

Value of Micronuclei Count as a Predictor of Cytotoxic Damage in Smoked and Smokeless Tobacco Users

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Abstract

Background: Tobacco usage in the smoked and smokeless forms has reached epidemic proportions in the Asian sub-continent often leading to oral cancer which is the sixth most common cause of cancer related deaths globally. Micronuclei (MN) count is a promising indicator for the cytotoxic effects of tobacco usage. Our study was aimed at establishing a correlation between the MN counts among various tobacco habits.

Methods: Exfoliated cells were collected over a period of three years from 2015 to 2017, from a total of 400 individuals consisting of 100 individuals each of smokers, gutkha chewers, khaini chewers and controls. PAP staining was done and the number of cells with micronuclei was counted under 40 X, by two independent examiners. The mean MN count was compared using the ANOVA test for statistical analysis.

Results: Significant increase in micronuclei count was observed in individuals with tobacco habit. Mean value of epithelial cells with micronuclei \pm SD was 1.58 ± 0.24 for controls, 7.51 ± 0.59 for smokers, 13.95 ± 0.97 for khaini chewers and 15.45 ± 1.17 for gutkha users.

Conclusion: The MN count can be used as an early indicator for susceptibility to Oral squamous cell carcinoma (OSCC), as a non-invasive early detection tool for mass screening, for patient education as well as to check for efficacy of treatment.

Keywords: Micronuclei; Exfoliated cells; Smokers; Khaini users; Gutkha users

Introduction

Use of tobacco in smoked and smokeless forms is widely prevalent in all parts of the world and reaches epidemic proportions in the Asian sub-continent. Tobacco may be smoked or used alone/mixed with additives and chewed/kept in the buccal/labial sulcus. Oral cancer is the 11th most common cancer worldwide [1] and among the top ten most common in India [2]. Globally, oral cancer is the sixth most common cause of cancer associated deaths [3]. Greater than 90% of oral malignancies arise from the epithelium [4] and a vast majority is associated with tobacco habit.

The oral cavity has the distinction of being the only mucosal site that can be examined visually with the naked eye to see for any morphological changes due to tobacco habits. The emphasis on early diagnosis and treatment depends on the morphological alterations observed.

Micronuclei (MN) have been defined as a microscopically visible, round to oval cytoplasmic chromatin masses next to the nucleus [5]. MN formation is the result of segregation defects due to chromosomal instability causing chromatin to be excluded from the reforming nucleus [6].

Our study was aimed at assessing the extent of MN formation in individuals with tobacco habits and to establish a correlation between the various habits and to determine the existing cytotoxic damage present even in the absence of significant clinical manifestations that were noticeable to the individual, thereby giving a false sense of well-being.

Materials and Methods

Study sample

All individuals gave a written consent for participation. All

individuals were interviewed for awareness of presence of any lesion associated with the habit, type of habit, duration and intensity of habit, dietary habits, systemic and local disease history and family history.

400 individuals consisting of 100 each of controls, smokers, khaini chewers and gutkha users were included in our study which was carried out over a period of three years from Jan 2015 to Dec 2017.

Inclusion criteria

- Smokers who were smoking 5 or more, filtered cigarettes per day for a minimum of 1 year.
- Tobacco users who were using khaini, 1 packet or more per day for more than 6 months.
- Gutkha users who were using flavored gutkha 1 packet or more per day for more than 6 months.
- Control individuals were healthy individuals who had never consumed tobacco in any form.

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Exclusion criteria

- Any history of chronic systemic disease e.g. Diabetes, hypertension, heart disease.
- Any history of medication for chronic illness or recent antibiotic intake.
- Any history of oral lesions e.g. recurrent aphthous, herpes or poor oral hygiene.
- Present history of stress.
- Any history of radiation or chemotherapy.
- Malnutrition and vitamin deficiency.
- Tea drinkers having more than two cups of tea per day.

To avoid any confounding factors individuals using alcohol based products or consuming alcohol more than 60 ml twice a month were not included in the study. Any individual having multiple habits were also not included.

Cell sampling

Before sampling, all individuals were asked to rinse thoroughly with tap water. Exfoliated cells were collected from the buccal mucosa of controls, site of placement in khaini or gutkha users and palatal mucosa of smokers. The cells were collected by gently rubbing the mucosa with a pre moistened wooden spatula. The cells were spread onto pre cleaned glass slides, allowed to air dry and fixed with Biofix spray (Biolab Diagnostics (I) Pvt Ltd) and then stained with Rapid - PAP stain (Biolab Diagnostics (I) Pvt Ltd). 1000 cells per slide were counted under high power (x40) using the battle field method (Figure 1). Only cells which were not fragmented and not overlapping were counted. 2 blind examiners carried out the count.

Results

There was observed a statistically significant increase in MN count when controls were compared to individuals with a history of tobacco use. The number of MN increased with an increase in duration and intensity of habit (Table 1).

Discussion

Case selection has to be done keeping in mind that confounding factors have the potential to induce expression of MN, such as; exposure to physical and chemical mutagens, radiation, consumption of alcohol, tea and coffee, food habits, stress, medication, viral infections suffered

S. No.	Group	n	Mean \pm SD
1	Controls	100	1.58 \pm 0.24
2	Smokers	100	7.51 \pm 0.59
3	Khaini chewers	100	13.95 \pm 0.97
4	Gutkha chewers	100	15.45 \pm 1.17

Table 1: Statistical analysis was done using the ANOVA test.

in the last 3 months, vaccination and hereditary diseases, use of anticonvulsants and antibiotics, oral infections, pregnant or lactating women. Collection of cell samples can be done using metal spatula, moistened wooden spatula, cotton swab, tooth brushes or cytobrushes. Though cytobrushes have shown ideal results, the cost factor makes the wooden spatula suitable for large number of samples. Casterelli et al. have observed that vigorous scraping lead to higher MN count suggesting a decreasing gradient of MN presence from the basal to the superficial layers [7].

Various staining procedures have been used such as acridine orange, propidium iodide, Giemsa and PAP. However PAP staining remains the preferred stain due to its DNA specificity.

Criteria for identification of MN were first given by Heddle and Countryman in 1976 as:

- Diameter less than 1/3rd the main nucleus
- Non-refractivity (to exclude small stain particles)
- Colour same as or lighter than the nucleus (to exclude large particles)
- Location within 3 or 4 nuclear diameters of a nucleus (to make frequency measurements meaningful)
- No more than 2 MN associated with one nucleus.

Numerous studies have been done on MN count and most substantiate that there is an increase in MN formation due to tobacco habits however the quantification varies significantly. The increase in MN count varies from 2 to 8 times when control group count is compared to tobacco users. Similarly, the count of the control group itself varies from 0.39 [8] to 2.70 [9]. Such findings further underline the significance of standardization of protocols for MN count to be used for effective comparison between study populations.

We found a significant increase in MN frequency in tobacco users (Figure 1) when compared to controls. Contrary to the findings of Sarto et al. and Piyathilake et al. we found that the MN formation was more in users of smokeless tobacco as compared to individuals with smoked tobacco usage [10,11]. An increase in MN count was also associated with smokeless tobacco as studied by Desai et al. Roberts DM and Stich et al. however not in accordance with Ozkul et al. who found no statistically significant variation between users of smoked and smokeless forms of tobacco [12-15]. We also found a positive correlation between MN frequency and gutkha chewing, which was increased when compared to controls.

Conclusion

Sufficient evidence exist that tobacco in smoked or smokeless form causes cytogenic and genotoxic damage to epithelial cells. The usage of tobacco products in any form is detrimental to oral health. The tobacco users have to be made aware of these dangers and there can be no better way than to practically demonstrate the effects of tobacco. Being noninvasive, economical and rapid, easy to carry out and highly reproducible, the MN count can be repeatedly obtained from the

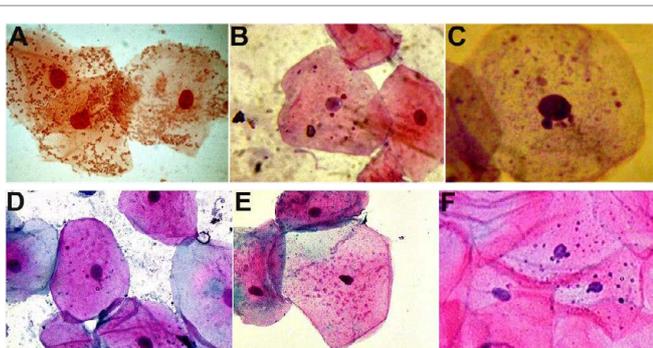


Figure 1: (Rapid PAP stain X40) Increased number of Micronuclei observed in exfoliated cells obtained from the buccal mucosa of individuals with the habit of Tobacco smoking and chewing.

same patient for longitudinal studies and for checking the efficacy of treatment. Moreover it can be used as a reliable tool for mass screening and early detection, patient education and motivation.

Compliance with Ethical Standards

Disclosure of potential conflicts of interest

The author of this article has not received any research grant, remuneration, or speaker honorarium from any company or committee whatsoever, and neither owns any stock in any company. The author declares that she does not have any conflict of interest.

Research involving human participants and/or animals

All procedures performed on the patients (human participants) involved were in accordance with the ethical standards of the institution and/or national research committee, as well as with the 1964 Helsinki declaration and its later amendments and comparable ethical standards.

Ethical approval

Although this article does not contain any new studies with human participants or animals performed by the authors, nevertheless Ethical approval was obtained from the hospital before conducting this evaluative study.

Informed consent

Informed consent was obtained from all the individual participants in this study.

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