Original Article

Assessing the Quality of Long-Term Stored Tissues in Formalin and in Paraffin-Embedded Blocks for Histopathological Analysis

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Abstract

Introduction: Formalin is the most commonly used fixative which enables for long-term storage of specimens and preserves morphologic features allowing the microscopic evaluation for future research analysis. Archival collections of the tissue serve as a reliable tool for diagnostic research purpose. They have an important role in on-going patient care, allows for evaluation of recurrent cases for diagnostic purpose and rare case specimens can also be used as an educational tool as well as for further biomedical research purposes. However, studies assessing quality and their usefulness for such purposes are scanty. Hence, the present study is aimed at evaluating and comparing the tissue changes after long-term storage in formalin as well as in paraffin-embedded blocks. **Methodology:** Three study groups include specimens stored in formalin for a minimum of 5 years (long-term fixed tissue) and their corresponding paraffin-embedded old tissue blocks along with freshly fixed tissues taken as controls which were subjected to routine histopathological procedures and were assessed for macroscopic and microscopic evaluation. Chi-square test and Z-proportion tests were considered for statistical analysis. **Results:** Prolonged storage of the tissues in formalin showed variation in color and consistency, difficulty in cutting during grossing with inadequate sectioning characters, loss of tissue integrity and architecture, and inadequate nuclear and cytoplasmic details. **Conclusion:** On histological analysis, prolonged formalin-stored specimens showed deleterious effects than archival blocks. Hence, it can be proposed that tissues are better preserved in paraffin blocks rather than in formalin for further biomedical research purposes.

Keywords: Archival specimens, fixation, formalin, paraffin blocks, prolonged formalin-fixed tissues

Introduction

Formalin-fixed specimens, formalin-fixed paraffin-embedded (FFPE) blocks, and histological slides are commonly used for histological analysis and pathological diagnosis worldwide and are suitable for storage for long periods. These serve as the most valuable resources for molecular biological analysis. Use of FFPE for immunohistochemistry eliminates the need for fresh or frozen tissues.[1,2] Archival tissue preservation either in fixative solutions or paraffin-embedded blocks is very important and essential as they serve as a reserve bio-bank for further molecular studies. Purpose of preservative solutions is to stabilize the specimen and prevent it from deteriorating. Specimens can last for years if stored in good, well-maintained, and favorable protective microenvironment around the specimens.[3]

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Fixative should be readily disposable or recyclable and support long-term storage giving excellent microtomy of the paraffin blocks.[4] These stored specimens are the wonderful resource for research, educational tool, and retrospective investigation for unusual and recurrent cases, and the potential exploration using them with newer markers can facilitate newer hypothesis. This fact makes the choice of fixative a crucial aspect of the success of histopathology and tissue storage. However, data regarding the usefulness of the long-term stored tissue in formalin and in paraffin wax for histological analysis

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are scanty. Hence, the present study is aimed at evaluating and comparing the tissue changes after long-term storage in formalin as well as in paraffin-embedded blocks.

Methodology

51 tissue specimens stored in formalin for minimum of five years (long-term fixed tissues - LFT) which were apparently in good condition and five year old paraffin embedded tissue blocks of same cases (old tissue blocks - OTB) were retrieved from archives of Department of Oral Pathology and Microbiology. Macroscopic details of the tissues of OTB were retrieved from old grossing details of department records. 51 freshly fixed tissues (FFT) submitted to the department were used as control group.

The grossing of LFTs and FFTs was performed noting macroscopic features such as color, consistency, and cutting efficiency. Both were processed using automated tissue processor (19-h) and subsequently embedded in tissue embedding station. Three sections from each sample of LFT, FFT, and OTB of 5–6 μ thickness were prepared using motorized rotary microtome Leica RM 2165. New disposable stainless steel microtome blades were used for all the tissue blocks for sectioning and noted the presence or absence of ribbon formation and persistence of folds in the water bath at 56°C.

Of the three sections prepared of LFT, FFT, and OTB, one each was stained with Haematoxylin and eosin (H and E), Van Gieson, and Mallory stain. Microscopic evaluation was done using Research Microscope BX51 for the following criteria:

1. Tissue integrity (fold, disintegration, and tear)
2. Tissue architecture (epithelium and connective tissue – present/absent)
3. Identification of tissue components (keratin, fibers – present/absent)
4. Cellular and nuclear details (identifiable, identifiable but not-clear, and nonidentifiable).

Integrity and architecture of the tissue were analyzed under × 4 magnification, while nuclear and cytoplasmic details were studied under × 40 magnification.

Statistical analysis

Chi-square test was carried out for comparison between the study groups, i.e., OTB, LFT, and FFT. Z-proportion was carried out for the presence of keratin between H and E and Mallory stain. $P \leq 0.05$ was set for statistical significance. SPSS version 16 (Chicago, SPSS Inc.) software was used for all analysis.

Results

Macroscopic features

Color

Predominant color changes were noted in FFT and LFT [Table 1]. Variation in the color of the tissues after long-term fixation was observed with nearly one-third of the cases showing difference in the color, while two-third of the cases remained the same [Table 2].

Table 1: Color of the specimen in three study groups

Color	*n* (%)		
	OTB (details from old record)	LFT	FFT
Predominantly grayish	23 (45.098)	25 (49.019)	25 (49.019)
Predominantly creamish	28 (54.90)	26 (50.98)	26 (50.98)
Total	51 (100)	51 (100)	51 (100)
Chi-square test	0.320		
P	0.571 (NS)		

OTB: Old tissue blocks (details from old record), LFT: Long-term formalin-fixed tissue, FFT: Freshly fixed tissues, NS: Not significant

Consistency

Maximum percentage of softness and friability of tissue was observed in LFT. More than half of the cases showed variation in consistency when we compared OTB (old record) with respective LFT [Tables 3 and 4].

Cutting efficiency

Cutting efficiency of the tissue during grossing of the LFT and FFT was categorized as easy or difficult which showed difficulty in cutting LFT sections compared to FFT [Table 5].

Sectioning characters

Sectioning characters were assessed during microtomy based on the presence or absence of ribbon formation and persistence of folds in the water bath after microtomy. More number of cases in LFT showed persistence of folds than OTB and FFT. Failure to form ribbon was noted in both OTB and LFT, while all samples of FFT had ribbon formation [Table 6 and Figure 1a,b].

Microscopic features

Effect on subsequent staining and histological appearance in LFT and its corresponding OTB and FFT was observed in the sections for the following features:

Tissue integrity

Tissue integrity was evaluated in H and E-stained slides based on the presence of folds, tissue disintegration, and tear in tissue sections. Among the three groups, integrity is shown to be good in the sections from FFT followed by OTB and least in LFT. Tissue disintegrity was found to be maximum in LFT sections [Table 7 and Figure 2a,b].

Tissue architecture

Tissue architecture was assessed based on the presence or absence of epithelium and connective tissue in H and E-stained slides among the three study groups. Presence of epithelium was found to be maximum in FFT followed by OTB and least with LFT [Table 8]. Connective tissue was present in 100% of all the cases.

Identification of tissue components

Tissue components was assessed based on the identification and

Table 2: Variation in the color of the tissues after long-term fixation

Color	Shades of gray to shades of cream	Shades of cream to shades of gray	Shades of gray	Shades of cream
OTB (details from old record) versus LFT (%)	8 (15.68)	10 (19.60)	15 (29.41)	18 (35.29)
Total	18 (35.29%) cases showed a change in the color		33 (60.78%) cases did not show the change in the color	
Chi-square test	0.353			
P	0.552 (NS)			

OTB: Old tissue blocks (details from old record), LFT: Long-term formalin-fixed tissue, NS: Not significant

Table 3: Consistency of specimen in three study groups

Consistency	*n* (%)		
	OTB (details from old record)	LFT	FFT
Hard	5 (9.80)	1 (1.96)	0
Firm	45 (88.23)	26 (50.98)	50 (98.03)
Soft	1 (1.96)	18 (35.29)	1 (1.96)
Friable	0	6 (11.76)	0
Total	51	51	51
Chi-square test	242.111		
P	<0.001**		

**indicates highly significant. OTB: Old tissue blocks (details from old record), LFT: Long-term formalin-fixed tissue, FFT: Freshly fixed tissues

presence/absence of keratin in the epithelium using Mallory stain and fibers in the connective tissue using Van Gieson stain among three study groups using routine H and E stain as the standard. Van Gieson stain revealed the presence of connective tissue fibers in all samples in all the three study groups [Figure 3a and b]. Presence of keratin was found in maximum number of cases from FFT, followed by OTB and least in LFT using H and E and Mallory stain [Table 9 and Figure 4a, b].

Cellular and nuclear details

Cellular and nuclear details were assessed in H and E-stained slides by categorizing them as identifiable, nonidentifiable, and identifiable but not clear. Features were readily identifiable in OTB than in LFT and 5.9% of cases of LFT were not clear [Table 10 and Figure 5a-c]. All samples in the FFT showed clear cellular and nuclear details.

A statistically significant difference in the variation of macroscopic features such as consistency, cutting efficiency, and sectioning characteristics and in microscopic features such as tissue integrity, tissue architecture, tissue components, and cellular-nuclear features was found between the three study groups and also between OTB and LFT.

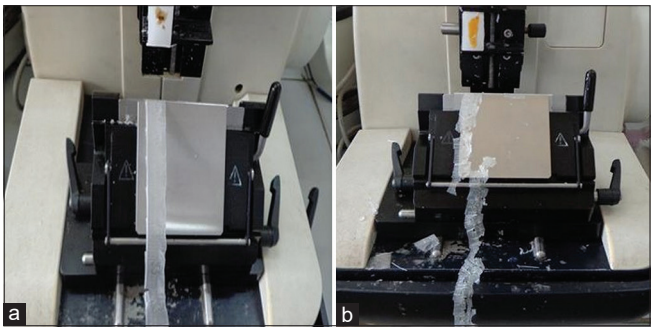

Figure 1: (a) Adequate ribbon formation in freshly fixed tissue; (b) inadequate ribbon formation in long-term fixed tissue

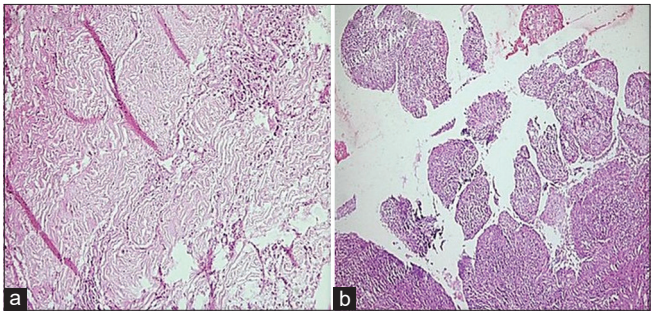

Figure 2: (a) Section showing folds in long-term fixed tissue (H and E, ×10); (b) Section showing tear and disintegrated tissue in long-term fixed tissue (H and E, ×10)

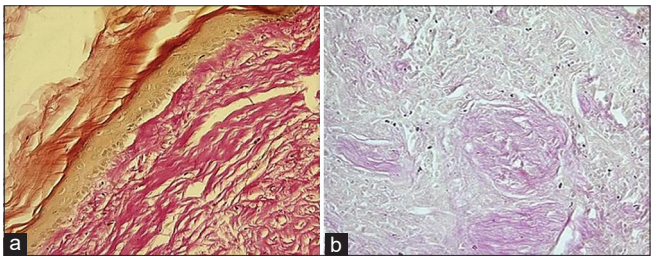

Figure 3: (a) Section of old tissue block demonstrating collagen fibers after staining with Van Gieson (×20); (b) Section of long-term fixed tissue demonstrating collagen fibers after staining with Van Gieson (×20)

Discussion

Proteins and nucleic acids are the principal macromolecules of a cell. Their shape and size in and around cells determine the tissue structure. Preserving the cells and tissues in a natural state is the most essential part of a histological technique for understanding cellular-subcellular structures and functions, which is achieved by immersing the tissues in fixatives immediately after their separation from the body. Fixatives act by inactivating lysosomal enzymes, prevent putrefaction and autolysis, and also make the macromolecules resistant to the dissolution by water and other liquids, thereby stabilizing the cell.[4,5] In surgical pathology, neutral-buffered formalin has been considered as the "gold standard" fixative since years. This enables for long-term storage

Table 4: Variation in the consistency of the tissue after long-term fixation in formalin

Consistency	Variation in the consistency of the tissue after long-term fixation in formalin, *n* (%)					
	Hard to firm	Firm to soft	Firm to friable	Soft to firm	Firm	Hard
OTB (details from old record) versus LFT	4 (7.843)	18 (35.29)	6 (11.76)	1 (1.96)	21 (41.17)	1 (1.96)
Total	29 (56.86% of cases changed the consistency)				22 (43.13% of the cases remained the same)	
Chi-square test	108.667					
P	<0.001**					

**indicates highly significant. OTB: Old tissue blocks (details from old record), LFT: Long-term formalin-fixed tissue

Table 5: Cutting efficiency during grossing of long-term formalin-fixed tissue and freshly fixed tissues

	Cutting efficiency during grossing, *n* (%)	
	LFT, *n* (%)	FFT, *n* (%)
Easy	34 (59.6)	50 (98.03)
Difficult	23 (40.4)	1 (1.96)
Chi-square test	32.980	
P	<0.001**	

**indicates highly significant. LFT: Long-term formalin-fixed tissue, HS: Highly significant, FFT: Freshly fixed tissues

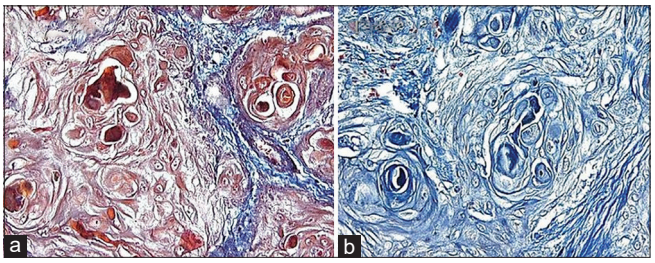

Figure 4: (a) Section of old tissue block demonstrating keratin after staining with Mallory stain (×20); (b) section of long-term fixed tissue unable to demonstrate keratin after staining with Mallory stain (×20)

of specimens, thereby aiding in preserving the morphologic features and allowing microscopic evaluation for diagnostic and future research analysis.[1] Even after adequate sampling, portions of large specimen are often left untouched. In most laboratories, remaining tissues are commonly stored in formalin, thereby leading to accumulation of large number of specimens in laboratory archives. These tissues which are stored for their possible future use in research require allocation of laboratories, resources in terms of space, and labor for maintenance. In spite of regular maintenance, it can be assumed that between subsequent changes of solutions, there will be an increase in the concentration of formic acid and fall in formalin concentration, leading to various changes in the tissue. Paraffin blocks and mounted slides are the other means of laboratory archives.[1,6,7]

However, studies regarding the usefulness of the long-term stored tissue in formalin and in paraffin wax for histological analysis are scanty. The results of our study could not be compared with any other studies since, to the best of our knowledge, there are no studies reported in the English literature evaluating the macroscopic quality, except one study mentioning about staining reproducibility of microscopic features.[8]

Macroscopic features

Color

Change in color of the tissue was noted between OTB (old record) and LFT. This can be attributed to preservative solutions which are generally solvents that tend to discolor over time, and bleaching of the tissues might occur after prolonged fixation.[3] However, the mechanism of change in the specimen color is uncertain, but color change may also be implicated to the deterioration of formaldehyde itself to methanol and formic acid.

Consistency

Change in the consistency of tissue from firm to soft and fragile can be attributed to rapid evaporation of formalin than water from fixative fluid, which could have occurred either due to formalin gas evaporating as a result of change in atmospheric temperature and pressure or when the containers are opened while accessing specimens during long storage.[3]

Cutting efficiency

During grossing, it was found to be difficult in LFT as compared to FFT; the difference could probably be due to the fact that LFTs in our study were friable, which made the cutting difficult, and also the soft tissue that lacks firm support preserved for a long time is known to shrink or desiccate and deform.[9]

Sectioning characters

Failure to form ribbon was observed to be more in OTB than in LFT and FFT. The OTBs evaluated in the study were stored over a period of more than 5 years in room temperature in closed chambers. Such a long period of storage can induce alterations in the physical and certain chemical properties of paraffin wax. This could be the possible reason for the maximum number of ribboning failure in OTB in our study in comparison with freshly used wax in the other two groups.

Persistence of folds was observed in the maximum number of cases in LFT followed by OTB and FFT. In support of this, Wick *et al*. reported that friable tissue may possibly shed small fragments float freely on the surface of the water.[10] This friability of the tissue which is also observed in the macroscopic features of our study could possibly be the cause for loss of ribboning and persistent tissue folds while sectioning in LFT.

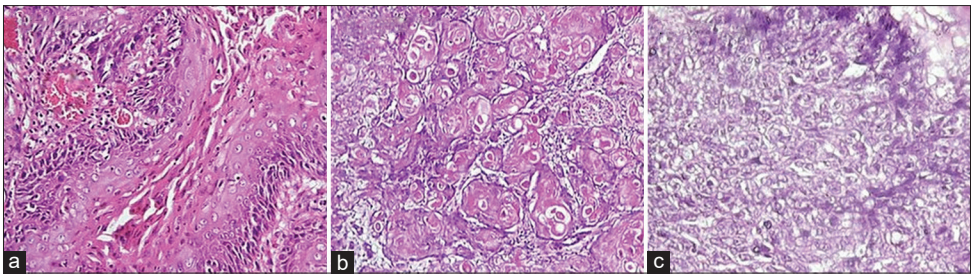

Figure 5: (a) Section showing nuclear and cytoplasmic details from freshly fixed tissue (FFT) which is easily identifiable (H and E, ×20); (b) section showing identifiable nuclear and cytoplasmic details from old tissue block (H and E, ×20); (c) section showing nuclear and cytoplasmic details from long-term fixed tissue which lack clarity (H and E, ×20)

Table 6: Sectioning characteristics of three study groups

Sectioning criteria	Ribbon formation		Persistence of folds	
	Present	Absent	Present	Absent
OTB, *n* (%)	43 (84.30)	8 (15.70)	16 (31.40)	35 (68.60)
LFT. *n* (%)	45 (88.20)	6 (11.80)	20 (39.20)	31 (60.80)
FFT, *n* (%)	51 (100)	0 (0.00)	13 (25.50)	38 (74.50)
Chi-square test between 3 groups	8.18		2.22	
P	0.017*		0.329 (NS)	
Chi-square test between OTB and LFT	53.686		12.706	
P	<0.001**		<0.001**	

*indicates significant, **indicates highly significant. LFT: Long-term formalin-fixed tissue, HS: Highly significant, FFT: Freshly fixed tissues, OTB: Old tissue blocks (details from old record), NS: Not significant

Table 7: Comparison of tissue integrity among three groups

Characteristics	*n* (%)		
	OTB	LFT	FFT
Good	43 (84.3)	28 (54.90)	47 (92.15)
Fold	3 (5.88)	5 (9.80)	2 (3.92)
Tear	4 (7.84)	5 (9.80)	1 (1.96)
Disintegrate	0	8 (15.68)	1 (1.96)
Disintegrate and fold	1 (1.96)	1 (1.96)	0
Tear and disintegrate	0	2 (3.92)	0
Tear and fold	0	2 (3.92)	0
Total	51 (100)	51 (100)	51 (100)
Chi-square test between 3 groups		30.76	
P		0.002*	
Chi-square test between OTB and LFT		15.78	
P		0.015*	

*indicates significant. LFT: Long-term formalin-fixed tissue, FFT: Freshly fixed tissues, S: Significant, OTB: Old tissue blocks (details from old record)

Microscopic features

Tissue integrity

Maximum cases of tissue disintegrity, tear, and folds were noted in LFT when compared to OTB and FFT. This loss of tissue integrity is possibly caused due to friability of tissue that was observed in 11.76% of the cases from LFT in our study.

Tissue architecture

Presence of the epithelium was found to be maximum in FFT with OTB and least in LFT. Connective tissue was present in 100% of all the cases. According to studies, long-term formalin fixation can cause increased oxidation of the bonds, leading to the alteration in cellular protein in terms of increased polymerization and also nuclear proteins due to hydrolytic action.[9,11] This will lead to loss of cellular architecture which has been confirmed by loss of immune reaction of certain proteins in long-term FFTs. All these factors support our finding of loss of tissue architecture.

Tissue components

Keratin was demonstrated using H and E and special stain Mallory in the maximum number of cases of FFT, followed by OTB and LFT. The difference in the identification of keratin was found to be statistically significant using H and E and Mallory stain. According to our observation, all three study groups demonstrated keratin at variable levels. Although less, 67% of the cases of LFT demonstrated keratin, which might indicate that keratin is resistant to degradation after long-term storage.[2]

Nuclear and cytoplasmic features

They were found to be superior in FFT followed by OTB when compared to LFT using H and E. Formaldehyde on prolonged storage achieves acidic pH which is known to cause greater damage to DNA.[4,5] Apart from this, additional hydrolytic reactions due to the presence of water could also damage DNA.[9] Methanol which is formed on prolonged storage of formalin is known to compete with water for hydrogen bonds, leading to weakness in localized points in the DNA; this could also be one of the reasons for degradation of DNA.[9] Prolonged fixation

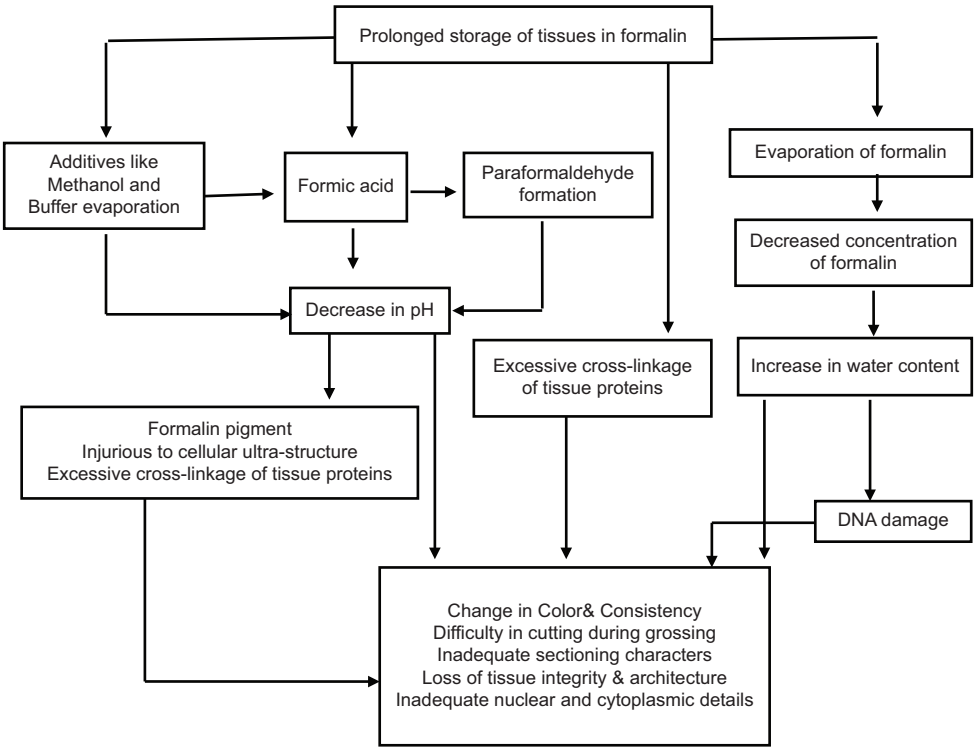

Figure 6: Long term formalin-fixed tissues showing deleterious effects

Table 8: Tissue architecture among three groups - epithelium

Epithelium	*n* (%)		
	OTB	LFT	FFT
Present	38 (82.60)	33 (71.73)	46 (100)
Absent	8 (16.66)	13 (27.08)	0
Total	46 (100)	46 (100)	46 (100)
Chi-square test between 3 groups		14.240	
P		<0.001**	
Chi-square test between 3 OTB and LFT		35.294	
P		<0.001**	

**indicates highly significant. LFT: Long-term formalin-fixed tissue, FFT: Freshly fixed tissues, OTB: Old tissue blocks (details from old record)

Table 9: Identification of keratin by H and E and Mallory stain in study groups

Stain	Keratin, *n* (%)			
	H and E		Mallory	
	Present	Absent	Present	Absent
OTB	30 (75)	10 (25)	27 (67.5)	13 (32.5)
LFT	27 (67.5)	13 (32.5)	24 (60)	16 (40)
FFT	39 (97.5)	1 (2.5)	38 (95)	2 (5)
Chi-square test between 3 groups	12.19		14.18	
P	0.002 *		0.001*	

*indicates significant. LFT: Long-term formalin-fixed tissue, FFT: Freshly fixed tissues, OTB: Old tissue blocks (details from old record)

also causes excessive crosslinking of cytosolic and membrane proteins that could possibly result in deranged display of cytoplasmic features that were observed in LFT cases. Similar reduction in staining quality with weaker stainability and microscopic findings were observed in liver, kidney, and other organs assessed in rat tissues stored for 30 years.[8]

Conclusion

Archival tissue blocks (OTB) could demonstrate all the characters similar to FFT in terms of staining and other handling properties superior to the tissues and sections obtained in the LFT group. Although OTB tissues were comparable to FFT, we encountered difficulty in the ribboning formation. As old paraffin-embedded tissue blocks were not stored in temperature-regulated cabinets and were exposed to environmental temperature changes, paraffin wax properties may have been altered.

Formalin, though a good fixative, has deleterious effects on the specimen [Figure 6]. Any formalin-free fixatives which may serve as choice of material for tissue preservation for long term are yet to be elicited. If alternatives are not available, then a regular change of the solution and pH maintenance has to be done in the containers having specimens stored in formalin fixative for longer duration. Alternatively properly stored FFPE tissue blocks serve as a good archival tissue preserving method for long-term purpose of biomedical and histopathological research in prospective studies. Since routine histopathological analysis is found to be better in archival paraffin-embedded tissue blocks, it can presumably be a source for molecular analysis too.

Table 10: Nuclear and cytoplasmic details among three study groups in H and E stain

H and E stain	Nuclear and cytoplasmic details, *n* (%)		
	OTB	LFT	FFT
Identifiable	46 (90.20)	17 (33.30)	51 (100)
Identifiable but not clear	5 (9.80)	31 (60.78)	0
Nonidentifiable	0	3 (5.90)	0
Total	51 (100)	51 (100)	51 (100)
Chi-square test between 3 groups		69.90	
P		<0.001**	
Chi-square test between OTB and LFT		35.13	
P		<0.001 **	

**indicates highly significant. LFT: Long-term formalin-fixed tissue, FFT: Freshly fixed tissues, HS: Highly significant, OTB: Old tissue blocks (details from old record)

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Conflicts of interest

There are no conflicts of interest.

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