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Original article

Effect of formalin fixation on DNA: A Time-Based Approach

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ABSTRACT

Introduction: Tissue preservation is important. From it forensic scientist may extract sufficient DNA for profiling. The most commonly used preservative found in the literature is formalin. However, it causes severe side-effects on its users as well as the environment; we need to find its replacement.

Method: This study examined whether formalin could preserve soft tissues (fresh) stored at 4°C for 100 days and how DNA can be extracted from it. Qualification and Quantification of the presevred samples were done.

Result: The results revealed that tissues preserved in formalin failed to generate sufficient quantity of DNA for profiling whereas tissues preserved in normal saline did so.

Conclusion: The study concluded that there is a need to find an alternative to formalin which can preserve the tissue samples well and enable DNA profiling.

Keywords: DNA quantification; Formalin; Fixation; Normal saline; Preservative; Tissue Preservation.

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INTRODUCTION

Tissue fixation is an initial and important step for processing of a specimen for histological and DNA examination and requires that the tissue is kept in a safe medium that prevents it from degradation for a long period of time.[1,2] Well preserved tissue is crucial in biological sciences studies. It plays an important role in the Court of Law where decisions are made based on the facts and the evidence.[3] There is a wide range of nucleic acid extraction methods available from homemade procedures to commercially-available kits. Various commercial kits have their own efficiency of recovery of nucleic acids. Some kits recover quantifiable DNA yield, while others recover lesser DNA concentration. Literature shows that the method used for extraction of nucleic acids affects DNA yield.[4,5]

The literature reveals a number of preservatives, out of which formalin is the most common. It is generally used in the concentration of 10%. Though, various

concentration of formalin has been debated upon in the past.[6,7] Due to its low price, good application effect and easy availability, it is among the most commonly used fixative worldwide. However, cross-linking of proteins and nucleic acids by formalin resulted in hampered analysis and also restricted the study of proteomics to frozen tissues. It also hampers the study of rare disease subtypes.[8]

The OSHA regulation standard has declared it as "hazardous" because of its carcinogenic nature and ill-effects on the environment and its users.[9] Additionally, it has been observed that formalin denatures the DNA and mRNA and does not allow full profile to get generated during DNA profiling.[10] This has raised the quest for some less toxic or formaldehyde-free preservative which has fixative properties comparable to that of formalin.[9,11]

Nucleic acid extraction is an important step in evaluation of tissue for their source and integrity. DNA can be extracted from a wide range of sources which include human as a biological source in the form of hairs, nails, whole blood, buccal cells etc. It has also been reported that archival unstained bone marrow slides resulted in good DNA yield. A properly preserved tissue enables appropriate extraction of nucleic acids from them.[12]

Nucleic acid isolation has some standard protocols which require specific reagents. These reagents are now being commercially available in the form of DNA extraction kits.[13] Carlsson in his study reported different extraction kits to evaluate the quantity and quality of DNA and RNA which can be extracted from FFPE prostate cancer biopsies.[4] Additionally, the Organic Extraction is amongst the most widely used method of DNA Isolation.[14]

METHOD

This study has been approved by Institutional Ethics Committee, Civil Hospital, Gurugram. The informed consent from the legally acceptable representative of the deceased has been obtained in written. In the study, twenty-five soft tissues from five different organs were obtained from a deceased individual. The deceased were admitted into the Mortuary 24 hours after death and the tissue samples were then collected during post-mortem. Those organs were heart, lung, liver, kidney and brain. Tissues were further cut into small segments (n=50) using laboratory protocols. Twenty-five samples were placed immediately into containers having 10% neutral buffered formalin (NBF) and rest twenty-five different tissue samples were placed in containers having solution of normal saline. The weight of each sample has been decided to be approximately 1-2 gram as the tissues taken in this study are particularly soft tissues. The tissues were preserved at 4ºC for 100 days in NBF and normal saline to assess how efficiently DNA quantification can be done in such a condition.

Autosomal STRs were amplified using Power Plex 21TM PCR amplification kit. PCR Amplified products were subjected to electrophoresis in ABI 3500XL genetic analyzer. The GeneMapper ID-X 1.4 software was used for STR analysis.

DNA Isolation

Tissues preserved in the NBF solution and normal saline solution were taken out from the refrigerator and an appropriate size was cut from it for analysis. They were placed in a 50 ml tube and firstly washed with tap water and then washed with Milli Q water three-four times in order to completely remove the formalin. Cut piece was taken out from the tube with the help of forceps and placed in a Petri dish. Piece was finely chopped using a surgical blade. Finely chopped pieces of tissue were transferred to aliquots where Phenol Chloroform Extraction Method has been applied to the tissues. Forensic buffer, protease K and Sodium dodecylsulphate (SDS) were then added to the aliquots and kept at 56ºC in water bath overnight. Multiple number of times centrifugation was done after adding required reagents and in the end, intense washing was done. Last step was to add Tris EDTA to the sample and then placed in thermo mixer at 56ºC for 30 minutes.

Quantitation

After isolation, all the samples were quantified by Quantifier Trio kit. The PowerPlex 21 System is a multiplex STR system for human identification applications including forensic analysis and relationship testing. The system allows co-amplification and fluorescent detection of 21 loci (20 STR loci and Amelogenin).

Table 1: RT-PCR Findings of tissues preserved in Formalin.

Tissue Type	RT-PCR Value
Heart	0/0.06/0.00
Lung	0/2.24/-
Liver	-/0.28/-
Kidney	0/0/0
Brain	0/0.47/0
Heart	0/0.08/0.00
Lung	0/2.21/-
Liver	0/0.30/-
Kidney	0/0.01/0
Brain	0/0.78/0
Heart	0/0.09/0.01
Lung	0.1/2.20/-
Liver	0/0.29/-
Kidney	0/0.01/0
Brain	0/0.52/00
Heart	0/0/0
Lung	0/2.1/-
Liver	-/0.27/-
Kidney	0/0/0
Brain	0/0.48/0
Heart	0/0.06/0.01
Lung	0.1/2.1/-
Liver	-/0.27/-
Kidney	0/0.01/0
Brain	0/0.42/0

Table 2: RT-PCR Findings of tissues preserved in Normal Saline.

Tissue Types	RT-PCR Values
Heart	1.35/1.69/0.0
Lung	0.22/0.61/0.0
Liver	0.49/1.17/0
Kidney	0/0.01/0
Brain	1.45/3.07/0
Heart	1.21/1.63/0.1
Lung	0.24/0.66/0.2
Liver	0.69/1.21/0
Kidney	0/0.02/0.01
Brain	1.56/2.07/0
Heart	1.22/1.57/0.0
Lung	0.32/0.61/0
Liver	0.66/1.31/0
Kidney	0/0.04/0.0
Brain	1.76/2.57/0
Heart	1.34/1.78/0
Lung	0.24/0.76/0
Liver	0.98/1.89/0.0
Kidney	0/0.01/0
Brain	1.56/3.07/0.1
Heart	1.89/1.96/0
Lung	0.15/0.67/0.0
Liver	0.59/1.31/0
Kidney	0/0.02/0
Brain	1.87/1.57/0.0

RESULT AND DISCUSSION

F. Blum in the year 1893 accidentally discovered fixation by formalin. Protein-protein cross links along with intermolecular cross linking of proteins with DNA and RNA takes place in formalin preserved tissues. However, as per chemical testing, on coming in contact with uncharged amino acid groups, formaldehyde makes extremely reactive methylols.[15] Due to this, it is said that the tissues get rigid for histological and immunohistochemical studies.

Results in this study revealed that the tissues preserved in formalin at 4ºC for 100 days were not able to generate complete profile due to binding or inhibition. The values obtained in RT-PCR has been mentioned in Table 1 and 2. On the other hand, the reference samples kept in normal saline at 4ºC showed good yield even after 100 days.

The failure of amplification in formalin-fixed tissue could be due to inhibitions and quantity of the DNA amplified was found to be poor. The most affected area of DNA was large size marker more than 300 base pairs. In case of formalin, it is also observed that some of small sized markers were able to generate partial profile which are markers ranging from 80 base pairs to 160 base pairs. However, large size markers above 240 base pairs could not produce the profiling of the deceased individual as they could not be amplified.

Moreover, samples preserved in normal saline yielded sufficient quantity of DNA which has been represented by almost all the genetic markers of different sizes. In fact, larger markers above 210 base pairs also showed better amplification in normal saline under the preservation conditions. (Figure 1) Whereas, in case of formalin, they could not produce any result. Though, as few reported studies, it is essential to add methylene bridges between proteins

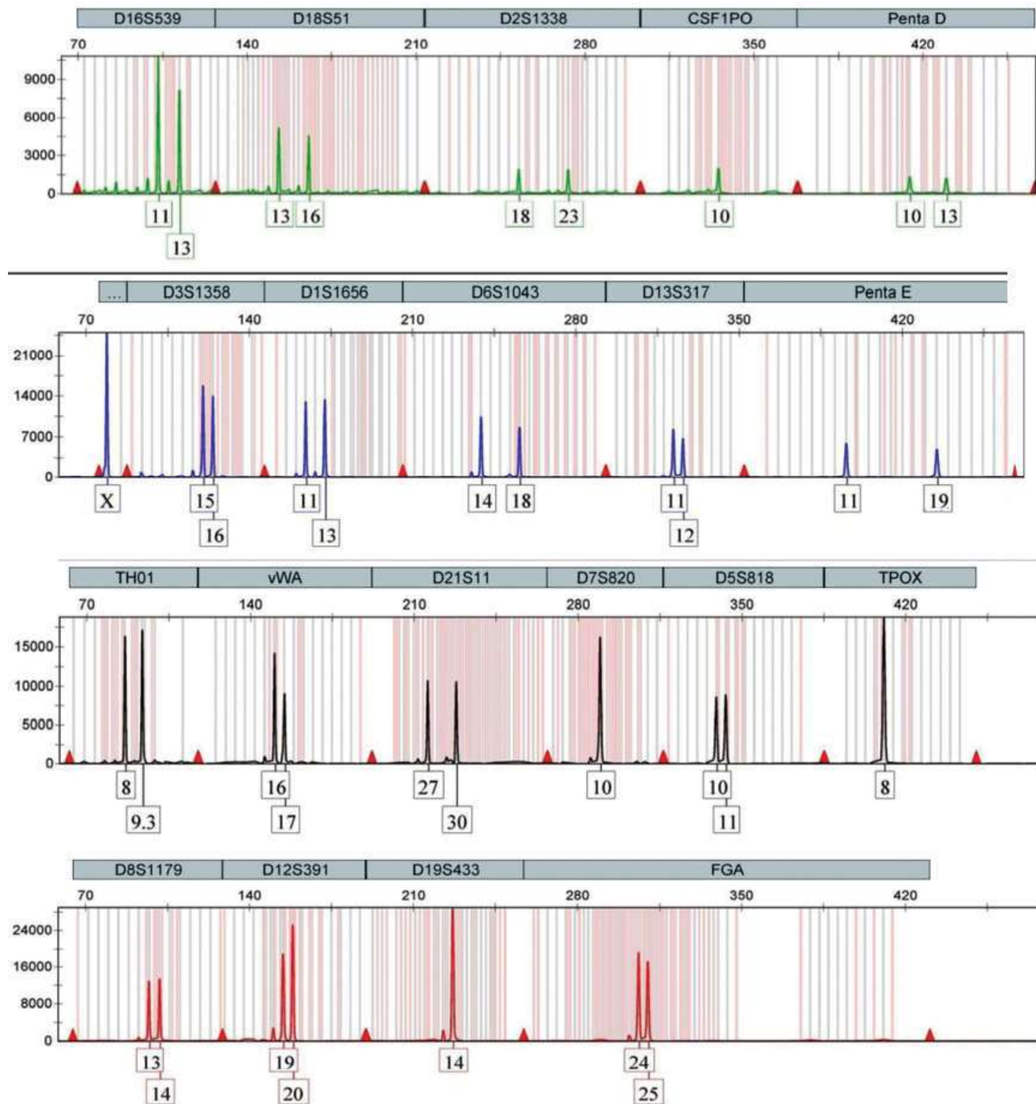

Fig. 1: DNA profile obtained from tissues preserved in normal saline.

and nucleic contents and CH_2OH to bases of nucleic acid for easy nucleic acids extraction, especially in case of RNA. Otherwise, fixation in formalin yields in poor quality RNA due to degradation. Moreover, high yield of RNA can only be extracted from fresh tissues.[16-18]

Some researchers believe that by washing and dehydration, formaldehyde can be completely removed from the formalin-fixed tissue. Actually, when washed multiple times, only loosely bound formaldehyde gets removed. Remaining formaldehyde cannot be removed merely by washing, though if a tissue is kept in water for a prolonged time, formaldehyde slowly starts to get removed. However, such intense washing is not a good idea for the purpose of histochemistry and histology.[15] However, it is interesting to know that a study revealed that the quantity and quality of extracted products gets affected by the type of kit used for the extraction method and the nucleic acids amount is also dependent on formalin fixed paraffin embedded tissue age and origin.[4,5]

Sodium chloride being an inexpensive and readily available substance have found place in fixation of tissue, even in field conditions in tropical countries which lack cooling and freezing facilities and

hospitals lacking service of pathology. Preservation of molecular structure of tissues by sodium chloride has not been explained in the past. It can be a sodium specific, chloride anion and hyperosmolarity effect. N-formyl-methionyl-leucylphenylalanine gets stopped in hypertonic saline which raises intracellular calcium ion. Additionally, dehydration of cells in osmotic medium and intercellular matrix can also be considered.[19] Saraj's research suggests that saturated sodium chloride solution can be used as an alternative to formalin as it gives same histological features as formalin in tissue fixation.[20] Tissue preserved in normal saline has been proven to yield RNA better than formalin-fixed tissues. Since tissues fixed in formalin severely affect the RNA so normal saline can be considered as a safer alternative which protect the histomorphology as well as the RNA of the tissue. The integrity of membrane of cell is guarded by normal saline which results into inhibiting the release of intracellular RNase. However, some unexplored facts may also be responsible for avoiding RNA degradation in normal saline preserved tissues.[21]

In this study, formalin preserved tissues were found unable to generate sufficient or complete profile in the above-mentioned condition. Though some of the small sized markers can be identified but large sized markers showed no peak. It happened because of alterations and cross-linking of nucleic acids with proteins which changes the bonding of DNA and RNA. It even led to complete breakage of the DNA. The sample size taken in this study is relatively small to conclude a definite result so it is suggestive that more number of samples can be considered to come to a conclusive result. Moreover, the type of DNA kit used for extraction also affects its overall analysis. Different commercial kits can be used to see if variance occurs in the data. The factors i.e. temperature and duration of preservation can also limit the research data. These two parameters can be explored to obtain varied results.[4,15]

CONCLUSION

In our study, the reference sample yielded good quantity of DNA which shows that normal saline can be used to preserve tissue under the defined conditions. Normal saline is an easily available, handy and cheap alternative to formalin. So, it can be safely considered to be used in the laboratories for nucleic acid extractions.[21] Some of the tissues were found to be dissolved in normal saline hence it is suggested that the tissue preserved in normal saline should be clean prior to preserve in normal saline to avoid contamination. Furthermore, more studies are needed to determine the actual reliability and safety of using normal saline for genetic analyzes.

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Conflicts of interest: The authors declares there is no conflicts of interest.

Ethical Approval

The ethical approval for conducting this research study has been obtained from Institutional Ethics Committee, Civil Hospital, Gurugram, Haryana, India.

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