MATERIAL AND METHOD

It is a case controlled study which includes 60 subjects comprising of those who are habitually consuming smokeless tobacco and those who have no such habit.

The subjects will be divided into four groups according to their age which are as follows below 15 years of age, 15-30 years of age, 30-45 years of age and above 45 years of age.

The age and sex of the control group of subjects comprising of individuals without any adverse habit will be matched.

Inclusion criteria-

Subjects consuming tobacco (smokeless) for more than 3 years

Exclusion criteria

Those consuming drugs (other than medication)

Subjects reporting with acute or chronic illness

Any subject who is undergoing treatment for acute/chronic illness or for oral lesions.

A written consent shall be obtained from all individuals

An oral screening will be done using toluidine blue for suspected lesions

(According to Warnakulasuriya KA et al 1996 Toluidine blue (also known as tolonium chloride) is a vital dye that may stain nucleic acids and abnormal tissues. It has been used as an aid to the identification of mucosal abnormalities of the oral cavity. Many surgeons prefer to use Toluidine blue to demarcate the lesion prior to excision.)

A biopsy of the same will be taken for histo-pathological examination.

A cytomorphological examination shall be done using exfoliative cytology
(Giunta J et al 1969, Sciubba JJ et al 1999, Svirsky JA et al 2002, Eisen D et al 2005 advocated that exfoliative cytology for clinically suspicious lesions is detected by visual examination. Any suspicious lesion shall be examined using brush biopsy however these lesions would need confirmation by histopathology, as the use of brush cytology does not provide a definitive diagnosis. But it may be useful in those lesions that are not clinically suspicious, but would still need to be kept under surveillance. Being non-invasive, there is more chance of compliance with screening by this method in the general population.)

Before sampling, each subject will be asked to rinse his mouth thoroughly with tap water.

Exfoliated buccal cells shall be obtained by gently scraping the inside of both cheeks with a moistened wooden spatula.

Cells shall be smeared on to a clear glass slide and fixed with 95% ethanol for a minimum of 15 mts.

A minimum of smears from each individual will be taken so that at least 50 cells per smear are obtained.

The cells will be stained with Feulgen Fast Green (as it has been shown to be most consistent and DNA specific).

Cells will be analysed for cellular diameter, nuclear diameter and nucleo-cellular ratio.

The Feulgan Fast green technique is as follows:

This technique involves mild acid hydrolysis with 1M hydrochloric acid at 60°C to break the purine-deoxyribose bond, the resulting exposed aldehydes are then reacted with Schiff’s reagent to stain the DNA red-purple in color.

Solutions
(a) 1 M hydrochloric acid Hydrochloric acid (conc.) 8.5 ml Distilled water 91.5 ml (b) Schiff reagent (c) Bisulfite solution 10% potassium metabisulfite 5 ml 1M hydrochloric acid 5 ml Distilled water 90 ml

Bring all sections to water.

Rinse sections in 1M HCl at room temperature.

Place sections in 1M HCl at 60°C

Rinse in 1M HCl at room temperature, 1 minute.

Transfer sections to Schiff’s reagent, 45 minutes.

Rinse sections in bisulfate solution, 2 minutes, repeating twice again.

Rinse well in distilled water.

Counterstain if required in 1% light green, 2 minutes.

Wash in water.

Dehydrate through alcohols to xylene and mount.

Staining with acridine orange the protocol is as follows

ACRIDINE ORANGE

Stock reagents
0.1M Citric Acid (Citric Acid, anhydrous (FW 192.1); 1.921g per 100ml dH2O) 0.2M Dibasic Sodium Phosphate (Sodium Phosphate, dibasic anhydrous (FW 142.0) 2.839g per 100ml dH2O) Triton X-100 (Baker) 0.5M EDTA, disodium salt (SIGMA) NaCl (FW 58.44) Acridine Orange (Polysciences or SIGMA) Sucrose, anhydrous (FW 342.3; SIGMA)

Stock Buffer I
20mM Citrate-Phosphate, pH 3.0, 0.1mM EDTA, 0.2M Sucrose, 0.1% Triton X-100 (To 125ml dH2O add 40µl 0.5M EDTA, 26.48ml 0.1M Citric Acid, 6.85ml 0.2M Dibasic Sodium Phosphate, 13.69g Sucrose, 0.2ml Triton X-100. QS to 200ml and 0.2µ filter. Store at 4oC)
Stock Buffer II
10mM Citrate-Phosphate, pH 3.8, 0.1M NaCl (To 150ml dH2O add 9.92ml 0.1M Citric Acid, 5.46ml 0.2M Dibasic Sodium Phosphate, 1.7g NaCl. QS to 200ml and 0.2m filter. Store at 4°C)

Alcohol-fixed smear to distilled water will be taken.

Then diluted acridine orange solution at pH 6.0 for 3 minutes will be used to stain.

It will be rinsed in pH 6.0 buffer for 1 minute.

Then differentiated in 0.1 M calcium chloride solution for ½-1 minute.

It will be washed in phosphate buffer and the same will be mounted.

DNA will be stained in yellow-green.

RNA, some mucins will be stained in red.

100 cells from each sample will be focused under fluorescent microscope and number of Micronucleated cells (MN) will be counted by a single observer.

(Sample collection


Exfoliated buccal mucosal cells can be collected using a wooden tongue-depressor, a metal spatula, toothpicks or toothbrushes, or a cytobrush moistened with water or buffer to swab or gently scrape the mucosa of the inner lining of one or both cheeks113,114

Palve DH et al 2008

Different diagnostic methods, such as routine histopathology, exfoliative cytology, and immunohistochemistry, are available today. Of these, oral exfoliative cytology is particularly valuable for mass screening purpose. It has been shown to have a sensitivity of 94%, specificity of 100%, and an accuracy of 97%.115

Preparation and staining of slide
the cytobrush used to collect buccal cells was shaken in a centrifuge tube containing saline solution (Hank’s basic or other buffer solution) to release the cells, and the tube is then centrifuged to wash the cells in a buffer solution or a fixative. This washing procedure helps to remove bacteria and cell debris, which confound the scoring. Buccal cell smears have been prepared by spreading the cells on a clean slide transferred either by careful dropping with pipette or by cytocentrifugation followed by fixation. Commonly used fixatives include 80% methanol, absolute ethanol, or a methanol–glacial acetic acid mixture.

Numerous staining methods have been used, among them DNA-specific stains are preferred for staining nuclei, MN, and other nuclear anomalies in buccal exfoliated cells. Feulgen–Fast Green staining is favoured by many investigators because of its DNA specificity and a clear transparent appearance of the cytoplasm, which enables easy identification of MN. Other stains include fluorescent dyes, such as diamidino2-phenylindoleDAP acridine orange, Hoechst and propidium iodide, May–GrunwaldGiemsa (Giemsa) stain.

Scoring criteria

Heddle 1981, Tolbert et al 1991

Heddle 125 initially described the well-established basic criteria for MN. However, the criteria for identifying cells for inclusion into the MN frequency count were not provided. Later Tolbert et al.85 developed the criteria for choosing the cells and this is being most widely applied. It consists of the following parameters: 1. Intact...
cytoplasm and relatively flat cell position on the slide; 2. Little or no overlap with adjacent cells; 3. Little or no debris; 4. Nucleus normal and intact, nuclear perimeter smooth and distinct.

Micronuclei will be scored when chromatin structure colour, intensity is similar to/weaker than main nucleus and included within the same cytoplasm.

Staining with MGP stain

First, the patients used a mouthwash with 0.9% sodium chloride solution for 15 minutes, and the area to be smeared was wiped of excessive saliva and surface debris. The smears will be obtained with a wooden tongue spatula scraped firmly across the mucosa and the cells were scattered on a dry glass slide, and then fixed in 95% ethanol for 12 hours.

After keeping slides in an incubator (48°C) for 24 hours, methyl green-pyronin staining (Merck, Darmstadt, Germany) will be performed. Methyl green (1.5g) and pyronin (2.5g) will be dissolved in 200 ml of distilled water.

After which 60 ml of distilled water and 20 ml phosphate buffer (M/10), PH5, will be mixed and added to the above solution. Excess stain will be blotted from the slides. Slides will be placed in an alcoholic solution (25% ethanol and 75% butanol) for 2 min, dipped in xylene for a further 2 min, and will be finally mounted with Entellan (Merck). The PH of the solution to be reconfirmed after each slide is stained.

In this method, pyronin stains RNA red, while methyl green stains DNA green.

All sections will be examined at a magnification of x 1,000 under a light microscope equipped with a digital camera.

(Jahanshah.S et al 2012 concluded that the MGP Y staining technique is a valuable tool for establishing a link from premalignant lesion to malignant lesion.)

Staining with Papanicolou Stain
Slide fixed in alcohol
Absolute alcohol for 2mts
70% alcohol 2mts
50% alcohol in 2mts
Tap water 2mts
Stain in haematoxylin 4mts
Rinsing with tap water
Differentiation in acid alcohol 5sec
Blue in tap water
Dehydrate in absolute alcohol
Stain in orange g 10secs
Rinse in absolute alcohol
Stain in E.A 50 for 2mts
Rinse in absolute alcohol
Clear in xylene

(Ayyad et al 2006, Shally et al 2014- used PAP stain for buccal analysis under field conditions and concluded that it was a preferred method of detecting micronuclei in oral epithelia due to clarity and transparency of cells.)