ORIGINAL ARTICLE

Comparative Study of Crystal Violet Stain and Haematoxylin and Eosin Stain in the Assessment of Mitotic Figures in Dysplastic and Malignant Lesions of Oral Cavity

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Abstract:

Background: Mitotic figures are valuable tool in assessing cellular proliferation and act as a prognostic indicator in dysplastic and malignant lesions of oral cavity. Routinely used Haematoxylin and Eosin (H&E) stain has limitations in clearly distinguishing mitotic figures. *Aim and Objectives:* To compare the mitotic count in Crystal Violet (CV) stain and H&E stained sections of dysplastic and malignant lesions of oral cavity and to evaluate the efficacy of CV stain in assessing mitotic count in these lesions. *Material and Methods:* Study sample constituted formalin fixed and paraffin-embedded tissue sections, diagnosed on histopathology as dysplastic and malignant lesions of oral cavity (n = 70). For each case two slides of serial sections were cut, one was stained with H&E and the other with CV stain. The number of mitotic figures under 400× magnification in 10 microscopic fields was recorded and average value was calculated for both stains. Data obtained was statistically analyzed by using unpaired t-test. *Results:* An average mitotic count in 21 cases of epithelial dysplasia was 0.75 per high power field on H&E stain and 1.07 on CV stain. In 49 cases of Squamous cell carcinoma, it was 2.57 per high power field on H&E stain and 3.35 on CV stain. There was significant increase in mitotic count in CV stained sections when compared with H&E stain with a statistically significant difference showing $P<0.001$ in both dysplastic and malignant lesions. *Conclusion:* CV stain can be better alternative in assessing mitotic count, as it is cost-effective and simple procedure.

Keywords: Crystal Violet Stain, Epithelial Dysplasia, Mitotic Figures, Squamous Cell Carcinoma

Introduction:

In the Indian subcontinent, oral, pharyngeal and laryngeal cancers are more common and also significantly prevalent [1]. Epidemiological studies have revealed that in India, 10% of all cancer cases are cancers of the oral cavity and out of them squamous cell carcinoma is the commonest cancer amounting to 90-95% cases [1]. The National Cancer Registry Programme of the Indian Council of Medical Research has reported that up to 80,000 new oral cancer cases occur annually in India [2]. This highlights the significance of timely identification and management of dysplastic and malignant lesions of oral cavity [3]. Common risk factors for Oral Squamous Cell Carcinoma (OSCC) are tobacco chewing, smoking [2] and alcohol [4]. Dysplasia refers to disordered proliferation of cells characterized by loss of uniformity, loss of orderly arrangement and increased abnormal mitoses. A dysplastic epithelium possesses increased risk of malignant transformation. Mitosis is a process of nuclear division which causes the replicated DNA molecules of each chromosome to divide into two nuclei. Mitotic Figures (MFs) are the chromosomal

arrangements that are seen in different phases of cell division. Increased mitoses are indicative of rapid cell growth [4].

Occurrence of mitosis does not stipulate whether the tissue is non-neoplastic or neoplastic. More important morphological feature of dysplasia and malignancy is presence of atypical and bizarre MFs with tripolar, quadripolar or multipolar spindles [5]. Therefore, identifying and quantifying abnormal MFs, is a significant aspect of histological grading schemes and are used to categorize the grades of these lesions. Routinely used Haematoxylin & Eosin (H&E) stain has limitations in clearly distinguishing MF from other mimickers of mitosis such as apoptotic bodies [6].

Various authors have tried newer staining methods to assess MFs. Few studies were done to assess the efficacy of special stains like giemsa, toluidine blue and Crystal Violet (CV) stains in evaluating MFs [1, 7]. In some studies it was observed that there was a notable rise in mitotic count in sections stained with CV as compared to conventional H & E stain [7, 8].

Hence, the present study was done to evaluate the efficacy of CV stain in assessing the mitotic count in dysplastic and malignant lesions of oral cavity by doing comparison of mitotic count in CV and H&E stained sections of dysplastic and malignant lesions of oral cavity.

Material and Methods:

A prospective cross-sectional study was done on tissue sections of clinically suspected dysplastic and malignant lesions of oral cavity received in the Histopathology section from 1st December, 2017 to 30th June, 2019. The study was approved by Institutional Ethics Board (Ethical clearance number IEC/Ref/NO-142/17, Date 14.11.2017). Histologically diagnosed cases of dysplastic and malignant lesions of oral cavity were included in the study. Cases where tissue was not sufficient for further processing were excluded. Two slides of serial sections were prepared from the paraffin blocks which were processed according to standard procedure of tissue processing. One slide was stained with commercially available Alum haematoxylin stain and 1% Eosin Y stain solutions. H&E staining was done as per standard protocol. Another slide was stained with commercially available 1% CV stain.

H&E Staining Procedure:

Sections were de-paraffinized, followed by 3 changes of xylene for 5 minutes and rehydrated through graded alcohol (100%, 90% and 80%). Sections were brought to water and kept for 5 minutes. Then stained with Alum haematoxylin for 10 minutes and again rinsed in water for 5 minutes. Then sections were transferred to 1% Acid alcohol for 5-10 seconds followed by rinse in water for 10-15 minutes. Counterstaining was done with 1% Eosin Y for 10 minutes after that sections were rinsed in water for 1-5 minutes. The sections were then air dried, cleared with xylene for 10-15 minutes and mounted with Dibutylphthalate Polystyrene Xylene (DPX).

Crystal Violet Staining Procedure:

Sections were de-paraffinized, followed by 3 changes of xylene for 5 minutes and rehydrated through grades of alcohol (100%, 90% and 80%). Sections were brought to water and kept for 5 minutes. Then, sections were stained with 1% aqueous CV stain for 15 seconds. Then sections were differentiated in 2% Acetic acid (dip for

5–10 seconds) and again were rinsed in water for 5 minutes. The sections were then air dried, cleared with xylene for 10-15 minutes and mounted with DPX.

Both H & E stained and crystal violet stained slides were examined under a binocular compound light microscope (Olympus MLXi Plus microscope) under 40×, 100× and 400× magnifications. Both H&E and crystal violet stained slides were studied by two separate observers without any exchange of information between them. Observations made by each observer regarding the number of MFs in 400× magnification in 10 microscopic fields were recorded separately and the average value was calculated for both observations. The areas selected for counting of MFs included the cellular part of the tissue. The areas showing necrosis, inflammation, tissue folds and calcifications were not considered for counting.

MFs were identified by using criteria given by Diest *et al.* [9]:

- Nuclear membrane should be absent.
- Clear, hairy extensions of nuclear material (condensed chromosomes) should be present. They may appear clotted, in a plane or in separate clots.
- Two clearly separated chromosome clots that are arranged parallel to each other should be counted separately.

These criteria helped to distinguish mitosis from other commonly seen nuclear changes like pyknotic nuclei, apoptosis and karyorrhexis.

Statistical Analysis:

The difference of the means of analysis variables between two independent groups and two time points in the same group were tested by unpaired and paired t-test. If the *P*-value was < 0.05, then the results were considered to be statistically significant. Data were analyzed using SPSS software v.23.0 and Microsoft office 2007.

Results:

Total 70 cases were included in the study. Study group were in the age range of 22-90 years with maximum number of cases in the range of 41-60 years. Mean age was 55.70 yrs. The gender preponderance was skewed towards male with 51 male cases. Most common site of involvement was tongue amounting to 27 cases (38.6%), followed by buccal mucosa amounting to 16 cases (22.9%) (Table 1).

Commonest clinical presentation was ulcero-proliferative growth amounting to 35.7%, followed by ulcers with irregular margins in 30.0% cases. In 24.3% cases exophytic growth and in 10% cases hypertrophied mucosa was noted.

Out of 70 cases 49 cases were histopathologically diagnosed as squamous cell carcinoma amounting to (70%) and 21 cases were diagnosed as epithelial dysplasia (30%). Out of 21 cases of Oral Epithelial Dysplasia (OED), 12 cases (57.1%) were of mild dysplasia, 5 cases (23.9%) were moderate dysplasia and 4 cases (19%) showed severe dysplasia. Out of 49 cases of OSCC, 31 cases (63.3%) were moderately differentiated, 11 cases (22.4%) were well-differentiated and 7 cases were poorly differentiated (14.3%).

In our study, a significant increase was observed in the identification of MFs in the crystal violet stained sections of all 70 cases, when compared with gold standard H&E stain. Difference between mitotic count of H&E stain and CV stain was statistically significant having $p<0.001$ (Table 2).

Table 1: Demographic Profile of Subjects

Characteristics		N = 70	Percentage
Gender	**Male**	51	72.86
	Female	19	27.14
Age (years)	**21-40**	17	24.29
	41-60	28	40.00
	61-80	20	28.57
	81-100	05	7.14
Site of involvement	**Tongue**	27	38.6
	Buccal mucosa	16	22.9
	Hard palate	9	12.9
	Lower lip	8	11.4
	Retro molar trigone	4	5.7
	Gingival Buccal sulcus	3	4.3
	Floor of mouth	2	2.8
	Upper alveolus	1	1.4

Table 2: Comparison of Average Mitotic Figures between Haematoxylin & Eosin Stain and Crystal Violet Stain (n=70)

Average MFs per high power field		Mean ± SD	*p*-value
Observer 1	H&E	1.95±2.06	<0.001
	CV	2.56±2.23	
Observer 2	H&E	2.09±2.17	<0.001
	CV	2.76±2.31	
Average	H&E	2.02±2.11	<0.001
	CV	2.66±2.26	

In cases of OED, as well as cases of OSCC, a significant increase was seen in the count of MFs on examination of the crystal violet stained sections in comparison to H&E, by both observers 1 and 2. When the average value of MFs counted by both observers was analyzed, a significant increase in mitotic count was again appreciated in CV stain, for both OED and OSCC. Difference between mitotic count was statistically significant having $p<0.001$ (Table 3).

When 12 cases of mild dysplasia and 5 cases of moderate dysplasia were evaluated for MFs in 10 HPF and average value was taken for the findings of observer 1 and 2, a significant increase ($P=0.001$) was noted in the identification of MFs in crystal violet stained sections. When 4 cases of severe dysplasia were evaluated, an increase was observed in the identification of MFs in crystal violet stained sections. However the difference was statistically not significant (Table 4).

In the present study, 11 cases of well-differentiated OSCC, 31 cases of moderately differentiated OSCC and 7 cases of poorly differentiated OSCC were separately evaluated for MFs in 10 HPFs and average value of findings of observers 1 and 2 were taken and it was observed that mitotic count was significantly increased in crystal violet stained sections ($P=0.016$, $P<0.001$ and $P=0.029$ respectively) (Table 5).

Table 3: Comparison of Mitotic Figures between Haematoxylin & Eosin Stain and Crystal Violet Stain in Lesions of Oral Epithelial Dysplasia and Oral Squamous Cell Carcinoma (n=70)

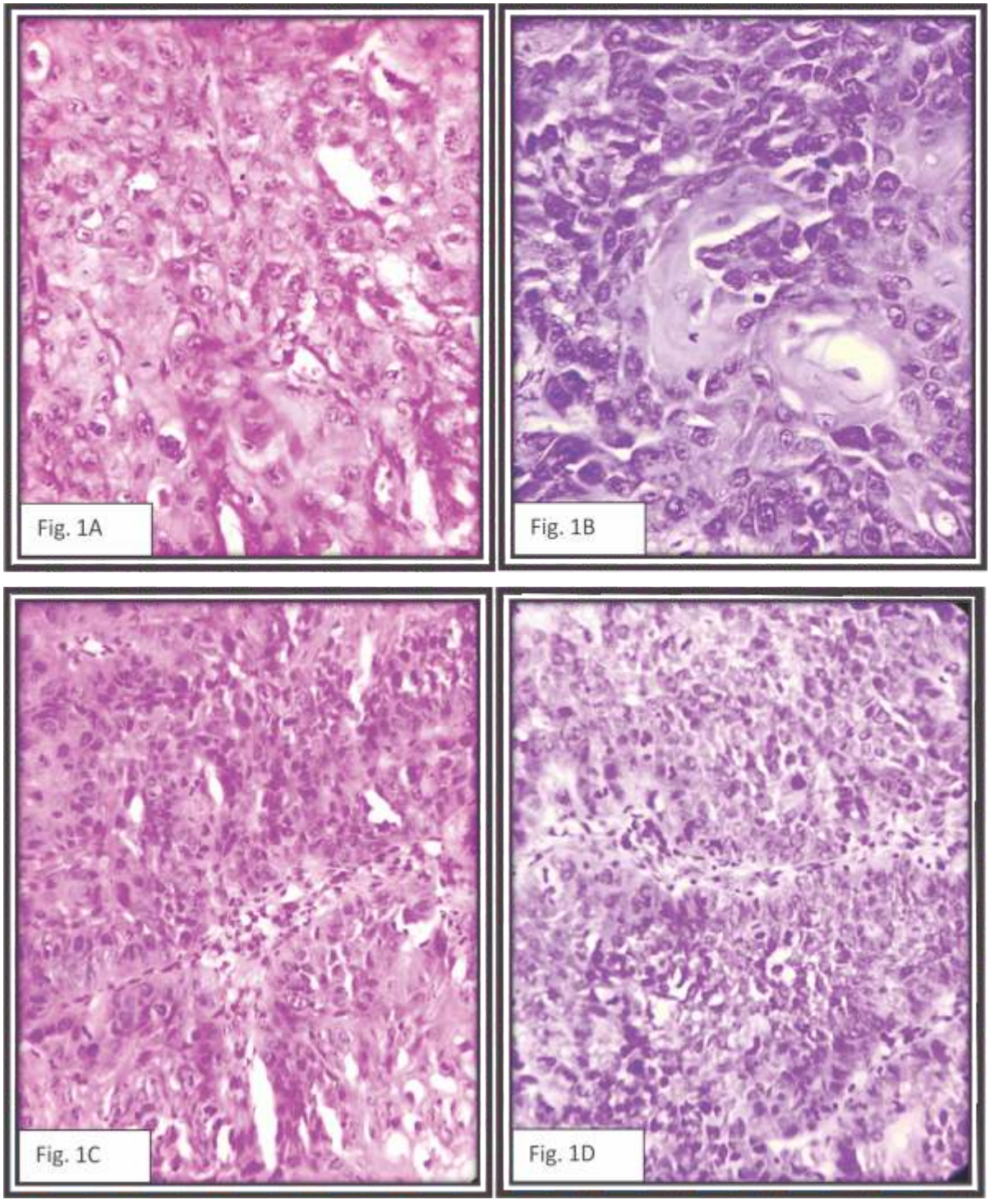
Average MFs per high power field		Epithelial Dysplasia (Mean±SD)	*p*-value	Squamous Cell Carcinoma (Mean±SD)	*p*-value
Observer 1	H&E	0.70±0.87	<0.001	2.48±2.20	<0.001
	CV	0.96±0.92		3.24±2.28	
Observer 2	H&E	0.79±0.93	<0.001	2.65±2.32	<0.001
	CV	1.16±0.94		3.45±2.39	
Average	H&E	0.75±0.89	<0.001	2.57±2.25	<0.001
	CV	1.07±0.93		3.35±2.32	

Table 4: Comparison of Mitotic Figures in Haematoxylin & Eosin Stained Slides with Crystal Violet (CV) Stained Slides in Various Grades of Oral Epithelial Dysplasia (N=21)

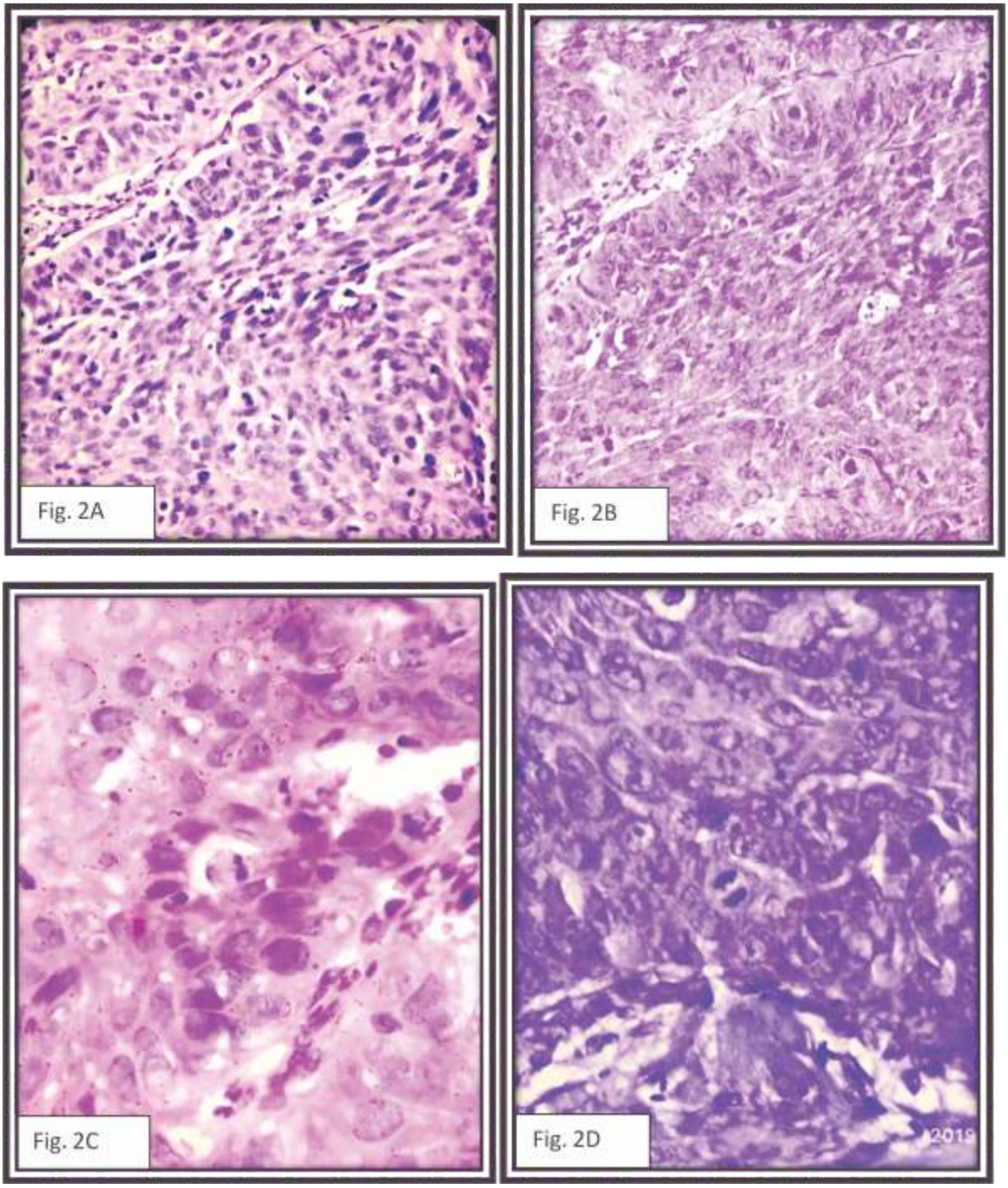
Average mitotic figure per high power field		Mild Dysplasia (Mean± SD)	*p*-value	Moderate Dysplasia (Mean± SD)	*p*-value	Severe Dysplasia (Mean± SD)	*p*-value
Observer 1	H&E	0.23±0.11	0.001	0.98±0.58	0.019	1.75±1.45	0.171
	CV	0.41±0.13		1.24±0.51		2.28±1.28	
Observer 2	H&E	0.3±0.15	<0.001	1.04±0.83	0.001	1.95±1.36	0.151
	CV	0.64±0.21		1.34±0.85		2.5±1.11	
Average	H&E	0.28±0.12	0.001	1±0.68	0.001	1.85±1.39	0.149
	CV	0.53±0.15		1.3±0.68		2.4±1.19	

Table 5: Comparison of Mitotic Figures in Haematoxylin & Eosin Stained Slides with Crystal Violet Stained Slides in Various Grades of Oral Squamous Cell Carcinoma (N=49)

Average MFs per high power field		WD SCC (Mean± SD)	*p*-value	MD SCC (Mean± SD)	*p*-value	PD SCC (Mean± SD)	*p*-value
Observer 1	H&E	2.31±2.59	0.010	1.87±1.34	<0.001	5.43±2.51	0.022
	CV	3.09±2.76		2.53±1.43		6.64±1.65	
Observer 2	H&E	2.31±2.5	0.025	2.02±1.44	<0.001	6±2.61	0.070
	CV	3.22±2.72		2.75±1.66		6.9±1.78	
Average	H&E	2.31±2.54	0.016	1.95±1.38	<0.001	5.71±2.53	0.029
	CV	3.15±2.73		2.64±1.53		6.77±1.69	

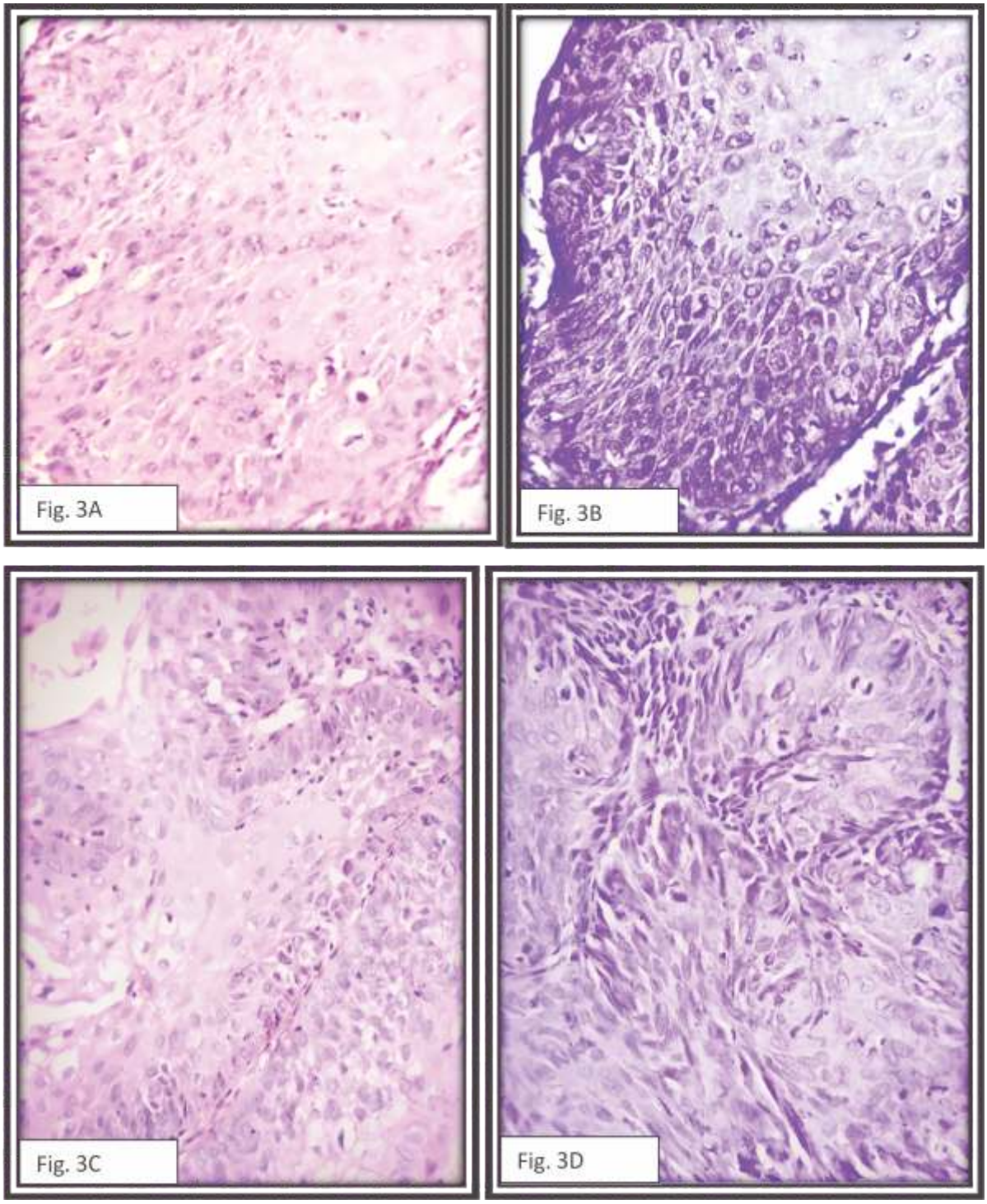


Figs. 1A-B: Showing MFs in Well Differentiated OSCC (A-H&E, 400×& B-CV, 400×).
Figs. 1C-D: Showing MFs in Metaphase Stage in Moderately Differentiated OSCC (C - H&E, 400×& D - CV, 400×).



Figs. 2A-B: Showing Atypical Mitoses in Poorly Differentiated OSCC (H&E, 400×) (CV, 400×).

Fig. 2 C-D: Showing MFs in Anaphase Stage (C-H&E, 1000X, D-CV, 1000×).



Figs. 3A-B: Showing MFs in Mild OED (A-H&E, 400×, B-CV, 400×).
Figs. 3C-D: Showing MFs in Severe OED (C-H&E, 400×, D-CV, 400×).

Discussion:

A variety of techniques, such as microscopy, IHC, flow cytometry, nucleotide radio labeling, and morphometry have been used for years to study MFs [1]. In spite of being more precise, these newer methods are less practical for routine use due to high cost and prolonged duration [5]. Routinely used H&E stain, show most of the histological structures of tissue and generally provide satisfactory morphological features for the diagnosis of lesions. But it has limitations in clearly distinguishing MF from other mimickers of nuclear abnormalities. Therefore, special stains are important and can act as an adjunct to H&E stain in accurate counting of mitotic figures [6-7]. In some studies, CV stain has been used for assessment of MFs. Since CV is a basic dye; it demonstrates greater sensitivity and strong affinity to extremely acidic chromosomal material of a cell undergoing mitosis. Also being a metachromatic dye, there is production of stable intermediates on reaction. Hence, there is clear staining of the chromosome, which helps in clear demonstration of MFs [9]. Silverman *et al*., [10] observed that there is an increase in occurrence of oral dysplasia and malignancy in patients older than 40 years of age. Longer use of tobacco, smoking or alcohol abuse in this age group causes increased duration of contact of the noxious agent with the tissues [11, 12]. In our study also, majority of the patients amounting to 75.71% were more than 40 years of age. In some studies an increase in the incidence of OSCC in young adults has been noted. Awareness among people and availability of newer and better methods of diagnosis may be the reason behind early diagnosis in younger patients of OSCC [13]. In our study, in 18.5% cases age of presentation was less than 40 years. In our study, the male: female ratio was 2.7: 1. This is similar to studies conducted by various authors [13, 14]. This high proportion of oral cancers in males may be attributed to exposure of tobacco related habits [15]. However, recently rise in the incidence of oral dysplasia and malignancy in females due to increased exposure to tobacco related habits has been noted [15]. This explanation may hold true in our study, as in the present study history of tobacco related habits were noted in female patients also. In the present study out of 70, 19 cases (27.1%) were females. In a study done by Roychoudhury *et al*., [16] and Hirata *et al*., [17] the most common site for OSCC was tongue. In the present study also tongue was the commonest site of involvement, observed in 27 cases (38.6%), and followed by buccal mucosa. In the Indian population most commonly involved sites of oral cavity carcinoma are buccal mucosa, edentulous alveolar ridge, hard palate, tongue and lips [18].

The site of dysplastic and malignant oral lesions mostly depends on the type of smoking habit, the quantity and the quality of tobacco used [19]. In a study done by Tandon *et al*., [1] crystal violet (87.6%) showed better diagnostic efficiency than H&E stain (81.3%). Radhakrishnan *et al*., [20] found that CV stained sections had significant increase in mitotic count than H&E in different grades of OSCC. Kesarkar *et al*., [21] concluded that MFs were better identified in CV stained slides compared to H&E stained slides and hence there was an increase in the mitotic count. In our study, the mean mitotic count was 2.02 (SD=2.11) in H&E stained sections and 2.66 (SD=2.26) in CV stained sections. Thus, a statistically significant

Table 6: Comparison of Mean Mitotic Figure Count between Haematoxylin & Eosin and Crystal Violet Stain in Squamous Cell Carcinoma of Oral Cavity with Other Studies

Studies	Tandon *et al.* [1]	Jadhav *et al.* [6]	Ankle *et al.* [10]	Present study
Number of cases	20	30	15	49
Mean MF (H&E)	6.30	4.3	5	2.57
Mean MF (CV)	8.9	6.7	7.9333	3.35
***p*-value**	<0.001	<0.01	0.0443	<0.001

(*P*<0.001) increase was noted in the mitotic count, in CV stained sections, when compared with H&E. In present study, the average mitotic count for 21 OED cases was 0.75 (SD=0.89) in H&E stained sections and 1.07 (SD=0.93) in CV stained sections. Thus, a significant increase (*P*<0.001) was observed in the identification of MFs in CV stain as compared to H &E stain. Some authors correlated mitotic count attained by using 1% CV and the IHC marker Ki67LI, in cases of OSCC. They concluded that 1% CV stain has an advantage over H&E stain. A positive correlation was also seen between Ki67LI and mitotic frequency index in CV stain [22]. In the present study, amongst the 49 cases of OSCC, the average mitotic count was 2.57 (SD=2.25) in H&E stained sections and 3.35 (SD=2.32) in crystal violet stained sections (Table 6). A statistically significant increase (*P*<0.001) was observed in the identification of MFs per HPF in crystal violet stained sections in comparison to H&E. These findings were correlating with other author studies where CV stained slides showed significantly increased mitotic count in comparison to H&E [1,5,9-10]. Radhakrishnan *et al*. [20] observed that in all three grades of OSCC, CV showed increase (*P*>0.01) in mitotic count than H&E. The limitation of our study was that, the sample size for cases of moderate OED, severe OED, and poorly differentiated OSCC were less.

Conclusion:

Mitotic count has a significant role in the evaluation and grading of histopathological diagnosis of dysplastic and malignant lesions of the oral cavity. It facilitates in assessing the prognosis of the tumour and will also help in treatment planning. In the present study, statistically significant increase (p<0.001) in mitotic count was noted in CV stained slides. Hence we conclude that CV can be used as an adjunct to routine H&E stain for localization and assessment of MFs in dysplastic and malignant lesions of the oral cavity.

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