Original Article

Comparitive efficacies of a natural fixative with a conventional fixative

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Abstract

Introduction: The quest for formalin substitutes has long been going on due to its health hazards. Honey has been recognized as a safe substitute for formalin. However, we explored jaggery as a natural substitute for formalin. The aim of this study was to compare the tissue fixation abilities of jaggery syrup (30%) with that of 10% neutral-buffered formalin (NBF) and to determine the best fixative among both.

Materials and Methods: A study was conducted with 65 pathological tissues. Each specimen was divided into two equal parts. One part was fixed in 30% jaggery solution (Group A), while the other half was fixed in 10% NBF solution (Group B). 24 h tissue fixation was attained at room temperature followed by evaluation of pre- and post-fixation, tissue shrinkage, weight difference and ease of sectioning, followed by evaluation of conventional processing and staining. The histomorphological assessment for each slide was made based on evaluation of cellular outline, cytoplasmic details, nuclear details, staining quality and overall morphology under light microscopy. Each criterion was rated on a scale of 1–4. Nominal categorical data between the groups were compared using Chi-squared test.

Results: The preservation of tissue specimen by jaggery syrup was comparable to that of formalin and surprisingly overall nuclear detail of the tissue was better than conventional formalin fixative.

Conclusion: Jaggery can be successfully adopted in routine histopathology laboratories in place of formalin.

Keywords: Fixative, formalin, jaggery

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INTRODUCTION

Biopsy of any tissue is a key to its final diagnosis as it plays a pivotal role to arrive at a final diagnosis.[1] This biopsied tissue needs to be fixed for further procedures. Fixation is the basic step to study pathology and plays a major role in preventing autolysis and degradation of tissue and its components so that they can be examined anatomically and evaluated microscopically following sectioning.[2] Hence, it has been considered as a crucial step for preparing tissues for histopathology.[3] Errors in fixation cannot be improved at a later stage, and the final product can only be as good as its primary fixation.[4] Formalin is considered the gold standard fixative in routine haematoxylin and eosin (H&E) procedures because it is economical, easily available; feasible and provides rapid fixation with an ease of processing.[5,6] Despite all these benefits, the health and safety threats associated with formalin usage are a concern.

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Ideally, any fixatives should completely recover the DNA and mRNA details of any tissue which is essential for many test of molecular biology, but unfortunately, this is not achieved by formalin.[7]

Many chemicals used in routine laboratory procedures have detrimental effects on an individual's health, and formalin is amongst these chemicals as it has high levels of toxicity.[8] Formalin exposure, even for a short span of time, is extremely irritating to the eyes, nose and throat and can lead to breathlessness and coughing.[9] Long-term exposure causes serious allergic responses in the skin, eyes and respiratory tract.[10] According to the 11th report on carcinogens by Environment Health and Safety Information (EHSI), formalin was classified as 'reasonably anticipated to be a human carcinogen.' It is also associated with nasal and lung cancer and has a probable connection to leukemia and brain cancer.[11] Furthermore, the International Agency for Research on Cancer (IARC) has declared formaldehyde to be a carcinogen and that it is associated with causing nasopharyngeal cancer.[12]

Due to its health hazards, the search for formalin replacements has been on-going for years. This quest has been further motivated by the Occupational Safety and Health Administration (OSHA), which asserts that formalin is unsafe and encourages its replacement with less perilous substances.[6] The health concerns with formalin can be avoided by using alternative fixation methods and fixatives. Studies have shown that honey, sugar and jaggery preserve tissue morphology similar to formalin and cause no difficulties in routine processing and H&E staining.[5,6,13] There are innumerable benefits of using honey, sugar and jaggery in tissue fixation. These substances are harmless, eco-friendly, well suited for laboratory processing and staining, and require no additional equipment.[6] Jaggery is widely available and cost effective when compared to honey, especially in countries like India, Pakistan, Bangladesh, Sri Lanka and Myanmar. Indian Ayurvedic medicine has considered jaggery to be beneficial in treating lung and throat infections. In addition, it protects the body from damage at molecular and cellular levels due to its rich phenolic content and antioxidant activity.[14]

Few studies have been conducted to elucidate the use of jaggery as a fixative. Hence, this study aimed to compare, contrast and evaluate the efficacy of jaggery-fixed paraffin-embedded tissue (JFPT) sections stained with H&E to formalin-fixed paraffin-embedded tissue (FFPT) sections stained with H&E.

MATERIALS AND METHODS

A cross-sectional study was performed to test and compare the hypothesis that JFPT sections stained with H and E are better or at par with that of conventional FFPT sections stained with H and E. The study was carried out on 65 pathological specimens (50 soft tissues and 15 hard tissues). The surgically excised tissues were divided into two bits immediately after surgical removal and were placed in two different vials, one containing 30% jaggery solution (Group A) and the other containing 10% neutral-buffered formalin (NBF, Group B). Group A sections were proceeded for jaggery fixation and Group B for conventional formalin fixation. The hard tissues were taken for decalcification after 24–48 h of jaggery and formalin fixation followed by normal processing and H and E staining.

Evaluation criteria

The tissue sections were initially assessed to observe:

- Pre- and post-fixation weight and volume change in tissue samples
- Pre- and post-fixation change in tissue stability/shrinkage [Table 1].
- The slides were evaluated histomorphologically [Table 2].

Results on categorical measurements are presented in frequency and percentage (%). For all statistical tests, significance is assessed with the $P < 0.05$ being considered statistically significant difference.

RESULTS

It was found that Group A in total gave 95.4% of positive results, which were at par or almost similar to that of Group B which gave 96.7% of positive result. Surprisingly, overall nuclear detail of JFPT was better than FFPT sections. There was no significant difference in pre- and post-measurements for evaluating tissue shrinkage

Table 1: Tissue shrinkage and rating criteria

Tissue shrinkage (mm)	Rating (each criterion was rated on a scale of 1-4)
>0.6	1. Poor
0.5-0.6	2. Satisfactory
0.4-0.5	3. Good
0.3-0.4	4. Excellent

Table 2: Histomorphological criteria and rating score

Each criteria was rated on a Scale 1 to 4 (1= Poor, 2=Satisfactory, 3= Good, 4=Excellent)
a. Cellular outline
b. Cytoplasmic detail
c. Nuclear detail
d. Staining quality
e. Overall morphology

and weight difference. Overall result showed that the differences were not found to be statistically significant [Table 3 and Graph 1].

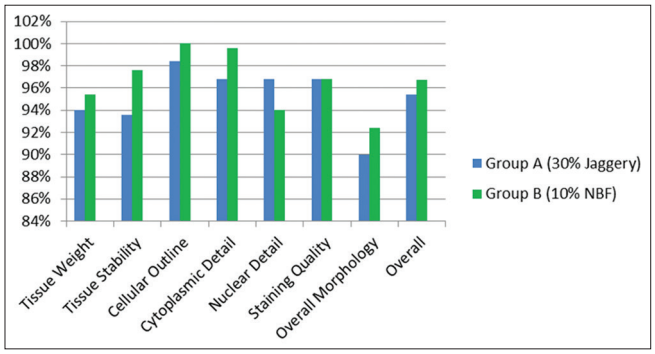
DISCUSSION

Any innovative technique must provide facts of prognostic or therapeutic significance beyond the current gold standard.[15] The word biopsy originates from the Greek terms bios (life) and opsis (vision): vision of life.[16] Thus, a histopathological technique always aims at producing good microscopic-stained detail of tissues, which closely resemble tissues structures to its life-like condition.[17] The biopsy tissue should be immediately transferred and fixed in suitable fixative solution.[18] For optimum fixation, 10% NBF is considered as an ideal fixative and the amount of fixative should not be <20 times the total volume of the specimen.[19] Fixation modifies the physicochemical state, along with redox and membrane potentials of the tissue sample which alter the reactivity of cellular components with the stain.[20] Formaldehyde was primarily explored in 1859 by a Russian chemist Alexander Mikhaylovich Butlerov and the amazing fixing performance has voted formalin to be fixative of choice till date.[21] It has been recognized by all means as a satisfactory chemical to be designated as impeccable fixative.[22] Despite its numerous advantages, formalin has many disadvantages. IARC categorizes formaldehyde as a human carcinogen which can lead to nasopharyngeal cancer.[23] Lu *et al.* established strong evidence backing up genotoxic and cytotoxic genre of inhaled formaldehyde involved in carcinogenetic changes in respiratory nasal epithelium.[24]

Well ahead, several studies have proven that honey is a safer substitute to formalin in routine histochemical and immunohistochemical staining procedures.[25] However, honey is not commonly available, so it is impractical to use honey on a larger scale because of its expensiveness. Thus, keeping in mind the hazards of formalin and expensiveness of honey and sugar, we urge to explore substances that can overcome these shortcomings.[5] Various studies related to honey, sugar and jaggery conducted till date demonstrate that these natural fixatives satisfy almost all the requirements, to be called as an ideal fixative.[5,6] The decision of choosing jaggery was motivated by the above-mentioned properties of jaggery as well as its composition similarity to honey and sugar, which have already proven their ideal fixation quality. Studies conducted by Patil *et al.*[5,6] on animal tissues concluded that jaggery is at par and meets all the ideal properties of a fixative and it fixes the biological tissue in the same manner as that of the formalin. Our study is the first of its kind as no other previous studies have been undertaken on pathological human soft and hard tissues where jaggery has been taken as a fixative and then compared with that of formalin.

To begin with, we tried to standardize the jaggery by experimenting changed concentrations (10%, 20%, 30% and so on). Jaggery at higher concentrations gave rise to tissue shrinkage and loss of architecture. A concentration of 30% for jaggery syrup gave the best outcomes during pilot study. Earlier works on jaggery have also revealed that 30% of jaggery can satisfactorily fix the tissues.[5-7] All these natural preservatives including jaggery grows molds with time; hence, it is logical to add thymol crystals as an antimicrobial agent and if possible it is advised to preserve the solution in refrigerator. The same concept was followed in the present study for better storage of the prepared solution. The probable mechanism of fixation by jaggery may be comparable to that of sugar and honey[5-7] [Figure 1]. The pH of jaggery in our study ranged between 4.5 and 5.5 which were in favor of the explained mechanism.

In total, jaggery-fixed samples showed brownish tint, although there was no meddling with subsequent staining. The overall result in case of staining quality was similar i.e., 96.8% with insignificant *P* value, proving the equivalent staining quality. The tissues fixed with jaggery showed neither



Graph 1: Overall scores of each parameter in terms of percentage

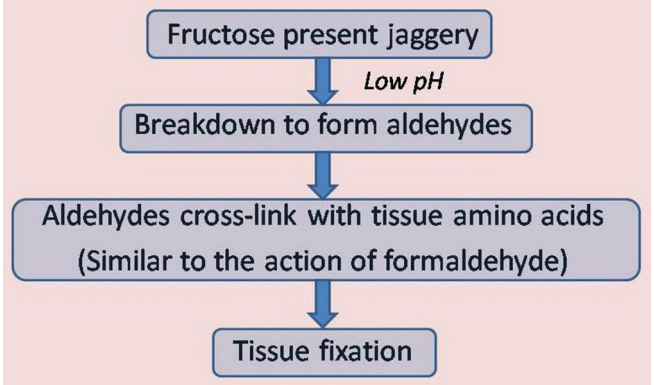


Figure 1: The possible mechanism of fixation by jaggery

Table 3: Overall scores of each parameter

Evaluation parameters	Results of Group A in percentage (%)	Results of Group B in percentage (%)	Chi square Combined results of Groups A & B	P-value Combined results of Groups A & B
Tissue Weight	94	95.4	2.122	0.112
Tissue Stability(Shrinkage)	93.6	97.6	2.00	0.368
Cellular Outline	98.4	100	0.638	0.727
Cytoplasmic Detail	96.8	99.6	1.766	0.41
Nuclear Detail	96.8	94	3.733	0.155
Staining Quality	96.8	96.8	1.612	0.447
Overall Morphology	90	92.4	2.568	0.277

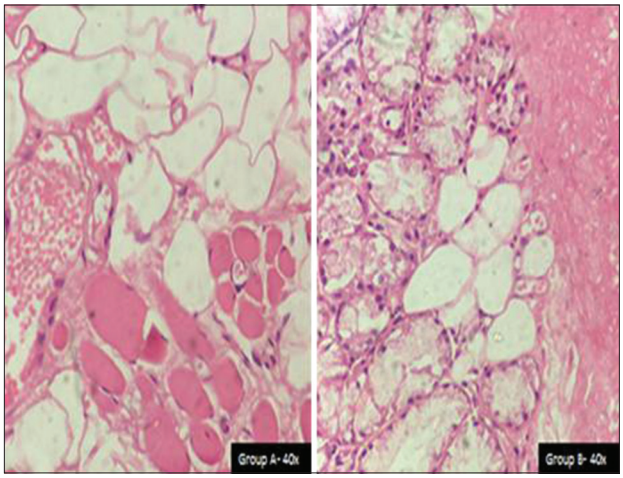


Figure 2: Jaggery-fixed paraffin-embedded tissue stained with H & E (Group A) with that of formalin-fixed paraffin-embedded tissue sections stained with H & E (Group B) depicting staining quality of soft tissue

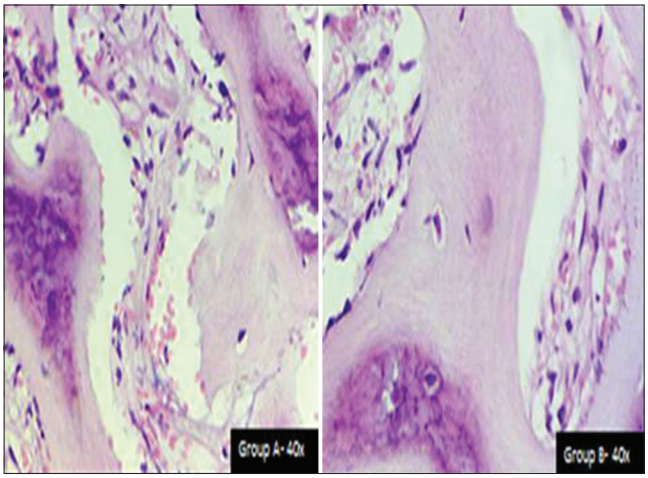


Figure 3: Jaggery-fixed paraffin-embedded tissue stained with H & E (Group A) with that of formalin-fixed paraffin-embedded tissue sections stained with H & E (Group B) depicting staining quality of hard tissue

any viable tissue shrinkage nor any observable change in volume and weight, when compared with its counterpart, i.e., the formalin-fixed tissue. The only problem faced with usage of this natural fixative was during tissue sectioning. The tissue fixed with jaggery was hard as compared to formalin-fixed tissue resulting in mild difficulty during sectioning, which can be easily overlooked, considering the enormous benefits of using jaggery as a fixative. Jaggery surprisingly showed better nuclear detailing (96.8%) as compared to formalin (94%). The reason behind this can be contributed to the acidic nature of jaggery which resulted in better staining of nucleus. Overall fixation quality of jaggery (95.4%) was at par with formalin (96.7%) both for soft and hard tissues [Figures 2 and 3].

In summary, jaggery surpassed both our anticipations and the established results shown by honey and sugar. We found no difficulty in making diagnoses of tissues submitted for histopathological examination that were fixed with JFPT compared with FFPT. Introducing jaggery syrup as a tissue fixative enlists several advantages including the facts that jaggery is non-toxic, eco-friendly, well suited for laboratory processing and staining, and requires no supplementary equipment. Jaggery is widely available and much more reasonable compared to other chemical or natural standard fixatives. Furthermore, jaggery can be used as a natural substitute, particularly when formalin is not available. Jaggery has all the innovative potential to be a brilliant standby for formalin. However, these findings necessitate more research into its wide-scale applications.

CONCLUSION

Great strides have been made in histopathology that have facilitated a rapid unravelling of altered microscopic quality of fixed tissue as well as enlightening any deleterious effects of chemical fixatives. The fixation field has been dominated by formalin since the 19th century. However, in the last 20 years, formalin has been found to be toxic and carcinogenic. Natural substitutes like honey, sugar and jaggery share properties that are similar to formalin in terms of fixation with no health hazards per se; hence, these may be a boon when compared with formalin. Thus, we conclude that jaggery syrup is an acceptable standby for formalin and a breakthrough in the area of tissue fixation.

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Conflicts of interest

There are no conflicts of interest.

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