

4.0 DISCUSSION

4.1 Introduction to discussion

The diagnosis and monitoring of many diseases, infections, inflammation and neoplasms is facilitated by cytopathology.^[1] In neoplasia, cells are studied especially to identify primary, recurrent and metastatic malignant neoplasms and their precursors.^[1] A cytologic diagnosis has many benefits.^[2] Fine needle aspirations (FNA) is safe, easy to perform, minimally invasive, inexpensive, and well tolerated by patients. It remains an excellent diagnostic procedure for preliminary diagnosis.^[2, 21]

One of the constraints of the conventional FNA smear is the limited material available for adjuvant diagnostic investigations including immunocytochemistry.^[3-13] The cell block technique employs the retrieval of small tissue fragments from a FNA specimen which are processed to form a paraffin block. Several articles in the literature have determined that this method of analysis increases the cellular yield and improves diagnostic accuracy.^[3-13]

The ability to obtain numerous tissue sections allows for multiple immunostains and other studies to be performed akin to paraffin sections produced in histopathology.^[3]

The aim of this study was to compare the cytomorphology and immunocytochemistry on paired FNA smears and cell block specimens with a view to establishing the cell block technique as a diagnostic procedure in the cytology laboratory.

4.2 Cellularity and needle rinse versus dedicated aspiration

Kim et.al., (2008) ^[58] have stated that the success of obtaining an adequate FNA sample depends on the skill of the aspirator, lesional characteristics, the number of aspirated samples and the accuracy of lesion and needle localization. ^[16, 17] Inadequate FNA smears are those that cannot be interpreted for a variety of reasons; namely, sparse cellularity, thickly smeared areas with poor cell distribution on the slide and poor fixation. ^[3]

Material for cell block samples can be obtained by rinsing needles in cell block fixative solution after preparing the FNA smear slides or by obtaining an aspiration dedicated for the cell block sample in addition to the needle rinses. In our setting, FNA's are performed by radiology interns, medical officers, pathologists, cytotechnologists and nurses whose experience and skill varies. Consequently, material for cell block was usually aspirated after at least 3 to 4 aspirations for the conventional direct FNA smear, which was given priority. This may have contributed to a more traumatized and consequently less well preserved specimen. In many cases (20/50), the syringe containing residual FNA sample was rinsed into cell block fixative solution and a dedicated pass was not performed. In these cases, 65% (13/20) had suboptimal cellularity (score 0 and 1). In 30/50 cell block samples, a dedicated needle aspiration was placed directly into the cell block vial containing Shandon's Formal-Fixx™ fixative and 60% (18/30) showed adequate cellularity (score 2 and 3). Eight per cent (4/50) of the cell block samples (obtained by either dedicated aspiration or needle rinse) were acellular but none (0/50) of the FNA samples (obtained by dedicated needle aspiration only) scored zero for cellularity. Of these samples, 4% (2/50) were obtained by rinsing the needle and the hub

of the syringe and 4% were obtained by performing one dedicated aspiration for the cell block sample. Comparison of cell block samples that was obtained by dedicated needle aspiration only illustrated the benefit of a dedicated needle aspiration. Although, there were no FNA samples (0/30) that scored zero for cellularity only 3.3% (1/30) of cell block samples (with a dedicated needle pass) was acellular. This demonstrates that a dedicated needle aspiration for cell block improves cell yield. Mayall et al ^[16] have found that the success of producing cell blocks of adequate cellularity depended on the high cellularity of the aspirate and a dedicated needle aspiration. In the setting of this study, a dedicated needle pass in addition to needle rinses is recommended. Indeed, both the cellularity and morphology is expected to improve if not one but several dedicated needle passes are performed for cell block purposes.

Kaur and Sivakumar (2007) ^[56] found that the proportion of unsatisfactory aspirates (from breast, thyroid, lymph node, salivary glands and soft tissue lumps) by pathologists which was determined by inadequate cellularity for interpretation was much lower compared to medical officers. As a result, they advocated that medical officers be offered hands-on training in aspiration techniques, use of the cytospin smears and cell block in bloody thyroid aspirates. In this study, 54% (27/50)) of the samples for cell block preparation and direct FNA smears were collected by pathologists (registrars and consultants), 34% (17/50) by radiology registrars, 8% (4/50) by medical officers and 4% (2/50) by nurses. Table 3.8.1 refers. However, results from this study indicate that the proportion of cell block samples with inadequate cellularity (grading score = 0; n = 4/50), were 11% (n = 3/27) for pathologists (registrars and consultants) and the same for both

medical officers and radiology registrars (11%, $n = 1/9$). None of the sample collected by the nurses ($n = 0/2$) were inadequate. The proportion of suitable samples comprising higher cellularity (grading score of 2) were highest among the nurses (50%, $n = 1/2$), followed by the radiology registrars, (35%, $n = 6/17$), the pathologists (30%, $n = 8/27$) and the medical officers (22%, $n = 2/9$). The proportion of cell block samples with the highest cellularity grading score of 3 were as follows: 50% ($n = 1/2$) by nurses, 22% ($n=2/9$) by medical officers, 19% ($n = 5/27$) by pathologist, 17% ($n = 2/12$) by radiology registrars. To maintain proficiency, personnel procuring specimens should be properly trained on how to perform an FNA. This training should be standardised as far as possible. This is particularly relevant regarding radiology registrars, some of which receive minimal training, if any, on how to perform an FNA and collection of cell block samples. It is also recommended that ongoing competency assessment of all personnel performing FNA is instituted, together with re-training if indicated.

4.3 Cellular preservation

The preservation of cell morphology refers to the presence of crisp, well-preserved, clear nuclear chromatin, nuclear margin, cytoplasm contents and cytoplasmic membrane.^[51]

The deterioration in cell morphology and loss of cell detail is due to cell distortion and shrinkage caused by inadequate fixation.^[34] As a result, cellular degeneration makes the interpretation of FNA smears difficult.^[51] In this study, morphology was well preserved in 100% (50/50) of FNA samples and in 88% (44/50) of cell block samples. When the assessment of morphology was compared in the cell block samples that had a dedicated

pass (n=30) with that of FNA samples; all FNA samples (100%) displayed the presence of morphological preservation (score 1+) compared to 90% (27/30) cell block samples.

The cellular degeneration suffered by the cell block samples could be explained by the work of Kingsbury et al. (1995).^[17] This team stated that prolonged tissue hypoxia reduced pH in tissues which resulted in a lower yield of quantifiable nucleic acids. In relation to the current study, the delay in fixation of the cell block samples could have led to cellular hypoxia which resulted in degeneration. According to the Shandon Package Insert for Cytoblock Cell Block Preparation System, “cell degeneration could appear to look like inadequate fixation”.^[18]

Due to the limitations discussed, sub-optimal preservation of cellularity (23/50), morphology (41/50) and architecture (28/50) in cell blocks was observed early on in the study in many of the samples. Nevertheless, it was decided to continue with the use of Formal-Fixx fixative since the remainder of the samples (27/50, 9/50 and 22/50 respectively) displayed optimal preservation, illustrated by higher grading scores. Hanley et al., (2009)^[57] have stated that the preservation of antigenicity of tumour cells is essential for accurate immunocytochemical analyses and the use of an ideal fixative and optimal tissue processing parameters is crucial in this regard. Since optimal preservation was observed in the remainder of the cell block samples, the fixative and the tissue processing schedule used was deemed suitable. The sub-optimal preservation and discrepant aberrant immunocytochemical staining observed could have been due to the pre-fixation time lag.

Thapar and colleagues (2009) ^[32] have achieved better preservation of architectural patterns in their cell block samples compared to the FNA effusion smears using a different method of cell block preparation: fixation for 1 hour in 1:1 solution of 10% alcohol:formalin followed by routine paraffin section tissue processing. In contrast, using the Shandon Cytoblock Kit only 44% (22/50) of cell block samples displayed the preservation of architectural patterns. This could have been due to the delayed fixation or the chemical action of the reagents in the Shandon Cytoblock kit that cause the cells to clump together, thereby forming a gel matrix. Nevertheless, an exhaustive survey of the literature has not revealed any contradictory information on the efficacy and versatility of the Shandon Cytoblock kit. ^[25-32] Therefore, optimisation of the fixation procedure is required in this cytology unit in this regard.

4.4 Logistical issues

Inference drawn from Bardi & Schwartz, 2007 ^[58] indicates that the mindset of both patients and aspirators is of equal importance. Some patients did not consent to an extra needle aspiration for the cell block sample while many aspirators were reluctant to perform additional needle aspirations despite patients' informed consent. This was due either to the patient not being able to withstand the procedure, risk of causing a pneumothorax in patients undergoing lung FNAs, lack of amenability of the mass to FNA, time constraints, inexperience or merely not being willing to do so. Although not statistically evident, samples collected for the cell block technique may have been disadvantaged by not receiving a dedicated aspiration. ^[16] All of the above could have

contributed to the poor cellularity and architectural preservation of cell blocks ^[16] and a poor agreement between the two methods of sample preparation. Mayall et al ^[16] reported that the success of obtaining cell blocks with a high adequacy rate was attributed to being able to perform a re-aspiration (dedicated aspiration) for the sole purpose of collecting material for a cell block, rather than dividing the first aspirate in two. Patients and staff procuring FNAs need to “buy in” to the concept that the cell block is a valuable technique that may contribute significantly to an accurate diagnosis. Education and motivation is required in this regard.

4.5 Immunostaining

The results obtained for the immunostains of the cell block samples were comparable to other studies ^[59] with respect to fewer aberrant staining results and much less background staining. Non specific aberrant staining was observed in the FNA negative control of the CK7, CK20, TTF1, synaptophysin and Hepar-1 immuno stains. This phenomenon was not displayed in the respective cell block negative controls and in the paired AE 1/3 immunostains. A possible explanation for this could be that, “not all antigens are susceptible to anoxic degradation or diffusion just as not all antigens are equally affected by fixation”. ^[37] The FNA samples most affected by background and anomalous staining were some liver FNA samples, samples containing preproteinaceous debris and predominantly necrotic or very thickly smeared samples ^[19] indicating that the problem was intrinsic.³⁷ Kung et al. (1990) ^[59] made a similar observation with regard to stronger staining intensity, lack of non specific background staining and lack of aberrant staining in cell block samples especially with cytokeratin stains.

A poor agreement (K-statistic 0.22) between methods was obtained for the CK7 immuno-stain. However this was the only test that displayed a statistically significant difference (p-value 0.02) between methods. A statistically significant difference was not obtained in immunocytochemical staining between the two methods of sample preparation for the following tests: CK20, TTF-1, Synaptophysin, Hep1 and AE1/3. This could be due to the small sample size of the respective tests: AE1/3, n =7, Hep-1, n =8, synaptophysin, n =10 and TTF1, n = 18. The asymmetry obtained was a random event. Although an adequate sample size (n =44) was obtained for CK20, the asymmetry (K-statistic 0.20) lacked statistical significance (p-value 0.14).

Discrepant results were obtained for one sample, a FNA from the liver.

Immunocytochemistry was performed on the FNA smear and tumour cells were positive for CK7 and synaptophysin while CK20 was negative. This cytokeratin profile is observed in 56% of neuroendocrine carcinomas of the lung.^[38] All of the ICC tests performed on the cell block sample were negative. Although all tests were repeated the results remained unchanged. A possible explanation could be the sub-optimal preservation of antigenicity of tumour cells in this sample which was somehow more susceptible than the others.^[37] This could have been due to destaining the cytology slide which results in high levels of background staining and loss of conventionally stained cytological material.^[16]

Sidham et al ^[6] have reported that FNA smears stained with haematoxylin-eosin or the Papanicolaou method displayed superior morphology compared to immunostaining. However, destained FNA smears did not display good immunostaining.^[6] In this study, the poor agreement in CK7 immunostain between FNA smears and cell block samples could be due to false negativity of cell block samples due to lower cellularity or false positivity of FNA samples.^[60] The former seems very likely due to logistical issues of this study, while the latter seems unlikely yet possible, although many measures were taken to preclude endogenous enzyme activity and the risk of false-positive results when performing ICC on tissue containing the presence of peroxidase (endogenous biotin) as in FNA samples.^[60] The use of a peroxidase – blocking step and the Envision detection system (Dako, cat.# 5007) was incorporated into the staining procedure to circumvent false positivity and non-specific staining. This system is polymer based and does not depend on the affinity of the glycoprotein avidin for biotin, a low molecular weight vitamin.^[35] As a result unwanted background staining is reduced especially in biotin-rich liver tissue.^[35] The heavily stained background in many of the FNA smears is due to the disruption of cytoplasmic contents resulting in the release of cytoplasmic antigens.^[3] When immunostains are performed on these FNA smears a high background is observed, which is in fact a reflection of “true” staining of disrupted cytoplasmic contents rather than background staining in the true sense.^[3] The dissimilarity in immunostaining between the two methods of sample preparation is further displayed in Figure 3.5.3.2 where the poor agreement is illustrated in the distribution of the 44 samples across the various ICC grading score categories. Despite this trend, a huge benefit of performing ICC on cell block samples was further illustrated by the lack of severe background

staining (score 3) in all cell block samples (0/44) in comparison to 14% (6/44) of FNA samples in CK7 (Table 3.6.1).

A similar result was obtained when this comparison was performed between cell block samples that obtained a dedicated needle aspiration with that of FNA samples (n = 28) (K = 0.27; p = 0.12). The bias towards samples prepared conventionally (FNA smears) was illustrated (Figure 3.5.3.3) by a dominant deviation of FNA samples from the diagonal in comparison to the cell block samples. Only 18% (5/28) FNA samples had absent / negative staining (score 0) compared to 39% (11/28) cell block samples. More total FNA samples (57%) (16/28) had higher CK7 ICC grading scores (scores 5 and 6) than cell block samples (25%) (7/28). This bias is further displayed in Figure 3.5.3.4 where the dissimilarity is illustrated in the distribution of the 28 samples across the various ICC grading score categories for CK7.

Miller et al ^[3] reported that a potential pitfall present on immunostained cytological smears leading to erroneous staining is the trapping of reagents in the three-dimensional cell clusters. In FNA smears the cytoplasm covers the nucleus, therefore a cytoplasmic stain will in some instances show nuclear staining in addition to cytoplasmic staining. Interpretation of these smears should be confined to areas where the cells are present in monolayers in order to render definitive results. ^[3] This phenomenon was not observed in the cell block samples since they are two- dimensional. On sectioning the cell blocks the cytoplasm does not cover the nucleus hence the lack of nuclear staining. This facilitates ease of interpretation of immunostains on cell block samples. ^[3]

However, due to the delay in fixation of cell block samples in this study, the results for the preservation of cytomorphology in cell block samples were not comparable to similar studies undertaken.^[25-32] Consequently in our setting, both smears and cell blocks are needed, the former to assess morphology, and the latter for optimal immunocytochemistry results.

4.6 Standardisation and Quality Assurance of Immunostains

More recently, Colasacco et al. (2010),^[24] advocated that more stringent documentation of procedure and use of the relevant controls in immunocytochemistry of cytopathologic studies would ensure more valid and reproducible results. Although the College of American Pathologists realises that it is not always a pragmatic approach to maintain separate positive control samples for every possible combination of fixation, processing and specimen type;^[24] the use of cell block samples for immunohistochemistry instead of FNA samples would be easier to implement to ensure compliance with this standard. In our setting, the positive controls for both split-slide ICC and the cell block IHC comprised of routinely processed formalin fixed paraffin embedded (FFPE) tissue. Both cell block and positive tissue control samples should be processed identically to ensure accuracy of procedure and ultimately the diagnosis. An ad-hoc committee on Immunohistochemistry Standardisation, affiliated with the College of American Pathologists have discouraged the use of non-formalin based fixatives and or alternative fixation methodologies.⁶¹ This is due to the performance data of the IHC assay using other fixatives being limited and extrapolation from existing data being unreliable.^[61]

Cell blocks from FNA of liver which were fixed in 10% neutral buffered formalin (NBF) were successfully produced by Axe et al (1986)⁶² with optimal cellularity, preservation and successful IHC enabling sub-classification of the tumours studied.

4.7 Other techniques

Many other methods (agar, plasma, thromboplastin)^[4, 13, 14, 15, 16] to prepare cell blocks have been utilised successfully by other teams which is also aimed at recovering sparse material obtained during the FNA procedure. The use of the Shandon Cytoblock Cell Block Preparation System proved beneficial in previous studies undertaken in this department and a review of the literature did not reveal any negative views on its use.^[25, 27, 28, 29, 30, 31] Consequently it was utilized in this study since it was possible to capture small groups of cells which would have otherwise been impossible to achieve with routine tissue processing. An improved technique of cell block preparation and cell capture has been devised by Varsegi and Shidham (2009).^[5] It may be beneficial to incorporate this method into the current method. Using the Varsegi technique increases the chance of capturing individually scattered cells with the use of Histogel (Thermo Shandon), thus preventing the histotechnologist from cutting too deeply into the block and risk missing the area with the cells of interest. Although not statistically evident, samples collected for the cell block technique may have been disadvantaged by not receiving an initial dedicated aspiration, which may have compromised the cellularity. In this regard, obtaining material from dedicated needle aspirations for cell block should be explored to improve cellular yield. A novel technique by Dagg et al. (1992)^[25] was successfully performed utilizing the Shandon Cytoblock Kit in a modified manner for cell

block preparation. A similar technique was personally observed at the John's Hopkins Medical Institute, Department of Cytopathology, Baltimore, USA during training attended in July-August 2009. Using 10% unbuffered formalin as the collection medium for cervical cells and 10 minutes of centrifugation at 3000rpm, the pellet was resuspended by vortexing in 4 drops of Reagent 2. The use of Reagent 1 was omitted. The sample was then centrifuged and the embedding cassette containing the cell disc was prepared according to the manufacturer's instruction. Thereafter, the samples were processed as for routine histology. On embedding, the cell disc was folded in half to obtain maximum thickness for microtomy. Although this procedure was not tested in the current study, it remains a possibility in the future.

Since sufficiently cellular cell block samples may not always be possible, another alternative technique in the preparation of cell blocks could be employed. ^[63] Nga et al. (2005) ^[63] proposed the use of a "cytoscape" technique in which cell blocks were created from previously stained Papanicolaou smears after thorough photographic documentation. They found that with this new technique the difficulties associated with non-diagnostic cell block samples were circumvented. Considering the limiting factors encountered in the current investigation, one of the major advantages of exploring this technique would be that an additional needle pass for the cell block sample would not be necessary thus obviating the associated problems.

Future research in this regard would include the use of 10% neutral buffered formalin (NBF) as the fixative of choice in preparing cell block samples for

immunohistochemistry (IHC) with a decrease in the time lapse between sample collection and fixation.

Table 4.1: Comparison of cell block preparation methods

Name of technique	Method of action (very brief 1-2 lines)	Advantages	Disadvantages	Comments
Plasma ^[13] Thromoplastin / thrombin-based [4, 14]	Small cellular fragments are captured by the clotting action of the plasma.	Good cellularity obtained.	Success depends on high cellularity of the aspirate.	-
Rapid cell block technique ^[15]	Automated system. Deposits needle rinse at one plane.	Higher yield of tissue fragments obtained.	Automated system. Expensive. Success depends on high cellularity of the aspirate.	Cost effectiveness must be assessed before routine implementation.
Agar ^[16]	Molten agar is used to capture small cellular fragments after hardening at 4°C.	Good cellularity obtained.	Success depends on high cellularity of the aspirate.	-
Shandon Cytoblock system ^[25, 27, 28, 29, 30, 31]	A cell button is formed by gelling action of Reagent 1 and 2 available in the kit. This results in the capture of small cellular fragments.	Cost effective for our setting. Previously utilized successfully in our department.	Success depends on high cellularity of the aspirate.	Available in kit form thereby providing convenience, consistency and reliability of reagents and consumables used.

Table 4.2: Advantages and disadvantages of cytology preparation methods

Direct Smear (FNA sample)	Cell Block Sample
Samples with low cellularity can be used.	High cellularity samples needed.
Limited material (slides) available.	Paraffin wax block produced enable multiple serial tissue sections.
<u>Immunocytochemistry:</u> Antibody panels unlikely (Split slide ICC: Same cells may not be present on all sections of slide) Background staining severe. Prior staining may affect results. Controls and test samples must be prepared identically, not easily procured.	<u>Immunohistochemistry:</u> Antibody panels possible (Same area of interest can be followed on sample due to multiple serial sections). Less / no background staining. No prior staining done. Identically prepared positive tissue controls can be easily obtained.

5.0 Conclusion and recommendation

In this study, the Papanicolaou stained FNA smear was the better method for routine diagnoses due to superior preservation of nuclear and cytoplasmic characteristics ^[64] whilst the cell block technique was more suited to immunocytochemical analyses. Our findings would suggest that the cell block samples are best used as an adjunct for ICC and not for primary cytological diagnoses. The degeneration of cells in the cell block samples may be attributed to a delay in immersing the cell block specimen into fixative immediately after collection, and variation in FNA technique amongst personnel. This variation in technique may have contributed to either the success or failure of obtaining adequate cell block samples which is largely dependant on the skill of the aspirator and cellularity of the aspirate.^[16,17] Therefore, material should be placed in cell block sample fluid as soon as possible.

The work performed in this study was undertaken in a similar manner to proposed methodologies ^[26-32] but due to the limitations and logistical issues discussed above led to different outcomes. Consequently, corrective and preventative measures in vulnerable areas, such as FNA technique and cell block sampling, must be implemented in order to overcome these variations to successfully obtain cell block samples of diagnostic value.

The role of cell block preparation in diagnostic cytopathology is without doubt of immense significance as it allows for multiple special investigations and consequently a more refined cytological diagnosis. The methodology used could be improved on by

modifying techniques and correcting the limitations posed in this study viz. exploring the use of 10% neutral buffered formalin (NBF) as the fixative of choice in preparing cell block samples for immunohistochemistry (IHC), with a decrease in the time lapse between sample collection, fixation and standardization of FNA technique amongst personnel. Direct FNA smears and cell blocks complement each other,^[22] and our results indicate that both are needed in the diagnostic work-up of patients; the former to assess morphology, and the latter for optimal immunocytochemistry results. In resource constrained settings, the cost implications of performing both conventional and paraffin blocked smears on all FNA material warrants further evaluation. This study has been of immense benefit to this cytology unit and will prove valuable to other laboratories who are considering implementation of cell block technique. This study has demonstrated that it is not possible to obtain a good cell block by using previously published articles and the manufacturers' instructions alone. All aspects of the FNA process including proper specimen collection, fixation, cell block preparation procedures and immunochemistry techniques need to be assessed, continually monitored and improved if necessary to ensure good results.