Open Access Maced J Med Sci **electronic publication ahead of print,**
published on September 13, 2019 as https://doi.org/10.3889/oamjms.2019.452

ID Design Press, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences.
https://doi.org/10.3889/oamjms.2019.452
eISSN: 1857-9655
Basic Science

Comparison of Fixation Methods for Preservation Cytology Specimens of Cell Block Preparation Using 10% Neutral Buffer Formalin and 96% Alcohol Fixation in E-cadherin and Ki-67 Immunohistochemical Examination

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Citation: Ireka Y, Agustina H, Aziz A, Hernowo BS, Suryanti S. Comparison of Fixation Methods for Preservation Cytology Specimens of Cell Block Preparation Using 10% Neutral Buffer Formalin and 96% Alcohol Fixation in E-cadherin and Ki-67 Immunohistochemical Examination. Open Access Maced J Med Sci. https://doi.org/10.3889/oamjms.2019.452

Keywords: Immunohistochemistry; Cell blocks; 96% alcohol; 10% NBF

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Received: 17-May-2019; **Revised:** 19-Aug-2019; **Accepted:** 20-Aug-2019; **Online first:** 13-Sep-2019

Funding: This research was funded by an internal grant for Padjadjaran University batch 2 (4851/UN6.C/LT/2018)

Competing Interests: The authors have declared that no competing interests exist

Abstract

BACKGROUND: Cytological and molecular examinations are among the most important examinations in cancer diagnosis. 96% alcohol is a fixative solution commonly used by clinicians for cytological samples because of its accessibility and affordability. Cellblock preparation from cytology specimen may increase morphology detail and may be used for further biomarker analysis. E-cadherin is an adhesion protein expressed in the cell membrane of most carcinoma. Ki67 is a protein expressed in nuclei of malignant cells that used as a proliferation marker.

AIM: This study was designed to investigate the effect of fixation duration in 96% alcohol on protein preservation for immunohistochemistry (IHC) evaluation compared to 10% neutral buffered formalin (NBF) as the gold standard.

METHODS: Twenty-five fine-needle aspiration biopsy (FNAB) specimen diagnosed as carcinoma were fixed in 10% NBF and 96% alcohol for 1 hour, 6 hours, 24 hours, 48 hours and 72 hours. Cell blocks preparation were made from those 6 groups of specimens. E-cadherin and Ki67 IHC were done to cell blocks section and evaluated. The data were statistically analysed using the Friedman test with p-value < 0.05 of a significant level.

RESULTS: There were significant differences between E-cadherin and Ki67 expression in cell block preparation from 96% alcohol-fixed cytology specimen for 1 hour, 6 hours, 24 hours, 48 hours and 72 hours to 10% NBF (p = 0.0001).

CONCLUSION: The result indicated that 96% alcohol is not suitable as a fixative solution for cell block preparation in E-cadherin and Ki-67 IHC examination.

Introduction

Cytological and molecular examinations are among the most important examinations in cancer diagnosis. Recently, cytology testing is becoming more frequent as the less invasive sampling technique develops. With the development of personalised medicine in the treatment of cancer, a molecular examination is a very important examination. The cell block (CB) offers many advantages, over other cytological preparations, particularly for diagnostic and immunohistochemical testing. One of the important points to making a good cell block is a fixation. Ten percent NBF is universal fixative for optimal preservation of cellularity, cytomorphology, and architecture in the cell block. It also provides optimal fixation for FNAB material for cell block sample [1]. 10% NBF as a gold standard. However, there are also disadvantages to using NBF as a fixative, including the handling of formalin, since formaldehyde is considered a carcinogen and crosslinking agent [2].

The most commonly used fixatives for diagnostic pathology and cytologic specimens are 10% NBF and 95% ethanol [3]. In our institution, 96% alcohol used by clinicians as a fixative for cytology specimens because of its accessibility and affordability. To our knowledge, no study has examined the effect of 96% alcohol fixative agent in cell block preparations from FNAB sample. So, the

author wants to know the duration of 96% alcohol fixation that will greatly affect the preservation of protein molecules.

In this study, to see the effect of fixation, we were using IHC to analyse how the preservation of protein molecules. To analyse the preservation of antigens in cell block preparations, IHC was carried out using E Cadherin, for proteins located in the cell membranes, and expressed in most carcinomas [4]. Other IHC examination was Ki67 as a marker of proliferation in tumour cells and expressed in proteins located at nuclei and generally associated with tumour cell proliferation and malignant potential of the tumour [5].

Our study assessed alcohol 96% with fixation time: 1 h, 6 h, 24 h, 48 h and 72 h interacted with tumour cell block, which might cause protein denaturation. Based on experience, where cytology samples obtained from clinicians, 96% alcohol had been fixed within a few hours, due to a late and delayed transportation or laboratory sample process from late Friday surgery during the weekend, and the waiting time for a long holiday weekend. Yamashita-Kashima *et al.* discovered that the time to and length of fixation of tumour specimens could affect HER2 IHC and fluorescence in situ hybridisation (FISH) scores [6].

This study was designed to investigate the effect of fixation duration in 96% alcohol on protein preservation for immunohistochemistry (IHC) evaluation compared to 10% neutral buffered formalin (NBF) as the gold standard.

Material and Methods

This research has ethical clearance from the Health Research Ethics Committee Padjadjaran University with number 1150/UN6.KEP/EC/2018.

Cytologic Specimens and Cell Block Preparation

In total, we used 25 fresh surgical specimens; all tumours are carcinoma (ovarian carcinoma, invasive breast carcinoma of no special type (NST), Papillary thyroid carcinoma). FNAB was performed in a tightly controlled manner in the surgical pathology gross room at Dr Hasan Sadikin Hospital / RSHS. For each specimen, 6 separate FNABs were performed, sampling the same area. Because we were working with large surgical pathology specimens, we were able to sample the same general area of the tissue without sampling the same needle track in with the tissue that has been disrupted by prior needle pass. We used 23-gauge needles from Terumo medical corp., with a 10-cc slip-tip syringe and using the standard FNAB technique [7]. The first FNAB was rinsed and fixed with 10% NBF centrifuge for 7 minutes at 3000 revolutions per minute, decant supernatant, add 10% NBF then the specimen was submitted for processing. The five FNABs fixed with 96% alcohol were processed according to the duration of fixation 1 hour, 6 hours, 24 hours, 48 hours, 72 hours then centrifuged for 7 minutes at 3000 rpm respectively. After that, the cells block was prepared from residues.

IHC of Cell Block Section

Immunohistochemical (IHC) techniques were performed according to Agustina *et al., [8]*. IHC staining on the samples was performed manually using a labelled streptavidin-biotin immunoperoxide complex method, using the Starr Trek Universal HRP Detection system (Biocare Medical, Concord, CA, USA). Each cell block was cut into 4-µm slices and examined on L-lysine coated glass slides and baked at 60°C for one hour on a standard histology hotplate. Deparaffinized using xylene and rehydrated using an alcohol solution than brought to water. Antigen retrieval used a decloaking chamber (DC2008INTL, Biocare Medical, USA) in EDTA (pH 8.0), followed by cooling at room temperature for 20 minutes. Sections were then treated to block endogenous peroxidase, stained with primary antibodies, and incubated for 1 hour at room temperature. Detection was done by horseradish peroxidase polymer-based detection system (Biocare Medical) and diaminobenzidine chromogen and counterstained with haematoxylin. The primary antibodies were E-cadherin (G10) sc-8426 from Santa Cruz Biotechnology, inc (Santacruz, CA) and KI-67(SP6) from Cell Marque (Rocklin, CA, USA).

IHC Analysis and Interpretation

To analyse antigen preservation in the cell block, we used immunostaining E-cadherin that represent antigen in membrane and Ki-67 in nuclei. The expression for E-cadherin in the membrane of cancer cells was score with histologic score (also known as histoscore) scheme [9]. The intensity of staining was categorized as 0 (negative), 1 (weak), 2 (moderate), or 3 [10]. The percentage of positive cells were scored as 0 (negative), 1 (< 20%), 2 (20% < 50%), 3 (≥ 50%-80%), 4 (> 80%). A histoscore was generated as the product of the intensity and the area of the staining. The histoscore was than dichotomised into weak expression (histoscore 0-4) and strength of expression (histoscore 6-12). All procedures were done by 2 assessors pathologist (BSH and TI). Both of whom were blind to the fixative used.

To analyse immunostaining of Ki-67 for the antigen located in nuclei. The number of Ki-67-positive cells was counted using image analysing

software QuPath according to Zhong, *et al.,* and Laurinavicius *et al*., [11], [12]. The image-analysis software automatically counts the nuclei of cells that have an intensity that exceeds the predetermined threshold level. The advantages of using quantitative analysis are a time-saving alternative to manual counting method, reduce the variability of the pathologist in counting the tumour cell [13]. Histoscore was calculated with 40 cut-off points, ≤ 40 was weak, > 40 was strong [14].

Statistical Analysis

The quantitative comparative analysis method is applied in this study, six paired groups with an experimental design to obtain a good preservation cell and an optimal Immunohistochemistry (IHC). P ≤ 0.05 is considered statistically significant. The data obtained were recorded on a special form and then processed using SPSS program ver. 22.0 for Windows (IBM Corp., Armonk, NY, USA).

Results

Tumour Characteristic

The clinical characteristics of the carcinomas summarised in Table 1. In total, we used 25 fresh surgical specimens; all tumours are diagnosed as carcinoma.

Table 1: Characteristic sample from each tumour

Samples	N = 25
Ovarian Carcinoma	10
Invasive breast carcinoma of no special type (NST)	5
Papillary Thyroid Carcinoma	10

Immunohistochemistry of E-cadherin

E-cadherin is a membrane protein expressed on the cell membrane, commonly known as epithelial cell marker [15]. We evaluated the E-cadherin expression on different tumour tissues through IHC examination, on cell blocks fixed with 10% NBF, compared to cell blocks fixed with alcohol 96% in different fixation time. E-cadherin histoscore showed that 10% NBF fixation gives strong result 100%. Meanwhile, alcohol 96% fixation tends to exhibit a histoscore decrease, as shown in Table 2. P-value showed a significant difference between 10% NBF and alcohol 96% fixation.

From the results of statistical tests on the E-cadherin Histoscore in Table 2, information was obtained that P-value = 0.0001 was smaller than 0.05 (P-value < 0.05) which meant that it was significant or statistically significant thus it could be explained that there were differences between E-Cadherin Histoscore in 10% NBF fixation group and all of 96% alcohol duration fixation.

Table 2: Histoscore comparison of E-cadherin expression between 10% NBF fixation and alcohol 96% with a various fixation time

Variable	10% NBF N = 25	96% Alcohol					P-value
		1 h	6 h	24 h	48 h	72 h	
		N = 25	N = 25	N = 25	N = 25	N = 25	
Histoscore E-cadherin							0.0001**
Weak	0	2	7	11	16	16	
Strong	25	23	18	14	9	9	
P-value		0.157	0.008**	0.0001**	0.0001**	0.0001**	

Description: Categorical data on p values are calculated (with) Friedman test. **Significant difference was determined by p < 0.05.

From the results of statistical tests on the E-cadherin Histoscore in Table 2, information was obtained that P-value = 0.157. The P-value in the Histoscore was greater than 0.05 (P-value > 0.05) which means it was not statistically significant so it could be explained that there was no difference between E-Cadherin Histoscore in 10% NBF fixation group and 1 hour of 96% alcohol fixation.

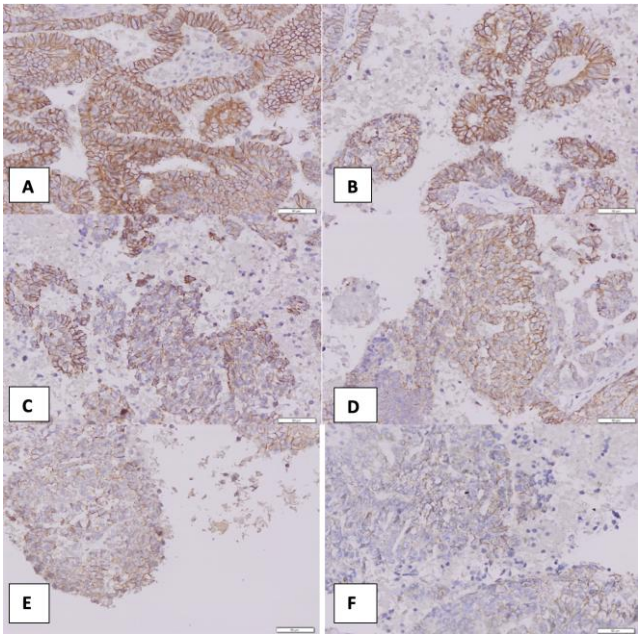

Figure 1: Representative images of E-cadherin expressions on immunohistochemistry staining; A) Cellblock section fixed with 10% NBF; B), C), D), E) and F) Cellblock sections fixed with 96% alcohol for 1 h, 6 h, 24 h, 48 h, and 72 h respectively (x 200)

From the results of statistical tests on the E-cadherin Histoscore in Table 2, information was obtained that P-value = 0.008 was smaller than 0.05 (P-value < 0.05) which meant that it was significant or statistically significant thus it could be explained that there were differences between E-Cadherin Histoscore in 10% NBF fixation group and 6 hours of 96% alcohol fixation.

We used McNemar statistical analysis test to reveal the significant difference between 10% NBF and alcohol 96% fixation in each fixation time. The results showed that short duration of fixation time has no significant difference between both fixation method

(p > 0.05) meanwhile longer duration exhibit significant difference (6 h, 24 h, 48 h, 72 h).

E-Cadherin expressions on IHC staining represent antigen in the membrane. The strong of expression (Figure 1A and 1B) and weak expression (Figure 1C, 1D, 1E and 1F).

Immunohistochemistry Ki-67

To analyse the preservation of antigen in cells block, we also performed immunostaining for the nuclear protein Ki-67 (shown in Figure 2) [5]. The histoscore comparison on Ki-67 expression in FNAB samples fixed with 10% NBF and 96% alcohol with different fixation time showed a significant gradual decrease in a time-dependent manner for both fixation methods (Table 3; P-value = 0.0001).

Table 3: Histoscore comparison on Ki-67 expression between 10% NBF fixation and 96% alcohol with a various fixation time

Variable	10% NBF	96% Alcohol					P-value
		1 h	6 h	24 h	48 h	72 h	
	N = 25	N = 25	N = 25	N = 25	N = 25	N = 25	
Histoscore Ki-67							0.0001**
Weak	6	12	20	24	25	25	
Strong	19	13	5	1	0	0	
P-value		0.003	0.0001**	0.0001**	0.0001**	0.0001**	

Description: Categorical data on p values are calculated (with) Chi-square test; **Significant difference was determined by p < 0.05.

Statistical analysis indicated that Ki-67 histoscore fixed with 10% NBF compared to 1h and 6h of 96% alcohol showed a significant difference as well as to longer alcohol fixation time. IHC staining result on (96% alcohol-fixed) FNAB samples demonstrated the gradual reduction of Ki-67 staining, as listed in Figure 2.

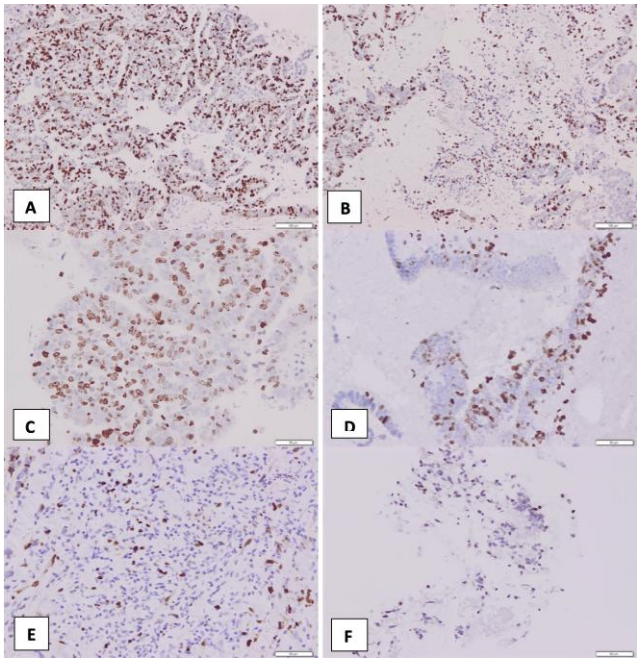

Figure 2: Representative images of Ki-67 expression on immunohistochemistry staining; A) Cellblock section fixed with 10% NBF; B), C), D), E) and F) Cellblock sections fixed with 96% Alcohol for 1 h, 6 h, 24 h, 48 h, and 72 h respectively (x 200)

Immunohistochemical expression of Ki-67 shows a strong association with tumour cell proliferation growth.

Discussion

Fixative agents are essential in diagnostic pathology. Ten percent NBF is commonly used in histopathology to fix tissue because it provides optimal fixation for FNAB material for cellblock samples. However, NBF has carcinogenic property as it has been categorised as 'carcinogenic to humans' (group1) by the International Agency for Research on Cancer (IARC, 2005) [16]. It has also become a major burden on the environment, and it crosslinks molecular groups [2]. Most laboratories in Indonesia use 96% alcohol as a fixative agent for cytology specimens because of its accessibility, and it's relatively inexpensive. Based on the literature, alcohol work as a coagulating agent which precipitates protein by breaking hydrogen bonds in the absence of protein cross-linking [17]. This research aims to determine the effect of 96% alcohol as a fixative agent in making cell blocks from cytology samples for further use in E-cadherin and Ki-67 immunostaining analysis. However, the effectiveness of using 96% alcohol as a fixative agent in making a cell block has not been evaluated and documented, and not supported by the literature.

Different fixation method can compromise the stability of protein expression in IHC staining. In our study, we compare the histoscore on E-cadherin as well as Ki-67 expression fixed with 10% NBF and 96% alcohol with various fixation time: 1 h, 6 h, 24 h, 48 h, and 72 h. Surprisingly we observed a gradual decrease of histoscore result corresponded to different 96% alcohol fixation time.

According to the previous study, Essen et al., discovered that tissues fixed in non-crosslinking alcohol-based fixatives could successfully be immunohistochemically stained for most antibodies following the usual NBF based protocols. Recently, alcohol-based fixative such as RCL2 and Boonfix have been proposed. Nonetheless, NBF-fixed tissues still provide significantly better immunostaining results (84% good staining) compared to RCL2 (66% good) and Boonfix-fixed tissue (60% good staining). The application of alcohol-based fixative may have additional benefits for molecular techniques, as they are expected to preserve DNA and RNA to a larger extent [2].

Moelans et al. found that alcohol-based fixative can replace NBF as the standard fixative agent, by saying that alcohol-based non-crosslinked fixative gives a better outcome in terms of preserving DNA and RNA, as well as providing quality and

applicability in molecular diagnostics. Nonetheless, despite the argument, NBF still provides a better result compared to alcohol-based fixative, and alcohol is unlikely to replace NBF universally [18]. This argument agrees with our findings, which 96% alcohol application showed a histoscore decrease in time-dependent fixation manner. In contrast, NBF fixation exhibited 100% and 76% histoscore on E-cadherin and Ki-67 immunostaining respectively.

Our results are consistent with Essen et al., the study that alcohol denatures proteins, showed by the decrease in histological results when fixed with different fixation time in a time-dependent manner.

The histoscore comparison of E-cadherin immunostaining showed no significant difference in the strong-weak category between fixation with 10% NBF and 96% alcohol for 1-hour. This result indicates that 1 hour might be optimum for fixation time to generate cell block.

Our results correspond with the study performed by Matsuda et al., compared the morphology and the quality and quantity of ribonucleic acid [19] and protein in paraffin-embedded tissues of nude mice implanted with human uterine cervical cancer cells, followed by fixation with commonly used fixatives, including 4% paraformaldehyde (PFA), 10% neutral buffered formalin (NBF), 20% NBF, and 99% ethanol (EtOH). The assay was then continued for IHC staining on E-cadherin, Ki-67, VEGF-A, HLA class 1, AE-1 protein expression. This study indicated that formalin fixation is better than alcohol fixation for RNA preservation in paraffin-embedded cancer cell implantation models. Their results showed that 90% of cells fixed by ethanol 99% showed that ethanol 99% cause cell shrinkage due to cell dehydration. Fixation with NBF 10% and NBF 20% showed good results on cell morphology quality. The 99% EtOH-fixed samples showed marked decreases of Ki-67 immunostaining compared with the formalin-fixed samples and showed a decrease of E-cadherin immunostaining to a lesser extent [20]. Su et al. found that formalin-based fixation is preferable to compare to ethanol 99% in analysing cell morphology. Gong et al., demonstrated significantly lower detection rates of Ki-67, PCNA, and p53 with the ethanol-based fixative ThinPrep as compared with formalin-fixed cell-block slides in malignant cases [21]. This is consistent with our study that IHC staining result on 96% alcohol-fixed FNAB samples demonstrated the gradual reduction of Ki-67 immunostaining. Our findings indicated that Ki-67 immunostaining results were incompetent for further analysis even from the 1hour fixation time with 96% alcohol.

Different from previous research, Denda et al., revealed ethanol-fixed smears, that Ki67 could be immunostained successfully with heat-induced antigen retrieval. The optimal antigen retrieval condition for each antibody must be individually determined. For the nuclear antigens, heat-induced antigen retrieval may allow access of the antibody to the DNA-binding protein epitopes, partly hindered by steric effects, because it can denature double-stranded DNA into single-stranded DNA. However, the role of antigen retrieval in the immunostaining of cytologic specimens is currently unclear and not yet optimised [3].

Ten percent NBF as the gold standard in the fixation process is routinely used in histology samples, as well as in IHC staining. This study shows that cell blocks fixated with 10% NBF showed good consistent results and were able to preserve cells obtained from cytology samples. Similarly, JH Williams suggests that some types of fixation include 10% normal saline, 10% NBF and 10% formalin showed consistent results both for cell preservation and immunohistochemistry [22]. According to Engel et al., specimens fixed with 10% NBF showed excellent results in preserving antigens and showing consistent results for immunohistochemistry [23].

In summary, we discover that the management of cytology samples using 96% alcohol fixation was not recommended as a fixative agent for making cell blocks and followed by IHC examination. Fixation with NBF 10% as the gold standard showed good results, and optimal histoscore values, hence it is recommended for sample fixation when making cell blocks, before IHC examination.

Acknowledgement

This research was funded by an internal grant for Padjadjaran University batch 2 (4851/UN6.C/LT /2018). The authors wish to thank technicians of histopathology and immunohistochemistry laboratories, for all technical support.

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