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COMPARISON OF STAINING OF MITOTIC FIGURES IN ORAL EPITHELIAL DYSPLASIA AND ORAL SQUAMOUS CELL CARCINOMA

Oral Pathology

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ABSTRACT

Appropriate cell division is essential to maintain tissue integrity. Mitotic rate is traditionally evaluated by counting mitotic figures on Hematoxylin and eosin (H&E)-stained tissue sections. This count has been used to predict aggressive behavior in several neoplasms.1 The abnormal excess of mitotic figures is commonly seen in oral epithelial dysplasia (OED) and oral squamous cell carcinoma (OSCC). Routine staining procedures often pose a problem in differentiating a mitotic cell from an apoptotic cell, deteriorating the reliability of histologic grading. Although various new methods have been recommended for identifying mitotic figures in tissues, the time factor and cost makes them less feasible. Special stains form an integral part of routine histopathology as an adjunct to Hematoxylin and Eosin, and give meaningful diagnostic information of the available tissues.

KEYWORDS

Mitotic figures, Special stains, Oral epithelial dysplasia, Oral squamous cell carcinoma

INTRODUCTION

Mitotic figures are defined as atypical, if they demonstrate an abnormal chromosomal distribution or an excessive number of mitotic spindles with a multipolar morphologic appearance. The abnormal excess of mitotic figures is commonly seen in dysplastic lesions and malignant neoplasms.[3]

Special stains like Crystal violet stain enhances the identification of mitotic figures even at lower magnification as compared to H&E stain. As Feulgen stain has high DNA specificity, a good contrast has been found in staining and mitotic figures could be appreciated even at low magnification. Relatively few studies have employed special stains for paraffin embedded sections of oral dysplasia and OSCC.[4] Thus, an attempt has been made to evaluate the usefulness of different stains in identifying mitotic figures and also to see for any variation in the number of mitotic figures in epithelial dysplasiaand oral squamous cell carcinoma.

MATERIALS AND METHODS

In this study, clinically and histopathologically confirmed cases of oral epithelial dysplasia and oral squamous cell carcinoma, 30 in each group were selected from archives of the Department of Oral Pathology and Microbiology. Control group included 30 normal mucosal tissue samples which were discarded during surgery for the removal of impacted molars or periodontal surgical procedures. Histopathologically stained tissues that were devoid of epithelium, insufficient tissue samples, cases with insufficient case details were excluded from the study.

All the tissue specimens were formalin fixed, routinely processed and paraffin embedded. Three sections, each of 4 microns thickness were made from each block for H&E stain, Crystal violet stain (Fraser FJ method modified method)[5] and Feulgen stain (Feulgen & Rossenbeck 1924).[6] The criteria given by van Deist PJ et al.,[7] was used to assign a structure as a mitotic figure in this study:

1) The nuclear membrane must be absent indicating that cells have passed the prophase.
2) Clear, hairy extensions of nuclear material (condensed chromosomes) must be present; either clotted (beginning metaphase), in a plane (metaphase/anaphase) or in separate clots (telophase).
3) Two parallel, clearly separate chromosome clots to be counted as if they are separate.

The slides were randomly selected and each slide was observed by two observers without any exchange of information. For counting and evaluation of mitotic figures, microscopic fields were selected starting from most mitotically rich area and then moving the stage to the next field, and continuing the selection to include 5 fields from each section. Average mitotic count of distinct visible mitotic figures from these 5 fields by each observer was calculated and recorded separately.

The mitotic count per field was calculated as:

$$\text{Mitotic count / field} = \frac{\text{Total number of mitotic figures observed}}{\text{Number of fields counted}}$$

Mean value of mitotic figures was calculated from average values of both observations.

Number of mitotic figures in each specimen using different stains were compared using one-way ANOVA and post hoc analysis. For interobserver reliability Cronbach's alpha test was done.

RESULTS

Mean age of group I (normal), II (oral epithelial dysplasia) and III (OSCC) were 56.1 ± 17.6, 65.2 ± 11.2, 68.7 ± 16.2 respectively. Association between age and study groups (I, II and III) was found to be statistically significant (p value<0.05). In study groups I, II and III, male: female ratios were 4:1, 2:1 and 3:2 respectively. In this study, normal tissues were obtained from gingiva (46.67%), retromolar area (40%) and alveolus (13.33%).

Most common sites of oral epithelial dysplasia were buccal mucosa (43.34%) and tongue (33.33%) and those of oral squamous cell carcinoma were tongue (26.67%), buccal mucosa (20%) and alveolus (20%). Chi square test was done and statistically significant association was found between the sites and study groups I, II and III (p value <0.005).

There was a statistically significant mean difference in the mitotic count between the three stains in all three study groups (Table 1).

Table 1: **Mean difference in mitotic count visible with the three stains in Normal oral mucosa, Oral epithelial dysplasia and OSCC**

Study group		Sum of Squares	df	Mean Square	F	Sig.
Normal Oral Mucosa	Between Groups	0.68	2	0.34	24.66	0.000*
Oral Epithelial Dysplasia	Between Groups	1.985	2	0.99	19.16	0.000*
OSCC	Between Groups	20.22	2	10.11	10.14	0.000*

*p value<0.005 is considered as statistically significant

A significantly increased mitotic count was observed in Feulgen-stained sections of normal oral mucosa, oral epithelial dysplasia and OSCC in comparison to 1% Crystal Violet and H and E-stained counterparts (Figure 1)

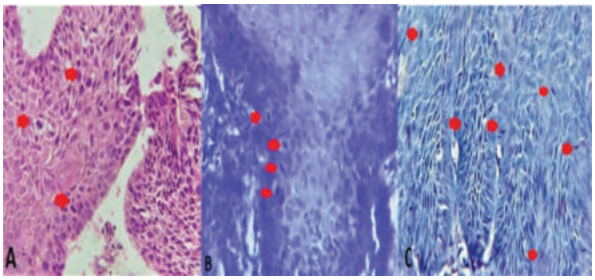

Figure 1: Photomicrograph showing mitotic figures in OSCC sections stained with H&E(A), Crystal violet (B) and Feulgen © (40x)

When frequency of mitotic figures was compared among the three study groups in all the three staining methods, there was a statistically significant mean difference between study groups (Table 2).

Table 2: Mean difference in mitotic count in Normal, Oral epithelial dysplasia and OSCC

Stain		Sum of Squares	df	Mean Square	F	Sig.
H&E	Between Groups	51.37	2	25.68	81.31	0.000*
Crystal Violet	Between Groups	29.62	2	14.81	81.76	0.000*
Feulgen	Between Groups	89.33	2	44.66	79.07	0.000*

*p value<0.005 is considered as statically significant

There was a significant increase in number of mitotic figures observed in group III in comparison with groups I and II irrespective of the stain used (p value<0.005). Reliability between mitotic count by two observers were analyzed and calculated using the Cronbach's alpha test and the results showed good internal consistency (0.989) between mitotic count by both observers in all three stains.

DISCUSSION

Dysplasia is characterized by the presence of certain histological and cytological features. Increase in mitotic index in dysplasia and carcinoma as compared to normal oral mucosa indicates an important kinetic change, possibly representing increased stem cell turnover.8

Even though Hematoxylin and eosin is the most widely used histological stain, it does not give precise view in identifying the mitotic figures. There are many other methods such as flow cytometry and IHC, but they seem to be expensive and time-consuming. Various selective stains such as Feulgen, Crystal violet, toluidine blue and Giemsa have been used for staining mitotic figures.9 Thus, special stains, which are much cost-effective, less time-consuming and less technique sensitive are used in enhanced assessment of mitotic figures of which two stains (Crystal violet and Feulgen) were used in this study along with H&E in order to find out the better stain for identification of mitotic figures.

Crystal violet has high affinity for the highly acidic nature of chromatin which is present in the mitotic cells. The mitotic cells stain magenta and standout against the light blue background when stained with this basic dye. In comparison with the studies by Ankle MR et al.2007, Jadhav KB et al.2012, and Tandon A et al.2016, which were carried out to compare staining of mitotic figures in oral epithelial dysplasia and squamous cell carcinomas, Crystal violet was proved to be a better stain over H and E. This may be attributed to the clear staining ability of chromosome, leaving the cytoplasm clear. The overall diagnostic efficiency of Crystal violet could also be explained due to the modification of the staining method by using 1N HCL at 60°C, therefore causing an increase in contrast due to the light staining of the cytoplasm, which is likely to be a result of the decreased RNA content following hydrolysis.5

In another study evaluating oral epithelial dysplasia and OSCC, Nikita G et al.2014, reported that Feulgen stains provided a greater contrast when staining mitotic figures as compared to H&E, toluidine blue, Giemsa and crystal violet stains; in addition, the mitotic figures were easier to see at lower magnification in comparison to the other stains. 10

Feulgen stain is a modified histochemical stain that is simple, cost-effective, reliable and allows for differentiation between apoptotic bodies, karyorrhexis and mitotic figures, thereby excluding false-positive results. The Feulgen reaction is a stoichiometric procedure; each fixed molecule of Schiff's reagent corresponds to a constant and equivalent portion of the DNA molecule. The biological activity of any cell is best reflected in the nucleus and the functional activity in the cytoplasm. Hence, Feulgen stain is a useful nuclear stain in examining and comparing dysplastic nuclear changes and in observing dysplastic changes of a cell. 11

CONCLUSION

As the staining methods of these special stains are easier, cheaper and more feasible, they can be used for the localization of mitotic figures and to assess proliferation, even in small scale laboratories. Hence, Feulgen and Crystal violet staining can be a rationalized step in the staining of mitotic figures compared to the usual H&E staining and can be employed as selective stains during routine histopathological procedures.

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