Comparison of staining adequacy between tissues stored in formalin and paraffin embedded blocks for prolonged duration

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Abstract

Introduction: Tissues submitted for histopathological diagnoses are routinely fixed in formaldehyde fixative which facilitates tissue storage for the extended period of time for further investigation or research purpose. The literature has reliable data relating to immunohistochemistry and molecular studies done using tissue fixed in formalin for longer duration and in paraffin-embedded tissue blocks. We have attempted to evaluate staining adequacy of archival specimens using hematoxylin and eosin (H&E) and trichrome stains.

Methodology: We compared staining adequacy in tissues stored in formalin for a minimum period of 5 years labeled as long term fixed tissues (LFT) and corresponding 5-year-old paraffin-embedded tissue blocks of same cases available in the archives designated as old tissue blocks (OTB), along with freshly fixed tissues (FFT) used as controls. Fifty-one tissue sections in each study group were stained with H&E, Mallory’s trichrome and Van Giesons’ and microscopic evaluation was carried out using Research Microscope B x 51.

Results: Staining adequacy of the tissue sections was found to be superior in FFT, followed by OTB and LFT using all three stains. Staining for H&E, was found to be superior as compared to trichrome stains.

Conclusion: Trichrome stains were poorly demonstrated in LFT indicating the possible alteration in protein structure and tissue architecture in LFT whereas H&E, stained LFT specimens showed relatively good staining character. Hence, OTB proved to be better as compared to LFT in terms of staining quality and hence archiving tissue in paraffin embedded tissue blocks serve as valuable bio-bank and source for prospective studies.

Keywords: Archival specimens, long-term formalin fixed tissues, paraffin embedded blocks, staining adequacy, trichrome stains

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INTRODUCTION

Histotechnique constitutes the pillar of diagnostic pathology, aiding histologic diagnosis of diseased tissue feasible for proper management. Pathologists routinely examine the section of tissues which have been fixed in formaldehyde, embedded in paraffin wax, sectioned and stained with hematoxylin and eosin (H&E). H&E, forms the mainstay of daily pathological diagnostic work which is a universal stain. The most widely used fixative in histopathology is formaldehyde as it is inexpensive, easily available, fairly convenient to store, and relatively safe.[1] Formalin enables long-term storage of specimens preserving their morphologic features[2] and allowing microscopic evaluation after routine and special histochemical staining. Hence, tissue stored in formalin can serve as a reliable tool for diagnostic and research purpose. Extended fixation in formalin is known to damage many antigens, secondary to the induction of molecular cross-linking of proteins. This can change the native three-dimensional protein conformations, thereby altering the normal three-dimensional structure of the epitope making it more difficult for the antibody to bind to its target. The amount of this adverse effect varies with the length of fixation, with increased duration of fixation leading to increased antigen damage, known as “antigen masking.”[3] Trichrome techniques do not produce optimal results with routine formalin-fixed tissues and prolonged tissue fixation in formaldehyde gives even less satisfactory results. This is due to the saturation of tissue groups with formaldehyde, and so leaving only a few groups available to react with the trichrome dyes.[4]

Prolonged formalin fixation is believed to result in decreased staining adequacy using routine H&E, staining and even inferior staining for trichrome techniques. Studies’ regarding the effect of prolonged formalin fixation on the immunohistochemistry (IHC) detecting different antigens is well-known. However, regarding the usefulness of the long term stored tissue in formalin and on paraffin wax block for histological analysis are scanty. This is the first study to the best of our knowledge done in human tissues for assessing the staining capability in archival specimens that were stored for more than 5 years using H&E, and trichrome stains.

METHODOLOGY

The study was conducted to evaluate and to compare the tissue specimens stored in formalin for a minimum period of 5 years and the corresponding 5-year-old paraffin-embedded tissue blocks of the same cases available in the archives of Department of Oral Pathology and Microbiology. Fresh biopsy specimens submitted to the department were used as controls. Fifty-one tissues stored in formalin for a minimum period of 5 years were labeled as long term fixed tissues (LFT) and corresponding 5-year old archival paraffin-embedded tissue blocks of the same cases were designated as old tissue blocks (OTB) along with freshly fixed tissues (FFT) routinely available in the department were used as controls. Standardized protocol was carried out for both LFT and FFT with 19-h automated tissue processing and embedding followed by motorized rotary microtome from which three sections of 5–6 micron thickness were prepared. Similarly, 5-year-old 51 corresponding archival blocks were obtained from the department and three sections from each sample were prepared. Staining was performed by taking equal number i.e., 51 in each group of LFT, OTB and FFT sections using H&E, Van Gieson’s and Mallory’s stain. For each and every case of LFT, OTB, one FFT was stained along. That is an equal number of cases and controls were taken and stained simultaneously. Microscopic evaluation was carried out using Research Microscope B × 51.

Staining adequacy was categorized as very good, good, satisfactory and inadequate and these characters were assessed based on identification of nucleus and cytoplasm, cellular outline, uniformity in staining and intensity of colors for H&E, and especially Mallory’s for keratin and epithelial components and Van Gieson’s stain for collagen and connective tissue components.

Staining adequacy was assessed by 3 observers and was categorized as in Table 1.

Statistical analysis

Chi-square test was applied for comparison between the study groups’ i.e., OTB, LFT, and FFT. Cohen’s kappa for intergroup variable was done.

RESULTS

Overall staining adequacy was found to be best in FFT followed by OTB and LFT in all the three stains. On comparing staining adequacy between 3 stains, H&E, [Figure 1a and b] and Mallory’s stain [Figure 2a and b] were better as compared to Van Gieson’s stain [Figure 3a and b] which showed better results in FFT. Both H&E, and Mallory showed very good staining quality in FFT, good in OTB and satisfactory and inadequate staining quality in LFT cases. Van Gieson’s stained best in FFT, good in OTB and satisfactory in LFT cases and also it was
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found that maximum number of cases were found to be good than very good in staining quality. Staining adequacy of the tissue sections from the FFT, OTB and LFT in H&E, [Figure 4a-c] Mallory’s and Van Gieson’s stain are tabulated in Table 2.

Staining adequacy was found to be very good in the maximum number of cases from the tissue sections of FFT for all the three stains followed by OTB and least in LFT. Category good was noted in the maximum number of cases from the tissue sections of OTB for all three stains followed by FFT and least in LFT. A Maximum number of cases from the tissue section of LFT showed satisfactory and inadequate staining with all three stains.

Chi-square test showed that the difference in staining adequacy using H&E, Mallory’s and Van Gieson’s stain in OTB and LFT to be statistically significant with a P value of 0.002 and 0.003 respectively. But in FFT sections, the difference in staining adequacy using all three stains was found to be statistically insignificant with a P value of 0.07.

Cohern’s kappa for intergroup variable was found to be 0.93 which shows strongly agreement.

DISCUSSION

Over fixed specimens often demonstrate alterations in the morphologic definition or antigenic integrity and in contrast, under fixation allows bacterial putrefaction to continue damaging the tissue sample.[3]

Studies have been done to evaluate the effect of prolonged formalin fixation on the immunohistochemical detection of different antigens and found that Immunoreactivity of cytokeratin 7, high-molecular-weight cytokeratin, and laminin was diminished by prolonged formalin fixation. However, immunohistochemical reactivity remained moderate to strong with up to 7 weeks of fixation for all other antibodies suggesting that prolonged formalin fixation has minimal effects on antigen detection for most commonly used antibodies.[4] Prolonged fixation of tissues for >4 years has shown reduction in the sensitivity for HCV detection compared to tissues fixed for 24 h and 3–4 days.[7]

Prolonged formalin fixation (>48 h) is reported to have detrimental effect on detecting antigens as demonstrated in some of IHC studies and other molecular studies are believed to be negatively affected in prolonged formalin-fixed tissues. Nevertheless, we have attempted to evaluate and compare

Table 1: Criteria selected to evaluate the adequacy of staining

| Category       | Percentage of area of the tissue sections taken up stain |
|----------------|----------------------------------------------------------|
| Very good      | >75                                                      |
| Good           | 50-70                                                    |
| Satisfactory   | 25-50                                                    |
| Inadequate     | <25                                                      |

Figure 1: (a and b) Hematoxylin and eosin, Stain in old tissue block (left) ×20 and in long term fixed tissue (right) ×20

Figure 2: (a and b) Mallory’s Stain in old tissue block (left) ×10 and in long term fixed tissue (right) ×20

Figure 3: (a and b) Van Gieson’s Stain in old tissue block (left) ×10 and in long term fixed tissue (right) ×20

Quality of formalin fixed tissues stored for 30 years in rat specimen for histopathological analysis reported fading in staining and few morphological artifacts and concluded that their results were favorable and comparable to their original histopathological reports.[8]

Prolonged fixation is known to have a detrimental effect on detecting antigens as demonstrated in some of IHC studies and also other molecular studies are believed to be negatively affected in prolonged formalin-fixed tissues.
the staining ability of prolonged formalin-fixed tissues with paraffin-embedded tissues belonging to of same cases using H&E, Mallory and Van-gieson’s stain.

Based on our observation, staining adequacy was found to be best in the sections from FFT, better in OTB when compared to LFT with H&E, Mallory’s and Van Gieson’s stain. A statistically significant difference in staining adequacy of the tissue sections between all the three study groups and also between OTB and LFT were found, suggesting that excess cross-linking of tissue proteins during prolonged fixation leads to less satisfactory staining. In support to this, it is well documented in the literature that formalin fixation is a time-dependent process in which increased fixation time would result in continued formaldehyde group binding to proteins to a point of equilibrium.[9] Studies have also shown that prolonged formalin fixation resulted in decreased antigenicity.[4] Formaldehyde on prolonged storage achieves acidic pH by the forming formic acid through oxidation, this lower pH of a fixative solution is known to cause greater damage to DNA within the nucleus.[2,10]

This reduction in staining quality in LFT from our study can be related to stainability and microscopic findings in liver, kidney and other organs assessed in rat tissues that were stored for 30 years.[8] Less satisfactory staining of prolonged formalin-fixed tissues than paraffin embedded blocks is due to excessive cross-linking of proteins and production of formic acid which lowers the pH of formalin fixative solution in which tissues are preserved for prolonged duration. Staining adequacy of the tissue sections for H&E, was found to be superior as compared to Mallory’s stain and Van Gieson’s stain. Also, tissue architecture and protein structure appeared to have altered in prolonged formalin stored tissues because trichrome stains which are based on the molecular size of dye and pore size of tissue was affected in LFT group.[4,11] This indicates that trichrome stains yield less favorable stainability due to saturation of tissue groups with formaldehyde leaving only few groups to react with trichrome dye. H&E, found to be superior in prolonged formalin stored tissues indicating that staining method based on acid-base property were not affected comparatively and yielded better results than trichrome stains.[4,11]

Treating formaldehyde fixed tissues with picric acid, mercuric chloride solutions or both enhances trichrome staining intensity and brilliance.[4] Lendrum et al. suggested “degreasing” the section for 24–48 h in trichloroethylene prior to staining improves the intensity of staining reactions. Satisfactory staining for trichrome techniques can be obtained with fixatives such as Zenker’s solution, formal mercury, Bouin’s fixative or picro-mercuric alcohol than formaldehyde fixation.[4]

**CONCLUSION**

Our observation show that prolonged storage of tissue in formalin did not affect the chemistry of tissues and
hence the staining techniques based on a chemical reaction like H&E, (acid-base property) were effective. But tissue architecture and protein structure are subjected to changes in long term storage hence, the trichrome stain were affected. Therefore, archival paraffin embedded tissue blocks proved to be better than preserving tissues in formalin for future microscopic analysis.

Scope of study
Individual tissue evaluation using IHC and PCR necessitates for efficacy of archival samples.

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Conflicts of interest
There are no conflicts of interest.

REFERENCES

1. Ulmer D. Fixation: The key to good tissue preservation. J Indian Soc Periodontol 1994;8:7-10.
2. Grizzle WE, Fredenburgh JI, Myers RB. Fixation of tissues. In: Bancroft JD, Gamble M, editors. Theory and Practice of Histological Techniques. 6th ed. Philadelphia: Churchill Livingstone Elsevier Ltd; 2008. p. 53-68.
3. Miller RT. Technical Immunohistochemistry: Achieving Reliability and Reproducibility of Immunostains; 2001. Available from: https://www.semanticscholar.org/paper/TECHNICAL-IMMUNOHISTOCHEMISTRY%3A-Achieving-and-of-Miller/8596d7b21017849d828c23214d828427eb3b50. [Last accessed on 2020 Jan 09].
4. Jones ML, Bancroft JD, Gamble M. Connective tissue stains. In: Bancroft JD, Gamble M, editors. Theory and Practice of Histological Techniques. 6th ed. Philadelphia: Churchill Livingstone Elsevier Ltd; 2008. p. 148.
5. Wick MR, Mills NC, Brix WK. Tissue Procurement, Processing, and Staining Techniques. Available from: http://assets.cambridge.org/9780521874106/excerpt/9780521874106_excerpt.pdf. [Last accessed on 2018 Aug 24].
6. Webster JD, Miller MA, Dusold D, Ramos-Vara J. Effects of prolonged formalin fixation on diagnostic immunohistochemistry in domestic animals. J Histochem Cytochem 2009;57:753-61.
7. Guerrero RB, Batts KP, Brandhagen DJ, Germer JJ, Perez RG, Persing DH. Effects of formalin fixation and prolonged block storage on detection of hepatitis C virus RNA in liver tissue. Diagn Mol Pathol 1997;5:277-81.
8. Ono Y, Sato H, Miyazaki T, Fujiki K, Kume F, Tanaka M. Quality assessment of long-term stored formalin-fixed paraffin embedded tissues for histopathological evaluation. J Toxicol Pathol 2018;31:61-4.
9. Thavarajah R, Mudimbaimannar VK, Elizabeth J, Rao UK, Ranganathan K. Chemical and physical bases of routine formaldehyde fixation. J Oral Maxillofac Pathol 2012;16:400-5.
10. Nowacek JM, Kiernan JA. Fixation and tissue processing. In: Kumar GL, Kiernan JA, editors. Special Stains and H and E. 2nd ed. California: Dako Publishers Ltd; 2010. p. 142-52.
11. Horobin RW. How do histological stains work? In: Bancroft JD, Gamble M, editors. Theory and Practice of Histological Techniques. 6th ed. Philadelphia: Churchill Livingstone Elsevier Ltd; 2008. p. 105-18.