

Evaluation of Feulgen, Crystal Violet and Mallory's PTAH Stains in Assessment of Mitotic Figures in 3 Grades of Oral Squamous Cell Carcinoma

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ABSTRACT

Introduction: Oral squamous cell carcinoma (OSCC) represents the most frequent of all oral neoplasms. Carcinogenesis is the result of genetic alterations in nuclear DNA, which is evident as deregulated mitosis and nucleoli numbers. Increased and abnormal mitotic figures are a crucial factor in grades of OSCC. Other than the routinely used stains, several other special stains is used to appreciate mitotic figures (MF).

Aims: This study was aimed to observe and compare mitotic figures in tissue sections of oral squamous cell carcinoma stained with Feulgen, 1% Crystal violet and Mallory's PTAH, to evaluate the efficacy of these stains in three different grades of oral squamous cell carcinoma and to search for a simple, cost effective and most effective staining technique to study mitotic cells.

Materials & methods: 20 archival samples each of well, moderate and poorly differentiated carcinoma was taken. Three sections of 4 µm thickness from each block was stained with Feulgen staining method and another section was stained with 1% Crystal violet stain, and Mallory's PTAH stain respectively. After mounting, the slides were observed for mitotic figures and average number of mitotic figures were counted under the same magnification for all the slides.

Results: Feulgen gave the best staining result, followed by Mallory's PTAH and Crystal violet. Total number of mitotic figures showed an increase with each grade of OSCC.

Conclusion: Feulgen was the most reliable, cost effective, easy and the most feasible staining technique to stain mitotic figures.

Key words: OSCC, Mitotic figures, Feulgen, Mallory's PTAH, 1% Crystal violet.

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INTRODUCTION :

Oral cancer includes a group of neoplasms affecting any region of the oral cavity, pharyngeal regions and salivary glands. However, this term tends to be used interchangeably with oral squamous cell carcinoma (OSCC), which represents the most frequent of all oral neoplasms. It is estimated that more than 90% of all oral neoplasms are OSCC. Worldwide, oral cancer accounts for 2%–4% of all cancer cases.¹ It is the sixth most common cancer worldwide.² Diverse malignant tumors of various cellular lineages originate in the oral cavity. Among these, squamous cell carcinoma (SCC) constitutes a significant proportion, comprising 95% of head and neck cancers. Oral squamous cell carcinoma (OSCC) has a striking global incidence and equally formidable mortality rates.³

Mitosis is a process of cell division in which the DNA molecules of each chromosome are divided into two nuclei followed by cytokinesis where a mother cell divides exactly into two identical daughter cells. Any defects during mitosis bring about abnormalities such as micronuclei, binucleation, pyknotic nuclei and increased or abnormal mitotic figures. Excessive proliferation of cells due to increased mitosis is the hallmark in oral pre-cancer & cancer. The number of mitosis seen in tissue sections is an important factor in grading malignancies and also in the determination of prognosis. So assessment of mitotic figures is routinely practised as a prognostic indicator in oral epithelial dysplasia (OED)

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and in oral squamous cell carcinoma (OSCC).⁴ Newer methods such as immunohistochemistry, flow cytometry, autoradiography, DNA ploidy measurement are available to assess the mitotic figures, but cost and time factor make them less feasible.⁵

Routine staining procedure may pose a problem in differentiating a mitotic cell from apoptotic cells which may weaken the reliability of histological grading.⁶ Mitosis are sometimes distinctly abnormal, that it appears as anarchic multiple spindles in tripolar or quadripolar forms.⁷ The distinction between a pyknotic nucleus, an apoptotic cell and a mitotic cell in a routinely

stained tissue section may pose a problem. Errors in identifying a mitotic cell can thus weaken the reliability of histological grading due to the loose use of morphologic criteria. Combination of stains and modification of the existing histochemical techniques can overcome these problems. A literature search revealed numerous selective stains like Crystal violet, malachite green with Crystal violet, toluidine blue, Feulgen and giemsa which highlight chromatin patterns. These stains have been used in brain tissue, uterus and breast carcinoma.^{5,8}

AIMS:

To observe and compare mitotic figures in tissue sections of 3 grades of oral squamous cell carcinoma and evaluate the efficacy of Feulgen, 1% Crystal violet and Mallory's PTAH in it.

Not many studies were done to compare the above stains; therefore it was an attempt to use Feulgen, 1% Crystal violet and Mallory's PTAH as selective stains for better appreciation of mitotic figures in tissues sections of oral squamous cell carcinoma and to arrive at a simple laboratory procedure, which was feasible to carry out on daily basis.

MATERIALS & METHODS:

The study sample included department archival tissues embedded in paraffin blocks diagnosed as well differentiated OSCC (n=20), moderately differentiated OSCC (n=20), poorly differentiated OSCC (n=20). Broader's grading system (1927) for OSCC was used to assess cases diagnosed of OSCC. The study was approved by Institutional Ethical Review Board (IRB).

Three serial sections of 4 micron thickness were made from each tissue specimen. Each section was stained with Feulgen, Mallory's PTAH and 1% Crystal violet stain (Fisher Scientific Company) respectively. Stain was prepared according to method given by Godkar *et al.* and Bancroft *et al.*^{9,10} After mounting, the slides were observed under microscope and photomicrographs were captured. The sections were studied under 4x, 10x and 40x magnification under a binocular microscope Olympus BX41.

The area of the sections selected for counting of mitotic figures

TABLE 1: Comparing the staining quality of 3 stains in well, moderate and poorly differentiated OSCC

LESION	N	Mean	Std. Deviation	F	p
WDSCC	FEULGEN	20	17.000	2.406	
	CV	20	14.875	2.549	
	MALLORY'S PTAH	20	16.000	2.212	3.947 .025
MDSCC	FEULGEN	20	21.600	2.850	
	CV	20	18.475	3.011	
	MALLORY'S PTAH	20	19.750	2.755	5.979 .004
PDSCC	FEULGEN	20	25.950	1.877	
	CV	20	22.375	1.546	
	MALLORY'S PTAH	20	23.125	1.432	26.774 .001

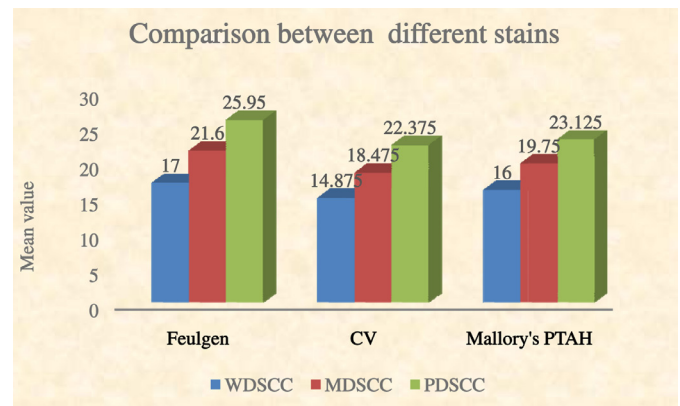
was the most invasive and the most cellular part of the tissue which made the inclusion criteria for this study. Mitotic figures were identified by using criteria given by Van Diest *et al.*¹¹

- ❖ The nuclear membrane must be absent indicating that the cells have passed the prophase.
- ❖ Clear, hairy extension of nuclear material (condensed chromosome) must be present – either clotted (beginning metaphase), in a plane (metaphase / anaphase) or in separate clots (telophase).
- ❖ Two parallel, clearly separate chromosome clots to be counted individually as if they are separate mitoses.

Exclusion criteria were as follows:

- ❖ Areas showing necrosis
- ❖ Inflammation
- ❖ Tissue folds and calcifications

Each slide was observed by two separate observers without any exchange of information regarding study sample details. Observations made by each observer regarding number of mitotic figures were recorded separately and average value was calculated for both observations. The entire stretch of epithelium was observed and the number of mitotic figures in each field was counted in a



GRAPH 1 : Comparison of staining quality of Feulgen, 1% Crystal violet and Mallory's PTAH in 3 grades of OSCC

TABLE 2 : Intercomparison of staining efficacy of each stain within 3 grades of OSCC

Tukey HSD

STAIN	(I) LESION	(J) LESION	Mean Difference (I-J)	Sig.
FEULGEN	WDSCC	MDSCC	-4.600	<0.001 vhs
		PDSCC	-8.950	<0.001 vhs
	MDSCC	PDSCC	-4.350	<0.001 vhs
CV	WDSCC	MDSCC	-3.600	<0.001 vhs
		PDSCC	-7.500	<0.001 vhs
	MDSCC	PDSCC	-3.900	<0.001 vhs
MALLORY'S PTAH	WDSCC	MDSCC	-3.750	<0.001 vhs
		PDSCC	-7.125	<0.001 vhs
	MDSCC	PDSCC	-3.375	<0.001 vhs

step ladder fashion to give the total number of mitotic figures in that particular tissue section.

The number of microscopic fields were defined and counted under same magnification for all the slides. Data obtained was statistically analyzed by using Kruskal Wallis test. P-value of <0.05 was considered as statistically significant. All the data analysis was done using the Statistical Package for Social Sciences (SPSS) [Version 17].

RESULTS:

A total of 60 cases of oral squamous cell carcinoma were retrieved, when compared with three stains, it was observed that, Feulgen stained the best among all three, followed by Mallory's PTAH and Crystal violet (fig 1, 2 and 3). The values shows high significance among the 3 grades of OSCC too. P value was 0.25 for well differentiated OSCC, 0.004 for moderately differentiated OSCC, and 0.001 for poorly differentiated OSCC. The P value seems to be very significant in case of poorly differentiated OSCC with all three stains as provided in Table 1.

The number of MFs was also compared among the three groups, it was seen that a significant increase in the number of Mitotic Figures was observed in sections stained with Feulgen stain as compared to 1% Crystal violet and Mallory's PTAH stains, especially in poorly differentiated squamous cell carcinoma. First graph shows significant mean value showing increased efficacy in staining mitotic figures with Feulgen stains, followed by Mallory's PTAH and Crystal violet.

A significant ($p < 0.001$) increase in the identification of MFs was observed in Feulgen stained sections as compared to the others. A very high significance was observed here. It was checked for any variation in the number of MFs in different grades of squamous cell carcinoma, which showed a significant increase in the number of MFs between well, moderate, poorly differentiated squamous cell carcinoma.

Multiple comparisons were made with Tukey's test between each grade of the individual stained slides, and there was increased mean difference when comparing well differentiated and poorly differentiated in Feulgen stains, followed by Mallory's PTAH and 1% CV as shown in Table 2.

Inter-observer variability was calculated to be 0.98 which showed high agreement between the observers.

DISCUSSION

Mitosis is a process where there is equal division of chromosomes and their genes into two identical groups & serves as the basis for cell proliferation. Cell proliferation is an uncontrolled event in vari-

ous neoplasms due to presence of abnormal & bizarre mitosis. It is an important factor to maintain tissue integrity. Various genetic alterations take place during cell proliferation.⁷ Therefore, the study of mitosis is very important to analyze the aggressiveness and prognosis of lesions. The significance of mitotic figures are: prognostication of various neoplasms, mechanisms behind observed chromosomal aberrations, assessing cellular proliferation and aid in histological grading.¹² Various authors like Mehta *et al*¹³ and Ankle *et al*⁵ have reported the presence of mitosis in oral precancerous and cancerous lesion.⁸

Special stains can be used for easy identification of mitotic figures since Haematoxylin and Eosin poses a problem, such as it cannot help to differentiate between mitotic cells and apoptotic cells and also does not give the clear picture of the various phases of cell cycle⁵.

A literature search reveals that various stains such as Crystal violet, malachite green with Crystal violet, toluidine blue, giemsa and Feulgen have been used to identify mitotic figures. Amongst which, Crystal violet and Feulgen are used to study the chromosomal pattern in cells based on acid hydrolysis of DNA.¹²

Crystal violet is a basic dye which has high affinity for the highly acidic nature of chromatin present in the mitotic cells. These mitotic cells stain magenta and stand out against the light blue background.¹⁴ Feulgen stain clearly stains the chromosome leaving cytoplasm clear and unstained due to hydrolysis of tissue sections by HCL at 60°C. This will help in easy identification of mitotic figures and also offer a more reliable counting.

However, in the present study, we noted that Feulgen was superior in staining MFs as compared to 1% Crystal violet. The purple-colored chromatin material against a green cytoplasm facilitated the distinct and highly selective identification of MFs even at low power. The use of Feulgen stain is reproducible, rapid, simple and feasible for the localization of MFs and can be routinely employed. The reason could be attributed to the qualitative comparison between Feulgen and the other stains

In the present study, we aimed to find out the most reliable, cost effective, simple and rapid technique to stain mitotic figures. It was observed that Feulgen gave the superior results compared to other two stains. Distinguishing mitotic figures from pyknotic nuclei, karyorrhexis, and apoptosis is important to prevent false positive results, and Feulgen provided the excellent morphological detail.^{7,12,15}

A similar study done by Jadhav KB, Sri Chinthu KK *et al*, evaluated a significant increase in number of MFs in oral epithelial dysplasia and OSCC in comparison with normal oral mucosa. There

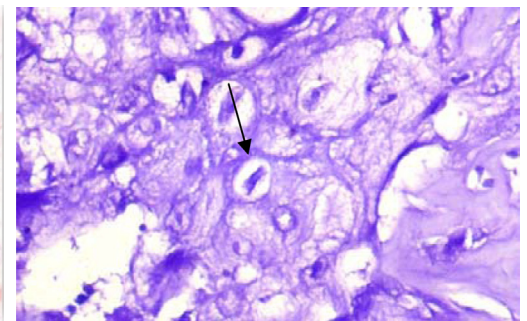
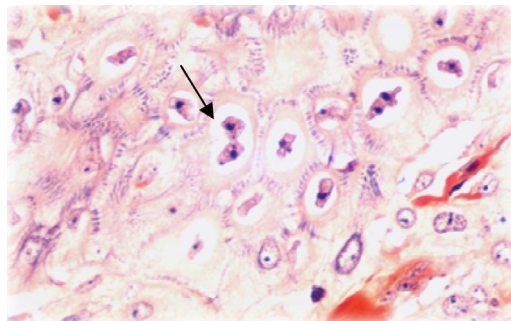
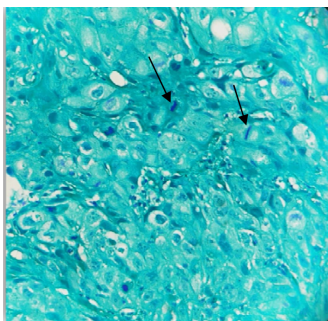


Figure 1: Feulgen staining (400x) Figure 2: Mallory's PTAH stain(400x)

Figure 3: Crystal violet stain (400x)

was a highly significant increase in number of MFs in Crystal violet stained tissue sections when compared with H and E stain.^{8,4}

Another study by Ankle M, Tandon A, Kesarkar K et al, found a statistically significant increase in the mean mitotic count in Crystal violet-stained sections of epithelial dysplasia and oral squamous cell carcinoma as compared to the H and E-stained sections. But no significant difference was found in the mitotic counts determined in dysplasia or carcinoma by either the Crystal violet or the H and E-staining techniques which was not in correlation with our study.^{5,16,17}

Crystal violet stain is a step ahead of the standard H&E stain.¹⁵ But the use of Mallory's PTAH in our study proved to be better than Crystal violet in providing the results. Till date no study was carried out to stain mitotic figures using Mallory's PTAH and no comparison was done with other special stains using this. Chatterjee S performed a study regarding Mallory's PTAH staining and to calculate mitotic indices and mean nucleoli counts. Her study was conducted using Mallory's PTAH staining technique as an alternative method to molecular markers or the AgNORs staining to analyse biological behaviour reflected by the mitotic activity and nucleoli numbers.¹⁸

Comparison of mitotic counts between various grades of oral squamous cell carcinoma also gave a positive significant result, with poorly differentiated OSCC showing statistically significant increase in mitotic figures in all the three stains.

CONCLUSION:

This is a study to compare the efficacy of Mallory's PTAH, Feulgen, and Crystal violet stains in 3 different grades of squamous cell carcinoma. Feulgen shows the best results in terms of efficacy of staining mitotic figures, followed by Mallory's PTAH and Crystal violet. So this study strongly supports the use of Feulgen stain for distinct and selective staining of mitotic figures. For detecting nuclear DNA, it is a reliable, cost effective, easy, feasible and specific histological method, if done carefully. In our study, we found all the stains as useful in identification of mitotic figures, even though Feulgen stains gave us the best result.

The authors intend to conduct this study with a larger sample size for quantification of mitotic figures with Feulgen, Mallory's PTAH and Crystal violet stain in oral squamous cell carcinoma.

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