

Buccal Cell Micronuclei in Patients with Oral Leukoplakia and Tobacco Pouch Keratosis: A Pilot Case-Control Study from Tamil Nadu, India

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ABSTRACT

Aim: This pilot study aimed to compare genetic damage, specifically micronuclei (MNi) in exfoliated buccal mucosal cells, in South Indians with oral leukoplakia (LPK) and tobacco pouch keratosis (TPK).

Background: LPK and TPK, two oral potentially malignant disorders (OPMDs) triggered by tobacco habits, eventually cause oral cancer. Exfoliated buccal cell micronucleus (BCMn) is an indicator of chromosomal damage resulting from tobacco-induced localized genotoxicity. However, the MN incidence reported by earlier studies varied depending on cohorts, demographics, tobacco forms used and OPMD types.

Methods: Exfoliated BCs were collected from 45 participants, aged 30 to 60, including 30 patients with clinically diagnosed LPK (n = 15) and TPK (n = 15) and healthy controls (n = 15) without oral mucosal lesions and no tobacco habits, and BCMN was detected using the Feulgen staining technique. A one-way ANOVA was applied to compare mean differences in MN between the three groups using the Tukey-Kramer Multiple Comparisons Test.

Results: The Mean BCMN index was significantly higher in the TPK group and LPK group as compared to the control group (p<0.001). However, there was no significant difference between TPK and LPK.

Conclusion: Compared to smoking, smokeless form (chewing tobacco) has a significant influence on localized genotoxicity, with an increase in MN occurrence dependent on habit duration.

Clinical Significance: The clinical significance of this pilot study lies in its potential to deepen the understanding of genetic damage in South Indian patients with OPMDs, particularly those with LPK and TPK. By comparing the occurrence of BCMN, the study may help identify early biomarkers for oral cancer risk, improve diagnostic accuracy, and inform targeted prevention strategies, especially in a region with a rising incidence of tobacco-related oral cancers.

Keywords: Oral Exfoliated Buccal Cells, Micronuclei, Leukoplakia, Tobacco Pouch Keratosis, Smoking and Smokeless Tobacco Use

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INTRODUCTION

Globally, tobacco use stands as one of the most significant threats to both public health and the environment^{1,2}. India ranks second in both tobacco production and consumption, providing a wide range of tobacco products at low prices^{2,3}. According to the Global Adult Tobacco Survey India (2016-17), nearly 267

million adults aged 15 years and older—representing 29% of the adult population—are tobacco users. The most common form of tobacco in India is smokeless

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tobacco, with popular products including khaini, gutkha, betel quid with tobacco, and zarda. Smoking forms include bidi, cigarettes, and hookah². In India, tobacco is a leading cause of death, responsible for approximately 1.35 million fatalities annually. It significantly increases the risk of several chronic diseases, including lung disease, cardiovascular disease, stroke, and, notably, various types of cancer². The habitual use of smoking and smokeless/chewing tobacco products makes oral cancer (OC) a major public health concern in India, particularly as its burden rises in Tamil Nadu⁴⁻⁶.

Tobacco smoking and chewing are primary risk factors for the development of oral potentially malignant disorders (OPMDs), such as leukoplakia (LPK) and tobacco pouch keratosis (TPK). LPK is classified as a white patch or plaque that serves as a premalignant lesion (PML) with the potential to progress to oral cancer (OC)⁷. TPK, also known as smokeless tobacco-induced keratosis (STK), manifests as a white mucosal lesion that develops at the site of tobacco contact⁸.

The genotoxic effects of tobacco are particularly evident in oral tissues at the primary site of exposure, where cellular and molecular alterations can reflect genetic damage. Exfoliated buccal cells (BCs) are frequently studied in evaluating tobacco-induced localized genotoxicity, due to their ease of collection and minimally invasive nature. In particular, the formation of micronuclei (MNI) in BCs has been widely investigated as a biomarker of genetic damage. The BC micronucleus (MN) cytome (BMNCyt) assay is widely utilized as a reliable tool for assessing genetic damage. Previous reports have been inconsistent, with varying MN incidence observed across different cohorts, ethnicities, demographics, types of tobacco use (smoking vs. smokeless), and various OPMDs. Therefore, this pilot study aimed to compare the occurrence of MN in exfoliated BCs among South Indian patients with LPK and TPK.

MATERIALS AND METHODS

Study design and centre

This pilot case-control study was carried out in the Department of Oral Medicine and Radiology with

technical assistance from the Department of Oral Pathology and Microbiology. This study received approval from the Institutional Ethics Committee of Rajas Dental College Hospital (RDCH/EC/15/2022).

Study participants

A total of 45 participants, aged 30 to 60 years, were enrolled following a detailed oral-visual clinical examination at the outpatient department of Rajas Dental College and Hospital in April 2022. The study participants were divided into three groups: Group I comprised clinically diagnosed LPK patients ($n=15$); Group II included patients with TPK ($n=15$); and Group III consisted of healthy control subjects with no history of tobacco use and no oral mucosal lesions ($n=15$). Written informed consent was obtained from all participants prior to enrollment. Demographic data, including age, gender, medical history, and oral habits, were collected from each participant.

Exfoliated cells collection and scoring of MN

The participants were instructed to rinse their mouths with clean water. Exfoliated BCs from patients with LPK and TPK were collected from the mucosa surrounding the oral cavity lesions using pre-moistened wooden spatulas. Similarly, BCs were collected from the healthy buccal mucosa of the control subjects. The BCs were mounted on pre-cleaned, grease-free, pre-coded slides and smeared immediately. The slides were initially stained with Feulgen reactive (Molychem, India) and then counterstained with Light Green SF Yellowish (Himedia Labs, India). They were examined under low ($\times 400$) and high ($\times 1000$) magnifications using a light microscope (Lawrence & Mayo, India). A total of 500 cells from each participant were subjected to double-blinded examination by two scorers to minimize bias. BCMN scoring was performed according to the method described by Tolbert et al. (1992)⁹, with the following criteria: (1) A rounded, smooth perimeter suggestive of a membrane; (2) Less than one-third the diameter of the associated nucleus, but large enough to discern shape and colour; (3) Feulgen-positive; (4) Staining intensity comparable to that of the nucleus; (5) Texture resembling that of the nucleus; (6) Located in the same focal plane as

the nucleus; (7) No overlap or bridging with the nucleus (Figure 1A, B).

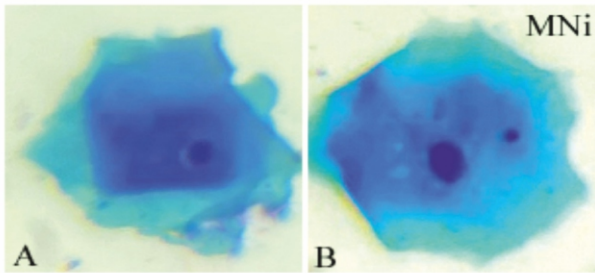


Fig 1: (A) – Normal differentiated BC, (B) mononucleated BC with MNi

Statistical analysis

Descriptive statistics were used to evaluate the demographic data and unpaired *t*-test was used to compare the habit duration. One-way analysis of variance (ANOVA) was performed using GraphPad PRISM 4 (GraphPad Software, Inc., San Diego,

California, USA), and the mean BCMN per cell was compared using the Tukey-Kramer Multiple Comparisons Test.

RESULTS

The study involved 45 participants, and their demographics are presented in **Table 1**. The participants were divided into three groups: LPK group consisted of 15 male patients, all of whom were smokers. The mean age was 42.60 ± 7.83 years, and the mean smoking duration was 16.66 ± 4.66 years. TPK group included 15 patients, with 12 males (80%) and 3 females (20%), all of whom were tobacco chewers. The mean age was 42.66 ± 7.89 years, and the mean duration of tobacco chewing was 19.53 ± 6.62 years. Control group: Comprised 15 healthy subjects, with 8 males (53.33%) and 7 females (46.66%), who had no tobacco-related habits. The mean

Table 1: Demographic data of study subjects

Variables	LPK	TPK	Control	Total	P value
<i>n</i>	15	15	15	45	
	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD		
Age (years) ^a	42.60 $\pm$ 7.827	42.66 $\pm$ 7.889	47.80 $\pm$ 6.763	-	<i>P</i> = 0.1062
Gender					
Males	15(100%)	12(80%)	8(53.33%)	35(77.77%)	
Females	0 (0%)	3(20%)	7(46.66%)	10(22.22%)	
Habits					
Smokers	15(100%)	-	-	15(100%)	
Tobacco chewers	-	15(100%)	-	15(100%)	
Habit duration (years) ^b	Mean $\pm$ SD	Mean $\pm$ SD			
	16.66 $\pm$ 4.655	19.53 $\pm$ 6.621	-		<i>P</i> =0.1810
					<i>t</i> = 1.372

^a One-way ANOVA using Tu key-Kramer Multiple Comparisons Test

^b Unpaired *t*-test

Table 2: Multiple group comparison of mean BCMN index in the study groups

Group	<i>n</i>	BCMNI		<i>P</i> value
		Mean $\pm$ SD	Mean difference	
TPK	15	1.333 $\pm$ 0.4880	0.3333	>0.05
LPK	15	1.000 $\pm$ 0.6547		
TPK	15	1.333 $\pm$ 0.4880	1.133	<0.001***
Control	15	0.2000 $\pm$ 0.4140		
LPK	15	1.000 $\pm$ 0.6547	0.8000	<0.001***
Control	15	0.2000 $\pm$ 0.4140		

*One-way ANOVA using Tukey -Kramer Multiple Comparisons Test

****p* < 0.05, significantly different from controls

age was 47.80 ± 6.76 years.

Statistical analysis revealed no significant difference in age between the groups ($P = 0.1062$) and no significant difference in habit duration between smoking and tobacco chewing ($P = 0.1810$).

Table 2 shows the mean BCMN index in the study groups. The TPK group (tobacco chewers) had a mean BCMN of 1.333 ± 0.4880 , while the LPK group (smokers) had a mean BCMN of 1.000 ± 0.6547 . The difference between the TPK and LPK groups was 0.3333, which was not statistically significant ($P > 0.05$). However, both the TPK and LPK groups had significantly higher BCMN compared to the Control group, which had a mean BCMN of 0.2000 ± 0.4140 ($P < 0.001$).

DISCUSSION

OC remains the leading cause of mortality worldwide, with oral cavity squamous cell carcinoma (OSCC) ranking as the sixth most common malignancy. The incidence of OSCC is notably elevated in South Asia¹⁰. Although OPMDs have a multi-factorial origin, tobacco use is the most frequently implicated risk factor. Strong evidence supports the association between the incidence of OPMDs and tobacco use, with a dose-dependent relationship observed due to smoking and chewing. The complex toxic effects of cigarette smoke are considered key contributors to the development of LPK and its potential for malignant transformation¹¹. Recent observations indicate that a history of tobacco smoking is significantly associated with the presence of LPK, with individuals who smoke being 1.32 times more likely to develop LPK. Similarly, the history of smokeless tobacco consumption was strongly linked to an increased risk of LPK, with users having a 318.6-fold higher risk compared to non-users¹².

Habitual tobacco use induces localized genotoxicity, causing genetic damage, thereby contributing to the progression from OPMDs to OC. The genotoxic effects of tobacco are particularly evident in oral tissues at the primary site of exposure, where cellular and molecular alterations can reflect genetic damage¹³.

In line with our study, previous research has reported an increase in the BCMN in North Indian LPK¹⁴ and TPK¹⁵ patients. The findings of this study reveal a significant association between tobacco use and increased BCMN. This observation is consistent with a previous study by Chandrasekar et al. which reported significantly higher BCMN in smokers and smokeless tobacco users compared to controls from the Western Ghats population of Tamil Nadu, South India¹⁶. Similarly, Himanta et al. also found significantly higher MN in smokeless tobacco users compared to smokers and controls from the Punjabi population¹⁷. A study by Soumya et al. reported higher BCMN in tobacco users from the Uttar Pradesh population¹⁸. Furthermore, Wagh et al. found that the mean number of BCMN was higher in smokeless tobacco users (2.76) compared to smokers (2.64) and controls (1.14) from the Gujarati population¹⁴.

Reports from other regions also support our observations. Ozkul et al. found no significant difference in BCMN between smokeless tobacco users (1.86%) and smokers (1.99%)¹⁹. Our findings contrast with those from other regions. Ping-An Wu et al. reported increased MN formation in heavy smokers compared to individuals with chewing tobacco habits, suggesting a stronger genotoxic effect in smokers²⁰.

For instance, research has suggested that antioxidant-rich diets or supplements may help reduce the incidence of MN in oral cells among individuals with tobacco habits²¹. These findings imply that dietary factors and lifestyle modifications could play a role in mitigating the genotoxic effects associated with tobacco use.

The present study has limitations, including a smaller sample size, and significant factors such as lifestyle and dietary habits were not assessed in the study population, which could potentially influence the occurrence of MN. These unconsidered variables may contribute to confounding effects, limiting the ability of the study to draw definitive conclusions. Future research should address these limitations by including larger cohorts, a more diverse sample, and accounting for lifestyle and dietary factors to better understand their role in the occurrence of MN in LPK and TPK.

CONCLUSIONS

In conclusion, when comparing smoking to smokeless tobacco use, specifically chewing tobacco, it has a significant impact on localized genotoxicity. Unlike smoking, which exposes the entire body to harmful substances, chewing tobacco primarily affects the areas it directly contacts, such as the mouth, gums, and oral mucosa. Studies show that the occurrence of MN, an index of genotoxicity, increases in individuals who use chewing tobacco. This increase is notably correlated with the duration of the habit, suggesting that the longer an individual uses chewing tobacco and retains it in the mouth, the higher the likelihood of experiencing genetic damage in localized tissues. This finding emphasizes the importance of increased awareness regarding the genotoxic risks associated with smokeless tobacco use and underscores the need for further research to elucidate its long-term impact on oral health.

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CONFLICTS OF INTEREST

The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

NA contributed to the experimental analysis, data acquisition, analysis, interpretation, drafted and critically revised the manuscript, RS, ACS, and SAJR contributed to conception and design, and critically revised the manuscript, and CSS contributed to conception and design, drafted and critically revised the manuscript.

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