcinoma(stage I) compared to normal as shown in this study is due to increase in nuclear contents for replication. **(Frost, 1997)**. A significantly increased nuclear area in malignant cells was also demonstrated by **Cowpe in 1984,** in his study of comparison of normal and abnormal (oral lesions like leukoplakia,lichen planus and oral cancer) oral mucosal squames. With radiation, the increase in nuclear area denotes good response to therapy as showed by **BM Biswal et al in 2010**. The increase in nuclear size is followed by nuclear fragmentation and then cell death. However, the differences in nuclear area between three stages of radiotherapy in study subjects was not found to be significant in our study. This may be due to marked inter-individual variability in radiation response **(V.Raj et al, 2010)** or the cellular changes may be significant only 8 weeks following radiotherapy **(D.Agarwal et al)**.

The present study demonstrated an increase in mean value of the nuclear diameter in the oral squamous cell carcinoma subjects compared to the controls. The mean value of the nuclear diameter at stage I was 9.46, at stage II 9.85 and 9.56 at stage III. The mean nuclear diameter of the control group was 9.1μm.

The difference in values between stage I and II, stage II and III and stage I and III did not show any statistical significance, the p values being 0.075, 0.069 and 0.704 respectively. However, all three stages showed a statistically significant difference from the control group (p values – 0.021, 0.001 and 0.008).

The increased nuclear diameter in study group subjects at stage I compared to control group is in concordance with various studies showing increased nuclear parameters due to increased nuclear contents for replication. In the study subjects, mean nuclear diameter was found to be increased at stage II compared to stage I and very little drop in the value at stage III. These changes were not statistically significant. Similar findings in oral squamous cell carcinoma patients were observed by **S.Silverman et al**, who showed an increase in nuclear diameter at 2 to 3 weeks and then a decrease though statistically significant changes were observed 8 weeks after onset of radiotherapy.

The mean cytoplasmic area of the control group was 2233.48 μm^2 and those of the study group at three stages were 2069.39, 2137.99 and 1924.03. There was a reduction in the mean cytoplasmic area in the study group when compared to the control group and this difference was found to be statistically significant with the difference between control and stage III being highly significant (p value – 0.007).

The difference when control compared with stage I and II were also statistically significant, the p values being 0.011 and 0.055 respectively. Among the three stages in the study group, the mean cytoplasmic area was found to be increased in stage II compared to stage I and a fall in the value at stage III again to a value less then stage I. However these differences were not found to be statistically significant.

The decreased cytoplasmic area in oral squamous cell carcinoma patients at stage I compared to normal healthy individuals is due to decreased ability of the cytoplasm to mature as the malignant cells show increased activity **(Frost et al, 1997)**. Also, the amount of cytoplasm made by the cell is less compared to the nucleoplasm and the decreased cytoplasmic area is a significant change occurring in an actively proliferating cell. **(Cancado et al, 2004)**. There is an increase in cytoplasmic area observed in stage II which may be due to cell enlargement which is an acute radiation change as observed by **HG Ahmed et al** in oral squamous cell carcinoma patients.. The reduction in value at stage III may be due to variable radiation response of tumor cells as reported by **BM Biswal et al in 2010** from his study in patients with squamous cell carcinoma of head and neck.

The mean value of the cell diameter observed in the present study in the control group was 54.37μm. There was a reduction in the mean values of cell diameter in all the three stages in the study group, the mean values being 51.92, 52.42 and 50.07 at stage I, II and III. These differences between control and the study group were found to be statistically significant (p values – control and stage I- 0.01; control and stage II – 0.043; control and stage III – 0.014). The mean cell diameter at stage II was increased compared to stage I with a reduction below stage I value in stage III. But these differences in the mean values did not show any statistical significance. (p values: stage I and II – 0.829; stage II and II – 0.192; stages I and III – 0.178).

The decrease in cell diameter observed in study subjects at stage I compared to controls is explained by the reduced ability of the cytoplasm to mature and reduced ability of the proliferating cell to form cytoplasm **(Frost et al, 1997)**.

The slight increase in stage II and later reduction observed is similar to the findings of **Silverman et al** who observed a slight cell enlargement at two to three weeks and later reduction, though statistically significant changes were observed only after 8 weeks after initiation of radiotherapy.

The present study showed an increase in nuclear cytoplasmic ratio in the study group compared to the control group. The mean nuclear cytoplasmic ratio of the control group was 0.03 while it was found to be 0.036, 0.039 and 0.04 at the three stages in the study group. It was found that the difference in mean nuclear cytoplasmic ratio between stage I and II and between I and III were statistically significant (p values: 0.033 and 0.02 respectively), though the difference between stage II and III was not statistically significant (p value – 0.329). When compared to the control group, the nuclear cytoplasmic ratio values were found to show a statistically significant increase in all three stages in the study group. (p values : control and stage I, II and II – 0.000).

There is a significant increase in nuclear cytoplasmic ratio in squamous cell carcinoma subjects compared to normal subjects in this present study, which is due to increased nuclear area and decreased cytoplasmic area, as pointed out in a study by **Cowpe in 1984**. The statistically significant increase in the ratio at stage II and the value maintained at stage III may be due to initial response of the tumor cells which may show changes later as observed by **Silverman et al** 8 weeks following therapy.

BACTERIAL ASSESSMENT

Streptococcus mitis

The present study revealed a mean S. mitis count of 161.43 x 10^6 CFU/ml saliva in the control group. In the three stages of the study group, the mean counts of the bacteria were observed to be more compared to the control group, though statistically significant difference was found only between control group and stage I, control and stage II (p values 0.000 and 0.017 respectively). The difference in count between control group and stage III was not statistically significant (p value – 0.802). Between the three stages of the study group, statistically significant reduction in the count of S.mitis with increasing radiation dose was observed, with mean value at stage I being 247.07 x 10^6 CFU/ml, at stage II 208.87 x 10^6 CFU/ml and 167.03 x 10^6 CFU/ml at stage III. A 'p' value of 0.000 was observed between stages I and II, stages II and III, stages I and III.

The mean count of Streptococcus mitis in the saliva of oral squamous cell carcinoma patients at stage I was observed to be much more than that of the control subjects, with a p value of less than 0.001. This observation is similar to the finding of **DL Mager et al in 2005** who found S.mitis to be increased in oral cancer subjects compared to cancer free subjects. During the course of radiotherapy, this study showed a decrease in the count of S.mitis. The count at stage III was observed to be close to the mean value of normal subjects but was higher than normal though statistically not significant.

In a study by **MP Kamath et al**, there was an decrease in count of Streptococcus pneumoniae following radiotherapy. **Meurman JH et al in 1997**, showed that there was an increased abundance of alpha hemolytic streptococci (mainly S. mutans) after radiotherapy, but present study showed a decrease of S. mitis with therapy. This can be explained by the fact that their study was performed as a five year follow up study in lymphoma patients undergoing radiotherapy but present study investigated the count during radiotherapy. Also, according to **ZY Shao et al**, there is no favorite shift in microbiota in patients during radiotherapy compared to preradiotherapy, which was shown by their study in which sequential analysis of plaque samples were done in head and neck cancer patients undergoing radiotherapy. They observed that different subjects showed predominance of different bacterial genera.

Prevotella melaninogenica

The present study showed increased count of P. melaninogenica in oral squamous cell carcinoma subjects at stage I compared to the control group. The mean count of the organism in the control group was 22.97 x 10^6 CFU / ml saliva and that in stage I was 49.33 x 10^6 CFU/ml. The mean counts in stage II and III were 33.93 x 10^6 CFU/ml and 26.30 x 10^6 CFU/ml respectively, which were observed to be more than the value obtained in the control group. But statistically significant difference was found only between control and and stage I, control and stage II (p values - 0.000 in both cases), while the difference between control and stage III was not statistically significant (p value- 0.149). Between three stages in the study group, there was a decrease in the count of P. melaninogenica, with increase in radiation dose.

The mean counts observed in the three stages were 49.33 x 10^6 CFU/ml, 33.93 x 10^6 CFU/ml and 26.30 x 10^6 CFU/ml. The difference between stages I and II, II and III, I and III observed were all statistically significant with p values of 0.000, 0.001 and 0.000 respectively.

The statistically significant variation in the count of Prevotella melaniogenica in oral cancer subjects before radiotherapy compared to normal controls is in agreement with the observation by **DL Mager et al** and **N Chocolatewala et al** who showed similar increase in the count of this organism in oral cancer subjects. In the present study, a decrease in the count of the organism was found with increasing dose of radiotherapy. This is in contrast to the study by **ZY Shao et al** who showed that there was an increase in abundance of P.melaninogenica in oral squamous cell carcinoma patients during radiotherapy. But the samples in this study were plaque samples from first molar teeth and not saliva as in our case. Also, specific bacterial species were cultured and counted in our study whereas several bacterial genera were studied by them by sequential PCR analysis of plaque samples. Moreover, according to **ZY Shao et al**, different bacterial genera can be abundant at different points during radiotherapy.

Capnocytophaga gingivalis

The mean count of Capnocytophaga gingivalis observed in this study in the control group was 4 x 10^6 CFU/ml saliva. The mean count of the bacterial species in the saliva of the study group subjects at three stages were more than that observed in the control group. The mean counts were 7.90 x 10^6 CFU/ml, 5.90 x 10^6 CFU/ml and 4.40 x 10^6 CFU/ml at stages I, II and III respectively.

This difference in counts between control and study group subjects were statistically significant (p values – 0.000 in stages I, II and III). The mean counts of the organism in the study group subjects in the three stages showed statistically significant differences at all stages (p values – Stage I and II – 0.000, stage II and III- 0.001, stage I and III – 0.000).

The increased count of C.gingivalis in oral squamous cell carcinoma patients compared to normal subjects observed in the present study is in concordance with the study by DL Mager et al in which an increase in the DNA count of the organism in saliva of oral squamous cell carcinoma patients was demonstrated. The count of the organism in the study subjects showed a decrease with radiotherapy at 2 weeks and 4 weeks. A study by **YJ Hu et al** on microbial shifts in head and neck cancer therapy patients showed that C. gingivalis was one of the most prevalent organisms in these patients and the count of the organism was said to vary significantly at different time points though the exact variation, increase or decrease is not specified.

In the present study, though there was a decrease in mean values of the counts of the three organisms with radiotherapy, there were irregular shifts seen in some patients like increase in count and unaffected counts with increase in radiation dosage. These changes, as they did not affect the mean values, were not reflected in the results. These variations in relative counts observed at different points during radiotherapy is supported by the study by **YJ Hu et al** who observed similar variations in relative abundance in genera Streptococcus, Prevotella, Capnocytophaga and Neisseria.

There were certain limitations in the present study in both cytomorphometric analysis and bacterial assessment. As far as cytology is considered, the number of cells analysed cytomorphometrically were 20 per slide whereas in other similar studies **(Ogden et al, 1989., Khandelwal et al, 2010., HG Ahmed 2011)**, 50 cells were examined for each smear. This was due to less yield of cells as the smears in our study were made from the saliva of patients using manual liquid based cytology technique whereas in other studies scrape smears were taken from the buccal mucosa. In the bacterial cultures, an improvised culture method was used in which a normal candle jar sealed with paraffin to prevent entry of air into the jar with anaerobic gas pack was used instead of McIntosh Filde's jar. Inspite of these limitations, the morphometric findings and bacterial assessment in this study is in agreement with other studies in oral squamous cell carcinoma subjects, using one or more of the parameters studied.

A search of the English language literature for combined cytomorphometric analysis and bacterial assessment in either oral squamous cell carcinoma or malignancy of any other region yielded no results (Search engine: google and pubmed, key words: cytomorphometry, bacteria, carcinoma, malignancy). Cytomorphometric analyses in various studies of oral squamous cell carcinoma were done in cells obtained by scrape smears from oral mucosal surfaces and not from exfoliated cells in saliva. Also, though bacterial assessment has been done in oral squamous cell carcinoma, there are no studies which quantify specific bacteria at various stages of radiotherapy. Hence, the findings in the present study using salivary samples with quantification of specific bacteria combined with cytomorphometric analysis in oral squamous cell carcinoma patients await further elucidation as no published articles are available for comparison at present.

SUMMARY

The present study was carried out in 30 patients diagnosed with oral squamous cell carcinoma planned for radiotherapy (study group) and 30 normal healthy individuals(control group). Salivary samples were collected from control group subjects and from study group subjects before and during radiotherapy. These samples were smeared and stained with PAP and examined for cytomorphometric changes using image analysis software (ProgRes Capture Pro 2.7) and the results tabulated. The samples were also cultured for three bacteria namely Streptococcus mitis, Prevotella melaninogenica and Capnocytophaga gingivalis and the counts tabulated. All the results were statistically analysed.

The analyses showed the following findings.

- There was statistically significant increase in the mean nuclear diameter (p value – 0.021), nuclear area (p value – 0.017) and nuclear cytoplasmic ratio (p value – 0.000) in the study subjects compared to normal subjects
- There was statistically significant decrease in cell diameter (p value – 0.01)and cytoplasmic area(p value – 0.011) in the study subjects compared to normal healthy subjects
- Of the five parameters of the cells(nuclear diameter, nuclear area, cell diameter, cytoplasmic area, nuclear cytoplasmic ratio) analysed in the study subjects at three stages, only nuclear cytoplasmic ratio showed a significant difference between values obtained before radiotherapy and at completion of 30 Gy (p value – 0.033)and 60 Gy (p value – 0.02)

- There was a statistically significant increase in the mean count of all the three species of bacteria in the study subjects before radiotherapy (stage I), compared to the control subjects (p values-0.000)
- There was a statistically significant decrease with increasing radiation dosage in the counts of all the three bacteria cultured
    - Streptococcus mitis- between stage I and II, stage II and III, stage I and III- p values 0.000 in each case
    - Prevotella melaninogenica - between stage I and II -p value 0.000, stage II and III p value- 0.001, stage I and III- p value 0.000
    - Capnocytophaga gingivalis - between stage I and II, stage I and III- p values 0.000 and stage II and III – 0.001

CONCLUSION

The changes in the cells during radiotherapy can be ascertained through cytomorphometric analysis which has made exfoliative cytology a reliable technique. The present study showed an increase in mean nuclear cytoplasmic ratio due to increased nuclear diameter and area and decrease in cytoplasmic area and cell diameter, in squamous cell carcinoma patients undergoing radiotherapy. This cytomorphometric change can be ascertained in patients undergoing radiotherapy to confirm a good radiation response. Also, there is a decreased culture counts of anaerobic bacteria Prevotella melaninogenica and Capnocytophaga gingivalis and facultative anaerobe Streptococcus mitis with increasing radiation dosage. This can be useful in the antibiotic therapy given in irradiated patients as infection is the cause for death in many cancer patients. This present study appears to be the first to quantify specific salivary bacteria during radiotherapy and to combine cytomorphometric analysis in oral squamous cell carcinoma patients. More studies with greater sample size are needed to know the predictive value of the above findings.

BIBLIOGRAPHY

- Abu Shara KA et al. Radiotherapeutic effect on oropharyngeal flora in patients with head and neck cancer. The Journal of Laryngology and Otology, 1993: 107; 222-227.
- Acharya S, Tayaar SA, Khwaja T. Cytomorphometricanalysis of the keratinocytes obtained from clinically normal buccal mucosa in chronic gutkha chewers. J Cranio Max Dis 2013;2:134-41.
- Agarwal D, Khan N, Siddhiqui SA, Afroz N. Assessment of various cytological changes for predicting radiosensitivity of oral cavity cancer by serial cytology. JK science in Oct-Dec 2011; 13(4):171-5.
- Ahmed HG, Al Elemirri D. Assessment of Oral Cytological Changes associated with exposure to chemotherapy and/or radiotherapy. Cyto Journal. 2009;6:8.
- Ahmed HG, Babiker AA. Assessment of Cytological Atypia, AgNOR and Nuclear area in Epithelial cells of normal oral mucosa exposed to Toombak and Smoking. Rare Tumors 2009;1(18):28-30.
- Ahmed, H. G., Diab, W. M. I., Abdulgafar, S. A., & Al-Hazimi, A. M. Role of quantitative nuclear cytomorphometric and NOR dots count in prediction of carcinogenic induced oral cellular proliferative activity. Oncocytology, 2011;1, 1-6.
- Anttila T, Koskela P, Leinonen M, Laukkanen P, Hakulinen T, Lehtinen M, *et al*. Chlamydia pneumoniae infection and the risk of female early-onset lung cancer. Int J Cancer 2003;107:681-2.

- Anuradha A, Sivapathasundram B. Image analysis of normal exfoliated gingival cells. Indian J Dent Res 2007;18:63-6
- Barret AP, Burkley DJ, Greenberg L, Michael J Earl. The value of exfoliative cytology in the diagnosis of oral herpes simplex infection in immunosuppressed patients. Oral Surg Oral Med Oral Pathol 1986; 62:175-78.
- Bayar S, Gold JS, Salem RR. Association of Streptococcus bovis bacteremia with colonic neoplasia and extracolonic malignancy. Arch Surg. 2004; 139: 760-5.
- Beale LS. Examination of sputum from case of cancer of the pharynx and adjacent parts. Arch Med 1860; 2: 44–6.
- Bhattathiri NV, Bharathykkutty C, Prathopan R, Chirayathmanjiyil DA, Nair MK. Prediction of radiosensitivity of oral cancers by serial cytological assay of nuclear changes. *Radiother Oncol* 1998; 49(1): 61-65.
- Bhattathiri NV, Bindu L, Remani P, Chandralekha B, Nair KM. 5. Radiation-induced acute immediate nuclear abnormalities in oral cancer cells: serial cytologic evaluation. Acta Cytol. 1998;42:1084-90.
- Biarc J, Nguyen IS, Pini A, et al (2004). Carcinogenic properties of proteins with pro-inflammatory activity from Streptococcus infantarius (formerly S. bovis). Carcinogenesis, 25, 1477-84.

- Biswal BM, Othman NH. Correlation of nuclear morphology and AgNOR score with radiation response in squamous cell cancers of the head and neck: A preliminary study. Malaysian Journal of Medical Sciences 2010: 17(3); 19-26.
- Bjorn Stenkvist, Sighild Westman-Naeser, Jan Holmquist. Computerized Nuclear Morphometry as an Objective Method for Characterizing Human Cancer Cell Populations. Cancer Res 1978; 38:4688-97
- Blaser MJ. Understanding microbe-induced cancers. Cancer Prev Res. 2008:1940–6207. CAPR-1908–0024.
- Cançado RP, Yurgel LS, Filho MS. Comparative analysis between the smoking habit frequency and the nucleolar organizer region associated protein in exfoliative cytology of smokers' normal buccal mucosa. Tob Induc Dis, 2004;15, 2(1): 43–49.
- Chocolatewala N, Chaturvedi P, Desale R. The role of bacteria in oral cancer. Indian J Med Paediatr Oncol 2010;31:126-31.
- Cowpe JG, Longmore RB, Green MW. Quantitative exfoliative cytology of abnormal oral mucosal smears. *J R Soc Med* 1988;81:509-13.
- Cowpe JG, Longmore RB, Green MW. Quantitative exfoliative cytologyof normal oral squames: An age, site and sex related survey. J R Soc Med 1985;78:995-1004.
- Cowpe JG. Quantitative exfoliative cytology of normal and abnormal oral mucosal squames: Preliminary communication. J R Soc Med 1984;77:928-31.

- Crowe SE. *Helicobacter* infection, chronic inflammation, and the development of malignancy. Curr Opin Gastroenterol 2005; **21**:32–8.
- D Maraki, J. Becker, A. Boecking. Cytologic and DNA-Cytometric very early diagnosis of oral cancer. J Oral Pathol Med 2004; 33: 398-404.
- D'Costa J, Saranath D, Dedhia P, Sanghvi V, Mehta AR. Detection of HPV-16 genome in human oral cancers and potentially malignant lesions from India. Oral Oncol 1998;34:413-20.
- DD Rosa, AC Pasqualotto, DW Denning Chronic mucocutaneous candidiasis and oesophageal cancer Med Mycol August, 28 (2007), pp. 1–7
- Dehio C. Bartonella-host-cell interactions and vascular tumour formation. Nat Rev Microbiol. 2005; 3:621–631.
- Dutta U, Garg PK, Kumar R, and Tandon RK. (2000). Typhoid carriers among patients with gallstones are at increased risk for carcinoma of the gallbladder. Am. J. Gastroenterol. 95:784-787.
- Einstein TB, Sivapathasundharam B. Cytomorphometric analysis of the buccal mucosa of tobacco users. Indian J DentRes 2005;16:42-6.
- Frost JK. Pathologic processes affecting from inflammation to cancer. Comprhensive cytopathology. 2nd ed. Philadelphia, USA: WB Saunders Company; 1997. p. 84-5.
- Gaetti-Jardim Junior, Elerson et al. Occurrence of yeasts, pseudomonads and enteric bacteria in the oral cavity of patients undergoing head and neck radiotherapy. Braz. J. Microbiol. [online]. 2011, 42 (3);1047-55 .

- Goregen M, Akgul HM, Gundogdu C. The cytomorphologicalanalysis of buccal mucosa cells in smokers. Turk J Med Sci 2011;41:205-10.
- Graham RM. The effect of radiation on vaginal cells in cervical 2. carcinoma; description of cellular changes. Surg Gynecol Obstet. 1947;84:153-65.
- Greenberg, Sprigs. Clinical and histologic changes of the oral mucosa in pernicious anemia. J Oral Surg 1981;52:38-42.
- Gupta J, Gupta K.K., Tiwari V, Karki R. Bacterial Stint in Oral Squamous Cell Carcinoma: A Review. Indian J Dent Sci. 2012 :5(4)
- Hande AH, Chaudhary MS. Cytomorphometric analysis of buccal mucosa of tobacco chewers. Rom J Morphol Embryol 2010;51:527-32.
- Hu YJ, Wang Q, Jiang YT et al (2013) Characterization of oral bacterial diversity of irradiated patients by high-throughput sequencing. Int J Oral Sci 5:21–25
- Jiro Inagaki et.al Causes of Death in Cancer patients. Cancer 1974:33; 568-573
- Joshi PS, Kaijkar MS. Cytomorphometric Analysis of Oral Premalignant and Malignant Lesions Using Feulgen Stain and Exfoliative Brush Cytology.. J Interdiscipl Histopathol. 2013; 1(4): 204-211.
- Kamath MP et al. Indian Journal of Otolaryngology and Head and Neck Surgery. 2002:54(2); 111-114
- Katz J, Wallet S, Cha S. Periodontal disease and the oral-systemic connection: "is it all the RAGE?". Quintessence Int. 2010 Mar;41(3):229-37.

- Khandelwal S, Solomon MC. Cytomorphological analysis of keratinocytes in oral smears from tobacco users and oral squamous cell carcinoma lesions - A histochemical approach. Int J Oral Sci 2010;2:45-52.
- Koyi H, Branden E, Gnarpe J, Gnarpe H, Steen B. An association between chronic infection with Chlamydia pneumoniae and lung cancer. A prospective 2-year study. APMIS 2001;109:572 – 80.
- Kumaresan G.D , Jagannathan N, Exfoliative Cytology – A Predictive Diagnostic Tool. International Journal of Pharmacy and Pharmaceutical Sciences 6 (5), 2014,1-3.
- Kumari R, Chaugule A, Goyal PK. Karyoanomalic frequency during radiation therapy. *J Cancer Res Ther* 2005;1:187-90.
- Kurago Z, Lam-ubol A, Stetsenko A, De La Mater C, Chen Y, Dawson D. Lipopolysaccharide squamous cell carcinoma-monocyte interactions induce cancer-supporting factors leading to rapid STAT3 activation. Head Neck Pathol. 2008; 2:1–12.
- Kuyama, K. , Matsumoto, T. , Morikawa, M. , Fukatsu, A. , Ichimura, M. , Wakami, M. , Fukumoto, M. , Kato, T. and Yamamoto, H. (2013) Morphometrical findings among dysplasias of oral, cervical and bronchial regions. *Open Journal of Stomatology*, **3**, 215-222.
- Lara-Tejero M, Galán JE. A bacterial toxin that controls cell cycle progression as a deoxyribonuclease I-like protein. Science 2000;290:354-7.
- Lax AJ, Thomas W. How bacteria could cause cancer: one step at a time. Trends Microbiol. 2002;10:293–299.

- Lax AJ. Bacterial toxins and cancer – case to answer? Nat Rev Microbiol. 2005;3:343–9.
- Lijinsky W, Saavedra JE, Reuber MD, Singer SS. Esophageal carcinogenesis in F344 rats by nitrosomethylethylamines substituted in the ethyl group. J Natl Cancer Inst. 1982;68:681–4.
- Littman AJ, Jackson LA, Vaughan TL. Chlamydia pneumoniae and lung cancer: epidemiologic evidence. Cancer Epidemiol Biomarkers Prev. 2005; 14:773–778.
- L. Kun et al. Impact of Radiotherapy on oral microflora in Oral carcinoma patients. seq 369 Oral diseases and oral cancer. 2005.
- Mager DL, Haffajee AD, Devlin PM, Norris CM, Posner MR, Goodson JM. The salivary microbiota as a diagnostic indicator of oral cancer: A descriptive, non-randomized study of cancer-free and oral squamous cell carcinoma subjects. J Transl Med. 2005; 3:27.
- Martin, D. & Gutkind, J.S. (2008). Human tumor-associated viruses and new insights into the molecular mechanisms of cancer. Oncogene, 27 Suppl 2, S31-42.
- Mehrotra R, Goel N, Singh M, Kumar D. Radiation-related cytological changes in oral malignant cells. *Indian J Pathol Microbiol* 2004;47: 343-47.
- Mehrotra R, Gupta A, Singh M, Ibrahim R. Application of cytology and molecular biology in diagnosing premalignant or malignant oral lesions. Mol Cancer. 2006; 5:11.

- Meurman JH et al. Five year follow up dtudy of saliva, mutans Streptococci, Lactobacilli and yeast counts in Lymphoma patients. Oral Oncol. 1997;33(6):439-43.
- Montalban, C., Santon, A., Boixeda, D. & Bellas, C. (2001) Regression of gastric high grade mucosa associated lymphoid tissue (MALT) lymphoma after *Helicobacter pylori* eradication. Gut, **49**, 584–587.
- Morrison LF, Hopp ES, Wu R. Diagnosis of malignancy of the nasopharynx. Cytological studies by the smear technic. Ann Otol Rhinol Laryngol 1949; 58: 18–32.
- Nadaf A, Bavle RM, Thambiah LJ, Paremala K,Sudhakara M, Soumya M. A phase contrast cytomorphometric study of squames of normal oral mucosa and oral leukoplakia: Original study. J Oral Maxillofac Pathol 2014;18:32-8.
- Nagy KN, Sondoki I, Nagy SE, Newman HN. The microflora associated with human oral carcinomas. Oral Oncol 1998;34:304-8.
- Naib, Z.M and Wills D. Cytological Examination Review Book, Vol. 1 (3rd edition). Little Brown and Company, Boston, pp 556-60
- Newman JV, Kosaka T, Sheppard BJ, Fox JG, Schauer DB. Bacterial infection promotes colon tumorigenesis in Apc(−/+) mice. J Infect Dis. 2001; 184:227–230.
- Ogden GR et al. Effect of radiotherapy on oral mucosa assessed by quantitative exfoliative cytology. J Clin Pathol 1989;42(9):940-943.
- Ogden GR, Cowpe JG, Green NW. Detection of field change in oral cancer using oral exfoliative cytologic study. Cancer. 1991; 68: 1611-5.

- Ogden GR, Cowpe JG, Wight AJ. Oral exfoliative cytology: Review of methods of assessment. *J Oral Pathol Med* 1997;26:201-05.
- Ogden GR, McQueen S, Chisholm DM, Lane EB. Keratin profiles of normal and malignant oral mucosa using exfoliative cytology. *J Clin Pathol* 1993;46:352-56.
- Panghal et al.: Incidence and risk factors for infection in oral cancer patients undergoing different treatments protocols. BMC Oral Health 2012 12:22.
- Papanicolaou GN, Traut HF: The diagnostic value of vaginal smears in carcinoma of uterus. Am J Obstet Gynecol 1941;1942:193-205.
- Parsonnet J. Bacterial infection as a cause of cancer. Environ Health Perspect. 1995;103:263–8
- Paul W. Montgomery. A study of exfoliative cytology of normal human oral mucosa. J Dent Res 1951;30(1):12-18.
- Pushalkar et al.: Comparison of oral microbiota in tumor and non-tumor tissues of patients with oral squamous cell carcinoma. BMC Microbiology 2012 12:144.
- Raj VM et al. Dose response relationship of nuclear changes with fractionated radiotherapy in assessing radiosensitivity of oral squamous cell carcinoma. J Clin Exp Dent. 2011; 3 (3): e193-200.
- Ramaesh T, Ratnatunga N, Mendis BR, Rajapaksa S. Exfoliative cytology in screening for malignant and premalignant lesions in the buccal mucosa. *Ceylon Med J* 1998;43:206-09.

- Ramesh T, Mendis BR, Ratnatunga N, Thattil RO. The effect of tobacco smoking and of betel chewing with tobacco on the buccal mucosa: a cytomorphometric analysis. J Oral Pathol Med 1999; 28(9):385-8.
- Rao VP, Poutahidis T, Fox JG, Erdman SE. Breast cancer: should gastrointestinal bacteria be on our radar screen? Cancer Res. 2007; 67:847–850.
- Rao VP, Poutahidis T, Ge Z, et al. Innate immune inflammatory response against enteric bacteria Helicobacter hepaticus induces mammary adenocarcinoma in mice. Cancer Res. 2006; 66:7395–7400.
- Raymond W.Monto, Rafeal A.Rizek, Gerald Fine. Observation on the exfoliative cytology and histology of oral mucous membranes in iron deficiency . Oral Surg Oral Med Oral Pathol 1961; 14(8):965-74.
- S. Mulki, P Shetty, P. Pai, Cytomorphological analysis in oral squamous cell carcinoma lesions and normal controls using rub and rinse technique. Clin Cancer Investig J, 3, 2014, 38-42.
- Salaspuro MP. Acetaldehyde, microbes, and cancer of the digestive tract. Crit Rev Clin Lab Sci 2003;40:183-208.
- Sciubba JJ, Goldenberg D Oral complications of radiotherapy. Lancet Oncol 2006;7:175-83.
- Shao ZY, Tang ZS, Yan C, et al. Effects of intensity-modulated radiotherapy on human oral microflora. J Radiat Res. 2011;52 6:834–839.

- Sharma P, Kumar N, Bahadur AK, Shukla DK: Quantitative analysis of radiation-associated cellular changes in oral cancer and their correlations with histologic grade and clinical stage: A multivariate evaluation of 43 patients. Anal Quant Cytol Histol 2005;27:111–117.
- Sheu BC, Chang WC, Cheng CY, et al (2008). Cytokine regulation networks in the cancer microenvironment. Front Biosci, 13, 6255-68
- Shukla VK, Singh H, Pandey M, Upadhyay SK, Nath G. Carcinoma of the Gallbladder - Is it a sequel of typhoid? Dig Dis Sci. 2000; 45:900–903.
- Silverman S, Sheline GE, Gillooly CJ. Radiation therapy and oral carcinoma. Radiation response and exfoliative cytology. Cancer 1967;20:1297-300.
- Sivapathasundharam B, Kalasagar MY. Yet another article on exfoliative cytology. J Oral Maxillofac Pathol, 2004;8(2): 54–57.
- Smitha T, Sharada P, Girish HC. Morphometry of the basal cell layer of oral leukoplakia and oral squamous cell carcinoma using computer-aided image analysis. J Oral Maxillofac Pathol 2011;15:26-33.
- Sugerman PB, Savage NW. Exfoliative cytology in clinical oral pathology. Austral Dent J 1996; 41(2): 71-3.
- Tran N, Rose B, O'Brien CJ. Role of HPV in the etiology of Head and Neck Cancer. Head Neck Oncol 2007;29:64-70.
- Veda Hedge. Cytomorphometric analysis of squames from oral premalignant and malignant lesions. J Clin Exp Dent 2011; 3(5):e441-4
- Vogelmann R, Amieva MR. The role of bacterial pathogens in cancer. Curr Opin Microbiol. 2007;10:76–81.

- Wagenlehner FM, Elkahwaji JE, Algaba F, Bjerklund-Johansen T, Naber KG, Hartung R, Weidner W. The role of inflammation and infection in the pathogenesis of prostate carcinoma. BJU Int. 2007a;100:733–737.
- Wagenlehner FME, Weidner W, Naber KG. Therapy for prostatitis, with emphasis on bacterial prostatitis. Expert Opin Pharmacother. 2007b; 8:1667–1674.
- Ward JM, Fox JG, Anver MR, et al. Chronic Active Hepatitis and Associated Liver Tumors in Mice Caused by a Presistent Bacterial Infection With a Novel Helicobacter Species. J Natl Cancer Inst. 1994; 86:1222–1227.
- Zheng JW, Qiu WL, Zhang ZY. Combined and sequential treatment 1. of oral and maxillofacial malignancies: an evolving concept and clinical protocol. Chin Med J (Engl). 2008;121:1945-52.
- Ziskin DE, Kamen P, Kittay I. Epithelial Smears of the Oral Mucosa. J Dent Res 1940; 20:386-94.

Annexure

Mean values for nuclear area in 30 study subjects (3 stages) and 30 controls

S.No	Stage I	Stage II	Stage III	Control
1	104.23	103.65	80.94	69.47
2	91.34	86.98	95.87	57.83
3	66.71	104.64	85.94	71.50
4	85.35	76.21	128.33	52.55
5	66.21	87.80	75.98	63.19
6	61.67	106.31	102.16	58.51
7	66.27	71.86	76.97	59.72
8	63.26	63.08	70.68	62.93
9	65.56	90.43	64.58	65.76
10	68.56	101.21	69.84	61.33
11	63.70	61.65	70.60	51.06
12	67.75	77.56	66.12	69.30
13	59.86	76.39	66.09	90.85
14	76.61	79.68	70.46	58.31
15	69.11	83.27	65.28	54.91
16	66.87	59.33	59.38	64.12
17	58.12	63.58	77.60	68.65
18	90.37	66.12	85.38	62.67
19	77.23	59.43	67.22	61.09
20	61.68	74.56	62.49	57.36
21	60.02	71.28	70.60	55.92
22	75.14	56.95	56.30	57.18
23	59.39	76.80	46.84	63.51
24	74.21	69.07	62.47	63.39
25	63.63	86.31	71.68	63.22
26	56.25	75.83	44.67	66.35
27	71.55	48.72	50.80	73.96
28	63.06	48.35	48.43	63.89
29	55.54	70.03	82.86	62.85
30	62.41	76.56	72.04	57.81

Mean values for cytoplasmic area in 30 study subjects(3 stages) and 30 controls

S.No	Stage I	Stage II	Stage III	Control
1	3,833.03	5,621.64	2,837.46	2,363.20
2	3,337.57	2,024.64	2,285.60	1,890.87
3	2,234.27	3,083.51	2,852.60	2,147.20
4	2,725.36	2,062.85	2,296.46	1,952.28
5	1,989.45	2,871.14	3,140.55	2,004.23
6	2,380.68	2,706.67	2,184.18	2,383.76
7	1,894.06	1,992.77	1,745.09	2,391.40
8	2,120.89	1,877.88	1,973.15	2,354.49
9	2,622.17	3,016.41	1,982.31	2,168.51
10	1,626.47	2,420.61	2,381.10	2,327.93
11	2,012.24	1,814.29	1,724.13	2,055.51
12	2,487.13	2,153.71	1,801.46	2,561.22
13	1,926.29	1,781.93	2,086.15	2,692.87
14	2,581.79	2,546.74	2,517.31	2,151.88
15	1,732.97	2,140.16	2,127.46	1,716.05
16	1,413.85	1,566.63	1,559.45	2,209.25
17	1,510.78	1,282.13	1,490.84	2,794.03
18	1,987.57	1,587.42	1,567.66	2,003.00
19	1,951.35	1,187.61	1,551.68	2,192.23
20	1,569.86	1,437.88	1,740.44	2,118.31
21	1,629.21	1,965.61	1,967.26	2,060.56
22	2,111.65	2,270.86	1,850.45	2,063.08
23	1,920.39	1,625.17	919.11	2,149.17
24	2,359.88	2,138.42	1,350.30	2,354.10
25	1,871.24	2,258.56	2,411.67	2,251.18
26	1,558.44	1,911.01	1,045.65	2,378.04
27	1,719.82	1,242.58	1,279.18	2,599.24
28	2,008.87	1,231.55	1,231.43	2,159.21
29	1,541.32	2,166.68	2,356.08	2,414.44
30	1,423.22	2,152.53	1,464.83	2,097.20

Mean values for nuclear diameter in 30 study subjects (3 stages) and 30 controls

S.No	Stage I	Stage II	Stage III	Control
1	11.17	11.43	10.27	9.42
2	10.51	11.82	10.68	8.36
3	9.01	11.44	10.49	10.02
4	10.40	9.59	12.43	8.44
5	9.22	10.57	9.99	9.29
6	8.95	11.78	11.40	8.78
7	9.24	9.51	9.98	8.90
8	9.21	9.01	9.51	9.16
9	9.22	10.82	9.18	9.34
10	9.33	11.29	9.51	9.16
11	9.11	8.94	9.58	8.23
12	9.46	9.86	9.18	9.64
13	9.06	9.99	9.38	10.84
14	10.09	10.05	9.52	9.00
15	9.56	10.16	9.42	8.47
16	9.27	8.89	8.76	8.93
17	8.73	8.98	9.86	9.43
18	10.68	9.30	10.43	9.04
19	10.06	9.03	9.36	8.85
20	8.93	9.77	9.25	8.83
21	8.96	9.72	9.78	8.65
22	9.90	8.70	8.76	8.87
23	9.00	9.73	7.91	9.15
24	10.02	9.49	8.90	9.35
25	9.23	10.47	9.58	9.17
26	8.59	9.81	7.55	9.35
27	9.72	7.96	8.18	9.79
28	9.24	8.05	8.04	8.88
29	8.73	9.45	10.37	9.06
30	9.25	9.95	9.56	8.67

Mean values for cell diameter in 30 study subjects (3 stages) and 30 controls

S.No	Stage I	Stage II	Stage III	Control
1	67.86	85.94	59.96	55.86
2	67.59	57.06	55.91	49.92
3	54.69	63.38	61.44	55.71
4	60.46	51.97	55.66	53.48
5	51.59	60.73	63.58	53.76
6	55.77	60.16	55.93	55.81
7	50.79	49.83	48.82	54.66
8	52.86	49.91	50.76	55.59
9	58.91	64.05	50.53	53.97
10	47.23	55.91	56.22	56.01
11	51.05	48.78	48.62	52.01
12	56.76	53.90	49.39	56.58
13	50.35	48.79	52.86	58.38
14	57.45	58.40	57.27	51.71
15	48.01	53.07	53.46	47.67
16	43.28	45.00	43.91	52.86
17	45.84	41.32	44.20	60.99
18	50.03	46.28	46.43	54.05
19	49.74	40.54	45.88	54.33
20	46.21	44.07	47.87	53.35
21	46.70	49.68	52.04	52.48
22	53.57	54.01	49.72	52.30
23	50.83	46.05	34.98	53.97
24	56.78	53.00	42.18	55.85
25	50.22	54.25	56.42	54.15
26	45.68	48.86	37.41	56.07
27	46.72	40.47	41.05	58.41
28	50.43	40.82	39.97	53.30
29	46.14	54.10	54.27	55.42
30	44.06	52.17	45.34	52.31

Mean values for nuclear cytoplasmic ratio in 30 study subjects(3 stages) and 30 controls

S.No	Stage I	Stage II	Stage III	Control
1	0.03	0.02	0.03	0.03
2	0.03	0.05	0.05	0.03
3	0.03	0.04	0.03	0.03
4	0.03	0.04	0.06	0.03
5	0.03	0.03	0.03	0.03
6	0.03	0.04	0.05	0.03
7	0.04	0.04	0.05	0.03
8	0.03	0.04	0.04	0.03
9	0.03	0.03	0.03	0.03
10	0.04	0.04	0.03	0.03
11	0.03	0.04	0.04	0.03
12	0.03	0.04	0.04	0.03
13	0.03	0.05	0.03	0.03
14	0.03	0.03	0.03	0.03
15	0.04	0.04	0.03	0.03
16	0.05	0.04	0.04	0.03
17	0.04	0.05	0.05	0.03
18	0.05	0.04	0.06	0.03
19	0.04	0.05	0.04	0.03
20	0.04	0.05	0.04	0.03
21	0.04	0.04	0.04	0.03
22	0.04	0.03	0.03	0.03
23	0.03	0.05	0.05	0.03
24	0.03	0.04	0.05	0.03
25	0.04	0.04	0.03	0.03
26	0.04	0.04	0.04	0.03
27	0.04	0.04	0.04	0.03
28	0.03	0.04	0.04	0.03
29	0.04	0.03	0.04	0.03
30	0.05	0.04	0.05	0.03

Annexure

Mean values for count of S.mitis CFU in 30 study subjects(3 stages) and 30 controls

S.No	Stage I	Stage II	Stage III	Control
1	280	240	208	126
2	330	160	133	28
3	283	212	209	201
4	386	260	223	160
5	228	171	161	46
6	313	292	260	58
7	242	231	193	63
8	211	201	192	106
9	293	271	182	42
10	209	168	151	103
11	282	263	143	221
12	283	174	169	183
13	194	179	142	262
14	238	212	173	205
15	289	262	245	212
16	261	244	202	195
17	189	103	110	253
18	294	281	193	199
19	194	181	160	100
20	312	294	216	288
21	168	172	133	138
22	305	293	124	289
23	169	171	138	157
24	176	139	112	107
25	306	269	221	189
26	260	242	146	151
27	169	120	93	177
28	184	146	121	103
29	193	168	127	238
30	171	147	131	243

Mean values for count of P.melaninogenica CFU in 30 study subjects(3 stages) and 30 controls

S.No	Stage I	Stage II	Stage III	Control
1	54	42	11	33
2	53	45	38	37
3	62	23	16	22
4	83	48	38	9
5	62	40	35	20
6	74	54	26	58
7	54	33	41	9
8	29	18	16	10
9	54	33	23	19
10	67	29	24	14
11	63	49	42	16
12	34	33	27	12
13	43	26	23	32
14	23	27	15	10
15	39	28	19	14
16	49	31	48	15
17	38	24	21	29
18	46	39	23	29
19	26	23	18	24
20	34	21	23	30
21	79	42	36	18
22	31	28	13	35
23	46	39	21	23
24	94	69	38	17
25	42	36	31	26
26	55	41	24	21
27	38	19	32	27
28	28	21	16	17
29	59	41	36	34
30	21	16	15	29

Mean values for count of C.gingivalis CFU in 30 study subjects(3 stages) and 30 controls

S.No	Stage I	Stage II	Stage III	Control
1	11	5	3	3
2	6	5	4	2
3	8	5	4	4
4	14	13	8	3
5	15	7	4	4
6	7	6	4	3
7	7	4	4	2
8	8	3	3	3
9	7	5	2	3
10	6	6	5	3
11	8	5	2	1
12	7	5	2	3
13	7	5	5	4
14	5	4	4	2
15	17	16	9	1
16	7	4	6	4
17	4	4	4	3
18	4	4	2	2
19	6	3	5	2
20	8	7	4	3
21	9	5	3	4
22	7	5	5	5
23	8	9	4	3
24	8	5	5	2
25	5	4	6	1
26	8	6	7	3
27	10	6	2	3
28	6	7	4	1
29	8	8	7	4
30	6	6	5	3