Alcoholic fixation over formalin fixation: A new, safer option for morphologic and molecular analysis of tissues

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Abstract

Formalin is a widely used fixative but there is potential public health risks to exposure. Besides, alcoholic fixation is advantageous over formalin fixation because of faster fixation, optimal preservation and safer workplace environment. Following fixation by EMA and 10% neutral buffered formalin (NBF), we analyzed the tissue morphology, antigenic stability, DNA and RNA quantity with quality (OD value). The findings of EMA fixing on both the tissue morphology and molecular characterization, were satisfactory. Specially, EMA was faster in penetration of tissues than NBF, fixed ideally as early as 8 h of fixation whereas improper fixation was evident for NBF. In Hematoxylin and Eosin (H & E) staining, better cellular details with stronger affinity for staining were observed. In immunohistochemistry, better antigenic stability was reported for EMA-fixed tissues. The nucleic acid analysis revealed that total genomic DNA and RNA yield from EMA fixed tissues were significantly higher (P < 0.05) with superior quality than NBF fixed tissues. Our results suggest that EMA could be a potential alternative to NBF for fixation and preservation of tissues. These data provide new insights into an option for a safer working environment to support study and research.

Keywords:
Fixation
Formalin
Alcohol-based fixative
Tissue morphology
Nucleic acid

1. Introduction

Fixation is a physico-chemical phenomenon and reactions involving gradual diffusion of fixative into the tissues. It is an essential step in the evaluation and study of biopsy tissue specimens. It aids in the preservation of the tissue’s cellular architecture and composition during processing. Fixation also maintains the spatial connection of proteins, carbohydrates, and other bioactive moieties to the cell, allowing them to be examined (Woodyard, 2011). For more than a century, the fixative of choice in routine histopathology has been a 10% solution of formalin (4% formaldehyde) diluted in water or in a buffered solution. Formalin maintains chemical activity and cellular antigenicity in tissues by forming covalent bonds between biological macromolecules (Warmington et al., 2000). As a result, formalin-fixed paraffin-embedded (FFPE) tissues are now the most widely used fixatives in the world.

However, formalin is not always the ‘gold standard’ fixative when other downstream biochemical and molecular analyses are considered (Panzacci et al., 2019). In reality, the benefits of formalin as a histological preservation are outweighed by a variety of drawbacks, the most prominent of which are diminished immunohistochemical reactivity and rapid nucleic acid breakdown (Cox et al., 2006; Moelans et al., 2011). Although the degree of epitope modification differs with molecular targets, the crosslinking mechanism of formalin changes protein folding, decreasing the total availability of epitopes that can be bound by antibody (Bogen et al., 2009; Hayat 2000; O’Leary et al., 2009; Otali et al., 2009; Paavilainen et al., 2010). Several proteolytic or heat-induced antigen retrieval methods have recently become commercially available.
available to address this problem, restore normal protein folding, and improve the accessibility of epitope on fixed tissues (Fowler et al., 2011; Paavilainen et al., 2010). Even though their mechanisms of action are unknown, these antigen retrieval techniques are currently considered the standard procedures for achieving high-quality staining. As a result, antigen retrieval methods are often analytical and require high-level optimization.

The baleful effects of formaldehyde have been scrutinized in public with all emotions and sensibility. Anatomists, technicians in histology and embalming laboratories, as well as medical students during their practical courses when they need to do the dissection of specimens, are all rigorously exposed to formaldehyde, which in certain cases reach to the threshold for inflammation of the eyes and upper respiratory tract. There is no debate about the acute toxicity of formaldehyde and the incidence of contact dermatitis (Pabst, 1987). The use of ethanol and methanol as a fixative, on the other hand, is a relatively non-toxic alternative to formalin. Ethanol and methanol are coagulating fixatives that break the hydrogen bonds to precipitate proteins. While both ethanol and methanol have demonstrated their potential tissue fixation quality through routine use in cytological preparation, acting alone, both ethanol and methanol can cause tissue shrinkage and brittleness. Nucleic acid preservation as well as histological and immunohistochemical studies are possible with an alcohol-based fixative (molecular fixative, MF) (Hostein et al., 2011).

Denaturation of the target antigens’ binding site can result in the use of an alcohol-based fixative. As a result, antigen retrieval testing for each antibody is recommended, as it has been shown to produce a better immune reaction (Magaki et al., 2019).

Milcheva et al. (2013) found that after one hour of fixation in modified methacarn fixative, RNA extracted from the specimens still had intact rRNA subunits, while 24 h of exposure to alcohol-based fixative resulted in total loss of rRNA subunit detection on an agarose gel. Alcohol-based tissue fixatives, such as ethanol and methanol, are more efficient preserving agents than buffered formalin. These fixatives allowed the recovery of higher quality DNA by controlling dehydration rather than cross-linking (Duval et al., 2010). Alcohol-based fixatives work by precipitating proteins, which does not disguise their antigenicity and eliminates the need for antigen retrieval on slides. It has long been recognized that alcohol supplemented with acetic acid results in strong tissue morphology preservation (Buchwalow et al., 2010). Both methanol and ethanol combined with glacial acetic acid, with either routine or manual processing of the tissue specimen, provide an excellent and reliable tool for research studies that aim to investigate tissue morphology, immunohistochemical detection of proteins, and molecular analyses in normal and pathologically changed tissues, particularly when tissue morphology, immunohistochemical detection of proteins, and molecular analyses in normal and pathologically changed tissues are investigated. The higher immunoreactivity of the alcohol-fixed tissue parts necessitates lower antibody concentrations, resulting in cost savings (Milcheva et al., 2013). In this research, the output of the alcohol-based fixative, EMA, in tissue morphology, protein, and nucleic acid preservation was assessed using morphometric and molecular analysis.

2. Materials and methods

2.1. Ethical approval

As per the standardized instruction, all of the animals received human care. These research interventions have been permitted and carried out in compliance with animal welfare recommendations and used as defined by the Animal Welfare and Experimental Ethics Committee, Bangladesh Agricultural University, Mymensingh, Bangladesh (Protocol Number: AWECC/BAU/2019–05).

2.2. Sample collection

Fresh tissue samples from the liver (right lobe) and brain (cerebral cortex) of adult black Bengal goats (Capra hircus) were collected and rinsed into normal saline. Each tissue sample was fixed for 24 h into the alcohol-based fixative, EMA (a combination of ethanol, methanol and acetic acid = 3 : 1 : 1) and 10% neutral buffered formalin (NBF). After fixation, tissues were subjected to gross evaluation.

2.3. Histological analysis of tissues

After fixation, liver and brain tissues were processed for histological study by Hematoxylin and Eosin (H & E) staining. The tissues were rinsed in 70 % alcohol and then processed. The processing schedule was 70 % alcohol, 80 % alcohol, 90% alcohol, two changes of 100 % alcohol and two changes of xylene (1 h each) and three changes of graded paraffin wax for 45 min each. Following embedding, each block was coded. The paraffin blocks were sectioned at thickness of 6 µm, and glass slides with tissue sections were dried in a slide warmer at 40 °C. The sections were stained using the set up as xylene-I, II & III (3 min each); 100 % alcohol, 95 % alcohol, 80 % alcohol & 70 % alcohol (3 min each); deionized water with gentle shaking (10 min); Harris Hematoxylin (10 min); deionized water with gentle shaking (10 ins); Eosin-Y (2 min), 70 % alcohol, 80 % alcohol, 95 % alcohol and 2 changes of 100 % alcohol (3 min each); 50 % xylene + 50 % alcohol and xylene-I, II & III (3 min each). Then the sections were cover slipped using DPX mounting medium and examined under microscope.

2.4. Immunohistochemical analysis of tissues

For immunofluorescence study, paraffin embedded tissue sections were deparaffinized in xylene-I, II & III (for 3 min each), rehydrated in 100 % ethanol, 90 % ethanol, 80 % ethanol, 70 % ethanol and distilled water (for 5 min each). Then changes in PBT (Phosphate Buffered Saline with 0.01% Tween-20) (for 5 min each), 3% H2O2 in PBT (for 10 min), washing in PBT - for 5 min) followed by blocking with 10% BSA (Bovine Serum Albumin) in PBT (for 30 min). After blocking, tissue sections were incubated overnight at 4 °C with mouse monoclonal anti-actin antibody, 1:100 dilution (Cat. no. I55, Developmental Studies Hybridoma Bank, Iowa, USA) and mouse monoclonal anti-Ki67 antibody (Cat. no. SC-23900) that can be used as the general markers for immunohistochemical analysis.

1:200 dilution (Santa Cruz Biotechnology, USA). Then washed for 3 times in PBT (for 5 min each) and incubated with Alexa Fluor (Alexa Fluor® 488 and Alexa Fluor® 594) conjugated donkey anti-mouse IgG (Cat. no. A21202) + IgM secondary antibody (Cat. no. R-37115), 1: 200 dilution (Invitrogen, USA) at room temperature for 2 h. After washing for 3 times in PBT (5 min each), tissue sections were dehydrated in 70 % ethanol, 80 % ethanol, 90 % ethanol and 100 % ethanol (for 5 min each) followed by three changes in xylene-I, II & III (for 3 min each). Finally, tissue sections were cover slipped with fluorescence anti-fade mounting medium (Vector laboratories, USA). A negative control (without primary antibody) was maintained to avoid background intensity.

2.5. Nucleic acid analysis of tissues

For extraction of genomic DNA and RNA from fixed liver and brain tissues, we used Pure Link DNA and RNA extraction Mini Kits (Thermo Fisher, USA) and their protocols. Briefly, for RNA extraction, 50 mg of fixed tissues (liver and cerebrum) were taken into
sterile microcentrifuge tube and washed in PBS for 3 times (20 min each). Then 300 µl Binding Buffer (L3) was added to the tube immersing the tissues, homogenization of the tissue was done by using tissue homogenizer, centrifugation for 5 min at 12000Xg of the Lysate was done, the supernatant was transferred into a fresh tube, finally for clear sample 300 µl of 70% Ethanol was added to the supernatant and mixed well by vortexing and isolated the RNA by eluting with 50 µl of distilled water. For DNA extraction, 25 mg of fixed tissues (liver and cerebrum) were taken into sterile microcentrifuge tube and washed in PBS for 3 times (20 min each). 180 µl PureLink® Genomic Digestion Buffer and 20 µl Proteinase K was added to the tube, mixed well by vortexing. Then incubated at 55 °C for 4 h until the lysis is completed. Centrifuged the lysate at 12000Xg for 3 min at room temperature to remove any particulate materials. Then 20 µl RNAase A to the lysate was added and then vortexed for mixing well and incubated for 2 min at room temperature. Then 200 µl PureLink® Genomic Lysis buffer was added to the tube and mixed well by vortexing to obtain a homogenous solution. Again, centrifugation was done at 12000Xg for 3 min. The supernatant was removed, 200 µl of 70% Ethanol was added to the tube and centrifuged briefly at 12000Xg for 5 min and finally elution was done by using 50 µl of distilled water. Total genomic DNA yield (ng/µl) along with quality (OD value 260/280) was measured using UV-Spectrophotometer (Bio-Lab, South Korea).

2.6. Imaging and data analysis

The images were taken by using Nikon fluorescence microscope at 20X magnification.

2.7. Statistical analysis

The data were analyzed by Student’s t-test using the Statistical Package for Social Sciences (SPSS) software, Version 20. Significant was considered at the level of 5% confidence interval.

3. Results

3.1. Gross evaluation of fixed tissues

After 24 h of fixation, gross examination of liver and brain tissues revealed that alcohol-based fixative, EMA fixed tissues ideally (hard and turned grey-white) whereas NBF fixed tissues turned red-brown and remained soft. Current investigation showed that EMA was faster in penetration of tissues than NBF. It fixed tissues ideally as early as 8 h of fixation but improper fixation was evidenced in case of NBF. NBF showed mild shrinkage with incomplete fixation but EMA did not show any shrinkage with complete fixation (Fig. 1). These results indicated the differential diffusion capacity of fixatives in the tissues over the given period of time. Of these, EMA was faster and better preserver of gross tissue morphology.

3.2. Histological evaluation of fixed tissues (H & E)

Histological study of tissues (H & E staining) revealed that EMA preserved histo-architectures better than NBF. It preserved better histologic structures with nice staining affinity of cellular details- cell boundaries, nuclei and cytoplasm (Fig. 2). However,
pale staining of cellular and intercellular structures were observed in case of NBF. These results were supportive of alcoholic fixative, EMA as a better preserver of histo-architectures than aldehyde fixative, NBF.

3.3. Immunohistochemical evaluation of fixed tissues

In case of EMA fixed liver tissues, strong emissions of fluorescence signals were detected from the hepatocytes, interstitial tissues and hepato-portal vessels. Similar signals were observed in the neuronal cells of cerebral cortex. However, weak/poor signals were evident in both cases of formalin fixed tissues (Fig. 3). These results revealed better antigenic stability of EMA fixed tissues than NBF.

3.4. Total genomic DNA yield and quality from fixed tissues

We used Pure Link DNA extraction Mini kit (Thermo Fisher, USA) for extraction of genomic DNA from the fixed liver and brain tissues. Total genomic DNA yield (ng/μl) along with the quality (OD value 260/280) was measured using UV-Spectrophotometer (Bio-Lab, South Korea). Total DNA yield from EMA fixed liver and brain tissues was recorded as 295.48 ± 3.85 ng/μl with OD value 1.82 ± 0.01 and 171.97 ± 12.38 ng/μl with OD value 1.81 ± 0.01, respectively. On the otherhand, total DNA yield from NBF fixed liver and brain tissues was 61.84 ± 5.63 ng/μl with OD value 1.22 ± 0.01 and 35.77 ± 3.52 ng/μl with OD value 1.23 ± 0.08, respectively. These results indicated that EMA fixed tissues produced significantly higher (P < 0.05) quantity of genomic DNA with superior quality than that of NBF (Figs. 4a, 4b, 5a and 5b).

3.5. Total genomic RNA yield and quality from fixed tissues

We used Pure Link RNA extraction Mini kit (Thermo Fischer, USA) for extraction of genomic RNA from the fixed liver tissues. Total genomic RNA yield (ng/μl) along with quality (OD value 260/280) was measured using UV-Spectrophotometer (Bio-Lab, South Korea). Total RNA yield from EMA fixed liver and brain tissues was recorded as 257.50 ± 6.92 ng/μl with OD value 2.03 ± 0.01 and 130.05 ± 9.04 ng/μl with OD value 2.02 ± 0.02, respectively. Contrary for NBF fixed liver and brain tissues, total RNA yield was 38.09 ± 2.87 ng/μl with OD value 1.88 ± 0.02 and 19.95 ± 3.97 ng/μl with OD value 1.72 ± 0.09, respectively. These results indicated that EMA fixed tissues produced significantly higher (P < 0.05) quantity of genomic RNA with superior quality than that of NBF (Figs. 6a, 6b, 7a and 7b).

4. Discussion

Considering the safety concerns and public health risks, it is needed by the researchers to adopt alternative solutions for the histopathological researches to reduce the risk factors associated...
with the exposure to formalin. Despite its widespread use, the choice of formalin for tissue fixation in histopathology laboratories around the world is not justified by its superior performance, but rather by the need to harmonize diagnostic criteria (Zanini et al., 2012). Many researchers have demonstrated that formalin, in fact, like all chemical fixatives, elicits protein modification and hampers the extraction of intact nucleic acids (Bogen et al., 2019; Cox et al., 2006; Gillespie et al., 2002; Hayat et al., 2000; Moelans et al., 2011; O’Leary et al., 2009; Otali et al., 2009). Therefore, it is critical to find a suitable alternate to formalin for ethical issues and the safety of the users. As a result, the scientific community is concentrating its efforts on finding alternative fixatives to formalin; alcohol-based fixatives are among the most promising candidates.

Fixation times are also a critical parameter in terms of biomolecule preservation for optimum histomorphological study, as the use of complex fixatives remains a critical point for the optimization of analysis process. This aspect has been investigated by a few authors, like Goldstein et al., and Taziji et al., found that under- or over-fixation of tissues with NBF results in poor immunohistochemical staining efficiency (Goldstein et al., 2007; Taziji et al., 2009).

![Fig. 3. Immunofluorescence staining of goat liver tissues with anti-actin (A, B) and brain tissues with anti-Ki67 (C, D) antibodies. (A, C) EMA fixed tissues; (B, D) NBF fixed tissues. Scale bar in all images (A-D) = 50 μm.](image1)

![Fig. 4. a. Genomic DNA yield (ng/μl) from fixed liver tissues of goat (*P < 0.05.), b: OD value (260/280) of yielded DNA.](image2)
Yaziji et al., 2008) and Chung et al., additionally found that under- or over-fixation of tissues with NBF results in poor nucleic acid preservation (Chung et al., 2018). Some other studies have found conflicting results when it comes to the issue of over-fixation duration (De Marzo et al., 2002; Shi et al., 2007; Wester et al., 2000; Dapson et al., 2007). Insufficient information regarding minimum tissue fixation times has been gathered (Dapson et al., 2007; Kalkman et al., 2014). In regards to the aforementioned criteria, there is a research gap. Herein lies the need for our research and the significance of using alcohol-based alternative fixatives in histomorphological studies.

This research attempted to assess the output of an alcohol-based fixative (EMA) that, hopefully, fixed tissues within 24 h of fixation without causing major morphological changes. However, the presence of formalin-fixed tissues differed from that of alcohol-based fixatives because it induced simultaneous dehydration and fixation. Alcohol-based fixatives coagulate proteins rather than masking antigenic sites as formalin does (Bancroft and Gamble, 2008). These fixatives are faster in penetration of tissues as all these components of EMA (ethanol, methanol and acetic acid) have higher coefficients of diffusibility than formalin (Kok and Boon, 1990). That’s why, EMA preserved better general tissue mor-
The alcohol-based fixative act by precipitation of proteins, which do not mask their antigenic sites. From our immunofluorescence study of tissue sections we have got the best stability of actin and Ki67 antigens in case of EMA fixed tissues which resulted strong signals. Current findings are in line with Holstein et al., 2011 who established that the alcohol-based fixatives have higher stability of fixed tissues, resulting in strong signals. On the contrary, formalin fixation showed weaker signals than the EMA in the similar tissue samples and following the same protocols. In comparison to the 10% NBF-fixed tissues, EMA was highly consistent with the antibody used in our study, showing the expected subcellular localization of the matching proteins. We assume that these results are largely depending on the susceptibility of individual epitope to formalin which results in cross linking and masking epitope sites, quite a similar statement was provided by Werner et al., 2000. As a result, formalin requires unmasking, although alcohol-based fixatives do not. Milcheva et al., 2013 suggested that the increased immunogenicity of the alcohol-fixed tissue sections necessitates lower antibody concentrations, resulting in economic benefits as well being more feasible.

Bostwick et al., 1994 and Moelans et al., 2011 stated some of the advantages of alcohol-fixation methods which included short fixation time, optimal preservation of DNA, RNA and proteins and a safer workplace environment which was also observed from our study as well. We found that EMA may fix tissues as early as 8 h after fixation, whereas NBF takes 24 h, which strongly support the findings of Bostwick et al., 1994. Thus, EMA gives advantage of shorter fixation time over formalin. Advanced molecular methods requiring high-quality nucleic acids have been developed in recent years to aid pathologists in diagnosis. Chung et al., 2018 suggested that the recovery of nucleic acids from FFPE tissues remains difficult with low recovery and poor quality. Our study demonstrated that alcohol fixation is preferable to formalin fixation regarding the quantity and purity of nucleic acid recovered from EMA-treated tissues. A recent work of Chung et al., 2018, revealed that nucleic acid integrity is well conserved from 1 to 6 months after 70% ethanol fixation, whereas NBF-fixation results in rapid fragmentation. Moreover, previous studies showed that nucleic acids extracted from FFPE are of high quality and slightly fragmented (Dotti et al., 2010; Gillespie et al., 2002; Milcheva et al., 2013; Moelans et al., 2011; Perry et al., 2016; Srinivasan et al., 2002). Findings of the current research support the growing body of scientific evidences linking low-risk chemical substances like alcohol and acetic acid to accelerate tissue fixation outcomes. There are no scientific reasons to justify the extensive use of formalin for tissue processing in clinical and research facilities, given the risks connected with its usage. Standardization of formalin-free methods and harmonization of diagnosis in pathology department in the whole world, should urgently aim to formalin substitution and the development of safer alternative protocols (Bostwick et al., 1994). Pathology departments all over the world are working to standardize formalin-free methods and harmonize diagnoses. For the sake of the purpose the researchers should focus on replacement of formalin and the safer alternative practices should be developed as soon as possible.

5. Conclusions

Based on the results, we suggest that alcohol-based fixative (EMA) could be a potential alternative to NBF for faster and optimal preservation of tissues to maintain cellular and molecular integrity. Many strict rules and regulations to formalin use have been introduced worldwide by public health authorities for protecting people. But the most acceptable form of prevention would be avoiding any injudicious use of formalin and its substitution with safer and cheaper alcohol-based alternatives, for example, EMA.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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Author contributions

Authors contributed to conception or design (MAS, NS, ZH); data acquisition, analysis, or interpretation (MAS, SB, MA, ZH); drafting the manuscript (MAS, UA, MA, ZH) and critically revising the manuscript (NS, SB, MA). All authors gave final approval and agreed to be accountable for all aspects of work in ensuring that questions relating to the accuracy or integrity of any part of the work.

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