 DOI: 10.18231/2394-6792.2017.0088

Evaluation of a New Compound Fixative: A step towards limited formalin exposure

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Abstract

Introduction: The aim of the study was to minimize formalin exposure in histopathology laboratory and analyze the fixation characteristics of a group of formalin containing compound fixatives with reduced concentration of formaldehyde.

Materials and Method: A minimal formalin containing fixative was prepared with varying concentrations of formalin, ethanol, glycerin and hypotonic saline. The pH of the fixative was maintained below 7.2 to 7.4. Multiple human tissue materials of varying sites, organs, and lesions were utilized. Tissue slices were immediately fixed in the prepared compound fixative. A comparative analysis of fixation and staining qualities were done.

Result: There is no significant difference between 10% NBF and new fixative at 8 and 10 hours fixation and the new fixative is comparable to 10% NBF in preserving cytoarchitectural features at 8 and 10 hours of fixation. A total of 32 out of 35 cases had a maximum score 9 at 8 and 10 hours fixation. The formaldehyde vapor from the compound fixatives were qualitatively measured and found to be 3 times less in the laboratory atmosphere.

Conclusion: The present study demonstrates that a minimal formalin containing fixative can be easily prepared in the laboratory and they are suitable for histopathological examination of routine surgical specimens. The effectiveness of this new compound fixative is comparable to conventional formalin fixation with an improved air quality of the working laboratory and considerably reduced formalin vapor density.

Keywords: Compound fixative, Cytoplasmic features, Formalin toxicity, Formalin vapor, Histopathology, Nuclear features.

Manuscript Received: 7th March, 2017 **Manuscript Accept:** 9th May, 2017

Introduction

As a popular fixative formalin has its own advantages, its low cost, its ability to help with long term storage of tissue, its ability to help preserve morphological features, and the fact that it allows special histological stains. However, the toxicity of formalin is emerging as a major deterrent in its continued usage in the laboratory practice. The formation of DNA, protein cross links denotes a permanent signature of exposure to formalin in tissues. Fixation is one of the most important steps in the practice of diagnostic pathology. Even in this modern age where many things have changed, formaldehyde continues to be the leading tissue fixative. However, the toxicity of formalin is emerging as the main reason for the call to abolish it as the commonest fixative used in laboratories.(1) Initial report from the IARC (International Agency for Research on Cancer) link formaldehyde exposure and leukemia. These reports were further highlighted in a report issued in 2012 by the same agency. The agencies that monitor formaldehyde exposure in the national and international level set stringent limits for formaldehyde exposure. The above said limit ranges from 0.016 ppm TWA (time weighted average) to 2 ppm for STEL (short term exposure limit).(1) An attempt has been made in this study to minimize formalin exposure by reducing the formalin concentration in a new compound fixative.

Materials and Method

A minimal formalin containing fixative was prepared with varying concentrations of formalin, ethanol, glycerin and hypotonic saline. The pH of the fixative was maintained between 7.2 to 7.4. Ethanol as a dehydrant fixative, it will produce cell shrinkage. To overcome this, hypotonic saline was added. Glycerin was added to minimize evaporation. Methylene blue was added to monitor the color of fixatives and subsequent dehydrants and to avoid the tendency to smell the solutions. The prepared solutions were light blue in color. Fixation was done at 3 different fixation times. Multiple human tissue materials of varying sites and lesions were utilized. Tissue slices were immediately fixed in the prepared compound fixative and fixation time was titrated between 7 to 10 hours.

New compound fixative was prepared with 10% Formalin 7 ml, Ethanol 20 ml, Glycerin 5ml, Methylene Blue 0.05g, Buffer - 4g of Sodium dihydrogen phosphate monohydrate, 6g of Anhydrous disodium hydrogen phosphate. The PH adjusted between 7. 2 - 7.4 and reconstituted in 0.7% hypotonic saline to 100 ml. Fixation of tissues in the above solution was done in 7,8 and 10 hours. Conventional Tissue processing was completed in 9 Hours. Processed tissues were embedded in paraffin wax. Then the sections were taken at 4 micron thickness and stained with routine hematoxylin and eosin. Stained slides were studied under light microscope. Fixation artifacts, staining characteristics, architecture, nuclear and cytoplasmic

details were analyzed by two independent pathologists. Combined nuclear, cytoplasmic and architectural features were scored between 0- 9. A combined total score of 9 was given to nuclear, cytoplasmic and architectural features of all the tissues well fixed in 24 hours conventional 10% NBF (Neutral Buffered Formalin) which is considered as absolute fixation.

Nuclear features were assessed based on following features - nuclear and nucleolar preservation, nuclear size, regularity of the nuclear membrane, chromatin pattern whether fine, coarse, granular/ reticular pattern and mitotic figures. A nuclear Score 3 was given to tissues fixed in compound fixatives with similar nuclear features to tissues fixed in conventional 10% NBF. Score 2 was given to sections with 1 to 2 less defined nuclear features. Score 1 was given to sections with more than 2 less defined nuclear details. Score 0 was given to sections with poor preservation of details which was unsuitable for diagnosis.

Cytoplasmic features were assessed by color of cytoplasm, abundance, cytoplasmic granules and mucin differentiation**.** A cytoplasmic score of 3 was given to tissues fixed in compound fixatives with similar cytoplasmic features to tissues fixed in conventional 10% NBF i.e. absolute fixation. Score 2 was given to sections with cytoplasmic shrinkage with less prominent cytoplasmic granules and considered as suboptimal fixation. Score 1 was given to sections with more than 2 less defined cytoplasmic details. Score 0 was given to sections with poor preservation of details which was unsuitable for diagnosis.

Architectural features were assessed based on shrinkage artifacts, distortion, cracking and formalin pigments. An architectural score of 3 was given to tissues fixed in compound fixatives with similar architectural features to tissues fixed in conventional 10% NBF i.e. optimal fixation. Score 2 was given to sections with 1 to 2 less defined architectural features. Score 1 was given to sections with more than 2 less defined nuclear details. Score 0 was given to sections with poor preservation of details which was unsuitable for diagnosis. The nuclear, cytoplasmic and architectural scores of each tissue were added to get a total score of 0 - 9.

Table 1: Scoring system

Total fixation Score	Quality of fixation
8 – 9	Good fixation
6 -7	Sub-optimal
4 -5	Poor
< 4	Unsuitable

The fixation time used in each fixation schedule was evaluated and compared with conventional fixation procedures. The results were tabulated and analyzed by Mann-Whitney U test. P value < 0.05 is considered as statistically significant.

The concentration of formaldehyde vapor in our new compound fixative was compared with conventional 10% NBF by using Schiff's reagent. The No. 1 Whatman filter paper was soaked in Schiff's reagent and dried in air. Two glass beakers of 9 cm in length and 7.5 cm in diameter were taken and labeled as beaker A and beaker B. 10 ml of 10% NBF was taken in beaker A and 10 ml of new fixative was taken in beaker B. Both beakers were closed by Whatman paper (Schiff's reagent soaked) and allowed to stand. The time taken for the filter papers to change color into pink/magenta was noted.

Result

A total of 35 specimens was fixed in new fixative. Among them, 14 cases (40%) were the uterus and cervix followed by breast 7 cases (20%), thyroid 5 (14.29%), gastrointestinal tract specimens 3 (8.58%), soft tissue 2 (5.71%), lymph node 2 (5.71%), ovary (1) and testis (1). Cellular, nuclear and architectural features were compared to that of tissues (i.e. Same 35 specimen) fixed in 24 hours conventional 10% NBF (i.e. Absolute standard fixation). Macroscopically, tissues fixed in our new compound fixative were light blue in color and the texture of tissues after fixation was same as tissues fixed in conventional 10% NBF.

Nuclear features of tissues fixed in the new fixative were compared with conventional 10% NBF. At 7 hours fixation more than half the cases showed nuclear shrinkage, less prominent nucleoli and mitotic figures and they got score 2 At 8 hours fixation, only one specimen (lymph node) showed nuclear shrinkage compared to 10% NBF and got score 2. At 10 hours fixation, all the 35 specimens scored 3. All the other 34 specimens received scores 3. (Table 2)

Table 2: Comparison of nuclear features - new fixative and conventional 10% NBF

Fixatives	Score				P Value*
	3	2	1	0	
10% NBF	35	Nil	Nil	Nil	n/a
Fixative1, 7 Hrs	15	20	Nil	Nil	<0.0001
Fixative 1, 8Hrs	34	1	Nil	Nil	0.325
Fixative 1,10 Hrs	35	Nil	Nil	Nil	n/a

*Mann Whitney U test

There is a significant difference in nuclear features at 7 hours fixation compared to conventional formalin and it is suboptimal in preserving nuclear details. There is no significant difference between 10% NBF and new fixative at 8 and 10 hours fixation. New fixative is comparable to10% NBF in preserving nuclear features at 8 and 10 hours fixation (Fig. 1, 2, 3 & 4).

Cytoplasmic features were compared between new fixative and conventional 10% NBF. At 8and 10 hours fixation, 32 cases fixed in new fixative have received scores 3 as they preserves cytoplasmic color, granules and mucin differentiation well. (Table 3)

Table 3: Comparison of cytoplasmic features - New fixative and conventional 10% NBF

Fixatives	Score				P Value*
	3	2	1	0	
10% NBF	35	Nil	Nil	Nil	n/a
Fixative1, 7 Hrs	10	25	Nil	Nil	<0.0001
Fixative 1, 8Hrs	32	3	Nil	Nil	0.083
Fixative 1, 10 Hrs	32	3	Nil	Nil	0.083

*Mann Whitney U test

There is no significant difference between 10% NBF and New fixative at 8 and 10 hours fixation. Hence the new fixative is comparable to 10% NBF in preserving cytoplasmic features at 8 and 10 hours fixation (Fig. 1, 2, 3, 4). But there is a significant difference in 7 hours fixation, hence it is inferior to 10% NBF in preserving cytoplasmic details.

Architectural features were assessed based on shrinkage artifacts, distortion, cracking and formalin pigments. Architectural features were compared between tissues fixed in new fixative and 10% NBF. At 7 hours of fixation, more than half of the specimens received suboptimal score. 33 specimens fixed in 8 hours (Fig. 1, 2) and 10 hours (Fig. 3, 4) got optimal score 3. Two cases received scores 2 because of shrinkage artifacts and distortion. (Table 4)

Table 4: Comparison of architectural features - New fixative and Conventional 10% NBF

Fixatives	Score				P Value*
	3	2	1	0	
10% NBF	35	Nil	Nil	Nil	n/a
Fixative 1, 7 Hrs	12	23	Nil	Nil	<0.0001
Fixative 1, 8Hrs	33	2	Nil	Nil	0.160
Fixative 1, 10 Hrs	33	2	Nil	Nil	0.160

*Mann Whitney U test

There is a significant difference between 10% NBF and new fixative at 7 hours fixation. There is no significant difference at 8 and 10 hours fixation. Hence new fixative is comparable to 10% NBF in preserving architectural features at 8 and 10 hours fixation. 32 out of 35 cases had a maximum score 9 at 8 and 10 hours fixation. Three had a score of 7 at 8 and 10 hours of fixation.

The formaldehyde vapor from the compound fixatives were qualitatively measured and compared with conventional 10% NBF by Schiff test. In this test, filter paper over beaker A (10 % NBF started to change color in 10 minutes and completely changed to magenta color in 25 minutes. Whereas filter paper over beaker B (New fixative) started to change color in 55 minutes and completely changed magenta in 90 minutes indicating a significant reduction (nearly 4 times less) in the formaldehyde concentration with new fixative.

This study also shows increased formalin pigments in tissue sections when the prepared new fixative solutions were stored for more than 10 days. In addition, it was found to evaporate less formaldehyde vapor than 10% NBF and fixation time is considerably reduced to 8 hours. However, the effectiveness of this fixative and its impact on histochemical reactions and demonstration of immunomarkers are yet to be evaluated.

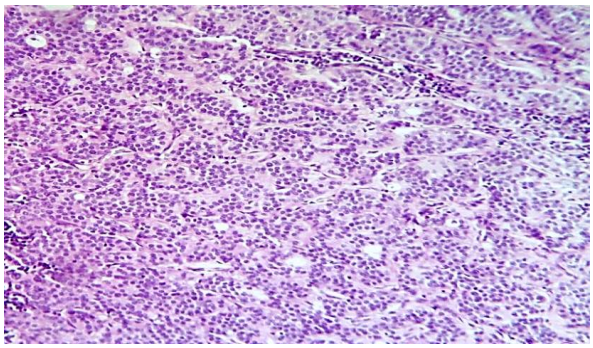

Fig. 1: Photomicrograph showing histopathological features of Metastatic carcinomatous deposits lymph node, – New fixative, 8 hours. H&E, (10x)

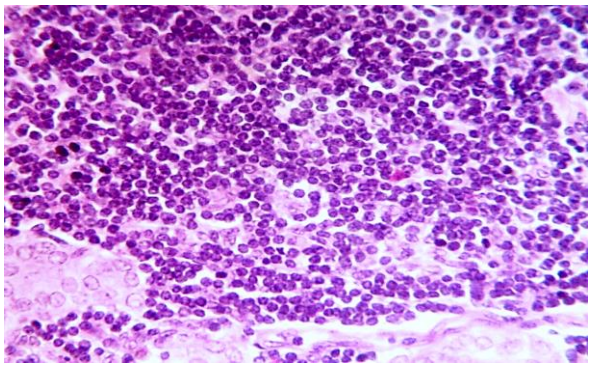

Fig. 2: Photomicrograph showing histopathological features of the Metastatic carcinomatous deposits node, – New fixative, 8 hours. H&E, (40x)

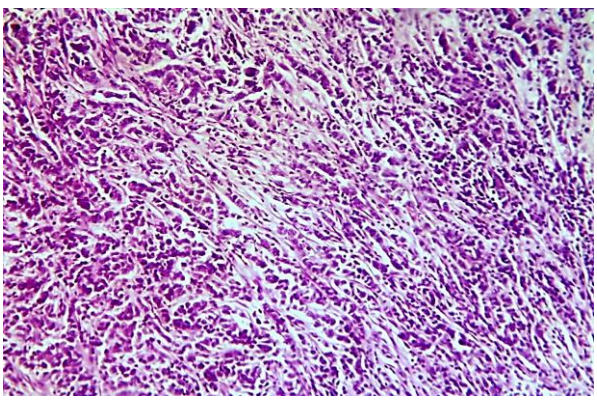

Fig. 3:Photomicrograph showing histopathological features of Invasive ductal carcinoma breast – New fixative, 10 hours. H&E, (10x)

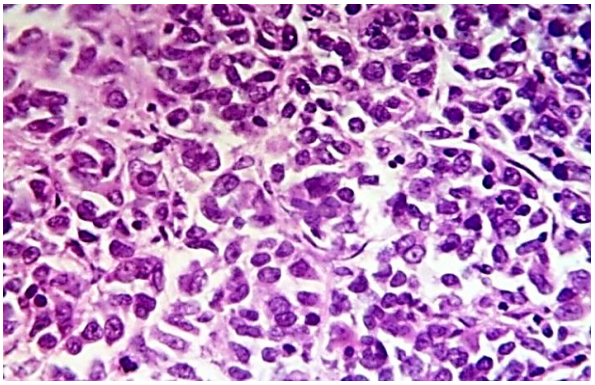

Fig. 4: Photomicrograph showing histopathological features of Invasive ductal carcinoma breast, – New fixative, 10 hours. H&E (40x)

Discussion

Fixation is a very important step in histopathological analysis as it preserves tissues in a lifelike manner. Formalin is considered the gold standard fixative and has been used for over 100 years. Another fact, under consideration is that pathology laboratories use huge quantities of formalin and often do not give due importance to its toxic hazards. Technicians and pathologists are constantly exposed to a dilute solution of formaldehyde and its vapor. As the exposure occurs every day, the role of formaldehyde as a chemical carcinogen must be given due consideration.[2,3,4]

Over the past years, many pathology laboratories have tried to replace formalin with other less toxic alternatives, but the results obtained have not been satisfactory, due to factors like alterations in cellular structure The study by Cathy.B.Moelans et al found that tissues fixed in Finefix and RCL2 were to be paler when compared to specimens fixed with NBF.[5] The study by Cristina Zanini et al showed that tissues fixed in PAGA, ZBF, Z7, RCL2 and CellBlock (alternative fixatives) do not change the color in a similar manner as formalin.[1] Tissues fixed in our fixatives were light blue in colour and do not interfere with macroscopic analysis. Another factor is that the odour associated with compound fixatives is less irritant than 10% formalin.

Cristina Zanini et al found that tissues fixed in alternative fixatives were suitable for microtomy[1,6] but Cathy.B.Moelans et al reported that tissue fixed using RCL2 were soft and slippery, making cutting difficult.[5] Tissues fixed by using new compound fixatives are found to be suitable for microtomy and there is no difficulty in cutting in this study. Rate of fixation time depends on the rate at which diffusion of fixative into the tissue occurs and the rate at which chemical reactions with various components occurs.[7] The study by Cathy. B. Moleans et al, penetration speed of alcohol based fixatives was found to be faster than 10% NBF.[1] In the present study, new fixative shows there is no significant difference between tissues fixed at 8 and 10 hours fixation comparable to conventional NBF fixed tissues.

In the study by Cristina Zanini et. al, nuclear features were better preserved in alcohol based fixatives.[1] L. Benerini Gatta et al – Bouin fixative showed higher resolution in the nucleus.[8] In the study by Cathy. B. Moelans et al demonstrated highest score for nuclear and cytoarchitectural features tissues fixed in NBF and lowest for FineFIX.[5] On comparing nuclear features of new fixative and 10% NBF, the present study indicates that there is no significant difference between them at 8 and 10 hours fixation and both are comparable in preserving nuclear features. In a study by Cristina Zainini et al, alcohol based fixatives showed shrinkage artifacts, especially when the concentration of alcohol is more than 50%. Fixatives containing zinc also had shrinkage artifacts.[1] On comparing architectural features, our study found that there is no significant difference between fixative 1 and conventional formalin at 8 and 10 hours fixation.

In the present study, we have been trying to minimize formalin exposure in the histopathology laboratory by reducing formalin concentration. In this fixative formalin concentration was reduced from 10 to 7%. Alcohol concentration was 20%. To minimize the evaporation of absolute ethanol, glycerol was added. Ethanol is a dehydrant coagulative fixative, it removes water molecules from tissues leads to shrinkage of cells. To overcome this defect, 0.7% hypotonic saline was used to reconstitute the solution, Methylene blue was added to monitor spillage and contamination of subsequent dehydrants in processing. The pH of the solution was maintained between 7.2 to 7.4 by adding sodium dihydrogen phosphate monohydrate and anhydrous disodium hydrogen phosphate. In our study, we have analyzed the fixation characteristics and cytomorphological features of minimal formalin containing compound fixatives.

Conclusion

As formaldehyde is a group 1 human carcinogen, it should be replaced by less toxic fixatives in histopathology laboratory. The present study demonstrates that minimal formalin containing fixatives can be easily prepared in the laboratory and they are suitable for histopathological examination of routine surgical specimens. In this study, we have taken into account only the histomorphological features of H & E stained sections. Tissue characteristics in special histochemical and immunohistochemistry reactions were not taken into account. However, for a routine diagnostic histopathology using H & E stain, the effectiveness of this compound fixatives is comparable to conventional formalin fixation with an improved air quality of the working laboratory and considerably reduced formalin vapor density.

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