DOI: https://doi.org/10.53350/pjmhs2023173179

ORIGINAL ARTICLE

Nuclear and Nucleolar Morphometric Changes in Methyl Green Pyronin (MGP) Stained Oral Epithelial Cells In Cigarette Smokers, Passive Smokers and Non Smokers

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ABSTRACT

Aim: To compare nuclear and nucleolar morphometric changes in methyl green pyronin stained oral epithelial cells in cigarette smokers, passive smokers and non smokers
Study design: A comparative cross sectional study
Place and duration: Carried out in Postgraduate Medical Institute, Lahore. Duration was 6 months, March 2022 to August 2022.
Methods: 120 subjects were equally divided into three main groups, cigarette smokers, passive smokers and non smokers, each fulfilling the inclusion and exclusion criteria. Buccal smears were collected by exfoliative cytology of mucosa and then stained by methyl green pyronin stain. The quantitative nuclear morphometric parameters i.e., nuclear and nucleolar diameter and area, and number of nucleoli, were measured.
Results: Themedian of all nuclear parameters of smokers was significantly higher as compared to passive smokers and non-smokers in MGP stain ($p \leq 0.05$). Further, effect of duration of smoking on morphometric variables showed significant ($p < 0.05$) results amongst the smokers in MGP stain. However, no significant differences were seen in all morphometric parameters with respect to frequency of cigarette smoking.
Conclusion: The study confirms nuclear morphometric changes in oral epithelial cells of cigarette smokers indicating that smoking does influence cellular alterations. The simplicity of cytology technique with the use of methyl green pyronin stain is valuable screening tool for detecting early oral mucosal changes in high risk patients.
Keywords: smoking, exfoliative cytology, nuclear morphometric parameters, methyl green pyronin stain.

INTRODUCTION

In Pakistan the frequency of tobacco intake is high going up to 33% amongst adult males[1]. Various forms of tobacco are being used like cigarettes, water-pipe, chewable tobacco and tobacco snuff, depending on the cultural and social backgrounds of the consumers. In a study conducted previously, one out of every five males in Pakistan has either smoked more than 100 cigarettes or had made use of water pipe in his life[3].

In the whole world, one of the most commonly occurring malignancies is oral squamous cell carcinoma (OSCC)[8].

Various factors contribute to the occurrence of OSCC, but tobacco consumption in different forms is a significant risk factor[12]. It has been estimated that tobacco related deaths are increasing constantly and are likely to go from 5.4 million during 2005 to 6.4 million during 2015 and 8.3 million in 2030[10].

This meagre survival rate amongst oral cancer patients can be credited to a number of factors; as is common with other types of cancers and malignancies, however,one major factor is lack of early detection. If it can be detected at early stages, its spread can be curbed better. Therefore, an easy way out to this problem would be to detect potentially malignant lesions at their initial stage [2].
Regardless of the new techniques in surgery, radiation, and chemotherapy, over the past several decades, the five year survival rate of oral cancer has not enhanced much and still stays at about 50 to 55 percent[15].

Smoke from cigarettes is one of the most widespread pollutants, due to the high frequency of tobacco use worldwide. It is to be noted here that the detrimental effects of smoking are not just felt by active smokers but by nonsmokers as well who are exposed to cigarette smoke at various places, like at work, at public places, at school and in homes[16].

Aim of the study was to compare nuclear and nucleolar morphometric changes in methyl green pyronin stained oral epithelial cells in cigarette smokers, passive and non smokers

Received on 06-09-2022
Accepted on 16-02-2023

METHODOLOGY

This cross sectional, comparative study was conducted at Postgraduate Medical Institute, 6-Birdwood Road, Lahore, Lahore General Hospital, Nawaz Sharif hospital, Lahore during from the period March 2022 to August 2022. 120 subjects of either gender were enrolled.
Smokers: Subjects with age range 20-50 years, Males/Females, Subjects smoking 20 cigarettes daily for at least 10 years and Subjects without any oral lesion.
Passive smokers: Subjects with age range 20-50 years, Males/Females, Subjects who are lifelong non smoker with self-reported histories of contact with environmental tobacco smoke at work or at home or both for at least 1 hour per day for at least 3 yearsand Subjects without any oral lesion
Non smokers: Subjects with age range 20-50 years, Males/Females, Subjects who are lifetime nonsmokers and had never been frequently exposed to environmental tobacco smoke in workplace or at homeand Subjects without any oral lesion were considered as control.
Exclusion criteria

- Alcoholics.
- Subjects with other habits like gutka, betel nut or paan chewing.
- Subjects with chronic debilitating diseases i.e. tuberculosis, bleeding disorders, diabetes mellitus etc.
- Subjects on drug therapies that may cause oral mucosal changes like anticancer drug, antibiotics, NSAIDS
- Had not undergone chemotherapy and/or radiotherapy in the previous month.
- Subjects with poor oral hygiene
- Subjects wearing dentures.
- Patients with already existing malignant lesion.

Smears were taken by the candidate from clinically normal buccal mucosa of subjects i.e., smokers, passive smokers and non-smokers using a wooden spatula moistened in distilled water. The

scrapings were then transferred onto a clean glass slides marked with the patient's reference number beforehand, and spread finely and homogeneously over the middle third of the slide. For each subject, two slides were prepared for MGP staining. The completed proforma was entered into computer using SPSS-21.

RESULTS

In this study, forty smokers, forty passive smokers and forth healthy control were included. Kruskal Wallis test showed significant difference in all nuclear parameters among study groups in MGP staining. For multiple comparisons, Mann Whitney U test with Bonferroni correction was used. With MGP staining, the median of all nuclear parameters of smokers was also significantly higher as compared to passive smokers and non-smokers. Significant difference was also observed between passive and non-smokers with respect to mean large nuclear diameter and mean nuclear area whereas no significant difference was observed in mean small nuclear diameter between passive smokers and non smokers.

Kruskal Wallis test showed that there was significant difference in all other nucleoli parameters among groups with MGP staining. For multiple comparisons, Mann Whitney U test with Bonferroni correction was used. With MGP staining, the median of all nucleoli parameters of smokers was also significantly higher as compared to passive smokers and non-smokers whereas no significant difference was observed between passive smokers and non smokers.

Table 1: Comparison of nuclear parameters among smokers, passive smokers and non smokers with methyl green pyronin (MGP) stain.

Stain	Parameter a	Smoker	Passive smoker	Non-smoker	p-value
MGP	Mean Large Nuclear Diameter (µm)	11.14 (10.96 - 11.19)	10.26 (10.21 - 10.30)	10.17 (10.13 - 10.21)	0.000*
	Mean Small Nuclear Diameter (µm)	7.04 (7.01 - 7.10)	6.45 (6.39 - 6.51)	6.40 (6.40 - 6.42)	0.000*
	Mean Nuclear Area (µm2)	62.31 (60.1 - 63.1)	52.45 (51.97 - 53.20)	51.78 (51.57 - 52.03)	0.000*

[a]Values are given as median(IQR) [b]p- value is generated by Kruskal Wallis test *p- value ≤ 0.05 is considered statistically significant.

Table 3: Multiple comparison of nuclear parameter among groups with methyl green pyronin (MGP) staining

Variable	Groups	Test statistics	Std. error	Std .test statistics	p-value
Mean large nuclear diameter (µm)	Non smokervs, passive smoker	25.375	7.767	3.267	0.003*
	Non smokervs, smoker	72.688	7.767	9.358	0.000*
	Passive smoker vs, smoker	47.312	7.767	6.091	0.000*
Mean small nuclear diameter (µm)	Non smokervs, passive smoker	15.775	7.745	2.037	0.125
	Non smokervs, smoker	67.888	7.745	8.765	0.000*
	Passive smoker vs, smoker	52.112	7.745	6.729	0.000*
Mean nuclear area(µm2)	Non-smoker vs, passive smoker	22.825	7.775	2.936	0.010*
	Non-smoker vs, smoker	71.412	7.775	9.185	0.000*
	Passive smoker vs, smoker	48.588	7.775	6.249	0.000*

*p-value ≤ 0.05 is significant.

Table 4: Comparison of nucleoli parameters among smokers, passive smokers and non smokers with methyl green pyronin (MGP) staining

Stain	Parameter a	Smoker	Passive smoker	Non-smoker	p-value b
MGP	Mean Number of Nucleoli per cell	0.910 (0.88 - 0.92)	0.53 (0.49 - 0.55)	0.54 (0.51 - 0.55)	0.000*
	Mean Large Nucleolar Diameter (µm)	1.74 (1.64 - 1.78)	1.33 (1.32 - 1.34)	1.33 (1.32 - 1.34)	0.000*
	Mean Small Nucleolar Diameter (µm)	0.95 (0.93 - 0.97)	0.83 (0.82 - 0.84)	0.83 (0.83 - 0.83)	0.000*
	Mean Nucleolar Area (µm2)	1.45 (1.42 - 1.51)	0.91 (0.89 - 0.92)	0.91 (0.90 - 0.92)	0.000*

[a]Values are given as median(IQR) [b]p - value is generated by Kruskal Wallis test *p-value ≤ 0.05 is considered statistically significant

Table 6: Comparison of nucleoli parameters among groups with methyl (MGP) staining

Variable	Groups	Test statistics	Std. error	Std .test statistics	p-value
Mean Number of Nucleoli per cell	Passive smoker vs, Non-smoker	-2.975	7.740	-0.384	1.000
	Passive smoker vs, smoker	61.488	7.740	7.944	0.000*
	Non smokervs, smoker	58.512	7.740	7.559	0.000*
Mean large nucleoli diameter (µm)	Passive smoker vs, Non-smoker	-.550	7.689	-0.072	1.000
	Passive smoker vs, smoker	60.275	7.689	7.839	0.000*
	Non smokervs, smoker	59.725	7.689	7.768	0.000*
Mean small nucleoli diameter (µm)	Passive smoker vs, Non-smoker	1.275	7.587	-0.956	1.000
	Passive smoker vs, smoker	60.638	7.587	7.993	0.000*
	Non smokervs, smoker	59.362	7.587	7.825	0.000*
Mean nucleolar area(µm2)	Passive smoker vs, Non-smoker	-2.475	7.723	-0.320	1.000
	Passive smoker vs, smoker	61.238	7.723	7.929	0.000*
	Non smokervs, smoker	58.762	7.723	7.608	0.000*

*p-value ≤ 0.05 is significant

DISCUSSION

In this study, forty smokers, forty passive smokers and forty healthy controls were studied. Buccal smears were preferred to be collected with a wooden tongue spatula as it collects sufficient cells with relatively less trauma to the patient and is cost-effective.

In this study, the nuclear and nucleolir diameters and area and also the number of nucleoli per cell of oral epithelial cells were measured. Results of multiple comparisons amongst all three groups in MGP stain showed that the median of all nuclear parameters of smokers was significantly higher as compared to passive smokers and non-smokers. This may be because of various reasons like tobacco use or increase in tobacco content as stated by Hande and Chaudhary (2010)[9]. Significant difference was also observed between passive and non-smokers with respect to median large and small nuclear diameter whereas no significant difference was observed in median nuclear area between passive smokers and non smokers in MGP stain.

Various other researchers in the past also studied nuclear parameters by cytomorphometrical analysis. Einstein and Sivapathasundharam (2005)[7] had studied the effects of smoking and betel quid chewing on the oral mucosa and reported a rise in the average values of nuclear diameter (ND), and a decline in cytoplasmic diameter values of cells of smokers and also of individuals with both these habits. Recently, the results of various studies regarding the nuclear morphometric parameters i.e., increase nuclear diameters or areas of buccal cells of tobacco users are in accordance with the results of this present study[14,4].

With regards to the effects of passive smoking on buccal mucosal cells, little work have been done till now. However, recently Cavalcante et al. (2017)[6] carried out a research in which analysis of metanuclear alterations was performed in exfoliated buccal cells of children exposed to passive smoking and urban air pollutants. The results showed that children exposed to cigarette smoke presented higher levels of nuclear abnormalities than children who were not usually exposed to this type of mutagenic agent. These results are comparable with the results of this present study where epithelial cells of passive smokers do show increased values of nuclear morphometric parameters compared to non smokers.

Regarding the nucleolar parameters, Kruskal Wallis test showed that there was significant difference in nucleoli parameters among groups with methyl green pyronin staining. Because of the size and rigidity of nucleoli, they are particularly attractive cell structures for quantification[5]. In present study the number of nucleoli was determined by counting when seen under light microscope. It was found that median of mean number of nucleoli was significantly higher in smokers being 0.90(0.88 - 0.92) in MGP stainand 0.53(0.49 - 0.55) in MGP stain in passive smokers and 0.54(0.51 - 0.55) in MGP stain in non smokers. These results can be related to the work done by Mohtasham et al., (2010)[11] who found that mean number of nucleoli tends to progressively increase from normal buccal mucosa to dysplastic mucosa and in oral cancer. However, passive smokers andnon smokers showed the same results.

Multiple comparisons among three groups showed that withMGP staining the median of all other nucleoli parameters of smokers was significantly higher as well as compared to passive smokers and non-smokers whereas no significant difference was observed between passive smokers and non smokers. However, these results were in contrast to the work done by Salehinejad et al. (2012)[13] who did not come across any considerable gap regarding the diameters, number and areas of the nucleoli in the smoker and non smoker groups in their study.

CONCLUSION

Dimensions of all the quantitative nuclear morphometric parameters is higher in smokers as compared to passive smokers and non smokers. Hence it is emphasized that nuclear morphometric analysis of buccal smears using methylgreen pyronin stain is an important technique to assess the influence of tobacco on buccal mucosa. The simplicity of cytology technique with the use of methyl green pyronin stain will make it a valuable screening tool for detecting early oral mucosal changes in high risk patients.

Conflict of interest: Nil

REFERENCES

1. Ahmad, K., Jafary, F., Jehan, I et al. 2005. Prevalence and predictors of smoking in Pakistan: results of the National Health Survey of Pakistan. *European journal of cardiovascular prevention & rehabilitation,* 12: 203-208.
2. Al Bulushi, N. M., Macpherson, L. M., Worlledge-Andrew, H et al. 2016. A protocol for a systematic review of clinical guidelines and published systematic reviews on the early detection of oral cancer. *Translational Research in Oral Oncology,* 1: 2057178X16658308.
3. Alam, S. E. 1998. Prevalence and pattern of smoking in Pakistan. *J Pak Med Assoc,* 48: 64-6.
4. Babuta, S., Garg, R., Mogra, K et al. 2014. Cytomorphometrical Analysis of Exfoliated Buccal Mucosal Cells: Effect of Smoking.
5. Boon, M., Schleicher, A., Wijsman-Grootendorst, A et al. 1988. Staining of the nucleolus with protein and RNA stains for automatic measurement of nucleolar size in paraffin sections. *Stain technology,* 63: 289-297.
6. Cavalcante, DN, Sposito JCV, Crispim BA et al. 2017. Genotoxic and mutagenic effects of passive smoking and urban air pollutants in buccal mucosa cells of children enrolled in public school. *Toxicology Mechanisms and Methods,* 27: 346-351.
7. Einstein, T. & Sivapathasundharam, B. 2005. Cytomorphometric analysis of the buccal mucosa of tobacco users. *Indian journal of dental research: official publication of Indian Society for Dental Research,* 16: 42-46.
8. Ferlay, J., Shin, H. R., Bray, F et al. 2010. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *International journal of cancer,* 127: 2893-2917.
9. Hande, A. H. & Chaudhary, M. S. 2010. Cytomorphometric analysis of buccal mucosa of tobacco chewers. *Rom J Morphol Embryol,* 51: 527-32.
10. Mathers, C. D. & Loncar, D. 2006. Projections of global mortality and burden of disease from 2002 to 2030. *PLoS medicine,* 3: e442.
11. Mohtasham, N., Mahdavi-Shahri, N., Salehinejad, J et al. 2010. Detection of nucleoproteins in squamous cell carcinoma, and dysplastic and normal mucosa in the oral cavity by methyl green-pyronin staining. *Journal of oral science,* 52: 239-243.
12. Ramaesh, T., Mendis, B., Ratnatunga, N et al. 1999. The effect of tobacco smoking and of betel chewing with tobacco on the buccal mucosa: a cytomorphometric analysis. *Journal of oral pathology & medicine,* 28: 385-388.
13. Salehinejad, J., Mahdavi-Shahri, N., Mohtasham, N et al. 2012. Comparative assessment of nuclear and nucleolar cytochemical parameters of oral epithelial cells in smokers and non-smokers by methyl green-pyronin staining. *Journal of Dental Materials and Techniques,* 1: 19-23.
14. Seifi, S., Feizi, F., Mehdizadeh, M et al. 2014. Evaluation of cytological alterations of oral mucosa in smokers and waterpipe users. *Cell Journal (Yakhteh),* 15: 302.
15. Silverman, S. 2001. Demographics and occurrence of oral and pharyngeal cancers: the outcomes, the trends, the challenge. *The Journal of the American Dental Association,* 132: 7S-11S.
16. Wunsch Filho, V., Mirra, A. P., López, R. V. M et al. 2010. Tobacco smoking and cancer in Brazil: evidence and prospects. *Revista Brasileira de Epidemiologia,* 13: 175-187.