Improved Preservation of Histomorphology Resulting from Combined Effect of Tris-Hcl Buffered Formalin Fixative and Controlled Fixation Time

Kenneth C. Onyegbula[1]*, Olugbenga S. Olunaike[2], Gideon T. Oluwaloye[2]
[1]Department of Biomedical Laboratory Science, Faculty of Basic Medical Sciences, College of Medicine, University of Ibadan, Nigeria
[2]Department of Medical Laboratory Sciences, School of Public and Allied Health, Babcock University, Ilishan-Remo, Nigeria

Abstract. Formalin fixation is rife with standardization problems which are possible sources of pre-analytical errors. This study therefore attempts to identify a formalin-based fixative and fixation time that collectively provides the best balance of preservation of tissue morphology using mouse liver as a model. Fifty (50) albino mice aged 6-8 weeks of both sexes were sacrificed by cervical dislocation and their liver were harvested, pooled together and randomly divided into control and experimental groups. Control groups were fixed in 10ml of 10% formalin for 2, 6, 10, 14, 18, 22 and 24 hours at room temperature respectively, while each of the experimental group was fixed separately in 10ml of tris-Hcl buffered 10% formalin at pH 7.2, 7.4, 7.6 and 7.8 respectively for the same duration at room temperature and thereafter processed for general tissue morphology. Nuclear, cytoplasm and cell membrane integrity were assessed as indices of the combined efficacy of fixative and fixation time. Morphology was scored using a four-point grading scale with 1 being poor and 4 being excellent. Tissues fixed with 10% formalin were generally poorly preserved, whereas, tissues fixed with tris-Hcl buffered 10% formalin at pH of 7.2 for 18 and 22 hours and pH 7.4 for 24 hours were excellently preserved. Morphological quality of tissues declined at higher pH values. Combination of tris-Hcl buffered formalin at pH (7.2 / 7.4) for 18, 22 / 24 hours provide an excellent formalin-based fixative and fixation time for adequate tissue preservation for histopathology analysis.

Keywords: Buffer, fixative, fixation, morphology, preservation

Introduction

The preservation of the structural integrity of tissues is one of the most important aspects of histological and histochemical studies, thus an ideal fixative and fixation regimen should preserve the original structure of the tissue as good as possible and should be able to provide an equivalent close to the natural state. This demand can be accomplished by fast penetration of the fixation fluid into the tissue, thus avoiding autolysis and guaranteeing rapid conservation (Meyer & Hornickel, 2010; Singh *et al*., 2019).

Despite raising serious issues because of its toxicity and allergic reaction on respiratory system, eyes and skin of the laboratory personnel, coupled with the fact that it has been labeled as class-1 carcinogen (IARC, 2016; Sarsilmaz, 2018; Vaid *et al*., 2019), formaldehyde fixation and paraffin-embedding remains the most widely used technique for processing tissue specimens for pathologic examination, the study of tissue morphology and archival preservation. It was first identified as a fixative more than a century ago. It can be used for virtually all tissue types, inexpensive, readily available and unlike ethanol as a fixative, does not cause excessive tissue shrinkage or distortion of cellular structure (Srinivasan *et al*., 2002; Sinha *et al*., 2017; Fox *et al*., 1985; Chesnick *et al*., 2010).

Formaldehyde halts tissue degradation by reacting with a wide range of amino acids to form methylol adducts which subsequently undergo a dehydration reaction to form a schiff

*Corresponding Author: ORCID id: 0000-0001-8177-3440.

base, additionally, the protein N-terminal amine can be converted to a stable 4-imidazolidione adduct (Metz *et al*., 2006; Metz *et al*., 2004; Chesnick *et al*., 2010). One other important drawback to the use of formaldehyde as a universal fixative that requires attention is the penetration-fixation paradox (Fox *et al*., 1985; Chesnick *et al*., 2010). In a study, a positive correlation between fixation time and distance travelled in human tissues was established (Start *et al*., 1992a; Start *et al*., 1992b). Kinetic studies also show that formaldehyde binds to tissue components faster at pH 7.0 than at low pH (Helander, 1994).

In this study, we compared the morphology of liver samples fixed with 10% formalin at room temperature for 2, 6, 10, 14, 18, 22 and 24 hours to liver samples fixed with tris-Hcl buffered 10% formalin (pH 7.2, 7.4, 7.6 and 7.8) at room temperature for the same time regimen.

Materials and Methods

Research Location and Experimental Animals

This research was carried out at the oral pathology laboratory of the Department of Oral Pathology, University College Hospital, Ibadan, Nigeria. 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 experimental fixatives.

Fixation of Liver Samples

Extracted liver samples were fixed in 10% formalin (control fixative) for 2, 6, 10, 14, 18, 22 and 24 hours respectively at room temperature and in the various test fixatives which comprises tris-Hcl buffered 10% formalin at pH 7.2, 7.4, 7.6 and 7.8 for 2, 6, 10, 14, 18, 22 and 24 hours respectively at room temperature. 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 efficacy of fixative and fixation time. A four-point grading scale with 1 being poor and 4 being excellent was used to score the slides.

Results and Discussion

Table 1. Histomorphological grading of tissues fixed for 2 hours

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

Note: Grading was on a scale of 1 (poor) to 4 (excellent). THBF (tris-Hcl buffered 10% formalin).

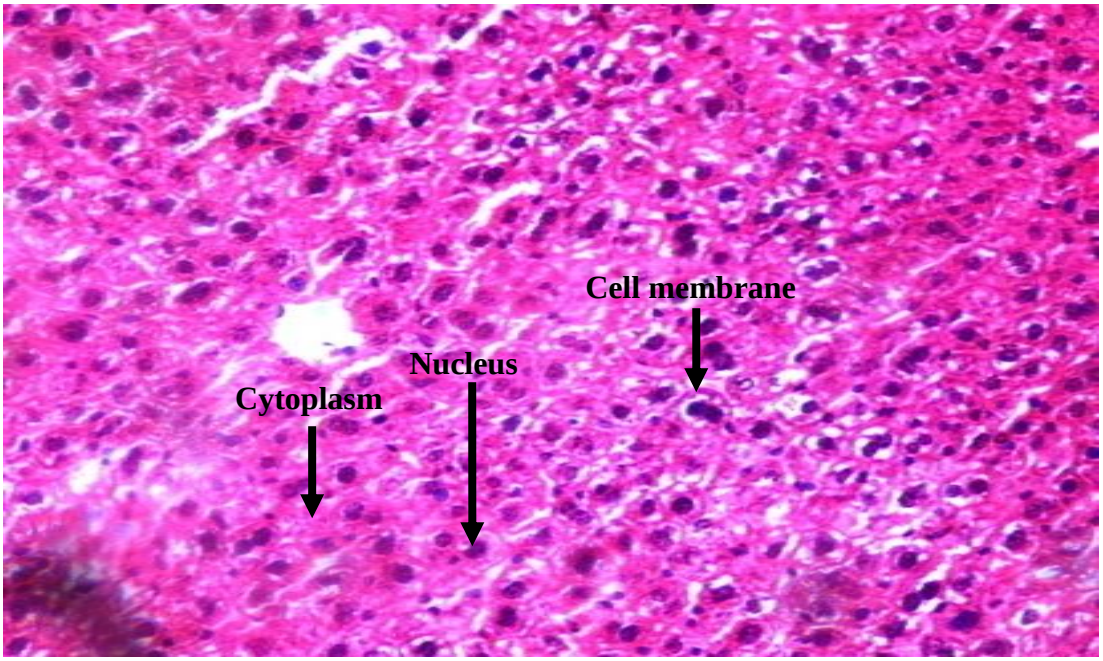

Figure 1. Selected photomicrograph of section of mouse liver fixed with 10% formalin for 18 hours (mag x400)

Histomorphology of Tissues Fixed for 2 Hours

Histomorphological grading of tissues fixed for 2 hours at room temperature is as presented in Table 1. Nuclear, cytoplasm and cell membrane details were assessed as indices of the combined efficacy of fixative and fixation time. Grading was on a four-point scale with 1 being poor and 4 being excellent. Tissues fixed with 10% formalin shown in Figure 1 had a combined nuclear, cytoplasm and cell membrane score of 3, while tissues fixed with tris-Hcl buffered 10% formalin had the highest combined nuclear, cytoplasm and cell membrane score of 6 at pH 7.4 and 7.8.

Table 2. Histomorphological grading of tissues fixed for 6 hours

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

Note: Grading was on a scale of 1 (poor) to 4 (excellent). THBF (tris-Hcl buffered 10% formalin).

Histomorphology of Tissues Fixed for 6 Hours

Histomorphological grading of tissues fixed for 6 hours is as presented in Table 2. Nuclear detail, cytoplasm detail and cell membrane detail were assessed as indices of the combined efficacy of fixative and fixation time. Grading was on a four-point scale with 1 being poor and 4 being excellent. Tissues fixed with 10% formalin also had a combined nuclear, cytoplasm and cell membrane score of 3. Tissues fixed with tris-Hcl buffered 10% formalin also had the highest combined nuclear, cytoplasm and cell membrane score of 6 at pH 7.4 and 7.8 respectively.

Table 3. Histomorphological grading of tissues fixed for 10 hours

Fixative	Nucleus	Cytoplasm	Cell membrane	Total
10% formalin	1	1	1	3
THBF (pH 7.2)	1	1	1	3
THBF (pH 7.4)	2	1	4	7
THBF (pH 7.6)	1	1	4	6
THBF (pH 7.8)	1	1	4	6

Note: Grading was on a scale of 1 (poor) to 4 (excellent). THBF (tris-Hcl buffered 10% formalin).

Histomorphology of Tissues Fixed for 10 Hours

Histomorphological grading of tissues fixed for 10 hours is as presented in Table 3. Nuclear detail, cytoplasm detail and cell membrane detail were assessed as indices of the combined efficacy of fixative and fixation time. Grading was on a four-point scale with 1 being poor and 4 being excellent. Tissues fixed with 10% formalin also had a combined nuclear, cytoplasm and cell membrane score of 3, whereas, tissues fixed with tris-Hcl buffered 10% formalin had a highest combined nuclear, cytoplasm and cell membrane score of 7 at pH 7.4.

Table 4. Histomorphological grading of tissues fixed for 14 hours

Fixative	Nucleus	Cytoplasm	Cell membrane	Total
10% formalin	1	1	1	3
THBF (pH 7.2)	4	1	4	9
THBF (pH 7.4)	1	2	2	5
THBF (pH 7.6)	1	2	4	7
THBF (pH 7.8)	1	1	4	6

Note: Grading was on a scale of 1 (poor) to 4 (excellent). THBF (tris-Hcl buffered 10% formalin).

Histomorphology of Tissues Fixed for 14 Hours

Histomorphological grading of tissues fixed for 14 hours is as presented in Table 4. Nuclear detail, cytoplasm detail and cell membrane detail were assessed as indices of the combined efficacy of fixative and fixation time. Grading was on a four-point scale with 1 being poor and 4 being excellent. Tissues fixed with 10% formalin also had a combined nuclear, cytoplasm and cell membrane score of 3 compared to tissues fixed with tris-Hcl buffered 10% formalin which had the highest combined nuclear, cytoplasm and cell membrane score of 9 at pH 7.2.

Table 5. Histomorphological grading of tissues fixed for 18 hours

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

Note: Grading was on a scale of 1 (poor) to 4 (excellent). THBF (tris-Hcl buffered 10% formalin).

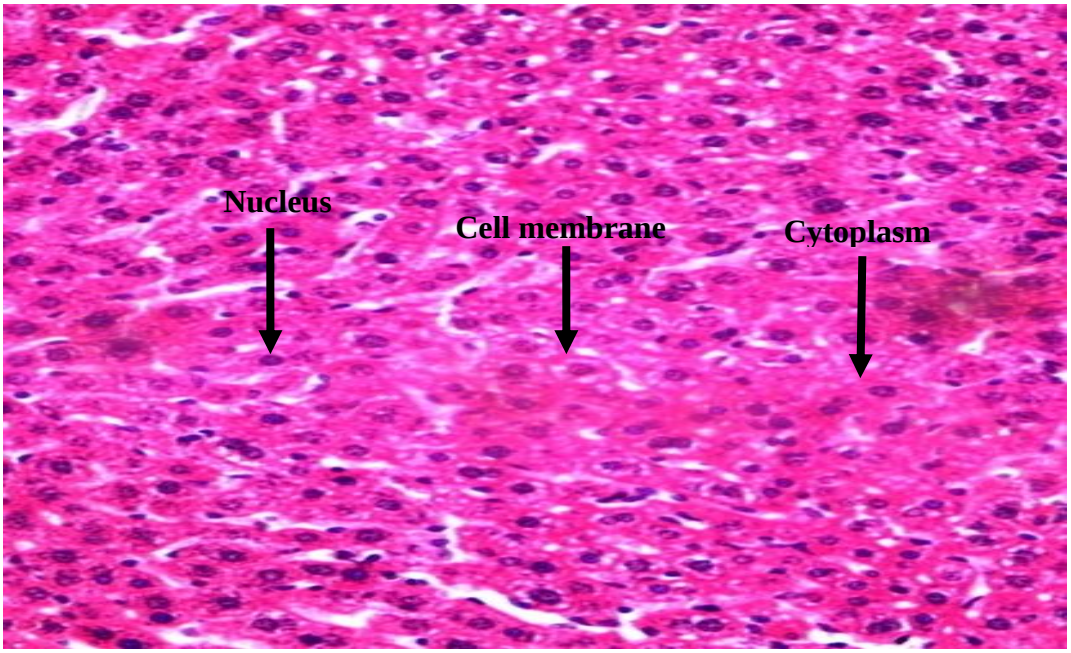

Figure 2. Selected photomicrograph of section of mouse liver fixed with tris-Hcl buffered formalin at pH 7.2 for 18 hours (mag x400)

Histomorphology of Tissues Fixed for 18 Hours

Histomorphological grading of tissues fixed for 18 hours is as presented in Table 5. Nuclear detail, cytoplasm detail and cell membrane detail were assessed as indices of the combined efficacy of fixative and fixation time. Grading was on a four-point scale with 1 being poor and 4 being excellent. Tissues fixed with 10% formalin also had a combined nuclear, cytoplasm and cell membrane score of 3, however, tissues fixed with tris-Hcl buffered 10% formalin had the highest combined nuclear, cytoplasm and cell membrane score of 12 at pH 7.2 shown in Figure 2.

Table 6. Histomorphological grading of tissues fixed for 22 hours

Fixative	Nucleus	Cytoplasm	Cell membrane	Total
10% formalin	1	1	1	3
THBF (pH 7.2)	4	4	4	12
THBF (pH 7.4)	2	4	4	10
THBF (pH 7.6)	4	3	4	11
THBF (pH 7.8)	1	2	2	5

Note: Grading was on a scale of 1 (poor) to 4 (excellent). THBF (tris-Hcl buffered 10% formalin).

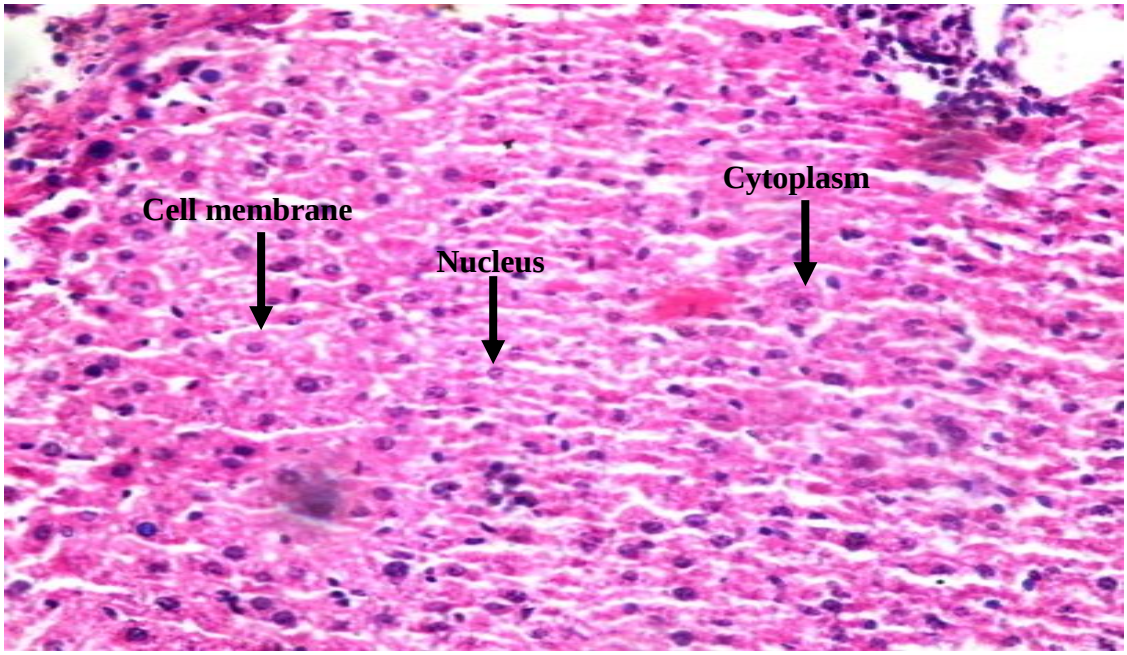

Figure 3. Selected photomicrograph of section of mouse liver fixed with tris-Hcl buffered formalin at pH 7.2 for 22 hours (mag x400)

Histomorphology of Tissues Fixed for 22 Hours

Histomorphological grading of tissues fixed for 22 hours is as presented in Table 6. Nuclear detail, cytoplasm detail and cell membrane detail were assessed as indices of the combined efficacy of fixative and fixation time. Grading was on a four-point scale with 1 being poor and 4 being excellent. Tissues fixed with 10% formalin also had a combined nuclear, cytoplasm and cell membrane score of 3. Tissues fixed with tris-Hcl buffered 10% formalin had the highest combined nuclear, cytoplasm and cell membrane score of 12 also at pH 7.2 shown in Figure 3.

Table 7. Histomorphological grading of tissues fixed for 24 hours

Fixative	Nucleus	Cytoplasm	Cell membrane	Total
10% formalin	1	1	1	3
THBF (pH 7.2)	2	4	4	10
THBF (pH 7.4)	4	4	4	12
THBF (pH 7.6)	4	3	4	11
THBF (pH 7.8)	1	2	2	5

Note: Grading was on a scale of 1 (poor) to 4 (excellent). THBF (tris-Hcl buffered 10% formalin.

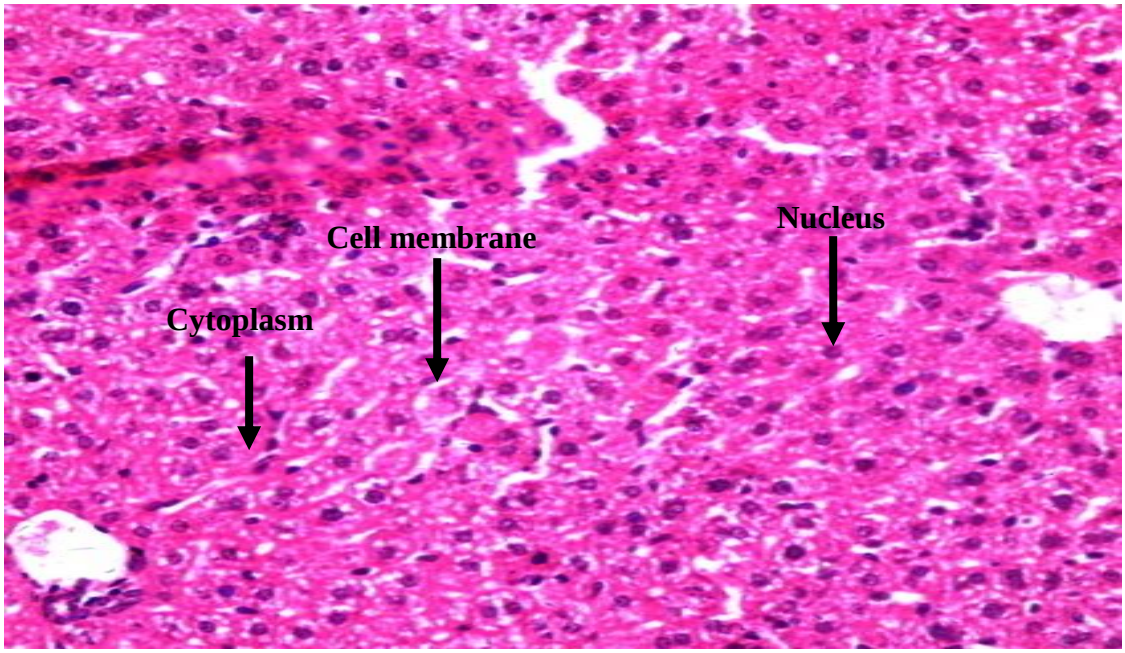

Figure 4. Selected photomicrograph of section of mouse liver fixed with tris-Hcl buffered formalin at pH 7.4 for 24 hours (mag x400)

Histomorphology of Tissues Fixed for 24 Hours

Histomorphological grading of tissues fixed for 24 hours is as presented in Table 7. Nuclear detail, cytoplasm detail and cell membrane detail were assessed as indices of the combined efficacy of fixative and fixation time. Grading was on a four-point scale with 1 being poor and 4 being excellent. Tissues fixed with 10% formalin also had a combined nuclear, cytoplasm and cell membrane score of 3, however, tissues fixed with tris-Hcl buffered 10% formalin had the highest combined nuclear, cytoplasm and cell membrane score of 12 at pH 7.4 shown in Figure 4.

One of the most important aspects of histological and histochemical studies is the preservation of the structural stability of tissues. It has been observed that fixation modifies the physico-chemical state, including other parameters such as redox and membrane potentials of the tissue, thereby changing the reactivity of cellular components with the stain (Meyer *et al*., 2010; Tokuda *et al*., 2018). Formalin fixation, followed by paraffin embedding is frequently used to preserve tissue specimens for pathological examination due to the high quality of the resulting tissue blocks, its low cost and ease of archival storage. To be effective, the first part of the fixation process must be rapid specimen collection, whereby, several factors have to be taken into account to obtain representative results. Thus samples should be taken from freshly dead animals and the time period between tissue removal and immersion in the fixation solution kept as short as possible. Furthermore, a successful fixation process would depend on the composition of the fixation solution used (Meyer *et al*., 2010; Tokuda *et al*., 2018).

Formalin belongs to the chemical group of aldehydes, and fixation with these chemicals is a complex process which includes a rapid penetration that stops autolysis, followed by covalent binding and cross-linking. These three parts of fixation proceed simultaneously, but at very different rates. For instance, penetration is 12 times faster than binding. In a review, it was stated that formaldehyde causes a decrease in the overall positive electrical charge of tissues, which is related to the amount of acid generated during fixation and lowering of the pH. In their opinion the change of charge or lowering of the iso-electric point of proteins as a consequence of fixation with aldehyde is bound to alter the physical nature of proteins with a resulting increase or decrease in the number of sites available for stain uptake (Meyer *et al*.,

2010). In contrast, Bouin's fluid which aside from formalin, also contains picric acid and glacial acetic acid has the advantage of penetrating into tissues more rapidly and evenly and is less damaging to the conformation of proteins than formaldehyde alone, thus guaranteeing excellent tissue structure and avoiding tissue shrinkage. They suggested that bad qualities of tissue preservation, which sometimes may occur in tissues fixed with buffered formalin could originate from the high pH level (7.0-7.4) of the solution, whereas, Bouin's solution fixes at a pH level of 6.0 to 5.5 (Meyer *et al*., 2010).

Earlier in another article, it was emphasized that maximum tissue fixation occurs in the pH range 4.0 to 5.5 and that no increase in tissue stabilization was observed above pH 5.5. They concluded that the increased amount of formaldehyde bound at higher pH levels only blocks numerous reactive groups (Puchtler & Meloan, 1985). Kinetic studies however show that formaldehyde binds to tissues components faster at pH 7.0 than at low pH (Helander, 1994).

In this study however, we demonstrate that the general tissue morphology which takes into account the nuclear, cytoplasm and cell membrane structures of mouse liver fixed with 10% formalin alone for 2 to 24 hours at room temperature were poorly preserved but excellently preserved with tris-Hcl buffered 10% formalin at pH 7.2 for 18 and 22 hours and pH 7.4 for 24 hours at room temperature. We also demonstrate that fixation of tissues for less than 18 hours on the one hand and at higher pH levels of 7.6 and 7.8 on the other hand with tris-Hcl buffered 10% formalin were unfavorable for tissue morphology preservation.

Conclusion

In histology practice, despite the influx and introduction of newer non-formalin based fixatives, 10% formalin (buffered or un-buffered) has remained the universal fluid for preserving the morphology of tissues in life-like manner for subsequent processing. In this study, we have shown that in contrast with 10% formalin which produced poor general tissue morphology preservation, tris-Hcl buffered 10% formalin produced excellent general tissue morphology preservation. We have also shown that for excellent general tissue morphology preservation, tissue samples should be allowed to fix in tris-Hcl buffered 10% formalin at pH 7.2 for 18 to 22 hours or at pH 7.4 for 24 hours thereby reducing the time needed for tissue fixation. This study has also contributed to the controversy surrounding the appropriate pH for formalin or non-formalin based fixation fluids.

Acknowledgments

This research did not receive any form of funding from funding agencies.

Competing interests

Authors declare that there are no competing interests.

Authors' contributions

KCO was responsible for the concept, design, control and supervision of the project; Data collection and processing were done by KCO, OSO and GTO; Analysis and interpretation of results was done by KCO; Literature review and writing of article was done by KCO; All authors have critically reviewed and approved the final draft and are responsible for the extent and similarity index of the manuscript.

Availability of data and materials

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

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