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Original Article

Pathology Section

Histomorphological Comparison of Tissues Fixed in Conventional Formalin and Eco-friendly Jaggery Solution: A Cross-sectional Study

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ABSTRACT

Introduction: Tissue fixation is an essential step in the microscopic preparation of tissues to preserve them by preventing autolysis, bacterial putrefaction, and increasing the tissue's refractive index. The conventional fixative used is 40% formaldehyde. Short-term exposure to formaldehyde can cause irritation to the eyes and respiratory tract, leading to breathlessness and coughing. The International Agency for Research on Cancer (IARC) has classified formaldehyde as a Class 1 human carcinogen capable of potentially causing various neoplasms, including nasopharyngeal carcinoma. Therefore, an innovative approach is being explored to replace formalin with harmless and eco-friendly jaggery. Jaggery possesses cytoprotective, antioxidant, and tissue-preservative properties. At a low pH, the fructose in jaggery breaks down into aldehydes, which cross-link with tissue amino acids, resulting in tissue fixation similar to formaldehyde.

Aim: To compare the histological and gross morphological features of tissues fixed in formalin and jaggery.

Materials and Methods: This cross-sectional study was conducted in Department of Pathology of a tertiary care centre. Surgical specimens obtained fresh, which were not suspicious of malignancy, were included. A sample size of 23 was selected. Surgical specimens already placed in formalin were excluded. A 30% jaggery solution was prepared by dissolving 300 grams of finely powdered jaggery in 1000 mL of distilled water, which was then filtered using filter paper. A 40% formaldehyde solution was used to prepare a 10% formalin fixative. Tissue bits from each specimen were cut into two halves and placed in formalin and jaggery separately. After 24 hours of fixation, the tissue bits were evaluated for gross morphological features, including tissue shrinkage, consistency, and colour. Tissue shrinkage was classified as mild, moderate, or marked, while consistency ranged from soft to firm to hard. The colour of the specimens varied case by case. Stained slides from jaggery-fixed and formalin-fixed tissues were assessed for histological parameters, such as nuclear details, cytoplasmic details, cellular outline, and overall staining quality. A blinded method was used to compare the stained slides using a microscope. Ratings were assigned to each case on a scale of 1-4. The data were statistically analysed using R software.

Results: When comparing the formalin-fixed and jaggery-fixed specimens, no significant differences were observed in gross morphological features. All jaggery-fixed specimens appeared brown grossly. Histological features also showed no significant difference (p-value >0.05) except for cytoplasmic details. Therefore, it was observed that tissue preservation using the jaggery solution was comparable to that of formalin.

Conclusion: The tissue-preservative properties of jaggery are on par with formalin. Hence, jaggery solution can be used as an eco-friendly substitute for formalin. Further research with larger sample sizes can pave the way for the effective replacement of hazardous formalin with natural jaggery.

Keywords: Fixative alternative, Formalin substitute, Natural fixative

INTRODUCTION

Histopathological examination involves the demonstration of tissues at the microscopic level [1]. Tissue fixation is crucial in preventing autolysis, bacterial putrefaction, and increasing the refractive index of the tissue [2,3]. For decades, 40% formaldehyde has been the conventional fixative due to its easy availability, long-term preservation capabilities, and efficiency. In 1859, Russian chemist Butlerov AM et al., discovered formaldehyde. Subsequently, in the 19th century, Ferdinand Blum, while working on formaldehyde for disinfection, accidentally found that formalin can be used to fix tissues [2]. The mechanism of formalin fixation is based on cross-link formation, and formalin is recognised by its pungent odour [4].

Formalin is a known carcinogen and allergen, exhibiting high levels of toxicity [4]. Additionally, other synthetic substances such as alcoholic fixatives, non alcoholic fixatives, fixatives for nucleic acids, and fixatives containing <10% formalin are commercially available [5]. Short-term exposure to formaldehyde can cause irritation to the eyes, nose, and throat, leading to breathlessness and coughing. Concentrations above 0.1 ppm in the air can result in dermatitis on the skin, as well as eye redness and watering. Inhalation of vapour at low levels can lead to headaches, while at higher levels, formalin exposure can cause metabolic acidosis, tachypnoea, jaundice, proteinuria, haematuria, and acute renal failure. Prolonged exposure may result in severe allergic responses in the skin, eyes, respiratory tract, neurotoxicity, pulmonary disorders, hepatotoxicity, teratogenicity, and carcinogenesis [6,7]. The IARC and The US National Toxicology Program have classified formaldehyde as a class 1 human carcinogen with the potential to produce various neoplasms, including nasopharyngeal carcinoma [5,6]. Formaldehyde can interfere with Deoxyribonucleic Acid (DNA) and Ribonucleic Acid (RNA) recovery, potentially impacting molecular biology. It can enter the human body through inhalation, ingestion, and inoculation. Due to its health hazards, there has been an ongoing quest for formalin substitutes [6].

Natural substitutes like jaggery, honey, and sugar have been utilised by researchers to mitigate the impact of formalin on health [6,8-11]. Bhattacharya A et al., in their study utilised 100% buffered formalin, 100% honey, 100% sugar syrup, and 100% jaggery syrup [2]. Kuriachan D et al., used 20% solutions of honey, jaggery, and sugar

along with 10% formalin [6]. In a cross-sectional study, Sinha N et al., compared the fixative efficacy of a 30% jaggery solution with 10% neutral-buffered formalin [1]. These substances are non hazardous, inexpensive, readily available, cost-effective, and compatible with routine processing and staining [12].

Cost-effective and eco-friendly jaggery is preferred here, as jaggery has shown better fixative efficacy than sugar and is more affordable compared to expensive honey. Jaggery possesses cytoprotective, antioxidant, and tissue-preservative properties. Jaggery, a traditional Indian sweetener produced from sugarcane, is a natural mixture of sugar and molasses [5,13]. Due to its rich phenolic content and antioxidant activity, jaggery provides protection at molecular and cellular levels [4]. At low pH, the fructose present in jaggery breaks down into aldehydes and cross-links with tissue amino acids, producing tissue fixation similar to formaldehyde [2,6]. Natural sweeteners like honey, sugar, and jaggery preserve tissue morphology similarly to formalin and do not pose difficulties in routine processing and staining [5,14,15]. A recent review article by Lum A et al., systematically reviews the properties of various natural fixatives and compares their promising results [16]. The quest for safer fixatives has led to the use of newer non formalin compounds for morphologic and molecular analysis [17,18].

However, all natural fixatives have only been tested on small tissue biopsies. Studies on larger specimens, the effects of long-term fixation, the use of various special stains, immunohistochemistry, and molecular studies may be conducted to address the gaps in the literature. This study aims to compare the gross morphological and histological features of tissues fixed in jaggery and formalin.

MATERIALS AND METHODS

A cross-sectional, institution-based study was conducted in the Department of Pathology at Malankara Orthodox Syrian Church Medical College, Kolenchery, Kerala, India, from January 2019 to July 2019. The total number of study samples in this study was 23. The study was approved by the Institutional Ethics Committee (IEC) and was assigned the IEC number: MOSC/IEC/340/2019. Patient consent was taken.

Inclusion criteria: Surgical procedure specimens obtained fresh which were clinically, radiologically, and intraoperatively not suspicious or definitive of malignancy were included in the study.

Exclusion criteria: Surgical specimens that have already been placed in formalin were excluded from the study.

Sample size estimation: A total of 23 samples were collected using convenient sampling.

Data collection procedures and instruments used: Specimen collection started after obtaining permission from the Institutional Review Board and IEC. Specimens were obtained immediately after the surgical procedure in the operation theatre before being placed in formalin. A 30% jaggery solution was prepared by dissolving 300 grams of finely powdered jaggery in 1000 mL of distilled water and filtering it using filter paper. The jaggery used was Truefarm organic jaggery. The pH was checked and maintained at 5.5. Thymol crystals were added to prevent molds. The container was tightly capped, sealed, and refrigerated [4]. A 40% formaldehyde (Nice Chemicals Private Ltd.,) was used to prepare 10% formalin by mixing 100 mL of 40% formaldehyde and 900 mL of distilled water.

One tissue bit was taken from the main unfixed specimen and cut into two halves to be placed in formalin and jaggery separately. The main specimen underwent routine fixation and processing. After 24 hours of fixation, the tissue bits under study in jaggery and formalin were evaluated for the following gross morphological features: tissue shrinkage, consistency, and colour. Tissue shrinkage was quantified as mild, moderate, or marked. Consistency ranged from soft to firm and hard [4]. The specimen colour varied from case to case. Subsequently, these specimens were subjected to conventional tissue processing, embedding, microtomy, and staining with Haematoxylin and Eosin. The histotechnician falsely numbered the slides so that the investigator would not be able to distinguish between jaggery-fixed and formalin-fixed tissues. Stained slides from jaggery-fixed and formalin-fixed tissues were evaluated for the following histological parameters: nuclear details, cytoplasmic details, cellular outline, and overall staining quality based on a rating scale ranging from 1 to 4 [1,4,19,20].

Rating scale

- Poor
- Satisfactory
- Good
- Excellent

The investigator conducted an evaluation and comparison of the stained slides in a blinded manner. After evaluating each case, the false number on the slide was removed, and the ratings were recorded in the case study form.

Quality control: A 30% jaggery solution was prepared using Truefarm organic jaggery, and 10% formalin from nice chemicals was used throughout the study. The specimens in the study were subjected to the same protocol of tissue processing and staining by an experienced histotechnician. Routine quality control measures of the histopathology lab were followed.

STATISTICAL ANALYSIS

Quantitative variables were summarised using the mean and standard deviation if the data followed normality; otherwise, the median and interquartile range were used. The Wilcoxon matched-pair test was used to determine if there was a significant difference in histological features between tissues fixed in formalin and jaggery. Comparison of gross morphological parameters did not require any statistical test, as all the values were similar in both groups.

RESULTS

When comparing the formalin and jaggery-fixed specimens, gross morphological features such as tissue shrinkage and consistency did not show any significant difference. There was no shrinkage of tissues and no change in consistency observed after 24 hours for both jaggery and formalin. However, a brownish tint was present over all the jaggery-fixed specimens grossly. Some tissues appeared light brown, while others were dark brown, depending on the natural colour of the tissues. This brownish tint was not observed in the formalin-fixed specimens [Table/Fig-1].

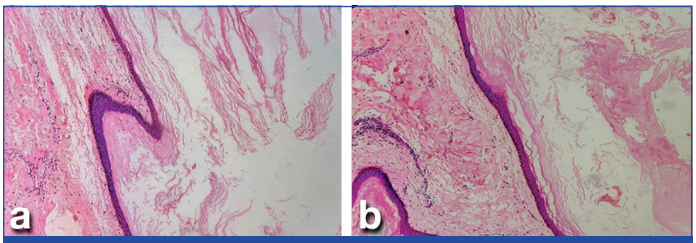
[Table/Fig-1]: Comparison of gross morphology.

Histopathological procedures, including automated tissue processing, embedding, and microtomy, were performed similarly in both jaggery and formalin-fixed specimens. No modifications or changes were made upon the introduction of a new substitute for formalin. The jaggery-fixed tissues were as firm as the usual formalin-fixed tissues after the automated tissue processing. The histotechnician encountered no difficulties during embedding and microtomy with the jaggery-fixed specimens.

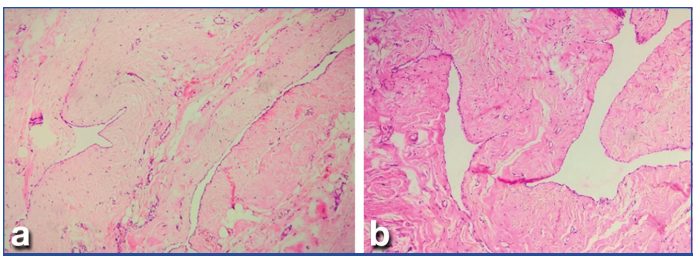
It was observed that features such as cellular outline, nuclear details, and overall staining quality of tissues fixed in jaggery showed no significant difference (p-value >0.05) compared to formalin. However, cytoplasmic details showed a slight difference with a p-value <0.05. Therefore, cytoplasmic details were better visualised with the formalin fixative, while all other histological parameters were similar for both formalin and jaggery [Table/Fig-2-4]. Thus, it was concluded that the preservation of tissue specimens by the jaggery solution was comparable to that of formalin. Nuclear details were crisp and well appreciated, and cellular outlines were discernible. Epithelial cells were better appreciated than connective tissues in the jaggery-fixed specimens. In one case with skeletal muscle, striations were not clearly appreciated in the jaggery-fixed tissue [Table/Fig-5a], while they were clearly visible in the formalin-fixed tissue [Table/Fig-5b]. Additionally, spores were noted in some microscopic sections fixed in jaggery [Table/Fig-6].

Histological parameters	Group and median (Q1, Q3)	Wilcoxon matched pair test	p-value
Cytoplasmic details	Jaggery- 3 (3,4) Formalin- 3 (3,4)	-2.24	0.025
Cellular outline	Jaggery- 3 (3,4) Formalin- 3 (3,4)	0.00	1
Nuclear details	Jaggery- 3 (3,4) Formalin- 3 (3,3)	0.00	1
Overall staining quality	Jaggery- 4 (3,4) Formalin- 4 (3,4)	-0.45	0.66

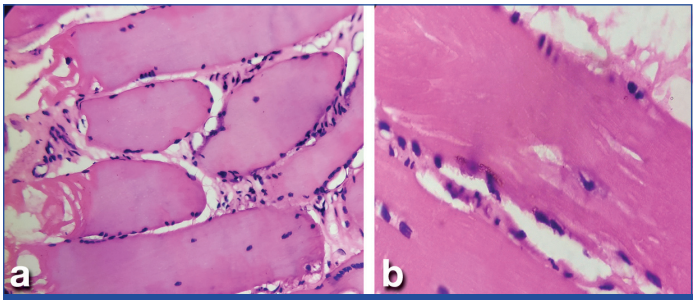
[Table/Fig-2]: Statistical analysis of histological parameters.



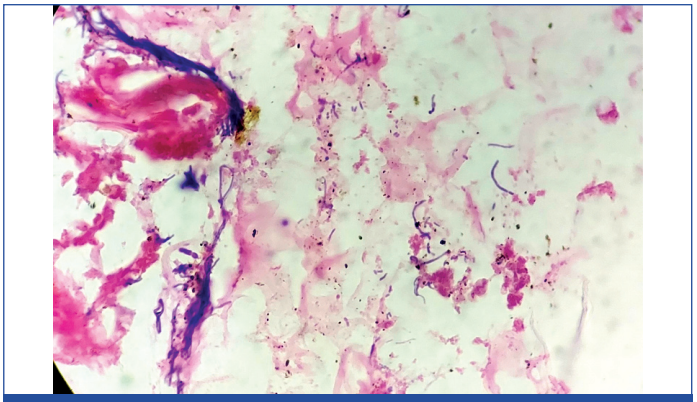
[Table/Fig-3]: a) Epidermal cyst (Formalin fixed 40x, H&E); b) Epidermal cyst (Jaggery fixed H&E, 40x).



[Table/Fig-4]: a) Hernial sac (Formalin fixed 40x); b) Hernial sac (Jaggery fixed 40x).



[Table/Fig-5]: a) Jaggery fixed, 40x; b) Formalin fixed, 100x.



[Table/Fig-6]: Spores in jaggery fixed, H&E, 40x.

DISCUSSION

In this study, it was striking to notice a brownish tinge over the specimens fixed in the jaggery solution. The degree of brown colour varied among different tissues, possibly influenced by the natural colour of the specimen. However, this did not pose any problems during further staining or microscopic interpretation. This particular finding of a brownish tint was also observed by Sinha N et al., and Bhattacharya A et al., Kuriachen D et al., [1,2,6]. Bright OA et al., pointed out that even tissues fixed in honey had a brown tint [21]. It was observed that there was no difference in the degree of shrinkage and change in the consistency of tissues in both jaggery and formalin-fixed specimens. However, Sinha N et al., found that jaggery at higher concentrations led to tissue shrinkage and loss of architecture [1], while Kuriachen D et al., showed no tissue shrinkage or observable changes in volume and weight [6].

The histotechnician did not encounter any difficulties during processing and sectioning of both jaggery and formalin-fixed tissues in the present study. In contrast, Sinha N et al., Bhattacharya A et al., and Kuriachen D et al., reported difficulties during sectioning and discontinuity in sections [1,2,6].

This study demonstrated similar nuclear details, cellular outline, and overall staining quality for both jaggery and formalin-fixed tissues. The jaggery-fixed specimens exhibited crisp nuclear features and intense staining properties. A study by Sinha N et al., and Patil S et al., showed better nuclear detailing for jaggery compared to formalin, with the overall fixation quality of jaggery being comparable to formalin [1,4]. Kuriachen D et al., observed that the jaggery fixative provided good overall staining [6]. Intense staining with eosin in jaggery-fixed specimens was also noted by Bhattacharya A et al., [2]. The acidic pH of the jaggery solution may be attributed as the reason for this [22]. However, cytoplasmic details were superior in formalin-fixed sections. The exact reason for this needs to be validated in a study with a larger sample size.

One advantage of the study was that it did not require any special equipment or staining protocols. In rural areas, during emergencies requiring a biopsy, these natural alternatives can be used if formalin fixative is unavailable. This study did not encounter any difficulties during embedding or sectioning with jaggery-fixed tissues, whereas Kuriachen D et al., observed fragility during sectioning with jaggery-fixed specimens [6]. Similarly, in this study, spores were observed in some sections taken from jaggery-fixed tissues.

Limitation(s)

No study is without drawbacks and limitations. Some difficulties encountered in jaggery-fixed tissue included a brownish tint over specimens, the presence of spores in sections, and inferior cytoplasmic details compared to formalin. The brownish colour of the specimens can obscure gross findings. Therefore, this issue may be addressed by the addition of certain chemical agents without affecting the quality of microscopic sections. The development of spores can be prevented by the inclusion of additional antimicrobial

agents and refrigeration. Efforts to enhance the quality of cytoplasmic details in jaggery-fixed specimens need to be explored further in larger research studies. This study had a small sample size and only included benign and small specimens.

This research suggests that a modified fixative based on a jaggery solution could potentially provide a natural alternative to the hazardous formalin fixative.

CONCLUSION(S)

The tissue preservation properties of jaggery are comparable to formalin. Therefore, jaggery solution may serve as an eco-friendly alternative to formalin. This study found that the fixative effectiveness of natural jaggery is nearly equivalent to that of formalin. Natural substances like jaggery as a fixative offer great potential for histopathology, presenting excellent qualities to be a novel natural substitute for formalin. The use of cost-effective and non hazardous jaggery could help laboratories become more environmentally friendly. A larger study with a greater sample size and a variety of tissues is needed for proper validation. Further research involving larger samples could pave the way for the successful replacement of hazardous formalin with natural jaggery. The introduction of natural substitutes has the potential to contribute to the development of an eco-friendly environment.

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