Original Research Paper

Pathology

LEISHMAN GIEMSA COCKTAIL, A CYTOLOGICAL STAINING COMPARABLE TO PAPANICOLAOU STAIN FOR ORAL CANCER DIAGNOSIS.

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ABSTRACT Oral scrape cytology was carried out on 103 patients with clinically diagnosed cases of squamous cell carcinoma. Patients clinically presented as ulcer lesion in the buccal mucosa, palate, tongue or a growth in the oral cavity or sub mucosal Fibrosis were included in this study. Ulcer lesion was the commonest followed by growth in the oral cavity.

Cytodiagnosis revealed squamous cell carcinoma in 89.3% cases followed by dysplasia 10.67%. Majority of cases were in the age group of 5thdecade and squamous cell carcinoma was maximum in 7th decade.

Diagnostic efficacyof Pap, MGG and LG cocktail were recorded and found that sensitivity was 93.7%, 93.2% and 95% respectively.

LG cocktail sensitivity was better than Pap & MGG and specifically was comparableto Pap.

LG Cocktail was less time consuming, single, one step procedure, cost effective and can also be used in mass screening programme.

INTRODUCTION:

Oral cancer is the most common malignancyworldwide and delay in diagnosis undoubtedly increases both morbidity and mortality. [1]

Oral cancer found more commonly among men than women. There is conclusive evidence that tobacco use is causally associated with oral cancer. Other risk factors for oral cancers are alcohol use, Herpes virus infection,Candidiasis, Syphilis, Nutritional deficiency, Poor dental hygine,Immune deficiency and genetic factors. [2]

Exfoliative cytology is a valuable aid for screening of malignant and potentially malignant oral lesions. It is an easy, economical, non invasive and feasible method for detection of malignancies.

PAP (PapanicoLaou Stain) a polychromatic stain developed by George N. Papanicolaou, the father of cytopathology in 1942 and subsequently modified by him in 1954 & 1960 is used in exfoliative cytology. The procedure of staining is time consuming, multiple steps, expensive & associated with drying artifacts. [3,4]

In addition to Pap many laboratories are using MGG for cytological diagnosis. Leishman's stain is also used by somecytopathologists with certain limitations.

To overcome these limitations the combination of leishman's and Giemsa stain (LG cocktail) was used in the present study. This combination of stain is very simple, easy to use and cost effective. LG cocktail (1:1 proportion) technique was quicker, one step method and gave comparable results.

This present study was done for evaluation and comparison of Pap, MGG and LG cocktail staining in exfoliated cells of oral malignancy. Leishman's stain, a good nuclear stain gives intense metachromasiato extra cellular ground substance. Giemsa stain, a good cytoplasmic stain when mixed with the Leishman's stain, the LG cocktail provides a moderate metachromasia to the ground substance and brilliantly stained cellular components.

MATERIAL AND METHODS:

This prospective study was conducted in the department of Pathology in cytology section of SCB Medical College, Cuttack over a period of two years. It comprised of 100 healthy control and 103 clinically diagnosed squamous cell carcinoma patients referred to the cytology section of Dept. of Pathology for oral scrape cytological examination.

Clinically diagnosed cases of SCC not ready to undergo biopsy procedure were excluded from the study.

Clinically diagnosed patients of SCC came to the cytology section of pathology department for evaluation.

A detailed clinical history was taken and local examination of the lesion was done as regards the number (single / multiple) site, size, types of lesion (Ulcerative, whitish patch, SMF, growth). Then the patients were subjected to scrape cytology examination of the lesion. After taking an informed consent, the sample was collected by rigorous scraping in a scalpel blade and smears were prepared over a clean, dry glass slides (3 slides). One smear was fixed immediately by 95% ethanol. Other two slides were air dried. The fixed slide was stained with Pap stain. One air dried smear stained with MGG and other air dried smear with LG cocktail. Standard staining procedures were used for Pap , MGG and LG Cocktail.

LG Cocktail was prepared by one unit volume of filteredgiemsa mixing with an equal volume of distilled water to prepare giemsa working solution. Equal volume ofLeishmanstain mixed with an equal volume of working giemsa (1:1) to prepare the LG cocktail.The air dried smear were flooded with LG cocktail and left for 1 minute. An equal volume of buffer (PH 6.8) was added and left for 5 minutes with gentle blowing. Then slides were washed in tap water ,dried, cleared and mounted in DPX.Nuclear and cytoplasmic details are evaluated by seeing the well stained cells.Cytoplasmic details were evaluated based on transparency and nature of cell membrane. Nuclear details were assessed based on the nature of chromatin, vesicularity, membrane integrity and scored. [5]

Cytoplasmic scoring

0	Not preserved
1+	Non transparent with intact cell membrane
2+	Non transparent masking nuclear details
3+	Transparent,intact cell membrane without masking nuclear details

Nuclear scoring

0	Poor preservation
1+	Smudgy
2+	Fair preservation & chromatin granularity not appreciable
3+	Excellent preservation with crisp chromatin

The stained slides were evaluated for cytological diagnosis by a single examiner. All the cytological smears that showed any evidence of malignancy were undergone biopsy of the lesion; HE stained and correlated with cyto diagnosis.

OBSERVATION:

A total number of 103 clinically diagnosed cases of SCC were subjected to scrape cytology of which 92 were found to be cytologically as SCC and 11 were dysplasias and subjected to biopsy. History of Tobacco consumption seen in 57 Males and 46 Females. Maximum number of cases were seen in 5th decade followed by 7th decade. Dysplastic lesion were most common in 5th decade and SCC in 7th decade. Maximum no. of patients,41 (39.80%) presented as ulcer on buccal mucosa, gum and alveolar margin,of which 36cases were SCC and 5 cases dysplasia .Out of 35(33.98%) cases of growth in oral cavity, 31 were SCC.

Table No.-1 Correlation between cytology and Histology

Type of Lesion	No	Comparable Diagnosis in H.P.	Diff. in Diagnosis	% of Positivity
Mild dysplasia	04	03	1 (IL)	75%
Moderate Dysplasia	04	03	1 (IL)	75%
High Grade Dysplasia	03	02	1 (IL)	66.66%
S.C.C	92	92	---	100%

Table shows correlation between cytodiagnosis and histological interpretation. In Mild, Moderate and High grade dysplasias there were 75, 75 & 66.66% positivity respectively. 100% positivity is there for S.C.C. out of 4 cases of mild dysplasia 1 case was found to be inflammatory lesion (IL) by HP.

Out of 4 cases of moderate dysplasias, 1 case was found to be IL by HP. Out of 3 cases of high grade gradedysplasias, 1 case was found to be inflammatory lesion by HP.

Table No – 2 CLINICAL SITES OF S.C.C (n = 103)

Sites	Frequency	Percentage
Alveolobuccal complex(ABC)	53	51.45
Tongue	30	29.12
Hard palate	5	4.85
Floor of mouth	13	12.62
Lip	02	1.94

Above Table shows sites of squamous cell carcinoma. Alveolobuccal complex was the most common site (51.45%) followed by tongue (29.12%) and least common was lip(1.94%).

Table 3 (a)Cytoplasmic staining for Pap,MGG,LG Cocktail in tests

Cytoplasm	Mean	Std deviation
Pap	2.5	0.604
MGG	2.18	0.666
LG Cocktail	2.53	0.571

Table 3(b) Nuclear Staining for Pap,MGG,LG Cocktail in tests

Nucleus	Mean	Std.Deviation
Pap	2.37	0.624
MGG	2.05	0.771
LG Cocktail	2.48	0.636

Table – 3(c)HISTOLOGICAL DIAGNOSIS (n=103)

Types	Frequency	Parcentage
Well-differentiated S.C.C	70	67.96
Moderately diff (S.C.C)	19	18.44
Poorly diff (S.C.C)	03	2.91
Mild dysplasia	02	1.94
Moderate dysplasia	03	2.91
High grade dysplasia	03	2.91

Table3c shows histological diagnosis of different oral lesions. Out of 103 cases subjected to biopsy Well-differentiated S.C.C being the most common lesion (67.96%). Poorly differentiated carcinoma being least common constituting 2.91%.

COMPARISON OF DIAGNOSTIC EFFICACY OF PAP, MGG AND LG Cocktail stains Table – 3(d)

Stain	Sensitivity (%)	Specificity (%)	Negative Predictive value
PAP	93.7	88.76	76.2
MGG	93.2	88	84.7
LG Cocktail	95	88.27	76

Every cytological diagnosis is recorded and complied to yield the p value..

It can be seen from the above table no. 3a,b,c,d that the sensitivity of LG Cocktail is higher than Pap while specificity was comparable and both were better than Pap while specificity was comparable and both were better than MGG.

DISCUSSION:

Oral cancer is the 6th most common cancer in the world (park, 2007)& commonest cancer in India. In contrast to the gradually decreasing incidence of oral cancer in western countries,however in India and other south east Asian Nations the incidence is progressively in the rise (*Mac Farlandet al*[6] 1996). This incidence can be attributed to the wide spread habit of tobacco abuse among Indians. Global burden of oral cancer related mortality is 8% & 4.7% on males and females respectively (WHO-2003).

This calls for a wide spread screening of high risk population groups. In this contest scrape cytology presents itself as an efficient & cost effective technique. However as a diagnostic procedure, its accuracy & responsibility falls a little short ofbiopsy. Therefore, to obtain optimum results, cytology should be combined with histology to yield the highest percentage of accuracy resulting in an early diagnosis of precancerous and cancerous lesions.

This prospective study was undertaken with an effort to evaluate the efficacy of Papanicolaou, May-GrunwaldGiemsa, and LG cocktail and subsequently using in combination with biopsy to obtain a reliable diagnosis of oral cancer in high risk population groups. 103 no. of patients with clinically diagnosed cases of oral S.C.C. presented to the cytology section of Dept. of Pathology.After cytological diagnosis, histological correlation was done in all cases. S.C.C.-92 cases, Mild Dysplasia-03 cases, moderate Dysplasia-03 case, High grade Dysplasia-2 cases and 3 cases of inflammatory lesion proven by histopathology.

Out of 103 no. of cases of clinically diagnosedcases of S.C.C 57 cases were found to be in Male and 46 cases were Females with H/O tobacco consumption, showing a male predominance, smokeless tobacco is the most common form of abuse among adult & adolescent male Sinha el al[6] as well as in female and smoking habit is seen in male patients. Maximum patients were in 5th decade. In each age group there was overall male predominance. Maximum no. of male patients were detected in 5th decade but maximum no. of female seen both in 5th& 7th decade. Cases diagnosed cytologically as dysplasia were seen mostly within age range of 41-50 years .

Incidence of squamous cell carcinoma by cytological examination was found to be 89.32% and all were histologically correlated. 100% cyto-histocorrelation were observed in S.C.C. showed a high efficacy

of scrape cytology. The high incidence is due to the fact that the study population only included clinically diagnosed cases of S.C.C.

In all age ranges males outnumbered females. Male: female=1.23:1. Patients presenting as ulcer buccal mucosa, gum 41 (39.80%), growth oral cavity 35(33.98%), Ulcer palate 16(15.53%), SMF 11(10.67%)

The maximum number of patients presented with oral ulcer of which 5 cases were diagnosed as dysplasia, 36 no. of caseswere diagnosed as S.C.C. Total no. of dysplastic lesion diagnosed by cytology on our study was 05 (1 mild, 2 moderate, 2 High grade dysplasia) table -1 The next target clinically diagnosed series was growth in the oral cavity (33.98%).31 cases were cytologically diagnosed as S.C.C., 4 cases were dysplasia (2 mild, 1 moderate, and 1 high grade dysplasia.)

Out of 11 dysplastic lesions diagnosed in cytology, all were subjected to biopsy and 8 cases were confirmed histologically as dysplasia (mild, moderate, high grade).

The present study showed that S.C.C of oral cavity present most commonly in the alveolobuccal complex, followed by tongue and least common being the lip.table-2.Well-differentiated carcinoma being the commonest (70%), poorly differentiated carcinoma being least common. Table no.-3. Out of 11 number of cases 8 were diagnosed as dysplasia on HP.

It was observed that the cytoplasmic staining in test group was better appreciated with Pap and LG cocktail stains when compared to MGG. Pap stain was better than LG Cocktail, but the difference was statistically insignificant .

However, for nuclear staining, it was observed that LG Cocktail gave comparatively better results followed by Pap and MGG in test group. Cytoplasmic staining of LG Cocktail was better than MGG in the present study which was in accordance to the study by *Das et al*[8]

The results for cytoplasmic staining for Pap and MGG were similar and comparable to a study by *Idris and Hussain[9]*. However, in a study by *Sujathanetal*[5]. Comparing Pap and MGG stains, MGG was better cytoplasmic stain and Pap a better nuclear stain, and they suggested the use of both stains to increase efficacy. Though the nuclear transparency of Pap was absent in LG Cocktail, the chromatin granularity and vesicularity was better appreciated in air dried Lg cocktail stained smears. This is in accordance with *Gabryaletal*[10] and *Shetty et al*[11].

The sensitivity of LG Cocktail was 95%, which was higher than PAP and specificity was 88.27% .

Finally, the cytological diagnosis of MGG and LG cocktail stained smears were compared with the histopathology reports. It was observed that no statistically significant difference was found between the diagnostic ability of Pap and LG cocktail stains, while MGG stain gave slightly inferior results[11].

Out of 92 cases diagnosed as S.C.C., the number of cases diagnosed by Pap and LG cocktail were almost identical (p=0.158). Pap vsMGG(p=0.001), MGG vs LG cocktail(p=0.001). Hence no statistical significant difference was observed between the diagnostic ability of Pap and LG cocktail stain. Both were better than MGG, this was in accordance with the study by *Shetty et al*[11].

The overall observations of the present study were that the LG cocktail is comparable to Pap stain, which is in accordance with the study were that the study by *Gabryalet al*[10], and superior to MGG stain both in staining characterstics and diagnostic ability.

We mixed Giemsa (a good cytoplasmic stain) working solution with Leishman's stain to prepare the LG cocktail. When the smears were stained with this cocktail, the results were excellent as the cocktail provide a panoptic picture to smear along with perfect (moderate) metachromasia to the ground substances & brilliantly stained cellular components[10].

One can use the range of dilutions on preparing the Giemsa working solution from the stock solution. The same applies to the mixing of Leshman's&Giemsa working solution. However, we observed that the best results were achieved of Giemsa solution, with the Leishman's at a 1:1 ratio. The use of buffer enhances the results. The results were excellent as LG cocktail provides a moderate metachromasia to the ground substance and brilliantly stained cellular component[10].

Additionally, the nuclear enlargement and variation in nuclear size is exaggerated in air-dried smears which is helpful in cytological diagnosis. If the background staining is too intense, it may also prevent adequate visuallisation of cell cluster [11]. In the present study too, MGG-stained smears showed a more intense metachromasia when compared to LG cocktail and sometimes obscured cellular detail.

Our study also revealed that the cytomorphologic features of malignant cells were better seen since the nuclear metachromasia of these malignant cells was better detected in LG cocktail-stained smears [10]. Cytoplasmic granules ,intracellular and extracellular mucin were stained brilliantly.

All the stains in question in this study fulfill the criterias. But LG cocktail stain would be better in the sense that; it was simple, cost effective and safe, taking lesser time than other two stains, having a higher sensitivity than other two stains[10]. For a stain to be utilized in a mass screening programme, in addition to good staining characteristics, the technique must be easy, rapid and economical. The time required for staining with Pap stain, i.e. for fixation and staining is about 45 minutes. The staining procedure requires multiple steps, large volumes of alcohol and expensive stains[11].

The fixing and staining procedure for MGG takes about 45 minutesand the cost is higher than the LG cocktail. However, the LG cocktail staining procedure of air-dried smears require no additional fixation as in MGG stain and can be completed in less than 10 minutes, with the least expenditure[10,11]. Moreover, the staining technique is designed for staining a number of slides and not indivisual slides. Though Rapid Pap kit is available for faster turnaround time of approximately 5 minutes, it still requires multiple steps and is very expensive when compared to the above stains.

However, with a few limitations like evaluation by a single examiner, subjectivity in scoring the sensitivity and specificity of the LG cocktail staining technique need to be further evaluated[152].

Within the limitations of the study, the LG cocktail staining technique was found to give results comparable to the Pap stain. Keeping in mind the added advantages of a single step procedure, cost effectiveness and speed of the technique, the study supports the idea of utilizing this method for early detection of oral cancer, especially in mass screening[10].

LG Cocktail needs no prior fixation of air-dried smears(stain contain fixative), requires only 1 solution (easy to transport) and can be used with tap water in place of buffer, the LG Cocktail can stain smears for long intervals. The LG Cocktail stain is being used in the field(cancer screening and detection camps in villages with the underprivileged) and has been used in various laboratory routinely for the last 2 years, providing consistently high-quality staining[10].

SUMMARY AND CONCLUSION:
Scrape cytotology was carried out on 103 clinically suspected squamous cell carcinoma patients presenting as oral lesions. Smokeless tobacco was the most commonly associated addiction.

Patients clinically presented as ulcer in the buccal mucosa, palate, tongue, growth in the oral cavity and SMF.Ulcer in the oral cavity was common followed by growth in the oral cavity. Cytodiagnosis revealed SCC (89.32%), Dysplasias(10.67%). Histologic corelation was done,in dysplasia it was 72.2% and in SCC it was 100% positivity. Overall corelation was 97.08%.

Squamous cell carcinoma occurs mosty in alveo buccal complex,and most common type was well differentiated SCC.

Diagnostic efficacy of Pap,MGG and LG Cocktail were corelated and the sensitivity was 93.7,93.2 and 95% respectively.
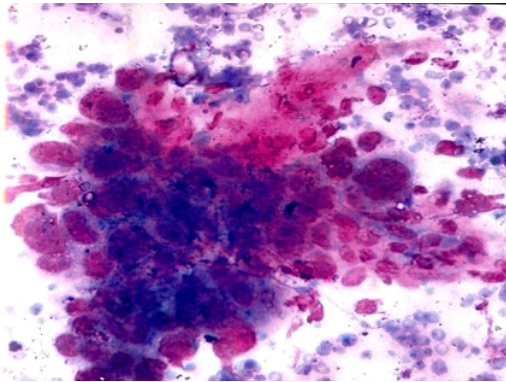
No statistical difference was observed between the diagnostic ability of Pap and LG Cocktail stains.

In conclusion LG Cocktail staining is a simple,cost effective,less time consuming and can best be used for oral cancer screening programme.
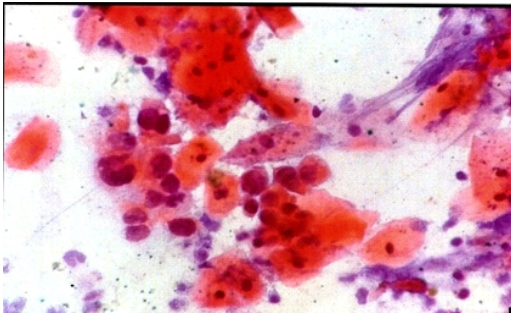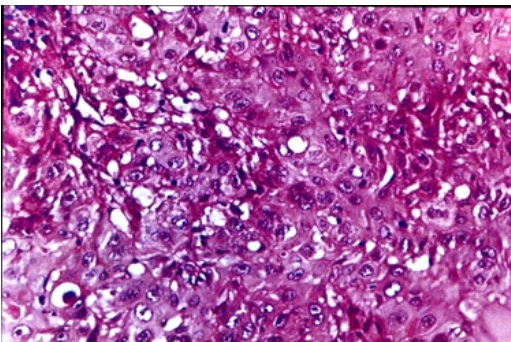
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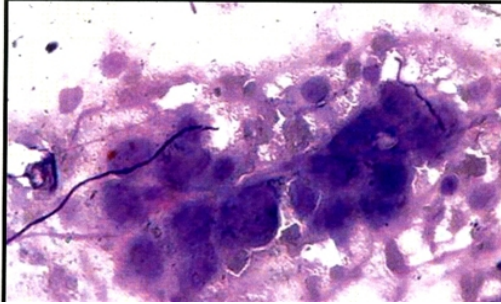
LG Cocktail x400

MGGx400

PAPx400

HP as SCC (MD),H&E x400

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