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Original

Effectiveness of 70% ethanol as a tissue fixation agent in preservation of human tissue morphology

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Abstract. Formaldehyde is the preferred fixative in routine histopathology sample processing. However, the use of formaldehyde is demoted by a variety of drawbacks, including potential health risks, tissue shrinkage, artefactual pigmentation, etc., impacting the quality of the stained sections and affecting certain diagnostic techniques. Alcohol-based fixatives are relatively non-toxic and, therefore, a safer option for tissue fixation. The present study was to assess the effectiveness of 70% ethanol as a tissue fixative in preserving human tissue morphology at different time durations. A total of 40 histology samples including thyroid, breast, appendix, uterus, gallbladder, kidney, etc. of comparable size (thickness; 0.5 – 1 mm) were fixed in 10% formal saline (One part of formaldehyde: nine parts of distilled water: 8.5g of NaCl) and 70% ethanol (6.65 parts of 95% ethanol: 3.35 parts of distilled water) for 24 hours, and two weeks respectively for the assessment of histology on Hematoxylin and Eosin (H & E)-stained tissue sections according to a semi-quantitative scoring system. Ethanol fixation resulted in better preservation of nuclear details, uniformity in H & E staining, and the absence of artefactual pigment depositions. However, preservation of cytoplasmic details and extracellular components was poor in ethanol-fixed sections compared to that of formal saline. The nuclear (24 hour; p=0.174, two-weeks; p=0.000) and cytoplasmic shrinkage (p=0.000 in both 24 hours and two weeks) was more significant in ethanol fixation. Ethanol fixation for two weeks led to worsened nuclear vacuolization, nuclear shrinkage, cytoplasmic cohesiveness, uniformity, texture, cell border preservation, and cytoplasmic shrinkage/swelling in tissues. In conclusion, the results revealed that 70% ethanol fixation was effective in 24 hour fixation of human tissues, however, there were limitations in fixation in 70% ethanol for two weeks. Further studies are warranted for the development of alternative ethanol-based fixatives for applications requiring extended tissue fixation.

Keywords. Alcohol-based fixatives; Formalin; Hematoxylin and Eosin staining; Long-term fixation; Short-term fixation

INTRODUCTION

Chemical fixation is crucial in preserving tissue morphology and cellular information for microscopic analysis. Formaldehyde has been the fixative of choice in routine histopathology for centuries. Quick penetration through cell walls and membranes, affordability, and long-term preservation of tissue samples are the main reasons for the wide use of formalin as a potential fixative [1]. Formaldehyde is often used as 10% formalin, 10% formal saline or 10% neutral buffered formaldehyde for tissue fixation.

The use of formalin as a tissue fixative is demoted by a variety of drawbacks, in which diminished immunohistochemical reactivity and fast nucleic acid breakdown are the most prominent. Pure formaldehyde is a vapor that forms a solution containing 37–40% formaldehyde when completely dissolved in water and is reported to have carcinogenic effects. Therefore, the potential health risks associated with the rigorous exposure to this toxic chemical have become a serious issue for its wide usage [2-4]. Moreover, formalin causes tissue shrinkage, impacting the quality of the stained sections and affecting certain diagnostic techniques. Further, it generates various tissue artifacts, which can lead to erroneous pathological interpretations [5].

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Alcohol-based fixatives are relatively non-toxic and, therefore, a safer option for tissue fixation for morphological and molecular analysis. Alcohol does tissue fixation via the denaturation and dehydration of cellular proteins and membranes and thereby facilitates the maintenance of tissue integrity by preventing autolysis and preserving molecular and cellular details [5]. Reduced tissue shrinkage, shorter fixation time, better preservation of nucleic acids, and improved antigen retrieval are the additional advantages reported [6]. Yet, the majority of histopathology sample processing laboratories use formalin as the fixative in routine practice. Moreover, the tissue samples are preserved in formalin for longer durations, especially in state sector hospitals in limited resource settings due to the huge workload, even though the ideal duration of fixation is 24 hours. However, the adverse effects associated with the long-term storage of tissues in formalin are a potential challenge for accurate histopathological diagnosis. Therefore, assessment of the effectiveness of the particular alternatives of formalin at different fixation durations is beneficial.

Even though numerous studies have been carried out on tissues excised from laboratory animal models for the assessment of the effectiveness of potential alternatives suggesting better fixation effects of alcohol-based fixatives, no study has been reported on the effectiveness of 70% ethanol alone on histomorphology under H & E staining of human tissues to date. The alcohol-based fixatives evaluated in previous studies were absolute ethanol or mixtures of ethanol and other chemicals, such as formalin, methanol, acetic acid, glycerol, glacial acetic etc. to produce specific effects [1,5]. Further, most of the reported pre-clinical studies which have been carried out in experimental animals had focused on the effect on molecular level diagnosis and the studies reported on H & E staining, which is the most common procedure of histopathological diagnosis in routine practice are limited. More importantly, assessment of the effectiveness of tissue fixation by 70% ethanol derived from laboratory-grade ethanol with 95% purity which is produced as a byproduct of sugar refining would be a cost effective and sustainable option for a resource-limited country like Sri Lanka. The adequate supply of 70% ethanol is often possible with the availability of laboratory-grade ethanol with 95% purity from the local distilleries of sugar refineries in the country at a low price compared to formalin and it may reduce the reliance on imported chemicals as well. Therefore, the present study was undertaken to assess the effectiveness of 70% ethanol derived from laboratory-grade ethanol with 95% purity, as a potential tissue fixative in preserving human tissue morphology at different time durations.

MATERIAL AND METHODS

Chemicals: Formaldehyde (39.1% w/v) (Sigma-Aldrich, St. Louis, MO) and laboratory-grade ethanol with 95% purity (Sevanagala Sugar Refinery, Sri Lanka) were used in the preparation of 10% formal saline and 70% ethanol fixatives respectively. Hematoxylin (Harris Hematoxylin) and Eosin (Eosin-Y) were purchased from Thermo Scientific, USA and were used in H & E staining.

Fixatives: The two fixatives; 70% ethanol (6.65 parts of 95% ethanol: 3.35 parts of distilled water) and 10% formal saline (One part of formaldehyde (39.1%): nine parts of distilled water: 8.5g of NaCl) were prepared and stored at room temperature for tissue fixation.

Tissue Samples and fixation: An evaluation study was carried out at the Histopathology Laboratory, District General Hospital, Matara over three months for assessment of the effectiveness of 70% ethanol as a tissue fixation agent on human tissue morphology against 10% formal saline. Ethical clearance was obtained from the Ethics Review Committee of the Faculty of Allied Health Sciences, University of Ruhuna (Ref No. 2023.12.360). Human tissues frequently excised during surgical procedures such as the uterus, ovary, fallopian tube, thyroid, parotid gland, appendix, breast, gallbladder, cervix, kidney, axillary lymph nodes, scrotal

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wall, placenta, and bronchial cysts, were used in the present study.

The tissue specimens collected from the surgical theatres of the District General Hospital Matara, General Hospital Kamburupitiya, and National Hospital Galle in fresh state were immediately transported to the laboratory and comparable pieces of tissue sections (approximate thickness of 0.5 – 1 mm) were fixed in 70% ethanol and 10% formal saline. The tissue-to-fixative volume was maintained exceeding a 1:10 ratio, and the specimens which include muscular, connective, glandular tissues etc. were stored at room temperature. After fixation for 24 hours, one half of the tissue pieces fixed in each 70% ethanol and 10% formal saline fixatives were processed using an automated tissue processor (Sakura Tissue- Tek VIP 5 Jr Tissue processor, Japan) with a 30 – 45 min/station protocol. The processed tissues were embedded in paraffin wax and sectioned for histological assessment (Biobase rotating microtome, China). The same procedure was followed to prepare H & E-stained tissue sections for the remaining halves of tissues after two weeks of fixation in the respective fixatives.

Assessment of histology: H & E staining was performed for each tissue sample using the automated tissue stainer (TEK DRS 2000 automatic slide Stainer, Japan), following the standard procedure of Bancroft and Gamble [7]. Histomorphology examination was carried out according to a modified version of a pre-validated semi-quantitative scoring system, which includes parameters for the assessment of the physical quality of tissue blocks, quality of microscopic preservation, quality of H & E staining, and fixation artifacts [8]. Tissue disruption, adhesion, cracking and thickness of the sections were evaluated under physical quality of tissue blocks. Tissue disruption was evaluated by the presence of artifacts such as wrinkles and chatters, and proper tissue adhesion was ensured by the absence of features such as tissue detachment from the slide, wrinkling or tearing of the tissue sections.

The quality of microscopic preservation was examined in H & E-stained sections by the assessment of preservation of nuclear and cytoplasmic details. Preservation of nucleolus and chromatin details defined nuclear envelop, vacuolation, shrinkage or swelling were examined under nuclear details. Preservation of uniformity, cohesiveness, texture, vacuolation, defined cell border, shrinkage/swelling, and preservation of extracellular component and muscle (collagen/elastin) were evaluated under cytoplasmic details. The cytoplasmic cohesiveness and texture were assessed by the eosin staining in the present study. With reference to the texture, the presence of subtle variations in cytoplasmic eosin staining, indicating the presence of different cytoplasmic components were considered as good quality. Uniform, consistent eosin staining, with distinct cell membranes were considered as well-cohesive cytoplasm.

The quality of H & E staining was assessed through the uniformity and the intensity of nuclear and cytoplasmic staining. Microscopic observations were carried out blindly by two independent investigators in the research group. The investigators were trained for the evaluation of tissue sections by a Consultant Histopathologist (a member of the research group) at the beginning. An average score of the independent investigators were considered in each parameter and the findings were confirmed by the Consultant Histopathologist.

Each tissue section was examined and recorded across ten random fields for the selected features based on a 4-point scale of 1= Poor, 2= Satisfactory, 3= Good, 4= Excellent. Accordingly, higher scores were allotted for good quality sections with respect to the selected features, whereas poor quality sections were given lower scores. The results of the test tissue sections fixed for 24 hours and two weeks in 70% ethanol and 10% formal saline were compared against a reference slide of the each original tissue. The reference slides used were the H & E-stained tissue sections prepared by the respective reference laboratory after 24-hours of fixation period in 10% formal saline.

Data analysis: Statistical analysis was carried out using IBM SPSS software version 26. Average histological scores were

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calculated for each parameter separately on H & E-stained sections fixed in ethanol and formal saline during semi-quantitative assessment of histology. Kruskal–Wallis test was used for the multiple comparison of data of semi-quantitative assessment. $p<0.05$ was considered statistically significant.

RESULTS

Physical properties of histology specimens: A total of 40 histology samples were collected including thyroid glands (n=8), thyroid carcinomas (n=2), kidney tissues (n=1), appendix (n=8), breast tissues (n=6), breast fibroadenoma (n=3), scrotal wall tissue (n=1), gall bladder (n=2), cancer tongue tissue (n=1), uterus (n=4), cervix (n=1), ovaries (n=1), fallopian tissue (n=1), and a bronchial cyst (n=1). The findings on the physical characteristics of the histology specimens fixed in 10% formal saline and 70% ethanol are shown in Table 1 and Figure 1.

Table 1: Physical properties of tissues following fixation in 10% formal saline and 70% ethanol

Features	10% Formal saline		70% Ethanol	
	24 hours	Two weeks	24 hours	Two weeks
Color	Dark brown gray/white	Dark brown – gray/white	Slightly reddish-gray/white	Slightly reddish–gray/white
Size	No shrinkage/ swelling	No shrinkage/ swelling	No shrinkage/ swelling	No shrinkage/ swelling
Hardness of the tissue	Normal	Normal	Normal	Normal

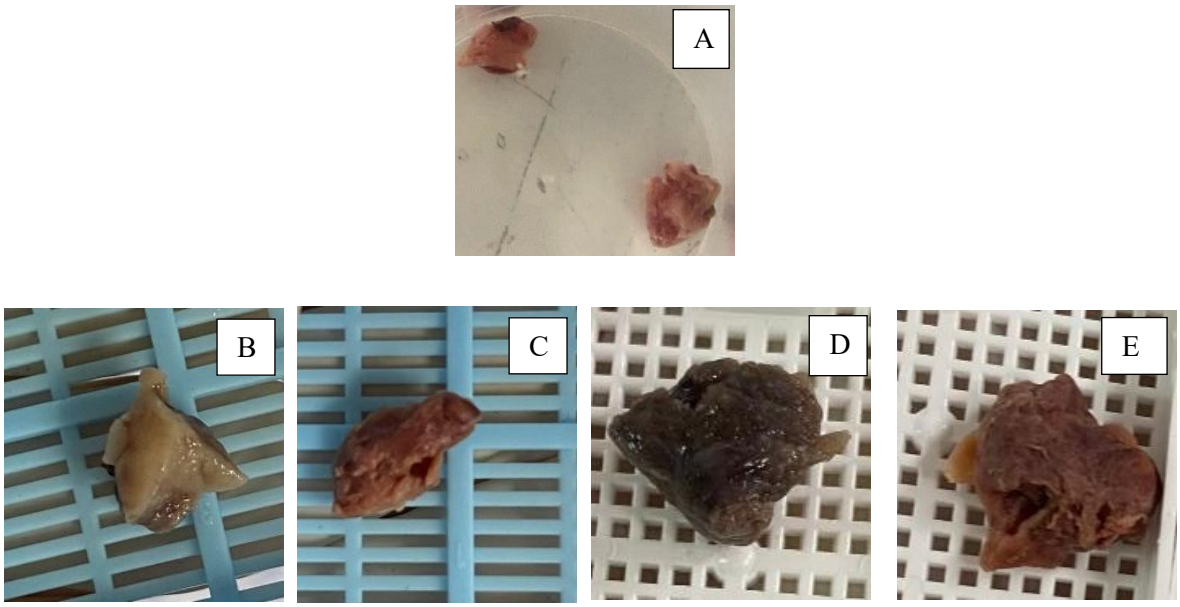

Figure 1: Physical appearance of thyroid tissue sections at the grossing (A), 24 hours after fixation in formalin (B) and 70% ethanol (C), and two weeks after fixation in formalin (D) and 70% ethanol (E) (Scale bar: 10 mm).

Histomorphology: A total of 200 H & E-stained sections (24-hour and two-week 10% formal saline and ethanol fixed sections of 40 samples and their reference slides) were examined for the assessment of the effectiveness of 70% ethanol and 10% formal saline in tissue fixation. The findings are shown in Table 2 and Figure 2. When comparing the total histological scores of the tissue sections prepared after fixing for 24 hours and for two weeks in 10% formal saline and 70% ethanol with the reference slide, a statistically significant difference in scores was observed only with reference to the sections prepared following two weeks of fixation in 70% ethanol ($p=0.032$).

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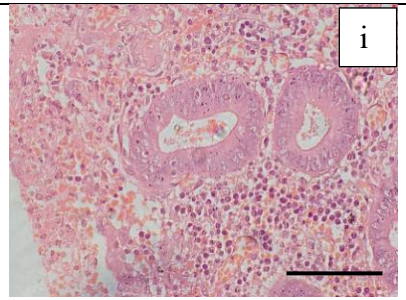
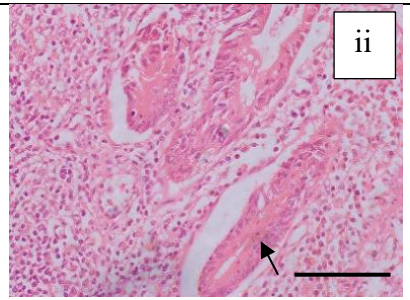
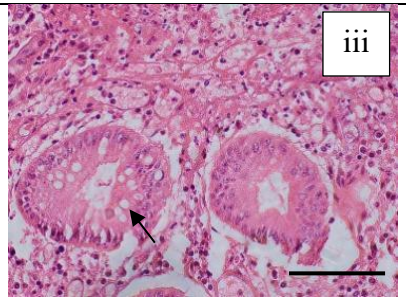
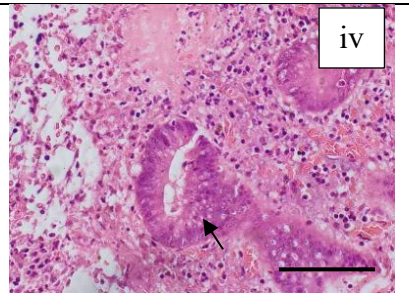
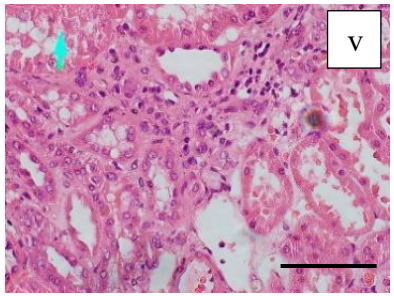
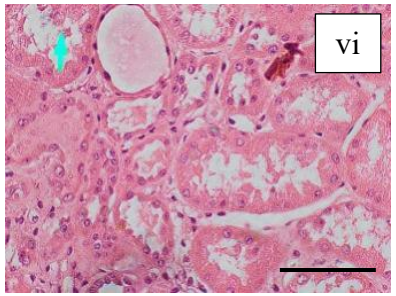
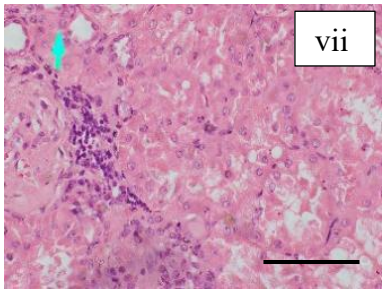
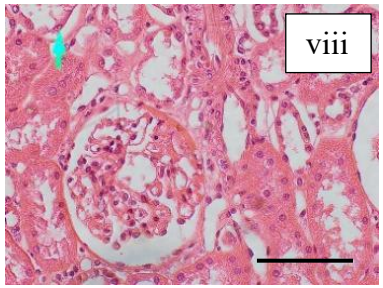
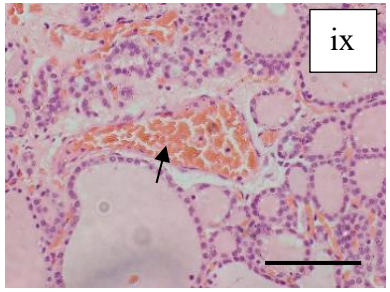
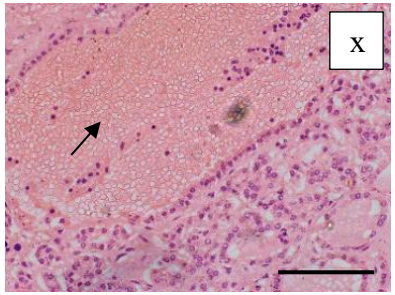
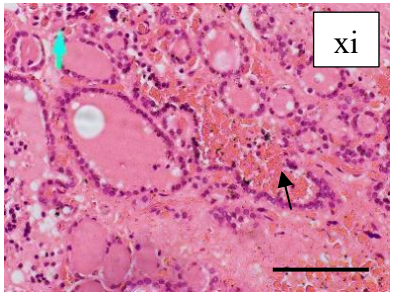
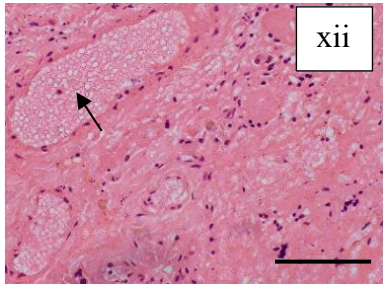
Features	24-hour formalin	24-hour ethanol	2- week formalin	2- week ethanol
Preservation of cellular details				
Preservation of nuclear details				
Extra cellular component preservation (preservation of red cells)				

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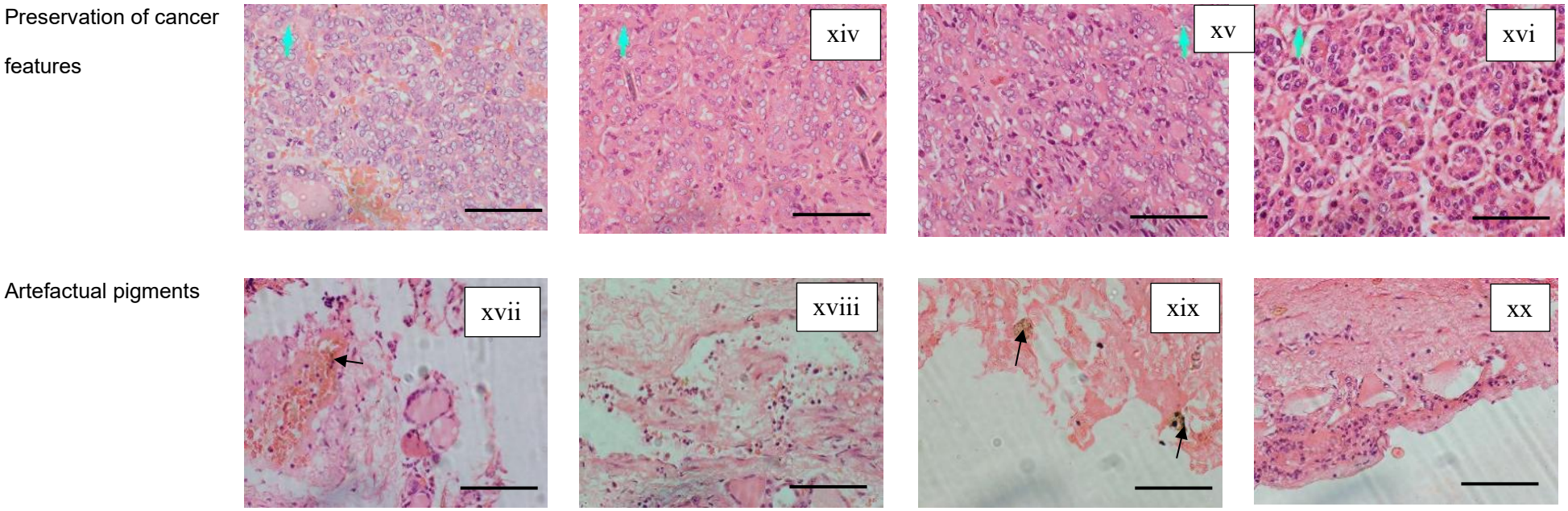

Figure 2: Histological features of tissue sections prepared following fixation in formalin and 70% ethanol for a period of 24-hours and two weeks (x400 magnification, Scale bar: 50 µm)). The photomicrographs of the appendix (i-iv), kidney tissues (v-viii), normal thyroid tissues (ix-xii and xvii-xx) and thyroid carcinoma (xiii-xvi) are shown in the figure and the arrows in the respective photomicrographs indicate cytoplasmic vacuolation (iii, iv), loss of nuclear details (v-viii), differences in preservation of red blood cells (ix-xii), preservation of nuclear and cytoplasmic changes observed in thyroid carcinoma (xiii-xvi) and formalin pigment deposition (vii, ix).

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Table 2: Semi-quantitative assessment of histology following fixation in 10% formal saline and 70% ethanol

Parameters	**10% Formal saline**				**70% Ethanol**				**Reference slide (24-hour in 10% formal saline)**
	24-hour	**P values**	**2-weeks**	**P values**	**24-hour**	**P values**	**2-weeks**	**P values**	
Physical properties									
Disruption	3.47±0.71	0.996	3.15±0.80	0.371	3.20±0.97	0.550	3.05±0.84	0.133	3.42±0.74
Adhesion	3.75±0.63	0.840	3.78±0.48	0.712	3.65±0.66	1.000	3.87±0.33	0.220	3.70±0.68
Cracking	3.65±0.53	0.491	3.40±0.66	0.332	3.37±0.67	0.244	3.12±0.82*	0.013	3.60±0.59
Proper section thickness (3 µm)	3.90±0.37	0.995	3.87±0.40	1.000	3.87±0.40	1.000	3.92±0.26	0.936	3.87±0.40
Nuclear details									
Preservation of nucleolus	3.42±0.63	1.000	3.50±0.67	0.955	3.62±0.58	0.412	3.65±0.57	0.308	3.45±0.63
Preservation of chromatin details	3.35±0.69	1.000	3.55±0.63	0.163	3.67±0.57*	0.022	3.67±0.61*	0.016	3.35±0.69
Preservation of nuclear envelop	3.52±0.59	0.999	3.62±0.62	0.950	3.65±0.57	0.873	3.65±0.86	0.873	3.55±0.59
Vacuolation	3.40±0.67	0.992	3.40±0.70	0.992	3.57±0.67	0.822	3.37±0.66	0.965	3.45±0.63
Shrinkage/ swelling	3.75±0.49	1.000	3.57±0.59	0.174	3.57±0.59	0.174	3.12±0.88*	0.000	3.77±0.47
Cytoplasmic details									
Cohesiveness	3.20±0.51	0.566	3.00±0.59*	0.037	2.75±0.74*	0.000	2.72±0.64*	0.000	3.22±0.47
Uniformity preserved	3.17±0.50	0.602	2.87±0.64*	0.011	2.85±0.89*	0.028	2.65±0.86*	0.001	3.25±0.49
Texture shown	3.35±0.57	0.746	2.92±0.79*	0.016	2.72±0.84*	0.001	2.47±0.75*	0.000	3.37±0.58
Vacuolation	3.62±0.49	1.000	3.42±0.71	0.306	3.55±0.55	0.932	3.62±0.49	1.000	3.60±0.49
Cell border defined	2.85±0.76	1.000	2.87±0.93	1.000	3.20±0.75	0.242	3.10±0.84	0.563	2.85±0.80
Shrinkage/swelling	3.62±0.49	0.850	3.17±0.63*	0.003	2.92±0.57*	0.000	2.72±0.72*	0.000	3.60±0.49
Extracellular component and muscle (collagen/elastin) preservation	3.57±0.50	0.878	3.45±0.55	0.372	2.40±0.74*	0.000	2.35±0.83*	0.000	3.60±0.49
H &E staining									
Uniformity of H & E staining	3.32±0.57	0.999	3.42±0.67	0.812	3.45±0.71	0.699	3.50±0.75	0.461	3.32±0.57
Nuclear staining	3.37±0.54	0.999	3.40±0.54	0.984	3.27±0.59	0.935	3.35±0.57	1.000	3.37±0.54
Cytoplasmic staining	3.35±0.57	0.999	3.52±0.55	0.355	3.35±0.62	0.999	3.47±0.59	0.608	3.35±0.57
Artefactual pigment deposition	3.35±0.80	0.940	3.35±0.66	0.581	3.87±0.40*	0.000	3.95±0.22*	0.000	3.35±0.80
Total histological score	69.03±4.93	1.000	67.27±6.06	0.617	66.55±5.54	0.276	65.38±6.74*	0.032	69.08±4.91

Semi-quantitative assessment was carried out across ten random fields for the selected features based on a 4-point scale of 1= Poor, 2= Satisfactory, 3= Good, 4= Excellent with reference to quality. Results are expressed as mean ± standard deviation (SD). Statistical significance was defined as *$p<0.05$ compared to the respective reference tissue section

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Interestingly, neither sections fixed in 10% formal saline (for both 24-hours and two weeks) nor ethanol for 24-hours showed significant differences in total scores compared to the reference slide of each tissue ($p>0.05$). During multiple comparisons of tissue sections, the sections prepared after 24 hours of fixation in 10% formal saline also showed a significant increase in the total histological scores compared to the sections prepared following two weeks of fixation in 70% ethanol ($p=0.36$).

None of the tissue sections showed statistically significant changes in scores compared to the reference tissues regarding the physical properties assessed, including tissue adhesion, tissue disruption, and section thickness in the present study ($p>0.05$). With reference to the preservation of nuclear details, significant increase in preservation of chromatin details was observed in tissue sections prepared both 24 hours ($p=0.022$) and two weeks ($p=0.016$) following fixation in 70% ethanol and significant shrinkage of nuclei was observed in the tissue sections prepared two weeks following fixation in 70% ethanol ($p=0.000$) when compared to the reference sections. All the other parameters, including preservation of nucleolus, the appearance of the nuclear envelope, and nuclear vacuolization, showed comparable results to the reference tissue sections. However, the tissues preserved in 70% ethanol exhibited higher scores than that of the respective formalin-fixed tissue sections and the reference tissue sections with respect to preservation of nucleolus, chromatin details, nuclear envelop, and absence of vacuolation.

Several features of cytoplasmic preservation, including cytoplasmic cohesiveness, uniformity, texture, cytoplasmic shrinkage/swelling, and preservation of extracellular components, showed comparatively low scores in tissue sections prepared from specimens fixed in ethanol for 24-hours and two weeks durations compared to the respective formalin-fixed specimens. However, the cell border showed high scores in ethanol-fixed tissues fixed for 24 hours as well for two weeks when compared to both respective formalin-fixed sections and the reference sections.

With reference to the uniformity in staining and artefactual pigment deposition, the ethanol-fixed tissues showed higher scores both in 24 hours and two weeks of 70% ethanol fixation compared to the respective formalin-fixed tissues. The ethanol-fixed tissues showed statistically significant changes in artefactual pigment deposition as well ($p=000$). Relatively higher deposition of artefactual pigments was observed in the tissue sections fixed in 10% formal saline for 24 hours (Figure 2; xvii) and two-weeks (Figure 2; xix), showing lower scores compared to 70% ethanol. Yet, the nuclear and cytoplasmic staining showed comparable results in both ethanol and formalin-fixed sections.

Two weeks of fixation in ethanol showed increased histological scores for adhesion and section thickness compared to the 24-hour ethanol fixed tissues. Yet, the histological scores for tissue disruption and cracking of tissues following two-week fixation were relatively low in two weeks fixation in ethanol. Similarly, the scores for nuclear vacuolation, nuclear shrinkage, cytoplasmic cohesiveness, uniformity, texture, preservation of cellular border, cytoplasmic shrinkage/swelling and extracellular component preservation were decreased during long-term fixation in ethanol compared to its short-term fixation. However, the changes were not statistically significant ($p=0.997$). Yet, long-term fixation in ethanol resulted in increased scores for uniformity of staining, quality of nuclear and cytoplasmic staining, and artefactual pigment deposition compared to short-term fixation. However, none of the fixation methods or periods affected the preservation of cancer cells adversely. Histopathological features of cancerous tissues such as prominent nucleoli, irregular nuclear size and shape, changes in chromatin condensation patterns, nuclear vacuolation, altered cytoplasmic staining intensity were observed in cancer tissues fixed for both 24 hours and two weeks in the selected fixatives in the present study.

DISCUSSION

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Formalin remains the gold standard for tissue fixation due to its ability to effectively cross-link proteins, thereby preserving tissue morphology with high fidelity over long periods [9]. Formalin facilitates better preservation of cellular structures and reduces the risk of morphological changes during tissue processing [10]. Yet, given the safety concerns and public health risks, researchers often seek alternative solutions for histopathology sample processing to minimize the risks associated with formalin fixation. Therefore, the present study was carried out to assess the suitability of using 70% ethanol instead of formalin-based fixatives for tissue fixation.

The effectiveness of ethanol in tissue fixation has been investigated in some studies and the results obtained were controversial. However, almost all the studies have been carried out using experimental animals [3, 4, 7], and this is the first study which is reported on human tissues to date.

The concentration of a fixative plays a significant role in its effectiveness. For example, the role of ethanol in fixation is primarily to dehydrate tissues, and different concentrations can affect the rate and extent of dehydration. In general, high concentrations of ethanol can lead to more rapid dehydration but may also cause excessive shrinkage or distortion of tissue structures. Conversely, low concentrations might result in slower dehydration but potentially better preservation of tissue morphology [10]. In the present study, the effectiveness of 70% ethanol in tissue fixation was evaluated by considering the use of particular concentration in the first step of tissue processing as the initial ethanol concentration of the ethanol series. Further, the use of 70% ethanol in animal tissue preservation has been well documented [6].

The study evaluated the effectiveness of 70% ethanol as a tissue fixation agent with a focus on both short-term and long-term applications. Ideally, the histology specimens should be processed 24 hours after fixation, and therefore, the short-term effectiveness of the fixatives was assessed following 24 hours of fixation. However, tissue processing following 24-hour fixation is not possible with the large number of test requests received for routine histopathology laboratories in Sri Lankan healthcare setting. Therefore, the long-term effect was evaluated following two weeks of fixation in the selected fixatives in the present study.

Assessment of the physical properties of the tissue sections fixed in 10% formal saline and 70% ethanol for the period of 24 hours and two weeks revealed differences in the color of tissues. No shrinkage/ swelling was observed in either tissue affecting the size of the specimens. Further, the hardness of the sections was normal. However, it is worth noticing that the tissue sections fixed in 10% formal saline and 70% ethanol for the investigations in the present study were small biopsies from the original entire specimens. Hence, more variations in the histological scores could be possible due to small specimen sizes. To minimize the potential errors that could occur with the fixation of small biopsies rather than the entire specimen in the selected fixatives, the H & E-stained sections fixed in ethanol and formal saline for 24 hours and two weeks were compared against a reference slide of the particular tissue prepared from the original tissue in which the entire specimen was fixed in 10% formal saline by the reference laboratory.

The results of the semi-quantitative assessment revealed that short-term fixation (24 hour- fixation) in 70% ethanol is quite effective in maintaining the structural integrity of human tissues. During 24-hour fixation, tissues preserved in 70% ethanol exhibited better preservation of nuclear details as indicated by higher scores than that of the respective formalin-fixed tissue sections and the reference tissue sections. Better preservation of nucleolus, absence of vacuolization and reduced nuclear shrinkage were observed in two weeks ethanol fixed tissue sections. Interestingly, the tissues fixed in 70% ethanol for 24 hours and two weeks showed comparable scores with reference to the preservation chromatin details, and nuclear envelop. However, a significant increase in nuclear shrinkage was observed in ethanol-fixed tissues, two weeks following fixation, compared to the reference

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tissues as depicted by reduced histological scores.

The features, including cohesiveness, preservation of uniformity, texture, cytoplasmic shrinkage, and extracellular component preservation showed relatively lower scores in ethanol-fixed tissues indicating comparatively poor tissue preservation compared to formalin. Histological scores for cytoplasmic shrinkage and preservation of extracellular components were significantly lower (p=0.000) in ethanol-fixed tissues than that in reference tissues further substantiating poor fixation by ethanol. However, cell borders were well defined in ethanol fixed tissues in 24 hours as well as two weeks when compared to respective formalin-fixed sections as well as to the reference sections as depicted by higher scores.

Increased nuclear and cytoplasmic shrinkage by ethanol might be attributed to the dehydrating effect of ethanol [6]. The dehydration process reduces tissue water content and halts enzymatic activities that could otherwise lead to tissue degradation. The relative shrinkage by ethanol fixation has been reported in previous studies as well [11].

Better uniformity in H & E staining and absence of artefactual pigment depositions could be observed during 24 hour and two weeks of fixation with 70% ethanol as demonstrated by increased scores in the present study. The brown color formalin pigments could be observed following both 24 hour and two-weeks of fixation in 10% formal saline. The reaction of formalin with blood or the acidic nature of the formalin solution due to oxidation of formaldehyde to formic acid might be the possible reasons for appearance of formalin pigments in H & E-stained tissue sections. Even though this effect could be overcome by using a buffered formal saline solution, it was avoided in the present study to simulate the actual current practice in the state hospital set-up. However, nuclear and cytoplasmic staining showed comparable results to the respective formalin-fixed sections indicating no adverse effect of ethanol fixation on nuclear and cytoplasmic staining during the H & E procedure.

Further, two-week fixation in ethanol led to improving the physical qualities of the sections as demonstrated by increased scores concerning adhesion and section thickness compared to the 24-hour ethanol fixed tissues. Yet, the results showed increased disruption and cracking of tissues following two-week fixation. However, the values were not statistically significant in either situation. Similarly, nuclear vacuolization, nuclear shrinkage, cytoplasmic cohesiveness, uniformity, texture, preservation of cell border, cytoplasmic shrinkage/swelling and extracellular component and muscle preservation were worsened during two weeks fixation in 70% ethanol compared to its 24-hour fixation, as shown by relatively reduced scores. Prolonged exposure to ethanol might have led to significant morphological distortions due to the progressive dehydration effect of ethanol, which continued to remove water from tissues even after the initial fixation phase. The continuous loss of water over time might cause increased brittleness of tissues and make tissues more prone to structural distortion [10].

Interestingly, the quality of the H & E-stained sections increased due to two weeks of fixation in ethanol compared to 24 hours fixation. Long-term fixation in ethanol showed increased scores in uniformity of staining, quality of cytoplasmic staining, and artefactual pigment deposition even compared to reference sections.

The use of ethanol as a potential fixative is more advantageous in a resource-poor setting like Sri Lanka. The significant increase in the imported price of absolute ethanol with the economic crisis of the country has adversely affected routine histopathology sample processing in current healthcare settings. Yet, the possibility of having 95% ethanol as a byproduct from sugar factories in Sevanagala and Palewatte has become a better solution for the practical issues associated with the use of ethanol in histopathology tissue processing. Even 95% ethanol purchased from the sugar factories is

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cheaper than formalin, and its usage is quite economical and sustainable to the country. Therefore, the findings of the present study would generate new vistas for the effective and sustainable use of rectified spirit of sugar refineries as well. Further, the potential safety to the healthcare personnel in using ethanol compared to formalin is an additional advantage of using 70% ethanol as a potential fixative in routine histopathology sample processing.

Limitations

The size of the specimens tested in the present study were small biopsies excised from the original entire specimens. However, the outcome might be different in the actual situation where entire specimen is preserved in the respective fixation agent, rather than a small piece from the original tissue.

Even though the accepted best practice is to preserve the specimens in the respective fixative for 24 hours before the commencement of tissue processing, it is not often possible with the large number of test requests received for routine histopathology laboratories in Sri Lankan healthcare setting.

CONCLUSIONS

Fixation of tissues in 70% ethanol for 24 hours was proven to be effective however, an extension of the duration of fixation worsened nuclear vacuolation, nuclear shrinkage, cytoplasmic cohesiveness, uniformity, texture, preservation of cell border, and cytoplasmic shrinkage/swelling of tissue sections. The study highlights the effectiveness of 70% ethanol fixation in the short term and the need for alternative fixation methods for applications requiring extended tissue fixation, considering potential safety, efficacy, and cost-effectiveness associated with ethanol fixation. Further studies are warranted for the development of alternative ethanol-based fixatives for the long-term fixation of human tissues in limited resource settings.

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References

1. Haque Z, Rahman MA, Zahirul M, et al. Alcohol-based fixatives can better preserve tissue morphology than formalin. International Journal of Morphology 2020;38(5):1371-1375.
2. Patil S, Rao RS, Ganavi BS, et al. Natural sweeteners as fixatives in histopathology. Journal of Natural Science, Biology and Medicine 2015;6(1):67-70.
3. Warmington AR, Wilkinson JM, Riley CB. Evaluation of ethanol-based fixatives as a substitute for formalin in diagnostic clinical laboratories. Journal of Histotechnology 2000;23(4):299-308.
4. Bosetti C, McLaughlin JK, Tarone RE, et al. Formaldehyde and cancer risk: a quantitative review of cohort studies through 2006. Annals of Oncology 2008;19(1):29-43.
5. Rahman MA, Sultana N, Ayman U, et al. Alcoholic fixation over formalin fixation: A new, safer option for morphologic and molecular analysis of tissues. Saudi Journal of Biological Sciences 2022;29(1):175-182.
6. Perry C, Chung JY, Ylaya K, et al. A buffered alcohol-based fixative for histomorphologic and molecular applications. Journal of Histochemistry and Cytochemistry 2016;64(7):425-440.
7. Bancroft JD, Gamble M. Theory and Practice of Histological Techniques. 2008; Elsevier, UK.
8. Rolls G, Farmer N, Tarbet F. Assessing the Quality of Tissue Processing and the Performance of PelorisTM using the Leica Microsystems Scoring System. Leica Microsystems 2008. https://api.semanticscholar.org/CorpusID:611170 4
9. Panzacchi S, Gnudi F, Mandrioli D, et al. Effects of short and long-term alcohol-based fixation on Sprague-Dawley rat tissue morphology, protein, and nucleic acid preservation. Acta Histochemica 2019;121(6):750-760.
10. Troiano NW, Ciovacco WA, Kacena MA, et al. The effects of fixation and dehydration on the histological quality of undecalcified murine bone specimens embedded in methylmethacrylate. Journal of Histotechnology 2009;32(1):27-31.
11. Howat WJ, Wilson BA. Tissue fixation and the effect of molecular fixatives on downstream staining procedures. Methods 2014;70(1):12-19.