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ORIGINAL RESEARCH

# An Exploration for the Most Congruous Stain for Valuation of Micronuclei

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## ABSTRACT

**Objectives and Aims:** The present study was carried out to evaluate and to compare different staining techniques for the assessment of MNi and to identify the most suitable stain for mass screening procedures.

**Materials and methods:** A total of 15 patients visiting the outpatient department of oral pathology and microbiology, Chhattisgarh dental college and research institute, Rajnandgaon who were diagnosed clinically with leukoplakia were taken up for study.

**Results:** Results obtained through One Way ANOVA suggesting that mean micronuclei found in a sample vary significantly on the basis of special stains used. The F ratio of 3.05, which is statistically significant at .05 level, confirms this finding.

**Conclusion:** According to the above study PAP stain was found to be the most suitable stain followed by LG cocktail and MGG stains for the evaluation and assessment of micronuclei.


**Keywords:** Micronuclei, MNi, PAP stain

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## INTRODUCTION

Oral squamous cell carcinomas are characterized by complex karyotypes that involve many chromosomal deletions, translocations, and structural abnormalities. Cells from these types of tumors often have errors in chromosome segregation that lead to the formation of a lagging chromosome or chromosome parts that become lost during the anaphase stage of cell separation and are excluded from the reforming nuclei. The laggards are observed in the cytoplasm as micronuclei.[1,2] The buccal cell micronucleus (MN) assay was first proposed in 1983[3] and it continues to gain popularity as a biomarker of genetic damage in numerous applications. Micronucleus assays provide information on the cytogenetic damage in the tissues, that are targets of human carcinogens and from which carcinomas can develop.

Significantly higher frequencies of MNs have been observed in exfoliated buccal cells, from people who are exposed to organic solvents, antineoplastic agents, diesel derivatives, polycyclic aromatic hydrocarbons, lead-containing paints and solvents, and drinking water which is contaminated with arsenic.[4] Recent studies have also suggested the genotoxicity and the cytotoxicity of the urban air pollution and ozone during the summer season, particularly in places with high ambient levels.

The lifestyle factors that are associated with genetic damage include smoking, alcohol consumption, and diet, especially vitamin deficiencies and supplementation.[5] A majority of the studies which reported a significant increase in MNs in the buccal mucosa cells, which were related to a risk of oral cancer, were performed in subgroups of subjects with specific lifestyle habits, i.e., chewers of betel quids (areca nut, betel leaves, slaked lime, and tobacco) from India, Taiwan, and Philippines; reverse smokers from India and Philippines; snuff dippers from Canada; users of Khaini tobacco (tobacco mixed with slaked lime) from India, and other similar practices.

However, little attention has been given, until now, to the effect of different staining procedures on the results of micronuclei assays. An evaluation of the literature shows that a variety of different stains are used in micronuclei studies. Among the deoxyribonucleic acid-specific stains, the ones which are most widely used are Feulgen and acridine orange; in some experiments, 4',6-diamidino-2-phenylindole and propidium iodide were also used. About 30% of the studies on epithelial cells were conducted by using nonspecific stains (Giemsa, May Grunwald's Giemsa (MGG), and less frequently Orcein).[6]

Hence, the present study was carried to evaluate and to compare different staining techniques for the assessment of MN and to identify the most suitable stain for mass screening procedures.

## MATERIALS AND METHODS

A total of 15 patients visiting the outpatient Department of Oral Pathology and Microbiology, Chhattisgarh Dental

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College and Research Institute, Rajnandgaon, who were diagnosed clinically with leukoplakia, were taken up for study.

A thorough case history was taken from all the patients. There was no reported systemic or chronic disease. All the patients' habit history was recorded including the content, frequency of consumption, and the duration.

The patients were duly informed and a written consent from each patient was obtained.

Before sampling, the patients were asked to rinse his/her mouth with tap water. A disposable prewet wooden spatula was used to obtain the oral exfoliated cells from the lesional tissue, and three slides were prepared for each patient.

The first slide was fixed using Biofix and was stained with Papanicolaou (PAP).

The second slide was air-dried and was stained with MGG.

The final slide was air-dried and stained with Leishman Giemsa (LG) cocktail stain.

The stained slides were cleaned, dried, and mounted using DPX mounting media (mixture of distyrene, a plasticizer, and xylene used as a synthetic resin mounting media) and were examined first under 40× and later under oil immersion for the evaluation and counting of micronuclei. A total of 100 cells per slide were examined for the frequency of micronuclei.

The frequency of micronuclei was noted for each slide based on the criteria described by Sarto et al[11] and Tolbert et al.[4]

The data so obtained were subjected to analysis of variance (ANOVA) summary.

## RESULTS

In the present study (Table 1 and 2), a total of 15 patients diagnosed clinically with leukoplakia were selected. $X^2$ (df = 2) = 6.23, $p < 0.05$. Chi square of 6.23 is significant at 0.05 level indicating that PAP is more useful in identifying the micronuclei followed by LG and May Grunwald stain.

Results obtained through one-way ANOVA suggest that mean micronuclei found in the sample vary significantly on the basis of special stains used. The *F* ratio of 3.05, which is statistically significant at the 0.05 level, confirms this finding.

This result is also confirmed by Least Significant Difference Test presented in Table 3.

**Table 1:** Descriptive statistics (mean and SD) of micronuclei in oral exfoliated epithelial cells in patients with leukoplakia on different special stains

| Special stains | Number of samples | Micronuclei | | |
|---|---|---|---|---|
| | | Mean | SD | Standard error |
| PAP | 15 | 4.00 | 1.00 | 0.25 |
| Leishman Giemsa | 15 | 3.73 | 0.96 | 0.24 |
| May Grunwald | 15 | 3.13 | 0.99 | 0.25 |
| F = 3.05, p < 0.05 | | | | |

PAP: Papanicolaou; SD: Standard deviation

**Table 2:** Analysis of variance summary

| Source | df | Sum of squares | Mean squares | F | Sig. |
|---|---|---|---|---|---|
| Between groups | 02 | 5.911 | 2.956 | 3.05 | 0.05 |
| Within groups | 42 | 40.667 | 0.968 | | |
| Total | 44 | 46.578 | | | |

**Table 3:** Least significant difference test with significance level 0.05

| Mean (I) | Mean (J) | Mean difference (I − J) |
|---|---|---|
| PAP | Leishman Giemsa | 0.26 |
| | May Grunwald | 0.86 |
| Leishman Giemsa | PAP | −0.26 |
| | May Grunwald | 0.60 |
| May Grunwald | PAP | −0.86 |
| | Leishman Giemsa | −0.60 |

PAP: Papanicolaou

## DISCUSSION

In the last 15–20 years, MN assay has been applied to evaluate chromosomal damage for biologic monitoring of human populations exposed to a variety of mutagenic and carcinogenic chemical or physical agents, such as antineoplastic drugs used for hospital staffs, areca nut chewers, arsenic in drinking water, dioxin as fertilizers, ethylene oxide, formaldehyde, lead oxide, solvents, benzene, ozone, polycyclic aromatic hydrocarbons, chlorants, toxic gases, pesticide mixtures, toluene, hexane, acetone, methyl-ethyl-ketone, 2-trans-hexol, and all forms of tobacco. A broad range of baseline MN frequencies have been reported (0.05–11.5 MN/1,000 cells), with the majority of values between 0.5 and 2.5 MN/1,000 cells.[7] There is no clear pattern of the variations among laboratories from different countries. Many studies report a statistically significant elevation of MN levels in exposed individuals compared with control groups, although the observed effects are relatively small, ranging between 1.1- and 4-fold, and many other studies report changes that were not statistically significant. A 12-fold increase in MN frequency was observed in a study by Make it Kumar V.[8] This increase was unusually large, but has been confirmed by independent analyses using a different staining procedure.

For a stain to be utilized in a mass screening program, in addition to good staining characteristics, the technique must be easy, rapid, and economical. The time required for staining with rapid PAP stain, i.e., for

fixation and staining, is about 3 minutes. The staining procedure requires multiple steps, large volumes of alcohol, and expensive stains. The fixing and staining procedure for MGG takes about 45 minutes,[6] and the cost is higher than the LG cocktail. The LG cocktail staining procedure of air-dried smears requires no additional fixation as in MGG stain and can be completed in less than 10 minutes, with the least expenditure. Some disadvantages of MGG stain include tendency to precipitate, high background staining, and preparation of fresh solution every day. Moreover, the staining technique is designed for staining a number of slides and not individual slides.[9,10] Though rapid PAP kit is available for faster turnaround time of approximately 5 minutes, it still requires multiple steps and is very expensive when compared with the above stains.[11]

However, with a few limitations like evaluation by a single examiner, subjectivity in scoring the sensitivity and specificity of the LG cocktail staining technique needs to be further evaluated. Within the limitations of the study, the PAP staining technique was found to give the most accurate results when compared with the LG Cocktail and MGG stain. Keeping in mind the added advantages like clarity and accuracy of the technique, the study supports the idea of utilizing the PAP staining technique followed by LG cocktail and MGG for early detection of oral cancer, especially in mass screening programs.

## CONCLUSION

According to the above study, PAP stain was found to be the most suitable stain followed by LG cocktail and MGG stains for the evaluation and assessment of micronuclei.

The study also supports the idea of utilizing assessment of micronuclei for early detection of oral cancer, especially in mass screening programs.

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