Current Journal of Applied Science and Technology

40(40): 42-49, 2021; Article no.CJAST.78682
ISSN: 2457-1024
(Past name: British Journal of Applied Science & Technology, Past ISSN: 2231-0843, NLM ID: 101664541)

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

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Authors' contributions

This work was carried out in collaboration between both authors. Author KCO was responsible for the concept, design, control and supervision of the project; Data collection and processing were done by Author KCO and GTO; Analysis and interpretation of results was done by author KCO; Literature review and writing of article was done by author KCO; Both authors have critically reviewed and approved the final draft and are responsible for the extent and similarity index of the manuscript.

Article Information

DOI: 10.9734/CJAST/2021/v40i4031601

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/78682

Original Research Article

Received 09 October 2021
Accepted 18 December 2021
Published 20 December 2021

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^{o}C, 30^{o}C, 35^{o}C, 40^{o}C, 45^{o}C, 50^{o}C, 55^{o}C and 60^{o}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.

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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.

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.

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 (Fig. 1A). No other fixative produced excellent tissue morphology at this temperature.

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)

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).

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

Fixative	Nucleus	Cytoplasm	Cell membrane	Total
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 (Fig. 1B) with no other fixative producing excellent preservation of tissue morphology at this temperature.

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 (Fig. 1C). No other fixative produced excellent tissue morphology at this temperature.

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 (Fig. 1D). No other fixative produced 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)

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

Fixative	Nucleus	Cytoplasm	Cell membrane	Total
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)

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 (Figs. 1E, 1F and 1G). No other fixative produced excellent preservation of tissue morphology at this temperature.

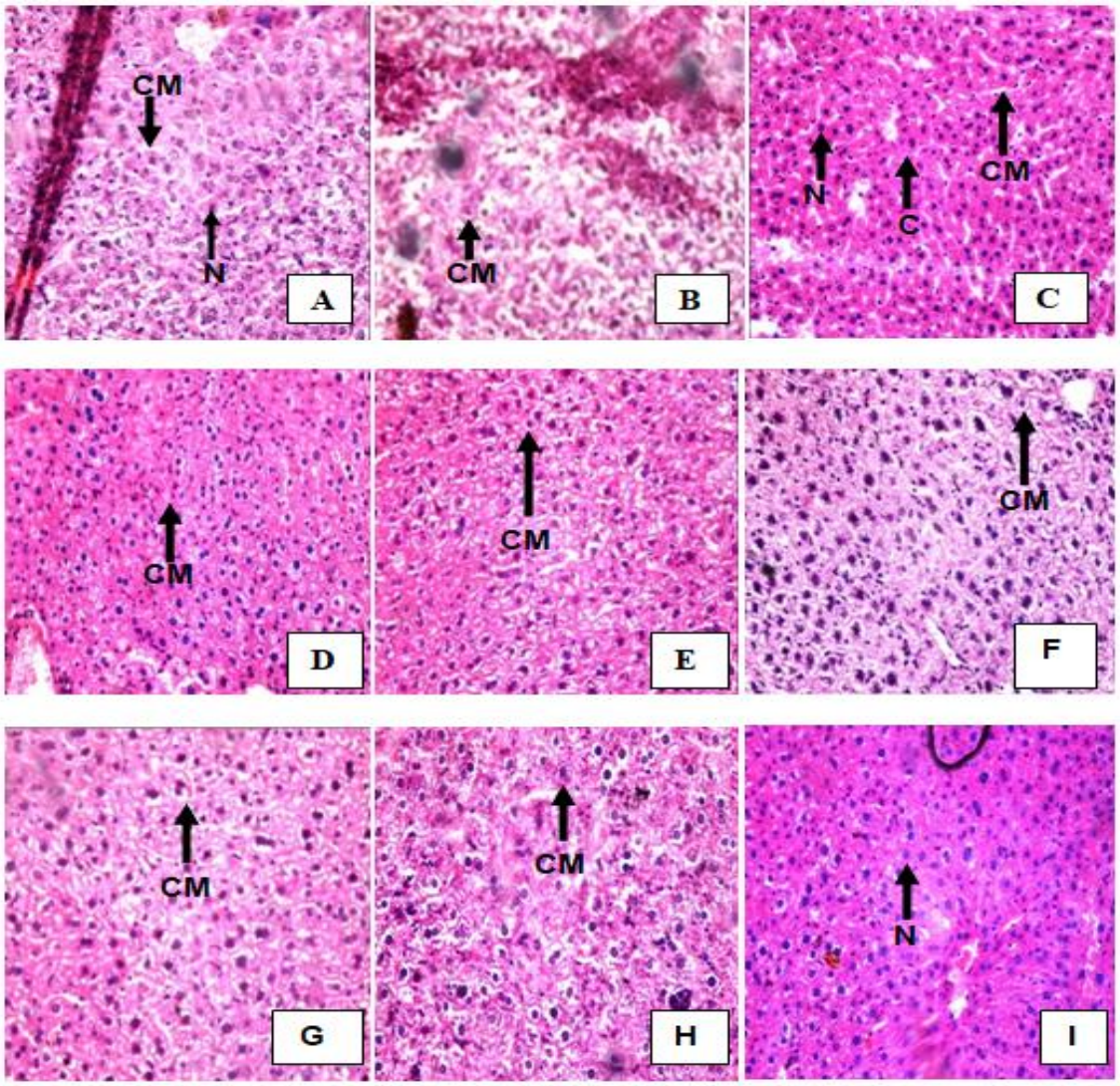

Fig. 1. Showing selected photomicrographs of sections of mouse liver fixed in: (A) 10% formalin at 35°C (Excellent nuclear N and cell membrane CM preservation) (x400); (B) 10% formalin at 40°C (Excellent cell membrane CM preservation) (x400); (C) phosphate-buffered 10% formalin (pH 7.2) at 45°C (Excellent 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)

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

Fixativ	Nucleus	Cytoplasm	Cell membrane	Total
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)

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)

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 (Figs. 1H and 1I). No other fixative produced excellent preservation of tissue morphology at this temperature.

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

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

ACKNOWLEDGMENTS

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

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Rahman A, Sultana N, Ayman U, Bhakta S, Afrose M, Afrin M, et al. Alcoholic fixation over formalin fixation: A new, safer option for morphologic and molecular analysis of tissues. Saudi Journal of Biological Sciences. 2021;29:175-182. DOI.org/10.1016/j.sjbs.2021.08.075.
2. Meecham A, Miranda E, Morris H, Hair J, Oien K, Gerrard G, et al. Alternative tissue fixation for combined histomorphological and molecular analysis in a clinically representative setting. Histochemistry and Cell Biology. 2021;156:595-607. DOI.org/10.1007/s00418-021-02029-1.
3. Gatta LB, Cadei, M, Balzarini P, Castricano S, Paroni R, Verzeletti A, et al. Application of alternative fixatives to formalin in diagnostic pathology. European Journal of Histochemistry. 2012;56(2):e12. DOI.org/10.4081/ejh.2012.e12.
4. Tayri-Wilk T, Slavin M, Zamel J, Blass A, Cohen S, Motzik A, et al. Mass spectrometry reveals the chemistry of formaldehyde cross-linking in structured proteins. Nature Communicatons. 2020;11:3128. DOI.org/10.1038/s41467-020-16935-w.
5. Sompuram SR, Vani K, Messana E, Bogen SA. A molecular mechanism of formalin fixation and antigen retrieval. American Journal of Clinical Pathology. 2004;121: 190-199.
6. Berrino E, Annaratone L, Miglio U, Maldi E, Piccinelli C, Peano E, et al. Cold formalin fixation guarantees DNA integrity in formalin fixed paraffin embedded tissues: Premises for a better quality of diagnostic and experimental pathology with a specific impact on breast cancer. Frontiers in Oncology. 2020;10:173. DOI.org/10.3389/fonc.2020.00173.
7. Titford ME. Formalin fixation. Archives of Pathology and Laboratory Medicine. 2012; 136(2):137-138. DOI.org/10.5858/arpa.2011-0363-LE.
8. Baloglu G, Haholu A, Kucukodaci Z, Yilmaz I, Yildirim S. The effects of tissue Fixation alternatives on DNA content-A study on normal colon tissue. Appleton Immunohistochemistry of Molecular Morphology. 2008;16:485-492.
9. Srinivasan M, Sedmark D, Jewell S. Effect of fixatives and tissue processing on the content and integrity of nucleic acids. American Journal of Pathology. 2002;161 (6):1961-1971. Doi.org/10.1016/s0002-9440(10)644720.
10. Uneyama C, Shibutani M, Masutomi N, Takagi H, Hirose M. Methacarn fixation for genomic DNA analysis in microdissected, paraffin-embedded tissue specimens.

Journal of Histochemistry and Cytochemistry. 2002;50(9):1237-1245. DOI.org/10.1177/002215540205000911.

11. Delfour C, Roger P, Bret C, Berthe M. RCL2, a new fixative, preserves morphology and nucleic acid integrity in paraffin-embedded breast carcinoma and microdissected breast tumor cells. Journal of Molecular Diagnostics. 2006;8(2):157-169.
12. Elzabbal MHE, Hussain G, Abdellaziz MS, Imam MS, Abdalhmeed M. Effect of fixatives temperatures on subsequent Histochemical staining. Sudan Journal of Medical Sciences. 2013;8(3):147-150.
13. Bamisi OD, Alese MO. Effect of various fixatives and temperature on the quality of glycogen demonstration on the brain and liver tissues. Annals of Diagnostic Pathology; 2020. DOI.org/10.1016/j.anndiagpath.2020.151604.
14. Gnanadesigan M, Soest G, White S, Scoltock S, Ughi G, Steen F, et al. Effect of temperature and fixation on the optical properties of artherosclerotic tissue: a validation study of an ex-vivo whole-heart cadaveric model. Biomedical Optics Express. 2014;5(4):1038-1049. DOI.org/10.1364/BOE.5.001038.

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Peer-review history:
The peer review history for this paper can be accessed here:
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