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Comparison of Staining of Mitotic Figures By Haematoxylin And Eosin, Crystal Violet And Giemsa Stains In Oral Squamous Cell Carcinoma

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Abstract

Context: Increased mitotic figures because of excessive proliferation of cells are considered as hallmark in cancer and precancer. Inaugurating an easy way to differentiate mitotic figures and defects in mitosis will help in classifying and understanding their biological potential.

Aims: To compare the number of mitotic figures present in normal oral mucosa and in oral squamous cell carcinoma in Heamatoxylin and eosin, crystal violet and Giemsa stained section

Settings and Design: Study sample include control groups comprised of tissue specimen from oral mucosa of healthy volunteers (n=15) and archival tissue embedded in paraffin blocks diagnosed as oral squamous cell carcinoma (n=31). Three serial sections of each tissue specimen were stained separately with H&E, giemsa and 1% crystal violet stain

Statistical analysis used: The groups were compared by one way analysis of variance (ANOVA) and the significance of mean difference between the groups was done by Tukey’s post hoc test after ascertaining normality by Shapiro-Wilk’s test and homogeneity of variances by Levene’s test. A two-tailed (α=2) p value less than 0.05 (p< 0.05) was considered statistically significant.

Results: An increase in the number of mitotic figures was observed in crystal violet staining followed by giemsa

then H&E but with a non-significant result. Whereas for defects in mitosis, mean values was nearly equal for crystal violet, H&E followed by giemsa with non-significant result.

Conclusions: The routine stains along with selective stains are equivalent to visualize the various phases of mitotic figures and mitotic defects.

Keywords: Anaphase; Crystal Violet;Eosine;Giemsa Stain; Haematoxylin; Metaphase; Squamous Cell Carcinoma; Prophase;Telophase.

Keymessages: The routine stains along with selective stains are equivalent to visualize the various phases of mitosis and mitotic defects.

Introduction

Oral cancer ranks 3rd most common malignancy among all the cancers in India. Annually nearly 80,000 cases are diagnosed as oral cancer. Head and neck cancer is a major cause of concern in Asia, especially in the Indian subcontinent (1, 2). Squamous cell carcinoma is the most common oral cancer, and some oral carcinomas are preceded by precursor lesions that can present as leukoplakia, erythroplakia, or erythroleukoplakia (3, 4).

Genetic damage is designated by an increased and abnormal mitosis which is an important feature in precancer and cancer. Thus for prediction of precancerous and cancerous lesions, identification and quantification of mitotic cells forms an indivisible part of the histological grading systems used. Therefore, mitotic figure counting is extensively used to assist in pathological judgment (5).

Mitosis is a process where a parent cell divides into two identical daughter cells. The various phases of mitosis are prophase, metaphase, anaphase and telophase. Defects of mitosis result in micronuclei, binucleation, broken egg appearance, pyknotic nuclei, and increased number of mitotic figures or abnormal mitotic figures. These are commonly seen in oral epithelial dysplasia and squamous cell carcinoma (5).

Several selective stains such as crystal violet, malachite green with crystal violet, toluidine blue, and Giemsa stain have been used for staining of mitotic figures according to various literature surveys. There are very few studies which have been conducted on the oral cavity(5).

Crystal violet is a basic dye which has affinity for the acidic chromatin of mitotic cells. It stains mitotic cells magenta against a light blue background (5). Giemsa's stain belongs to Romanowsky group of stains which were formerly designed to incorporate cytoplasmic (pink)staining with nuclear (blue) staining.

They were originally developed for blood films and bone marrow films, but cell smears, cellular imprints cytospins preparations of different origin and thin tissue sections work equally well (6).

In the present study giemsa is used in combination with Leishman stain. It is easy, one-step technique and cost-effective. Leishman stain is a good nuclear stain. It gives an intense staining of extracellular ground substance while it leaves individual cells and 3-dimensional clumps under-stained when used alone. When incorporated with Giemsa stain, considered as good cytoplasmic stain, the Leishman giemsa (LG) cocktail provides a moderate metachromasia to the ground substance and it brilliantly stained cellular components (7, 8).

The present study is an attempt to compare the staining of mitotic figures and defects in mitosis by the Haematoxylin and eosin stain; Giemsa stain and Crystal violet stain in oral squamous cell carcinoma.

Thus, the outcome can provide with simple, speedy, cheaper, and most feasible staining technique for even a small scale laboratory in order to localize mitotic figures and to assess proliferation.

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Subjects and Methods

The total numbers of 46 Formalin Fixed Paraffin embedded tissue blocks were evaluated, of which 31 samples were of previously reported cases which were diagnosed as Oral Squamous Cell Carcinoma in the Department of Oral Pathology and Microbiology, and 15 fresh biopsy specimens of control group were included in the present study. An ethical clearance was obtained from the institutional ethical committee headed by the head of the institution.

A customized case history proforma was obtained for each case. The individuals from the control group were informed prior to the procedure and a signed informed consent was obtained from each. The clinical details of all the subjects were tabulated.

Inclusion criteria: Formalin fixed paraffin embedded tissue blocks of reported cases which were diagnosed as Oral Squamous Cell Carcinoma with good morphology and Blocks with sufficient tissue.

Exclusion criteria: Patients with systemic disorders such as diabetes mellitus, hypertension, bleeding disorders, etc., was excluded from the study. Diabetes mellitus and hypertension will affect the rate of mitosis through altered metabolism of cell and oxygen supply, respectively (9). The area showing necrosis, inflammation, tissue folds and calcifications were not considered for counting.

From each sample, 3 - 4 micrometre thick three serial sections were cut by using soft tissue microtome. First section was stained with routine haematoxylin and eosin stain, second section with giemsa stain and third section by 1% crystal violet stain. The area selected for counting of mitotic figures included the most invasive part and the most cellular part of the tissue. Mitotic figures like prophase, metaphase, anaphase, telophase and defects in mitosis like pyknotic nuclei, binucleation, micronuclei, broken egg appearance were counted under a magnification of x400 in step ladder fashion in ten high power fields in high power fields in Motic Pentahead microscope (Model: BA400) using Moticam 2500 (5.0M Pixel USB2.0) camera by single observer.

Mitotic figures were identified by using criteria given by Van Diest et al., 1992 (10).

i. The nuclear membrane must be absent indicating the cells have passed the prophase.

ii. 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).

iii. Two parallel, clearly separate chromosome clots to be counted individually as if they are separate mitoses.

We not only counted the total number of mitotic figures, but noted the phases of the cell cycle (prophase, metaphase, anaphase and telophase).

Statistical Analysis

The further data was summarized on the Microsoft excel software. They were further analyzed statistically with the help of SPSS software version 18. Data were further summarized as Mean ± SD (standard deviation). The groups were compared by one way analysis of variance (ANOVA) and the significance of mean difference between the groups was done by Tukey's post hoc test after ascertaining normality by Shapiro-Wilk's test and homogeneity of variances by Levene's test. A two-tailed (α=2) p value less than 0.05 (p<0.05) was considered statistically significant.

Results

The 31 tissue specimens of Oral Squamous Cell Carcinoma (OSCC) ranges from 21 to 80 years, of which 24 were males and seven, were females. Among 31 cases of OSCC, 14 were Well Differentiated Squamous Cell carcinoma (WDSCC) cases, 14 were Moderately Differentiated Squamous Cell carcinoma (MDSCC) and

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three were Poorly Differentiated Squamous Cell carcinoma (PDSCC). For control, 15 samples were selected ranges from 10 to 50 years, six were male and nine were females. The mitotic figures, defects in mitosis and total mitotic count (mitotic figures + defects in mitosis) were found absent in controls thus not analyzed and compared.

On evaluation of mitotic figures, the mean value (± SD) of prophase in H&E, Giemsa and Crystal violet stain groups ranged from 6.52 ± 3.60, 8.10 ± 4.17 and 10.06 ± 4.42, respectively with a statistically significant result with p value of 0.004. The mean value (± SD) of metaphase of H&E, Giemsa and Crystal violet groups ranged from 11.39 ± 6.03,10.71 ± 6.17 and 12.39 ± 5.59, respectively. The mean value (± SD) of anaphase of H&E, Giemsa and Crystal violet groups ranged from 0.84 ± 1.13, 0.45 ± 0.77 and 0.68 ± 0.94, respectively. The mean value (± SD) of telophase of H&EE, Giemsa and Crystal violet groups ranged from 0-1 with mean (± SD) 0.06 ± 0.25, 0.06 ± 0.25 and 0.03 ± 0.18, respectively. The mean value (± SD) of overall mitotic figures in H&E, Giemsa and Crystal violet stains are 18.81 ± 9.41, 19.32 ± 10.20 and 23.16 ± 10.12, respectively with a non-significant result.(Table 1)

On intercomparison of mean value of different mitotic figures in three different stains, only prophase showed a statistically significant result with p value of 0.003 in H&E versus crystal violet stain. (Table 2)

On evaluation of defects in mitosis, micronuclei and broken egg appearance were found absent thus not analyzed statistically. The mean value (± SD) of pyknotic nuclei of H&E, Giemsa and Crystal violet groups ranged from 0 3.61 ± 1.65, 4.00 ± 1.65 and 4.16 ± 1.57, respectively. The mean value (± SD) of binucleation of H&E, Giemsa and Crystal violet groups ranged from 1.39 ± 1.12, 0.94 ± 0.85 and 0.87 ± 0.88, respectively. The mean value (± SD) of overall defects in mitosis counts (pyknotic nuclei + binucleation) of H&E, Giemsa and Crystal violet groups ranged from 5.00 ± 2.07, 4.94 ± 2.13 and 5.03 ± 2.26, respectively with a non-significant result. (Table 3)

On intercomparison of mean value of various defects in mitosis in three different stains, none of the defects showed any significant result. (Table 4)

The mean values (± SD) of total mitotic counts (mitotic figures + defects in mitosis) in H&E, Giemsa and Crystal violet stains ranged from 23.81 ± 10.78, 24.26 ± 11.52 and 28.19 ± 11.72, respectively with a non-significant result. (Table 5)

On intercomparison of total mitotic count within three different stains, H&E vs. giemsa, H&E vs. crystal violet and giemsa vs. crystal violet showed non-significant result with p value of 0.987, 0.286 and 0.363 respectively. (Table6)

Discussion

The division of cells maintains the tissue integrity. Increased and abnormal mitosis indicate genetic damage, which is an important feature in precancer and cancer (5). For the assessment of the proliferative activity of a tumor, mitotic figure counting is a widely used technique (11). Thus, for classification, grading and predicting prognosis of tumor, mitotic count or mitotic index is regularly used. Few investigators have proposed a poorer prognosis for malignant tumors with high mitotic index than that for tumors with reduced mitotic count (12).

Literature search revealed numerous studies demonstrating selective stains like crystal violet, giemsa, toluidine blue, feulgen stain and malachite green in tissues like brain, uterus and breast carcinoma, but however very few studies were evident in relation to oral mucosa (5, 13). Routinely used Haematoxylin and Eosin present a problem in eliminating the erroneous inclusion of mitotic cell from apoptotic cell and pyknotic nucleus. This may

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further deteriorate the reliability of histological grading due to the slack use of morphologic criteria (5, 13). Hence in the present study along with H&EE, giemsa and crystal violet was used to compare for its efficacy in demonstrating mitotic figures and defects in tissues of oral squamous cell carcinoma.

In the present study, the mean count of prophase and metaphase was maximum in crystal violet stain. Jadhav KB (13) also demonstrated similar results. It can reasonably be anticipated because of the occurrence of chromosomes clump in the equatorial plane which may help in easy identification of metaphase. However, anaphase and telophase showed highest number in H&EE. Contrastingly, Jadhav KB (13) demonstrated an increase in mean count in crystal violet stain followed by H&EE. The mean value of overall mitotic figures was highest in crystal violet which was in correlation with Jadhav KB (13) and (5).The significantly increased mitotic counts with 1% crystal violet stain suggest that, this crisp stain provides easy identification of mitotic figures even at a lower magnification as compared to an H&EE stained sections. It also eliminates false positive result of pyknosis, apoptosis and karyorrhexis as mitotic figures (5). Crystal violet stain offers a more reliable mitotic counting as it clearly stains the chromosomes leaving cytoplasm clear and unstained, due to hydrolysis of tissue sections by 1N HCl at 60°C for 10 minutes, which helps in easily identification of mitotic figures (13).

In the present study, on intercomparison between various stains, none of the mitotic figures showed significant value except for prophase in H&EE V/s Crystal violet. Contrastingly Jadhav KB (13) demonstrated an significant increase in mean value of metaphase. In the present study, when the mean mitotic defects were analyzed, pyknotic nuclei were highest in crystal violet and binucleation in H&EE. However, overall defects in mitosis in all the stains showed almost similar mean value. On intercomparison of mitotic defects among the three different stains showed non-significant results. No literature was available to compare the results. However several studies are available on oral exfoliated cytological smears. Rimpu K et al, (14) demonstrated increased in frequency of binucleated cells with increasing dosage of radiotherapy when stained with giemsa. Ambroise MM (15) showed significant binucleated cells in routine Pap smears in invasive squamous cell carcinoma.

In the present study, on evaluation of total mitotic count, crystal violet staining showed highest number. Intercomparison among the different stains showed non-significant results.The literature search revealed no studies on giemsa staining for mitotic figure count in OSCC. However, one study was conducted by Palaskar SJ (16) wherein mitotic figures were counted in giemsa stained sections in epithelial dysplasia and was compared with H&E and crystal violet stain. They demonstrated that mitotic figures were enhanced with giemsa and crystal violet as compared to H&E. In the present the staining quality of giemsa and crystal violet stains are equivalent to H & E for identifying the mitotic figures. This may be because of difference in the methods followed or ph difference of the staining solution, temperature and duration of tissue blocks stored.

Conclusion

It can be concluded from the present study that the routine stains along with selective stains are equivalent to visualize the various phases of mitosis and mitotic defects. Since excessive proliferation of cells due to increased mitosis is one such outcome, which is the hallmark in precancer and cancer. Thus for standardization of mitotic figure counting there is a need to carry out further studies involving larger sample size and with various other selective stains. And further studies can also be carried on

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the significance of mitotic count on histopathological grading of oral squamous cell carcinoma.

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Legend Figures and Tables

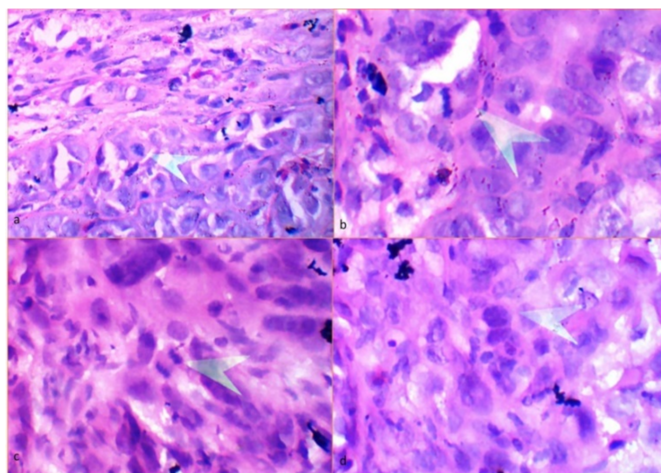

Figure 1: Histopathological photomicrograph shows mitotic figures, prophase (a), metaphase (b), anaphase (c) and telophase (d). (Haematoxylin and Eosin stain, X 40

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magnifications)

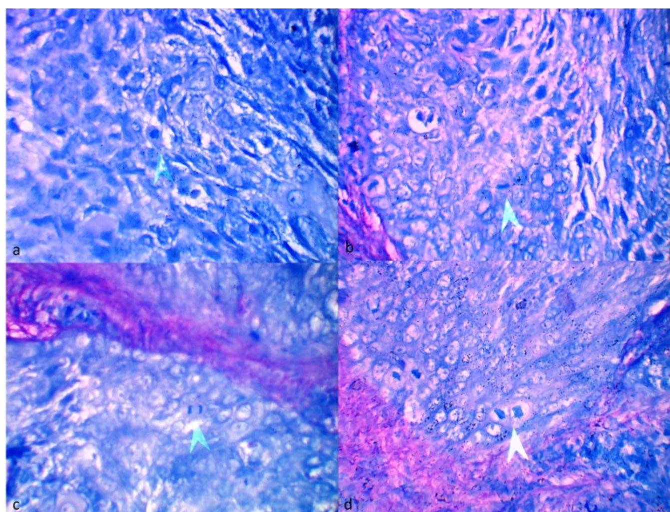

Figure 2: Histopathological photomicrograph shows mitotic figures, prophase (a), metaphase (b), anaphase (c) and telophase (d). (Giemsa Stain, X40 magnification)

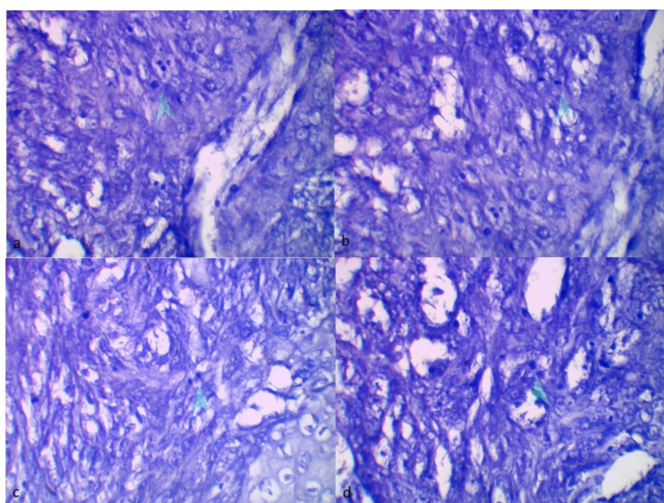

Figure 3: Histopathological photomicrograph shows mitotic figures, prophase (a), metaphase (b), anaphase (c) and telophase (d). (Crystal violet stain, X 40 magnification)

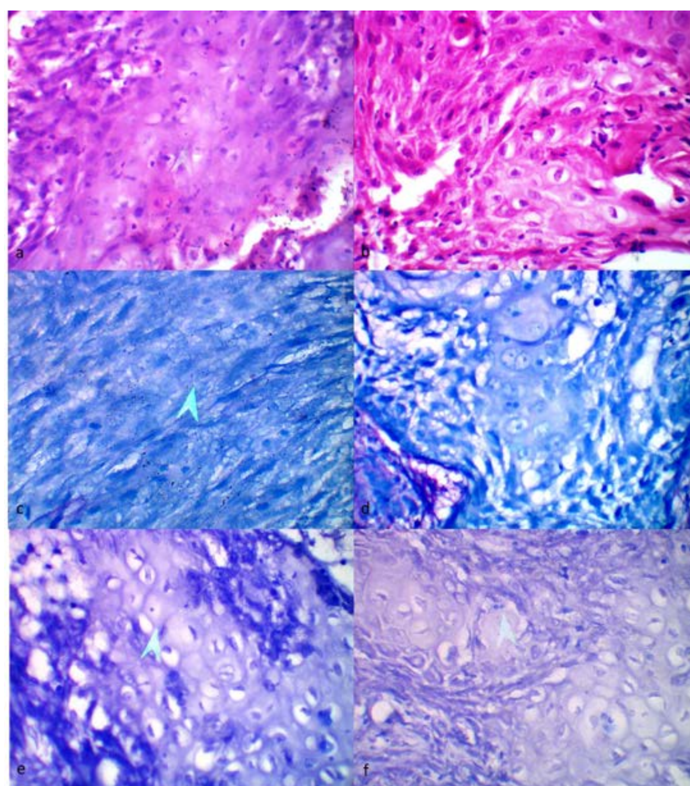

Figure 4: Histopathological photomicrograph shows defects in mitosis in Haematoxylin and eosin, giemsa and crystal violet stain (X40 magnification) showing pyknotic nuclei (a,c,e) and binucleation (b,d,f) respectively.

Table 1: The mean values of the various mitotic figures in OSCC under various stains

Mitotic figures	H&E (Mean ± SD)	Giemsa (Mean ± SD)	Crystal violet (Mean ± SD)	p value
Prophase	6.52 ± 3.60	8.10 ± 4.17	10.06 ± 4.42	0.004
Metaphase	11.39 ± 6.03	10.71 ± 6.17	12.39 ± 5.59	0.537
Anaphase	0.84 ± 1.13	0.45 ± 0.77	0.68 ± 0.94	0.284
Telophase	0.06 ± 0.25	0.06 ± 0.25	0.03 ± 0.18	0.815
Total	18.81 ± 9.41	19.32 ± 10.20	23.16 ± 10.12	0.174

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Table 2: Intercomparison of H&E, Giemsa and Crystal Violet stains among the various phases of mitosis

Comparisons	Prophase (p value)	Metaphase (p value)	Anaphase (p value)	Telophase (p value)	Total (p value)
H&E vs. Giemsa	0.283	0.895	0.255	1.000	0.977
H&E vs. Crystal violet	0.003	0.785	0.786	0.844	0.200
Giemsa vs. Crystal violet	0.144	0.509	0.624	0.844	0.284

Table 3: The mean value of the various defects in mitosis in OSCC under various stains

Defects in mitosis	H&E(Mean ± SD)	Giemsa (Mean ± SD)	Crystal violet (Mean ± SD)	p value
Pyknotic nuclei	3.61 ± 1.65	4.00 ± 1.65	4.16 ± 1.57	0.397
Binucleation	1.39 ± 1.12	0.94 ± 0.85	0.87 ± 0.88	0.075
Total	5.00 ± 2.07	4.94 ± 2.13	5.03 ± 2.26	0.984

Table 4: Intercomparison of H&E, Giemsa and Crystal violet stains among the various defects of mitosis

Comparisons	Pyknotic nuclei (p value)	Binucleation (p value)	Total
H&E vs. Giemsa	0.618	0.158	0.992
H&E vs. Crystal violet	0.383	0.092	0.998
Giemsa vs. Crystal violet	0.919	0.962	0.983

Table 5: The mean total mitotic count in OSCC under various stains under various stains

H&E (Mean ± SD)	Giemsa (Mean ± SD)	Crystal violet (Mean ± SD)	p value
23.81 ± 10.78	24.26 ± 11.52	28.19 ± 11.72	0.251

Table 6: Intercomparison of H&E, Giemsa and Crystal violet stains among the total mitotic count

Comparisons	Total Mitotic count (P value)
H&E vs. Giemsa	0.987
H&E vs. Crystal violet	0.286
Giemsa vs. Crystal violet	0.363

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