

## Original Research Article

# Phosphate-buffered Formalin Fixative Under Controlled Fixation Temperature Yielded Excellently Preserved Histomorphology

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### ABSTRACT

**Aim:** Using mouse liver as experimental model, this study attempts to identify a formalin-based fixative and fixation temperature that jointly provides the best balance of preservation of tissue morphology. **Methodology:** Liver samples from fifty (50) albino mice aged between of 6 to 8 weeks consisting of both male and female was harvested following cervical dislocation and randomly distributed into control and experimental groups. Control samples were fixed in 10ml of 10% formalin at 25°C, 30°C, 35°C, 40°C, 45°C, 50°C, 55°C and 60°C respectively for 24 hours, while experimental samples were each fixed in equal volume of phosphate-buffered 10% formalin (pH 7.2, 7.4, 7.6 and 7.8) at the same temperature and time duration regimen and processed for general tissue morphology. Nuclear, cytoplasm and cell membrane morphology were assessed as evidence of the combined effectiveness of fixative and fixation temperature. Morphology was scored using a four-point grading scale with 1 being poor and 4 being excellent.

**Results:** Nuclear, cytoplasm and cell membrane morphology were excellently preserved in tissue fixed with phosphate-buffered 10% formalin (pH 7.2) at 45°C. Tissue fixed with 10% formalin at 35°C exhibited excellent nuclear and cell membrane morphology, while excellent preservation of cell membrane morphology were observed in tissues fixed with 10% formalin at 40°C, phosphate-buffered 10% formalin (pH 7.4) at 55°C and 60°C, (pH 7.6) at 50°C and 55°C and (pH 7.8) at 55°C respectively. Furthermore, excellent preservation of nuclear morphology was observed in tissue fixed with phosphate-buffered 10% formalin (pH 7.8) at 60°C.

**Conclusion:** Phosphate-buffered 10% formalin at a temperature of 45°C and pH 7.2 provide an excellent formalin-based fixative and fixation temperature that adequately preserves the microanatomy of tissue for histopathology examination.

*Keywords: buffered, fixative, fixation, morphology, preservation*

## 1. INTRODUCTION

For over a century, histologists have used formalin to prepare tissue for sectioning and microscopic examination [1,2]. Despite this long history of use, the chemistry of formaldehyde's interactions with tissue constituents is understood less completely [3].

Tissue proteins, for example are customarily thought to be simply cross-linked by formaldehyde [4], but they can take part in more chemical reactions with formaldehyde than just cross-linking. Protein amino groups are not the only targets of formalin as evidenced by the observations that peptides lacking amino groups can be reversibly fixed [5] and non-

peptides such as nucleic acids also appear to be altered by fixation in an incompletely understood mechanism [6].

The action of formaldehyde in tissues is further complicated by the fact that formaldehyde in solution is in fact mostly not formaldehyde but rather, it is in equilibrium with a large excess of its nonreactive hydrate (methylene glycol), such that only a small fraction of the molecules in formalin are reactive formaldehyde species which accounts for the often repeated observation that formalin penetrates tissues rapidly but fixes them slowly [6]. However, with these mechanistic reservations formalin still remains an overwhelmingly popular choice of fixative in clinical laboratories regardless of the existence of multiple alternative fixation strategies [7,8].

In this study, we compared the histomorphology of mouse liver samples fixed with 10% formalin at 25°C, 30°C, 35°C, 40°C, 45°C, 50°C, 55°C and 60°C for 24 hours to mouse liver samples fixed with phosphate-buffered 10% formalin (pH 7.2, 7.4, 7.6, and 7.8) at 25°C, 30°C, 35°C, 40°C, 45°C, 50°C, 55°C and 60°C respectively for 24 hours.

## **2. MATERIALS AND METHODS**

### **2.1 Research Location**

This research was conducted at the oral pathology laboratory of the Department of Oral Pathology, University College Hospital, Ibadan, Nigeria.

### **2.2 Experimental Animals**

A total of fifty (50) albino mice were purchased from the Department of Zoology, University of Ibadan, Nigeria. The ages of the animals ranged between 6 and 8 weeks and were a mixture of both sexes. The animals were sacrificed by cervical dislocation within the day of purchase and the extracted liver samples were immediately immersed in the fixatives.

### **2.3 Fixation of Liver Samples**

Extracted liver samples were fixed in 10% formalin which served as control fixative and in the various test fixatives comprising phosphate-buffered 10% formalin (pH 7.2, 7.4, 7.6 and 7.8) for 24 hours at 25°C, 30°C, 35°C, 40°C, 45°C, 50°C, 55°C and 60°C respectively. The fixed tissues were thereafter processed for general tissue morphology using standard histology procedures. Nuclear, cytoplasm and cell membrane morphology were thereafter assessed as indices of the combined effectiveness of fixative and fixation temperature. A four-point grading scale with 1 being poor and 4 being excellent was used to score the slides.

## **3. RESULTS**

### **3.1 Histomorphological Grading of Tissues Fixed at 25°C**

Histomorphological quality of tissues fixed at 25°C for 24 hours is as presented in Table 1. Nuclear, cytoplasm and cell membrane morphology were assessed as indices of the combined efficacy of fixative and fixation temperature. Grading was on a four-point scale with 1 being poor and 4 being excellent. None of the fixatives under investigation produced excellent nuclear, cytoplasm and cell membrane morphology at 25°C.

**Table 1. Histomorphological grading of tissues fixed at 25°C**

Fixative	Nucleus	Cytoplasm	Cell membrane	Total
10% formalin	1	1	1	3
PBF (pH 7.2)	2	3	3	8
PBF (pH 7.4)	1	1	1	3
PBF (pH 7.6)	1	2	2	5
PBF (pH 7.8)	1	2	3	6

Grading was on a scale of 1 (poor) to 4 (excellent). PBF (Phosphate buffered 10% formalin).

### 3.2 Histomorphological Grading of Tissues Fixed at 30°C

Histomorphological quality of tissues fixed at 30°C for 24 hours is as presented in Table 2. Nuclear, cytoplasm and cell membrane morphology were assessed as indices of the combined efficacy of fixative and fixation temperature. Grading was on a four-point scale with 1 being poor and 4 being excellent. None of the fixatives under investigation produced excellent nuclear, cytoplasm and cell membrane morphology at 30°C.

**Table 2. Histomorphological grading of tissues fixed at 30°C**

Fixative	Nucleus	Cytoplasm	Cell membrane	Total
10% formalin	1	1	1	3
PBF (pH 7.2)	2	3	3	8
PBF (pH 7.4)	1	2	2	5
PBF (pH 7.6)	1	2	2	5
PBF (pH 7.8)	1	2	3	6

Grading was on a scale of 1 (poor) to 4 (excellent). PBF (Phosphate buffered 10% formalin).

### 3.3 Histomorphological Grading of Tissues Fixed at 35°C

Histomorphological quality of tissues fixed at 35°C for 24 hours is as presented in Table 3. Nuclear, cytoplasm and cell membrane morphology were assessed as indices of the combined efficacy of fixative and fixation temperature. Grading was on a four-point scale with 1 being poor and 4 being excellent. With the exception of 10% formalin that selectively produced excellent nuclear and cell membrane morphology at 35°C (Figure 1A). No other fixative produced excellent tissue morphology at this temperature.

**Table 3. Histomorphological grading of tissues fixed at 35°C**

Fixative	Nucleus	Cytoplasm	Cell membrane	Total
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10% formalin	4	1	4	9
PBF (pH 7.2)	1	1	1	3
PBF (pH 7.4)	1	2	2	5
PBF (pH 7.6)	1	1	2	4
PBF (pH 7.8)	1	1	1	3

Grading was on a scale of 1 (poor) to 4 (excellent). PBF (Phosphate buffered 10% formalin).

### 3.4 Histomorphological Grading of Tissues Fixed at 40°C

Histomorphological quality of tissues fixed at 40°C for 24 hours is as presented in Table 4. Nuclear, cytoplasm and cell membrane morphology were assessed as indices of the combined efficacy of fixative and fixation temperature. Grading was on a four-point scale with 1 being poor and 4 being excellent. 10% formalin selectively produced excellent cell membrane preservation (Figure 1B) with no other fixative producing excellent preservation of tissue morphology at this temperature.

**Table 4. Histomorphological grading of tissues fixed at 40°C**

Fixative	Nucleus	Cytoplasm	Cell membrane	Total
10% formalin	2	1	4	7
PBF (pH 7.2)	1	1	2	4
PBF (pH 7.4)	1	1	3	5
PBF (pH 7.6)	1	1	2	4
PBF (pH 7.8)	1	1	3	5

Grading was on a scale of 1 (poor) to 4 (excellent). PBF (Phosphate buffered 10% formalin).

### 3.5 Histomorphological Grading of Tissues Fixed at 45°C

Histomorphological quality of tissues fixed at 45°C for 24 hours is as presented in Table 5. Nuclear, cytoplasm and cell membrane morphology were assessed as indices of the combined efficacy of fixative and fixation temperature. Grading was on a four-point scale with 1 being poor and 4 being excellent. Tissues fixed with phosphate-buffered 10% formalin (pH 7.2) produced excellent preservation of nuclear, cytoplasm and cell membrane morphology (Figure 1C). No other fixative produced excellent tissue morphology at this temperature.

**Table 5. Histomorphological grading of tissues fixed at 45°C**

Fixative	Nucleus	Cytoplasm	Cell membrane	Total
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10% formalin	1	2	2	5
PBF (pH 7.2)	4	4	4	12
PBF (pH 7.4)	1	1	2	4
PBF (pH 7.6)	1	2	3	6
PBF (pH 7.8)	3	3	3	9

*Grading was on a scale of 1 (poor) to 4 (excellent). PBF (Phosphate buffered 10% formalin).*

### 3.6 Histomorphological Grading of Tissues Fixed at 50°C

Histomorphological quality of tissues fixed at 50°C for 24 hours is as presented in Table 6. Nuclear, cytoplasm and cell membrane morphology were assessed as indices of the combined efficacy of fixative and fixation temperature. Grading was on a four-point scale with 1 being poor and 4 being excellent. Phosphate-buffered 10% formalin (pH 7.6) selectively produced excellent cell membrane preservation (Figure 1D). No other fixative produced excellent preservation of tissue morphology at this temperature.

**Table 6. Histomorphological grading of tissues fixed at 50°C**

Fixative	Nucleus	Cytoplasm	Cell membrane	Total
10% formalin	2	1	1	4
PBF (pH 7.2)	1	1	1	3
PBF (pH 7.4)	1	1	2	4
PBF (pH 7.6)	1	1	4	6
PBF (pH 7.8)	1	1	1	3

*Grading was on a scale of 1 (poor) to 4 (excellent). PBF (Phosphate buffered 10% formalin).*

### 3.7 Histomorphological Grading of Tissues Fixed at 55°C

Histomorphological quality of tissues fixed at 55°C for 24 hours is as presented in Table 7. Nuclear, cytoplasm and cell membrane morphology were assessed as indices of the combined efficacy of fixative and fixation temperature. Grading was on a four-point scale with 1 being poor and 4 being excellent. Tissues fixed with phosphate-buffered 10% formalin (pH 7.6, 7.4 and 7.8 selectively produced excellent preservation of cell membrane respectively (Figures 1E, 1F and 1G). No other fixative produced excellent preservation of tissue morphology at this temperature.

**Table 7. Histomorphological grading of tissues fixed at 55°C**

Fixative	Nucleus	Cytoplasm	Cell membrane	Total
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10% formalin	2	1	1	4
PBF (pH 7.2)	2	2	3	7
PBF (pH 7.4)	3	1	4	8
PBF (pH 7.6)	1	2	4	7
PBF (pH 7.8)	2	1	4	7

*Grading was on a scale of 1 (poor) to 4 (excellent). PBF (Phosphate buffered 10% formalin).*

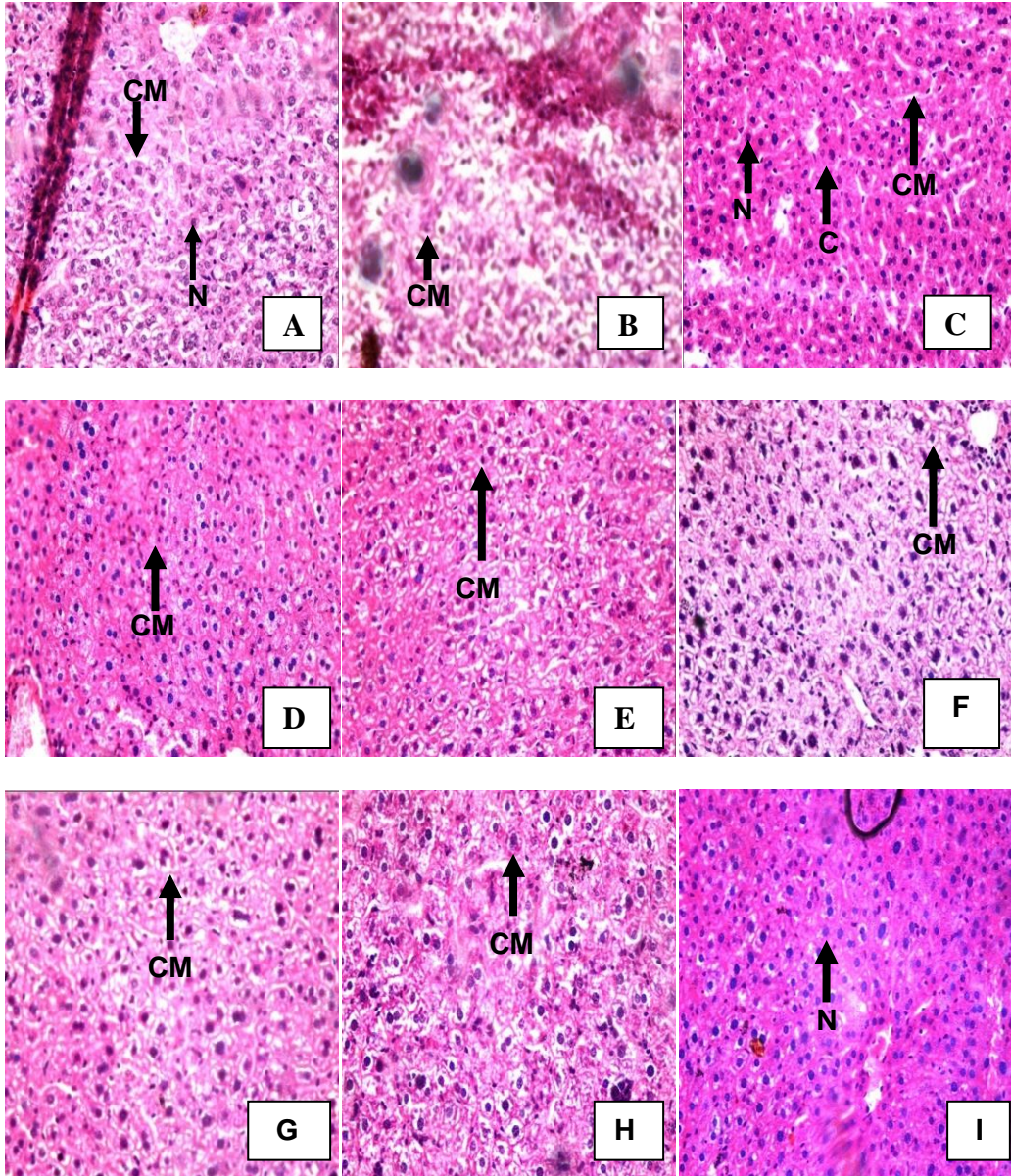
### 3.8 Histomorphological Grading of Tissues Fixed at 60°C

Histomorphological quality of tissues fixed at 60°C for 24 hours is as presented in Table 8. Nuclear, cytoplasm and cell membrane morphology were assessed as indices of the combined efficacy of fixative and fixation temperature. Grading was on a four-point scale with 1 being poor and 4 being excellent. Phosphate-buffered 10% formalin (pH 7.4 and 7.8) selectively produced excellent cell membrane and nuclear morphology respectively (Figures 1H and 1I). No other fixative produced excellent preservation of tissue morphology at this temperature.

**Table 8. Histomorphological grading of tissues fixed at 60°C**

Fixative	Nucleus	Cytoplasm	Cell membrane	Total
10% formalin	1	1	1	3
PBF (pH 7.2)	1	1	1	3
PBF (pH 7.4)	1	2	4	7
PBF (pH 7.6)	2	1	1	4
PBF (pH 7.8)	4	3	3	10

*Grading was on a scale of 1 (poor) to 4 (excellent). PBF (Phosphate buffered 10% formalin).*



**Fig. 1. Showing selected photomicrographs of sections of mouse liver fixed in: (A) 10% formalin at 35°C (Excellent nuclear N and cell membrane CM preservation) (x400); (B) 10% formalin at 40°C (Excellent cell membrane CM preservation) (x400); (C) phosphate-buffered 10% formalin (pH 7.2) at 45°C (Excellent nuclear N, cytoplasm C and cell membrane CM preservation) (x400); (D) phosphate-buffered 10% formalin (pH 7.6) at 50°C (Excellent cell membrane CM preservation) (x400); (E) phosphate-buffered 10% formalin (pH 7.6) at 55°C (Excellent cell membrane CM preservation) (x400); (F) phosphate-buffered 10% formalin (pH 7.4) at 55°C (Excellent cell membrane CM preservation) (x400); (G) phosphate-buffered 10% formalin (pH 7.8) at 55°C (Excellent cell membrane CM preservation) (x400); (H) phosphate-buffered 10% formalin (pH 7.4) at 60°C (Excellent cell membrane CM preservation) (x400); (I) phosphate-buffered 10% formalin (pH 7.8) at 60°C (Excellent nuclear N preservation) (x400).**

## **4. Discussion**

The living cell is in a fluid or a semi-fluid state and fixation involves some chemical modification of tissue proteins and constituents, a necessary event to prevent their loss during tissue processing [9]. For histological assessment, tissue fixation and subsequent paraffin embedding are routinely employed because of the ease of handling tissues and subsequent staining, as well as the good preservation of morphology. Usually, formaldehyde-based fixatives are used for this purpose [10]. Formalin-fixed, paraffin-embedded tissues represent the most abundant supply of archival material for clinical and molecular analysis [11]. Aside from the choice of fixative, the temperature of the fixative has also been shown to affect subsequent histochemical staining of tissues [12]; demonstration of cellular components [13] and optical properties of cadaver tissues [14]. In the long sequence of steps between procurement of the specimen and cover slipping the stained slide, fixation is the single most influential factor. Nearly all other steps can be reversed to ameliorate a problem, but contrastingly, errors in fixation are permanent. Technicians, pathologists and research workers must therefore decide on the most appropriate method.

The objective of this study was to identify a formalin-based fixative and an appropriate fixation temperature that collectively provide the best balance of preservation of tissue morphology. Using mouse liver as a model, we determined that combination of fixative and fixation temperature play critical role in optimizing tissue morphology. The morphology associated with each fixative and fixation temperature was evaluated on a four-point grading system with 1 being poor and 4 being excellent. Nuclear, cytoplasm and cell membrane detail were assigned equal weight.

Based on this scale, excellent nuclear, cytoplasm and cell membrane morphology was obtained with 10% phosphate-buffered formalin (pH 7.2) at 45°C. With 10% formalin at 35°C, excellent nuclear and cell membrane morphology was obtained; however, cytoplasm was poorly preserved. Furthermore, excellent preservation of cell membrane morphology and poor preservation of nuclear and cytoplasm morphology were obtained with 10% formalin at 40°C, 10% phosphate-buffered formalin (pH 7.4) at 55°C and 60°C, 10% phosphate-buffered formalin (pH 7.6) at 50°C and 55°C, 10% phosphate-buffered formalin (pH 7.8) at 55°C. In addition, excellent preservation of nuclear morphology and poor preservation of cytoplasm and cell membrane morphology was obtained with 10% phosphate-buffered formalin (pH 7.8) at 60°C.

## **5. CONCLUSION**

This study has shown that as against 10% formalin which exhibited restrictive preservation of selected component parts of the cell, phosphate-buffered 10% formalin on the other hand was non-selective in its preservation of general tissue morphology. We have also shown that in choosing a formalin-based fixative in conjunction with the appropriate fixative temperature that provides excellent preservation of general tissue morphology, phosphate-buffered 10% formalin (pH 7.2) at 45°C fulfills this criterion. This study has also added its voice to the debate surrounding the suitable pH for formalin-based fixative fluids.

## **AVAILABILITY OF DATA AND MATERIALS**

Data used in this study were presented in the main text.

## **CONSENT (WHERE EVER APPLICABLE)**

Not applicable.

## **ETHICAL APPROVAL (WHERE EVER APPLICABLE)**

Not applicable.

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