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Qualitative analysis of histochemical and immunohistochemical properties of EGF (ethanol, glycerol, and formalin) fixative

Buvaneswari V[1], Deepadevi G[2], Peter Samidoss A[3], Muthu Prabha MS[4], Vinoth Kumar V[5]

[1,3,4]Assistant Professor, Department of Pathology, Government Theni Medical College, Theni, [2]Assistant Professor, Department of Pathology, Government Erode Medical College, Erode, [5]Assistant Professor, Department of Community Medicine, Government Medical College, Kallakurichi, Tamil Nadu, India

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ABSTRACT

Background: Fixation is essential in histopathology to preserve tissue integrity before analysis, with neutral buffered formalin being the gold standard for decades because of its effective protein cross-linking properties. **Aims and Objectives:** This study aimed to evaluate the histochemical and immunohistochemical properties of ethanol, glycerol, and formalin (EGF) fixative and to compare and analyze the impact of fixation using conventional formalin and EGF fixative on special histochemical stains and immunohistochemical studies. **Materials and Methods:** A minimal formalin containing EGF fixative was prepared with a specific concentration of EGF and hypotonic saline. Tissue specimens were collected directly from the surgical operation theatre and were fixed in EGF fixative and formalin fixative. After fixation and processing, the tissues were subjected to various Histochemical stains, including periodic-acid Schiff (PAS), Alcian blue-PAS, Alcian blue, Masson's Trichrome, Van Gieson, and Reticulin, along with immunohistochemical staining for E-cadherin, human epidermal growth factor receptor 2/neu, and Ki-67. Comparative analyses were done for both fixatives on histochemical and immunohistochemistry stains. **Results:** This study assessed the efficacy of EGF fixative and is compared with conventional formalin for histochemical and immunohistochemical stains which was found to be effective. This minimal formalin-containing EGF fixative was qualitatively comparable to conventional formalin fixative for histochemical and immunohistochemical stains. **Conclusion:** EGF fixative is a safe alternative to conventional formalin for routine histochemical and immunohistochemical staining, offering a means to mitigate the health risks associated with formalin exposure among health-care professionals.

Key words: Fixation; Histochemical stains; Immunohistochemical staining; Ethanol, glycerol, formalin fixative; Formalin; Tissue preservation

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INTRODUCTION

Fixation is a crucial step in tissue preparation for histopathology, as it conserves tissue before analysis.[1] Ideal fixative should penetrate a tissue quickly, be isotonic, cause minimum loss of physical and chemical alteration of tissue and its components, be rapid in action, and allow isolation of macromolecules, including protein and DNA, without significant biochemical alterations.[2] Neutral buffered formalin (NBF) has been the gold standard fixative in surgical pathology for decades.[1] Formaldehyde has been the most used fixative for more than 150 years.[3] Formaldehyde acts by cross-linking of proteins in tissues by creating methylene bridges among them.[4] The advantages of formalin are low-cost, easy to prepare in laboratory, it preserves morphology of tissues allowing it do special stains and immunohistochemistry (IHC) studies in Formalin fixed tissues.[2] However, formalin toxicity is a serious and

Address for Correspondence:
Dr. Vinoth Kumar V, Assistant Professor, Department of Community Medicine, Government Medical College, Kallakurichi, Tamil Nadu, India. **Mobile:** +91-9551332114. **E-mail:** vinoth7799@gmail.com

major deterrent to its prolonged and continuous use in laboratory practice.[5] The formaldehyde was classified as carcinogenic to humans (group 1) by the International Agency for Research on Cancer and, therefore, represents a risk to anyone handling the solution.[2]

Another consideration is that the pathology laboratory uses much formalin, and hazards are often underestimated because pathologists and technicians are continuously and daily exposed to formaldehyde solution.[6] Furthermore, daily exposure is seen in large quantities, so we must not underestimate the risk of formaldehyde as a chemical carcinogen.[7] Even at relatively low levels, formaldehyde causes allergic reactions and skin irritation in some individuals.[6] Formaldehyde exposure was associated with increased risk for Hodgkin's lymphoma, multiple myeloma, and leukemia, particularly myeloid leukemia, which increased with peak and average intensity of exposure as mentioned by International Agency for Research on Cancer in 2012.[7]

Over the past 20 years, several laboratories have attempted to replace formalin with other less toxic fixatives.[1] Other less toxic alcohol-based cross-linking fixatives and non-cross-linking fixatives have been proposed as NBF alternatives.[2] However, the results of replacing formalin were unsatisfactory owing to cellular morphology and antigenicity. The search for an alternative to formalin fixation, which offers better technical performance and greater protection for health workers, is unavoidably needed.[8]

In this study, an attempt to reduce formalin exposure in the histopathology laboratory was made by reducing the concentration of formalin. A minimal formalin-containing compound fixative was prepared by varying concentrations of ethanol, glycerol, formalin (EGF), and hypotonic saline.[6] In the previous study by Muthuselvi et al., it was proven that our EGF fixative is suitable for histopathological studies of routine surgical specimens and that we can easily prepare these minimal formalin-containing fixatives in the laboratory.[9]

In recent years, efforts have been made to develop fewer toxic alternatives to formalin fixation that retain the ability to preserve tissue morphology and antigenicity for histochemical and immunohistochemical analysis. To address these risks, we explored the potential of a minimal formalin-containing fixative EGF–comprised of ethanol, glycerol, formalin and hypotonic saline in varying concentrations. This fixative was designed to reduce formalin exposure while maintaining the necessary tissue preservation characteristics.

Aims and objectives

This study aimed to evaluate the histochemical and immunochemical properties of EGF fixative and to compare and analyze the impact of fixation using conventional formalin and EGF fixative on special histochemical stains and immunohistochemical studies.

MATERIALS AND METHODS

Fixatives

Fixatives were stored and used at room temperature. A compound EGF fixative with minimal formaldehyde was prepared using EGF and hypotonic saline. The concentrations of ethanol, glycerol, and formalin used were 30%, 5%, and 6%, respectively. To prepare 100 mL of EGF fixative, all the necessary solutions were added. The pH of the fixative was maintained at 7.2–7.4 by adding sodium dihydrogen phosphate monohydrate and anhydrous disodium hydrogen phosphate. Hypotonic saline was prepared by dissolving 3 g sodium chloride in 300 mL of distilled water, of which 270 mL was mixed with 120 mL of distilled water. Ethanol induces cell shrinkage, as it is a dehydrative fixative. Hypotonic saline was then added to overcome these side effects. Glycerol was added to minimize evaporation. To monitor the color of the fixative, methylene blue was added to avoid the tendency to smell the solution.

Tissue sampling and tissue fixation

Multiple human tissue samples from various sites such as the liver, aorta, lymph nodes, colon, small intestine, breast, skin, and stomach with normal morphology as well as lesions (carcinoma, inflammatory) were freshly collected directly from surgical theaters and brought to the Department of Pathology. For this study, each biopsy was split into two and was fixed in EGF fixative and formalin. The duration of the fixation process was 8 h at room temperature. Tissues were processed using increasing concentrations of isopropyl alcohol (60–100%), xylene, and wax and then embedded in paraffin. Microtome sections (4 μm thick) were stained in hematoxylin and eosin stain and further subjected to histochemical and Immunohistochemical studies.

Histochemistry

Periodic-acid Schiff (PAS), Alcian blue, Alcian blue-PAS, Masson trichrome, Van Gieson, and Reticulin were applied to tissues fixed in EGF fixative and formalin fixative. Standard deparaffinization, rehydration, and manual staining protocols were followed for each stain, including hydration using graded alcohols, specific dye applications, counterstaining, and mounting with dibutyl phthalate in xylene.

IHC

The proteins investigated were E-cadherin, Ki67, and human epidermal growth factor receptor 2 (HER2/neu). The manual method of immunohistochemical analysis was performed on 4 μm sections of fixed, paraffin-embedded tissue. After deparaffinization and hydration, specific antigen retrieval was applied. All samples were processed according to the standard protocol.

Microscopic evaluation

A blinded evaluation was carried out by two experienced pathologists for the histochemical and immunohistochemical stains. Qualitative assessment of the staining in all histochemical stains and immunohistochemical staining was graded. The results were tabulated and analyzed with an unpaired T-test. $P<0.05$ is considered statistically significant.

Histochemical evaluation

The slides stained with histochemical stains were assessed for parameters composed of non-specific staining, background staining, crispness of nuclear stain, crispness of cytoplasmic stain, staining of necrotic tissues, uniformity of staining, changes seen after excessive staining, and excessive washing.[10] All these were taken into consideration for grading, and slides were graded[5] as 0, 1, and 2.

Grade 0 – inadequate for diagnostics-insufficient.

Grade 1 – Quality reasonable for diagnostics-intermediate.

Grade 2 – Quality good for diagnostics-optimal.

Immunohistochemical evaluation

The slides stained with IHC were graded based on the positivity of the stain (nuclear, membranous, and cytoplasmic), uniform distribution, intensity of the stain, background staining, and non-specific staining.[4] All the above-mentioned criteria were assessed, and stained slides were graded[2] as 0, 1, and 2.

Grade 0 – inadequate for diagnostics-insufficient.

Grade 1-Quality reasonable for diagnostics-intermediate.

Grade 2 – Quality good for diagnostics-optimal.

Statistical analysis

Data were collected, entered, and double-checked using a Microsoft Excel spreadsheet. The data were presented as frequencies and percentages and were analyzed using MS Excel and SPSS. A qualitative assessment of all histochemical and immunohistochemical stains was performed, and the results were tabulated and analyzed using an unpaired t-test. Statistical significance was set at $P<0.05$.

RESULTS

In total, 39 specimens were fixed in both the conventional and EGF fixatives. Among them, 11 (28%) cases were colon, followed by 7 cases (18%) were stomach, 7 (18%) cases were liver, 4 cases (10%) were aorta, 4 (10%) cases being breast, 4 (10%) skin cases, 1 (3%) lymph node, 1 case (3%) small intestine.

Out of 39 specimens, 33 specimens were stained with histochemical stains. Out of that 33 specimens, seven specimens were stained with Alcian blue, seven specimens were stained with Reticulin, five specimens were stained with Alcian blue – PAS, five specimens were stained with PAS, four specimens were stained with Van Gieson, and five specimens were stained with Masson trichrome.

IHC staining for E-cadherin in two breast specimens helped assess cell adhesion, with loss of expression indicating invasive ductal carcinoma. HER2/neu staining in two breast specimens revealed HER2 overexpression, which suggests aggressive breast cancer and the potential for targeted therapy. Ki-67 staining in two colon specimens evaluated tumor proliferation, with a high Ki-67 index indicating rapid cell growth and a more aggressive tumor, whereas a lower index suggested a less aggressive cancer with a potentially better prognosis.

On comparing NBF with EGF fixative stained with PAS stain, Out of five cases, three cases show non-specific staining, background staining, crisp nucleus and cytoplasm, uniformity of staining, staining of necrotic tissue was well demarcated. Extracellular components staining in EGF fixative were comparable to conventional formalin, earning a grade 2 score. In two cases, nuclear details and cytoplasmic details in EGF fixative were not crisp when compared to conventional formalin but interpretation was still possible, and were given grade 1. There is no significant difference between EGF fixative and conventional formalin with PAS stain ($P=0.105$) (Figure 1).

On comparing NBF with EGF fixative stained with Alcian blue, out of seven cases, four cases of non-specific staining, background staining, crisp nucleus, crisp cytoplasm, uniformity of staining, staining of necrotic tissue was well demarcated. In extracellular components staining, there is no change in stain intensity even after excessive staining and excessive washing. These four cases of EGF fixative are comparable to conventional formalin and given a grade 2 score. In three cases, uniformity of staining and crisp cytoplasmic details was not comparable to conventional

Table 1: Comparison of conventional formalin versus EGF fixative with PAS stain, Alcian blue stain, Alcian blue–PAS stain

Specimen	Histological diagnosis	Formalin grade	EGF grade	P-value
Stomach with PAS stain (n=5)	Signet ring cell carcinoma	2	2	0.105
	Moderately differentiated adenocarcinoma	2	1	
	Mucinous adenocarcinoma stomach	2	2	
	Poorly differentiated adenocarcinoma	2	1	
	Moderately differentiated adenocarcinoma	2	2	
	Mean±SD	2±0	1.6±0.48	
Colon with Alcian blue stain (n=6)	Moderately differentiated adenocarcinoma rectum	2	2	0.09
		2	1	
		2	2	
	Well-differentiated adenocarcinoma rectum	2	2	
	Moderately differentiated adenocarcinoma	2	2	
	Well-differentiated adenocarcinoma	2	1	
Small intestine with Alcian blue stain (n=1)	Normal histomorphology	2	1	
	Mean±SD	2±0	1.57±0.49	
Colon with Alcian blue–PAS stain (n=3)	Moderately differentiated adenocarcinoma	2	2	0.105
		2	2	
		2	1	
Stomach with Alcian blue–PAS stain (n=2)	Moderately differentiated adenocarcinoma	2	1	
	Signet ring cell carcinoma	2	2	
	Mean±SD	2±0	1.6±0.48	

EGF: Ethanol, glycerol, formalin, PAS: Periodic-acid Schiff, SD: Standard deviation

formalin, but the interpretation is still possible and was given grade 1 (quality reasonable for diagnostics). EGF fixative and conventional formalin with Alcian blue stain show no significant difference (P=0.09) (Table 1).

Five cases were stained with Alcian blue–PAS stain, three cases in EGF fixative had non-specific staining, background staining, crisp nucleus, crisp cytoplasm, uniformity of staining, staining of necrotic tissue was well demarcated, staining of extracellular components in EGF fixative comparable to conventional formalin earning grade 2 score. Two cases showed non-uniformity of staining, no crisp cytoplasmic details, and interpretation of slides was still possible.

There is no significant difference between EGF fixative and conventional formalin with Alcian blue–PAS stain (P=0.105) (Table 1).

Seven cases stained with reticulin stain showed that four cases in EGF fixative had non-specific staining, background staining, crisp nuclei, and cytoplasm, along with well-demarcated necrotic tissue. These cases demonstrated no change in stain intensity after excessive staining and washing, earning a grade 2 score, comparable to conventional formalin. In three cases, non-uniform staining and non-specific staining were noted, resulting in a grade 1 score; however, interpretation remained possible. There was no significant difference between EGF fixative and conventional formalin for Reticulin stain (P=0.09) (Figure 2).

Four cases stained with Masson trichrome revealed that three cases in EGF fixative exhibited non-specific staining,

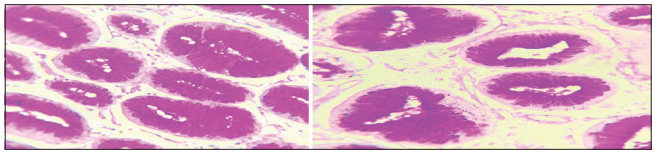

Figure 1: Histochemistry. Photomicrograph of representative sections from adenocarcinoma stomach, stained with PAS stain fixed in a. Conventional formalin (left) b. EGF fixative (40 x)

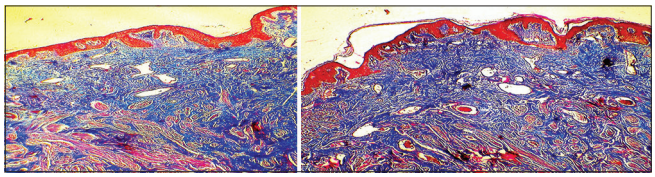

Figure 2: Histochemistry. Photomicrograph of representative sections from skin stained with Masson trichrome stain fixed in a. Conventional formalin (left) b. EGF fixative (10 x)

background staining, crisp nuclei, and cytoplasm, along with uniformity of staining and well-demarcated necrotic tissue. These cases showed no change in stain intensity after excessive staining and washing, earning a grade 2 score comparable to conventional formalin. Two cases received a grade 1 score due to non-uniform staining and lack of crisp cytoplasmic details; however, interpretation remained possible. There was no significant difference between EGF fixative and conventional formalin for the Masson trichrome stain (P=0.105) (Table 2).

Four cases were stained with Van Gieson stain. In two cases in EGF, fixative shows non-specific staining, background staining, crisp nuclei, and cytoplasm were observed, along with uniformity of staining and well-demarcated necrotic tissue.

Table 2: Comparison of conventional formalin versus EGF fixative with Reticulin stain, Masson trichrome stain

Specimen	Histological diagnosis	Formalin grade	EGF grade	P-value
Liver with Reticulin stain (n=6)	Normal histomorphology	2	1	0.09
	Fatty change	2	2	
	Cirrhosis	2	2	
	Normal histomorphology	2	1	
		2	1	
Lymph node with Reticulin stain (n=1)	Fatty change	2	2	
	Metastatic ductal adenocarcinoma deposits	2	2	
	Mean±SD	2±0	1.57±0.49	
Skin with Masson trichrome stain (n=4)	Normal histomorphology	2	1	0.105
		2	1	
		2	2	
		2	2	
Liver with Masson trichrome stain (n=1)	Cirrhosis	2	2	
	Mean±SD	2±0	1.6±0.48	

EGF: Ethanol, glycerol, formalin, SD: Standard deviation

Table 3: Comparison of conventional formalin versus EGF fixative with Van Gieson stain, IHC-E-cadherin marker, IHC-HER2/neu marker, IHC-KI 67 marker

Specimen	Histological diagnosis	Formalin grade	EGF grade	P-value
Aorta	Normal histomorphology (n=4)	2	1	0.09
		2	1	
		2	2	
		2	2	
	Mean±SD	2±0	1.5±0.5	
Breast with IHC-E-cadherin marker	Invasive ductal carcinoma breast (n=2)	2	1	0.299
		2	2	
	Mean±SD	2±0	1.5±0.5	
Breast with IHC-HER2/neu marker	Invasive ductal carcinoma breast (n=2)	2	1	0.299
		2	2	
	Mean±SD	2±0	1.5±0.5	
Colon with IHC-KI 67 marker	Moderately differentiated adenocarcinoma (n=2)	2	1	0.299
		2	2	
	Mean±SD	2±0	1.5±0.5	

EGF: Ethanol, glycerol, formalin, IHC: Immunohistochemistry, HER2/neu: Human epidermal growth factor receptor 2, SD: Standard deviation

These cases showed no change in stain intensity after excessive staining and washing, earning a grade 2 score comparable to conventional formalin. The other two cases received a grade 1 score due to non-uniform staining and less crisp cytoplasmic details; however, interpretation remained possible. There was no significant difference between EGF fixative and conventional formalin for the Van Gieson stain (P=0.09) (Table 3).

Two cases were stained with an E-cadherin IHC marker. One case in EGF fixative demonstrated membranous positivity comparable to formalin, with equal intensity and uniformity, earning a grade 2 score. The other case exhibited lower intensity and non-uniformity compared to conventional formalin, resulting in a grade 1 score; however, interpretation is still possible. EGF fixative and conventional formalin for E-cadherin staining show no significant difference (P=0.299) (Table 3).

Two cases were stained with HER2/neu marker. One case in EGF fixative showed membranous positivity comparable to formalin, with equal intensity and uniformity, earning a grade 2 score. The other case exhibited lower intensity and non-uniformity compared to conventional formalin, resulting in a grade 1 score; however, interpretation was still possible. There was no significant difference between conventional formalin and EGF fixative for HER2/neu staining (P=0.299). Two cases were stained with a Ki67 marker. One case showed nuclear positivity comparable to formalin, with equal intensity and uniformity, earning a grade 2 score. The other case had lower intensity and non-uniformity compared to conventional formalin, resulting in a grade 1 score; however, interpretation was still feasible, and there was no significant difference between conventional formalin and EGF fixative for Ki67 staining (P=0.299) (Figure 3).

DISCUSSION

In our study, we aimed to compare the quality of EGF fixative in histochemical and immunohistochemical

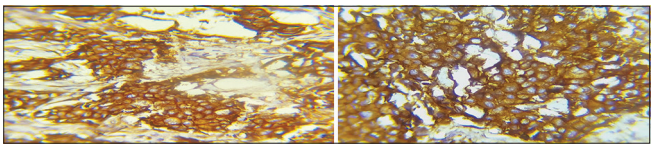

Figure 3: Immuno Histochemistry. Photomicrograph of representative sections from carcinoma breast stained with HER2NEU stain a. Conventional formalin (left) b. EGF fixative (40 x)

procedures with conventional formalin. We analyzed 39 tissue specimens, each fixed in both EGF and formalin for 8 h, and subjected them to various staining techniques, including PAS, Alcian blue, Alcian blue–PAS, Masson trichrome, Van Gieson, and Reticulin stains. Bancroft and Stevens provided an understanding of the microscopic evaluation of tissues fixed in EGF and conventional formalin.[11] Benerini Gatta et al. explored multiple fixatives and reported that tissues fixed in formalin exhibited good PAS staining, whereas other alternatives, such as green fix and UPM, showed varied results. They found that in Alcian blue staining, Holland was the best fixative.[1]

Moelans et al. also found that conventional formalin yielded excellent results for Alcian blue staining, scoring 100%, followed by Fine FIX and RCL. For PAS stain, they found that conventional formalin offered good results with a score of 100%. That was followed by Fine FIX (polyvinyl alcohol, glycerol, and ethanol), which gave 93%, and next by RCL2 (ethanol, acetic acid, and complex carbohydrate) with 89%.[2]

In our study, we compared the efficacy of EGF fixative versus conventional formalin in staining tissues with PAS, Alcian blue, Alcian blue-PAS, Masson trichrome, Van Gieson, and Reticulin stains. Four out of seven cases were stained with Alcian blue, three out of five cases were stained in PAS stain, three out of five were stained with Masson trichrome stain, two out of four cases were stained with Van Gieson, four out of seven cases were stained in Reticulin stain, three out of five cases were stained in Alcian blue–PAS cases showed comparable results to those fixed in formalin.

Tissues fixed with EGF exhibited crisp nuclear and cytoplasmic details, less non-specific background staining, and well-demarcated necrotic tissue staining. These cases were given a grade of 2, whereas the rest of the cases stained with the above stains with fixation in EGF fixative lack crisp cytoplasmic details, showing less intensity and non-uniformity of staining, all other parameters were well correlated with conventional formalin fixative. These cases were given grade 2, but the overall interpretation remained feasible without significant differences. There is no significant difference between EGF fixative and conventional formalin fixative.

Benerini Gatta et al. found that in Masson trichrome staining, formalin and UPM gave good results with respect to color, positivity, and contrast. Cymol, Greenfix, and Hollande fixatives were superior, but Bouin fixed tissues showed less definition. EGF fixative shows comparable results as with conventional formalin fixative in Alcian blue–PAS, PAS, Reticulin, Masson trichrome, Van Gieson, and Alcian blue staining. The tissues fixed with EGF fixative showed less non-specific staining, background staining was minimal in tissues fixed with EGF fixative, there was crisp nuclear stain and crisp cytoplasmic stain, and staining of necrotic tissues was well demarcated. There is no change in staining intensity even after excessive staining and excessive washing in water, and interpretation was quite possible. Benerini Gatta et al. found that in Masson trichrome staining, formalin and UPM gave good results for color, positivity, and contrast. Cymol, Greenfix, and Hollande fixatives were superior, but Bouin's fixed tissues showed less definition.[1]

In our study, staining with Masson Trichrome, PAS, Alcian Blue, Alcian Blue-PAS, Reticulin, Van Gieson, Masson trichrome, and EGF fixative were found comparable with formalin fixative. In a study by Molens et al., formaldehyde substitute fixatives, FineFIX (polyvinyl alcohol, glycerol, and ethanol), and RCL2 (ethanol, acetic acid, and complex carbohydrate) were used to compare the immunohistochemical properties of various strains. They used cytokeratin AE 1/3, CAM 52, vimentin, CD45, estrogen receptor (ER), progesterone receptor (PR), S100, and chromogranin A p63. Slides were analyzed and graded from 0 to 2. Overall, conventional formalin was proven to be the best, with a score of 100%, followed by RCL2 70% without pre-treatment, FineFIX 68%, and F-solv 60%. Chromogranin A was found to be optimal for all fixatives. The S100 was found to be suboptimal for all alternatives. RCL2 and Fine FIX showed less than adequate staining of the ER, regardless of pre-treatment.[2]

In a study by Nadji et al., 33% (23 out of 70 total antibodies) of clinical antibodies and UMFIX (methanol and polyethylene glycol) showed better results than standard NBF.[11] Van Essen et al. used 85 *in vitro* clinical antibodies and proved that fixation using 10% NBF provided better staining in 84% of the antibodies, while RCL2 provided better staining in 66%.[12]

Each two cases were stained with IHC marker HER2/neu, E-cadherin, and ki67, respectively. One case in each marker gave results comparable with conventional formalin and given grade 2. In one case, in each marker given grade 1, all the parameters are comparable to that of conventional formalin fixative except for less intensity and non-uniformity of staining, which were observed when

compared with conventional formalin. The interpretation was still possible with the EGF fixative. In all cases stained with EGF fixative, it was observed that there was minimal non-specific staining and minimal background staining. Thus, EGF fixative can be used for immunohistochemical staining.

A minimal formalin containing EGF fixative used in the study was proved to be comparable to conventional formalin with respect to Hematoxylin and Eosin for routine histopathological study.[2] This study assessed the efficacy of EGF fixative for histochemical and immunohistochemical staining and which was found to be effective. It was qualitatively comparable to conventional formalin fixative for histochemical and immunohistochemical staining techniques.

Limitations of the study

In this study we used EGF fixative which is relatively costlier when compared to Formalin. We are able to include only small number of samples due to the sample needed to be freshly collected from surgical theatres and also the study was time constrained, also and limited tissues are used because as it is done in Histochemical stains that will stain positive only in specific tissues. Because of time constrain we did IHC only in limited specimens.

CONCLUSION

Formalin toxicity has been known for decades, and a huge quantity of formalin is being used by the pathology laboratory, and its continuous exposure causes serious side effects, which is a limiting factor for formaldehyde usage. However, there is a lack of a perfect alternative fixative that replaces the formaldehyde fixation to overcome its toxic effects. This EGF fixative was proven to be comparable with conventional formalin with respect to Hematoxylin and Eosin for routine histopathological use in a previous study.[6] In this study, we have assessed the efficacy of EGF fixative in histochemical and immunohistochemical reactions. There were occasional minor dissimilarities in specimens fixed in EGF fixative when compared to conventional formalin. Overall, it was found that the effectiveness of EGF fixative was comparable to that of conventional formalin fixation for histochemical and immunohistochemical staining. This novel EGF fixative can be used for routine histochemical and immunohistochemical staining in addition to routine histopathological staining. Alternative fixatives such as EGF fixative with minimal formalin could greatly help in reducing the risks associated with laboratory exposure of health care professionals to formalin.

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Authors Contribution:
BV- Manuscript preparation, performed the procedure; **DG-** Editing manuscript; **PSA-** Protocol review, review manuscript; **MPMS-** Literature review, data collection, data analysis; **VKV-** Study design, review manuscript.

Work attributed to:
Department of Pathology, Government Theni Medical College, Theni, Tamil Nadu, India.

Orcid ID:
Dr. Buvaneswari V - https://orcid.org/0009-0002-5862-9146
Dr. Deepadevi G - https://orcid.org/0000-0003-3123-8196
Dr. Peter Samidoss A - https://orcid.org/0009-0005-4609-4115
Dr. Muthu Prabha MS - https://orcid.org/0009-0009-9363-9180
Dr. Vinoth Kumar V - https://orcid.org/0009-0003-9653-695X

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