

Alcohol-Based Fixatives can Better Preserve Tissue Morphology than Formalin

Fijadores a Base de Alcohol Pueden Preservar Mejor la Morfología Tisular que la Formalina

Ziaul Haque¹; Md. Asabur Rahman¹; Md. Zahirul Islam Khan¹; Mohammad Tufazzal Hussan² & Md. Mahmudul Alam³

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SUMMARY: Fixation is a crucial step in processing of tissue specimen for preservation of cellular architecture and composition of cells. Alcohol-based fixatives are considered some of the most promising alternatives to formalin. We evaluated the performance of alcohol-based fixatives (EthMeth and methacarn) and formalin as a comparator fixative in the research laboratory. Following 24 hours of fixation, tissue morphology and cellular details of the liver, spleen and brain (cerebral cortex) were evaluated. Morphological characteristics were evaluated by gross observations and analyzing cellular details, tissue architecture and overall staining characteristics (Hematoxylin and Eosin). EthMeth and methacarn fixation gave generally comparable and satisfactory results on the tissue morphology and subsequent identification of tissue characteristics. Particularly, tissues were well preserved and all nuclear as well as cytoplasmic details were clearly visible. However, formalin fixed tissues showed some peculiarity such as improper fixation, mild shrinkage, and alterations of tissue components. These results confirm that alcohol-based fixation is the superior alternative to formalin for preservation of tissue morphology. However, it is required to standardize the formalin-free methods and harmonize diagnosis in the laboratory worldwide.

KEY WORDS: Fixation; EthMeth; Methacarn; Formalin; Tissue morphology.

INTRODUCTION

Fixation is an essential step to prevent autolysis and degradation of tissues, while preserving morphology and cellular details for microscopic evaluations. It helps to preserve cellular architecture and composition of cells in the tissue to allow them to withstand subsequent processing. Fixation also preserves the proteins, carbohydrate and other bio-active moieties in their spatial relationship to the cell, so that they can be studied.

An ideal fixative is expected to harden tissue components and prevent decomposition, putrefaction, and autolysis. Formalin has been used as the “gold standard” fixative for study and researches. But the employees in these settings may be at risk for high levels of exposure to formalin. In particular, anatomists, histologists, pathologists, and medical students are the individuals most frequently exposed to formalin in dissection lectures and laboratories (Cohen *et al.*, 1998; Sarnak *et al.*, 1999). Because of this issue, the selection of a particular fixative generally warrants multiple

and careful considerations. To date, no ideal fixative has been found, i.e., a safe fixative that perfectly preserves cellular morphology and yet does not modify the specimen composition so as not to change the reactivity of the chemical moieties therein for subsequent detection.

For decades, 10 % formalin (4 % formaldehyde), diluted in water or in a buffered solution has been considered as a fixative of choice in routine histopathology to preserve tissue specimens for long periods of time at reasonable cost. Formaldehyde is a toxic, potent carcinogen for public health (Baan *et al.*, 2009; National Research Council, 2011). Formalin creates covalent bonds between biological macromolecules and ensures chemical activity conservation in tissues (Warmington *et al.*, 2000). With a fast penetration, formalin is a slow and reversible fixative that requires 24 to 48 hours to completely bind to tissues (Srinivasan *et al.*, 2002; Buesa, 2008). It causes cross linking of cellular proteins that masks antigenic sites for subsequent tests and

¹ Department of Anatomy and Histology, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh.

² Department of Anatomy and Histology, Faculty of Animal Science and Veterinary Medicine, Patuakhali Science and Technology University, Babugonj, Barisal-8210, Bangladesh.

³ Department of Surgery and Obstetrics, Faculty Veterinary Science, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh.

degrades nucleic acids rapidly (Cox *et al.* 2006; Moelans *et al.*, 2011a,b). Several proteolytic or heat-induced antigen retrieval methods have been recently become available through commercial sources, with the purpose of restoring normal protein folding and to improve epitope accessibility on fixed tissues (Paavilainen *et al.*, 2010; Fowler *et al.*, 2011). All alcoholic fixatives are fast, less toxic, act by coagulation and don't mask antigenic sites (Stanta *et al.*, 2006). Fast fixation, optimal preservation of DNA, RNA and proteins and a safer workplace environment, are some of the advantages of alcoholic fixation over formalin fixation (Bostwick *et al.*, 1994; Moelans *et al.*, 2011a). But use of formalin substitutes has been restricted to a few laboratories, and reports about them are scarce. Therefore, the present study is designed to examine the efficacy of presumably less toxic alcohol based fixatives as potential substitute to formalin.

MATERIAL AND METHOD

The research experiment was conducted in the Department of Anatomy and Histology, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh-2202, as per approval of the Animal Care and Ethics Committee.

Preparation of fixatives. Two different alcohol-based alternatives to formalin such as EthMeth (100 % Ethanol: 3 parts and Methanol: 1 part); Methacarn (100 % methanol: 6 parts, chloroform: 3 parts, glacial acetic acid: 1 part) and 10 % formalin were prepared and stored in the laboratory at room temperature for fixation of tissues.

Gross evaluation of tissues. A total of 15 adult, healthy male black Bengal goats (*Capra hircus*) were anaesthetized with pentobarbital sodium @ 15-20 mg/kg body weight (I/M) and every effort was made to minimize suffering of the animals. All the animals were bled to death by giving incision on the right common carotid artery. Fixation was performed by perfusion through the carotid artery for gross evaluation of the tissues.

Histochemical evaluation of tissues. Fresh liver, spleen and brain samples were collected at the Department of Anatomy and Histology, Bangladesh Agricultural University, Mymensingh. Each tissue sample was divided into 2 equal pieces and fixed into the fixatives for 24 hours at room temperature. After fixation, tissues were processed for histochemical study. The tissues were rinsed in 70 % alcohol and then processed. The processing schedule was 70 % alcohol for 45 minutes, 80 % alcohol for 30 minutes, 2

changes of 95 % alcohol for 30 minutes each, 2 changes of 100 % alcohol for 45 minutes each, 2 changes of xylene for 45 minutes each, and 4 changes of graded paraffin wax for 30 minutes each. Following embedding, each block was coded. The paraffin blocks were sectioned at 4 µm, and 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 minutes each); 100 % alcohol, 95 % alcohol, 80 % alcohol & 70 % alcohol (3 minutes each); deionized water with gentle shaking (10 minutes); Harris Hematoxylin (10 minutes); deionized water with gentle shaking (10 minutes); Eosin-Y (2 minutes), 70 % alcohol, 80 % alcohol, 95 % alcohol and 2 changes of 100 % alcohol (3 minutes each); 50 % xylene + 50 % alcohol and xylene-I, II & III (3 minutes each). Then the sections were coverslipped using DPX mounting medium (Lerner Laboratories, Pittsburgh, Pa) and examined under microscope.

Imaging and data analysis. The images were taken by using Nikon photomicroscope and data were analyzed by student's t-test.

RESULTS AND DISCUSSION

In this study, a group of experienced tissue morphologists examined a variety of tissue specimens fixed with formalin and 2 alcohol-based formalin substitutes (EthMeth and methacarn) without encountering significant alterations in gross morphology. Despite the fact that none of the morphologists knew which fixative had been used for section, this group of unbiased observers rated formalin and alternatives to find the best overall fixative.

On gross examination after 24 hours of fixation, all of the alcohol-based fixatives fixed tissues properly (hard and turned grey-white) except for those fixed in formalin, which turned red-brown and remained soft. The alcohol-based fixatives penetrate and fix faster than formalin. There was minor tissue contraction in case of EthMeth and formalin but not evident for Methacarn. EthMeth showed mild shrinkage with incomplete fixation which was more remarkable in formalin. Methacarn showed no shrinkage with almost complete fixation which fixed ideally. Among the fixatives, methacarn was the best preserver of tissue morphology (shape, size and consistency) followed by EthMeth and formalin. These results indicate the graded diffusion power of different fixatives in the tissues during the given period of time.

The alcohol-based fixatives preserved tissue blocks sectioned well but formalin fixed tissue blocks sectioned

poorly. Microscopic examination of tissues was used to compare the overall effectiveness of each fixative. For this purpose, the stained tissue sections of liver, spleen and brain on the slide was examined to rate adequacy of fixation applying a scale, 1= poor, 2 = fair, 3 = good, 4 = very good and 5= excellent. The slides were rated on the basis of 5 parameters: cellular outline, cytoplasmic detail, nuclear detail, overall morphology, and overall staining (Table I). In this blind study, alcohol-based fixation provided the highest histomorphologic quality for tissue stained with hematoxylin and eosin (H & E). The overall histologic score for alcohol-based fixatives was significantly ($p < 0.05$) higher than formalin (Table II). When individual factors in fixation were studied, alcohol-based fixatives performed better than others (Table I). The methacarn provides greater staining intensity

compared with EthMeth and formalin. In case of formalin, weak preservation with cellular degeneration and weak affinity for staining (H & E) were evident. However, methacarn preserved better cellular and intercellular structures with nice staining affinity to nuclei and cytoplasm, showing no cellular disintegration or degeneration (Figs. 1 to 3). The degree of pale staining of nuclei and cytoplasm with more or less cellular/intercellular disintegration/degeneration were found in other fixatives. However, it was highest in the formalin fixed tissues (Figs. 1 to 3). These results are suggestive of alcoholic fixative as a better preserver of tissue morphology than formalin.

Alcohol-based fixatives (EthMeth and methacarn) fixed tissues ideally without significant alterations of tissue

Table I. Histological scores for formalin and alcohol-based fixatives.

Parameters	Formalin	EthMeth	Methacarn
Cellular outline	2.65	2.86	3.00
Cytoplasmic detail	2.50	2.88	3.08
Nuclear detail	2.80	2.96	3.14
Overall morphology	2.84	2.90	3.10
Overall staining	2.64	2.93	3.00

1 = poor, 2 = fair, 3 = good, 4 = very good, 5= excellent.

Table II. Overall histological scores for formalin and alcohol-based fixatives.

Fixatives	Mean	Variance	p-value
Formalin	2.686	0.018	
EthMeth	2.906	0.001	0.015
Methacarn	3.064	0.003	0.002

1 = poor, 2 = fair, 3 = good, 4 = very good, 5= excellent.

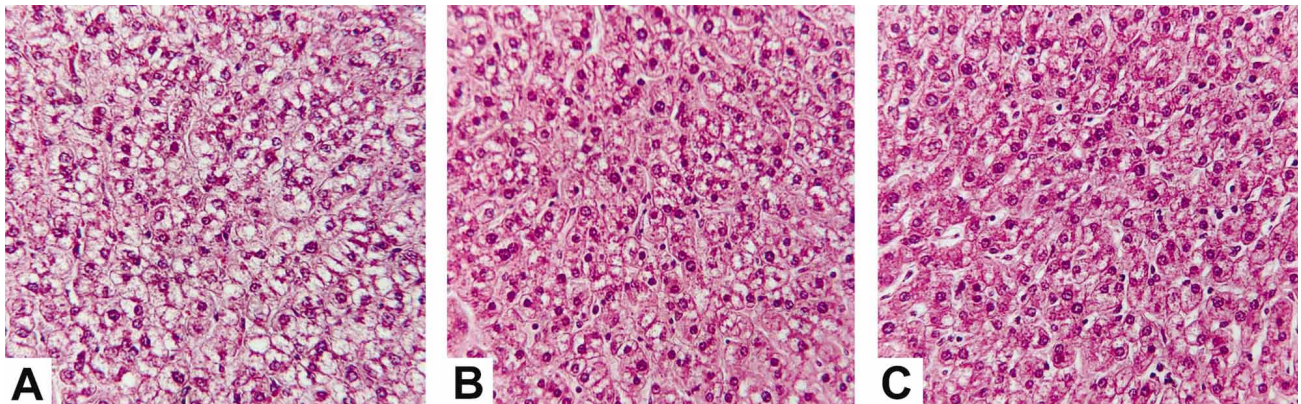


Fig. 1. Hematoxylin and Eosin (H & E) staining of liver (40X). (A) Formalin fixed tissue (B) EthMeth fixed tissue (C) Methacarn fixed tissue.

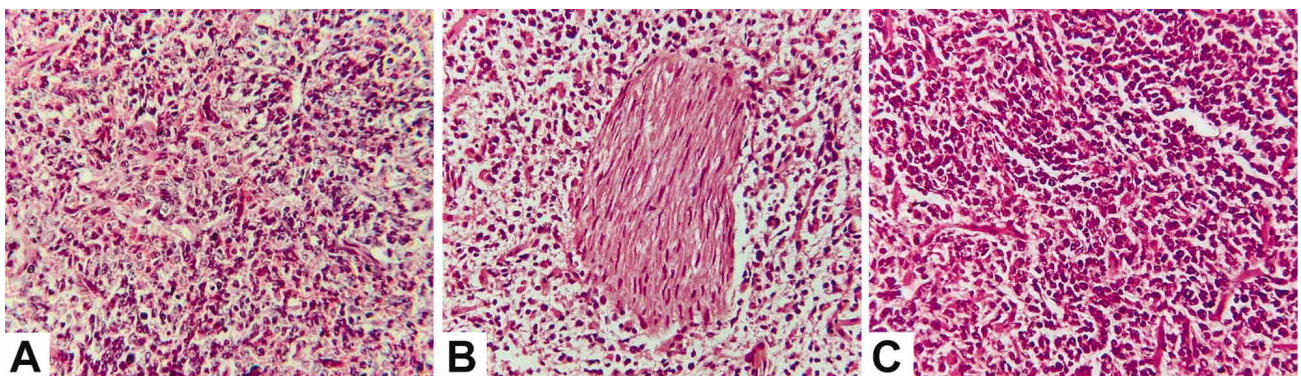


Fig. 2. Hematoxylin and Eosin (H & E) staining of spleen (40X). (A) Formalin fixed tissue (B) EthMeth fixed tissue (C) Methacarn fixed tissue.

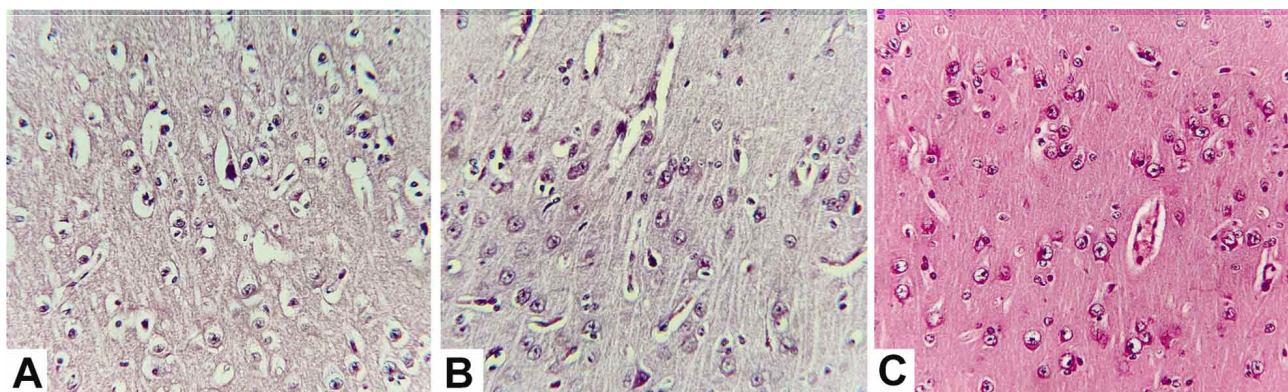


Fig. 3. Hematoxylin and Eosin (H & E) staining of cerebral cortex (40X). (A) Formalin fixed tissue (B) EthMeth fixed tissue (C) Methacarn fixed tissue.

morphology. However, tissue appearance of formalin fixed tissues differed from alcohol-based fixatives as it caused dehydration and fixation simultaneously. Alcohol-based fixatives act by coagulation of proteins and do not mask antigenic sites as formalin does (Bancroft & Gamble, 2008). These fixatives are faster in penetration of tissues as all these components of EthMeth and methacarn (ethanol, methanol and acetic acid) have higher coefficients of diffusibility than formalin (Kok & Boon, 1990). Both EthMeth and methacarn preserved better general tissue morphology (gross and histomorphology) than formalin. Our results are in agreement with Buesa.

CONCLUSIONS

In our study, methacarn fixation preserved the best gross and histomorphology (H & E) followed by EthMeth and formalin. Thus, alcohol-based fixatives can be potential alternative to formalin for preservation of tissue morphology. However, morphologists will have to familiarize themselves with a different set of microscopic details associated with alcohol-based fixatives.

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RESUMEN: La fijación es un paso crucial en el procesamiento de muestras de tejido para preservar la arquitectura celular y la composición de las células. Los fijadores a base de alcohol se consideran algunas de las alternativas más prometedoras a la formalina. Evaluamos el rendimiento de los fijadores a base de alcohol (EthMeth y metacarn) y formalina como fijador comparativo en el laboratorio de investigación. Después de 24 horas de fijación, se observó la morfología del tejido y los detalles celulares del hígado, bazo y corteza cerebral. Se evaluaron las características morfológicas mediante observaciones generales y analizando detalles celulares, arquitectura de tejidos y características generales de tinción (hematoxilina y eosina). La fijación de EthMeth y metacarn dio resultados generalmente comparables y satisfactorios en la morfología del tejido y la posterior identificación de las características del mismo. Particularmente, los tejidos estaban bien conservados y todos los detalles nucleares y citoplasmáticos eran claramente visibles. Sin embargo, los tejidos fijados con formalina mostraron cierta peculiaridad, tal como una fijación inadecuada, la contracción leve y alteraciones de los componentes del tejido. Estos resultados confirman que la fijación a base de alcohol es la mejor alternativa a la formalina, para preservar la morfología del tejido. Sin embargo, es necesario estandarizar los métodos sin formalina y armonizar el diagnóstico en los laboratorios.

PALABRAS CLAVE: Fijación; EthMeth; Metacarn; Formalina; Morfología del tejido.

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Corresponding author:
Professor Dr. Ziaul Haque
Department of Anatomy and Histology
Faculty of Veterinary Science
Bangladesh Agricultural University
Mymensingh-2202
BANGLADESH

Email: zhaqueah80@bau.edu.bd

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