OBSERVATIONS ON THE STAINING TECHNIQUES EMPLOYED IN STUDY OF EXFOLIATIVE CYTOLOGY

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After the epoch-making invention of the microscope by Antan van Leeuwenhoek, the foundation of cellular pathology by Rudolph Virchow, the pupil of Johannes Mueller, and of the many distinguished personalities who followed, like Cameron, the invention of a special staining method by G. N. Papanicolaou, spotlighted the importance and utility of exfoliative cytology.

Exfoliative cytology, a new avenue, provided a rapid technique for an early diagnosis, and concentrated attention upon individual cells in search for disease. After Papanicolaou (1917) made his first examination of vaginal smears, reviving the forgotten method of Lataste (1892-93), this science has broadly been applied in two fields: (i) In the diagnosis of malignancy, and (ii) in the study of the physiology and endocrinology of the female sex organs. The cytodiagnosis is now being practised as a routine investigation at many clinics all over the world. Workers in this field have stressed the simplicity and the rapidity of cytological studies besides their utility. Many new facts have emerged from focussing attention upon individual cells, specially from those organs hidden from view and which therefore cannot be studied without operative procedures. Yet in our country these techniques have neither been widely introduced nor practised.

The criticism, common doubts and even the total denial of the method are understandable in the context of the new invention not yet tried extensively, and the disparity of the results obtained by the few workers in this field. A little analysis shows, however, that the principal reasons for all the confusion and unfounded skepticism are the inadequacy of the staining methods employed, lack of uniformity of the techniques used, and the conditions under which such studies were made.

When we decided to introduce Papanicolaou's technique of staining the exfoliated cells in our laboratory, it soon became apparent why these

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procedures have not been very popular. There are a number of difficulties that confront a worker who wishes to introduce and establish these techniques in the clinical laboratories, for example, (i) the considerable quantities of alcohol needed for this purpose, (ii) the non-availability of suitable stains locally, (iii) the unsatisfactory working of the staining solutions prepared in laboratory, etc.

Aim and Object

It was, therefore, the first intention of the authors to try and find out a less expensive and more simplified procedure for the pathologist and the clinician, to help and encourage them to establish this as a routine technique. It had, therefore, to be a method which satisfied two main criteria, viz., easy availability and low cost, so as to be within the reach of an average clinical laboratory.

Materials and Methods

To establish and learn these staining techniques, random smears were collected to begin with and Papanicolaou's technique was tried out. His original technique has been modified by numerous workers in various countries. It became apparent that to the Papanicolaou's technique, which was referred to by various authors, many of them had introduced numerous minor modifications which, according to the author concerned, gave better results.

The technique followed by us is as follows: The preparation of various solutions is appended at the end of the article.

(1)

95% ethyl alcohol	10 dips	1 change
50% ethyl alcohol	10 dips	1 change
Distilled water	10 dips	3 changes
Harris haematoxylin	1½ minutes	
Distilled water	rinse well	3 changes
Alcoholic ammonia	45 seconds	
75% ethyl alcohol	10 dips	2 changes
95% ethyl alcohol	10 dips	2 changes
Orange G-6	3 minutes	
95% ethyl alcohol	1 dip	2 changes
EA 36	3 minutes	
95% ethyl alcohol	2 dips	2 changes
Absolute ethyl alcohol	2 dips	2 changes
Xylol	1-4 hours (until cleared)	2 changes

Mount in neutral Canada balsam.

(2) The slides were also stained by an alternative full Trichome technique of Shorr as described by De Allende and Orias. The procedure in this method is as follows:

1. Fix immediately, avoiding drying, in equal parts of 95 per cent alcohol and ether, for at least 2 minutes; longer fixation is not harmful.
2. Pass through 80 per cent, 70 per cent, 50 per cent alcohol and distilled water, dipping the slide about 10 times in each.
3. Stain 1½ minutes in Harris's hematoxylin.
4. Dip twice in distilled water and 10 times in a weak solution of ammonia (0.5 c.c. in 200 c.c. of distilled water).
5. Stain 1 minute in the Biebrich Scarlet-Orange G. solution. Wash rapidly in distilled water.
6. Leave in the mordanting solution 1 minute. Wash in distilled water.
7. Stain 2 minutes in the Fast Green solution.
8. Without washing, place directly in 1 per cent acetic acid for ½ minute.
9. Wash in distilled water and pass through 50 per cent, 70 per cent, 80 per cent, 95 per cent, absolute alcohol; xylol; 1-4 hours and 2 changes, mount in Canada balsam or an equivalent cement, with a cover slip.

Fixation

The fixative used in both the techniques was a mixture of equal parts of ethyl alcohol and ether. Fixing property of this solution becomes impaired with passage of time as the high volatility of ether makes preservation of original 1 : 1 ratio of ether-alcohol fixative difficult. It is therefore desirable to prepare fresh solution of ether and alcohol before use. Generally, slides were kept in the fixative overnight and stained next morning. This procedure ensures proper fixation and adequate adherence of smears to the slide.

Results

The Papanicolaou's technique gave consistently good results. However, the dyes available locally sometimes did not dissolve fully even in alcohol and thus the actual strength of staining solution was always less than that prepared, after filtering it. Therefore, the time interval for which a smear is immersed in that particular dye solution, in each dye, had to be modified. We believe that each worker has to find out for himself and standardise the suitable time intervals for immersion of smears in different solutions of the stains prepared by him, as the time fixed by any other laboratory will often be found unsuitable. The time schedules mentioned by us gave satisfactory results, with our dyes. The orange 6 dye proved to be a delicate one and was easily washed out of the cells by alcohol. It is therefore advisable to rinse the smear only momentarily in alcohol after the orange 6 stain and before immersing the smear in EA 36.

It became clear soon that considerable quantities of alcohol were needed in the Papanicolaou's technique.

The difficulties and inconveniences in obtaining and storing absolute alcohol, its cost, and the limited quantities that could be made available, led to a search for an alternate method of staining which would give comparable results and yet be less expensive and require less alcohol. Moreover, staining by Papanicolaou's technique with locally available dyes was not as satisfactory as with the imported dyes of American make which could be obtained only with difficulty due to import restrictions, and cost more. Only thus by making it less expensive and complicated for the clinician and the pathologist was it possible to establish cytology as a routine investigation. Therefore, Shorr's trichrome technique, as described by De Allende and Orias was adopted. This procedure needs much less alcohol and all the dyes are locally available. The colour-differentiation of the different cells was good and permitted the fullest estimation of the hormonal influences on the epithelium. Staining of the nucleus was generally better in the Papanicolaou's which was therefore preferable for diagnosis of malignancies, where the nuclear detail is so important. For the endocrinology of the female sex organs, Shorr's full technique is quite adequate. De Allende and Orias, Cheema and Malkani among others have used this technique. The authors believe that a simple but equally efficient staining procedure utilising less costly and more easily attainable materials should have wider use.

Shorr's single stain technique was found inferior for permanent preparations or for research work. It can, however, be used for a routine outpatient laboratory for rapid diagnosis and when smears are not required to be stored permanently.

Summary

Procedures of staining for exfoliative cytology are described in detail. Their comparative utility is discussed. Shorr's full trichrome technique should have wider use for hormonal work as it is much less expensive, and quite satisfactory. Papanicolaou's technique is ideal for all cytological work but is expensive as it requires larger quantities of alcohol.

Appendix I. Preparation of Stains for Papanicolaou's Stains.

I. Orange G-6 (OG-6):

Prepare a 0.5% solution of orange G in 95% distilled ethyl alcohol. Add phosphotungstic acid .015 gm.

We prepare 200 c.c. of this stock solution which when stored in the refrigerator keeps well for 3 months.

From this stock solution 60 c.c. are put into a coplin jar and used for daily staining. The covered coplin jar with the stain is kept in the refrigerator when not in use. It is renewed once a week, i.e. the used stain is discarded and the coplin jar is filled with another 60 c.c. from the stock solution.

II. EA 36:

Prepare 200 c.c. each of the following solutions:

1. A 0.5% solution of Light Green S.F. Yellowish in 95% distilled ethyl alcohol.

2. A 0.5% solution of Bismarck Brown in 95% distilled ethyl alcohol.
3. A 0.5% solution of Eosin Yellowish in 95% distilled ethyl alcohol.

These solutions are kept in the refrigerator. They keep well for about 2 months. From these stock solutions EA 36 for daily use is prepared as follows:

Light Green S.F.	27 c.c.
Eosin Yellowish	27 c.c.
Bismarck Brown	6 c.c.
Acid phosphotungstic	0.12 gm.
Lithium carbonate (saturated aqueous solution)	1 drop

This solution is filtered into a coplin jar. When the stain is not being used the covered coplin jar is kept in the refrigerator; the stain is prepared freshly once a week.

III. Harris Haematoxylin:

Haematoxylin	1 gm.
Ammonium or potassium alum	20 gms.
Distilled water	200 c.c.
Mercuric oxide	0.5 gm.

1 gm. of haematoxylin is dissolved in 10 c.c. of distilled absolute ethyl alcohol. This is mixed with a solution of 20 gms. of alum in 200 c.c. of distilled water. Bring this mixture to boiling then add 0.5 gm. of mercuric oxide. Boil until a dark blue colour appears. Then cool in water bath.

This stock solution is kept in a coloured bottle at room temperature, away from the light. 60 c.c. is put into a coplin jar. This is filtered daily before it is used. Fresh haematoxylin is added every few days to keep the level in the staining jar high. It does not have to be renewed completely more often than once or twice in a year (depending on the number of slides processed).

IV. Alcoholic ammonia is prepared by adding 3 c.c. of ammonia to 97 c.c. of 70% alcohol.

Appendix II. Preparation of Shorr's Stains.

Haematoxylin:

To two parts of the stock solution of Harris's haematoxylin, add one part of saturated solution of ammonia alum (15 grams of ammonia alum, 4 c.c. of glacial acetic acid, 100 c.c. of distilled water). Add an excess of ammonia alum crystals. Filter from time to time as used. Each batch of staining solution will suffice for about 1000 smears. The solution does not deteriorate during use.

2. Biebrich Scarlet—Orange G

Biebrich Scarlet (water soluble)	1 gram
Orange G	0.4 gram
Distilled water	100 c.c.
Glacial acetic acid	1 gram

3. Fast Green

Fast Green	0.75 gram
Distilled water	100 c.c.
Glacial acetic acid	0.75 gram

4. Mordant

Equal parts of a 5 per cent solution of phosphomolybdic acid and a 5 per cent solution of phosphotungstic acid, both in distilled water.

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