Queries

Query No. 1: No mention of OSMF in the introduction part.

Ans: Introduction about OSMF is added on page no. 1 paragraph 2

Query No. 2: Tobacco chewing is a habit, whereas OSMF is a disease. How can these two be compared.

Ans: Tobacco chewing is one of the etiological factor for oral potentially malignant Disorders (OMPDs) like OSMF. In the present study, an attempt has been made to evaluate the association of levels of salivary thiocyanate and presence of micronuclei between tobacco cheweing- a habit and OSMF- a disease. As per review of literature,this is the first study carried out to study the correlation of levels of salivary thiocyanate with occurrence of number of micronuclei in tobacco chewers and OSMF Patients.

Query No.3: It makes no sense to put pictures of basic instruments and chemical bottles. This is waste of time and eye wash.

Ans: It is as per MUHS guidelines for dissertation writing.

Query No.4: The details of statistical test used for analysis of the data has been described in 12 pages, which is again a waste of time.

Ans: It is as per comparative groups and as per MUHS guidelines for dissertation writing.

Query No.5: The dissertation lacks photomicrographs of cytological of three groups

Ans: The dissertation is having cytological photomicrographs of Micronuclei in tobacco chewers and in OSMF patients. Control group photograph added.on page no.35

Query No. 6: The author is not clear about etiology of OSMF.

Ans: OSMF has multifactorial etiology. Numerous studies have been conducted to assess the main etiological factor causing this debilating disease. Oral habits like tobacco chewing in the form of betel quid are the most prevalent in Indian population. Other etiological factors like arecanut chewing ,betenut,capsicum,chillies etc.

Query No.7: The author is not clear about disease caused by tobacco consumption.

Ans: The tobacco use is one of the greatest threat to the global health today. Developing countries already account for half of all death related to tobacco. Tobacco use is one of the risk factor to several general chronic diseases like lung cancer, chronic bronchitis and oral diseases like OSMF and we are concern about oral diseases.

Query No. 8: In tobacco chewers , the author has reported the increase in number of micronuclei and also increased in the levels of salivary thiocyanate. Then why there were no clinical manifestations.

Ans: Group B (Tobacco chewers without any lesions) selected in such a way that indivuals having tobacco chewing habit without any lesion(as per inclusion crerteria)Hence there are no clinical manifestations. Increase in levels of salivary thiocyante and increase in number of micronuclei is not associated with clinical manifestations.It is only a biochemical findings and cytological findings whether these can be used as biomarker.

Query No.9: The basic concept of study is irrevalant.

Ans: As explained in ques No.8 from research point of view, levels of salivary thiocyanate in tobacco chewers and OSMF patients is compared only for biochemical purpose and assessing micronuclei for possible tissue damage.

Title of synopsis approved by MUHS, Nashik.

Query No.10: There are observable flaws in bibliography, for example ref no.9 and 12 are repeat refrences, ref no.23 is incomplelety written.

Ans: Refrences has been corrected. Reference no .23 which becomes Ref..No.22 is completely written.

ESTIMATION AND CORRELATION OF THE LEVELS OF SALIVARY THIOCYANATE WITH OCCURRENCE OF MICRONUCLEI IN TOBACCO CHEWERS & ORAL SUBMUCOUS FIBROSIS PATIENTS- A BIOCHEMICAL AND CYTOLOGICAL STUDY.

DISSERTATION SUBMITTED TO

MAHARASHTRA UNIVERSITY OF HEALTH SCIENCES, NASHIK

IN THE PARTIAL FULFILLMENT OF REGULATIONS FOR THE AWARD

OF THE DEGREE OF

MDS

IN

ORAL PATHOLOGY & MICROBIOLOGY

BRANCH VI

2021

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LIST OF ABBREVIATIONS

Abbreviation	Full Form
OSMF	Oral submucous fibrosis
SCN	Salivary thicyanate
MN	Micronuclei
PMDs	Potentially malignant disorders
OSCC	Oral squamous cell carcinoma
H_2O_2	Hydrogen Peroxidase
ClO-	Hypochlorite
mm	Millimeters
WHO	World Health Organization
ICD	Indian Statistical Classification of Diseases
GATS	Global Adult Tobacco Survey
ANOVA	Analysis of variance
SD	Standard deviation
P-value	Probability value
NS	Statistically not significant
HS	Statistically Highly significant
S	Statistically significant
ES	Effect Size

INTRODUCTION

Oral cancer is a serious and growing problem in many parts of the globe. In high risk countries such as Sri Lanka, India, Pakistan & Bangladesh oral cancer is the most common cancer in men & may contribute upto to 25% of all new cases of cancer.[21] Many oral cancer are preceded by clinically evident premalignant mucosal changes that give a warning of risk and present an opportunity for detection and preventive measures. The key to diagnosis is the early detection of mucosal changes that may represent disease and which are not variations to normal. Early dectection of potentially malignant disorders like oral submucous fibrosis promise to improve the survival and the morbidity of patients suffering from these conditions.[20]

Oral submucous fibrosis(OSMF) is an insidious, chronic disease which was first described in the early 1950s. It is characterized by changes in the connective tissue fibres of the lamina propria and deeper part leading to stiffness of the mucosa

and restricted mouth opening.[22] The etiology of OSMF is multifactorial. OSMF can lead into squamous cell carcinoma, a risk that is further increased by concomitant tobacco consumption .[2]

Tobacco use is one of the single most leading cause of preventable deaths globally causing more than 5 millions death/year.[1,2] Tobacco consumption is positively correlated with the accumulation of DNA damage, and exposure to tobacco-related chemical carcinogens is proved to have direct damaging effects on the cellular DNA in the oral cavity[4] . Tobacco is known to decrease the body's immune response and is a well-known cause for the development of lesions like oral submucous fibrosis which is a potentially malignant disorder, and can cause significant morbidity and mortality in patients.[1]

Salivary thiocyanate which is a metabolic product of cyanide, is an anion found in organic and inorganic compounds. It is a normal constituent of body fluids such as serum, saliva, urine and tears. Range of thiocyanate concentration in normal group is from 0.5 to 2mM with an average of 1mM.[3]Thiocyanate ion is derived endogenously as a detoxification product of the reaction between cyanide and thiosulfates in the liver. Determinig thiocyanate (SCN) levels in saliva is one of the biochemical test for establishing the incidence or prevalence of tobacco consumption.[12] Direct contact between saliva and tobacco makes a measurement of salivary thiocyanate a simple, non-invasive alternative to serum and tissue testings.[4]

High levels of thiocyanate which are present in tobacco can cause structural alterations in the DNA of target cells, leading to genomic instability in the form of chromosomal abnormalities & it can be observed as changes in chromosomal

structure, chromosomal number, sister chromatid exchange and **MICRONUCLEI(MN)**.[4] The consequences of carcinogenic insult to the oral tissues result in chromosomal damage in early cell divisions which can be examined in routine cytopathological smears as micronuclei, before the development of clinical manifestations. The buccal cell micronucleus (MN) assay was first proposed in 1983 and it continues to gain popularity as a biomarker of genetic damage in numerous applications.[9] These are extranuclear cytoplasmic bodies induced in the oral exfoliated cells by a variety of substances including genotoxic agents in tobacco smoke.[4] Cytological study of oral cells is a nonaggressive technique that is well accepted by the patient,and its application in early diagnosis of potentially malignant disorder(PMDs) is well established.[20]

In addition, establishment of a correlation between the salivary thiocyanate levels and cytological changes in the form of MN may pave the path for its future use as a biomarker in prevalence studies and screening procedures and thus may improve the treatment plan, management, and prognosis of the patient to a greater extent[4].

The present study aimed to estimate levels of salivary thiocyanate with occurance of micronuclei in tobacco chewers and OSMF patients.

AIM & OBJECTIVES

The present study will be an attempt to estimate and correlate the levels of salivary thiocyanate with occurance of micronuclei in tobacco chewers and Oral submucous fibrosis(OSMF) patients with the following aim:

AIM

To estimate and correlate the levels of salivary thiocyanate with occurrence of micronuclei in tobacco chewers and oral submucous fibrosis patients using exfoliative cytology.

The aim will be fulfilled with help of following objectives:

Objectives:

- To estimate and compare the levels of **salivary thiocyanate** among **control group**, **tobacco chewers** group and group of **oral submucous fibrosis** patients.

- To evaluate & compare the **micronuclei** in oral mucosal epithelium using **cytological smear** of **control group**, **tobacco chewers** group and group of **oral submucous fibrosis** patients.

- To correlate the levels of **salivary thiocyanate** with **micronuclei** in **control group**, **tobacco chewers** group and group of **oral submucous fibrosis** patients.

REVIEW OF LITERATURE

Oral cancer is one of most common causes of morbidity and mortality nowdays. In developing countries, both smoking and smokeless tobacco have cancer causing behaviour that continues to be increasing the global burden of oral cancer. The World Health Organization (WHO) estimated that the proportion of deaths that result from tobacco related diseases would rise in India from 1.4% of all in 1990 to 13.3% of all deaths in 2020. According to a report of Economic and Social Council, the models presented in 2002 showed that the number of persons consuming tobacco is also likely to rise. The majority of the oral cancers preceded by the potentially malignant lesion sand conditions (Potentially Malignant Disorders PMDs). These lesion clinically show premalignant mucosal changes that give a warning of risk at hand an opportunity for detection and preventive measures.[6]

The regular use of tobacco slowly deteriorates the general health of a person which is found to be a common cause of tobacco addiction, which leads to illness,

disability and death of a person.[19] The person who consumes more tobacco is at a greater risk for developing oral precancer like condition (mostly OSMF), oral cancer.[19]

Salivary tests have the advantages of easy, noninvasive sampling and have good stability. The main significance is half-life of SCN is 14 days. A salivary SCN levels can be easily accessed by a simple biochemical test that can play a critical role in indicating the incidence or prevalence rate of tobacco consumption. A salivary SCN levels can be easily accessed by a simple biochemical test that can play a critical role in indicating the incidence or prevalence rate of tobacco consumption. It can be used as biochemical indicator in the evaluation of potentially Malignant disorders (PMDs).[19]

Very few studies had been carried out in this regards, hence this study is carried out with the objective to find the correlation of salivary thiocyanate level with micronuclei in tobacco chewers and OSMF patients.

Salivary Thiocyanate

Thiocyanate is an end product of detoxification of hydrogen cyanide present in tobacco and tobacco products. Human saliva is a complex biofluid excreted by the salivary glands. Saliva composition includes air, water, proteins, peptides, amino acids, hormones, electrolytes, lipids, and other substances.[18] Thiocyanate is generated in the human organism during the digestion of food, mainly vegetables of the cabbage family as well as when drugs are ingested for the treatment of thyroid and hypertension.[18] In liver, tobacco consumption leads to the production of a toxic gas hydrogen cyanide (HCN), whose detoxification reaction between cyanide and

thiosulfate leads to production of salivary thiocyanate (SCN).[12,19] The SCN, which is produced in the liver is distributed to all of the extracellular fluids like saliva, cerebrospinal fluid, blood plasma, gastric juices and is slowly eliminated from the body through the urine excretion.[19] They prevent toxic accumulations of hydrogen peroxide (H_2O_2) and hypochlorite (ClO−), which may be carcinogenic or mutagenic.[3] The half life of SCN is reported to be approximately 14 days, that makes it as a reliable biomarker that confirms tobacco consumption characteristics in the population.[12] The thiocyanate concentration in saliva is a biochemical measure frequently used as an objective indicator of tobacco consumption.[5]

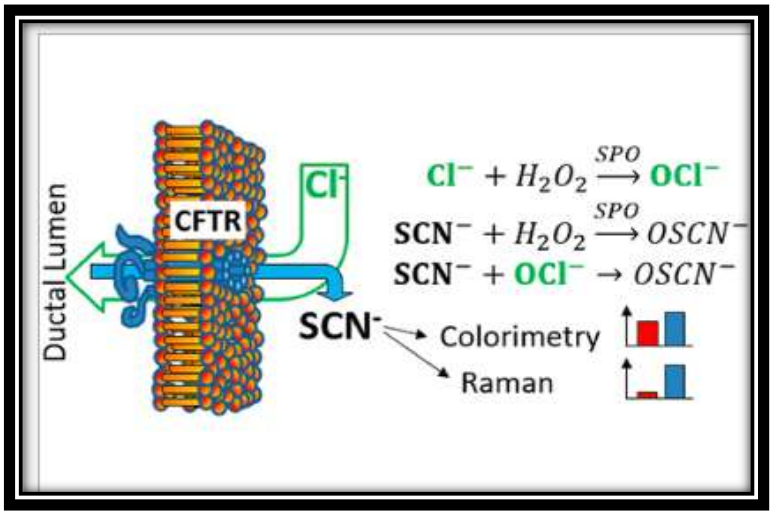


Fig No.1 Raman Spectroscopy Signature For SCN^- Provides An Indicator For Cystic Fibrosis Transmembrane Conduntance Regulator(CFTR) Function.[33]

Micronuclei

Micronuclei (MN) is a recently upgraded topic especially in the field of oral cancer. MN takes origin from chromosomes fragments or whole chromosomes, which lag behind at anaphase during nuclear divison. It could be argued that MN in

exfoliated oral epithelial cells represent a preferred target site for early genotoxic events induced by carcinogenic agents.[25]

Tobacco-specific nitrosamines have been reported to be potent calstogenic and mutagenic agents which are thought to be responsible for induction of chromatid/chromosomal aberration resulting in the production of micronuclei(MN). Oral cancer is characterized by complex karyotypes that involve many chromosomal deletions, translocations, and structural abnormalities.[9] Due to its association with chromosomal aberrations, MN has been used since 1937 as an indicator of genotoxic exposure, based on the radiation studies conducted by **Brenneke and Mather.**[16] The assay is reliable and technically easy to perform. The direct correlation between the MN formation and genomic damage make the MN assay an efficient alteration to the metaphase analysis.[6]

Early diagnosis of a potentially malignant lesions and sometimes cancerous lesions may improve the survival and the morbidity of patients, micronuclei (MN) are good prognostic indicators.[17]

Densen et al (1967)[13]: Studied the applicability to field epidemiologic studies of analysis of thiocyanate concentrations in one or more body fluids. Among 7 smokers than in that of 6 non-smokers were volunteers from the New York City Health Department; the remaining six smokers came from two other health organizations. They found that significantly greater concentrations of thiocyanate are found in urine, saliva & serum of smokers than in that of non-smokers.

Leupker et al (1981)[10]: Studied chemical measures of smoking habits among 1,419 eighth grade students. In that group 59.4% students admitted to regular smoking

of one pack/week had thiocyanide grater or equal to 100microgram/ml compared to 2.3% of non smokers at that level. It is found that chemical measures such as saliva thiocyanate shows promise to obtain accurate, quantitive information on smoking habits. It is not helpful in detecting very low level smokers, such as experimenters or those smoking only a few cigarrettess per month. It can be effectively used as an epidemilogial tool to evaluate smoking prevalance in populations and their changes overtime.

Tolbert et al (1991)[30]: Studied protocol for exfoliated micronucleus assay was field tested in a population exposed to genotoxic agent, snuff, at levels associated with significant increase in cancer risk among 38 female snuff users and female 15 nonusers recruited from a North Carolina Clinic in 1987. It is found that nuclear degenerative phenomenon can be difficult to distinguish from classical micronuclei.

Tsuge et al (2000)[8]: Studied the cyanide & thiocyanide levels were determined by head space gas chromatography & spectrophotometric koing method respectively among40 healthy adult volunteers. They found that there was statistical correlation were observed between the blood cyanide and the salivary thiocyanate levels. Then, they concluded that not only plasma and salivary thiocyanate levels but also blood cyanide level can be suitable indices for distinguishing smokers from nonsmkers.

Casartelli et al (2000)[35]: Studied the frequency of micronuclei was determined in exfoliated cells from normal oral mucosa, a preneoplastic condition (leukoplakia) and precancerous lesions with and without dysplasia, squamous cell carcinomas and sites of previous carcinomas that had been removed. They found that

micronucleus frequencies were increased in precancerous lesions as compared to normal mucosa and further increased in carcinomas, suggesting that micronuclei are a biomarker of neoplastic progression in this type of cancer. The micronucleus frequency did not vary with the sex or age of patients, while it did vary with the anatomic site of the lesions.

Sun et al (2000)[37]: Studied the micronuclei of exfoliated oral mucosa cells and oral mucosa cells in 119 patients comprising 59 simple hyperplasia, 32 mild and moderate oral leukoplakia and 28 severe oral leukoplakia and oral squamous carcinoma by Feulgen stain method were examined, and micronuclei of exfoliated oral mucosa cells in 100 normal persons were also examined. They found that Frequency of micronucleated cells of exfoliated oral mucosa cells in oral leukoplakia lesions was higher than that of normal persons.

Gimenez et al (2003)[5]: Studied the influence of salivary activity in evaluating adolescent tobacco consumption by determining the level of thiocyanate in saliva among 592 students aged 14–17 years. The results of a stepwise regression analysis did not show any significant effect of the salivary activity on SCN levels.

Halder et al (2004)[21]: studied the exfoliated oral mucosal cell micronuclei frequency among 50 patients with precancerous or malignant oral epithelial lesions were compared with 50 age and sex matched healthy controls without any oral lesions. They found that MN frequency was increased in preoperative cancer cases and decreased in postoperative cases, while in precancerous cases it was higher than in the controls.

Kamboj et al (2007)[38]: Studied the micronucleus assay among (group I) consisted of ten subjects with no abnormal oral habits and normal appearing mucosa between the age group of 15–20 years, 25 each of leukoplakia (group II) and SCC (group III). They found that showed a significant increase in the mean percentage micronucleated cells both in group II and group III when compared with group I in fluorescent and conventional staining.

Palve et al in (2008)[17]: Studied the clinic pathological correlation of micronuclei among 30 patients having oral squamous cell carcinoma with an age range of 24 to 75 years and 20 healthy control subjects, age and sex matched. They found that micronuclei frequency were found higher in squamous cell carcinoma patients than control subjects.

Palaskar et al (2010)[9]: Studied evaluation of micronuclei using Papanicolaou (PAP) and May Grunwald Giemsa stain in individuals with different tobacco habits among a total of 45 male subjects (15 smokers, 15 smokeless tobacco users and 15 non users/ non smokers). They found that Pap is a better stain as compared to MGG for counting micronuclei. Smokeless tobacco chewers showed an increased number of MNs as compared to the smokers, thus laying emphasis on the greater carcinogenic potential of tobacco which was used in the chewable form.

Anila et al (2011)[21]: Studied the frequency of micronucleated cells and micronuclei among 20 oral submucous fibrosis patients and to compare with 20 healthy individuals. They found that micronucleated cells and micronuclei in oral submucous fibrosis patients were statistically significantly elevated as compared to control group.

Jadhav et al (2011)[25]: Studied that to correlate the frequency of micronuclei in oral exfoliated cells among 16 clinically diagnosed cases of OSCC and 16 healthy subjects without any tobacco consumption habits formed control group. The cytosmears of both group were stained with rapid Papanicolaou stain. They found that the frequency of micronuclei three or four times in patients with OSCC as compared to patients in the control group and the difference was found to be highly significant.

Oliveria et al (2011)[40]: Evaluated the frequency of micronuclei in exfoliated cells from oral lesions previously identified by toluidine blue among d 20 removable prosthesis users with white lesions that were previously classified as toluidine positive or negative. They found that significant increase in the frequency of MN was observed in exfoliated cells from lesions compared to normal mucosal cells, and no relationship was seen with Toluidine blue (TB) staining.

Bansal et al (2012)[41]: Evaluated the micronuclei in exfoliated oral mucosal cells in individuals using various tobacco forms from the last 5 years among 75 healthy male subjects (25 smokeless tobacco users, 25 smokers, and 25 non-tobacco users) . They found that MN cells were found to be significantly higher in smokeless tobacco users than in smokers and controls.

Grover et al (2014)[20]: Studied diagnostic accuracy of micronuclei assay in Potentially malignant disorders of oral cavity among 30 controls with healthy mucosa and 45 cases as a patients with suspicious lesions, clinically diagnosed as potentially malignant disorders of the oral cavity were taken and separately stained with Papanicolaou(Pap) stain and Hematoxylin and eosin(H & E) stain. Micronuclei(MN) frequency was evaluated and based on the 50^{Th} percentile of all the MN(%) frequency

values obtained for both the groups. They found that the sensitivity and specificity with Pap stain was found to be 84% and 93% respectively, with diagnostic accuracy of 88% whereas with H & E stain, Sensitivity of 89% and specificity of 100% with diagnostic accuracy of 93% was observed.

Kalburgi et al (2014)[15]: Estimated, compare, and correlate the SCN levels in periodontally healthy, CP, smokers with CP and gutka chewers with CP subjects among 120 subjects with age 18-55 years, categorized as periodontally healthy (*n* = 30), CP (*n* = 30), smokers (*n* = 30), and gutka chewers (*n* = 30) .They found that the significant increase in salivary SCN levels among smokers and gutka chewers when compared to others, concluding that the analysis of salivary SCN levels could be used as an adjunctive means of diagnosis.

Katarkar et al (2014)[39]: studied the comparison the micronucleus (MN) assay in oral buccal mucosa cells with the comet assay in peripheral blood cells among 260 participants, including those with oral lichen planus (OLP; n = 52), leukoplakia (LPK; n = 51), oral submucous fibrosis (OSF; n = 51), oral squamous cell carcinoma (OSCC; n = 54) and normal volunteers (n = 52). They found that LPK shows significantly higher mean MNi frequency compared with OLP and OSMF.

Pradeep et al (2014)[43]: Studied the identification and quantification of micronuclei in the exfoliated cells of oral mucosa among 135 individuals with different tobacco related habits & buccal smears of 45 age and sex matched controls were obtained, stained using Giemsa stain and then observed under 100X magnification in order to identify and quantify micronuclei in the exfoliated cells of

oral mucosa. They found that MN count was more even in other groups when compared to normal control but to a lesser extent.

Pullishery et al (2015)[12]: Studied the salivary thiocyante, uric acid level, PH tobacco users and non users of age 35-44 years with their periodontal status. They found that presence of higher amount salivary thiocyanate level in tobacco users as compared to non tobacco users.

Hegde et al (2016)[3]: Carried out Estimation and correlation of salivary thiocyanate levels in periodontally healthy subjects, smokers, non-smokers, and gutka-chewers with chronic periodontitis among 40 systemically healthy subjects in the age group of 18–55 years that were further divided into four groups: Control, smokers, nonsmokers and gutka-chewers with chronic periodontitis and found that a significant increase in SCN levels in smokers with periodontitis as compared to non-smokers. They concluded that SCN can act as a reliable biochemical indicator for assessing smoking behaviour and possible tissue damage.

Kumar et al (2016)[14]: Studied the genotoxic effect of smoking and chewing tobacco on target tissue using Mn assay and to evaluate the prevalence of other nuclear anomalies associated with it and to determine the reliability of feulgen stain for MN assay over Papaincoloau (PAP) stain among tobacco habits (smoking and chewing) without lesion (n=30), individuals who were having tobacco habit (smoking and chewing) with PML (n=30) and apparently healthy subjects (n=30).They found that the individuals having tobacco habits (smoking and chewing) with lesion have high number of MN cells, thus supporting the assay to be used as a reliable biomarker to assess the genotoxic effect of tobacco in the oral mucosa and PAP stain can be used

to identify abnormal cytological changes resulting from mutagenic agent but not to interpret Mn.

Sangle et al (2016)[6]: Correlated the frequency of MN in oral exfoliated cells in clinically diagnosed cases of OSCC followed by a histopathological grading among 90 subjects (30 smokeless tobacco users, 30 smokers and 30 nontobacco users) consisted of clinically diagnosed cases of PMD's and OSCC were selected for the study. They found that MN index can be used as a biomarker/screening test among the high-risk groups particularly the smokeless tobacco users and PMD's. MN can be a candidate to serve as a biomarker for prediction of the grade of OSCC.

Somashekhar et al (2016)[23]: Studied the evaluation of micronuclei among 10 normal participants with no association of any habits and 10 betel quid chewers and 10 Potientially malignant disorders patients such as leukoplakia, oral lichen palnus and oral submucous fibrosis and 10 OSCC patients. They found that the median values of micronuclei were significantly higher in OSCC patients followed by Oral submucous fibrosis, Leukoplakia, Oral lichen Planus, and betel quid chewers as compared to normal participants.

Baldawa et al (2017)[4]: Evaluated the levels of salivary thiocyanate and its relation with the occurrence of micronuclei (MN) using exfoliative cytology in smokers and nonsmokers among one hundred and twenty patients by dividing them into 3 groups: nonsmoker group 1 (control) consisting of 40 patients, smokers group 2 with 4–10 filtered cigarettes per day consisting of 40 patients and smokers group 3 with 11–20 filtered cigarettes per day consisting of 40 patients. They found that as the

grade of smoking increased, the levels of salivary thiocyanate and occurrence of micronuclei increased.

Madiyal et al (2018)[1]: Studied Status of thiocyanate levels in the serum and saliva of non-smokers, ex-smokers and smokers among 20 non-smokers, 20 ex-smokers and 40 smokers. Smokers were divided into two groups based on the presence or absence of oral mucosal lesions. They found that the mean serum and salivary thiocyanate levels were increased significantly in smokers when compared to non-smokers and ex-smokers. The levels were not significantly different between ex-smokers and non-smokers and between smokers with tobacco related oral mucosal lesions and those without. Statistically significant correlation was seen between the serum and salivary levels of thiocyanate.

Prakruthi et al (2018)[11]: studied the estimation and correlation of salivary thiocyanate levels in young adult 35 smokers and 35 non-smokers and also to evaluate and correlate the cellular and nuclear changes in cytological smears with salivary thiocyanate level. They found that Salivary thiocyanate levels were significantly higher in smokers than controls and showed significant correlation with the number of pack years. Although the present study failed to reveal any significant correlation between salivary thiocyanate level and cytological alterations, few early alterations in the oral mucosa even in the absence of clinical manifestations were detected by exfoliative cytology.

Dash et al (2018)[42]: Compared the genotoxic effects of tobacco using micronuclei count among 200 individuals, divided into four groups. Group I: 50 subjects with history of tobacco chewing, group II: 50 subjects with a history of

smoking tobacco, group III: 50 subjects with a history of both tobacco chewing and smoking, and group IV: 50 subjects without any habits as controls (age-matched). They found that the significantly higher micronucleus frequency was found in smokeless tobacco users than in smokers and controls.

D'Cruz et al (2018)[2]: Estimated & compare he salivary thiocyanate levels among Smokers (25), Smokeless tobacco users (25), Passive smokers (25) and Non-Users (25). They found that Pairwise comparison of the groups showed that there was significant statistical difference among all pairs except between the passive smokers and smokeless tobacco users.

Madbhavi et al (2019)[24] : Studied the estimation and comparison of salivary thiocyante levels among 20 passive smokers and 10 active smokers aged between 18 years and 21 years, with a history of minimum of 1 cigarette/day, for minimum period of 3 months. They found that increased salivary thiocyante levels in active and passive smokers with increased number of cigarettes and duration of exposure.

Aggrawal et al (2020)[19]: Studied the comparison of salivary thiocyante among 30 clinically diagnosed case of oral leukoplakia between ages of 30 and 50 years with inclusion criteria of male gender and history of cigarette smokers with a habit of smoking 4-7 cigarettes per day for a minimum period of 4-5 years and 30 cigarettes smokers without leukoplakia group consisted of individuals with inclusion criteria of matched age and sex and history of cigarette smoking. They found that salivary thiocyante levels were found to be significantly higher in smokers with leukoplakia as compared to smokers without leukoplakia.

Benny et al (2020)[31]: Studied salivary thiocyante level among 25 smokers, 25 passive smokers , 25 smokeless tobacco users and 25 non-users. They found that Intergroup comparison showed statistically significant difference in thiocyanate levels of saliva. The passive smokers-smokeless tobacco users groups did not show any statistically significant difference.

Mohammed et al (2020)[44]: Evaluated the micronuclei scoring as a biomarker for early detection and screening of genotoxic effect of cigarette smoking in the peripheral blood T- lymphocytes among 148 individuals have participated in the study; 78 Current smokers and 70 never smokers. Cytokinesis-block micronucleus assay was performed for all the

MATERIALS & METHODS

The present study titled "Estimation and correlation of the levels of salivary thiocyanate with occurrence of micronuclei in tobacco chewers & oral submucous fibrosis patients- a biochemical and cytological study" was carried out in the Department of Oral Pathology & Microbiology as per the inclusion criteria. An informed consent was obtained from all the patients along with the explanation of the procedure that was performed.

The study was approved by the institutional ethics committee.

Type Of Study/Study Design:

Cross sectional, analytical, observational, comparative study

Study Duration: **18 months.**

MATERIALS & DATA COLLECTION TOOLS:

For collection of saliva:

- Clean-coded sterile container

For determining the levels of thiocyanate in saliva

- Test tube
- Trichloroacetic acid (20%)
- Hydrochloric acid (HCL)
- Bromine Water
- Pyridine-p-phenylenediamine
- Arsenious oxide
- Sphectrophotometer

For obtaining exfoliated cells

- Wooden spatula
- Standard slides
- Papanicolaou stain
- Binocular Microscope.

Sample Size

According to study by Baldawa PS et al. (2016), the authors obtained thiocyanate levels in patients in non-smokers, smokers with habit of 4-10 cigarettes

/day and smokers with 11-20 cigarettes /day. The descriptive statistics like mean and SD have been reported in the article. The data resulted into an effect size (ES) of 1.694. The proposed study also has three groups: normal, tobacco chewers without lesion and tobacco chewers with clinically diagnosed OSMF. Assuming above ES for the study, the estimated sample size was small. Hence, an effect size of 0.4 (the largest ES as per Cohen) was considered, which resulted into a sample of **90** (**30** in each group) was obtained, to detect the desired ES with 95% confidence and 80% power of test (F-test).

The formula used for estimation was:

$$n = \frac{(z_{1-\alpha/2\tau} + z_{1-\beta})^2}{ES^2}$$

where t is the number of possible comparisons, z 1-a/2t (2.409) is the standardized value for 5% error and for 3 paired comparisons, z 1-b (0.842) is the value for 80% power and ES is the effect size (0.4).

Sampling Technique:

The individuals will be selected randomly in each group subject to the fulfillment of inclusion and exclusion criteria and allocated as **Group A (Control Group)** and **Group B (Tobacco chewers without any lesion)** & **Group C (patients clinically diagnosed with OSMF).**

INCLUSION CRITERIA:

Group A: NORMAL PATIENTS

Sample size: 30

Inclusion criteria –

- No history of habits like tobacco or areca nut chewing, and smoking.
- Patients with no clinical lesions.

Group B: TOBACCO CHEWERS WITHOUT ANY LESION

Sample Size: 30

Inclusion criteria –

- Patients having tobacco chewing habit more than 5years & frequency more than 5 to 6 times per day.
- Tobacco containing product chewing habit.
- Patients with no clinical lesion.

Group C: OSMF patients

Sample Size: 30

Inclusion criteria –

- Patients who are clinically diagnosed with Oral Submucous fibrosis. (Irrespective of grades of OSMF)

EXCLUSION CRITERIA:

- Individuals with habits other than tobacco chewing like alcohol, smoking.
- Individuals suffering from any systemic diseases like diabetes mellitus, hypertension, salivary gland disorders .
- Patients with other potentially malignant disorders.
- Patients exposed to radiation.
- Patients with a history of any long term systemic diseases or acute infections.
- Patients who are on any medications like erythromycin, nitroprusside, chlorambucil, ifosfamide.

PLATE I

Fig No. 2 Diagnostic Instruments

Fig No.3 Sterile container for Saliva Collection

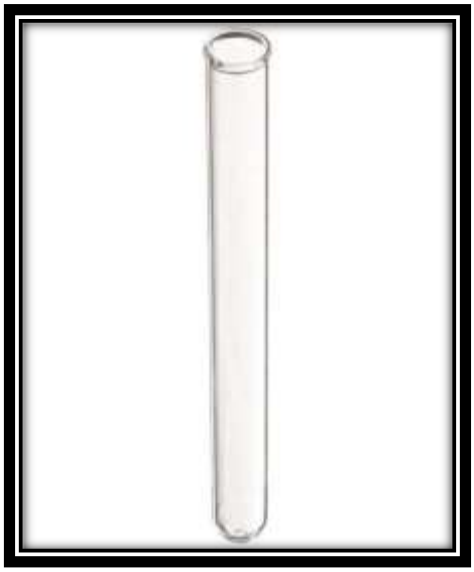


Fig No.4 Test Tube

PLATE II

Fig No. 5 Tirchloroacetic acid(20%)

Fig No. 6 Hydrochloric Acid

Fig No. 7 Bromine Water

Fig No. 8 Pyridine Phenylene amine

Fig No.9 Arsenic Trioxide

PLATE III

Fig No.10 Spectrophotometer

PLATE IV

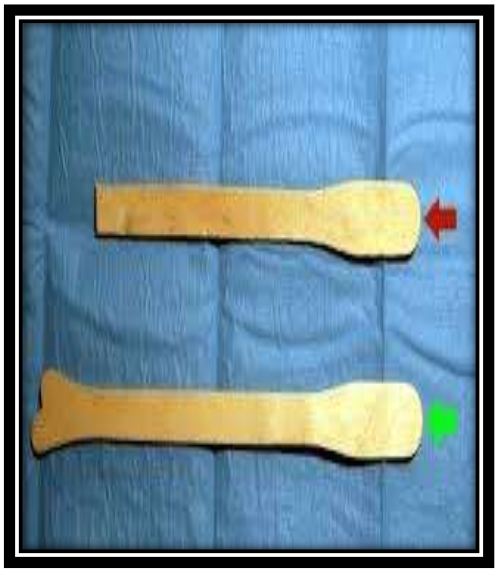


Fig No. 11 Wooden Spatula

PLATE V

Fig No. 12 Papanicolaou Stain (PAP Stain)

PLATE VI

Fig No. 13 Binocular Microscope

PLATE VII

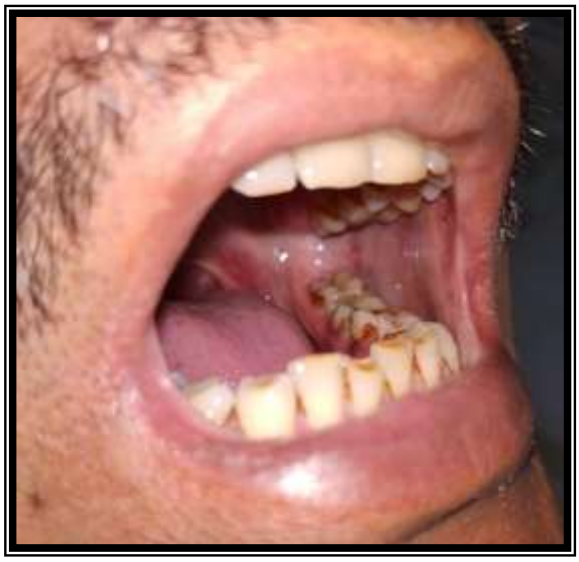
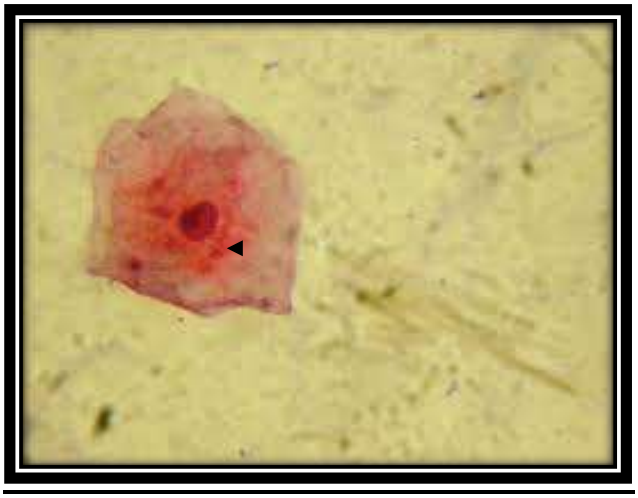
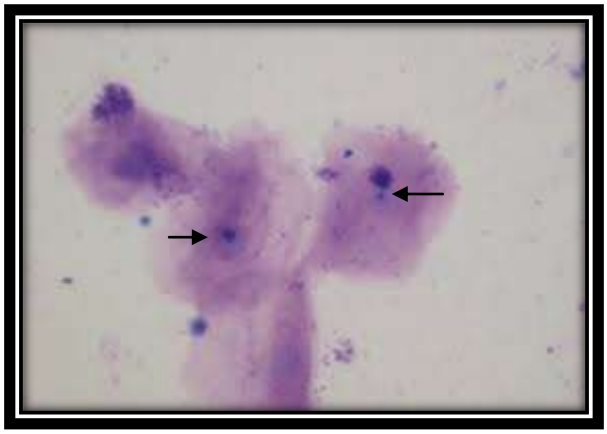


Fig No.14 Clinical Photograph Of OSMF

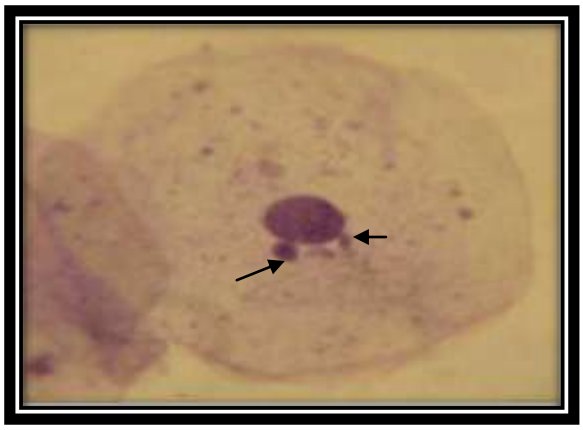
PLATE VIII



Micronuclei in control group



Micronuclei in Tobacco chewers



Micronuclei in OSMF Patients

Fig No15. Photomicrographs showing exfoliated cells with micronuclei

Papanicolaou stain -40x

Methodology

Method of collection of saliva[4]

Participants were asked to rinse their mouth gently with water, and 2 ml of unstimulated saliva was collected by spitting method in clean-coded sterile container. Saliva was then transferred into test tubes which were then frozen and stored at 4°C until analyzed for thiocyanate.

Procedure for determining the levels of thiocyanate in saliva[4]

Saliva was treated with trichloroacetic acid (20%) and ferric nitric acid reagent by the method of Denson. Twenty percent trichloroacetic acid solution was added to 1 ml of saliva in a test tube. The contents of the tube were mixed and allowed to stand for 10 min. Then, 3 ml of the filtrate was added to 2 ml of water, followed by 5 ml of ferric nitrate reagent. The color change produced was measured colorimetrically at 470 nm, and the results compared with a standard curve obtained by adding ferric nitrate solution to standard thiocyanate solutions. Using known standards of sodium thiocyanate, concentration was related to absorption.

Method of obtaining exfoliated cells[4]

Participants were asked to rinse their mouth gently with water, and oral mucosal surface scrapings were obtained using a wooden spatula. The cells were immediately smeared on pre-cleaned microscopic slides, and the smears were fixed with absolute alcohol.

Method of obtaining exfoliated cells [4]

Immediately after collecting the saliva samples buccal mucosal cells were collected using wooden spatula. Cytological smear were prepared and stained using Papanicolaou stain.

All the cytological smears were stained by papnicolaou .All the slides were observed under light microscope using low magnification (×100) for screening and high magnification (×400) for counting the MN.

Tolbert et al. developed the criteria for choosing the cells, and this is being most widely applied.

It consists of the following parameters:

- Cytoplasm intact and lying relatively flat
- Little or no overlap with adjacent cells
- Little or no debris
- Nucleus normal and intact, nuclear perimeter smooth.

This was correlated with the salivary thiocyanate levels.

Statistical Method Employed

The continuous parameter age was expressed in terms of mean, standard deviation, median and range, while the categorical parameter gender was summarized in terms of numbers and percentage for each study group. The comparison of mean salivary thiocyanate across three groups was performed using one-way analysis of variance. The pair wise comparisons were carried out using Tukey's post-hoc test.

Further, the comparison of number of micromuclei across study groups was performed using Kruskal-Wallis test and the paired comparisons was done using Wilcoxon rank sum test. Micronuclei were grouped into categories and the frequencies in each categories across groups were compared using Fisher's exact test. Also the mean salivary thiocyanate levels for each micronuclei category were compared using one-way analysis of variance. The correlation between salivary thiocyanate and micronuclei count for each group was obtained using Spearman's rank correlation coefficient.

All the analyses were performed using SPSS ver 20.0 (IBM Corp USA) and the statistical significance was tested at 5% level.

The methods used in the study are as below:

1. Measures of central tendency

If x_1, x_2,…x_n are the observations on a random variable X, then following measures of central tendency can be obtained:

- **Mean** for a set of observations is given by

$$\overline{x} = \frac{1}{n}\sum_{i=1}^{n} x_i$$

- **Median**: It is the middle value of a set of values when arranged in the increasing order of magnitude.

2. Measures of dispersion

- **Standard deviation** for a set of observations in given by

$$s = \sqrt{\frac{1}{(n-1)}\sum_{i=1}^{n}(x_i - \bar{x})^2}$$

where x_i = observation on each object

n = number of objects

- **Range** is the difference between maximum and minimum value of the variable.

3. Statistical inference tests

- One-way Analysis of variance

Analysis of variance (ANOVA) is used to test the significance of difference in the mean of three or more groups. The basic assumption is that the variable of interest is normally distributed in the population under study.

Here the interest is to test the null hypothesis that the population means are same, i.e.

$$H_0 : \mu_1 = \mu_2 = ...\mu_m$$

against the alternative H_1 that they are not same.

Some of the statistics computed to test the hypothesis are as below:

i) **Grand mean**: It is the mean of set of all observations in the studied groups and is given by:

$$\bar{x}_{GM} = \frac{1}{N}\sum_{i=1}^{N} x_i$$

ii) Total sum of squares: It is the sum of squares of each observation from the grand mean and is given by:

$$TSS = \sum_{i=1}^{N} (x_i - \bar{x}_{GM})^2$$

Total sums of squares is the sum of two components i.e., variation between groups and within groups.

iii) Between group sum of squares

$$SSB = \sum_{j=1}^{m} n_j (\bar{x}_j - \bar{x}_{GM})^2$$

iv) Within group sum of squares

$$SSW = \sum_{j=1}^{m} \sum_{i=1}^{n} (x_{ij} - \bar{x}_j)^2$$

The mean sum of squares is obtained by dividing the above sum of squares with the respective degrees of freedom, i.e. N-1, p-1 and $p(n$-1).

v) F-statistic: It is the ratio of between and within mean sum of squares

$$F = \frac{MS_{Between}}{MS_{Within}}$$

If the p-value based on F-statistic is greater than 0.05, H_0 is accepted, otherwise H_1 is accepted.

vi) Tukey's post-hoc test

After performing ANOVA, if alternative hypothesis H_1 is accepted, then the subsequent interest is to determine the pair wise significance of difference in the

means of study groups. This could be carried using Tukey's post-hoc test. The difference between the means of all groups are determined and compared with this critical difference called the honest significant difference (HSD). It is given by:

$$HSD = q\sqrt{\frac{MS_{within}}{n}}$$

where, q is the studentized range statistic derived from the tables, n is the sample size and the mean square value is from the ANOVA analysis. If the critical difference exceeds the absolute difference between any two sample means, then the corresponding means differ significantly.

Kruskal-Wallis test

The test is a non-parametric equivalent of one-way analysis of variance for comparing three or more groups. It is used for testing if the samples originate from same or different populations. The procedure for determining significance of difference across groups using the test is as below:

i) The $n_1, n_2, \ldots, n_k$ observations from k samples are combined into a single series of size n and arranged in order of magnitude from smallest to largest. The observations are then replaced by ranks from 1 assigned to smallest observation to n assigned to largest observation.

When two or more observations have same value, each observation is given a mean of the ranks for which it is tied.

ii) The ranks assigned to observations in each of the k groups are added separately to give k rank sums.

iii) The test statistic is defined as:

$$H = \frac{12}{n(n+1)} \sum_{j=1}^{k} \frac{R_j^{\ 2}}{n_j} - 3(n+1)$$

where k is the number of groups; n_j is the number of observation in j^{th} group; n is the total number of samples from all the groups and R_j is the sum of ranks from j^{th} group.

iv) When there are more than 5 observations in one or more groups, H is compared with the tabulated value of χ^2 with k-1 degrees of freedom.

Wilcoxon rank sum test

The test is a non-parametric equivalent of Student's t-test for independent samples, when the assumption of normality is violated. It evaluates the null hypothesis that the two populations are the same against alternative that particular population has larger values than the other. It involves computation of a test statistics based on ranked series. The observations are ranked according to magnitude irrespective of the two groups. The steps involved are as under:

i) Add the ranks for observations from group 1.

ii) Since sum of all ranks equal N (N+1)/2, the sum of ranks in group 2 is total sum minus the sum of group 1.

iii) A statistic U is defined as:

$$U_1 = R_1 - \frac{n_1(n_1+1)}{2}$$

where n_1 is the size of sample 1 and R_1 is the sum of ranks of sample 1. Equally valid formula for U is

$$U_2 = R_2 - \frac{n_2(n_2+1)}{2}$$

The smaller of U_1 and U_2 is for significance testing.

For large sample sizes ($N > 30$), U is approximately normally distributed, and the standardized value is given by

$$z = \frac{U - m_U}{\sigma_U}$$

where m_U and σ_U are the mean and standard deviation of U. The significance of z can be obtained from normal probability tables. Here m_U and σ_U are given by:

$$m_U = \frac{n_1 n_2}{2} \quad \sigma_U = \sqrt{\frac{n_1 n_2 (n_1 + n_2 + 1)}{12}}$$

4. Tests of association

Fisher's exact test

Fisher's exact test is a statistical test to determine if there are any non-random associations between two categorical variables.

If X and Y are the two categorical variables with m and n observed states respectively, then a m x n matrix can be generated with a_{ij} as the number of observations for i^{th} state of X and j^{th} state of Y. Accordingly, the row and the column sums R_i and C_j are

$$N = \sum_i Ri = \sum_j Cj$$

The conditional probability of getting the actual matrix given the particular row and column sums is given by

$$P_{cutoff} = \frac{(R_1!R_2!...R_m!)(C_1!C_2!...C_n!)}{N!\prod a_{ij}!}$$

This is a multivariate generalization of hypergeometric distribution. All possible matrices of non-negative integers consistent with rows and column sums are determined, and for each matrix the conditional probability using above expression is determined, such that the sum of probabilities is 1.

To determine the P-value of the test, the tables needs to be ordered by some criterion that measures dependence, and those tables that represent equal or greater deviation from independence than the observed table are the ones whose probabilities are added together. In a typical 2 x 2 case, the P-value of the test is simply the sum of P-values of matrices that are less than P_{cutoff}. In R package, the test can even be applied for *r* x *s* tables where *r* and *s* > 2.

5. Correlation

Spearman's rank correlation

The Spearman's rank correlation is defined as the Pearson's correlation coefficient between ranked variables. For n raw paired scores X_i and Y_i are converted into ranks rxi and ryi and rs is computed as:

$$r_s = \frac{\text{cov}(rg_x, rg_y)}{\sigma_{rgx}, \sigma_{rgy}}$$

If all ranks are distinct (no ties), then d_i = rgX – rgY is the difference between two ranks (i=1,2,…n). Then Spearman's rank correlation is defined as:

$$r_s = 1 - \frac{6\sum_i d_i^2}{n(n^2-1)}$$

Ethical Issue Involved-None

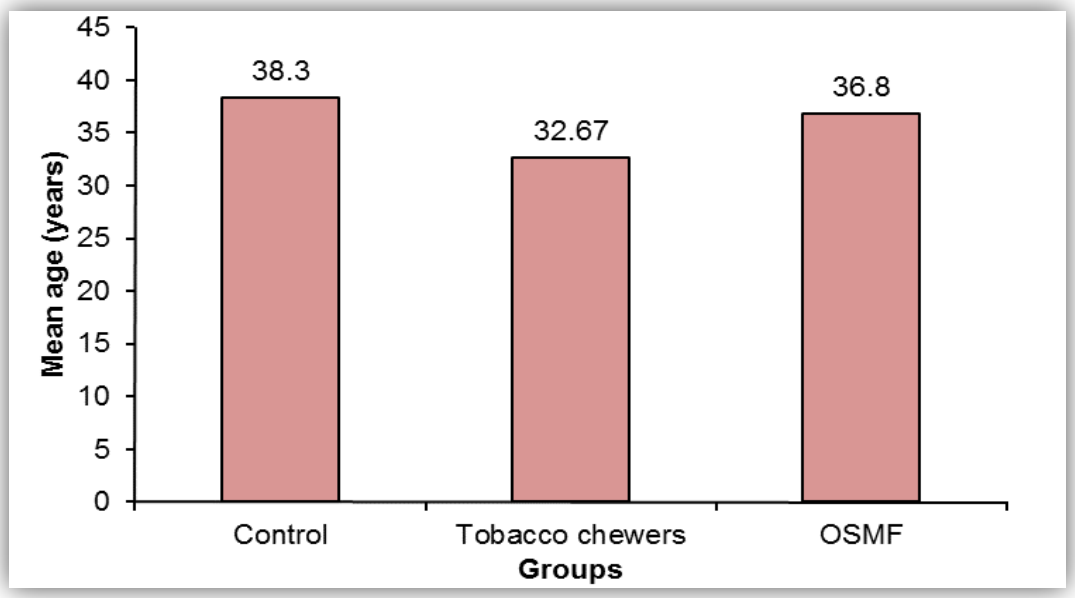
OBSERVATION & RESULTS

Table 1: Descriptive statistics for age of subjects in three study groups

Statistical parameter	Groups		
	Control	Tobacco chewers	OSMF
Mean	38.30	32.67	36.80
Median	36.50	30.00	35.00
SD	10.24	8.42	10.53
Minimum	23	22	21
Maximum	56	53	60

Table 1 provides the descriptive statistics for age of subjects included in three study groups. The mean age in the control group was 38.3 (SD: 10.24) years with minimum of 23 years and maximum 56 years. In the Tobacco chewers group, the mean was 32.67 (SD: 8.42) years with minimum of 22 years and maximum 53 years,

while in the OSMF group, the mean age was 36.8 (SD: 10.53) years with minimum age of 21 years and maximum 60 years.

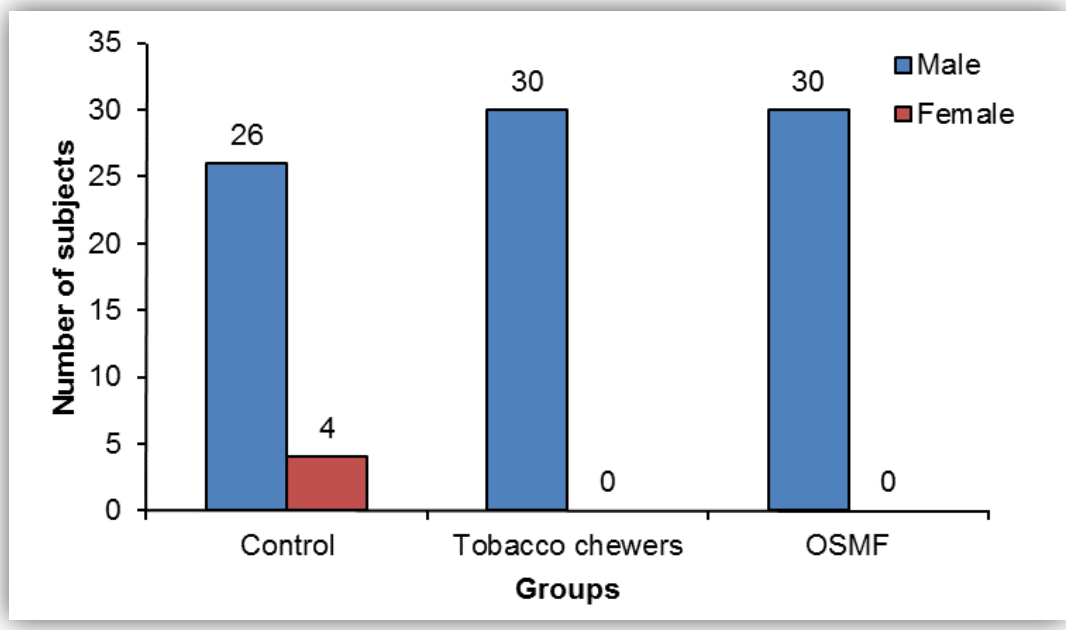


Graph 1: Column chart showing mean age of subjects in three groups

Table 2: Distribution of subjects as per sex in three study groups

Sex	Groups					
	Control		Tobacco chewers		OSMF	
	N	%	N	%	n	%
Male	26	86.7	30	100.0	30	100.0
Female	4	13.3	0	0.0	0	0.0
Total	30	100	30	100	30	100

Table 2 provides the distribution of subjects according to gender in three groups. In Control group, there were 26 (86.7%) males, while 4 (13.3%) females. In Tobacco chewers group there were 30 males and no females, similarly in SMS group there were 30 males and no females.



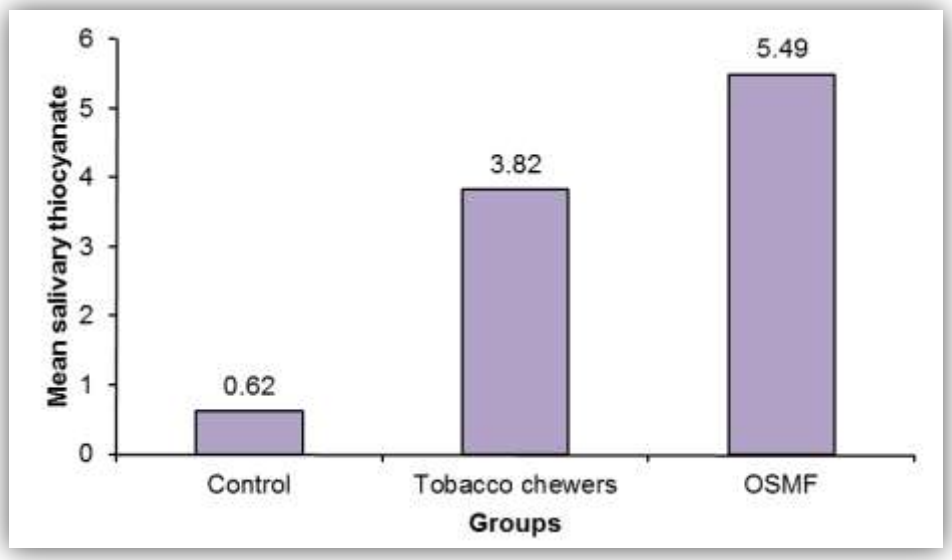
Graph 2: Column chart showing number of subjects in three groups

Table 3: Comparison of mean salivary thiocyanate levels across three groups

Group	n	Mean	SD	Median	P-value*
Control	30	0.62	0.31	0.56	< 0.0001 (HS)
Tobacco chewers	30	3.82	0.53	3.69	
OSMF	30	5.49	0.43	5.36	

*Obtained using one-way ANOVA; HS: Highly significant

Table 3 provides the comparison of salivary thiocyanate levels across three groups. The mean level in Control group was 0.62 (SD: 0.31), while in Tobacco chewer's group was 3.82 (SD: 0.53) and in OSMF group was 5.49 (SD: 0.43). The comparison of means across three groups was performed using one-way analysis of variance and resulted into a p-value < 0.0001, suggesting highly significant difference of means across groups. The paired comparison of means between groups using Tukey's post-hoc test also resulted into p-values < 0.0001, suggesting highly significant difference between each paired comparison.



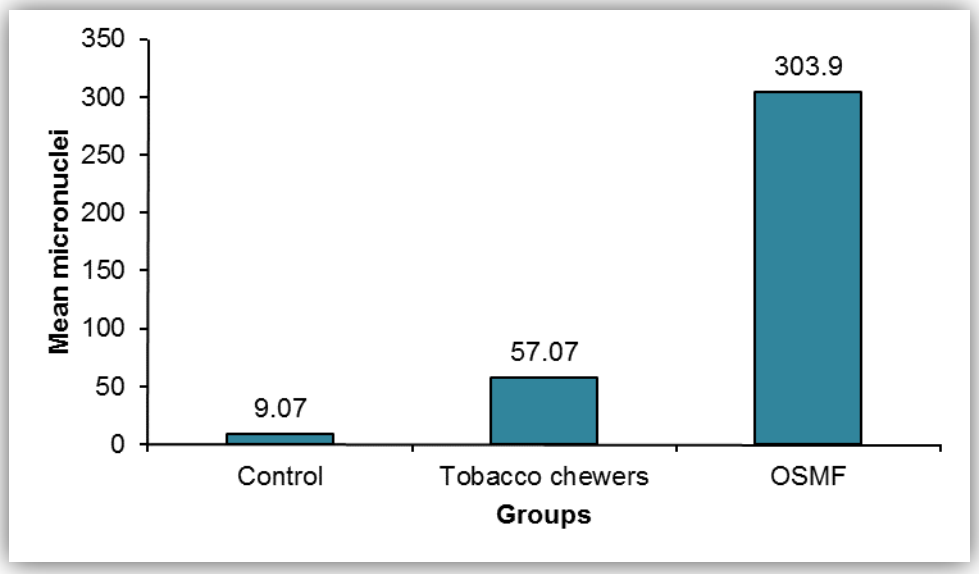
Graph 3: Column chart showing mean salivary thiocyanate Levels in three study groups

Table 4: Comparison of number of micronuclei across three groups

Group	n	Mean	SD	Median	P-value*
Control	30	9.07	7.93	8.50	< 0.0001 (HS)
Tobacco chewers	30	57.07	17.68	55.00	
OSMF	30	303.90	178.50	245.00	

*Obtained using Kruskal-Wallis test; HS: Highly significant

Table 4 provides the comparison of number of micronuclei across three groups using Kruskal-Wallis test. The mean in the Control group was 9.07 (SD: 7.93) and median 8.5, while in the Tobacco chewer's group, the mean was 57.07 (SD: 17.68) and median 55. In the OSMF group, the mean was 303.90 (SD: 178.5) and median 245. The difference in the distribution of micronuclei across three groups was statistically significant with a p-value < 0.0001. The paired comparison using Wilcoxon rank sum test with Bonferroni correction also resulted into statistically significant difference of micronuclei between groups with p-value < 0.0001.



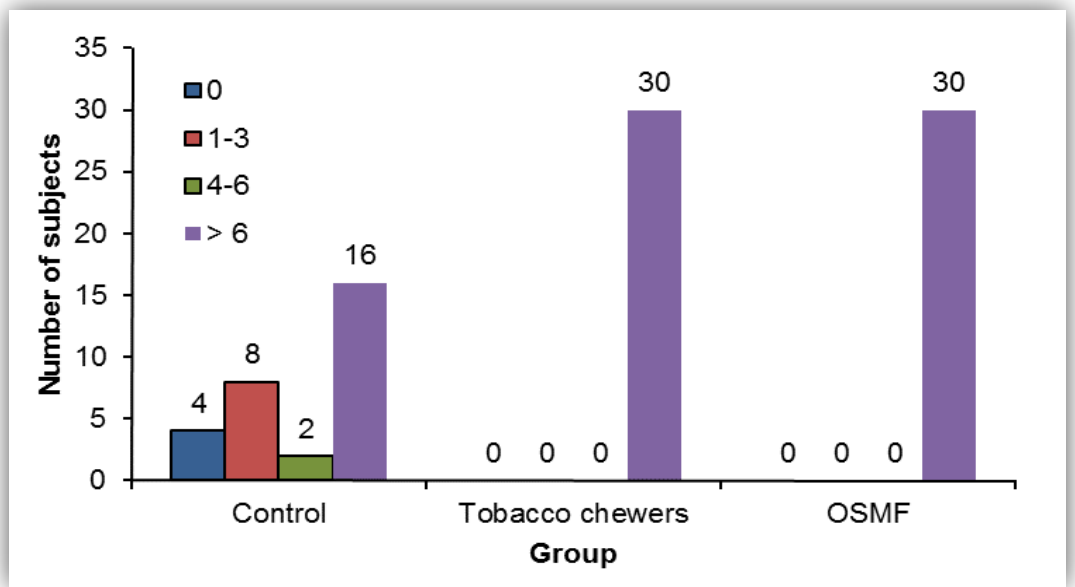
Graph 4: Column chart showing mean number of micronuclei in three study groups

Table 5: Distribution of subjects as per micronuclei in three study groups

Micronuclei group	Groups					
	Control		Tobacco chewers		OSMF	
	n	%	n	%	n	%
0	4	13.3%	0	0.0%	0	0.0%
1-3	8	26.7%	0	0.0%	0	0.0%
4-6	2	6.7%	0	0.0%	0	0.0%
> 6	16	53.3%	30	100.0%	30	100.0%
P-value* < 0.0001 (HS)						

*Obtained using Fisher's exact test; HS: Highly significant

Table 5 provides the number of subjects according to micronuclei categories in three study groups. In the control group, there were maximum 16 (53.3%) subjects with micronuclei > 6, followed by 8 (26.7%) in the range 1-3, 2 (6.7%) in the range of 4-6 and there were 4 (13.3%) cases with absence of micronuclei. In the Tobacco chewer's group as well as OSMF group, all the subjects had micronuclei count above 6. The comparison of the distribution of count across three groups showed statistically highly significant difference using Fisher's exact test as indicated by p-value < 0.0001.



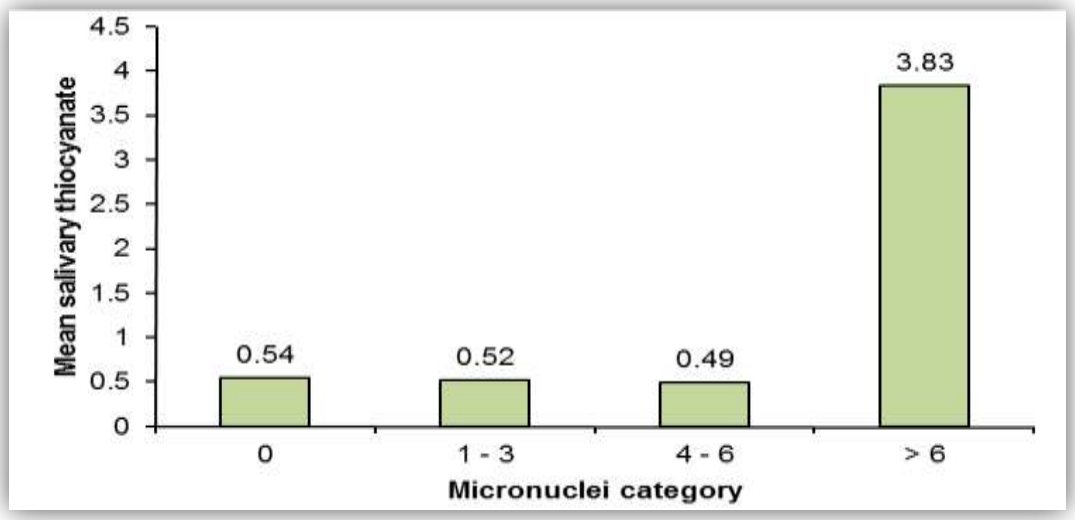
Graph 5: Column chart showing number of subjects as per micronuclei in each group

Table 6: Comparison of mean salivary thiocyanate levels across micronuclei categories

Micronuclei category	N	Mean	SD	Median	P-value*
0	4	0.54	0.22	0.51	< 0.0001 (HS)
1 – 3	8	0.52	0.33	0.48	
4 – 6	2	0.49	0.23	0.49	
> 6	76	3.83	1.84	4.19	

*Obtained using one-way ANOVA; HS: Highly significant

Table 6 gives the mean salivary thiocyanate levels for each micronuclei category. The mean corresponding to 0 micronuclei was 0.54 (SD: 0.22), for 1-3 category, the mean was 0.52 (SD: 0.33), for category 4-6, the mean was 0.49 (SD: 0.23) and for the category > 6, the mean was 3.83 (SD: 1.84). The difference in the mean levels of thiocyanate across categories was statistically highly significant with p-value < 0.0001 using one-way analysis of variance. The paired analysis using Tukey's test revealed statistically significant difference of >6 category from other categories.



Graph 6: Column chart showing mean salivary thiocyanate Levels according to micronuclei categories

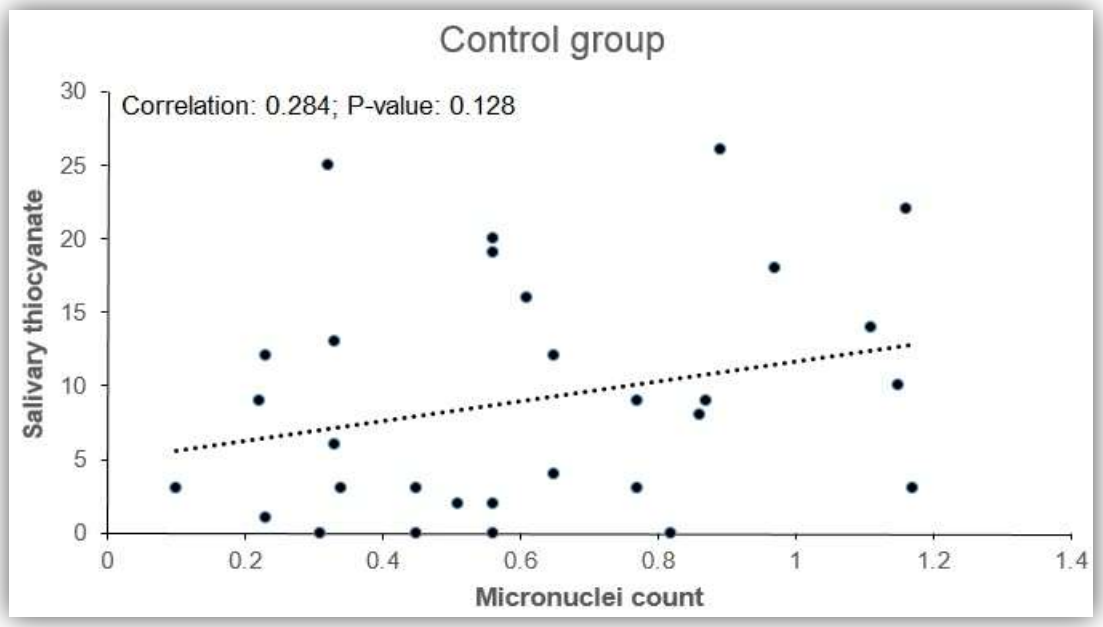
Table 7: Correlation of salivary thiocyanate Levels with micronuclei count in each study group

Group	N	Correlation coefficient*	P-value
Control	30	0.284	0.128 (NS)
Tobacco chewers	30	0.118	0.536 (NS)
OSMF	30	0.066	0.728 (NS)

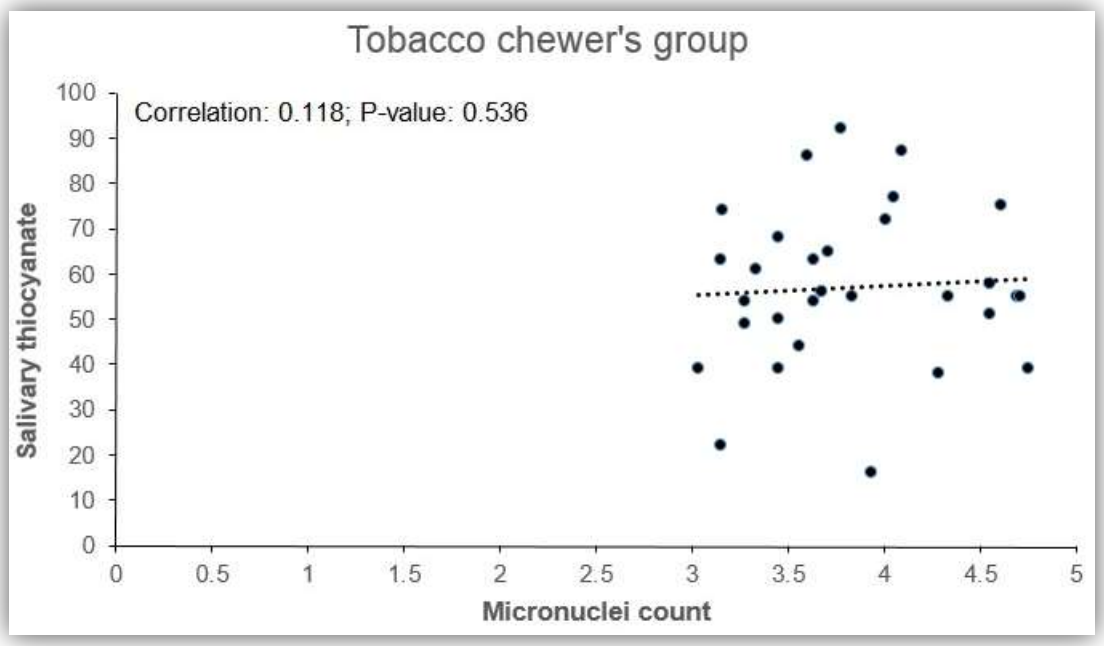
*Spearman's rank correlation; NS: Not significant

Table 7 shows the correlation of salivary thiocyanate levels with micronuclei count in each study group. It is evident from the table that in Control group, the correlation between the two was weak positive (0.284) and statistically insignificant (p=0.128). Further, in Tobacco chewer's group, the correlation was weak positive (0.118) and statistically insignificant (p=0.536). In the OSMF group, the correlation was again weak positive (0.066) and statistically insignificant (p=0.728).

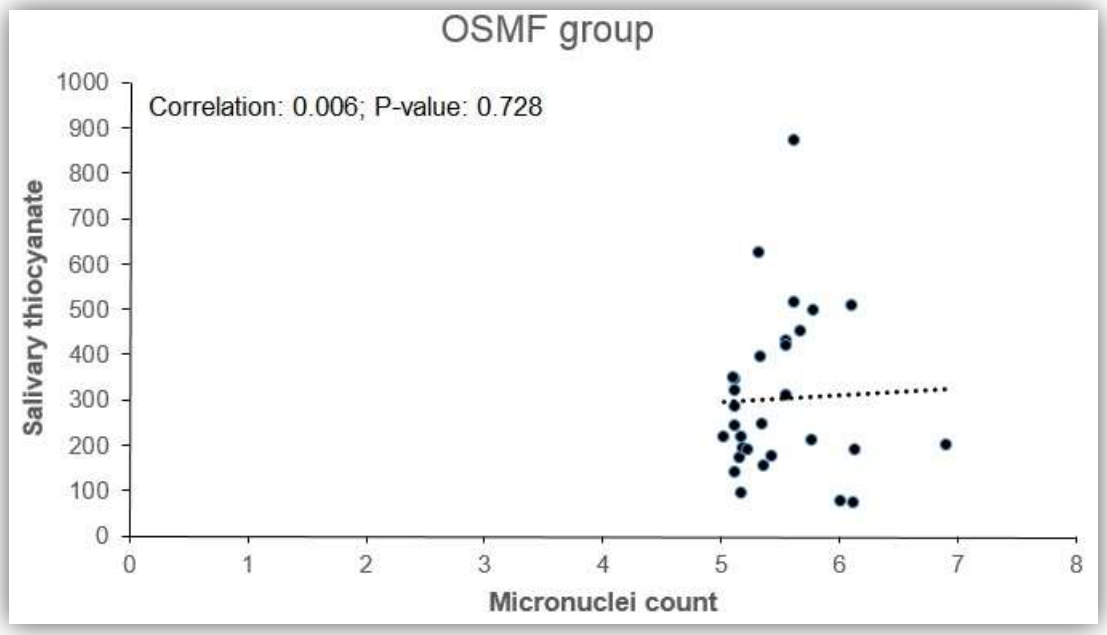
Correlation of micronuclei count and salivary thiocyanate levels in Control group, Tobacco chewers & OSMF Patients



Graph 7: Scatter plot showing correlation of micronuclei count and salivary thiocyanate levels in Control group



Graph 8: Scatter plot showing correlation of micronuclei count and salivary thiocyanate levels in Tobacco chewer's group



Graph 9: Scatter plot showing correlation of micronuclei count and salivary thiocyanate levels in OSMF group

HYPOTHESIS

Null Hypothesis:

Null hypothesis has been met with no correlation between levels of salivary thiocyante with occurance of micronuclei in tobacco chewers and OSMF Patient**s**

Altenate Hypothesis:

Alternate hypothesis has not been with no correlation between levels of salivary thiocyante with occurance of micronuclei in tobacco chewers and OSMF Patient**s**

DISCUSSION

Cancer is one of the most life-threatening diseases afflicting humankind. The word " oral cancer," in itself, generates fear among all human beings, to whichever strata of society they may belong. It is often diagnosed at an advanced stage because of the lack of early diagnostic markers, and therefore, the survival rate is markedly reduced despite the best available treatment options.[4] The majority of human cancers are caused by tobacco and synthetic and natural chemicals of occupational, environmental, medical, and dietary origin.[34]

Owing to the increasing tobacco availability and consumption in India, the **World Health Organization (WHO)** has considered it to be an 'epidemic'. Tobacco dependence has been classified as a disease by the **International Statistical Classification of Diseases and Related Health Problems, 10th revision (ICD-10)**. The tobacco consumption is rising by 3.4% every year and is the leading cause of head and neck cancers in India. Hence, tobacco has become a significant public health

concern in this country.[31] The **Global Adult Tobacco Survey (GATS)-India** 2010 has reported that about 275 million adults, who account for 35 % of the Indian population, consume some form of tobacco, the most prevalent being smokeless tobacco. There are about 206 million users of smokeless tobacco in India, which is the highest rate in the world.[31]

The regular use of tobacco slowly deteriorates the general health of a person which is found to be a common cause of tobacco addiction, which leads to illness, disability and death of a person.[19] The person who consumes more tobacco is at a greater risk for developing oral precancer like OSMF, various periodontal problems and other harmful oral conditions like acute necrotizing ulcerative gingivitis, oral candidiasis which usually shows a significant adverse effect on the outcome of oral care.[20] Tobacco consists of a mixture of combustion gases and particulate matter.[4] Hydrogen cyanide and carbon monoxide are among the many gases present in tobacco.[4]

Thiocyanate (SCN) present in the body fluids is partly because of the detoxification of hydrogen cyanide due to which the thiocyanate levels in the sera, urine, and saliva of tobacco chewers and OSMF patients are found to be higher in comparison control group. The half-life of thiocyanate is 10-14 days. Hence, the levels are less likely to vary within the span of a few days.[31]

Saliva is valuable in diagnosis of local diseases of salivary glands such as inflammatory conditions and autoimmune disease. A quantitative determination of specific constituent of saliva like thiocyanate (SCN) can be assessed, which acts as an indicator of tobacco consumption.[19]

Saliva plays a critical role in monitoring and regulating the health of oral cavity, and helps in keeping the integrity of the oral mucosa. As the saliva collection is non-invasive method to obtain so its popularity is increasing day by day in field in dentistry as a diagnostic fluid[19]. Saliva is considered to be analytical for maintenance of healthy oral mucosa.[24] Tobacco use leads to an alteration in the production or composition of saliva along with an alteration in the mucosal permeability and predisposes to oral cancer. SCN prevent toxic accumulations of Hydrogen Peroxide (H_2O_2) and Hypochlorite(ClO^-) , which may be carcinogenic or mutagenic. Salivary SCN reacts with the H_2O_2 in the oral cavity, leading to the elimination of the H_2O_2 . This reaction is catalyzed by the peroxidise. In the case of peroxidase inhibition, for example, when saliva is exposed to tobacco, H_2O_2 is not removed and leads to the production of free radicals. The free radicals are highly reactive and immediately react with neighbouring cellular macromolecules including DNA. This may result in malignant transformation.[3]

The present study was conducted among the three groups: Control group, Tobacco chewers and OSMF patients with sample size of 30 in each group. The number of cases showing the mean level in Control group was 0.62 (SD: 0.31), while in Tobacco chewer's group was 3.82 (SD: 0.53) and in OSMF group was 5.49 (SD: 0.43) was significantly higher in OSMF Group than tobacco chewers and control group. Thus it shows the difference in salivary thiocyanate levels in saliva in tobacco chewers and OSMF patients was found to be highly statistically significant.

According to Pullishery(2015)[12] salivary thiocyanate level was determined using spectrophotometric anyalysis. They found that the tobacco users had

significantly higher concentration of SCN levels than non-users. Similar results also seen in our study.

A study conducted **Kalburgi et al (2014)**[15] estimated the salivary thiocyante levels in healthy and different forms of tobacco users. They found that there is significant increase in level of salivary thiocyanate among smokers and gutka chewers as compared to others.

Similar findings were noticed by **Benny et al (2020)**[31] found significant difference in levels among tobacco users, non users and passive smokers.

Very few studies had been carried out in this regard. Thus, the present study showed the increase level of salivary thiocyante in tobacco chewers and OSMF patients.

A salivary SCN levels can be easily accessed by a simple biochemical test that can play a critical role in indicating the incidence or prevalence rate of tobacco consumption[20]. It can be used as biochemical indicator in the evaluation of potentially malignant disorders like OSMF.

These chemical carcinogens such as thiocyanate present in tobacco can cause structural alterations in the DNA of target cells, leading to genomic instability in the form of chromosomal abnormalities.[4]

Cytological alterations were assessed based on nuclear/cytoplasmic area ratio, nuclear and cellular pleomorphism, abnormal mitotic figures and number of micronuclei.[11] Micronucleus assay in exfoliated buccal cells is a useful and minimal invasive method for monitoring genetic damage in humans.[11] Many studies reveal

that the induction of MN cells by carcinogens and mutagens is a sign of the genotoxic effect of such substances. The damaged chromosomes, in the form of acentric chromatids or chromosome fragments, lag behind in anaphase when centric elements move toward the spindle poles. After telophase, the undamaged chromosomes as well as the centric fragments give rise to regular daughter nuclei. The lagging elements are included in the daughter cells, too, but a considerable proportion is transformed into one or several secondary nuclei, which are, as a rule, much smaller than the principle nucleus and are therefore called MN.[4]

These carcinogenic agents induce various kinds of DNA damage. Current predictive indicators of DNA damage are chromosomal aberration and micronuclei. A micronucleus is a small extra nucleus separated from the main nucleus and is generated during the cellular division when chromosomes fragments or divides late. Micronucleus assay has been used to determine the genotoxic and mutagenic potentials of various physical and chemical agents which could lead to the production of micronuclei. An early diagnostic test would be highly beneficial to check the progress of OSMF to squamous cell carcinoma as shown the pre-malignancy occur much before malignancy.[22]

In the present study, in the control group, there were maximum 16 (53.3%) subjects with micronuclei > 6, followed by 8 (26.7%) in the range 1-3, 2 (6.7%) in the range of 4-6 and there were 4 (13.3%) cases with absence of micronuclei. In the Tobacco chewer's group as well as OSMF group, all the subjects had micronuclei count above 6. The comparison of the distribution of count across three groups showed statistically highly significant. Similar results were found in the study

couducted by **Anila et al (2011)**[22] there is increase in number of micronuclei in OSMF patients were statistically significantly elev ated as compared to control group.

A study conducted by **Sangle et al(2016)**[6] assessed the frequency of micronuclei in exfoliated oral epithelial cells among potentially malignant disorders and OSCC patients. They found that frequency is three or four times higher in patients with OSCC as compared to PMDs patients. Similar results were found in our study.

A considerable number of studies conducted in the past have confirmed a significant increase in micronuclei frequency in oral exfoliated epithelial cells of potientially malignant disorders as compared to normal healthy individuals. The quantum of consumption of tobacco and the quality and volume of tobacco also act as factors responsible for the increased genotoxicity in the affected mucosa, thereby causing an increase in the MN cell counts of an individual.[4] Based on these findings, it was suggested that MN can be used as a biomarker in screening of PMDs. However no attention has been, until now, given to determine the overall accuracy of MN assay in diagnosis of potentially malignant disorders like OSMF.

Casartelli et al(2000)[35], concluded that the gradual increase in MN frequency from normal mucosa to precancerous lesion to carcinoma suggested a link of this biomarker with neoplastic progression. According to **Samanta and Dey (2012)**[36], the various possible explanations for MN formation in preneoplastic conditions include chromosomal aberrations, chromosome loss/breakage, mitotic apparatus dysfunctions, aneuploidy, and genetic instability.[36]

A study done by **Baldawa et al(2016)**[4] showed a contradictory findings that elevated levels of salivary thiocyante and occurance of micronuclei in smokers and

non smokers and found positive association between salivary thiocyante and occurance of micronuclei.

According to **Prakruthi et al (2018)**[11] ,the study showed an increase in level of salivary thiocyante among smokers as compared to non smokers. Cytological evaluation revealed increase in number of micornuclei in smokers than nonsmokers. The correlation between salivary thiocyanate levels and cytological changes showed insignificant results which is similar to our study.

Therefore, the present study showed the increase level of salivary thiocyanate with increase number of micronuclei but the correlation was found to be statistically insignificant. Levels of salivary thiocyanate when correlated with the cytological evaluation of number of micronuclei, revealed statistically insignificant result. Although thiocyanate has been found to have carcinogenic effects in the stomach.[11] but it failed to show significant cellular and nuclear alterations in the oral cavity, which may be explained by the differences in the local environment such as pH. (acidic in the stomach and buffered to stay neutral in the oral cavity)

Further scope is needed by taking into consideration duration & frequency of tobacco chewing habits & different grades of oral submucous fibrosis.

CONCLUSION

Oral cancer is certainly one of the most threatening disease that affect mankind. Many oral cancers are preceded by clinically evident premalignant mucosal changes that gives a warning of risk and present an opportunity for detection and preventive measures.

Saliva is mirror of the body. Biomarkers in saliva like thiocyanate(SCN) can be useful indicator of tobacco usage and potentially malignant disorders like OSMF. The SCN present in the body fluids is partially because of detoxification of hydrogen cyanide in tobacco chewers & OSMF patients. The elevated levels of SCN In the saliva may be responsible for excessive cancer risk through nitrosation process, which is a process of converting organic compounds into nitros derivatives which are potent carcinogens.

Tobacco habits in the form of chewing have mutagenic effects on human chromosomes which are indicated by increased frequency of MN in oral exfoliative cells. MN assay in oral exfoliated epithelial cells can be conveniently used as a biomarker for the screening of PMDs of oral cavity.

The present study demonstrated that there is sgnificant increase in the levels of salivary thiocyante in OSMF patients & tobacco chewers than control group. There is increase in number of micronuclei in OSMF patients & tobacco chewers than control group. But the correlation between levels of salivary thiocyanate with number of micronuclei is not statistically significant.

Oral exfoliative cytology is a useful method for detecting premalignant and malignant oral lesions. Oral exfoliative cytology is a simple, rapid, minimally invasive, and relatively painless method that is well accepted by patients and therefore suitable for population screening programs. Early detection and quantification of DNA damage in oral premalignancy or malignancy may help in management of the disease and improve survival rates.

Thus, these new biomarkers are noninvasive, painless and prove to be an efficient tool in screening a large population as well as in aiding motivation of individuals for withdrawal of tobacco habits. They can be extremely promising and should hopefully change the paradigm of oral cancer diagnostics.

Further studies are required to determine the value of these biomarkers in monitoring various treatment modalities and can also be used in finding out the risk of field cancerization.

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CASE HISTORY PROFORMA

DEPARTMENT OF ORAL & MAXILLOFACIAL PATHOLOGY

Case History No.: Date:

Patient's Name:

Age/Sex:

Occupation:

Address:

Phone No.:

Religion:

Economic Status:-Low/Middle/High

Martial Status: Married/Unmarried/Widowed/Divorced

Family Status: No Child/One/Two/More

Cheif Complaint:-

History Of Present Illness:-

Past Dental History:-

Past Medical History:-

Personal History:-

- a. Diet:
- b. Oral Hygiene Habits:
- c. Habits:-

Habits Particulars Frequency Duration Location
Chewing Tobacco/BetalNut
Guthka Chewing

Family History:-

Psychological Status:Normal/Average/Psychic

General Examination:-

Built:

Gait:

Temp:

Pallor:

Icterus:

Cyanosis:

Extra Oral Examiation:-

Lesion:

Site:

Size:

Colour:

No.

Extension:

Borders:

Surface Temperature:

Consistency:

Relation to underlying structure:

Tenderness:

Mobility:

Others:

b) Facial Symmetry:

C) Lips:

d) TMJ:

e) Lymph Node:

Intra Oral Examination:

Examination of lesion:

Nature of lesion:

Site:

Size:

Shape:

Colour:

Extension:

Borders:

Surface Texture:

Tenderness on palpation:

Consistency:

Relation to underlying structure:

Hard Tissue Examination:

Teeth Present:

Carious Teeth:

Provisnal Diagnosis:-

Investigations:

Hb: RBC's Count: TLC:

DLC:

P: E: B: L: M: Platelets:

Radiograph:

Histopathological:

a) Scrape Cytology:

b) FNAC:

c) Biopsy:

Any Other:

Final Diagnosis:

Treatment:

CASE RECORD FORM

Title: Estimation & correlation of levels of salivary thiocyanate in occurance with micronuclei using exfoliative cytology-A biochemical & cytological study.

Patient's I

D: _____

Age:_____

Sex:_____

Habit: _____

Group: A / B / C

1. **ESTIMATION OF THE LEVELS OF THIOCYANATE IN SALIVA**

SR. NO.	SALIVARY THIOCYANATE LEVEL IN mM

2. EVALUATION OF NUMBER OF MICRONUCLEI

SR. NO.	CYTOLOGY NUMBER	NO. OF MICRONULEI PER HIGH POWERED FIELD					LABELLING INDEX OF MICRONULEI (F1+F2+F3+F4+F5) /5
		FIELD 1	FIELD 2	FIELD 3	FIELD 4	FIELD 5	

3. CORRELATION BETWEEN SALIVARY THIOCYANATE LEVEL AND NO. OF MICRONUCLEI

SALIVARY THIOCYANATE LEVEL IN mM	LABELLING INDEX OF MICRONUCLEI

Confidential

Informed Consent Form

Estimation & correlation of levels of salivary thiocyanate in occurance with micronuclei using exfoliative cytology-A biochemical & cytological study.

Mr./Mas./Mrs./Ms_____

Resident of:_____

Age : _____ years, Sex:_____

Exercising my free will/choice, without any pressure/lure of incentive in any form, hereby giving my consent for the project to be conducted.

I acknowledge the doctor has informed me about the research project and explained the procedures in the best language known to me that is to be performed suitably and sufficiently to my satisfaction. I agree to let my salivary investigations to be taken as required. I agree to take part in this project and shall report to the dental hospital when called on given appointment dates and time. I am aware of the fact that I can opt out of the study at any time without having to give any reason and this will not affect my future treatment in the hospital. I shall co-operate with the doctors and paramedical staff, in all respect. I permit to publish my results/data in this study. I shall not be given any reimbursement or compensation.

I hereby record my consent for the participation in the said trial.

Patient's Name & Sign Investigator's Signature Witness Name & Sign

Date : _____ Date : _____ Date : _____

Time : _____ Time : _____ Time : _____

MASTER CHART SHEET I

Estimation of salivary thiocyante in Control Group

sr.No.	Age	Sex	Level of salivary thiocyanate in control group(mM)
1	29	M	1.17mM
2	32	M	1.15 mM
3	41	M	0.61 mM
4	32	M	0.32 mM
5	31	M	0.51 mM
6	29	M	0.23 mM
7	47	M	0.56 mM
8	56	M	1.16 mM
9	37	F	1.11 mM
10	34	M	0.22 mM
11	51	M	0.86 mM
12	44	M	0.33 mM
13	53	M	0.56 mM
14	28	M	0.89 mM
15	36	F	0.1 mM
16	42	M	0.45 mM
17	39	M	0.65 mM
18	27	M	0.87 mM
19	23	F	0.77 mM
20	37	M	0.97 mM
21	46	M	0.23 mM
22	24	M	0.65 mM
23	26	M	0.33 mM
24	54	F	0.31 mM
25	48	M	0.45 mM
26	52	M	0.56 mM
27	29	M	0.82 mM
28	30	M	0.34 mM
29	36	M	0.77 mM
30	56	M	0.56 mM

Estimation of salivary thiocyante inTobacco Chewers,

Sr.No	Age	Sex	Level of salivary thiocyanate in tobacco chewers(mM)
1	23	M	3.15 mM
2	45	M	3.27 mM
3	52	M	3.45 mM
4	26	M	4.61 mM
5	27	M	3.03 mM
6	29	M	3.6 mM
7	32	M	3.45 mM
8	45	M	3.33 mM
9	31	M	4.05 mM
10	34	M	4.55 mM
11	27	M	4.69 mM
12	24	M	4.71 mM
13	23	M	4.55 mM
14	22	M	4.33 mM
15	28	M	3.93 mM
16	53	M	3.63 mM
17	29	M	3.45 mM
18	27	M	4.01 mM
19	28	M	4.09 mM
20	31	M	3.63 mM
21	33	M	3.71 mM
22	34	M	3.56 mM
23	42	M	3.77 mM
24	43	M	3.83 mM
25	34	M	4.75 mM
26	28	M	3.16 mM
27	26	M	3.15 mM
28	27	M	3.27 mM
29	37	M	4.28 mM
30	40	M	3.67 mM

Estimation of salivary thiocyante in OSMF Patients

Sr. No.	Age	Sex	Level of salivary thiocyanate in OSMF Patients(mM)
1	23	M	5.16 mM
2	34	M	5.55 mM
3	47	M	6.12 mM
4	43	M	5.02 mM
5	26	M	5.35 mM
6	31	M	5.77 mM
7	33	M	6.01 mM
8	36	M	5.17 mM
9	42	M	5.55 mM
10	49	M	5.36 mM
11	60	M	6.9 mM
12	26	M	6.1 mM
13	45	M	6.13 mM
14	29	M	5.43 mM
15	33	M	5.12 mM
16	39	M	5.62 mM
17	44	M	5.12 mM
18	23	M	5.33 mM
19	39	M	5.78 mM
20	52	M	5.55 mM
21	43	M	5.12 mM
22	21	M	5.17 mM
23	25	M	5.18 mM
24	24	M	5.62 mM
25	30	M	5.32 mM
26	32	M	5.22 mM
27	33	M	5.11 mM
28	46	M	5.12 mM
29	37	M	5.67 mM
30	59	M	5.12 mM

MASTER CHART SHEET 2

Evaluation of Number of Micronuclei in Control Group

Sr.No	Age	Sex	No.of micronuclei in control group
1	29	M	3
2	32	M	10
3	41	M	16
4	32	M	25
5	31	M	2
6	29	M	1
7	47	M	19
8	56	M	22
9	37	F	14
10	34	M	9
11	51	M	8
12	44	M	13
13	53	M	20
14	28	M	26
15	36	F	3
16	42	M	3
17	39	M	4
18	27	M	9
19	23	F	9
20	37	M	18
21	46	M	12
22	24	M	12
23	26	M	6
24	54	F	0
25	48	M	0
26	52	M	0
27	29	M	0
28	30	M	3
29	36	M	3
30	56	M	2

Evaluation of Number of Micronuclei in Tobacco Chewers

Sr.No.	Age	Sex	No. Of micronuclei in tobacco chewers
1	23	M	22
2	45	M	49
3	52	M	50
4	26	M	75
5	27	M	39
6	29	M	86
7	32	M	39
8	45	M	61
9	31	M	77
10	34	M	58
11	27	M	55
12	24	M	55
13	23	M	51
14	22	M	55
15	28	M	16
16	53	M	63
17	29	M	68
18	27	M	72
19	28	M	87
20	31	M	54
21	33	M	65
22	34	M	44
23	42	M	92
24	43	M	55
25	34	M	39
26	28	M	74
27	26	M	63
28	27	M	54
29	37	M	38
30	40	M	56

Evaluation of Number of Micronuclei in OSMF Patients

Sr. No.	Age	Sex	No. Of micronuclei in OSMF Patients
1	23	M	171
2	34	M	309
3	47	M	72
4	43	M	218
5	26	M	247
6	31	M	212
7	33	M	75
8	36	M	219
9	42	M	429
10	49	M	153
11	60	M	202
12	26	M	506
13	45	M	191
14	29	M	175
15	33	M	243
16	39	M	516
17	44	M	344
18	23	M	396
19	39	M	496
20	52	M	421
21	43	M	322
22	21	M	96
23	25	M	193
24	24	M	872
25	30	M	625
26	32	M	191
27	33	M	347
28	46	M	286
29	37	M	451
30	59	M	139

Comparison of Salivary thiocyante level and no. of micronuclei in control Group

Sr. No.	Level of salivary thiocyanate in control group(mM)	No.of micronuclei in control group
1	1.17 mM	3
2	1.15 mM	10
3	0.61 mM	16
4	0.32 mM	25
5	0.51 mM	2
6	0.23 mM	1
7	0.56 mM	19
8	1.16 mM	22
9	1.11 mM	14
10	0.22 mM	9
11	0.86 mM	8
12	0.33 mM	13
13	0.56 mM	20
14	0.89 mM	26
15	0.1 mM	3
16	0.45 mM	3
17	0.65 mM	4
18	0.87 mM	9
19	0.77 mM	9
20	0.97 mM	18
21	0.23 mM	12
22	0.65 mM	12
23	0.33 mM	6
24	0.31 mM	0
25	0.45 mM	0
26	0.56 mM	0
27	0.82 mM	0
28	0.34 mM	3
29	0.77 mM	3
30	0.56 mM	2

Comparison of Salivary thiocyante level and no. of micronuclei in tobacco chewers

Sr. No.	Level of salivary thiocyanate in tobacco chewers(mM)	No.of micronuclei in tobacco chewers
1	3.15 mM	22
2	3.27 mM	49
3	3.45 mM	50
4	4.61 mM	75
5	3.03 mM	39
6	3.6 mM	86
7	3.45 mM	39
8	3.33 mM	61
9	4.05 mM	77
10	4.55 mM	58
11	4.69 mM	55
12	4.71 mM	55
13	4.55 mM	51
14	4.33 mM	55
15	3.93 mM	16
16	3.63 mM	63
17	3.45 mM	68
18	4.01 mM	72
19	4.09 mM	87
20	3.63 mM	54
21	3.71 mM	65
22	3.56 mM	44
23	3.77 mM	92
24	3.83 mM	55
25	4.75 mM	39
26	3.16 mM	74
27	3.15 mM	63
28	3.27 mM	54
29	4.28 mM	38
30	3.67 mM	56

Comparison of Salivary thiocyante level and no. of micronuclei OSMF Patients

Sr. No.	Level of salivary thiocyante in OSMF patients(mM)	N. Of micronuclei in OSMF Patients
1	5.16 mM	171
2	5.55 mM	309
3	6.12 mM	72
4	5.02 mM	218
5	5.35 mM	247
6	5.77 mM	212
7	6.01 mM	75
8	5.17 mM	219
9	5.55 mM	429
10	5.36 mM	153
11	6.9 mM	202
12	6.1 mM	506
13	6.13 mM	191
14	5.43 mM	175
15	5.12 mM	243
16	5.62 mM	516
17	5.12 mM	344
18	5.33 mM	396
19	5.78 mM	496
20	5.55 mM	421
21	5.12 mM	322
22	5.17 mM	96
23	5.18 mM	193
24	5.62 mM	872
25	5.32 mM	625
26	5.22 mM	191
27	5.11 mM	347
28	5.12 mM	286
29	5.67mM	451
30	5.12 mM	139