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ORIGINAL PAPER

Evaluation of micronuclei in oral squamous cell carcinoma and potentially malignant disorders via different staining techniques

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ABSTRACT

Introduction and aim. Oral squamous cell carcinoma (OSCC) and premalignant disorders (PMDs) are becoming common in India as the use of tobacco in different forms is increasing from a young age, and the prevalence of this disease is becoming more common in middle age. Identifying disease at earlier stages is an important measure for limiting disease incidence and improving patient prognosis. The micronuclei count can be a valid biomarker for screening suspected patients and can be helpful in educating patients about the discontinuation of treatment, diagnosing the disease in its early stages and planning a treatment for a better prognosis. Different stains that are nuclear specific can be used to identify micronuclei. The aim was to establish diagnostic efficacy of various staining techniques in OSCC and potentially malignant disorders on oral brush cytology smears with observation of micronuclei as a valid biomarker for evaluation of the disease.

Material and methods. Exfoliative cytology was done with oral brush and smears are obtained from 25 oral squamous cell carcinoma, 25 leukoplakia, 25 lichen planus, 25 oral sub mucous fibrosis patients and 15 samples with no disease. Each smear was stained with five different stains Papanicolaou (PAP), hematoxylin and eosin (H&E), toluidine blue, Leishman and Giemsa (LG) cocktail and Feulgen and observed for staining efficacy of micronuclei and cellular structures.

Results. The best stain to observe DNA content as micronuclei is Feulgen which gives clear and crisp details of micronuclei without giving any false count as it is nuclear specific stain. PAP can be the second choice stain. Micronuclei count is definitely increased in OSCC and PMDs compared to samples without disease confirming its use as biomarker.

Conclusion. Micronuclei count in oral brush cytology smears is a valid biomarker for evaluation of premalignant disorders and OSCC and can be used for detection of disease in individuals and for screening purposes of large populations at risk. Feulgen stain is best to study DNA content as micronuclei, on the other hand PAP can be used in large sampling investigations where there is lack of armamentarium.

Keywords. Feulgen stain, micronuclei, oral squamous cell carcinoma, potentially malignant disorders

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Introduction

Oral cancer is one of the major threats and economic burdens on the health care system. Oral squamous cell carcinoma (OSCC) is one of the ten most common cancers documented in the world; it accounts for more than 90% of all oral malignant lesions and is three times more common in men than in women, difficult to treat and often unbearable to the patients. According to the International Agency of Research on Cancer, the average yearly mortality rate is 145,000 worldwide.[1,2] The habits of tobacco chewing, smoking and alcoholism are major factors contributing to the development of potentially malignant disorders and their conversion into OSCC.[3] The course of conversion of potentially malignant disorders (PMDs) into malignancies is well documented; if left untreated.[4-6] PMDs earlier known as premalignant lesions and conditions were rephrased in combination with potentially malignant disorders by the WHO in 2005.[7] The prevalence of PMDs is approximately 4.5% worldwide. Among all PMDs, erythroplakia is most often associated with severe dysplasia, carcinoma in situ or frank carcinoma.[8]

The gold standard for diagnosing PMDs and OSCC is histopathological examination of the lesion. However, this approach requires additional armamentarium and time. Oral brush cytology or Exfoliative cytology for oral lesions is a newer and non-invasive methods which is not only equally predictable in diagnosing the severity and prognosis of pre malignant disorders and oral squamous cell carcinoma but also is an economical tool.[9] To screen a larger population and obtain quick results, sensitive and accurate screening is needed to identify individuals who are at risk for developing oral cancer. Exfoliative cytology or oral brush cytology is a valuable and economical tool for identifying individuals who are at risk and for monitoring lesions that may progress to malignancy.[10] Cytological smears are obtained from lesions in the oral cavity of diseased individuals and stained with different stains and techniques to observe cellular and nuclear changes to study and predict the course of the disease.[11,12]

The oral mucosa is the first line of defense against irritants inhaled or ingested as carcinogen-bearing substances, consequently causing buccal cells to metabolize carcinogens into reactive products.[4] These metabolites cause genetic damage, resulting in complex karyotypes that involve chromosomal aberrations and structural abnormalities. The chromosomal alterations appear as separated bodies from the nuclei and are observed in the cytoplasm as micronuclei.[13] Larger micronuclei result from aneugenic effects where the whole chromosome is excluded following damage to the spindle apparatus; on the other hand, smaller micronuclei result from structural aberrations. Micronuclei serve as effective biomarkers of genetic damage in exfoliated cells.[14]

The micronuclei in oral epithelial cells were first proposed as a biomarker in 1983 by Stich et al. It has been observed that micronuclei are significantly increased in individuals who are exposed to polycyclic aromatic hydrocarbons, as in smokers, alcoholics and individuals who habitually use tobacco in any form. The oral epithelium is structured with four layers having a stratum basale, i.e., a basal layer incorporating stem cells that may cause genetic damage. Genetically altered cells migrate to the surface layer during continuous cell renewal, are exfoliated from the superficial layer and are collected in cytological smears.[15]

Various stains are used to stain smears obtained from oral mucosa to study micronuclei. They can be DNA specific, as Feulgen is the most widely used for staining chromatin within the cell.[16] The other nonspecific stains used are Giemsa, Papanicolaou (PAP) and, less frequently, hematoxylin and eosin. Toluidine blue is another metachromatic basic dye that is used in oral cytology and fine needle aspiration cytology.[17]

Among these stains, PAP is the most widely used stain in exfoliative cytology because it provides promising nuclear and cytoplasmic staining results. Another advantage of PAPs is that they stain cells of different levels of maturity with different colors. With the development of the rapid PAP technique, PAP has gained popularity among pathologists for staining samples obtained via fine needle aspiration cytology (FNAC) and exfoliative cytology.

The Leishman-Giemsa cocktail is another newer staining technique that uses Leishman:Giemsa at a 1:1 ratio, yielding a deep blue color in the nuclear contents and a light blue hue in the cytoplasm.[18] Hematoxylin and eosin are the most easily available stains and are widely used because of their availability and feasibility.

The micronuclei assay is being established as a prognostic marker for oral cancer and potentially malignant disorders. Several studies have been performed to date to prove this fact.[19] The direct correlation between genetic damage and micronuclei makes the micronuclei assay an efficient adjunct to metaphase analysis.[13]

Several studies have confirmed increased micronuclei counts in desquamated oral squamous epithelium in individuals who are exposed to tobacco and other carcinogens encountering the oral cavity. In this study, we investigated the best stain for identifying nuclear anomalies and investigated whether micronuclei can be used as reliable biomarkers for identifying individuals who are at risk of transformation of potentially malignant disorders into malignant lesions.[20]

Aim

To assess the diagnostic efficiency of the Leishman-Giemsa cocktail, Feulgen stain, toluidine blue, Papanicolaou and hematoxylin and eosin staining of micronuclei

in oral smears of potentially malignant disorders and oral squamous cell carcinoma.

Material and methods

Source of data

The study was conducted in the Department of Oral Pathology and Microbiology at Career Post Graduate Institute of Dental Sciences and Hospital. Patients with oral squamous cell carcinoma, lichen planus, oral submucous fibrosis and leukoplakia were included in the study group of 25 patients with oral squamous cell carcinoma, 25 patients with lichen planus, 25 patients with leukoplakia, 25 patients with oral submucous fibrosis and 15 individuals with normal oral mucosa.

Methodology

Five smears were obtained from each patient with oral submucous fibrosis, lichen planus, leukoplakia or oral squamous cell carcinoma and from the individuals in the normal control group. The smears of each patient were stained with Leishman-Giemsa cocktail, toluidine blue, PAP stain, Feulgen and hematoxylin and eosin.

For each slide 1000 cells were counted to assess micronuclei under 100x magnification oil immersion in zig zag pattern on the prepared slide.

Toluidine blue staining steps

Aqueous 1% toluidine blue solution was prepared by mixing 1 gram of Toluidine Blue powder in 100 ml of distilled water.

The smears are dipped in isopropyl alcohol for fixation for 10 minutes. Then slides are dipped in 50% alcohol for 1 minute, then water washed. Smears are then stained with 1% aqueous Toluidine Blue solution for 45–60 seconds. Slides are water washed, air dried, clear with xylene and to be mount in DPX.[17]

Leishman-Geimsa cocktail steps

The unit volume of Giemsa stock is filtered and mixed with an equal unit volume of distilled water to prepare a Giemsa working solution (1:1 dilution from stock) (The dilutions can be changed according to one's preference, up to 1:7.). This cocktail (Garbyal's cocktail) can be used and stored just like Leishman's stain.

Air dried smears are flooded with the LG cocktail and left for 1 minute. An equal volume of buffer (Sorensen's phosphate), distilled water or tap water will be added. The slides were blown on gently and will be kept for 5–7 minutes and are mounted in DPX.[20]

Rapid PAP staining steps

We used commercially available rapid pap stain kit for staining of the smears.

Smears were fixed with Biofix spray and hydrated in tap water for 3-5 minutes, excess water was blotted out from the slide. Few drops of nuclear stain were added to the slide for 60 seconds and washed in running tap water. 3–5 drops of wash buffer were added and washed after 20 seconds. Then slides are dehydrated with rapid pap dehydrant for 60 seconds. Few drops of cytoplasm stain added for 60 seconds. Washed in water and dehydrated for 60 seconds, air dried and mounted in DPX.[17]

Feulgen staining steps

1N HCL was prepared from concentrated HCL (35–37%) assay, 8.6 ml of HCL mixed with 91.4 ml of distilled water.

Schiff's reagent: 1g basic fuchsin powder mixed in 200 ml of boiling distilled water. Solution is allowed to cool to 50 degrees centigrade and 2g of potassium metabisulphite is added with mixing. This solution is allowed to cool down to room temperature and to this 2 ml conc. HCL added with 2g of activated charcoal and left for overnight in the dark room. Solution is filtered through Whatman filter paper and stored in the dark container.

The slides were immersed in 1 M HCL at 60°C for 5 to 6 minutes, following by placement in 1 M HCL at room temperature for 1 minute. Then they were immersed in Schiff reagent for 30 minutes and then transferred to running tap water for 10 minutes.[6,10]

Hematoxylin and eosin staining steps

The conventional hematoxylin and eosin used for histopathological staining was used from our lab. Harris hematoxylin for nuclear staining and eosin Y for cytoplasmic staining was used in our study

Total staining procedure is done by following the steps, which included 6 slow dips under tap water, stained with Harris hematoxylin for 30 s, 6 slow dips under tap water, 6 dips in 95% Isopropyl alcohol, stained with EA-36 for 15 s, 6 dips in 95% isopropyl alcohol, 6 dips in 100% Isopropyl alcohol, 10 slow dips in xylene. Dehydrated, cleard and mounted.[14]

Scoring criteria

The criteria for assessing micronuclei will be followed as described by Tolbert et al.[21,22]

1. a rounded smooth perimeter suggested a membrane,
2. the diameter of the associated nucleus was less than one-third, but the nucleus was large enough to discern shape and color,
3. the staining intensity was similar to that of the nucleus,
4. the texture was similar to that of the nucleus,
5. the same focal plane as the nucleus,
6. the absence of overlap with, or bridging to, the nucleus.

Only those structures fulfilling the abovementioned criteria were recorded as micro nuclei.

Inclusion criteria

1. both male and female were included in the study,
2. age group of 20-60 years was considered,
3. cells included in smear counting were those with intact, clear cytoplasm,
4. cells included in smear counting with little or no debris,
5. cells included in smear counting with no overlap with adjacent cells,
6. the main nucleus was normal and intact.

Exclusion criteria

1. structures that resemble micronuclei were not included, as nuclear buds or broken egg (that are connected with main nucleus),
2. bi-nucleated cells that contain a smaller nucleus but has a diameter greater than 1/3 the other nucleus,
3. patients having any chronic diseases,
4. patients with syndromes,
5. child/infant are excluded from the study,
6. patients suffering from gastro esophageal reflux disease,
7. patients who are immune-compromised,
8. over and under stained slides.

Ethical approval

Ethical Ref. No. CPGIDSH/22/280. The duly constituted Ethical Committee for Post Graduate students of Career Post Graduate Institute of Dental Sciences & Hospital, Lucknow has approved and cleared the research project of Dr. Mohammad Imran Khan, Post Graduate Student of Batch 2020-21 in the speciality of Oral Pathology and Microbiology. The topic of the research project is as follows: Evaluation of The Diagnostic Efficiency of Leishman Giemsa Cocktail, Feulgen Stain, Toluidine, Papanicolaou and Hematoxylin & Eosin Stains in Oral Smears of Potentially Malignant Disorders and Oral Squamous Cell Carcinoma".

Statistical analysis

The results were analyzed using descriptive statistics and making comparisons among various groups. Categorical data were summarized as proportions and percentages (%) and quantitative data were summarized as mean±SD.

The following statistics were calculated in the present analysis: Kruskal-Wallis test (SPSS, IBM, Armonk, NY, USA). The significance level was taken as $p<0.05$

Results

On the basis of the observations made in the study on the various parameters, the following statistical results were obtained (Table 1, Figures 1–5).

For <1/3rd of the nuclei, the mean staining score for Feulgen was the maximum, while the staining score for H&E was the minimum. A significant difference was found in the mean staining score for the various stains ($p<0.001$), and the staining scores were calculated as Feulgen>PAP>L-G cocktail>toluidine blue>H&E.

Table 1. Comparison of staining scores among various stains for nucleus in the OSCC group

		L-G cocktail	Toluidine blue	H&E	PAP	Feulgen	p
<1/3rd of nucleus	Mean	4.6	3.7	3.4	7.9	8.9	z=72.65,
	SD	2.1	1.1	1.2	2.8	2.6	p<0.001
Smooth texture	Mean	3.2	2.6	2.4	5.6	6.6	z=58.76,
	SD	1.3	1.0	0.9	2.7	2.8	p<0.001
Same focal plane	Mean	3.8	3.2	2.9	6.2	7.4	z=66.33,
	SD	1.5	1.0	1.2	1.9	2.9	p<0.001
Round shape	Mean	3.2	2.8	2.4	5.7	6.7	z=61.59,
	SD	1.5	1.1	1.0	2.5	2.9	p<0.001
Same color	Mean	4.3	3.2	2.9	6.5	7.8	z=61.52,
	SD	2.2	1.0	0.9	2.3	3.3	p<0.001
Separated from main nucleus	Mean	4.0	3.3	3.0	6.4	7.5	z=58.70,
	SD	2.0	0.9	0.9	2.2	3.4	p<0.001

For the smooth texture, the mean staining score for Feulgen was the highest, while the staining score for H&E was the lowest. A significant difference was found in the mean staining score for the various stains ($p<0.001$), and the staining scores were calculated as Feulgen>PAP>L-G cocktail>toluidine blue>H&E.

For the same focal plane, the mean staining score of Feulgen was the maximum, while the staining score of H&E was the minimum. A significant difference was found in the mean staining score for the various stains ($p<0.001$), and the staining scores were calculated as Feulgen>PAP>L-G cocktail>toluidine blue>H&E.

For the round shape, the mean staining score for Feulgen was the highest, while the staining score for H&E was the lowest. A significant difference was found in the mean staining score for the various stains ($p<0.001$), and the staining scores were calculated as Feulgen>PAP>L-G cocktail>toluidine blue>H&E.

For the same color, the mean staining score of Feulgen was the maximum, while the staining score of H&E was the minimum. A significant difference was found in the mean staining score for the various stains ($p<0.001$), and the staining scores were calculated as Feulgen>PAP>L-G cocktail>toluidine blue>H&E.

For the separation, the mean staining score of Feulgen was the maximum, while the staining score of H&E was the minimum. A significant difference was found in the mean staining score for the various stains ($p<0.001$), and the staining scores were calculated as Feulgen>PAP>L-G cocktail>toluidine blue>H&E (Table 1).

For <1/3rd of the nuclei, the mean staining score of PAP was the maximum, while the staining score of total blue was the minimum. A significant difference was

found in the mean staining score for the various stains (p<0.001), and the staining scores were calculated as PAP>Feulgen>H&E>L-G cocktail>toluidine blue.

Table 2. Comparison of staining scores among various stains for nucleus in the leukoplakia group

		L-G cocktail	Toluidine blue	H&E	PAP	Feulgen	p
<1/3rd of nucleus	Mean	2.7	2.6	3.0	5.2	4.5	z=64.62, p<0.001
	SD	0.7	0.8	1.1	1.4	1.2	
Smooth texture	Mean	2.2	2.1	2.6	3.8	3.0	z=42.97, p<0.001
	SD	0.9	0.9	0.9	1.1	1.0	
Same focal plane	Mean	2.6	2.4	2.5	4.2	3.2	z=40.47, p<0.001
	SD	0.6	0.6	0.8	1.2	1.0	
Round shape	Mean	2.4	2.3	2.2	3.8	3.1	z=37.82, p<0.001
	SD	0.7	0.7	0.7	1.3	1.0	
Same color	Mean	2.3	2.0	2.2	3.9	3.5	z=54.78, p<0.001
	SD	0.7	0.6	0.9	1.2	0.9	
Separated from main nucleus	Mean	2.6	2.3	2.5	4.1	3.4	z=44.91, p<0.001
	SD	0.6	0.5	0.8	1.1	1.1	

For the smooth texture, the mean staining score of PAP was the maximum, while the staining score of toluidine blue was the minimum. A significant difference was found in the mean staining score for the various stains (p<0.001), and the staining scores were calculated as PAP>Feulgen>H&E>L-G cocktail>toluidine blue.

In the leucoplakia group, for the same focal plane, the mean staining score of PAP was the maximum, while the staining score of toluidine blue was the minimum. A significant difference was found in the mean staining score for the various stains (p<0.001), and the staining scores were calculated as PAP>Feulgen>L-G cocktail>H&E>toluidine blue.

For the round shape, the mean staining score of PAP was the maximum, while the staining score of H&E was the minimum. A significant difference was found in the mean staining score for the various stains (p<0.001), and the staining scores were calculated as PAP>Feulgen>L-G cocktail>toluidine blue>H&E.

In the leucoplakia group, for the same color, the mean staining score of PAP was the maximum, while the staining score of toluidine blue was the minimum. A significant difference was found in the mean staining score for the various stains (p<0.001), and the staining scores were calculated as PAP>Feulgen>L-G cocktail>H&E>toluidine blue.

In the leucoplakia group, for separation, the mean staining score of PAP was the maximum, while the staining score of toluidine blue was the minimum. A significant difference was found in the mean staining score for the various stains (p<0.001), and the staining scores were calculated as PAP>Feulgen>L-G cocktail>H&E>toluidine blue (Table 2).

For <1/3rd of the nuclei, the mean staining score of PAP was the maximum, while the staining score of the L-G cocktail toluidine blue was the minimum. A significant difference was found in the mean staining score for the various stains (p<0.001), and the staining scores were calculated as PAP>Feulgen>H&E>L-G cocktail=toluidine blue.

Table 3. Comparison of staining scores among various stains for nucleus in the lichen planus group

		L-G cocktail	Toluidine blue	H&E	PAP	Feulgen	p
<1/3rd of nucleus	Mean	1.9	1.9	2.2	3.6	2.6	z=31.32, p<0.001
	SD	1.1	0.9	1.1	1.3	1.0	
Smooth texture	Mean	1.6	1.7	2.0	2.9	2.2	z=29.96, p<0.001
	SD	0.9	0.7	0.9	0.9	0.8	
Same focal plane	Mean	1.7	1.8	1.9	2.8	2.2	z=21.82, p<0.001
	SD	0.9	0.8	0.8	1.0	0.9	
Round shape	Mean	1.4	1.6	1.9	2.7	2.1	z=29.77, p<0.001
	SD	0.9	0.8	0.9	1.0	0.9	
Same color	Mean	1.8	1.7	2.0	2.7	2.3	z=13.74, p=0.008
	SD	1.1	0.8	1.0	1.2	0.8	
Separated from main nucleus	Mean	1.8	1.7	2.0	3.1	2.1	z=26.65, p<0.001
	SD	1.1	0.9	1.0	1.0	0.8	

For the smooth texture, the mean staining score of PAP was the maximum, while the staining score of the L-G cocktail was the minimum. A significant difference was found in the mean staining score for the various stains (p<0.001), and the staining scores were calculated as PAP>Feulgen>H&E>toluidine blue>L-G cocktail.

In the lichen planus group, for the same focal plane, the mean staining score of PAP was the highest, while the staining score of the L-G cocktail was the lowest. A significant difference was found in the mean staining score for the various stains (p<0.001), and the staining scores were calculated as PAP>Feulgen>H&E>toluidine blue>L-G cocktail.

For the round shape, the mean staining score of PAP was the maximum, while the staining score of the L-G cocktail was the minimum. A significant difference was found in the mean staining score for the various stains (p<0.001), and the staining scores were calculated as PAP>Feulgen>H&E>toluidine blue>L-G cocktail.

In the lichen Planus group, for the same color, the mean staining score of PAP was the maximum, while the staining score of toluidine blue was the minimum. A significant difference was found in the mean staining score for the various stains (p=0.008), and the staining scores were calculated as PAP>Feulgen>H&E>L-G cocktail>toluidine blue.

In the lichen Planus group, for separation, the mean staining score of PAP was the maximum, while the staining score of toluidine blue was the minimum. A significant difference was found in the mean staining score for the various stains (p<0.001), and the staining scores were calculated as PAP>Feulgen>H&E>L-G cocktail>toluidine blue (Table 3).

Table 4. Comparison of staining scores among various stains for nucleus in the OSMF group

		L-G cocktail	Toluidine blue	H&E	PAP	Feulgen	p
<1/3rd of nucleus	Mean	2.5	2.5	2.4	3.6	2.8	z=17.39, p=0.002
	SD	0.8	1.0	1.0	1.2	1.5	
Smooth texture	Mean	2.1	2.0	1.8	2.5	2.2	z=8.57, p=0.073
	SD	0.8	0.8	0.8	0.9	1.3	
Same focal plane	Mean	2.2	2.1	2.0	2.9	2.4	z=15.39, p=0.004
	SD	0.6	0.8	0.7	0.8	1.3	
Round shape	Mean	2.0	2.1	2.0	2.6	2.4	z=11.23, p=0.024
	SD	0.8	0.6	0.7	0.7	1.4	
Same color	Mean	2.5	2.3	2.2	2.9	2.5	z=7.62, p=0.107
	SD	0.8	0.9	0.8	0.9	1.1	
Separated from main nucleus	Mean	2.5	2.2	2.1	2.8	2.4	z=8.44, p=0.077
	SD	0.8	0.9	0.8	1.0	1.3	

In the OSMF group, for <1/3 of the cells in the nucleus, the mean staining score of PAP was the highest, while the staining score of H&E was the lowest. A significant difference was found in the mean staining score for the various stains (p=0.002), and the staining scores were calculated as PAP>Feulgen>L-G cocktail=toluidine blue>H&E.

For the smooth texture, the mean staining score of PAP was the maximum, while the staining score of H&E was the minimum. However, no significant difference was found in the mean staining score of the various stains (p=0.073).

In the OSMF group, for the same focal plane, the mean staining score of PAP was the highest, while the staining score of H&E was the lowest. A significant difference was found in the mean staining score for the various stains (p=0.004), and the staining scores were calculated as PAP>Feulgen>L-G cocktail>toluidine blue>H&E.

Table 5. Comparison of staining scores among various stains for nucleus in the control group

		L-G cocktail	Toluidine blue	H&E	PAP	Feulgen	p
<1/3rd of nucleus	Mean	1.3	1.3	1.1	2.0	1.0	z=8.79, p=0.087
	SD	0.9	0.6	1.0	1.0	0.8	
Smooth texture	Mean	1.4	1.3	1.2	1.9	1.1	z=8.26, p=0.082
	SD	0.9	0.6	1.0	0.7	0.7	
Same focal plane	Mean	1.3	1.3	1.1	1.8	0.9	z=7.39, p=0.117
	SD	0.9	0.6	1.0	0.9	0.7	
Round shape	Mean	1.3	1.3	1.1	1.8	1.1	z=5.75, p=0.218
	SD	0.7	0.6	0.9	0.8	0.7	
Same colour	Mean	1.3	1.3	0.9	1.6	0.9	z=6.50, p=0.165
	SD	0.8	0.6	0.8	0.9	0.7	
Separated from main nucleus	Mean	1.3	1.1	0.9	1.5	0.9	z=7.81, p=0.099
	SD	0.9	0.5	0.7	0.7	0.7	

For the round shape, the mean staining score of PAP was the maximum, while the staining scores of the H&E and L-G cocktails were the minimum. A significant difference was found in the mean staining score for the various stains (p=0.024), and the staining scores were calculated as PAP>Feulgen>toluidine blue>L-G cocktail = H & E.

In the OSMF group, for the same color, the mean staining score of PAP was the highest, while the staining score of H&E was the lowest. However, no significant difference was found in the mean staining score (p=0.107).

In the oral submucous fibrosis group, for the separation, the mean staining score of PAP was the highest, while the staining score of H&E was the lowest. However, no significant difference was found in the mean staining scores of the various stains (p=0.077) (Table 4).

In the control group, no significant differences were observed in various parameters among the strains used in the study (Table 5).

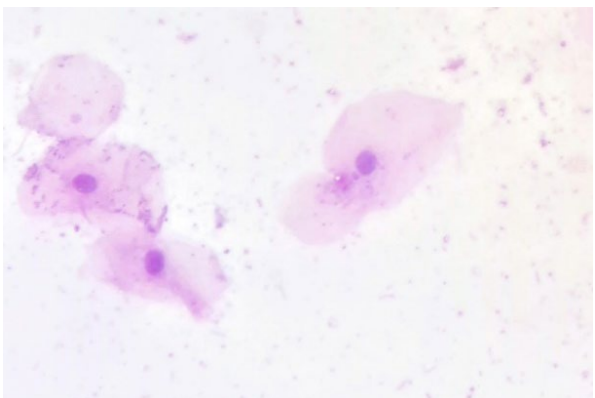

Fig. 1. Feulgen stain

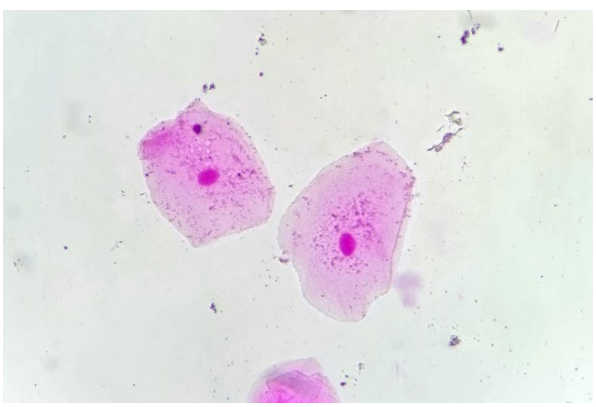

Fig. 2. PAP stain

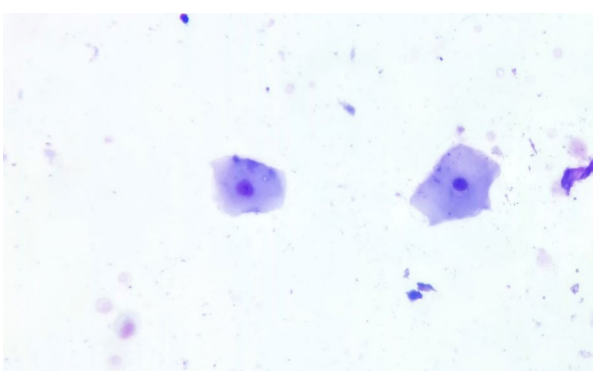

Fig. 3. LG cocktail stain

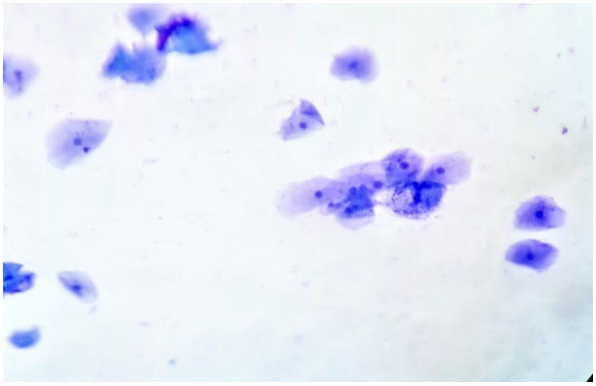

Fig. 4. Toluidine blue stain

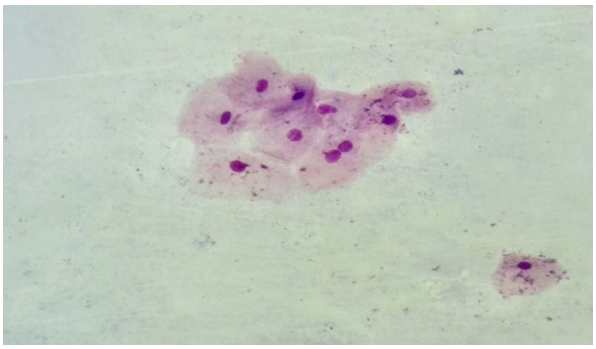

Fig. 5. H&E stain

Discussion

Oral cancer is the sixth most common type of cancer, with INDIA contributing to almost one-third of the total burden, and the second country having the highest number of oral cancer cases.[21] Studying pre-neoplastic diseases and oral malignancies via Exfoliative cytology is an economical, non-invasive and easy procedure for studying biomarkers of neoplastic changes in oral cells.[11] Biomarkers of genetic damage have immense potential to reveal the stage of the disease and are helpful in early diagnosis and progression of the disease. Among the various chairside investigations of lesions at an early stage, the micronuclei assay using Exfoliative cytology is a reliable procedure for diagnosing individuals who are at high risk of developing malignancy.[21,22]

Buccal cells are the first barrier for carcinogens inhaled or ingested and are capable of metabolizing carcinogens into reactive products. These carcinogens that cause genetic damage to stem cells in the basal layer are referred to as micronuclei during nuclear division. These chromosomal fragments or whole chromosomes that did not reach the spindle pole thus lag behind anaphase during nuclear division and are not incorporated into the daughter nuclei in telophase but are covered by a nuclear membrane resembling a small nucleus. Thus, the cells were termed micronuclei. As the oral epithelium is constantly maintained by cell renewal, which occurs at the basal layer by mitosis, damaged cells with disrupted nuclear division migrate to the surface along with other cells and are collected via Exfoliative cytology for evaluation.[23,24]

In our study, we chose nuclear specific stain, Feulgen, and nonspecific DNA stains, such as PAP, H&E, LG cocktail, and toluidine blue, to stain the smears obtained from exfoliative cytology to determine the micronuclei count in each individual smear.[20] Our study stands novel to the best our knowledge as nobody has done study on micronuclei count comparing with five stains and including all three commonly encountered pre malignant disorders as leukoplakia, lichen planus and oral sub mucous fibrosis compared with OSCC. Belgaumiet al. performed a study on 100 patients diagnosed with OSCC and 100 control patients and stained buccal smears with a PAP and LG cocktail. They concluded that, compared with the LG cocktail, PAPs gave almost equivalent results.[25,26]

Apurva A et al. compared PAP and LG cocktails to study oral neoplastic lesions and found no significant difference in the staining properties of the two agents, but we found a significant difference in the staining score of PAP compared with that of the LG cocktail.[27,28]

In addition, Gupta et al. conducted a study on 45 patients – 15 with OSCC, 15 with OSMF and 15 with leucoplakia. They found a significant increase in the mean micronuclei count, which was highest in OSCC patients, followed by OSMF and leukoplakia patients. They also concluded that the PAP stain was better than the LG cocktail and H&E stain.[29]

We found a significant increase in the mean micronuclei count in the PMD and OSSC groups compared with that in the control group as also reported in recent research by Bhatnagar et al. in which they also concluded that there is an increase in micronuclei count suggesting neoplastic changes as malignant changes progresses compared to histologically normal mucosa.[30]

Sarto et al. considered micronuclei size to be restricted to 1/5th of the parent nucleus.[31-34] Jadhav et al. considered micronuclei to be between 1/3 and 2/3 the size of the nucleus. We used the criteria suggested by Tolbert et al. for a micronuclei size <1/3rd of the parent nucleus.[21] A larger micronuclei result from the exclusion of the whole chromosome following damage to the spindle apparatus of the cell, whereas smaller micronuclei result from structural aberrations causing chromosomal fragments.[35]

These findings were consistent with those of studies performed by Sivasanskari et al., Dindgire et al., Grover et al., Saurabh et al., and Gunjan et al. (2019).[14,20,36-38]

In the OSCC group, as shown in Table 1, all the parameters included micronuclei shape, size, texture, same focal plane, smooth perimeter and separation from the main nucleus. On all of these parameters, in our study, we found the highest mean number of micronuclei with Feulgen, followed by PAP and LG cocktail.

Sharma D et al. performed a cytomorphometric analysis of 125 subjects divided into 25 patient groups, namely, healthy patients, smokers, smokeless tobacco users and OSCC patients, and evaluated variations in cellular area, nuclear area, cellular diameter, nuclear diameter and the nuclear/cytoplasmic ratio. They used Feulgen for staining smears and concluded that cytology was useful for DNA-specific staining for the early diagnosis of OSCC.[39]

Kumar et al. studied 15 confirmed cases of PMD, 15 confirmed cases of OSCC and 15 control subjects free of anu oral lesions. They used PAP staining for the evaluation of smears and reported a stepwise increase in the micronuclei count from the control group to the PMD group, after which the highest increase was observed in the OSCC group.[4]

Chaudhary M et al. studied histopathologically confirmed patients and divided them into three groups of patients with well-differentiated, moderately differentiated and poorly differentiated carcinoma and compared them with 10 patients in the control group.[22] They used PAP and Feulgen to study mean micronuclei and found an increased micronuclei count in OSCC in increasing order from well-differentiated to poorly differentiated OSCC. The authors commented that Feulgen is still the gold standard for studying micronuclei and that PAP can lead to misinterpretation of the micronuclei count, as it may not distinguish between true micronuclei and keratohyaline granules, bacterial clumps or artefacts.[40] (Figure 1 and 2).

The primary step in the Feulgen reaction is hydrolysis, in which purine bases are detached from DNA, exposing free aldehyde groups and leaving the DNA back bone intact; thus, the DNA becomes apurinic. When the cells are reacted with Schiff's reagent, bleached pararosalinine, which is a component of basic fuschins, binds to free aldehyde groups in apurinic DNA, acquiring a magenta color. The hydrolysis of RNA does not occur, and so RNA rings do not open or are less frequent than DNA rings; moreover, the RNA is degraded and washed off more easily. This is the reason why Feulgen is DNA specific.[41]

A statistically significant difference was observed among all of the stains. The mean staining score for Feulgen was the highest, followed by that for PAP, and the lowest mean staining score was for H&E. The increase in the mean micronuclei count in OSCC patients is attributed to increased abnormal nuclear content because of increased mitotic activity, chromosomal aberrations and disturbance of the cell cycle.

In smears obtained from Leucoplakia patients with reference to Table B, PAP staining gave the highest micronuclei count based on all of the criteria for selecting the micronuclei.

In a study comparing leucoplakia patients with those with habits of tobacco and habit-free controls, Mahimkar et al. reported similar results. They concluded that there is an overall increase in the micronuclei count in leucoplakia patients, indicating increased DNA damage and genetic alterations in the buccal cells of leucoplakia patients.[42]

This observation has been described by many other researchers, such as Grover et al., who conducted a study on 45 patients with PMD and stained smears with Feulgen, PAP and H&E. These authors found that in potentially malignant disorders, Feulgen had fewer micronuclei than PAP did, which is in correlation with our study on the mean micronuclei count in PMDs.[14]

According to Samantha et al., possible explanations for MN formation in preneoplastic conditions include chromosomal loss/breakage, mitotic apparatus dysfunction, aneuploidy and genetic instability. The mean staining score for PAP was the highest, followed by that for Feulgen. Toluidine blue was used to determine the minimum mean staining score.

Patil et al. compared PAP and toluidine blue in cervicovaginal cytology and found that toluidine blue was equivalent to PAP.[17,24]

Kohli et al. conducted a study on 200 subjects divided into four groups of 50 normal individuals (as controls), 50 with tobacco habits without leucoplakia, 50 with tobacco habits along with leucoplakia and 50 with OSMF. Potentially malignant disorders, including leukoplakia and OSMF, were studied. The authors used Feulgen, PAP and May Grunwald Giemsa stains for smear staining. The authors concluded that the number of micronuclei was greater in PMDs than in the control, and PAP had a greater micronuclei count than did Feulgen, which is in accordance with our study.[43] The lichen planus group, with reference to Table 3 and PAP, had the best mean staining scores for all the criteria for micronuclei. PAP was subsequently administered by Feulgen. Buajee et al compared oral lichen planus mucosa with normal mucosa and reported that the mean micronuclei frequency in OLP lesions was significantly greater ($p<0.01$) than that in normal-appearing mucosa adjacent to lesions and that in normal individuals.[23]

Compared with H&E staining, PAP was superior, as was the observation of Balaji et al.[44] Moreover, they compared PAP to H&E in oral lichen planus patients and found that PAP was better than H&E. Toluidine blue and LG cocktails had nearly the same effects. Figure (3,4&5). Ranjbar et al. performed a study on oral lichen planus and oral lichenoid reaction and reported increased mean micronuclei counts in OLP and OLR patients compared to those in normal mucosa. However, there were no significant differences between OLP and OLR ($p=0.67$ and $p=0.36$, respectively).[45]

In the OSMF group, with reference to Table D and PAP, the highest staining score was obtained for the micronuclei count, with PAP indicating the micronuclei size and shape and revealing the location of the focal

plane. No significant differences in texture, color or separation of micronuclei were observed among the strains, but for the evaluation of the mean staining score, PAP was the best stain, with the highest mean, and H&E was the lowest mean. Shreyas Shah et al. studied 60 patients with 30 OSMFs and 30 OSCC patients. They used PAP staining to study micronuclei and found a significantly increased number of micronuclei from the control to the OSMF-treated OSCC patients.[46]

Kumar M et al. studied a total of 90 participants and divided them into groups of 30 individuals each into one group with PMDs, one group with habits of tobacco use with lesions and the other group with habits of tobacco use without lesions. They used Feulgen and PAP for staining smears and found a high number of mean micronuclei in subjects with PMD lesions with PAP compared to Feulgen and suggested that PAP is DNA nonspecific. This high frequency of observation of micronuclei may not be true of micronuclei, as these are actually keratin granules that are found in degenerated cells with nuclear defects. These round cytoplasmic structures do not contain DNA and might resemble micronuclei.[26]

Kabiraj et al. screened lichen planus, leucoplakia and OSMF lesions with PAP using exfoliative cytology and found that 90% of the leukoplakic lesions were class II cytological features. Sixty-five percent of lichen planus had Class II features. In the oral submucous, 80% of patients had class II features.[47]

PAP had statistically significant effects on the micronuclei count, shape, texture, and separation in the PMD group because nonspecific DNA staining and nuclear anomalies, such as karyorrhexis, karyolysis, condensed chromatin, and binucleates, may be misinterpreted as micronuclei. Keratin granule formation may also be misinterpreted as the presence of micronuclei along with bacteria and small dye granules.[14]

Hence, in our study, there was a significant increase in the MN frequency in all the PMD and OSCC groups compared to that in the control group. When the nuclear staining properties were compared, the DNA-specific stain Feulgen was the best stain in the OSCC group, and PAP was the best stain in all the PMD groups, as were leukoplakia, lichen plans and oral submucous fibrosis. We did not find any statistically significant difference in nuclear staining properties in the OSMF group, although the mean staining score for PAP was maximal.

Based on our study, we can say that Feulgen is the best stain for observing MNs in malignant lesions but is technique sensitive. However, rapid PAP staining is less time-consuming than other methods and yields good results in terms of nuclear and cytoplasmic staining.

Conclusion

Facilitating exfoliative cytology to study biomarkers, such as micronuclei, in oral premalignant lesions and malignant lesions is a great tool for studying and helping in the early diagnosis of any cellular changes because of the presence of any carcinogen leading to the formation of premalignant lesions and the conversion of premalignant lesions into malignant lesions.

The micronuclei count has immense potential to be studied further for its significance in identifying premalignant lesion progress and thus can be a reliable tool for early intervention and treatment to reduce mortality and conversion to malignancy. In addition to PMDs and OSCC, the micronuclei assay has wide applications beyond these lesions to study genotoxicity in other tumors and lesions in humans, the effects of various chemicals or carcinogens on the human body, and the effects of drugs during and after treatment for these pathologies.

DNA specificity is important for studying nuclear content remarkably, and this approach helps to rule out other nuclear anomalies that can be misinterpreted by other non-DNA-specific stains and is still the gold standard for studying DNA-specific abnormalities and cytomorphology.

The staining properties of rapid PAP are good quality and less time consuming, providing satisfactory diagnostic reliability. Although the MN count is increased in PMDs and OSCC, additional studies are needed to determine whether the results of exfoliative cytology are equivalent to those of histopathological biopsy, which is the gold standard for diagnostic purposes.

For counting of micronuclei one has to be skilled enough to differentiate between micronuclei and other nuclear and cytoplasmic abnormalities and inclusion. Failing so can give false results. When doing PAP staining one has to be cautious because PAP stain gives higher micronuclei count which is sometimes false positive. Apart from these limitations micronuclei count is an excellent tool to study neoplastic changes with oral brush cytology. Although a newer technique yields equivalent results to PAP in nuclear and cytoplasmic staining, the LG cocktail can be used as a substitute for PAP for screening purposes. Toluidine blue is used in cervicovaginal cytology and as a vital stain in the oral cavity, its application in oral cytology still needs to be studied.

Declarations

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Author contributions

Conceptualization, M.I.K. and A.K.; Methodology, M.I.K.; Software, M.I.K.; Validation, M.I.K., S.S.K. and A.K.; Formal Analysis, A.K.; Investigation, M.I.K.; Resources, A.N. and K.A.; Data Curation, K.A. and S.L.; Writing – Original Draft Preparation, M.I.K.; Writing – Review & Editing, M.I.K.; Visualization, S.L.; Supervision, S.S.K.; Project Administration, AK.

Conflicts of interest

The authors have no competing interests.

Data availability

The data supporting the findings of this study are available from corresponding author (Mohammad Imran khan), upon reasonable request.

Ethics approval

The duly constituted Ethical Committee for Post Graduate students of Career Post Graduate Institute of Dental Sciences & Hospital, Lucknow has approved and cleared the research project (ethical ref. No. CPGIDSH/22/280).

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