

The correlation between micronuclei count and smoking behavior: A hospital-based study.

Correlación entre recuento de micronúcleos y hábito tabáquico: Un estudio hospitalario.

Lakshmi Balraj.¹
Tejavathi Nagaraj.²
UnniKrishnan K.³

Affiliations:

¹Sri Rajiv Gandhi College of Dental Sciences and Hospital, Bengaluru, Karnataka, India.

²Department of Oral Medicine and Radiology, Sri Rajiv Gandhi College of Dental Sciences and Hospital, Bengaluru, Karnataka, India.

³Manipal Academy of Higher Education, MAHE, Manipal, Karnataka, India.

Corresponding author: Lakshmi Balraj.
Sri Rajiv Gandhi College of Dental Sciences and Hospital, Bengaluru, Karnataka, India.
E-mail: lakshmi.balraj@gmail.com

Receipt : 04/16/2019 **Revised:** 07/17/2019
Acceptance: 03/14/2020

Cite as:

Balraj L, Nagaraj T & UnniKrishnan K.
The Correlation between Micronuclei Count and Smoking Behavior. A Hospital-Based Study.
J Oral Res 2020; 9(2):104-110.
Doi:10.17126/joralres.2020.016

Abstract: Background: Micronucleus is a microscopically visible cytoplasmic chromatin mass in the extranuclear vicinity, originating from aberrant mitosis, which consists of eccentric chromosomes that have failed to reach spindle poles during mitosis. The present study was designed to evaluate and compare cytogenetic changes in the buccal mucosa of smokers and non-smokers based on the occurrence of micronuclei. The study aimed to determine the correlation between the micronuclei count and the frequency and duration of smoking habit. **Materials and Methods:** Two groups (smokers and non-smokers) of 34 individuals each were examined. Cytological buccal smears were taken from participants using a moistened wooden spatula and stained with standard Papanicolaou stain. Presence of micronuclei was assessed at 40X magnification using a light microscope and a count per 500 cells was determined. The results of the study were analyzed statistically using Mann-Whitney U test, Spearman's rank correlation coefficient and Student t-test. **Result:** Smears from smokers showed a significant increase in the total number of micronuclei per 500 cell count compared to non-smokers. There was a strong positive correlation between the occurrence of micronuclei and the frequency and duration of smoking. A age-related increase in older age groups was also observed. **Conclusion:** The study reveals a strong positive correlation between the occurrence of micronuclei and the frequency and duration of smoking. This observation is vital in the utilization of the micronuclei detection in smears as a prognostic, educational and interventional tool in the management of patients with smoking habits.

Keywords: Mouth mucosa, chromosome aberrations, micronuclei, chromosome-defective, cytogenetic analysis, tobacco smoking.

Resumen: Antecedentes: El micronúcleo es una masa de cromatina citoplasmática microscópicamente visible en el área extranuclear, que se origina a partir de la mitosis aberrante, y que consiste en cromosomas excéntricos que no han podido alcanzar los polos del huso durante la mitosis. El presente estudio fue diseñado para evaluar y comparar los cambios citogenéticos en la mucosa bucal de fumadores y no fumadores en función de la aparición de micronúcleos. El estudio tuvo como objetivo determinar la correlación entre el recuento de micronúcleos y la frecuencia y duración del hábito de fumar. **Materiales and Métodos:** Se examinaron dos grupos (fumadores y no fumadores) de 34 individuos cada uno. Se tomaron frotis bucales citológicos de todos los participantes con una espátula de madera humedecida y se tiñeron con la tinción estándar de Papanicolaou. La presencia de micronúcleos se evaluó al microscopio

óptico con un aumento de 40X y se determinó un recuento por 500 células. Los resultados del estudio se analizaron estadísticamente utilizando la prueba U de Mann-Whitney, el coeficiente de correlación de rango de Spearman y la prueba t de Student. **Resultados:** Los frotis de fumadores mostraron un aumento significativo en el número total de micronúcleos por 500 células en comparación con los no fumadores. Hubo una fuerte correlación positiva entre la aparición de micronúcleos y la frecuencia y duración del tabaquismo. También se observó un aumento relacionado

con la edad en los grupos de mayor edad. **Conclusión:** el estudio revela una fuerte correlación positiva entre la aparición de micronúcleos y la frecuencia y duración del tabaquismo. Esta observación es vital en la utilización de la detección de micronúcleos en frotis como una herramienta pronóstica, educativa e intervencionista en el manejo de pacientes con hábitos de fumar.

Palabra Clave: *Mucosa bucal; aberraciones cromosómicas; micronúcleos con defecto cromosómico; análisis citogenético; tabaquismo.*

INTRODUCTION.

Oral cancer is among the ten types of malignant neoplasia with highest incidence worldwide and the age standardized incidence rate of oral cancer is 12.6 per 100,000 population in India.¹ Several risk factors are implicated in the development of oral cancer, of which the most common and established are tobacco smoking and betel quid chewing, acting either individually or synergistically together.²

Micronuclei (MN) results commonly from cell division defects, mitotic errors and anomalies in DNA replication or repair that generate acentric chromosome fragments.³ Ideally, cancer staging and histopathological grading are reliable for prognostication of oral squamous cell carcinoma. Micronucleus assay has been shown to have a sensitivity of 94%, specificity of 100% and an accuracy of 95% thus qualifying it as a good prognostic indicator.⁴ The micronucleus test serve for genomic damage risk assessment and is an important strategy for monitoring pre-neoplastic oral lesions, thereby guiding the therapeutic approach to be adopted.⁵

The present study was designed to evaluate the occurrence of micronuclei in the exfoliated squamous epithelial cells of the buccal mucosa of smokers and to study the correlation of micronuclei expression with the frequency and duration of smoking. This study also highlights the importance of the micronucleus assay that is an upcoming research domain in high risk population countries, where such simple user-friendly clinicopathologic techniques must be used to detect incipient cytogenetic changes.

MATERIALS AND METHODS.

This *in-vivo* study was conducted over a period of two years from January 2015 to December 2016. Based on the inclusion and exclusion criteria, a convenience sample of 68 male subjects between 20-60 years old

was selected. The subjects included in the study visited the Department of Oral Medicine and Radiology at the Sri Rajiv Gandhi College of Dental Sciences & Hospital, Bangalore, India to seek dental treatment. The study was explained to all participants in their native language, and only those who provided signed informed consent on an approved document participated in the study.

Demographic data and general history were taken from all participants and entered into a standard document. A detailed history consisting of chief complaint, history of past and present illness, and past medical history, if any was recorded. Emphasis was laid on recording oral use of smoking bidi and/or cigarettes along with the duration and frequency (per day), followed by a detailed clinical oral examination.

Patients with clinically evident changes in the oral cavity related to these habits (potentially malignant diseases, carcinoma), those who had quit the habit of smoking and those with a history of recent viral infection or having undergone radiation therapy were excluded from the study. Patients who resorted to other habits such as alcohol, smokeless tobacco or a combination of these were excluded from the study as the interaction between multiple cancer-causing products also causes nucleus degeneration and an appearance similar to micronucleus structure which would have confounded the study results.⁶ Male smokers were classified further into three groups based on the frequency and duration of smoking as follows:

Frequency:

- a) Less than five cigarettes or bidis/day
- b) Five-ten cigarettes or bidis/day
- c) More than ten cigarettes or bidis/day

Duration:

- a) Less than five years
- b) Five-ten years
- c) More than ten years

The selected subjects were asked to rinse their mouth gently with water. After briefing them about the procedure, the mucosal cells were scraped from both right and left buccal mucosa and from the labial mu-cosa using a slightly moistened wooden spatula.

The cells were immediately smeared on cleaned microscopic slides, fixed with commercially available alcohol spray fixative (BIOFIX) and the slides were marked and coded. On the same day, smears were stained with standard Papanicolaou (PAP) stain. The slides were assessed under 40x magnification using a light microscope; 500 cells per slide were examined in a

zig-zag pattern for the presence of micronuclei with the help of an eyepiece grid, which was then subjected to inter-observer evaluation by an oral pathologist at the Department of Oral Pathology. (Figure 1)

Micronuclei identification and scoring were performed following the criteria established by Tolbert *et al.*,⁷ The data was entered into a Microsoft Excel spreadsheet and statistically analyzed using Mann-Whitney, Spearman's correlation and Student t-tests using SPSS software (Statistical Package for Social Science, Ver.10.0.5). In all the tests, a *p*-value less than 0.05 was accepted as indicating statistical significance.

Figure 1. The microscopic examination of the stained slide with eyepiece grid showing micronuclei of buccal mucosa.

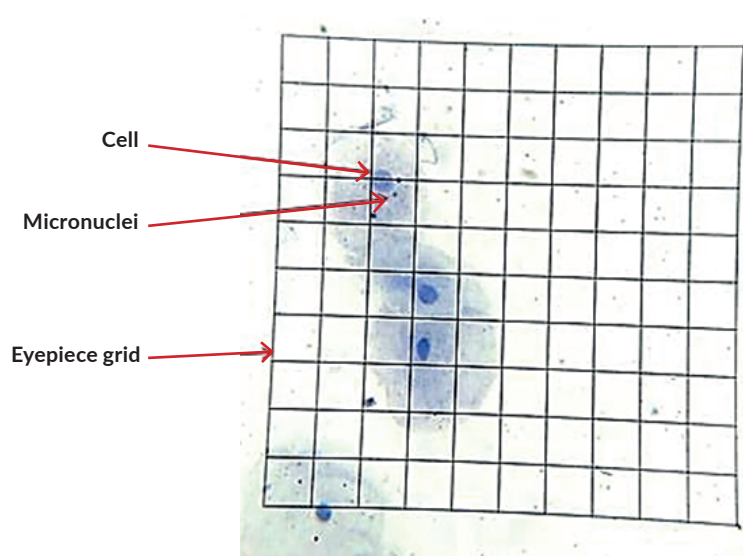


Figure 2. Comparison of mean micronuclei count according to smoking type.

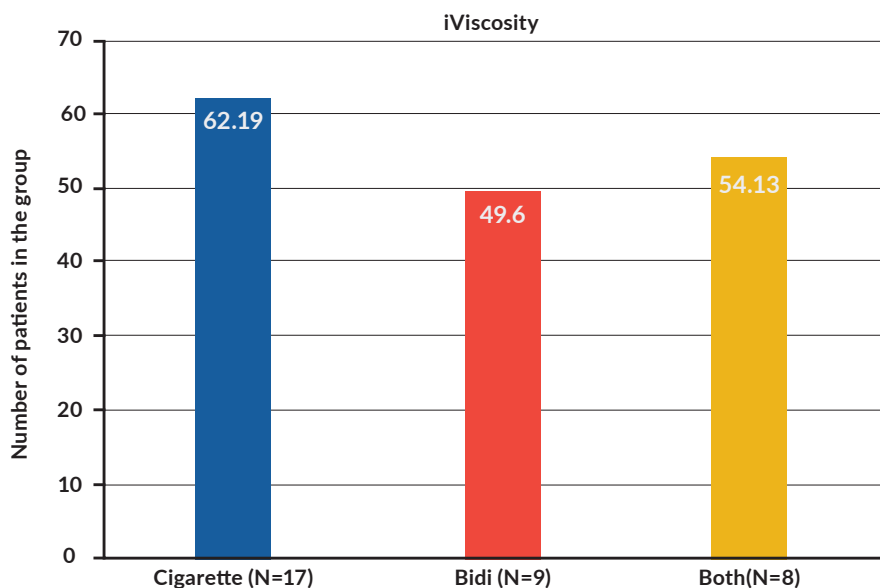


Table 1. Equilibrium analysis of age between smokers and non-smokers.

Group	N	MeanAge (in years)	SD	Min. Age	Max. Age	p-value*
Non-Smokers	34	40.7	12.482	21	60	0.935
Smokers	34	40.5	11.150	25	60	

*: Age equilibrium test, p-value not statistically significant. **N:** Number of patients in each group. **SD:** Standard Deviation. **Min:** Minimum. **Max:** Maximum.

Table 2. Distribution of micronuclei among smokers and non-smokers.

Group	N	N MN count	Mean MN count	S.D	Median	Min. MN count	Max. MN count	p-value*
Non-Smokers	34	7	4.35	9.779	0.00	0	37	<0.001
Smokers	34	34	56.59	11.147	56.00	36	78	

*: Mann-Whitney Test. **N:** Total number of patients in the group. **N MN count:** Number of patients in the group with MN expression. **SD:** Standard Deviation. **Min:** Minimum. **Max:** Maximum.

Table 3. Correlation between micronuclei counts versus duration of smoking and frequency of smoking.

	Correlation Coefficient r*	p-value
Duration (D)	0.608	<0.001
Frequency (F)	0.630	<0.001

*: Spearman's correlation test. **D:** Duration of smoking habit. **F:** Frequency (i.e. number of cigarettes or bidis smoked/day). (r = +.40 to +.69 Strong positive relationship).

Table 4. Mean micronuclei counts in relation to age in smokers.

Age Group	N	Mean MN count	SD	Min. MN count	Max. MN count	t-value*	p-value
21-40 years	19	49.47	7.933	36	68	36.325	<0.001
41-60 years	15	65.60	7.500	50	78		

*: Student t test. **N:** Number of patients in the group. **MN:** Micronuclei. **SD:** Standard Deviation. **Min:** Minimum. **Max:** Maximum.

RESULTS.

The present study included a total of 68 patients who were divided into two groups: 34 smokers and 34 non-smokers; a MN assay was conducted on their oral mucosa samples. Regarding age, 23.5% patients belonged were 21-30 years old, 32.4% were 31-40 years of age, 14.7% were 41-50 years old, and 29.4 % were 51-60 years old. Age equilibrium test showed that the mean age between the two groups was not statistically significant. (p=0.935) (Table 1)

Mann-Whitney test was used when comparing the distribution of micronuclei between the two groups – smokers and non-smokers. The majority of the smokers used cigarettes, and the cytological smears showed the presence of micronuclei for all the smokers, with a MN count ranging from 36 to 78. Micronuclei were also seen in the cytological smears of seven out of 34 members of the non-smoking group. (Table 2)

Patient history revealed diversity in the frequency and duration of the smoking habit. Accordingly, the

smokers' group was further divided into subgroups.

On cytological examination of the smears, it was observed that the mean micronuclei count in the group smoking more than 10 cigarettes/bidis per day was the highest (66.25); this was followed by those smoking 5-10 cigarettes/bidis a day (mean MN 60.1) and those who smoked less than 5 cigarettes per day (mean MN 49.56). When the duration of smoking habit was considered, cytological smears revealed that chronic smokers *i.e.* those with a history of smoking for over 10 years showed the maximum number of MN (mean=78).

This was followed by those who had been smoking for a period ranging from 5-10 years (mean=74) and by those smoking for less than 5 years (mean=58) respectively.

The Spearman's correlation test was used to study the correlation of micronucleus expression with the frequency and duration of smoking. The test indicated a strong positive correlation between MN count and duration and frequency of smoking. (Table 3)

The variance in the type of smoking was also taken into consideration. Cytological examination of the smears revealed that the mean MN count in cigarette smokers was the highest (mean=62.19), followed by those smoking both cigarettes and bidis (mean= 54.13); and the lowest numbers were found in those who solely smoked bidis (mean=49.60). However not all these groups have a similar number of members, with the cigarette-only smoking group having a higher number of individuals (Figure 2)

The Student t-test was used to determine whether there was a statistical difference in MN expression between age groups (21-40 years and 41-60 years) of smokers, with the group between 41-60 years having a higher MN count than the group between 21-40 years. ($p < 0.001$) (Table 4)

DISCUSSION.

Dr. George N. Papanicolaou was the first to introduce the PAP smear technique in 1928 in cervical tissues and this simple non-invasive technique can be replicated in the oral cavity for detecting carcinogenic changes.⁸

The frequency and the number of micronuclei are known to increase with carcinogenic stimuli, long before the development of clinical signs.

The effects of DNA damage or chromosomal exposure on the basal cell turnover rate influence the peak expression and hence the optimal timing between 7 to 21 days after exposure is required.⁹

Our study showed an increase in MN count among smokers compared to non-smokers, which is in accordance with the studies conducted by Motgi *et al.*,¹⁰ and Sherashiya *et al.*¹¹ However, micronuclei were also observed in the cytological smears of seven non-smokers out of a total of 34 (about 20% of the sample).

This could be because of cytogenetic aberrations in the buccal mucosa product of any other risk factor other than smoking, such as chronic inflammation, increased exposure to ionizing radiation or chemotherapeutic agents, vitamin deficiencies, or occupational and environmental exposures to harmful chemicals, among others.¹² Few exfoliative cytological studies of oral mucosa have been conducted. The subjects included in the MN assays conducted by Motgi *et al.*,¹⁰ and Pradeep *et al.*,¹³ resorted to various forms of tobacco products (both smoked and smokeless) and their combinations.

Increased MN count in patients with oral squamous cell carcinoma and other precancerous lesions have been demonstrated in studies conducted by Kamboj *et al.*¹⁴ and Buajeeb *et al.*¹⁵ The present study solely focused on determining the association between the habit of smoking and MN count; hence all the other factors such as the use of smokeless forms of tobacco or alcohol, and presence of clinically evident changes in oral cavity, all of which influence the MN count were excluded from the study. Gender bias was also omitted from the present study.

The MN assay done on peripheral lymphocytes in a study by Bonassi *et al.*,¹⁶ showed no increase in MN count in smokers with an exception in few cases where the subjects had occupational exposure. However our study excluded patients with occupational exposures and the MN assay was conducted on the oral mucosa. The oral epithelium is the first barrier during inhalation or ingestion and is capable of metabolizing proximate carcinogens to reactive products.

Approximately 90 percent of human cancers originate from epithelial cells. Thus, it can be justified that oral epithelial cells represent a preferred target site for early genotoxic events induced by carcinogenic agents entering the body via inhalation and ingestion and these cells can be easily collected from the mouth without causing much discomfort to patients.¹⁷

The highest MN mean was found in people with over ten years of smoking compared to the other groups. These results are in accordance to the study conducted by Naderi *et al.*,¹⁸ but in disagreement with the study

of Kamath *et al.*,¹⁹ who showed that individuals with a smoking history of 5 to 10 years had more micronuclei than those who had smoked more than ten years or less than five years.

In the present study, mean micronuclei count was also compared among two age groups. There was an increase in MN frequency in the group aged over 40 years and this effect could be due to the compounded genotoxic effect of tobacco on mucosal cells over a period of time.

It is well-known that the effects of genotoxic agents such as radiation and tobacco are cumulative and DNA damage is passed on to the daughter cells. The effects of these damages reflected by MN may lead to the development of pre-cancer and cancer.

Increased MN counts were found in people who smoke more than ten cigarettes per day when compared to those who smoke less than ten. We suggest that this could be due to the increased genotoxic effect of cigarette smoke on the oral mucosa and our results were in accordance with the previous studies conducted by Bonassi *et al.*,¹⁶ and Kamath *et al.*¹⁹

Lastly, the stain used for the smears of this cytological study was PAP stain, but other suitable stains include Giemsa, May-Grunwald, acridine orange, DAPI (4', 6-diamidino-2-phenylindole) and Feulgen.

Due to its specificity for DNA and contrast staining of cytoplasm, which helps micronuclei count, Feulgen-fast green staining is of higher interest among many researchers.¹² PAP staining method does not require complex pro-cedures such as cell culture or preparation of metaphase nor does it require DNA-specific staining.²⁰

According to a study by Palaskar *et al.*,²¹ for the routine MN assay, PAP is the most commonly used cytological stain, and it shows better staining results as compared to Romanowsky stain which is used widely in field studies.

Clinical significance

Micronuclei represent an "internal dosimeter" used to estimate exposure to genotoxic and carcinogenic agents.²² Using a MN assay, the results of the present study suggests that the frequency of micronuclei in the buccal mucosa of heavy smokers is significantly higher than that of nonsmokers with an almost 5-fold increase.

This inexpensive and non-invasive screening technique should be effectively performed as one of the clinical chair-side investigations in patients who have a history of risk factors associated to oral cancer.

The present study contributes valuable insights to the

area of exfoliative cytology in diagnostic dentistry with a further scope of research.

Limitations of the study

While designing and conducting the study, utmost care was taken to ensure the accuracy and reliability of the results obtained. However, the shortcomings of this study include a limited sample size which was obtained by convenience sampling and restricted to a particular geographical area.

Equational analysis of physical condition and disease between the groups was not performed in our study and further studies need to be conducted that include these criteria.

CONCLUSION.

Out of the different diagnostic methods available today, oral exfoliative cytology is particularly valuable for mass screening purposes for early diagnosis of oral cancer. Our study utilizes micronuclei assay to show a positive correlation between the MN count and duration and frequency of smoking. MN assays can be used to monitor, for prognosis and to motivate the individuals into cessation of the tobacco consuming habit, as an educative screening tool. More longitudinal studies are needed to be conducted to quantify and support this hypothesis.

Conflict of interests: Authors have no conflict of interest with this report.

Ethics approval: Ethical Committee approval obtained (Attached as Annexure 3). Patient consent obtained before participation.

Funding: None.

Authors' contributions: All authors contributed to this manuscript.

Acknowledgements: Special thanks to the teaching staff and postgraduate students at the Department of Oral pathology of Sri Rajiv Gandhi College of Dental Sciences for their contribution to the study. Our kind appreciation to Mr. Jagannath, for his prudent service in carrying out the statistical analysis of the study.

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