Introduction

Epithelial tissues form a barrier between the body and the environment. Depending on location, they perform various functions, but always serve to protect the internal tissues from environmental stresses, chemical damage, and bacterial infection.\(^1\)

A critical aspect of stratified keratinizing epithelia is that, the cells undergo a terminal differentiation program that results in the formation of a mechanically resistant and toughened surface composed of cornified cells that are filled with keratin filaments and lack nuclei and cytoplasmic organelles. In these squames, the cell membrane is replaced by a proteinaceous envelope that is covalently cross-linked to the keratin filaments, providing a highly insoluble yet flexible structure that protects the underlying epithelial cells.\(^1\)

Keratins are frequently the most abundant cellular proteins. They constitute the major component of the cytoskeleton of all epithelia; these intermediate filaments provide mechanical support for the cells and nucleus. Keratins have a number of distinct advantages for use as marker proteins to differentiate epithelial tumors from mesenchymal tumors, both histologically and immunohistochemically. In establishing a definitive diagnosis, it is sometimes advantageous to demonstrate histologically the degree of keratinization or the presence and/or absence of keratin through the application of a stain which discerns keratin.\(^2\)

In routine hematoxylin and eosin (H-E) staining, structures like collagen, amyloid, muscle, keratin and other extra...
cellular and intracellular secretions stain eosinophilic where differentiating one from other is difficult. For example, the histological demonstration of keratin is important in assessing the degree/ pattern of differentiation for squamous cell carcinoma.

In the past, Ayoub-Shklar A-S, Dane - Herman method, Schiff reagent after oxidation with performic acid, aldehyde-fuchsin, levafix red violet have been used to stain keratin. All these have advantages and disadvantages.

Papanicolaou (PAP) stain is a routinely used staining technique, commonly available in oral pathology laboratories. It is a multichromatic staining technique, which is used to differentiate cells in smear preparations for various body secretions.[3] The main use of orange G6 in the papanicolaou stain is to stain keratin.[4] Superficial cells with high content of keratin stain yellow-orange hue and parabasal cells stain green to blue in color.[3] Johnson and Klein et al, in 1956 reported the application of the papanicolaou stain to paraffin embedded sections, for demonstration of keratin.[10] Elzay et al have reported the modification of papanicolaou stain by adding phloxine-B on paraffin embedded sections to demonstrate keratin.[2] Phloxine-B, a red acid dye is a derivative of fluorescein with distinctly bluish shade.[11] It is used to stain mucin, prekeratin and keratin which appear distinctly red in color.

Although, the presence of keratin protein can be detected very specifically by immunohistochemical methods on paraffin embedded tissue sections, the antikeratin-antisera are not economical and are time consuming.[2] Hence this study was designed to demonstrate the efficacy of modified papanicolaou (Mod PAP) stain with phloxin embedded sections to demonstrate keratin. Phloxine-B, a red acid dye is a derivative of fluorescein with distinctly bluish shade. It is used to stain mucin, prekeratin and keratin which appear distinctly red in color.

MATERIALS AND METHODS

Since the study was planned to stain keratin in tissue sections, hematoxylin and eosin stained sections were reviewed for the presence of keratin in known keratin containing tissue sections and slides were selected.

The study group included:
- 20 cases of well differentiated squamous cell carcinoma (WDSCC)
- 17 cases of Verrucous carcinoma (VC)
- 15 cases of normal keratinized oral mucosa (NKOM)
- 8 cases of keratinized odontogenic keratocyst (OKC).

A total number of 60 paraffin embedded tissue blocks were retrieved from the archives for our study.

Method of collection of data

Three sections of 4 micron thickness paraffin embedded sections were taken. Sections were stained with 1) Hematoxylin and Eosin 2) Ayoub-shklar stains 3) Modified papanicolaou stain respectively.

Procedure for hematoxylin and eosin:
1. The deparaffinized sections in xylene were dehydrated in various grades of alcohol for 5 min each.
2. After water wash for 10 min, the slides were stained with Harris’s hematoxylin stain for 7 min.
3. Later, water washed for 10 min and after differentiation in acid alcohol, the slides were dipped in lithium carbonate for bluing for 5 min and were stained with eosin for 15 sec.
4. Dehydrated with graded alcohol, cleared in xylene and mounted.

Procedure for ayoub-shklar stain:
Materials used:
Solution: 1) 5% acid fuschin solution
   Acid fuschin – 5 gm
   Distilled water – 100 ml
2) Aniline blue orange G solution:
   Aniline blue (water soluble) – 0.5 gm
   Orange G – 2gm
   Phosphotungstic acid – 1 gm
   Distilled water – 100 ml

Staining procedure:
The sections were deparaffinized in xylene I and II for 5 min each.
1. Later dehydrated in various grades of alcohol i.e. 90% and 70% and water washed for 10 min
2. Acid fuschin solution was added for 3 min
3. Aniline blue OG was added directly to the sections for 30 min
4. Sections were transferred to 95% alcohol – 2 changes
5. Sections were dehydrated, cleared and mounted.

Procedure for modified papanicolaou stain:
Solutions used:
- Harris’s hematoxylin
- Orange G6
- Eosin – azure
- Phloxine – B (1% aqueous C.I no 45410)

Procedure for staining:
1. Deparaffinize sections through 2 changes of xylene, absolute alcohol, and 95% alcohol to water wash.
2. Stain in Harris’s hematoxylin for 6 min
3. Rinse in twice changes of tap water and dip in acid alcohol
4. Rinse thoroughly in tap water and dip in lithium carbonate
5. Wash in running tap water for 10 min, then rinse in distilled water
6. Stain in phloxine–B for 5 min
7. Rinse in distilled water and dehydrate
8. Stain in Orange G6 for 5 min
9. Rinse in 95% alcohol
10. Stain in eosin azure for 4 min
11. Rinse in 95% alcohol complete dehydration in absolute alcohol
12. Clear in xylene and mount.

Stained sections were evaluated by three oral pathologists independently and consensus was taken when required. The results were analyzed for efficacy of three staining technique and examined for:

1. Type of Surface keratin (para or orthokeratinized)
2. Amount of keratin present (keratin pearl as per the definition is the central focus of keratinization found within the concentric layers of abnormal squamous cells in epithelial islands).
   a. “More amount of keratin pearl” - more than 3 keratin pearls per field
   b. “Few areas of keratin pearl” - less than 3 keratin pearls per field
3. Pattern of staining (whether uniform or patchy).

Results were tabulated and statistically analyzed using Chi square test. $P$ value ≤ 0.05 was considered to be statistically significant.

**RESULTS**

In our study all the three staining procedures i.e., H - E stain, A/S stain and modified PAP stain showed clear, distinct keratin as pink, brilliant orange and magenta pink respectively.

All three stains showed similar results for staining surface keratin in normal mucosa, verrucous carcinoma, odontogenic keratocyst [Figures 1-9]. Whereas, WDSSC showed different results. Of 20 cases of WDSCC, in H-E stain 16 cases showed “more” amount of keratin pearls and only 4 cases showed “few” amount of keratin pearls. In both, A/S and modified PAP stain 15 cases showed “few” amount of keratin pearls and 5 cases showed “more” amount of keratin pearls. Results were statistically highly significant with $P= 0.000$ [Table 1].

When pattern of staining was evaluated, in H-E stain all 20 cases showed “uniform” staining pattern. In A/S stain, 12 cases showed “uniform” and 8 cases showed “patchy” staining pattern. In mod PAP stain, 11 cases showed “uniform” and 9 cases showed “patchy” staining pattern. Results were statistically significant when H-E was compared with A/S stain, $P= 0.002$ and when H-E was compared with mod PAP, $P= 0.001$ [Table 2] [Figures 10-15].

**DISCUSSION**

The oral epithelium represents the primary barrier between oral environment and the deeper tissues. It is lined by stratified squamous epithelium and consists of cells tightly
attached to one another and arranged in a number of distinct layers or strata. It maintains its structural integrity by a system of continuous cell renewal.\(^7\)

The epithelial surface of the masticatory mucosa, such as that of the hard palate and gingiva is flexible, tough and resistant to abrasion. This is caused by the formation of a surface layer of...
keratin and the process of maturation is called keratinization. Keratin constitutes the major component of cytoskeleton of all epithelia and provides a mechanical support for the cells and nucleus.\textsuperscript{7}
Keratin plays an important role as a marker protein in establishing a definitive histological diagnosis, like for e.g.; in grading of squamous cell carcinoma,[2] to differentiate between the epithelial and mesenchymal tumors and in certain conditions like when the epithelial component may be sparse and may be identified only by the presence of keratin reactivity.

Special stains are the stains that are used to visualize specific tissues and cellular structures. These are the dyes that bind to the cellular components either physically or by chemical bonds. Ayoub-Shklar, Dane Herman method,[2] Schiff’s reagent by oxidation with performic acid[8] are all special histochemical stains used to stain keratin specifically. These stains may highlight small foci of overt epithelial differentiation that sometimes is missed in routine H-E staining.

Papanicolaou stain is a routinely used cytopathological stain, available in most of the oral pathology laboratories. Johnson and Klein et al, in 1956 reported the application of the papanicolaou stain to paraffin embedded sections, for demonstration of keratin.[3] Later Elzay et al modified the routine papanicolaou stain by adding phloxine-B, which is a red acid dye that stains prekeratin and keratin distinctly red in color on paraffin embedded sections. Thus, the modified PAP stain can be used to stain keratin specifically.[3]

Thus, the aim of the present study was to stain the known keratin containing paraffin embedded tissue with modified papanicolaou stain, H-E stain and Ayoub-Shklar stain and to compare the efficacy of modified papanicolaou stain with that of H-E staining and Ayoub-Shklar staining technique, so as to design a staining procedure which is easy and effective for keratin.

In our study, all the three staining techniques showed similar findings in staining the surface keratin proteins in NKOM, OKC, and VC. In case of NKOM, the surface keratin which is normal or physiologic keratin stained distinctly and uniformly by all the three staining procedures as pink in H and E, brilliant red in A/S stain and magenta pink in modified PAP stain [Figures 1-3].

In normal keratinized oral mucosa, the cells in the basal layer, undergo a terminal differentiation program by a system of continuous cell renewal in which, cells produced by mitotic divisions in the deepest layers migrate to the surface to replace those that are shed. In the process, they form a tough, resistant and horny layer of squames called keratin.[7] This is a physiologic process of keratinization and is known as normal/physiologic keratin. Thus in the present study, all the three staining techniques showed uniform and distinct keratin layer in all cases of NKOM.

In a similar study done by Elzay et al,[2] the parakeratinized layer did not take up uniform stain in modified PAP stain when compared to orthokeratinized layer. These results were not consistent with our study where the parakeratinized layer was distinctly stained as magenta pink in color.

Odontogenic keratocyst is a developmental cyst derived from the dental lamina, which in turn is a derivative of oral ectoderm. As the name suggests, keratin is formed to a large extent in OKC. The cystic epithelium is lined by a regular keratinized stratified squamous epithelium, which are usually 5-8 cell layers thick with palisaded basal layer and without rete pegs. The form of keratinization is exclusively parakeratinized in about 80-90% but sometimes orthokeratinized or both forms are found in different parts of some cyst.[9]

In our study, the surface keratin as well as the keratin flakes in the lumen of OKC was stained distinctly in all the three staining procedures for all twenty cases [Figures 4-6]. The keratin produced by the cystic epithelium would have been in the similar manner produced by the physiologic/normal keratin in normal stratified squamous epithelium. As the cyst originates from the dental lamina, the signals to produce normal keratin though, in excess would have been maintained by the cystic epithelium and hence stained distinctly by the three staining procedures.

Verrucous carcinoma is a low grade variant of squamous cell carcinoma. Histologically the epithelial cells are well differentiated and show very few dysplastic features. Characteristically, cleft like spaces lined by thick layer of

---

### Table 1: Amount of keratin pearls in WDSCC

|          | H-E stain | A-S stain | Mod-PAP stain |
|----------|-----------|-----------|---------------|
| Few      | 4         | 15        | 15            |
| More     | 16        | 5         | 5             |
| Total    | 20        | 20        | 20            |

WDSCC - Well differentiated squamous cell carcinoma

### Table 2: Pattern of staining in WDSCC

|          | H-E stain | A-S stain | Mod-PAP stain |
|----------|-----------|-----------|---------------|
| Uniform  | 20        | 12        | 11            |
| Patchy   | 0         | 8         | 9             |
| Total    | 20        | 20        | 20            |

WDSCC - Well differentiated squamous cell carcinoma
parakeratin extend from the surface deeply into the lesion. The parakeratin lining the clefts with the parakeratin plugging is the hallmark of verrucous carcinoma.\[^{10}\]

In our study, all the three staining techniques showed similar results and stained keratin distinctly for all 20 cases [Figures 7-9]. As the epithelium in verrucous carcinoma is well differentiated and with few dysplastic features, the epithelial cells may undergo terminal differentiation in the same manner as the normal/physiologic keratin and hence take up the stain distinctly and appear as pink in H-E stain, brilliant orange in A/S stain and magenta pink in modified PAP staining procedures.

Squamous Cell Carcinoma is defined as a “malignant epithelial neoplasm exhibiting squamous differentiation as characterized by the formation of keratin and / or presence of intercellular bridges”. The WDSCC shows evidence of keratinization like individual cell keratinization and keratin pearl formation in the epithelial islands.\[^{10}\]

In our study, of the 20 cases of WDSCC when evaluated for the type of surface keratin, all the three staining procedures showed similar results. No statistical significance was found. When evaluated for the amount of keratin pearl, in H-E staining 16 sections showed “more” areas of keratin pearl and 4 sections showed “few” areas of keratin pearl. Whereas in both A/S stain and modified PAP stain only 5 sections showed “more” areas of keratin pearl and 15 section showed “few” areas of keratin pearl. Results were statistically significant for amount of keratin pearl when H-E stain was compared to both A/S and modified PAP stain [Table 1].

When evaluated for the pattern of staining, in H-E staining all 20 cases showed uniform staining but whereas, in A/S stain 12 cases showed uniform staining patterns and 8 showed patchy staining pattern. Results were statistically significant, when H-E stain was compared to A/S staining.

In case of Modified PAP stain, 11 sections showed uniform staining pattern and 9 sections showed patchy staining pattern. Results were statistically significant, when H-E stain was compared to modified PAP stain. But, no statistical significance was found when A/S stain was compared to Mod PAP stain [Table 2].

In our study, in both A/S stain and Modified PAP stain only 5 sections showed “more” areas of keratin pearl and all areas were not uniformly stained. Some of the keratin pearls stained only in the central core or only the periphery of keratin pearl was stained leaving the core unstained [Figures 10-12]. While, in other areas keratin pearl did not take up the special stain at all [Figures 13-15]. To rule out any technical errors, various sections of the same tissues were taken and stained simultaneously. Sections of WDSCC which also had surface epithelium were taken, surface keratin stained uniformly whereas, the keratin pearl did not take up uniform staining pattern in the same section though, the staining protocols were strictly followed.

The reasons for this difference in amount of keratin pearl and pattern of staining in WDSCC could be attributed to:

The keratin pattern in normal mucosa shows regional variations in accordance to the cornification process. Keratins like K1 and K10 are expressed in normal/physiologic cornified layer and takes up uniform staining. Whereas in case of WDSCC, the keratin pearls in few areas would have undergone normal cornification process as the physiologic keratin and stained uniformly and distinctly. Whereas in other areas the abnormal/ tumor keratin pearl, which did not stain, would have, some presently unknown biochemical differences in cornification process\[^{2}\] compared to the normal/physiologic surface keratin maturation and hence have no affinity for the special stains.

All the keratin pearls observed in H-E stain might not be true keratin pearl, as H-E stain is not a specific stain for keratin and stains all the cytoplasmic components and extracellular secretions giving a uniform eosinophilic appearance.

In the results obtained from our study via the three staining procedures such as H-E stain, A/S stain and modified PAP stain, all three stains stained surface keratin uniformly and distinctly in NKOM, OKC and verrucous carcinoma. Therefore, modified PAP stain was comparable with that of H-E stain and A/S staining for surface keratin. Whereas in case of WDSCC, A/S stain and modified PAP did not stain all the keratin pearls uniformly as in H-E staining procedures. As already stated, this tumor keratin in the epithelial islands of WDSCC would have some different cornification process compared to the normal/physiologic keratin and hence the special stains do not have an affinity for such tumor keratin.

Thus, the efficacy of modified PAP stain to stain surface keratin specifically is comparable with that of A/S and H-E staining technique. The modified PAP stain, stains keratin distinctly, yields nuclear detail, and uses readily available dyes compared to A/S stain, which do not have nuclear component. Although, routine H-E stain is a gold standard, it is not specific for keratin, while modified PAP is a polychrome stain which apart from staining keratin magenta pink, also imparts different hues of color to structures like nuclei – blue, collagen – green, bone – blue, muscle and erythrocytes as red in color. Hence, modified PAP stain can be used as an alternative special stain to A/S stain in staining surface keratin distinctly.

CONCLUSION

The present study, to the best of our knowledge, is the first study to document and compare H-E, modified PAP and A/S stain for keratin. Hence, our study adds to the limited literature on application of modified PAP stain on paraffin embedded tissue sections and it is comparable to A/S stain and H-E
stain to stain surface keratin. For the tumor keratin present in squamous cell carcinoma that is not stained by modified PAP and A/S stain, a more sensitive tool like immunohistochemical technique be applied to know the exact pattern of cytokeratin expression.

REFERENCES

1. Presland RB, Dale BA. Epithelial structural proteins of the skin and oral cavity: function in health and disease. Crit Rev Oral Biol 2000;11:383-408.
2. Elzay RP. A modification of Papanicolaou exfoliative cytology stain to demonstrate keratin in paraffin-block tissue sections. Oral Surg Oral Med Oral Pathol 1983;56:51-3.
3. Papanicolaou stain. Available from: http://en.wikipedia.org/wiki/Papanicolaou_stain. [Last accessed on 2011 Dec 22].
4. Orange G. Available from: http://en.wikipedia.org/wiki/Orange_G. [Last accessed on 2011 Dec 22]
5. Johnson Pergy L, Klein Morris N. Application of papanicolaou stain to paraffin section. Stain Technol 1956;5:223-5.
6. PhloxineB. Available from. http://chemicalland21.com/specialtychem/finechem/PHLOXINE%20B.htm. [Last accessed on 2011 Dec 22].
7. Tornec CD. Oral Mucosa. In: Tencate AR, editor. 5th ed. Tencate’s A.R Oral Histology. Singapore: Harcourt Asia Pte Ltd; 1999. p. 378.
8. Scott HR. Demonstration of keratin with aldehyde-fuchsin. Nature 1953;172:674-5.
9. Shear M. Odontogenic keratocyst. Cysts of the Oral regions. 3rd ed. Wright; Oxford: 1992.
10. Shafer-Hine-Levy. Benign and malignant tumors of oral cavity. Shafers Textbook of Oral Pathology. 5th ed. Delhi: Saunders: 2006.

How to cite this article: Ramulu S, Kale AD, Hallikerimath S, Kotrashetti V. Comparing modified papanicolaou stain with ayoub-shklar and haematoxylin-eosin stain for demonstration of keratin in paraffin embedded tissue sections. J Oral Maxillofac Pathol 2013;17:23-30.

Source of Support: Nil. Conflict of Interest: None declared.

Staying in touch with the journal

1) Table of Contents (TOC) email alert
   Receive an email alert containing the TOC when a new complete issue of the journal is made available online. To register for TOC alerts go to www.jomfp.in/signup.asp.

2) RSS feeds
   Really Simple Syndication (RSS) helps you to get alerts on new publication right on your desktop without going to the journal’s website. You need a software (e.g. RSSReader, Feed Demon, FeedReader, My Yahoo!, NewsGator and NewsCrawler) to get advantage of this tool. RSS feeds can also be read through FireFox or Microsoft Outlook 2007. Once any of these small (and mostly free) software is installed, add www.jomfp.in/rssfeed.asp as one of the feeds.