Intra-Oral Microscopy for Directed Biopsy—Increasing The Sensitivity

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Abstract
The diagnosis of a dysplastic pre-malignant or malignant lesion of the oral mucosa cannot be based solely on clinical findings. Therefore, histologic evaluation of a representative biopsy specimen is necessary. The choice for the site of biopsy, however, has so far remained a subjective choice that sometimes raises doubts about its representativeness. No simple and reliable method is yet available for selecting the most appropriate site for biopsy. Intra-oral microscopy (oral application of the colposcopy technique) of mucosal lesions seems to offer advantages in selecting the sites most representative of dysplastic changes for biopsy beyond routine clinical examination alone. This article highlights the application of intra-oral microscopy of the oral mucosa in the diagnosis of various oral pre-malignant lesions and conditions and its advantage in selecting the most representative biopsy site for the confirmation of the histopathological diagnosis of dysplasia.

Introduction
The incidence of various oral pre-malignant lesions and conditions and frank oral cancers is increasing steadily globally. In spite of advancement in early detection, the increasing mortality and morbidity related to oral cancers continues (1). At present, there are simple chairside methods for detection including staining with toluidine blue and exfoliative cytology; however, there is a high risk of false positives, with these well-known techniques. Moreover, the diagnosis of a dysplastic lesion of the oral mucosa cannot be based solely on clinical findings. A supplementary biopsy with a histological examination of the lesion is necessary to establish a definitive diagnosis (2). However, because the selection of a biopsy site is based solely on a clinical examination, it has remained a subjective choice and there always remains a possibility of the biopsy specimens being taken from unrepresentative sites. Hence, an appropriate biopsy result for the correct diagnosis of dysplastic pre-malignant or frank cancerous lesions is always dependent on the appropriate selection of a site most representative of the dysplastic features. Intra-oral microscopy (oral application of the colposcopy technique) of mucosal lesions seems to offer advantages in selecting the biopsy sites most representative of dysplastic changes beyond routine clinical examination alone.

Review of colposcopy
Colposcopy is an established technique for diagnosis in gynecology. Many pre-malignant and malignant lesions in gynecology have discernible characteristics that can be detected upon examination. The technique of colposcopy employs the use of a piece of equipment known as a colposcope that provides an enlarged view of the suspicious-looking areas, so that a physician may visually distinguish normal from abnormal areas of affected mucosa and take directed biopsies for further pathological examination (1). Various authors have attempted to adapt gynecologic methods of examination to the oral mucosa because of the similarities between oral mucosa and genital mucosa (3). However, relatively few reports in the literature describe the role of colposcopy in relation to oral mucosa and the diagnosis of oral mucosal lesions (Fig.1) (4).

Etymology
The word colposcopy is derived from the Ancient Greek: kolpos meaning "hollow, womb, vagina" and skopos meaning "look at". The procedure was developed in 1925 by the
Significance of intra-oral microscopy

To date, there is no reliable method applicable to the oral cavity that can replace a biopsy for a more definitive diagnosis of dysplasia (Fig. 2). Supplemental procedures such as exfoliative cytology, although suitable for screening large number of cases, carry the risk of both false-positive and false-negative results, thus making biopsy a mandatory procedure for the final confirmation of the diagnosis. Toluidine blue may be used to identify the site most suitable for biopsy, but studies indicate the risk of false-positive staining to be as high as 30% (2). Questions have also been raised regarding the risks associated with the use of toluidine blue staining because of its affinity towards DNA (5).

Intra-oral microscopy (oral application of the colposcopy technique) thus is emerging as an effective procedure for identifying visible clues suggestive of suspicious-looking areas of the mucosa. It offers advantages in selecting the most representative biopsy sites beyond routine clinical examination alone, despite being a simple chairside diagnostic adjunct (6).

Applications of colposcopy in gynecology

The main purpose of colposcopy is to detect intraepithelial and early neoplasia of the cervix, vagina, and vulva (7). Other indications of colposcopy include: to clarify the nature of clinically suspicious lesions; to display and localize the lesions suspected cytologically; as a part of the forensic examination of a sexual assault case; and to diagnose lesions due to human papilloma virus infection in immunosuppressed patients such as those with human immunodeficiency virus infection or a patient receiving a transplanted organ. In most cases, however, a colposcopic examination is indicated as an integral part of a gynecologic examination in concert with a cytological examination to further investigate a cytological abnormality on a Pap smear (8–10).

The image obtained is often considered the result of the reciprocal relationship between the epithelium and the underlying connective tissue stroma, wherein the epithelium acts as a filter through which both the incident light and the reflected light pass (11). The stroma appears red because of its rich vascularity. The redness of the stroma is transmitted through the epithelium and is visible through the colposcope. The intensity of color represents the ratio of reflected light to absorbed light and is related to the thickness of the epithelium, the optical density of the epithelium (i.e., the morphology and the organization of the epithelial cells), the vascularity and the nature of the underlying stroma, the amount of hemoglobin, and the concentration of the tissue chromophores (11–13).

Procedure

The representative areas are clinically examined for the selection of biopsy site. The outline of the lesion is marked with a black color pen and the biopsy site is then highlighted
with a red color pen with the help of a grid placed on the buccal mucosa. During clinical examination, clinical criteria for the selection of biopsy site for leukoplakia usually includes erythema, granular consistency and ulceration while for carcinoma buccal mucosa, erythema, induration and ulceration are considered to be the markers of dysplastic changes.

Following clinical examination, mucosa is wiped with saline. After the mucosa is wiped with saline, abnormal epithelium appears much darker than the normal epithelium. Using the green (or blue) filter and higher-power magnification, abnormal vascular patterns are evaluated. Then, 5% acetic acid is applied to the lesion for about 60s and the area that is estimated to have the most extensive cell changes based on colposcopic criteria is selected for biopsy. The area of the biopsy site is highlighted on the grid with a green color pen.

When the areas selected for biopsy by clinical criteria and colposcopy are superimposed (red and green areas), then only one common biopsy sample is obtained. When two different areas marked with red and green pens are selected from the same lesion, both are biopsied and subjected to histopathological examination (Fig.3). Biopsy specimens are taken with a 6-mm punch, wounds are closed (Fig.4), and histopathological examination is performed.

The routine procedure involves the application of 3% acetic acid to the suspicious-looking mucosal areas using cotton swabs; the areas of acetowhiteness correlate with higher nuclear density that is diagnostic of dysplasia. Areas of the mucosa that turn white after the application of acetic acid or that appear with an abnormal vascular pattern are often considered to be the most suitable areas for taking biopsy samples. If no lesions are visible, an iodine solution may be applied to help highlight areas of abnormality that are then used as the favoured sites for taking biopsy samples. Significant complications from a colposcopy procedure are not common, but may include bleeding, infection at the biopsy site, and failure to identify the lesion. Furthermore, Monsel’s solution and silver nitrate, used for inducing coagulation at bleeding points, may interfere with the interpretation of biopsy specimens (11,14,15).

The application of 3-5% acetic acid alters the epithelial surface. The effect is due to reversible coagulation of the nuclear proteins and cytokeratins (14,15). Burke et al (14) believed it to be due to a reversible osmolar change, resulting in cytoplasmic dehydration and cytoplasmic membrane collapse. This cellular change produces more reflected light, resulting in a dense acetowhite image. However, this acetowhite effect is transient; the speed with which it appears and disappears depending upon the number of cells present and their nucleo-cytoplasmic ratio. Immature metaplastic epithelium turns a shiny white color that disappears fast, whereas an opaque and longer lasting acetowhiteness is seen in areas of high-grade dysplasia or cervical intra-epithelial neoplasia.

**Grading and scoring systems**

Different grading and scoring systems have been devised
for colposcopic examination in gynecology. Some of the more commonly used criteria are described below.

A. Grading system of Coppleson and co-workers (16)

Coppleson and co-workers proposed three distinct gradations based on the histological diagnosis of the acetowhite areas as follows:

Grade 1 (insignificant, not suspicious): The acetowhite epithelium is usually shiny or semi-transparent, the borders are not necessarily sharp, with or without fine caliber vessels, often with ill-defined patterns, with the absence of atypical vessels, and with small intercapillary distance. The predicted histology is metaplastic epithelium (both immature and mature), acanthotic epithelium, sub-clinical papilloma virus infection (SPI), and low-grade cervical intra-epithelial neoplasia (CIN1).

Grade 2 (significant, suspicious): The acetowhite epithelium is seen with greater opacity and sharp borders, with or without fine caliber vessels, often with ill-defined patterns, with the absence of atypical vessels, and with small intercapillary distance. The predicted histology is high-grade CIN (CIN2 or 3).

Grade 3 (highly significant, highly suspicious): Very white or gray opaque epithelium is seen, sharply bordered and with dilated caliber, irregularly shaped, often coiled, occasionally presenting with atypical vessels, with increased but variable intercapillary distance, and sometimes irregular surface contour (micro-exophytic epithelium). The predicted histology is CIN3 or early invasive carcinoma. The latter diagnosis is considered more probable in the presence of atypical vessels and micro-exophytica.

B. Combined colposcopic index

Reid and Scalz (17) proposed a scoring system to predict the histologic diagnosis on the basis of four colposcopic features presented in Table 1.

C. Grading system of Burke and co-workers (14)

Burke and co-workers recommended a grading system to predict the underlying lesion as shown in Table 2.

Vascular patterns seen with the colposcope

The criteria described in colposcopic literature can also be used for selecting biopsy sites in relation to the oral mucosa. These include the surface pattern, color tone and opacity, and the clarity of demarcation of the mucosal lesions in addition to the vascular pattern and the intercapillary distance. The whiter and more opaque appearance of the lesions associated with dysplasia or carcinoma in situ can also be distinguished. In healthy oral or genital mucosa, two basic types of vascular patterns, hairpin capillaries (Fig.5a) and network capillaries (Fig.5b), can be seen (18).

<table>
<thead>
<tr>
<th>Colposcopic sign</th>
<th>Zero point</th>
<th>One point</th>
<th>Two points</th>
</tr>
</thead>
<tbody>
<tr>
<td>Margin</td>
<td>Condylomatous or micro-papillary contour, indistinct aceto whitening, flocculated or feathered margins, angular, jagged lesions, satellite lesions, and aceto whitening that extends beyond the transformation zone</td>
<td>Regular lesions with smooth, straight outlines</td>
<td>Rolled, peeling edges, internal demarcations between areas of differing appearance</td>
</tr>
<tr>
<td>Color (after application of acetic acid)</td>
<td>Shiny, snow-white color, indistinct aceto whitening</td>
<td>Intermediate shade (shiny gray)</td>
<td>Dull, oyster-white</td>
</tr>
<tr>
<td>Vessels</td>
<td>Fine-caliber vessels, poorly formed patterns, condylomatous or micro-papillary lesions</td>
<td>Absent vessels</td>
<td>Definite punctuation, mosaic</td>
</tr>
<tr>
<td>Iodine uptake</td>
<td>Positive iodine staining</td>
<td>Partial iodine uptake</td>
<td>Negative staining for significant lesion</td>
</tr>
</tbody>
</table>

HPV=human papilloma virus; CIN=cervical intra-epithelial neoplasia.

Scores of 0 to 2 are predictive of a minor lesion (HPV or CIN); Scores of 3 to 5 usually indicate a middle-grade lesion (CIN2); Scores of 6 to 8 are usually indicative of significant CIN (CIN 2 or 3).
Some principal abnormal findings include the following (19):

Punctuation: The tips of the terminal vessels in the stroma reach the surface of the epithelium through stromal papillae and appear as red dots prior to the application of acetic acid (Fig. 5c).

Fine punctuation: The finer the punctuation, the more likely the lesion is to be a low grade CIN or cervical intra-epithelial neoplasia.

Coarse punctuation: Coarse punctuation is suggestive of a high grade CIN or a frank malignant degeneration.

Mosaic: The vessels do not reach the epithelial surface and extend only partially into the epithelium, appearing as red lines surrounding blocks of epithelium. The appearance is further accentuated after the application of acetic acid (Fig. 5d).

Fine mosaic: The smaller, smoother, and finer the mosaic, the more likely the lesion is to be a low grade CIN or metaplasia.

Coarse mosaic: The coarser, wider, and more irregular the mosaic, the more likely the lesion is to be a high-grade CIN or invasive carcinoma.

Atypical vessels: These vessels appear to be running on or parallel to the surface of the epithelium and are of irregular caliber and are branching, appearing as coarse wide hairpins and commas, corkscrews, wastepaper, coarse and caliber tree-like and root-like forms, or spaghetti-like forms usually indicative of invasive carcinoma (16, 19, 20) (Fig. 5e).

Important points to be considered in the assessment and interpretation of abnormal colposcopic findings include surface contour and lesion margin; response to acetic acid; appearance of gland openings; iodine uptake; and keratosis and the varied vascular patterns, including the appearance of blood vessels (including atypical blood vessels) suggestive of the higher grades of dysplasia (16, 17, 19, 21).

### Intra-oral microscopy in oral epithelial dysplasia

An important area of application of intra-oral microscopy may well be in the diagnostic evaluation of the various oral pre-malignant lesions and frank oral cancers. Most of the patients with pre-malignant lesions and frank oral cancers show changes in the vascular patterns that can be easily recognized by direct intra-oral microscopy. Dysplastic areas could be mapped to indicate the full extent of epithelial change to guide biopsy and surgery. In a recent study, Pazouki et al. (22) found a close relationship between stromal vascularity and tumor progression in the oral mucosa. Moreover, because the equipment allows simultaneous viewing of surface cells as well as submucosal vessels, it is hoped that techniques will be developed for documentation without the need for vital staining. The comparative effects of different treatment modalities, such as radiation and chemotherapy, on the junction between the tumor and healthy tissue could also be studied (23).

Most patients with oral squamous carcinomas have a marked inflammatory infiltrate interfering with the evaluation of dysplastic lesions. In comparison, biopsy specimens selected by direct intra-oral microscopy overcome this most common cause for the decreased sensitivity of biopsy outcomes in these sets of patients (24). Direct intra-oral microscopy thus aids in the distinction between chronic inflammatory lesions, which can then be easily distinguished from dysplastic lesions by the assessment of the features noted in the examination.

### Table 2. Grading system of Burke and co-workers

| Grade | Surface | Margin   | Color        | Time                                | Vessels                               | Pathology                                      |
|-------|---------|----------|--------------|----------|-------------------------------------|----------------------------------------------|-----------------------------------------------|
| 1     | Flat    | Indistinet | Normal / slightly white | Appears slowly, remains for short time, disappears rapidly | Fine punctations with normal ICD | SPI, inflam, immat metapl, pregnancy, regeneration, repair |
| 2     | Flat    | Distinct | Whiter      | Average time to appear, remains for several minutes, disappears with average speed | Fine punctations or, mosaic with slightly increased ICD | SPI, CIN1 and CIN2                                |
| 3     | Raised  | Sharp    | Whitest     | Appears rapidly, stays for a longer time, disappears slowly | Coarse punctations or, mosaic with increased ICD, atypical vessels | CIN 3 and early invasive cancer |

*SPI=sub-clinical papilloma virus infection; inflamm=inflammation; immat metapl=immature metaplasia; ICD=inter-capillary distance.
Fig. 5. Vascular patterns seen in colposcopy.

a) Hairpin capillary pattern in healthy buccal mucosa.
b) Network capillary pattern in healthy buccal mucosa.
c) Punctation seen in leukoplakia.
d) Mosaic pattern observed in carcinoma buccal mucosa.
e) Atypical vessels seen in carcinoma buccal mucosa.
Limitations and controversies around colposcopy as a useful diagnostic adjunct in oral epithelial dysplasia

Complications from colposcopic procedures are exceedingly rare. Occasionally, bothersome bleeding can occur following biopsy. The most worrisome complication is inadequate or inaccurate evaluation leading to the missed diagnosis of invasive cancer. This obviously can lead to treatment delays and poorer outcomes. Keratosis can also mask the colposcopic finding of an endophytic and/or hyperkeratotic lesion by obscuring the vascular pattern, which is why a biopsy is necessary. Another complication is the overestimation of lesion severity by inexperienced practitioners. This can put the patient in a further train of diagnostic procedures and treatment courses that may not be necessary and has the potential for adverse sequelae.

This infrequent but preventable lack of adequate evaluation is the only real controversy surrounding the procedure today. Simply put, the question is who should be performing the exam and what training requirements must be met before instituting the procedure on patients. The learning curve undoubtedly is practitioner-dependent and currently no adequate studies have identified minimum criteria of standards. All practitioners performing this procedure should put safeguards in place to ensure their own competence and safety.

Conclusion

In conclusion, direct intra-oral microscopy shows promise as one of the most important diagnostic adjuncts that has an advantage for selecting the most representative biopsy areas. It may become the so-called gold standard in the diagnosis of dysplasia, thus increasing biopsy sensitivity and minimizing the possibility of false negative outcomes as a result of selection of inappropriate areas of suspicious-looking areas of the mucosa. Direct intra-oral microscopy also aids in the distinction among chronic inflammatory lesions that can be easily distinguished from dysplastic areas of the oral mucosa by the assessment of the features noted in the examination before the selection of the appropriate biopsy site. However, because there is a relative dearth of supporting research in this area, this method should be evaluated in further clinical studies and compared with the use of the various other available diagnostic adjuncts, including the more popular staining methods, to clearly draw support.

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