Evaluation of Papanicolaou Stain for Studying Micronuclei in Buccal Cells Under Field Conditions

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Objective

To compare Papanicolaou (Pap) and May-Grünwald Giemsa (MGG) stain as 2 techniques for staining for buccal mucosal cells to detect micronuclei (MN) in field studies.

Study Design

Eighty cytologic smears (2 per individual) were taken from the buccal mucosa of 40 cigarette smokers recruited at a rural village in Egypt. Forty smears were stained with Pap stain and 40 with MGG stain. All were assessed for cellularity and scored for MN.

Results

Pap stain was faster and easier to process and transport in the field study than was MGG stain. Regarding MGG smears, bacteria and cell debris masked the MN as compared to Pap smears, in which the fixative destroyed the bacteria and made the cell boundaries clearly demarcated. Using Pap stain, MN were seen easily in transparent cytoplasm.

Conclusion

Pap stain is the preferred method in field studies for scoring and detecting MN in cells of buccal mucosa. (Acta Cytol 2006;50:398–402)

Pap stain is the preferred method of detecting MN in oral cells from subjects in a field study....

Keywords: buccal mucosa, Papanicolaou smear, tissue stains, micronucleoli, Papanicolaou stain.

Exposure of a tissue to genotoxic carcinogens leads to an increase in chromosomal aberrations.[1-4] Consistent with this hypothesis, karyotypic anomalies and elevated DNA content have been observed in various preneoplastic lesions. Before particular karyotypic anomalies can be established, a long period of breakage and translocation of chromatids must occur.

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Such chromatid anomalies or chromosome deletions can lead to formation of micronuclei (MN), which are DNA-containing bodies in the cytoplasm without any structural connection to the main nucleus.[5] MN are small, extranuclear bodies separated from the main one, generated during cellular division by late chromosomes or by chromosome fragments because of their association with chromosomal aberrations.[4,6] The MN test is one of the current rapid, efficient and economical techniques used as an indicator of genotoxicity, as it provides a quantitative measure of the genotoxic action of carcinogens and mutagens.[7-12]

MN analysis of exfoliated cells is a convenient technique to study genotoxic changes directly in target organs affected by the toxicant.[2,4,13-30] For example, increased MN frequency was found in exfoliated oral (buccal) cells among smokers and tobacco chewers with oral carcinomas as compared to cancer-free subjects.[14-16] Epidemiologic studies have reported smoking habits to be associated with an increased occurrence of different kinds of oral carcinoma.[17-19] Comparisons between studies are difficult, however, because of differences in methodologies, especially with regard to staining techniques, microscopic magnification, number of cells counted and instruments used for sampling cells. Several investigators have used Feulgen stain and Fast green for scoring MN,[7,16,20-23] while others have used Papanicolaou (Pap) stain in scoring MN.[24-26] Others used May-Grünwald-Giemsa (MGG) stain in scoring MN in lymphocytes and buccal cells.[27,28]

Some of these staining methods require strict adherence to controlled laboratory conditions and are therefore difficult to apply in field studies, in which such conditions may be difficult to maintain. In our ongoing studies of tobacco health effects in rural villages in Egypt, for example, it is necessary to select a robust method of MN analysis that can be performed under field conditions of specimen sampling and preparation. The aim of this study, therefore, was to compare the Pap and MGG stain as 2 techniques for evaluating MN in buccal mucosa cells.

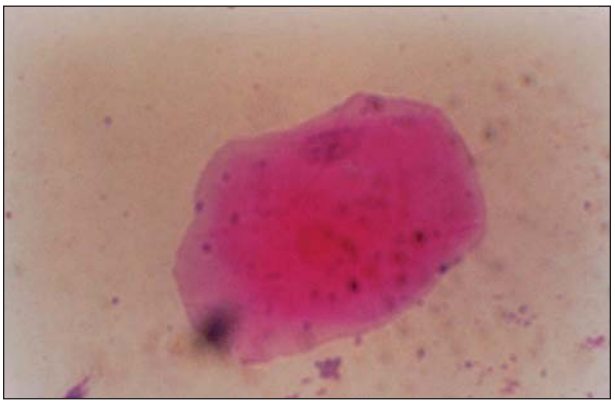

Figure 2 Buccal smear showing anucleated epithelial squamous cell (Pap stain, ×1,000).

Methods

A total of 40 cigarette smokers were selected from a rural village in Qaloubiya Governorate, Egypt; all met the eligibility criteria of being smokers for at least 5 years, smoking 10 or more cigarettes per day and smoking the same amount of cigarettes for the previous year. Exfoliated cells were obtained by scraping the tongue and buccal mucosa with a wooden spatula with a rolling motion.[4,29] The material obtained was immediately smeared onto the center of a clean glass slide. For each individual, 2 slides were prepared by smearing the oral scrapings on the slides for a total of 80 slides (40 slides stained with Pap stain and 40 with

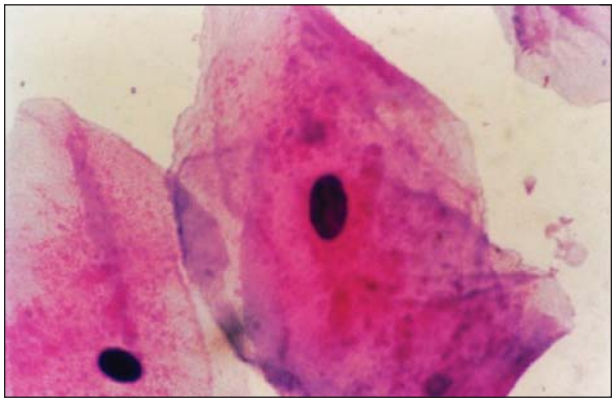

Figure 1 Buccal smear showing normal epithelial squamous cells (Pap stain, ×1,000).

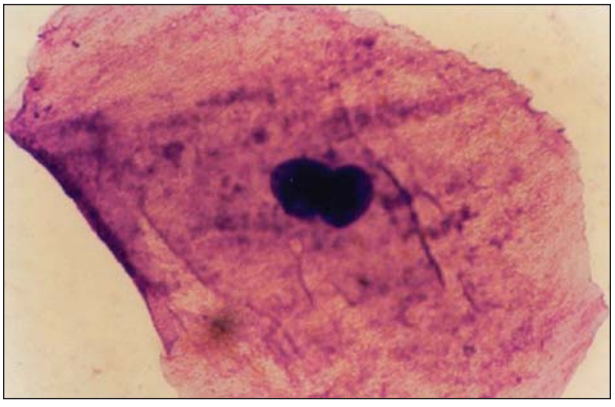

Figure 3 Buccal smear showing binucleated squamous cell (Pap stain, ×1,000, with phase contrast).

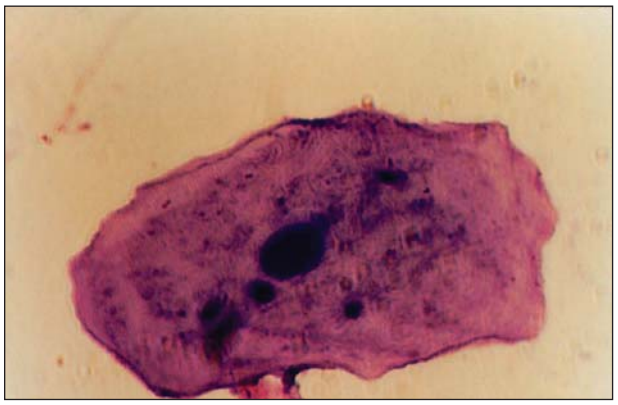

Figure 4 Buccal smear showing micronuclei (Pap stain, ×1,000, opaque with phase contrast.

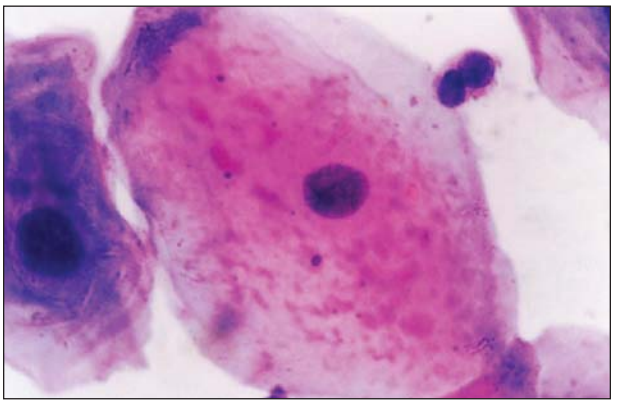

Figure 6 Buccal smear showing squamous cell with micronucleus and inflammatory cell on its lower border (MGG stain, ×1,000).

MGG stain).

Fixation

For MGG stain, the smears were received in the laboratory, allowed to air dry and then fixed in methanol (80%) at 0°C for 20 minutes.[28,30] For Pap stain, we used the wet fixation method in the field: immediate submersion of wet cell samples in 95% ethyl alcohol was performed.[30]

Staining

MGG Method. For this method we prepared fresh MGG stain (25 g/100 mL methanol) and then stained the slides in May-Grünwald stain for 3 minutes. (Staining time may vary depending on the concentration of stain.) We determined the optimal staining time with a trial slide before proceeding. Next we placed slides in dH_2O for 1 minute and stained with (10%) Giemsa stain (stock solution diluted 1:10 in Sorenson's buffer) for 7–10 minutes. (It is critical to determine the optimal staining time before proceeding.) The slides were washed 2 times in dH_2O, 3 minutes each, and then were air dried. We applied a coverslip with Permount.

Modified Pap Method. For this method we fixed the smears in 95% ethyl alcohol. Then we performed hydration with a running tap water wash. We applied nuclear stain (DNA related), Mayer's hematoxylin for 4 minutes, followed by a rinse in a stream of gently running water for 15 minutes. Dehydration by 70% and 95% ethyl alcohol was then performed with 10 dips each. Cytoplasmic stain (Orange green) was applied for 1 minute, followed by a rinse with 95% ethyl alcohol for 5 minutes. Cytoplasm and nuclear stain (RNA specific) by EA polychrome was applied for 1.5 minutes, followed by a rinse with 95% ethyl alcohol (2 times), 10 dips each. The next step was dehydration

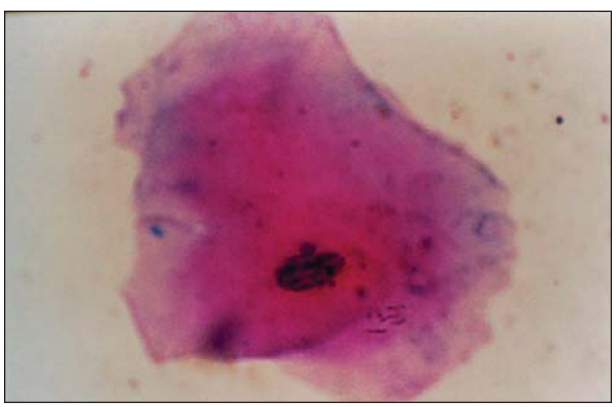

Figure 5 Buccal smear showing fragmented nucleus (karyorrhexis) (Pap stain, ×1,000).

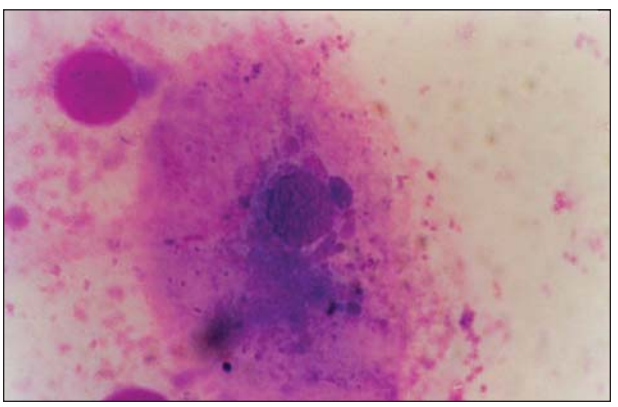

Figure 7 Buccal smear showing broken egg nucleus, bacteria and cell debris (MGG stain, ×1,000).

with absolute ethyl alcohol (2 times), 10 dips each, and then clearing by absolute ethyl alcohol and xylene (1:1) for 1 minute. The preparation was mounted with mounting medium.

MN Test. Screening for MN was performed under an oil immersion lens (×1,000) followed by phase contrast microscopy for confirmation of MN, according to established methods.[21,26,24] The opaque extranuclear-intracytoplasmic bodies were counted as MN, whereas binucleated cells, karyorrhexis, karyolysis and broken eggs were not considered MN.[6,22,29] At least 1,000 intact epithelial cells per individual were scored, and for each person we calculated the average percent micronucleated cells. In addition, we calculated the total number of MN (TMN) and the number of cells with MN (CMN) per individual since some cells had multiple MN.

Results

Pap stain was easier to read than the MGG stain and easier to process and transport in the field. Of 40 specimens (80 slides), 2 (4 slides) were not adequate (had < 1,000 cells per individual). Two specimens (4 slides) that were stained with MGG had bacterial colonies and cell debris masking the oral cells completely, so MN could not be detected. Figures 1–5 illustrate Pap smears, while 6–7 illustrate MGG smears.

Discussion

This study showed that under field conditions Pap stain is more practical than MGG stain. MGG stain requires slides to be air dried and then placed in methanol (80%) at 0°C. That was difficult to apply in the field and resulted in difficulties in transport. The methanol required by that method is not the substance of choice for fixation of cytologic specimens. Furthermore, the slides stained with MGG stain were full of bacteria, which often masked MN. They make the scoring of MN difficult and largely inaccurate.

Regarding Pap stain, the 95% ethyl alcohol used for fixation is the best fixative because it has a bactericidal effect. The alcohol allows the permeability of dyes across cell boundaries, permits cell adhesion to the glass surface and replaces cellular water. It also penetrates the cell rapidly and maintains morphologic integrity. Pap stain is preferred also as it consists of nuclear stain (hematoxylin) that stains all nuclear DNA, both intranuclear and extranuclear. It consists also of 2 counterstains that make the cytoplasm transparent and the cell well demarcated.

Many previous studies used other stains, such as Feulgen–fast green, for MN study.[21,7,23] This method of staining takes a long time (>4 hours), and the method of fixation is more difficult to use under field conditions. Its components are more expensive than those of Pap stain. Feulgen stain alone cannot delineate the cytoplasm: it needs 1% Fast green to demarcate the cytoplasm of cells. If the cytoplasm is too dark and the nucleus not clear and distinct, it needs destaining in ethanol until the optimal contrast is achieved.[21,29]

In summary, comparisons between 40 smears of buccal mucosa stained with Pap stain and 40 smears stained with MGG stain demonstrated that, and this conclusion is consistent with studies by Tsu,[26] Roberts[24] and Guzman et al.[25]

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