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ORIGINAL RESEARCH

Analysis of Fixative Properties of Three Eco-friendly Substances: A Comparison with Formalin

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ABSTRACT

Introduction: Ever since its initial use as tissue fixative in the 19th century, formalin has been the choice fixative in histopathology, providing efficient and economic tissue fixation. In recent years, there has been a search for suitable alternatives to this "golden fixative," following the reported harmful effects of formalin on humans. Honey has been quoted as having excellent fixative property, posing no hazard, being naturally occurring. Others like sugar and jaggery with composition similar to honey are also being explored of late, as substitutes to formalin.

Aim: To analyze the fixative properties of honey, jaggery, and sugar and compare the same with that of formalin, to assess the best fixative among them to seek the possibility of an eco-friendly substitute for formalin in routine histopathology.

Materials and methods: Bits of human gingival tissue were placed in four containers with 20% solutions of honey, jaggery, and sugar and 10% formalin. After 24-hour fixation followed by conventional processing and staining, the sections were analyzed under light microscopy for details, such as cell outline, nuclear and cytoplasmic staining, and uniformity of staining. Each criterion was assessed by two independent observers on a scale from 0 to 3; 0 for poor and 3 for excellent. The results obtained were analyzed using Kruskal–Wallis analysis of variance test.

Results and conclusion: Our study, aimed at assessing the fixation abilities of three eco-friendly substances, has yielded positive and promising results. The naturally occurring and highly economical substances like honey and jaggery were found to give better results than formalin, indicating a more prominent role for them in histopathology in the near future, thereby keeping alive the possibility of being used as substitutes to formalin in laboratories in the years to come.

Keywords: Fixative, Formalin, Honey, Jaggery, Sugar.

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INTRODUCTION

Preservation of tissues has been done from the ancient times for varying reasons. Ancient Egyptians and Incas believed that preserving their dead was necessary for resurrection.[1,2] In Christian tradition, preservation of body was done for temporary viewing until burial. Preservation was also done through burial in a particular type of soil in cold or dry heat in the right climate, by injecting colored waxes into organs, evisceration, etc.[3]

The use of formaldehyde as a biological reagent for tissue preservation occurred rather late in the history of laboratory technology due to its late manufacture by the chemical industry. Formaldehyde was discovered by Alexander M. Butlerov in 1859. In 1868, Van Hoffman developed a practical method for its synthesis from methanol.[4] It was first produced as an industrial reagent in France and Germany where its medical applications as an antiseptic to treat or prevent wound infections were explored. Issac Blum and Ferdinand Blum in 1893 further explored its antibacterial properties against bacterial species, including *Bacillus anthracis*, *Staphylococcus aureus*, and Proteus.

During the process, Ferdinand Blum reported that the skin of his fingers that had come in contact with the diluted solution became hardened. He also observed that the tissues of anthrax-infected mouse preserved in formaldehyde showed excellent staining results with hematoxylin and aniline dye.[5] This marked the beginning of the use of formaldehyde as a "fixative" for preserving tissue structure. Since then it has been the choice fixative providing efficient and economic tissue fixation and no laboratory or museum is now complete without tissues fixed in it.

But is it all well with formalin?

In the last few decades, the Occupational Safety and Health Administration has declared formaldehyde as a potential health hazard.[6] The International Agency for Research on Cancer (IARC) classifies formaldehyde as a Class 1 human carcinogen that can potentially produce different neoplasms including nasopharyngeal carcinoma.[7] Also, incomplete deoxyribonucleic acid (DNA) and mitochondrial ribonucleic acid (RNA) recovery which deleteriously affects many test results in molecular biology has forced researchers to find an alternative to formalin.

Fig. 1: Four substances analyzed for their fixative properties – honey, jaggery, sugar, and formalin

Fig. 2: Gross appearance of tissues fixed in honey, sugar, jaggery, and formalin (left to right)

Recent literature points to studies exploring the possibilities of naturally occurring substances like sugar, jaggery, etc., to fix tissues. These eco-friendly substances with little toxicity are also easily available. The aim of this study was to explore the possibility of finding alternative natural fixatives to formalin in routine histopathology. The objectives were to analyze the fixative properties of honey, jaggery, and sugar and compare the same with that of formalin to assess the best fixative among them.

MATERIALS AND METHODS

After obtaining the necessary clearance from the Institutional Ethical Committee, 40 fresh tissue specimens comprising bits of human gingival tissue obtained during gingivectomy procedures were fixed in 20% solutions of honey, jaggery, and sugar and 10% formalin for 24 hours (Fig. 1). Trial and error method was used to finalize the concentrations of the solutions for honey, jaggery, and sugar. At 20% concentration of the solutions in distilled water, they exhibited adequate flow, so as to make them easy to handle in the laboratory. At the end of 24 hours, the tissues fixed in each solution were checked for their consistency (Fig. 2) and were then subject to the conventional processing, microtomy, and staining with hematoxylin and eosin. The stained sections were analyzed under 20× and 40× objectives in a light microscope for assessing four histomorphologic criteria by two independent observers and were rated on a scale from 0 to 4 as shown in Table 1.

The scores for each criteria were tabulated for the 10 slides scanned for each of the four fixatives used. Total score for each fixative was calculated and the mean total score was also derived for each fixative. The mean scores were analyzed and compared using Kruskal–Wallis analysis of variance test. Interobserver variability was also determined by *post hoc* Games–Howell test.

Table 1: Histomorphologic criteria for assessment of fixative properties of the materials and their rating scale

Histomorphologic criteria	*Rating scale*
Cellular outline	1 – Poor
Nuclear staining	2 – Satisfactory
Cytoplasmic staining	3 – Good
Overall staining quality	4 – Excellent

RESULTS

Table 2 depicts the mean average score of all criteria for each of the fixatives used. Honey presented the highest mean score and sugar gave the lowest overall score. Comparison of "p" values of the fixatives revealed that both honey and jaggery showed significant difference with that of formalin ($p < 0.05$), both giving superior results to formalin. The scores for sugar did not show significant difference from that of formalin, with only sugar giving a marginally poorer result when compared with formalin. The tissues fixed with honey gave the best overall results showing excellent nuclear and cytoplasmic staining and cellular outline (Fig. 3).

Though the overall staining quality of jaggery was good and better than with formalin, the cellular and nuclear details were inferior to that of honey (Fig. 4). Tissues fixed in formalin gave a good nuclear staining, but overall staining was found to be inferior to that of honey and sugar (Fig. 5). With sugar, there was poor overall staining with lack of clarity of cell outlines giving the least satisfactory results of the four fixatives evaluated (Fig. 6).

DISCUSSION

Formalin, referred to as the "golden fixative" because of its advantages viz., easy availability, economical nature, good shelf life allowing long-term storage and above

Table 2: Total scores for each histomorphologic criteria for the four materials compared, along with the mean total scores

	Cellular outline	*Nuclear staining*	*Cytoplasmic staining*	*Overall staining quality*	*Mean total score*
Honey	51	54	51	53	52.25
Jaggery	46	46	46	47	46.25
Formalin	36	39	36	36	36.75
Sugar	32	30	31	33	31.5

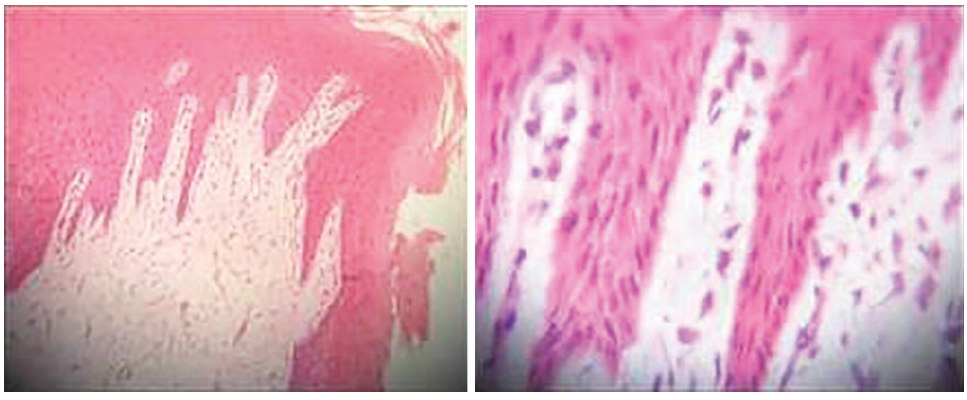

Fig. 3: Tissue fixed in honey showing excellent results (hematoxylin and eosin, 10× and 40×)

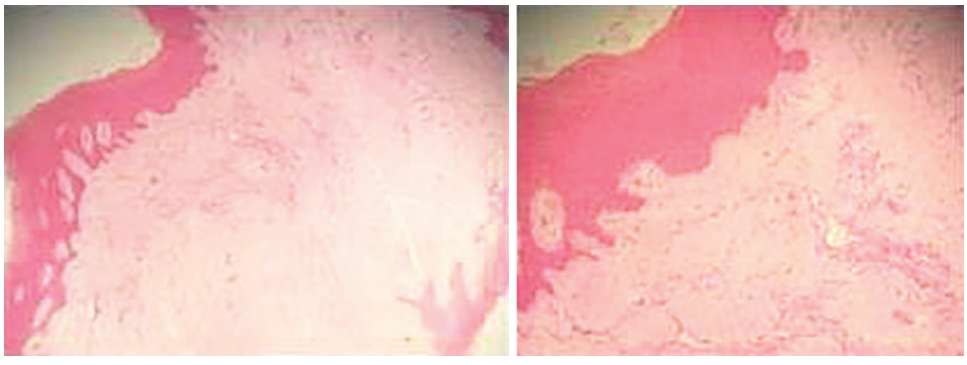

Fig. 4: Tissue fixed in jaggery (hematoxylin and eosin, 4× and 10×)

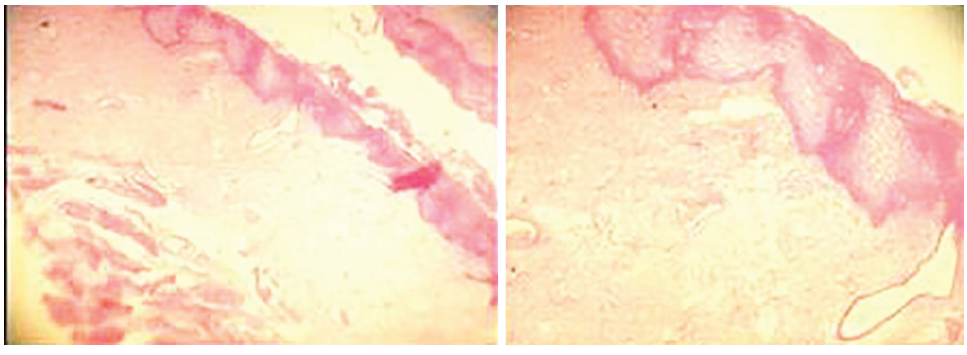

Fig. 5: Tissue fixed in formalin (hematoxylin and eosin, 4× and 10×)

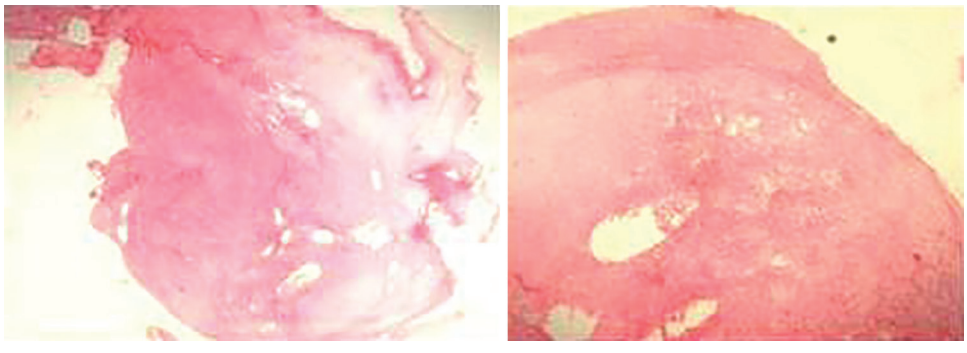

Fig. 6: Tissue fixed in sugar (hematoxylin and eosin, 4× and 10×)

all, the preservation of cellular architecture, helps us to understand tissue morphology for diagnosis. But on the contrary, comprehensive researches and large-scale human studies conducted internationally lead the IARC to classify formaldehyde as a human carcinogen under conditions of unusually high or prolonged exposure.[7,8] The US Natural Toxicology Program also reported formaldehyde as a known human carcinogen in its 12th Report of carcinogens.[9]

Evidence supports the linkage between formaldehyde exposure and nasopharyngeal cancer, nasal and paranasal

Table 3: Toxic effects of formalin on the human body categorized into acute and chronic effects

Acute exposure	*Chronic exposure*
Irritation • Skin contact causes dermatitis • At concentrations above 0.1 ppm in air, it irritates eyes causing redness and watery eyes • Inhalation of vapor at low levels, irritates nose, causes headaches and at higher levels leads to bronchitis and symptoms of asthma (reactive airway dysfunction syndrome)[14]	Neurotoxicity • Headaches, dizziness, sleep disorders, memory loss, amyotrophic lateral sclerosis[15]
Poisoning • Burns, ulceration, pain, nausea, vomiting, diarrhea, gastrointestinal hemorrhage • At higher levels causes metabolic acidosis, tachypnea, jaundice, proteinuria, hematuria, acute renal failure[16]	Cellular changes • Inflammation and metaplasia of mucosa[11]
	Pulmonary functional disorders
	Hematotoxicity • Reduced blood cell counts and hemoglobin[17,18]
	Teratogenic effect[19]
	Genotoxicity[20]
	Carcinogenesis

cancer, and myeloid leukemia.[10] The rapid chemical changes undergone by it after absorption is thought to be the reason for its unlikely influence in causing any carcinogenic change on organ sites beyond the upper respiratory tract. Formaldehyde enters the human body via inhalation of its gaseous form, ingestion, or dermal absorption.[11,12] It is converted to a nontoxic chemical called formate, which is excreted through urine or is exhaled out as carbondioxide.[13] Lu et al[11] have explained in detail the genotoxic and cytotoxic mode of action of formalin on the respiratory nasal epithelium. Formalin binds severely to DNA, RNA, and proteins, making it difficult to extract them in useful form for molecular tests.

The toxic effects of formalin on the human body, as reported by various authors, have been summarized in Table 3.

This prompted many researchers to start their quest for substances that could be used as efficient alternatives to formalin, but most importantly, were less toxic than formalin. A review of related literature shows quest for formalin alternative using materials, such as honey, sugar syrup, etc. Ancient Egyptians, Greeks, and Romans used honey for food preservation and mummification. It is the most ancient wound dressing known and it has continued to be used throughout the ages.[21] Dioscorides (50AD) wrote of honey being "good for sunburn" and "for all rotten and hollow ulcers," and its usage has continued into present-day folk medicine.[22] It has also been mentioned as a potent antibacterial agent.

Honey comprises a viscous saturated solution of sugars primarily glucose and fructose and about 20% water almost all bound up with the sugar molecules. Glucose oxidase which produces hydrogen peroxide and gluconic acids gives honey a pH typically 3.2 to 4.5. It contains minerals and phenol inibine which also contributes to its antioxidative and antibacterial effects.[23] Hydrogen peroxide is produced only in diluted honey as glucose oxidase is inhibited in undiluted honey. High osmolarity of undiluted honey and substances like phytochemicals also contributes to the antioxidative effects.[21] It is also reported to contain a wide range of amino acids, fatty acids, derivatives of tetracyclins, vitamins like ascorbic acid.[23] Honey, is easily available with no toxicity has been explored as an alternative to formalin.[24] At low pH (3.2–4.5), the sugars in these fixatives are converted into aldehydes which are thought to exert similar actions as formaldehyde.[25]

Jaggery and sugar have also been explored as safer alternatives to formalin. Jaggery has similar composition as that of Honey. It is a derivative of sugarcane. There is a cytoprotective and antioxidant property in jaggery because of which it could be used effectively for preserving tissues.[26] Sugar, like jaggery, is again a derivative of sugarcane juice. It creates an environment with low water activity inhibiting bacterial growth.[26]

These three natural occurring substances have been widely used in India as antibacterial, anti-inflammatory, and as food preservatives. They are produced abundantly in India. The advantages of these substances viz. their easy availability, economical nature because of which it could be used in large scale in screening camps, no known toxic effects, compatibility with routine laboratory procedures, and most importantly their ability to fix and preserve the tissue morphology led us to explore their fixative properties and compare the same with that of formalin.

Al-Maaini and Bryant[27] studied the fixative properties of honey and found that low concentrations of

honey, at room temperature, could fix tissues which were comparable to the tissue fixation produced by formalin. Similar studies by Gunter and Bryant[28] and Ozkan et al[29] further strengthened the comparable fixative properties of honey to those of formalin in routine histopathology procedures. Both jaggery and sugar are derived from sugarcane juice, and many earlier studies have reported their property of preservation. Nayaka et al[26] have reported the presence of cytoprotective and antioxidant property in jaggery.

With these three eco-friendly substances that were easily available in India being experimented as alternatives to formalin fixation in separate studies, we decided to compare the fixative properties of honey, sugar, and jaggery with that of conventionally used formalin, in order to choose the best potential alternative to formalin, among the three.

The lowest concentration of the natural fixatives which could effectively fix the tissues to give good microscopic detail was to be decided first. The literature has stated that concentrations as low as 20% for honey, jaggery, and sugar syrup at a pH of 4.5 to 5.5 could fix tissues similar to 10% formalin.[25] Another study illustrated 20% Omani honey with additives like 70% alcohol to enhance the rate of tissue penetration and acetic acid to soften the tissues could be used to give improved section cutting and enhanced staining qualities. Undiluted pure honey was thought to harden tissues between the second and third days of fixation and that the signs of autolysis and putrefaction were not seen in those tissues according to another study. Thus, by trial and error method, we standardized the optimum solution concentration to be 20% each for honey, jaggery syrup, and sugar syrup.

We compared the cellular outline, nuclear staining, cytoplasmic staining, and overall staining quality of each slide fixed in the four fixatives. On comparison of the mean average score for each fixative, we found that honey presented with the highest score, followed by jaggery, formalin, and sugar. Sugar gave the lowest overall score. Most of the previous studies have reported honey to produce comparable fixation as that of formalin,[25,26,28,29] but our study clearly indicates superior fixative quality of honey when compared with formalin. We found that jaggery too gave better results than conventional formalin, but its fixative property was not as good as honey. This was in contrast to a recent study by Patil et al[25] which reported that jaggery gave the best overall fixative property among the three natural substances compared. Only this study has been reported in literature, which compared the fixative ability of the four substances that we compared in our study. It stated that jaggery, honey, and sugar gave results comparable to formalin, with jaggery showing the most superior results. But from our study, we observed that honey presented with the best fixative property closely followed by jaggery. Both showed better fixation than formalin. Thus, our study clearly indicates that honey may be used as the best alternative fixative to formalin in routine fixation. Jaggery, too, could be used when honey was not available, and it can give results superior to formalin. Not much mention exists with regard to the fixative property of sugar in related literature, save a study which quotes promising result with honey, sugar, and jaggery with regard to tissue fixation.[25] We found that sugar gave the poorest fixation which was inferior to that of all other substances including formalin.

Analyzing the individual scoring criteria for the four substances, we found that the tissue fixed in honey gave the best overall results, giving excellent staining and cellular outlines. We observed that jaggery fixation gave a good overall staining quality, but clarity of cellular outlines was inferior to that of honey. With tissues fixed in sugar, we observed a poor overall staining and lack of clarity of cell outlines giving the least satisfactory results among the four substances evaluated for their fixative properties.

Both honey and jaggery—naturally occurring substances are nonhazardous, compatible with routine histopathology procedures and are financially viable, apart from being easily available in India.

But certain difficulties can be encountered during laboratory procedures with the three natural substances. These problems, along with their remedial measures, are summarized in Table 4.

However, other disadvantages like homogenization in the connective tissue as quoted in a study,[24] and artifacts, including viable spores, such as clostridia in honey that

Table 4: Problems encountered with use of natural fixative substances, along with their remedial measures

Problem	*Remedy*
Sedimentation of impurities in the solution (especially jaggery)	Filtration of solution
Immiscibility of natural fixatives with distilled water	Constant stirring of solution
Stickiness of natural fixatives to the tissue surface and difficulty in rinsing it off	Thorough washing under running tap water
Fungal growth in bottles of natural fixatives especially after 2 days of fixation	Thymol crystals could be used[25] But our specimens were fixed after 24 hours, which was not hindering with the result interpretation
Brittleness of tissue fixed in sugar syrup during microtomy	Careful cutting, using new blades for every block
Fungal growth on tissue surface within paraffin block	Applying wax coat over the tissue surface to protect tissue from exposure to the environment

is not well filtered, were not relevant in our study. Honey is difficult to be filtered because of its viscosity. Gamma irradiation makes it sterile as all organisms will be killed by this treatment.[26]

CONCLUSION

Naturally occurring formalin substitutes are a real boon, when the hazards of formalin on health are considered. These commonly available day-to-day substances have proved to be superior or comparable to the fixative property of formalin. In this novel attempt to select the best alternative to formalin, honey has emerged ahead of the rest, giving the best fixative property. Jaggery which came close behind also showed superior results than formalin. In our quest for "healthy fixatives," we conclude by stating that further research with larger samples can definitely pave the way for the replacement of the hazardous formalin with the more eco-friendly honey or jaggery. It would not be long before our laboratories become "green" and "eco-friendly."

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