Aceto-Orecin Aceto-Cresyl Fast Violet Technique for the Demonstration and Enumeration of Barr Bodies in Buccal Smears.

G.I.N. Ndubuka[1] and K.I. Nkuma-Udah[2]

[1]*Department of Histopathology, Abia State University Teaching Hospital. Aba*
Formerly at Department of Histopathology, Ahmadu Bello University Teaching Hospital. Zaria. Nigeria.
[2]*Department of Biomedical Technology, Federal University of Technology, Owerri, Nigeria*

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The chief staining methods facilitating the microscopic enumeration of Barr bodies in somatic cells have long been standardized in various laboratories. The two most popular techniques are the aceto- orecin and cresyl fast violet methods. A new technique Aceto-orecin Aceto-cresyl fast violet is an attempt to maximize the merit and minimize the demerits of the individual methods in a singly combination of two dyes. There was correlation observed between the three methods, with highest significance established by the new method.

Key words: Buccal cells, Barr body, Staining method, Demonstration, Enumeration.

1. Introduction

Barr body [1], is of the X chromosomes formed from random inactivation and condensation of one of the two female chromosomes in virtually all the somatic cells of female mammals. The inactive X appears as a facultative heterochromatic body existing visible during interphase as dark-staining, peripheral nuclear structure in a somatic cell nucleus of normal female but absent in male tissue [2]. It has normal size of about 1μ with average of 0.7-1.2μ in section of human, is preferentially located at the periphery of the cell nucleus and is considered heteropyknotic X [3]. In the segmented nuclei of granulocytes, it may form a characteristic appendage the so called drum-stick. Barr body (X-chromatin) can be seen well on the nuclear membrane of squamous epithelial cells of the epidermis and buccal mucosal cells as round, oblong, triangular, plano-concave, or flattened body lying adjacent to the nuclear membrane internally [4]. The distribution of Barr body present in an individual cell per se when there is more than one X-chromosome in the chromosomal structure can be understood by the knowledge of Lyon inactivation hypothesis [5]. Also molecular basis of X-inactivation is well explained, and is said to involve a unique gene called Xist [6]. Xist produce none coding of an RNA strand that is retained in the nucleus as it 'coats' the inactive X-chromosome and enhances a gene-silencing process by chromatin modification and DNA methylation. As inactive X is turned off by Xist allele and not shut off as believed earlier, means that up to 21% of genes on Xp, and about 3% on Xq may escape X inactivation.

The investigation of Barr bodies in cell nuclei allows provisional designation of the sex chromosome status of individuals hermophroditism, gonadal, and some complicated sex chromosome anomalies from easily accessible tissues as buccal mucosa, hair root and blood cells, whereas the use of amniotic fluid enables a prenatal sex diagnosis [7].

Identification and evaluation of Barr bodies can be carried out in living cells with the use of phase contrast microscope under favourable condition[2,8], Fluorescence microscopy using fluochrome such as acridine orange or quinacrine is possible [9]. As regards the cytological staining techniques for the demonstration of Barr bodies, the haematoxylin in the routine haematoxylin and eosin (H&E) stain (in fact all of the basic dyes) will stain Barr body, but many have a preference for what they consider to be more selective-stain i.e. Feulgen stain, cresyl fast violet, thionin, Papaincolaou technique, aceto-orecin, or Guard's stain. In principle, all methods

*Corresponding author email: chinmagideon@yahoo.com

which stain the chromatin of the entire cell nucleus and differentiate it from the nucleus are suitable. But methods which stain the chromatin deeply and trace the cytoplasm and the nucleoli are considered superior.

The most reliable histological method is Feulgen and it has been found to be laborious for Barr body. Particularly, well differentiated preparations are obtained by the thionin method [10], which is somewhat easier to perform than Feulgen and almost as reliable although like Feulgen it requires HCl hydrolysis before the actual staining. Owing to their specificity for the chromatin of the cell nucleus, the Feulgen and thionin methods have the advantage that X chromatin cannot be confused with other structures as in buccal smears contaminated with bacteria. In practice however, other stains such as cresyl violet [11], and carbol fuchsin, have proved suitable because they produced stronger contrast and are much simple to manipulate. Staining with aceto-orecin usually yields rather low contrast picture of X chromatin in comparison to the above mentioned stains.

This present study was aimed at divulging another cytological method for the demonstration and enumeration of Barr body in an environment denied of appropriate medical and biomedical technology transfer.

2. Materials and Methods

2.1. Sample Collection and Preparation.

Sample were sort among females in the University students, patients, and Health Workers, totalling one hundred plus two controls. These individuals voluntarily donated their cheeks on conversion. Twenty were in age grade 15-19 and were students; 50 in age group 20-29 were patients consulted at Out-Patient clinic of Medicine and 30 in age group 30-39 were various categories of Health Workers at Ahmadu Bello University Teaching Hospital (ABUTH), Zaria. All the donors were females that have never had child or been pregnant save for the positive control.

Buccal smears were obtained from the inner surface of the cheeks of all donors, the pregnant woman as a positive control, and also from a known father as a negative control. To ensure clean wholesome preparations the mouth was gorged with local gin two times and later rinsed thoroughly with distilled water followed by scrapping of the site with the rounded edge of Ayre's 2 or 3 times. These initial scrapings were discarded as they were charged with micro-organisms and occasional food particles. A fresh Ayre's spatula was next used to collect cells from the cleaned deep epithelial layers. These were spread fairly thinly on three grease-free twin frosts, graphite-labelled slides for each. These were in turn immediately placed in a choice fixative in a special plastic container with individual grooves for up to 10 slides.

2.2 Fixation

The choice of Fixative was 95% ethyl alcohol for wet fixation of cytological smears because it is as effective as, but more importantly cheaper and less hazardous than ether/alcohol mixture which it has supplanted world-wide. Excellent fixation coupled with preservation of cellular morphology was achieved by preventing air drying before placing in this fixative. Nevertheless, the preparation following alcoholic fixation were allowed to be air-dried before staining in order to make the cells adhere more firmly to the slide.

2.3 Staining

Preparation of stains

i. 0.5% cresyl fast violet (Moore and Barr 1955, Moore 1962)

Dissolve 0.5g cresyl fast violet in 100ml of distilled water. The stain is stable. The batch of stain is from Hopkin and Williams Revector microscopic colour index of stain- 959800.

ii. Aceto-orecin (Sanderson 1960)

To 100ml 50% acetic acid, add 2g orecin. Boil for 30 minutes (caution: *a container with a narrow neck is used, glass beads were added and heated slowly under hood)*. Filter before use. Store in a brown bottle and place in the refrigerator. The stain lasts for several weeks. The batch of stain is from BDH microscopic colour index of stain- 6850500.

iii. Aceto orecin/aceto-cresyl fast violet
Solution A
As for aceto-orecin above
Solution B
Aceto-cresyl fast violet
Dissolve 0.25g cresyl fast violet in 50ml 15% acetic acid. The stain is easily dissoluble.

Working Solution.
Combine solution B with warm solution A. Filter off deposit. Add 50ml 15% acetic acid. The stain is ready for immediate use. Label and store all. Stain in a brown bottle in the refrigerator, in which it is stable for a few weeks.

Staining methods.
i. Cresyl fast violet staining procedure by Moore 1962 was adopted.
ii. Aceto-orecin staining procedure by Sanderson 1960 was also adopted.
iii. Aceto-orecin/Aceto-cresyl fast violet method.
a) Immerse the preparation in aceto-orecin/aceto-cresyl fast violet for 5 minutes.
b) Differentiate in 95% alcohol- 5 dips.
c) Dehydrate in alcohol, clear in xylene and mount cover-slip in DPX mountant.

3. Results

The Barr bodies are present at the nuclear membrane only, and are approximately 1um in diameter and typically crescentric in shape. Barr body shapes may be round, oblong triangular, plano-concave or flattened but should be adjacent to the nuclear membrane internally. Cells selected and counted are those whose nuclear membranes are intact, without crenation or depression and the chromatins are without clump. Bacteria and other contamination can usually be readily distinguished from X-chromatin by focusing on different plane of microscope.

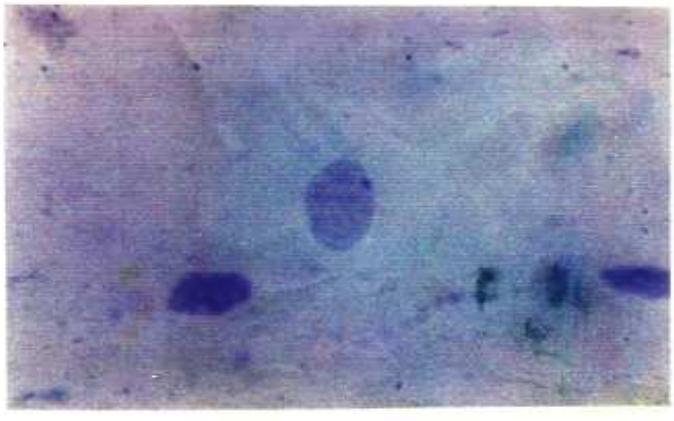

Figure 1: Barr Bodies stained with Aceto-orecin aceto-cresyl fast violet method

According to Poulding, additional criteria of cells selected for sex chromatin count should fulfil are, large, lightly-stained nucleus; no folding of the nuclear membrane, and nucleus not obscured by overlapping cells.

The oil immersion objective was used to examine the cell nuclei of the stained preparation. By applying the above principle, 200 cells from three preparations per donor were meticulously counted and scored. Those with Barr bodies were therefore expressed as a percentage of the total.

Example of calculation is thus:
Nuclei with X-chromatin = 80
Nuclei without X-chromatin = 120
Total nuclei counted = 200
Therefore, percentage of Barr body present
= 80/200 x100 = 40%.

Cresyl fast violet method:
Sex-chromatin stains – deeply violet
Cell cytoplasm stains – lightly violet
Micro-organisms – deeply violet
Barr body counted (EM) = 25%, (Kf) = 0%.

Aceto-orecin method:
Barr body stain – light brown
Cytoplasm stains – lightly stained
Micro-organism – lightly stained
Barr body count (EM) = 33%, (Kf) = 0%

Aceto-orecin/Aceto-cresyl fast violet method
Barr body – bluish to violet
Cell cytoplasm – extremely light to colourless
Micro-organisms – extremely light to colourless
Barr body count (EM) = 40%; (Kf) = 0%.
where EM = expectant mother; and Kf = known father.

4. Discussion

Fixing of fresh smears is usually aimed at discouraging loss of constituent and prevention of processes which would temper with geometrical forms of cell-to-cell border during the subsequent stages in staining procedure. More importantly a time, alcoholic fixations convert soluble cell components into derivatives which are insoluble and provides resistant to post mortem changes by autolysis apart from marauding bacteria and fungi[12]. Alcohol (95%) was the fixative of choice to achieve far reaching effects as in cytology and cytogenetics smear techniques. Since retention of substance is one of the necessary conditions required for histochemical demonstration; and Barr body a nuclear protein, the choice of 95% alcohol was to poorly checkmate the changes of biochemical reactivities of smeared cells. Therefore, the modification of nuclear enzyme active sites was considered excellently preserved because alcohol is a poor retainer and destroyer of whatever enzyme activity on any sample [13] unlike formalin. Consequently, nuclear thiols that are less modified have the unopened reactive sites exposed to staining reagents. It is the staining reagent molecules that convert the sulphur-rich protein (DNA and RNA), some of the thiol and disulphide groups by oxidation to anionic ions; hence the basophilic staining of the Barr body intensely and other nuclear proteins extremely light even colourless [14]. This staining complexity has always been known to affect staining time of alcohol fixed specimens [15]. When alcoholic fixative is considered alongside staining rate in our study, the mixture of dyes of different sizes [16], the different diffusions play a remarkable role than the fixative.

For Barr body to be intensely stained bluish violet in comparism to extremely light to colourless colour obtained of cell cytoplasm and micro-organisms is a factor of poor modification of large groups, and the resultant selectivity indicates incomplete blockade of amino groups due to perhaps slow diffusion rate of reagent into cells which may conversely give fast rate of reagent loss. Implicatively, the intensity apparent on Barr body is dependent upon reagent uptake and formation of covalent bond such that overall charge carried by the Barr body was altered radically to favour intense staining. This is observed in sections staining particular in the diagnosis of Madelung's disease[17].

The preparation of cresyl fast violet for Nissl granules in neuropathology and Barr body in cytogentics staining methods is in an aqueous media. This is because various dye interactions contribute to affinity when hydrogen bond clusters are broken by dye reagent, thereby enhancing interaction between dye molecules to form aggregates. The intention may also achieve the maintenance of hydrophobic bonding important for RNA and highly organised flattened fenestrated intercommunicating Nissl body [18] and same also to condensed chromatin Barr body. These provide for dye-dye interaction and reagent-tissue interaction at the reactive sites of smeared cells or section without selectivity. The acidification of aceto-orecin/aceto-cresyl fast violet reduces possibly the degree of dissolution of the two dyes as a new component is formed. The coarse granules were filtered off thereby expounding the interactions between dye-dye and reagent-tissue. The van der Waals' forces were increased as well as electrostatic bonds (Coulombic attractions) restored by fine staining molecules in the solution. During staining therefore, DNA or RNA interact appropriately with the negatively charged new dye. Also it must be remembered that Nissl granules with coarse membrane arrangement is as condensed Barr body, and will trap aceto-orecin/aceto-cresyl fast violet molecules and still allow permeable charged molecule to contribute in the intense staining. Hence acidification could possibly have helped in the formation of the new dye as well as enhance the rate of staining and staining property of the new dye.

Again for a staining procedure to be adopted routinely requires that mode of preparation is devoid of laboriousness and harm. Aceto-orecin/aceto-cresyl fast violet is more laborious to prepare when compared with aceto-orecin and twice so when cresyl violet is considered for same purpose. Truly, one has to contend with the pungent, irritant and choking vapour of aceto-orecin which stands as solution A

for aceto-orecin/aceto cresyl fast violet. But since there is no dangerous fume emanate from the mixture, it is what while staining preparation procedure. After all, Laboratory chemical hazards are observed with fumeless or odour producing chemical.

The staining time of dyes is dependent upon several factors, which include reagent-tissue affinities and the number of binding sites. Relatively, these are considered on high or low affinity of specimen to dyes. With alcohol fixed smear, affinity of cells and constituents to dye molecules is not absolutely dependent on charges as selective staining is achieved. This is because selective staining can still be possible even if dye-tissue affinities and the number of dye-binding sites of two structures are the same. Hence, rates of reagent uptake or rate of reaction or loss of staining molecules or the formation of new product might not be the same in any given two structures as in a smeared cell. We achieved selective staining by using a short period of 5 minutes as against 10 minutes from 0.5g aqueous solution of cresyl fast violet and 30 minutes from 2% acidified orecin. Considering that methods which use three or more acid dyes are affected by differing diffusion rates and, we exploit the wide variation in staining rates of the various acidophilic structures as advised by Richard W. Horobin[15]. Even at the short time, over staining was experienced and was remedied by differentiation in 95% alcohol 5 dips in a regressive staining. Over staining must have occurred from rapid penetration or clustered uncharged molecules. The rate control of reagent loss is usually critical even in practical staining methods particularly with tissue sections. Factors capable of affecting rate of reagent loss like thickness of smear, temperature of both reagent and smear, and stirring of the reagent solution, can alter the staining procedure[16]. Therefore, aceto-orecin/aceto-cresyl fast violet stained sex -chromatin a pleasant bluish violet while cytoplasm, micro-organisms, and debris remained either extremely light to colourless after differentiation (figure). The overall picture in this case favoured the conspicuousness and enumeration of Barr bodies.

The counts speak themselves. In descending order of merit, aceto-orecin/aceto-cresyl fast violet yielded 40%, against 33% harvested by aceto-orecin, and 25% gleaned from cresyl fast violet. This result was considered accurate because of the age range and number of Donors, controls, samples specificity, and reproducibility of staining reaction of aceto-orecin/aceto-cresyl fast violet.

In conclusion, Barr body count is a critical exercise in exfoliatve cytology. Indeed it is critical in the sense that upon its' result depends the verdict of male or female on patients with doubtful sex- especially in our environment which lack confirmatory chromosome studies. It follow from reason therefore that this new Barr body stain, aceto-orecin/aceto–cresyl fast violet, is better than the two usual routine stains; aceto-orecin and cresyl fast violet, because it is more reliable and gives a highest count. Seen in that light, aceto-orecin/aceto cresyl fast violet is suggested as a routine replacement for both aceto-orecin and cresyl fast violet stains for demonstration and enumeration of Barr body in exfoliative cytology.

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