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Cytomorphologic analysis of wet fixed and air dried oral buccal smears rehydrated with normal saline: A comparative study

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Abstract

Introduction: Cytology is useful in disease diagnosis. The science of cytopathology is currently well standardized with two major branches, exfoliative and aspiration biopsy. The limitations of cytological diagnosis, are shrinking day by day. Exfoliative cytology is the study of cells exfoliated from body cavities. These exfoliated cells, from oral buccal mucosa are analysed by making smears. Smears are immediately fixed in alcohol for a period of 20 minutes and then stained with Pap stain. H & E, and Pap stains, are generally regarded as the best stains for assessment of chromatin pattern in cytologic smears since they ensure maximum resemblance with the corresponding cells in tissue sections. Cytological smears are used nowadays for mass screening purposes in camps. Taking bottles of alcohol for fixing and ensuring to store them in tightly sealed copulin jars, with smears, may be difficult. Air-dried smears may be used to overcome such practical difficulties. Rehydration of these air-dried smears can be a good alternative to wet fixation.

Methodology: 20 healthy subjects were randomly selected and 2 oral buccal smears were taken from the same subject. One was fixed by routine alcohol wet fixation, the other was air dried for 24 hours, then rehydrated with saline for 20 minutes and fixed with alcohol. The slides were assessed after labelling. The scoring criteria was based on processing, preservation, nuclear details, cytoplasmic details and background staining. Two observers, blinded to the method of fixation, scored the slides, to avoid any bias.

Results: The air-dried smears rehydrated with saline were almost equal, or even superior, in few characteristics, to wet fixed smears. The ease of processing was equal in both, while the nuclear details and cytoplasmic details were superior in saline rehydrated smears. However, the background stain was slightly darker in saline rehydrated smears compared to wet fixed smears. A student paired t-test was also done ($p=0.639$).

Keywords: Wet fixation, Air dried rehydrated fixation, normal saline, buccal smear

Introduction

The study of the structural, bio- chemical, and functional changes in the body's cells, tissues, and organs that determine any diseased condition, is termed 'Pathology' ^[1]. Originally, the field of pathology is classified into general pathology and systemic pathology. General pathology deals with the cellular and tissue reactions to harmful stimuli while systemic pathology is associated with the adaptations and mechanisms that systemic organs adhere to, when afflicted with organ specific diseases ^[2]. There are other divisions in the field as well, including cytopathology or the study of cells, hematopathology or the study of blood related diseases, histopathology or the study of tissue related diseases, organ specific pathologies, forensic pathology etc. ^[3].

Cytopathology is a branch of pathology that involves studying diseases and their diagnosis. It is otherwise simply called 'cytology', the study of cells ^[4]. George Nicolas Papanicolaou, is known as the father of cytopathology, as he found, the same, in the year 1928 ^[5]. This field uses free cells and tissue fragments as samples, whilst the other fields use entire tissues. Cytopathological analysis involves two main methods, which are, exfoliative cytology (the study of exfoliated cells), and intervention cytology ^[6]. Fine needle aspiration, sediment cytology and imprint cytology are sub-divisions of the latter, referring to any procedure involving penetration into the cellular layers ^[7].

With the increased use of cytopathological procedures and tools for disease diagnosis, it has

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become well recognized that these procedures are safe if carried out systematically, economic and simple for diagnosing diseases and providing insight into clinicians as to how they must go about their treatment plan [8]. Cytopathological protocols are thus used as screening tools, and disease prognosis and diagnosis essentials. However, some hindrances associated with cytopathological studies include air drying artefacts which possibly could lead to false impressions of enlarged of the cells and nuclei, that only ends in false diagnosis, any marked inflammation present that might obliterate cellular details and presence of intervening blood elements, which require careful observation and exclusion [9].

Exfoliative cytology especially deals with the microscopic examination of exfoliated or desquamated cells from any mucosal or epithelial surface [10]. Samples could be gynaecological specimens from cervical mucosa, respiratory cytology, which includes bronchial washing, sputum, and bronchial brushing cytology. Urinary cytology includes samples collected from bladder washing and brushing. Body fluid cytology includes samples of pleural fluid, pericardial fluid, peritoneal fluid, and cerebrospinal fluid (CSF). Samples from the mucosa of the gastrointestinal tract are also used. Discharges from any site of the body also is examined to investigate infections and malignancies [11]. The simplest technique used however, is scrape cytology performed at bedside or bench-side [12]. Detection is also accurate in most cases with this sample. The materials required for sample collection include microscopic slides stored in containers, a cell harvesting instrument (wooden spatula, metal spatula, cytobrush, oral-CDX brush, tooth brush), fixative, clinical report form, slide marking pencil) followed by preparation of tissue site, smear taking and cell harvesting, staining, and finally assessment [13].

Following slide preparation, is a procedure termed fixation, which is a means of preventing degeneration of cells and tissue by intracellular autolytic enzymes so that they can be preserved in a state, as life like as possible [14]. Smears will be thus kept in the fixative solution or material for specific amounts of time. Fixation, thus, causes some physical and chemical changes in the cell that enables it to be stained. There are several fixation routines. Two common ones are wet fixation and air-dried smear rehydration [15]. Wet fixation is a procedure wherein freshly obtained smears are immediately placed in a liquid fixative like 95% alcohol, methanol, propanol, isopropanol etc. An alternative fixation method is the air-dried smear rehydration method. Here, smears will be air dried for about 24 to 48 hours. Following this is rehydration with hydrating agents like glycerine, normal saline, almond oil, coconut oil, etc. If glycerine is used, 2 drops will be placed on the smear and covered with a clean glass slide [16]. Next, this is wrapped in wax paper and sent to the laboratory in a suitable container. Transportation of smears may require fixatives like carbonated and spears coating fixative agents [17].

In this study, the Papanicolaou (PAP) stain was used.

Papanicolaou (PAP) stain is a universal stain used for gynaecologic and non-gynaecologic cytology smear. It is mostly employed for oral and cervical smear assessment. A well stained smear shows well stained nuclear chromatin with distinct staining [18]. Alternate staining methods in use currently are the Haematoxylin and Eosin (H&E) stain and Giemsa stain. H&E staining is the use of two histological stains together, which are, haematoxylin, a basic nucleus directed stain, and eosin, an acidic cytoplasmic stain. The nucleus is thus stained blue while the cytoplasm is stained pink. All other structures take up different shades by various combinations of these colours [19]. Giemsa stain, on the other hand, is specific to the phosphate groups in DNA, and attaches itself to regions of DNA with high amounts of adenine-thymine bonding [20]. G-banding, as it is also known, is used to create a karyogram or chromosome map and spot chromosomal aberrations. Here the nucleus is stained dark purple while the cytoplasm is stained pale blue [21].

The study was thus undertaken to assess which is more advantageous among the methods of fixation discussed.

Materials and methods

20 healthy subjects were selected on a random basis with 2 oral buccal smears taken from the same subject. One of pair was fixed in alcohol conventionally and the other was air-dried for 24 hours, after which it was rehydrated with normal saline for 20 minutes. This was followed by the usual routine of alcohol fixation. All 40 slides were thus sent to the two pathologists, who were not informed about the difference in fixation methods used, so as to prevent any bias when scoring the slides.

Scoring Criteria

Scoring was done based on processing and preservation, nuclear details, cytoplasmic details and background stains (Table 1). Scores 0 and 1 were considered as unacceptable whereas Scores 2 and 3 were considered as acceptable.

Table 1: Scoring criteria and interpretation²²

Category	Score	Interpretation
Preservation of cells	0,1,2,3	Washed out, satisfactory, good, very good
Nuclear details	0,1,2,3	Poor, moderate, good, very good
Cytoplasmic details	0,1,2,3	Washed out, uniform colour, no differentiation, good
Background stains	0,1	Stain deposit presence, absence

Results

The air-dried smears rehydrated with normal saline (Figure 1) were compared with the wet fixed smears (Figure 2). The morphological details were compared and scored according to the scoring criteria by two pathologists without any bias and the acceptable and Unacceptable scores were tabulated (Graph 1 and 2) and analysed statistically.

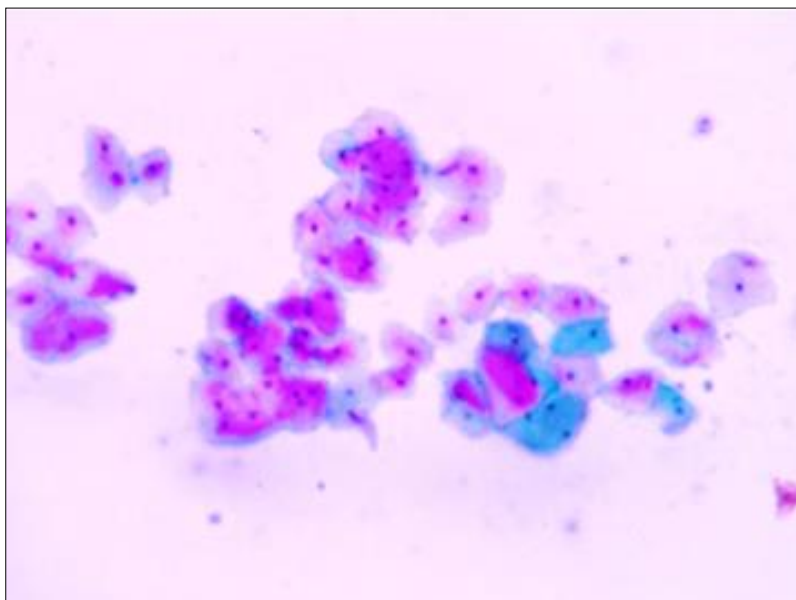


Fig 1: Photomicrograph of the Air-dried Normal saline treated cytological smear

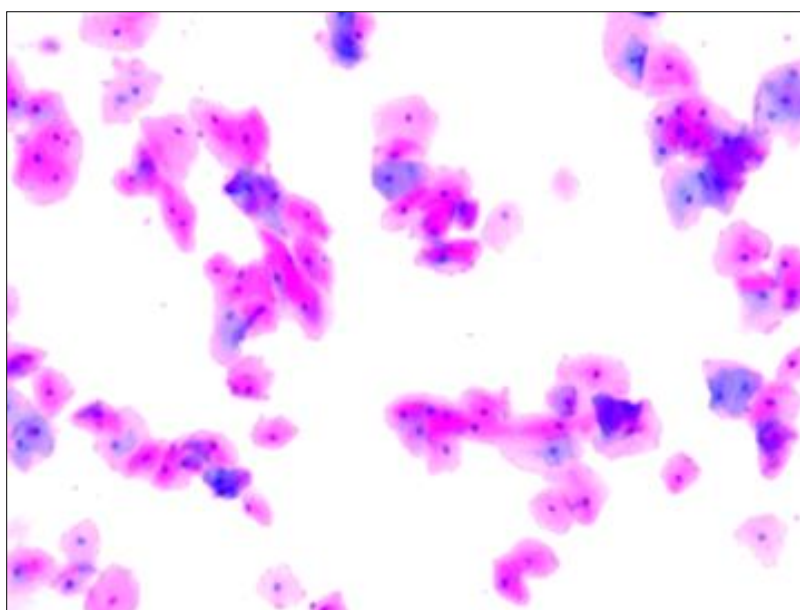
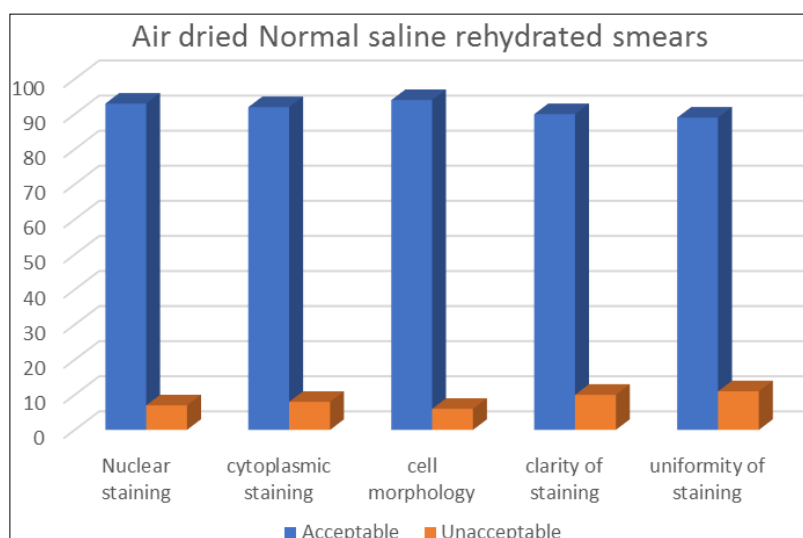
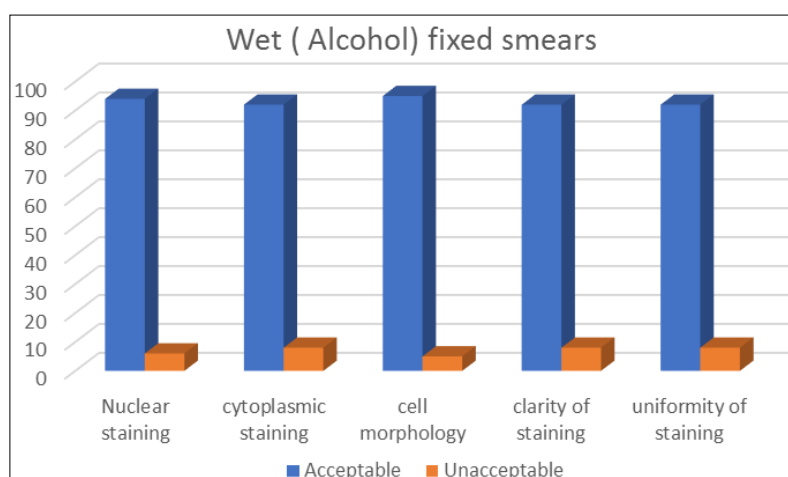


Fig 2: Photomicrograph of the Wet (Alcohol) fixed cytological smear



Graph 1: Acceptable and Unacceptable percentage for air dried & normal saline rehydrated smears



Graph 2: Acceptable and Unacceptable percentage for Wet fixed smears

Table 2: Statistical analysis (Mann-Whitney Rank Sum Test)

Group Name	N	Missing	Mean	Std Dev	SEM	P-Value
Acceptable (Normal saline treated)	5	0	91.6	2.074	0.927	0.248
Acceptable (Wet fixed)	5	0	93	1.414	0.632	

Discussion

Oral cytology is helpful in diagnosing diseases at the cellular level. It is a non-invasive method that can provide useful information about the structure and associated functions of exfoliated cells. Besides, it is simple, safe and cost effective, with smears ready in less than minutes, for diagnosis, when compared to the complexities associated with biopsy [22].

Smears are made with the collected exfoliated cells and then fixed. The smears taken in the present study, are from the buccal mucosa of the oral cavity. For a buccal smear, cells are collected by scraping the cheek with a spatula. The cells can be used for genetic testing, including the presence of the sample taken to the laboratory for evaluation [23]. In this preparation one can observe squamous epithelial cells from the oral mucosal stratified squamous non-keratinizing epithelium in addition to blood cells and bacteria [24].

For precisely half the smear samples, wet fixation was done using 95% alcohol. Besides, it is the fixative commonly and ideally used, recommended for the preservation of cytological specimens [25]. It replaces the water in the cells and causes the cells to shrink. Another mixture recommended by Papanicolaou himself, was the use of ether alcohol mixture, which is not currently in use on account of its safety hazards [26]. 100% methanol is also an acceptable substitute for 95% ethanol. It produces lesser cellular shrinkage compared to ethanol but is comparatively more expensive. Propanol and isopropanol are also used as they cause least cell shrinkage [27]. Before staining, at least 15 minutes of fixation in a suitable fixative is essential. Prolonged fixation for many days together will not affect the cellular morphology.

In this study, Naib mentioned but in passing, "If the smear is accidentally allowed to air dry, it can be rehydrated by placing it in tap water for a few minutes before fixation [28]."

In the present study, the efficiency of air-dried oral smears rehydrated with saline was thus, assessed, with the remaining half of the samples. In a study by Chan, the use of tap water, aqueous glycerine, and hypotonic saline are illustrated, which were not very satisfactory on account of

lysis of nucleated cells [29]. Normal saline, was rather satisfactory due to its ease of availability and ability to rehydrate the dried smears. With saline accessible to all medical centres, camps and clinics where at a time, numerous smears might require to be taken for mass screening, it is indeed an efficient alternative. Moreover, air drying of smears is advantageous when it comes to transporting smears without equipment and aids like jars or fixatives. With regard to the duration of air drying without compromising on subsequent rehydration, 30 minutes is most suitable, though smears air dried for up to one day may also be salvaged by rehydration, although less satisfactorily [30].

Fixation was followed by staining with Pap stain. This staining method gives a polychromatic transparent staining reaction with precise nuclear and cytoplasmic features distinguishing between basophilic and acidophilic cell components, thus obtaining a detailed chromatin pattern. The stain has 3 solutions with 6 dyes comprise the stain [31]. The first solution has a basic nuclear stain, Haematoxylin. The second solution has OG-6, an acidic cytoplasmic stain, with orange G and phosphotungstic acid. Orange G, an acid dye, stains keratin orange. Phosphotungstic acid, binds to proteins and acts as a mordant. The third solution has EA with a PH 4.5-5 which is polychromatic staining the cells green or blue, having 3 components; light green/fast green, eosin Y and Bismarck brown Y. The light green acid dye stains the cytoplasm of metabolically active cells. Eosin Y is another acid dye staining the cytoplasm of superficial cells and its components. Bismarck brown Y precipitates phosphotungstic acid, responsible for distinguishing staining by light green and eosin. EA has a low solubility in alcohol and hence is not washed away easily from cells. Pap staining is more extensively used, compared to H & E staining because it imparts different shades to different components of the cell. While nuclei stain blue, collagen is stained green, bone opus stained blue and, muscles and erythrocytes stain red.

Rupinder *et al.* in their study revealed that immersion of Pap smears in normal saline for 30 seconds lysed the red cells

effectively, giving a clearer background, retaining the squamous and glandular cells intact^[32]. Similar results were also obtained in other studies^[33]. Thus, with lesser background deposits, the disadvantage of darker background staining in air-dried smears, can be overcome. Air-dried buccal smears rehydrated with normal saline can therefore be effectively used as an alternative to wet fixed smears.

The necessity of air-dried smears is found where the practical difficulties associated with wet fixation is to be overcome. Alcohol, which is required in large quantities for wet fixation, needs to be transported, stored in air tight copulin jars, protected from sunlight exposure, and the assessment of the smear must be carried out immediately after removing from fixative and staining. Thus, if smears are to be collected from less equipped areas, rural areas, or even in mass camps, the poor availability of alcohol is an obstacle. Hence, a more efficient method would be to air dry collected smears, transport them to a more suitable location, and then carry on with the routine procedures for smear assessment.

Conclusion

Rehydration of air-dried smears is a suitable alternative to wet fixed smears. The cytologic features of saline rehydrated air-dried buccal smears were equal or superior to those of wet fixed smears. This method is useful, easier and avoids the disadvantages and practical difficulties associated to wet fixed smears.

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