

REVIEWS

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The Role and Application of Exfoliative Cytology in the Diagnosis of Oral Mucosa Pathology – Contemporary Knowledge with Review of the Literature

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A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article; **G** – other

Abstract

On the basis of the current available literature, the authors have presented a short description of cytological examination and its application in the oral mucosa disease diagnostic process. Furthermore, the advantages and disadvantages of this method are described. The available diagnostic tools used for oral smears were reviewed as well as more and more often available methods which aim at making the diagnosis process more accurate and more favorable for patients. Oral cytology analysis may, in the near future, be a very useful examination for patients in terms of diagnostics and monitoring, not only during the treatment but also afterwards. The authors would like to demonstrate what a beneficial tool this cytological examination could be as a fast and cheap cancer prophylactic test. This opinion is based on the fact that this cytological method has significantly improved the detection of uterine cervical cancer during a gynecological examination since the introduction of the Papanicolaou technique in the 40s (*Adv Clin Exp Med* 2014, 23, 2, 299–305).

Key words: oral mucosa, oral pathology, exfoliative cytology.

Exfoliative cytology is a diagnostic technique based on a microscopic evaluation of epithelial cells after a procedure of their fixation and staining. There are 2 methods in use: the indirect cell-collecting method, such as aspiration subjects with self-exfoliated cells, and the direct method, rub cells of the mucosal surface. The exfoliated cells are put into a preservative fluid and the samples are processed according to the manufacturer directions, after staining using the Papanicolaou method [1, 2].

Exfoliative cytology as a diagnostic tool is most popular in gynecology. Since cervix cytology (the Papanicolaou technique) appeared in the 1940's in diagnostics, the number of deaths from cervical cancer has decreased. Unfortunately, the role of exfoliative cytology in screening for oral cancer still isn't as successful as it is in the diagnostic process of the uterine cervix cancer.

The Papanicolaou technique is a multichromatic staining histological technique developed by George Papanikolaou and used to differentiate cells from smear preparations of various bodily secretions [3]. This is a polychrome staining method which comprises a nuclear stain (hematoxylin) and two counterstains (Orange G and Eosin Azure dyes). Hydration of the fixed smear is required for the cells to take up the hematoxylin, whereas dehydration prepares the smear for the counterstain dyes.

Advantages and Disadvantages of Exfoliative Cytology

Oral cytology examination in oral mucosa is still not popular. There are controversies with

regards to its real validity in oral mucosa pathologies. In the past, the controversies surrounded the use of oral exfoliative cytology (EC) in the management of oral malignancy, because of a large number of false-negative results and subjective interpretation of abnormal oral mucosa cells. The low sensitivity of oral cytology is related to various factors including inadequate sampling, procedural errors and subjective interpretation of the findings.

This technique is useful for preliminary diagnosis of many oral mucosal diseases but it is not a substitute for the routinely-used biopsy to obtain a definitive diagnosis. Lesions caused by reactive changes and inflammatory reactions are non-specific and non-diagnostic cytological findings. EC is not appropriate as a diagnostic tool for patients with clinical symptoms of desquamative gingivitis. It adds to the cost and delays the definite diagnosis [4, 5].

Although oral cytology has disadvantages, some authors underline that we can eliminate them and increase its diagnostic value. The best way to do that is by making the procedures clearer and by standardizing them. A quantitative technique increases the diagnostic ability of exfoliative cytology. It is precise, objective and reproducible. Quantitative methods include DNA cytophotometry (optical quantification of chemical substances incorporated into the DNA – the Feulgen stain) and cytomorphology (characterizing the proliferative activity of the cell populations of oral mucosal squame). Normal oral cells display a diploid DNA distribution, indicative of the non-replicating cell population. Malignant cells show a number of different DNA profiles such as diploid, polyploid, aneuploidy and hyperdiploid [6, 7]. One of the most handy and commonly used methods is DNA-image cytometry which, at the same time, is specific, sensitive and highly objective. It is performed for identification of early neoplastic epithelial cells in oral brushings. The presence of DNA-aneuploidy found in the cell is used as a sensitive and specific marker [4, 5]. Malignant lesions have abnormal DNA distribution histograms. Their cytomorphological values displayed significant variation, elevation in mean nuclear/cytoplasmic ratio (N/C), significant elevation in mean nuclear area (NA) and cytoplasmic area (CA). With age, the value of the nuclear-cytoplasmic ratio increases. Recently, the development of automatic-cytomorphometric methods, DNA content determination, tumor marker detection, and diverse molecular-level analyses, have aroused interest in exfoliative cytology [8].

Areas for Collecting Smear Samples and Instruments for Exfoliative Cytology

The most common areas for collecting smears within the oral cavity are the buccal mucosa, hard and soft palate line, dorsum of the tongue, bottom of the mouth and the lower labium region. The cells may be detached naturally (mouthwash sampling) or artificially (tool sampling). Tools for collecting smears should be easy to use in any location, not irritating and provide an adequate number of epithelial cells. The types used and compared were wooden, plastic and metal spatulas, dermatological curette, Fisherbrand sterilized polyester swab and different types of brushes (interproximal brush, cytobrush, oral CDx brush and Cytobrush Plus GT) [9, 10]. Cytobrush sampling is used more frequently; it maximizes the number of cells obtained, and facilitates their uniform distribution on the microscope slide. The brush is recommended as an adequate instrument due to its easy use in sampling and due to the quality of the oral cytology sample. The Oral CDx brush test used for exfoliative cytology is a simple, harmless and non-invasive procedure [11, 12]. It is a test for oral pre-cancer and cancer lesions. All authors agreed that brushes are best for oral cytology, being much more effective than other instruments. Scheifele et al. examined the sensitivity and specificity of the Oral CDx technique. In evaluation of 103 cases, the sensitivity amounted to 95.9% and 90.9% and false-negative rates from 1% to 4.1%. The specificity was 94.3%. The authors emphasized that the false positive results of the Oral CDx are possible in other oral lesions with a certain grade of epithelial atypia, e.g. inflammatory conditions. They concluded that the brush biopsy can be recommended, but the data still needs to be confirmed by larger controlled trials. Another tool, the Cytobrush Plus GT, is recommended for collecting cells from the lateral side of the tongue because it has been proven to be the only acceptable brush in this location [13].

Main et al. studied the suction sampling technique in which the device consisted of a collecting cup connected to a blood collection evacuation system. The vacuum (440 mm Hg) was applied for 5 seconds and the mucosa was drawn in against the filter, producing a monolayer imprint of cells. According to the authors, this suction sampling device has a simple construction and ease of clinical application. Using the vacuum tubes, the negative pressure applied was constant and reproducible on each sampling occasion. The procedure of sampling was fast and caused minimal inconvenience and discomfort to the patient [14].

Improvements in Exfoliative Cytology

Conventional cytopathology has improved the early detection of changes in individual exfoliated cells, but this methodology depends on subjective interpretations of the nuclear and cell's appearance, structure and stain uptake. A diagnosis made on morphology analysis is difficult when we have only collected low grade dysplasia or precancerous samples, because the structural changes are not yet visible. Evaluation of a high grade dysplastic specimen is also limited and quite difficult, because proper diagnosis depends on the presence of different than normal cells (morphologically) between numerous cells with no changes in their structures. Because of the development of instrument technology, it is possible now to study the inner biochemical structures of the cells and to also reveal changes in the composition caused by a pathological state, before they can be seen morphologically.

Papamarkakis et al. underscored the value of cytopathology with optical methods such as spectral cytopathology (SCP). According to their studies, the advantages of SCP are the possibility of monitoring the biochemical arrangement of particular cells, and analyzing the spectral information. This mathematical process provides an impartial, recurrent approach to help cytopathologists and to increase the standard of care for the patient [15].

Hayama et al. examined the efficiency of liquid-based preparations. These authors concluded that the technique of slide preparation can be enhanced by the use of liquid-based technology, which demonstrated a statistically significant improvement in smear thickness, in cell distribution and a reduction in cell overlapping, and the presence of blood [16].

Kujan et al. showed the same conclusions [17]. In their study, liquid-based cytology (LBC) demonstrated a statistically significant, 41% overall improvement in smear thickness and 66% in cell distribution, and a reduction in cell overlapping and the presence of blood. The cell morphology was better visualized in these liquid-based preparations and the material preserved in the liquid-fixative solution had a long storage life (material can be stored for additional analyses). Both the liquid-based preparation and conventional smear are diagnostically reliable but the liquid-based method showed an overall improvement on sample preservation, specimen adequacy, visualization of cell morphology and reproducibility. Since liquid-based cytology (LBC) was developed in the 1990's, various comparative studies have shown

its significant advantages over conventional cytology. Results obtained from uterine cervix exams showed that the liquid-based preparations reduce the problems related to sampling error, poor transfer and fixation of the cellular sample, and a significant reduction in false-negative results. The tongue and bottom of the oral cavity are the most frequent locations for oral cancer. Both have some differences in the structure, which can only be observed spectrally, an uncommon presentation of esophageal-type keratins and a large manifestation of collagen. These biochemical differences cannot be distinguished morphologically. Significant spectral differences of regular oral mucosa cells taken from five anatomical locations in the mouth were examined. It is proven that the cells which built the tongue and the floor of the oral cavity have different biochemistry than cells from other mouth structures. SCP (spectral cytopathology) differentiates cells by their exceptional biomolecules. SCP supplies a diagnostic workup so biomolecular abnormalities can be found, those which could lead from dysplasia and into neoplasia, before morphological compromise and this is the major prevalence over conventional method. A large number of cells from virally infected samples are morphologically normal. SCP is able to differentiate cells infected by viruses in the initial as well as in terminal stages (HSV infection). The spectral changes observed in cancerous progression and viral infections are similar. It can lead to the conclusion that primary spectral differentiation in those cases can suggest viral infection. This fact is notably meaningful since we know that viral infections (EBV and HPV) play an important role in oral cancer formation. The most useful advantage of SCP is repeatedly obtaining the same result of the examination regardless of the fatigue, experience and variability between observers. This technique is able to show subtle biochemical abnormalities of the cells which aren't reflected in their morphology yet [16, 17].

DNA-Image Cytometry and DNA Measurement Cytophotometry as Adjuvant Diagnostic Tools

Remmerbach et al. showed that DNA-aneuploidy is a sensitive and specific marker which can be used for early identification of atypical cells in oral brushings [18]. The study was based on 300 cytological diagnoses of 60 subjects with diagnosed cancer. The sensitivity of a cytological examination for the detection of cancerous cells was 95%. The specificity of this examination was 99.6% and positive and

negative prophetic values were respectively 98.3% and 99.8%. The prevalence of DNA-aneuploidy in cells taken from oral squamous cell carcinoma *in situ* and from invasive carcinoma was 96.6%. The sensitivity of DNA-aneuploidy in oral swabs for the confirmation of abnormal cells was 96.6%, specificity 100%, positive and negative prophetic values were respectively 100% and 99.2%. When we combine these two methods, the sensitivity will increase to 98.3%, specificity to 100% and positive and negative predictive values respectively to 100% and 99.6%. The authors concluded that DNA-image cytometry is a highly specific and sensitive as well as objective complementary method, helpful to demonstrate neoplastic marks in oral swabbed cells.

DNA measurement cytophotometry is a method in which nuclear and cytoplasmic measurement cytomorphology slides are fixed in equal parts 95% ethanol and diethyl ether, then stained with a Papanicolau stain. Recently, Sudbo et al. showed that the DNA content in tissue sections is of value for leukoplakia diagnosis. The histological changes in leukoplakia which possibly precede malignancy can't be reliably detected by exfoliative cytology (this study showed that 10 out of 16 lesions with histological evidence of epithelial atypia had normal cytology), which yields a false negative diagnosis. The keratinized surface of leukoplakia is probably responsible for the large number of false negative results. Positive smears were obtained from lesions with epithelial atypia which have nonkeratinized or ulcerated but not keratinized surfaces [19].

Hande et al. carried out buccal mucosa cytomorphometric analysis of tobacco chewers and concluded that cytomorphometric changes could be the earliest indicators of cellular alterations. They showed a progressive decrease in cellular diameter (CA), increase in nuclear diameter (NA) and increase in the ratio of nuclear diameter to cellular diameter (NA:CA) in smears taken from all tobacco users. This indicates that there could be a relationship between tobacco usage and quantitative alterations of cells. The concept of cellular or nuclear alteration on exposure to different forms of tobacco can be best explained by reviewing the nature of the cellular response to the end products of tobacco usage. The decrease of cellular diameter and increase of nuclear size are significant morphologic changes characteristic of actively proliferating cells [20, 21].

Age, Location and Gender-Related Study

Cowpe et al. collected gingival smears from 80 healthy patients divided into 4 age groups: 0–20, 21–40, 41–60, and over 60 years (40 male,

40 female) [22]. Image analysis software was used to measure the cytoplasmic (CA) and nuclear areas (NA). The authors found a meaningful difference in NA, CA, and NA:CA ratio in males as well as in females. In both major groups (males and females), the difference in NA:CA was significant irrespective of age. The dissimilarity in NA, CA, and NA:CA ratio with age, irrespectively of gender, was also significant. The authors also observed variations of the gingival smears according to age and sex. This fact can be helpful in creating a baseline to compare the same measurements taken from pathologically changed areas. Some authors suggest utilization of a quantitative method which uses the estimation of different parameters of the cell (nuclear area (NA), cytoplasmic area (CA), and nucleus-cytoplasmic area ratio (NA:CA)). Because of the precision, objectivity, reproducibility and non-invasiveness of these techniques, the early detection of oral cancer, using exfoliative cytology, might increase in the future. To obtain comparable and reliable data, a pre-examination specimen of regular oral squamous cells is required. Pathological cells can be further compared with baseline data, which can help with the evaluation and getting a higher positive rate for detection of malignant epithelial cells. It is important to create a baseline for these variables, to compare identical parameters from pathological samples. Healthy epithelial cells have a normal, morphologically unchanged nucleus. A suggestion has been made that cytomorphometric analysis or image analysis of exfoliated cells might be the best diagnostic tool for the evaluation and observation of cellular and nuclear abnormalities in cytological smears.

Cowpe et al. noted that usually the reduction in CA precedes NA reduction in tissues with malignant transformation [22]. Another conclusion was that samples of healthy mucosa obtained from the same person give the best control. The significant difference in NA, CA, and NA:CA ratio in different age groups of females was probably caused by a dissimilarity of hormones levels. Except the oldest age group, the NA and CA parameters were much higher in females than in males groups. The difference in NA:CA ratio between males and females was not statistically significant. The reduction in NA:CA ratio was similar for both genders in groups with advancing age. The conclusion of that research was that for making a baseline of normal oral squamous cells, the most important cellular parameters are measurements of nuclear, cell size and NA:CA ratio. The data suggested that inflammation is able to increase NA and to reduce cytoplasm but these features are usually found only in young cells and don't represent cellular atypia. The authors also

noted that the best control would be a sample taken from the healthy mucosa of the same patient. Exfoliative cytology is able to detect malignant changes in cells by the assessment ratio of nuclear to cytoplasmic size, using the planimeter method. Cellular diameter was highest in normal mucosa, lower in dysplastic lesions and lowest in oral squamous cell carcinoma.

In smears from leukoplakia and lichenoid lesions, Doseva et al. found hypodiploid or hyperdiploid, rather than diploid DNA profiles [23]. Mutation of the tumor suppressor gene p53 is another frequent genomic change in human cancers. According to most studies, p53 is not present in normal oral mucosa but in advanced changes and is detectable by immunohistochemical methods in squamous cell carcinoma (SCC) and potentially malignant lesions of the oral mucosa. Early diagnosis of oral cancer on the basis of p53 detection is not useful if exfoliative cytology doesn't reach basal epithelial cells. Early results indicate that quantitative cytology could be of great value for monitoring and follow-up of suspicious lesions and to provide an excellent additional diagnostic test for detecting early oral malignancy [24].

Oral Mucosa Smear Results in Correlation with Conditions such as Diabetes, Candida Infections and After Kidney Transplant

Kelesand et al. examined patients after kidney transplantation and found that there were alterations in the oral epithelial cells, detectable by microscopy and cytomorphometry [25].

Other authors' observations suggested that type 2 diabetes mellitus can produce morphological and functional alterations in oral epithelial cells detectable by microscopic and cytomorphometric analysis using exfoliative cytology, which can be used in the diagnosis of the disease. The authors showed morphological alterations in the buccal mucosal epithelial cells of the diabetic group like nuclear enlargement, karyorrhexis, binucleation and infiltration of polymorphonuclear leukocytes. The NA was significantly higher in the diabetic group but there was no difference in CA in these two groups, so the NA:CA ratio was significantly lower in the diabetic group. The conclusions were that exfoliative cytology is useful as an additional tool to help in the diagnosis of diabetes [26, 27].

Loss et al. examined patients with candidiasis and concluded that candida-infected epithelial cells have an increase in nuclear diameter (NA), perinuclear rings, discrete orangeophilia and cytoplasmic vacuoles and a decrease in cytoplasmic area (CA). There was also an increase of the NA:CA ratio. This study revealed that the oral mucosa of patients undergoing candida infection exhibited significant changes in the size and shape of the oral epithelial cells [28].

Discussion

Exfoliative oral cytology is a simple, pain-free, non-invasive, non-aggressive and rapid technique. The method is well tolerated by patients and is less stressful when compared to biopsy, so it can be widely used in population screening programs. Any dentist could perform an oral brushing and, up to now, no contradictions are known. In the examination of cells scraped from the surface of lesions, additional, different diagnostic techniques may be used. The most useful are cytomorphometry, DNA-cytometry and molecular analysis.

It would be important to make a characteristic base of exfoliative cytology in healthy groups of patients with normal, healthy oral mucosa (without inflammation), with no history of nicotine or alcohol abuse, in relation to sex, age and sites of oral mucosa from which the samples were collected. Then these characteristics of the cells of healthy oral mucosa could be easily compared to the samples from pathologically changed areas like the lichen planus, leukoplakia, recurrent aphthae, burning mouth syndrome (BMS), white-spotted, ulcerated lesions or other suspicious changes of oral mucosa [8, 22]. This will also be an important aid to reporting on the diagnostic precision of conventional oral exfoliative cytology.

Because of the development of a diagnostic procedure of malignant oral lesions called the "brush biopsy", the sample is collected with a particularly designed brush (complete penetration in the thickness of the mucosa) and collects representative material of the lesions. This method has been designed to collect cells from the upper to the basal layer of the epithelium. The malignancy or benignancy of cells taken from lesions is evaluated using a computer assisted analysis. Recently, the usage of oral exfoliative cytology as a diagnostic and prognostic tool, and also as an aid to check patients with oral pre-cancer and cancer lesions, has become more popular. Despite the improvement of methods and tools used for collecting and examining oral epithelial cells, this methodology is still unreliable for diagnosing oral cancer because

of frequent false positive and false negative results. The probable future role of exfoliative cytology is providing samples of DNA from biopsy, taken from proven oral cancer for genetic analysis, to help to understand the type of mutation, predict tumor behavior and its response to traditional and novel forms of therapy.

The p53 positive cells can be identified in oral cancer tissue using cytology but it is limited by the fact that all the lesions are clinically obvious because they are advanced already [8]. So oral cytology could be helpful in detecting malignant and premalignant lesions, long-term monitoring

of suspicious lesions and as part of the follow up protocol after oral cancer treatment.

It is important to mention, it shall never prevail over the classic biopsy and in all clinically suspicious lesions biopsy should be done.

Different changes in oral cells can also be seen in relation to Candida infection and diabetes.

Because of the continuing development of cytological techniques and improvements in cell collecting instruments and methods, there is now a big challenge for oral cytology to become a routine procedure in patients with oral mucosa problems.

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