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Research Article

HISTO-CYTOLOGICAL CORRELATION OF ORAL PREMALIGNANT AND MALIGNANT LESION IN SPECIMENS FROM TOLUIDINE BLUE STAINED BUCCAL MUCOSA

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ABSTRACT
Objective behind the study was to assess the efficacy of Toluidine Blue (TB) mouth rinse in the detection of neoplasia in oral cavity and to evaluate its usefulness in high risk groups. Correlation of the results of scrape cytology smear and histopathology of biopsy taken from the TB stained area was also done. This prospective study was carried out on 85 cases of suspected oral lesions. Complete clinical evaluation and all necessary investigations of patients were performed, local examination by visual inspection followed by toluidine blue staining as mouth rinse was done. Toluidine blue stained area were subjected to scrape cytology by using cytobrush and punch biopsy. Cytology smear were stained by papaniculoau stain and classified according to the 2001 Bethesda system for reporting cervical cytology. Biopsy specimen were stained by haematoxylin and eosin and classified according to WHO classification of tumors of the oral cavity and oropharynx. Sensitivity of toluidine blue staining found to be 92.4% and specificity of toluidine staining found to be 83%. Toluidine blue gives knowledge about lesion margins, helps the decision to biopsy, guide biopsy site of oral premalignant and malignant lesions. Toluidine blue used in addition to clinical examination increased efficacy in detecting premalignant and malignant lesions.

Keywords: *Toluidine Blue, Oral Scrape Cytology*

INTRODUCTION
Oral squamous cell carcinoma (OSCC) is a common malignancy worldwide. Despite advances in oral cancer treatment combining resection, chemotherapy and radiotherapy, the 5-year survival rate is around 50%. However, the prognosis of OSCC improves significantly if diagnosed early.
Approximately, 300000 new oral cavity cancer cases and 68000 deaths worldwide are expected annually. Although, the oral cavity is easily accessible for examination, oral SCC (OSCC) is frequently not diagnosed until symptomatic with an advanced stage of disease. Patients may not appreciate oral mucosal changes, and healthcare personnel may not perform a thorough head and neck and oral examination that leading to delay in recognition and diagnosis. Approximately, two thirds of OSCCs are diagnosed at stage 3 or 4 disease with spread to adjacent tissues and regional lymph nodes. Thus, there is a pressing need for early detection of oral premalignant lesions (OPLs) like leukoplakia, erythroplakia and oral sub mucus fibrosis (OSMF) and OSCC.
Toluidine Blue has been found to be a practical, rapid, inexpensive and effective adjunct diagnostic tool in mucosal diseases in high-risk patients facilitating early diagnosis. Toluidine Blue may help in detection of oral mucosa having molecular changes, which is with or without phenotypic changes of OPLs or OSCC. Toluidine Blue used in addition to clinical examination increases the efficacy in early detection of OSCC or premalignant lesions or both. Toluidine Blue is an adjunct to a detailed visual and digital head and neck examination and is useful in raising or confirming clinical suspicion. This stain is used as an oral rinse and if & when any part of oral mucosa retains the rinse a cytological examination followed by biopsy would detect maximum cases of OSCC and OPLs at a very early stage.
This study purports to examine the efficacy of toluidine blue as an adjunct for early detection of oral malignant and premalignant lesions in the patients attending the OPD of Gandhi Medical College associated hospitals.

Objectives
To assess the efficacy of Toluidine Blue in the detection of neoplasia in oral lesions.

Early detection of malignant changes in premalignant lesions.
To study the role of scrape cytology in the diagnosis of oral lesions & correlate the results of cytology with that of histopathology.
To evaluate usefulness in high risk group

Incidence Pattern (World Health Organization, 2002)

In India approximately 2- 2.5 million cancer cases are present at any given point of time, with around 7,00,000 new cases being detected each year. Nearly half of these cases die each year, age adjusted incidence rate per 1,00,000 population for all type of cancer for urban India ranges from 106.2 to 130.4 for men and 100 to 140.7 for women. Because of large population this provides an enormous disease burden for health services our country. Hence, the Government of India launched a National Control Programme in 1885 with following objects-

1. Primary prevention of cancer, particularly tobacco related cancer.
2. Early diagnosis & treatment of cancer
3. Extension and strengthening of other therapeutic services including pain relief & medical college.

In this sequence Government of India banned the smoking in public places like airlines, railways and hospitals.

In 2003, Indian Council of Medical Research (ICMR) reported that oral cancer is one of the commonest cancer in India (ICMR, 1992). There has been a substantial increase in the incidences of oral sub-mucous fibrosis; especially among youngsters; which further increased the incidence of the oral cancer. At present, oral cancer is the 4th common type of malignancy after lung, stomach and liver in males. It is the fifth common cancer after cervix, breast, stomach and lung cancer in females. Regional Cancer Centre (RCC) Kerala reported almost 14% oral cancer patients out of which 17.0 and 10.5% cases were in males and females, respectively. A significant number of oral cancer patients have been reported in Agra, Allahabad, Mainpuri, Varanasi and Moradabad belt of Uttar Pradesh.

Diagnosis (Lingen et al., 2008)

Optimal treatment and survival from oral cancer depend largely on adequate diagnosis and assessment of the primary tumour and its clinical extent. Physical examination should include inspection and palpation of all mucosal surfaces, bimanual palpation of the floor of the mouth and assessment of the neck for lymph node involvement. In examining a patient with an oral cavity tumor, the objective is to determine the type of tumor and the extent of disease. The diagnostic workup can be categorized into history, physical examination, biopsy, and imaging studies.

Currently available diagnostic technologies:

Standard Screening Test

• Conventional oral examination (COE)

Established Diagnostic Adjuncts

• Oral cytology
• Toluidine Blue (tolonium chloride)

Recently Available Light Detection Systems

• ViziLite, ViziLite Plus
• MicroLux DL
• VELscope

Toluidine Blue (Martin et al., 1998)

Supravital staining methods have long been used as an adjunct in the early diagnosis of malignant lesions. In 1928, Schiller reported the use of Lugol's iodine solution (iodine and potassium iodide) in carcinoma of the cervix uteri. Normal epithelium stains brown, whereas, carcinomatous tissue does not take up the stain. Morgenroth did not obtain reliable results by using the Schiller test.

Toluidine blue, an acidophilic, metachromatic dye belonging to the thiazine group, selectively stains acid tissue components (sulfate, carboxylate and phosphate radicals) such as DNA and RNA and its molecular weight is 305.84.

Canto (1999) evaluated four vital stains that had been used in patients with Barrett's esophagus: (1) Lugol's iodine solution, (2) Methylene blue, (3) Toluidine blue and (4) Indigo carmine. 1.5% to 4% Lugol's iodine solution was sprayed on the esophagus and within minute the normal whitish squamous mucosa will change to dark brown or greenish brown as a result of binding of the iodine to glycogen in nonkeratinized stratified squamous epithelium. Cells that are inflamed, dysplastic or malignant will not stain with Lugol's iodine. The sensitivity was 89%, specificity was 93% and accuracy was 91%. Toluidine blue has been used to diagnose inflammatory and malignant cells because of their increased nuclear- cytoplasmic ratio. The sensitivity was 98%, specificity was 80% for diagnosing Barrett' esophagus. Indigo carmine is a blue contrast stain and is not absorbed by cells. A 0.1% solution of indigo carmine was sprayed on columnar mucosa after delineation of the squamo-columnar junction with Lugol's iodine. These areas were examined by high magnification endoscope. Methylene blue is a vital stain taken up by actively absorbing tissues such as small intestine and colonic epithelium. It will not stain squamous or gastric mucosa. It has a sensitivity of 94%. He concluded that methylene blue appears to be highly accurate for selective staining of specialized columnar epithelium, which define Barrett's esophagus. Lugol's iodine and toluidine blue staining have not been shown to improve the diagnosis of dysplasia or cancer.

Kerawala *et al.,* (2000) did a prospective study of 11 patients undergoing surgery as primary treatment for intraoral mucosal squamous cell carcinoma. Mucosal surfaces were stained immediately preoperatively. Resections were performed to include a 1 cm margin of tissue of either normal clinical appearance or absent toluidine blue staining. All 14 resected margins were free from invasive squamous cell carcinoma. Totally, 16 areas of carcinoma in situ or dysplasia were identified at tissue margins, all of which occurred in clinically normal mucosa that had failed to stain with toluidine blue. They concluded that toluidine blue might be an adjunct in identifying invasive tumor at mucosal resection margins. It would appear to be of no benefit in delineating positive resection margins due to carcinoma in situ or severe dysplasia and therefore it may be of little value in decreasing the incidence of local recurrences.

Onofre *et al.,* (2001) selected 50 patients with potentially malignant epithelial lesions (PMELs) and superficial oral ulcerations suggestive of malignancy. The biopsy sites were selected on the basis of the clinical appearance of the lesion and the staining result. All retained stain, indicated 100% sensitivity of staining for detection of carcinoma in situ and invasive squamous cell carcinoma. In epithelial dysplasia, sensitivity was 50%. The overall specificity was 67%. The positive predictive value was 43.5% and negative positive value was 88.9%. They concluded that staining with toluidine blue is highly reliable for the detection of in situ carcinoma and invasive carcinoma. It is an adjunct to clinical judgment and not a substitute for either clinical judgment or biopsy.

Oral Exfoliative Cytology

Ogden *et al.,* (1991) compared the efficiency of the cytobrush with that of the wooden tongue spatula. The cytobrush has been used frequently in cervical cytology. Wooden spatula is used in oral exfoliative cytology usually. For 26 patients, 2 smears were collected from clinically normal mucosa from four sites in the oral cavity (dorsal tongue, ventral tongue, buccal mucosa and hard plate). The smears were graded for cell yield and dispersion on a three-point scale. Cytobrush produced significantly better dispersion for the dorsal tongue, ventral tongue and buccal mucosa and a better cell yield for tongue surfaces. No significant difference for cell yield or dispersion was found for the hard palate. They stated that the cytobrush is an effective instrument for use in exfoliative cytology of normal oral mucosa.

Ogden *et al.,* (1993) took 2 smears from biopsy confirmed oral cancers and from the contralateral mucosal site of 20 patients using a Cytobrush. Using a panel of antikeratin antibodies, the keratins expressed by these cells were identified using a standard immunocytochemical technique and assessed on a 3-point scale. The report establishes that the simple keratins 8,18 and 19 showed the most significant difference between smears taken from biopsy confirmed oral cancers and from normal oral mucosa. The simple keratins (K8, K18) are not expressed in oral mucosa and keratin 19 is limited to the basal cells. They stated that suprabasal expression of keratin 19 is associated with malignancy and keratins 8 and 18 are associated with oral cancer. They concluded that keratins detection within the smears from oral

lesions could be valuable in the diagnosis of oral cancer.

Risk Factors

1. *Tobacco* use has strongest link to oral cancer. In addition to cigarettes, tobacco use includes cigar and pipe smoking and smokeless tobacco (e.g. chewing tobacco, snuff).

Research has shown that tobacco causes damages to cells in the lining of the mouth, pharynx and larynx. Cells must grow more rapidly to repair this damage. Many of the chemicals found in tobacco cause damage to DNA, which tells the cells how to grow and repair the damage. In essence, tobacco both damages cells and inhibit the ability to grow new ones.

The National Institute of Health (NIH) estimates that approximately 85% percent of oral cancers are linked to some form of tobacco use.

Smokers are 6 times more likely to develop oral or upper throat cancers than nonsmokers. The risk of developing oral cancer increases with the amount of tobacco smoked, chewed or sucked and the duration of use.

Secondhand smoke, also known as environmental tobacco smoke (ETS), may contribute to the development of oral cancers. When nonsmokers are exposed to secondhand smoke, they can absorb nicotine and other chemicals similar to the way smokers do. The greater the exposure to secondhand smoker, the greater the level of compounds in the body. There are specific statistics as to the risk of oral cancer due to secondhand smoke exposure.

2. *Alcohol* use has the second strongest link to oral cancers. Drinking alcohol significantly increases the chances of developing oral, oropharyngeal and laryngeal cancers. The American Cancer Society (ACS) estimates that 75 to 80 percent of all patients with oral cancers are about 6 times more common in drinkers and nondrinkers.

Scientists are unsure if alcohol directly damages the DNA, but they have shown that alcohol increases the penetration of many DNA damaging chemicals into the cells. This is one reason why tobacco and alcohol use together causes such a large amount of DNA damage.

The risk factors for oral cancer change according to amount, types, and length of use for both alcohol and tobacco use. In addition, the combination of smoking and drinking also increases the risk.

Other risk factors are also associated with the developing oral cancers. They include:

3. *Age*: The likelihood of developing oral cancer increases with age. Approximately, 50 percent of all the cases are over the age of 65.

4. *Gender:* Oral cancers are twice as common in men as women. The highest occurrence is in men over age 50. This statistic may be related to higher incidence of alcohol and tobacco use by men.

5. *Mouth Irritation:* Poorly fitting dentures that cause long-term irritation of the mouth's lining is thought to be a risk factor for oral cancer. Poorly fitting dentures may allow causative cancer agents, such as alcohol and tobacco particles, to be trapped under them. However, many research studies have not shown any difference in the occurrence of oral cancer between denture wearers.

6. *Human Papilloma Virus (HPV) Infection:* Papilloma viruses are a large group of related viruses (e.g. herpes), some of which have a role in causing cancer. HPV is one of the most common causes of sexually transmitted diseases. One type of HPV (HPV-16) is thought to be linked to oral and oropharyngeal cancers. The links have been inconclusive at times, but a recent study made a link between HPV-16 infection and oropharyngeal cancers.

7. *Immune System Suppression:* For reasons that are not clear, individuals who are taking immunosuppressive drugs are more at risk for oral and oropharyngeal cancers. These drugs are taken to treat immune system diseases to prevent the rejection of a transplanted organ.

8. *Hereditary Factors:* Although, there are no hereditary factors clearly identified with the most forms of oral cancers, a recent study has identified genetic alterations in the tissue around certain oral tumors. These tissues were associated with the aggressiveness of tumors. Ex. 4q-, 6p-, LOH of 17p.

Oral cancer is the most frequent cancer among males and females in developing countries, where nearly 80% of cases occur (Parkin *et al.*, 1993). Indian registries display much higher age adjusted and truncated incidence rates than those reported by other registries in Cancer incidence in five continents (Parkin *et al.*,

1992). The age adjusted rates (per 100,000 person years) of oral cavity/ pharynx combined is 17.5 in Banglore, 26.8 in Bombay, 23.6 in Madras, 19.4 in Delhi, 10.8 in Barshi and 14.1 in Bhopal among males (NCRP, 1992). One of the most interesting features of oral cancers in India is that, they tend to be better differentiated and exhibit a higher proportion of verrucous type among them (Borges *et al.*, 1989).
Tobacco chewing has been described as an important cause of oral cavity cancer. Several case control studies have demonstrated the risk of oral cavity cancer among tobacco chewers. The risk estimates for tobacco chewer ranged from 4 to 15.9 in comparison to non-chewers of tobacco.

MATERIALS AND METHODS

The study was conducted in Gandhi Medical College and associated Hamidia Hospital Bhopal during the period extending from May 2011 to Sept 2012 prospective cases were assessed.
Patients attending ENT Out- patient department with suspected oral lesions or at high risk group were selected.
Previously, diagnosed cases of malignancy & Patient on chemotherapy or radiotherapy treatment for malignancy were excluded.
Patient's personal, present, past & clinical history was recorded on a Pre-standardized Performa. Thorough clinical examination of oral cavity was done in each case. Positive and negative findings were noted. The patients were asked to rinse mouth twice with water for 20 second to remove debries. It was followed by rinse the mouth with 1% acetic acid. This rinse is given for 20 second to remove any ropey saliva. 1% modified toluidine blue rinse is given for 20 second. This rinse was given when no obvious oral lesion visible, a second rinse with 1% acetic acid given to reduce the extent of mechanically retained stain. Finally, the patient is asked to rinse his mouth with water.
Taking sample with a cytobrush by scraping the surface of stained area. The sample material was spread on albumin coated glass slide, fixed with 95% ethyl alcohol & stain with Papanicolaou method. Then, punch biopsy is taken from same stained area (biopsy site selected on the basis of clinical appearance & dye retention), for histopathological examination.

RESULTS AND DISCUSSION

Observation

This study comprises 91 cases of Pre- cancerous and cancerous lesions of oral cavity collected from the ENT OPD, Gandhi Medical College and Hamidia Hospital, Bhopal between May 2011 to Sept 2012.
The salient observations made in this study are as follows:
The maximum number of cases i.e. 24 (26.3%) belonged to age group 41-50 years. Minimum number of cases belonged to age group 71 years and above i.e. 1(1.1%) case.
The next common age groups was 31-40 years comprised of 20 (22.0%) cases, 51-60 years comprised of 18 (19.8%) cases, 21-30 years comprised of 12(13.2%) cases, 61-70 years comprised of 11 (12.1%) cases and below 20 years age group comprised of 5 (5.5%) cases.
Out of 91 cases, 71 (78.1%) were males and 20 (21.9%) were females.
In the present series, 78 (85.8%) patients were having the habit of tobacco chewing either tobacco alone or with lime and betel nut. 43(47.2%) patients were started tobacco chewing before 25 years of age and 48 (53.0%) patients were started tobacco chewing after 25 years of age.
Although, 54 (59.3%) patients were having the habit of tobacco chewing and /or tobacco smoking.
39 (42.9%) patients were in habit of taking betel nut and /or pan masala.
In this study, 54 (59.3%) patients were having the habit of smoking either bidis or cigarettes. 48 (52.8%) patients were starts smoking between 15-24 years of age groups, 16 (17.6%) patients were having the habit of smoking at or above 25 years of age. Only 3 (3.3%) cases starts smoking before 15 years of age.
41 (45.0%) patients were having less than 30 years of duration of smoking and 26 (28.5%) patients were having more than 30 years of duration of smoking.
In this study, 38 (41.8%) patients were consumed less than 5 packs of bidis or cigarettes per day, and 29 (31.9%) patients were consumed more than 5 packs of bidis or cigarettes per day.

13 (14.3%) patients are ex-smokers, among them 7 (7.7%) cases were stopped smoking 1-10 years ago, 5 (5.5%) cases were stopped smoking 11-20 years ago and 1 (1.1%) case were stopped smoking more than 20 years ago.
24 (26.4%) patients found to be never smoked bidis or cigarettes.

Clinical Suspicion and Toluidine Blue Staining

Staining	Clinical Suspicion	Histo/Cyto Positivity	% Age
Without Toluidine blue staining	120	96	80%
With Toluidine Blue staining	91	85	93.4%

In the study, 18(19.8%) cases were negative for toluidine blue staining, 29 (31.8%) cases showed light blue staining, 25 (27.4%) cases showed blue staining and 19(20.8%) cases showed deep blue staining.
On cytological smear examination 6.5% cases were NILM, 26.4% cases were Pre-malignant, 48.35% cases were malignant and 3.2% cases showed features of a typical squamous cells. 15.3% cases of OSMF were showed normal buccal smears.
In this 85 cases were subjected for Histopathology examination, 31.8% cases showed light blue staining, 37.3% cases showed blue staining and 23.0% cases showed deep blue staining.
23.0% cases were premalignant, 59.3% cases were malignant. 3.3% cases showed areas of atypia and keratosis. 2.2% were ulcerative lesions and 4.4% cases were benign lesions which were showed light blue staining positivity.
47 (53.8%) cases out of 91 cases had lymph node metastasis at the time of presentation.
Out of 40 (85.7%) cases of well differentiated squamous cell carcinoma 29 (59.2%) cases present with lymph node metastasis in which 23 (46.9%) was positive for metastatic deposits.
Out of 7 (14.3%) cases of moderately differentiated squamous cell carcinoma 7 (14.3%) cases present with lymph node metastasis in which 6 (12.2%) cases was positive for metastatic deposits

Discussion

Cytological Evaluation Compared with Toluidine Staining

Toluidine blue is an acidophilic dye of the thiazine group that selectively stains acidic tissue components (carboxylates, sulfates and phosphate radicals) such as DNA and RNA. Its use in vivo is based on the fact that dysplastic and anaplastic cells contain quantitatively more nucleic acids than normal tissues. In addition, malignant epithelium may contain intracellular canals that are wider than normal epithelium, this is a factor that would enhance penetration of the dye and clinically appear as royal blue areas.
The correlation between the intensity of blue staining and the severity of dysplasia with toluidine blue staining patterns in different studies. Some reported only “a royal blue” intense stain as positive, while others reported any staining as positive. Shedd *et al.,* (1967) reported that all Oral Squamous Cell Carcinoma stained toluidine blue positive and that none of the Oral Squamous Cell Carcinoma lesions stained pale blue.
For cytological evaluation of smear, the Bethesda system of reporting cervical cytology was used. It was done because no other method was available in literature for reporting of oral cytology in detail. Out of 91 cases, 31.8% cases showed light blue staining, of these 2.2% cases showed false negative staining, 12.1% cases were reported as low grade squamous intraepithelial lesion and 14.3% cases were reported as high grade squamous intraepithelial lesion, 3.3% showed atypical squamous cells with features of dysplasia. 27.4% cases showed Blue staining which were reported as squamous cell carcinoma. 20.8% cases showed Deep Blue staining, of these 15.4% cases were found to be well differentiated squamous cell carcinoma and 5.5% cases were reported as moderately differentiated squamous cell carcinoma.
The data obtained from present study strongly suggest that it is important to do prior to sampling Toluidine blue staining and also classify the staining intensity as negative or positive (in varying shades

of blue i.e. light blue, blue, deep blue). This not only guides to appropriate sample site but also correlates with the possible diagnosis.

Histo-pathologic Evaluation Compared with Toluidine Blue Staining

Out of 91 cases, 85 cases underwent histopathological examination. 6.6% were found to be negative on toluidine blue staining and cytological smear examination.

31.8% cases showed Light blue staining, of these 1.1% case were ulcerative lesion, 3.3% cases were reported as oral submucous fibrosis, 9.9% cases were low grade oral intraepithelial neoplasia, 13.2% cases were reported as high grade oral intraepithelial neoplasia.

37.3% cases showed blue staining, of this 1.1% case was microinvasive carcinoma, 5.5% cases were reported as verrucous carcinoma, 27.4% cases were well differentiated squamous cell carcinoma, and 2.2% cases were reported as moderately differentiated squamous cell carcinoma.

13.2% cases showed deep blue staining, of these 2.2% cases were verrucous carcinoma, 15.4% cases were well differentiated squamous cell carcinoma and 5.5% cases were reported as moderately differentiated squamous cell carcinoma.

Efficacy of Toluidine Blue Staining

Clinically, it is difficult to pick up precancerous lesions. Toluidine staining gives important clue to detect these at a preclinical stage. 1% toluidine blue is an effective means in picking up malignant changes in premalignant lesions. In our study, toluidine blue staining was highly sensitive and efficient in detecting in malignant disease. Sensitivity in published data ranged from 93.5% to 97.8%, and specificity ranged from 73.3% to 92.9%.

False-positive associated with retention of dye in inflammatory and traumatic lesions have been extensively documented by Mashberg *et al.,* (1995, 1981) and Silverman *et al.,* (1984).

Conclusion

Toluidine Blue is recommended for the following reasons:

- It is useful in diagnosis of oral lesions and can be used as a screening procedure to decide further management.
- Assist in biopsy site selection and to accelerate the decision to biopsy.
- Multiple lesions can be studied simultaneously.
- Can assess the extent of a lesion and assess margins of Oral Pre-malignant Lesions/Oral squamous cell carcinoma.
- It is extremely useful in cases of debilitated, old patients in whom repeated surgical assault (biopsy and corrective surgery) is not desirable. In such cases only cytology can be done and diagnosis thus obtained along with clinical input can be utilized for management.

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