ORIGINAL ARTICLE

Comparing the efficacy of Leishman–Giemsa Cocktail Stain, Giemsa Stain, and Papanicolaou Stain in Potentially Malignant Disorders: A Comparative Study.

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ABSTRACT… Objective: To estimate the efficacy of the Leishman Giemsa cocktail stain, Giemsa stain, and Papanicolaou for the screening of potentially malignant lesions by exfoliative cytology. **Study Design:** Comparative study. **Setting:** Department of Oral Pathology, Isra Dental College, Faculty of Dentistry and Allied Sciences Hyderabad, Pakistan. **Period:** 1st February 2022 to 31st January 2023. **Methods:** A total of 90 participants were enrolled in the study, 30 subjects of normal healthy individuals were categorized in Group-I and 60 patients with diagnosed potentially malignant lesions (Oral submucous Fibrosis, Oral Lichen Planus, and Leukoplakia) were categorized in Group II. Participants were advised to rinse their mouth with water and the sample was taken by moving a brush clockwise and anti-clockwise over mucosa and the surface of the lesion. Three smears were prepared from each sample, one was fixed with biofix spray and stained with Papanicolaou stain, and other two smears were air-dried and stained with Leishman Giemsa and Giemsa stain. Stained slides were observed under a microscope and the slides were interpreted. **Results:** In Group-I Leishman Giemsa cocktail stain was highly significant compared to Papanicolaou and Giemsa stain, the mean value of the Leishman Giemsa cytoplasmic stain was 1.16, Papanicolaou was 1.0 and Giemsa was 0.52. Whereas in group II, the mean score of cytoplasmic staining of the Leishman Giemsa cocktail was 1.16, Papanicolaou was 1.10 and Giemsa was 0.67. There was no significant difference found in the mean score of cytoplasmic staining of Group-I and Group II respectively. **Conclusion:** According to current research study results, the Leishman Giemsa cocktail stain is a better staining technique for the screening of potentially malignant lesions along with the Papanicolaou stain.

Key words: Exfoliative Cytology, Giemsa, LG Cocktail Stain, Oral Potentially Malignant Lesions, Papanicolaou Stain.

INTRODUCTION

"Oral squamous cell carcinoma" is one of the major concerns of medical professionals due to its increasing prevalence and motility rate worldwide. Among all oral cancers oral squamous cell carcinoma (OSCC) is commonly found in Asian countries like Pakistan, Bangladesh, and India due to consumption of Alcohol, betel nuts, and other carcinogenic compounds. Oral cancers arise from potentially malignant lesions such as "Oral Submucous Fibrosis" (OSF), "Oral Lichen Planus (OLP) Leukoplakia", etc.[1] Premalignant disorders, also known as precancerous conditions or premalignant lesions, are abnormal tissue changes that have the potential to develop into cancer if left untreated, these conditions are characterized by cellular or tissue abnormalities that are considered a precursor to cancer but have not yet become invasive or malignant.[2] The management of premalignant disorders depends on the specific condition and its location. In some cases, close monitoring, regular screenings, and lifestyle modifications may be recommended. Other times, interventions such as surgical removal, topical treatments, or endoscopic procedures may be employed to eliminate or reduce the risk of progression to cancer.[3]

Early diagnosis of premalignant lesions ensures overcoming the chances of its transformation into malignant (OSCC), therefore it's important to find cost-effective and reliable diagnostic tools

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for the early detection of such lesions.[4] Moreover, diagnostic tools are essential for early detection, accurate diagnosis, treatment guidance, monitoring, and cost-effective management of diseases and conditions.[5] These play a vital role in improving patient outcomes, reducing complications, and ensuring optimal healthcare delivery. In such a way exfoliative cytology is proven a more appropriate technique without any surgical process.[6] Cytology is based on different staining which is useful to detect the shedding of cells from the oral cavity[7], Leishman stain, Giemsa stain, and Papanicolaou staining are widely used for the detection of these lesions[8], therefore the objective of this study was to associate and determine the efficacy of following stains for the early diagnosis of potentially malignant lesions.

The Papanicolaou stain, also known as the PAP stain or Pap smear, is a technique used to stain cells on a microscope slide for the purpose of detecting cellular variations.[9] The Papanicolaou stain is commonly used in the screening and diagnosis of cervical cancer and precancerous conditions.[10] During a Pap smear, a healthcare provider gathers cells from the cervix using a minor brush or spatula. It has been proven a critical tool in the early detection and prevention of malignancy, as it enables the identification of abnormal cells even before symptoms develop.[11] Early detection through Pap smears has significantly improved the prognosis for cervical cancer patients.[12]

The Giemsa stain is the group of stains known as Romanowsky stain which is commonly used for blood smears, but Giemsa stain is also widely used in histological and cytological staining techniques for the detection of cellular abnormalities However, some disadvantages include the tendency to precipitate, significant background staining, and the necessity to create fresh solutions every day. Leishman–Giemsacocktail (LG) is not commonly used for cytology, but it is an easy one-step procedure for screening oral lesions. Although there is a need for further modification for the diagnosis of such diseases.[13]

METHODS
This comparative study was conducted at the oral pathology Department Isra dental college, Faculty of Dentistry and allied sciences, Hyderabad Pakistan", from 1st February 2022 to 31st January 2023. The "Ethical Committee of Isra University Hyderabad" reviewed and approved the study protocols (IU/IDC/W.I.S.U.T/2022/282).

A total of 90 subjects were enrolled in the study, which included 30 subjects of normal healthy individuals having no obvious oral lesions or habits of consumption of tobacco, other tobacco and related substances, or other such substances were selected as the control group, and 60 diagnosed patients with potentially malignant disorders including 25 OSF, 15oral lichen planus, and 20 Leukoplakia patients. Written consent was taken from all subjects and informed about the research purpose. Patients with systematic disease, unable to participate and malignant disorders were excluded from the study.

Patients were advised to rinse their mouths with tap water to wash out food particles and other cellular debris. Exfoliated cells were collected from the buccal mucosa on the affected side using a brush clockwise and anticlockwise directions. Three smear slides were prepared from each sample and similarly, three smears were also prepared from samples of normal healthy individuals. One smear slide from all samples was fixed with Biofix spray (Commercially available) and stained with Papanicolaou and the other two were air-dried smears stained with LG cocktail and Giemsa stain.

Leishman-Giemsa (LG) cocktail was prepared by making a working solution of Leishman (Medline) into distilled water and an equal amount of Giemsa (Medline) stain into distilled water 1:1 ratio. The air-dried smear slide was stained with LG cocktail stain and Giemsa stain an equal amount of buffer was added with PH 6.8 for a maximum of 10 minutes. Slides were mounted by DpX and observed under a microscope (CH20 Olympus Japan).

The fixed smear was dipped into the Rapid

PAP nuclear stain for 60 seconds. This stain specifically targets the nuclei of the cells. 3 drops of Scott's tap water buffer were added to the slide. The slide was then washed by dipping it in the buffer for 10 seconds. Scott's tap water buffer is used to remove excess stains from the slide. The slide was dipped in Rapid PAP dehydrant for 30 seconds, and this step was repeated with a second change of dehydrant for another 30 seconds. The slide was then dipped in Rapid PAP cytoplasmic stain for 45 seconds. This stain targets the cytoplasmic components of the cells. The slide was washed in tap water to remove the excess stain. It was then dipped in saline, which is a saltwater solution commonly used for rinsing. Finally, the slide was left to air dry, allowing the water to evaporate completely.

The stained slides were examined for staining characteristics such as nuclear and cytoplasmic detail. A sufficiently cellular area was determined. Nuclear detail was graded based on the nature of the chromatin, pyknosis, vesicularity, and membrane integrity and was scored as follows: 0 poor preservation, 1+ smudgy, 2+ reasonable preservation but chromatin granularity not perceptible, 3+ excellent preservation with crisp chromatin. Cytoplasmic details were assessed as 0 not preserved, 1+ non-transparent with intact cell membrane, 2+ non-transparent masking nuclear details, and 3+ transparent, intact cell membrane without masking nuclear details. Intra observer variation was assessed by counting again after a 5-day break. Only the identical scores of both tests were used in the final analysis.[14]

RESULTS

The Study found significant results according to the research goals. A total of 90 subjects were enrolled in the study by dividing into two groups, 30 subjects in Group-I with normal healthy subjects and 60 patients with potentially malignant lesions including 25 patients of OSF, 15 Oral Lichen Planus, and 20 patients of Leukoplakia. Total male subjects were 20 (66.7%) in Group-I (Healthy subjects) and female were 10 (33.3%), while in Group II 38 (63.3%) were males and 22 (36.7%) were females. The mean age of male subjects in Group I was found to be 38.7 ± 10.3 years and Female was 32.5±12.2 years, whereas for Group II, the mean age of male subjects was 39.8 ± 12.2 years, and female was 34.21±10.3 (As shown in Table-I)

Variable	Frequency	Percentage	Mean Age (SD)
Group –I			
Male	20	66.7%	38.7 ± 10.3
Female	10	33.3%	32.5±12.2
Group-II			
Male	38	63.3%	39.8 ± 12.2
Female	22	36.7%	34.21±10.3

Table-I. Demographical features of subjects in Group-I and Group-II

In Group-I LG was compared with Papanicolaou and Giemsa stains, the mean value of cytoplasmic stain and the nuclear stain was calculated for each stain, the mean value of LG cocktail stain was calculated as cytoplasmic1.16, Nuclear stain 1.44, Papanicolaou was cytoplasmic stain1.0, nuclear stain=1.0 and Giemsa was cytoplasmic stain 0.52, nuclear 0.94. According to study results LG cocktail satin was highly significant compared to Papanicolaou and Giemsa stain in group I, whereas Papanicolaou was more significant than Giemsa stain. Cytoplasmic and nuclear scoring was also calculated for each stain, the PAP stain had a total score of 1136, the Giemsa was 943, and the LG stain score was 1221. The average nuclear and cytoplasmic staining score of Papanicolaou was 2.04, Giemsa had a score of 1.46, and LG had a score of 2.2. Thus, the LG cocktail stain was observed as significant ($P = <0.001$) as compared to the Papanicolaou stain, whereas Papanicolaou was observed as significant ($p < 0.05$) when compared with the Giemsa stain ($P > 0.05$) in Group I.

Cytoplasm and nucleus staining of three stains were also compared in potentially malignant lesions. The mean value was calculated for each stain, the mean score of cytoplasmic and nuclear staining was as following, LG cocktail stain cytoplasmic stain1.16, Nuclear stain 1.14, Papanicolaou was Cytoplasmic stain 1.10, Nuclear stain1.10, and Giemsa was C=0.67, C=0.46. There was also no significant difference found in the mean value of cytoplasmic staining values of Group-I and Group-II respectively. LG

and Papanicolaou were found more significant when compared to the Giemsa stain. In potentially malignant lesions, PAP scored 1198, Giemsa 750, and LG cocktail was 1294; the total possible points were calculated. The mean values for Giemsa stain (Giemsa), Papanicolaou stain (PAP), and LG stain (LG) were 2.0, 1.03, and 2.3, respectively.

According to the Friedman test, a statistically significant difference was found among the stains in both groups, Leishman Giemsa stain cocktail stain was found highly significant (<0.001) followed by the Papanicolaou stain, whereas Giemsa was observed as not significant as compared to other stains. T-test was applied to find a possible difference between both groups, but statistically no significant difference was found.

DISCUSSION

	Group-I				Group-II				
	Mean		Total Scoring	Mean of Total Scoring	Mean		Total Scoring	Mean of Total Score	P-Value
	Cytoplasm	Nucleus			Cytoplasm	Nucleus			
LG Cocktail	1.16	1.44	1221	2.2	1.16	1.14	1294	2.3	<0.001
Papanicolaou	1.0	1.04	1136	2.04	0.9	1.10	1198	2.0	<0.05
Giemsa Stain	0.52	0.94	943	1.46	0.67	0.46	750	1.03	>0.05

Table-II. Comparison of Mean mark of cytoplasmic and nuclear details in group I and group II, with PAP, Giemsa, and LG cocktail stains
*P<0.05; Significant; **P<0.001; highly significant, *P>0.05; not Significant

Exfoliative cytology is a useful tool for detecting malignant and potentially malignant oral lesions. It is a simple, inexpensive, non-invasive, and viable approach for detecting premalignant and malignant lesions.[15] Different stains are useful for the examination of cytology, Papanicolaou stain is a common stain used for exfoliative cytology but due to the multi-steps and time-consuming technique, it is always a struggle for researchers to find an easier method to detect premalignant lesions.[16]

This study was carried out in oral premalignant lesions and Normal cases (healthy subjects) by Papanicolaou stain, Leishman-Giemsa stain, and Giemsa stain. It was observed in our study Leishman-Giemsa stain is better than pap stain and Giemsa stain in the nuclear and cytoplasmic stain. In our results, according to T-test there was no significant difference observed in comparison of group I and Group II results. LG Cocktail shows significant results (P=0.001) in both Groups. Whereas Papanicolaou (0.008) stain had better staining results as compared to Giemsa stain. These study results are comparable with an Indian research study by Sindhu S.K et al (2018)[17] the study also reported Leishman-Giemsa cocktail stain highly significant results with a P value of <0.001, whereas Papanicolaou reported better staining results <0.001 as compared to Giemsa

in normal cases, potentially malignant lesions and malignant lesions (OSCC).

The current study showed the total mean score of the nuclear and cytoplasm staining Leishman-Giemsa cocktail stain 2.2 in Group-I (Healthy individuals) and 2.3 in group-II (Potentially malignant lesions), whereas Papanicolaou was 2.04 in group-I and 2.0 in Group-II, Giemsa stain was 1.46 in Group-I and 1.03 in group-II (Shown in Table-II). There was not any significant difference found in results of both groups. According to an Indian research study Belgaumi and Shetty (2013) Leishman Giemsa cocktail in comparison with Papanicolaou and May-Grünwald Giemsa stains was carried out in exfoliated cells for the detection of oral squamous cell carcinoma. According to Belgaumi study Leishman Giemsa stain found significant along with Papanicolaou stain as compare to May-Grünwald Giemsa stains. The P value obtained for the confirmed cases of squamous cell carcinoma in comparison for Pap vs MGG was 0.001, MGG vs LG cocktail was 0.001 and LG cocktail vs Pap was 0.157. Hence, no statistical significant difference was observed between the diagnostic ability of Pap and LG cocktail stains. Further LG cocktail is suggested an easy, cost-effective and one-step technique comparable to Pap staining.[18]

CONCLUSION

The study concluded that the Leishman Giemsa cocktail stain can be used as a screening tool for early detection of premalignant lesions. Whereas Papanicolaou stain can also be used for screening of premalignant lesions but due to the multi-step procedure and high-cost Leishman Giemsa cocktail would be proven more appropriate.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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2	Uzma Tariq	Slide interpretation, experimental work, Suggestions.	
3	Safiya Javed	Study design, Proforma design, Literature search.	
4	Surwaich Ali Channa	Results analysis and Discussion.	
5	Aswad Ahmed	Proofreading, Drafting in literature search.	
6	Arhama Surwaich	Drafting discussion, chapter Data analysis, drafting the manuscript.	