

NATURAL SUBSTITUTES FOR FORMALIN - CHEMICAL VERSUS NATURAL: A COMPARATIVE STUDY

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ABSTRACT

Objective: - To compare the tissue fixation ability of sugar and jaggery syrup with that of formalin using H & E stain and to determine the best fixative among the two.

Background: - Formalin has long been standard fixative for clinical routine worldwide. As a result of increasing concerns about the potential carcinogenicity of formaldehyde, attempts have been made to find safer alternatives. By exploring more economical, eco-friendly and readily available substances like jaggery and sugar has been suggested as natural substitute for formalin.

Materials and Methods: - Fifty tissue samples were obtained from gingivectomy (non-idiopathic) patients, cut into four pieces and placed in 10% formalin, 20% sugar syrup, 30% jaggery syrup and distilled water, 24 hours tissue fixation was attained at room temperature followed by conventional processing and staining. Tissue sections were assessed for cytoplasmic, nuclear details and staining quality under light microscopy and whole procedure was blinded.

Result: - Results were analysed by Kruskal Wallis ANNOVA test. Mean values for tissue fixed with sugar and jaggery syrup were similar to each other and closer to formalin. Among two natural fixatives, mean value of jaggery was superior. Preservation of tissue by jaggery gave good results than sugar after comparing with formalin.

Conclusion: - Jaggery syrup for tissue fixation yielded good results. Hence it can be successfully adopted in routine histopathology laboratories in place of formalin.

Key Words: - Formalin Substitutes, Jaggery Syrup, Natural Tissue Fixatives, Sugar Syrup.

Introduction

To fix the tissue, many approaches to fixation and types of fixatives have been developed and tested throughout the years. Fixation of tissue is an initial and important step in tissue processing for microscopical examination. It is a critical step in the preparation of tissues for histopathology. Fixation is aimed to preserve the tissues in a life – like state, prevent bacterial putrefaction, prevent autolysis and to increase the refractive index of the tissue.¹ Fixation is a complex series of chemical events and differs for different groups of chemical substances found in tissues. The current fixative of choice is formalin. Initially, the mechanism of formalin fixation was based on the formation of cross-links. Formalin has been established as a fixative for routine surgical pathology, and most clinicians are aware of it by its pungent odour.^{1,2}

Formaldehyde was first discovered in 1859 by the Russian chemist Alexander M Butlerov. Later it was Ferdinand Blum in 19th century who while working on formaldehyde for disinfection accidentally found it can “fix” the tissue and since then Formalin became fixative of choice in just a few years, and formalin-fixed paraffin-embedded tissue stained with haematoxylin and eosin (H & E) became “*Gold Standard*.” It has been said that, there is no other histopathology technique that provides so much information so quickly and for such a little cost.³

An optimal fixative should be nontoxic and allow for detailed morphologic analysis, high quality special histochemical and immune histochemical staining, and good

preservation of DNA and RNA at a reasonable price. Although formalin is cheap and enables long-term storage of surgical material, preserves morphological features well, allows special histologic stains and in combination with antigen retrieval, allows for reliable immune histochemical analysis. However formaldehyde was classified as “*Carcinogenic to humans*” (group 1) by the International Agency for Research on cancer and therefore represents a risk to anyone handling the solution. Furthermore, its cross-linking masks antigens, which may hamper immune histochemical analysis, and fragments nucleic acids, which impairs the extraction efficiency and quality of DNA and RNA. The OSHA (Occupational safety and Health Administration) regulation standard declaring it hazardous and advocating its substitution with less dangerous chemicals but none of them could meet the standards set by formalin. Bee honey has been shown to preserve tissue morphology similar to that by formalin. Since sugar & jaggery share similar composition with honey, they can also preserve the tissues, so in our study we explored the eco-friendly, economical and readily available substances like sugar and jaggery as substitutes for formalin.^{1,2,3}

Hence the present study is to compare the tissue fixation abilities of Sugar and Jaggery syrup with that of formalin using H & E stain and to determine the best fixative among the two.

Materials and Methods

Fifty tissue samples were obtained from normal gingiva i.e. from gingivectomy (non-idiopathic), operculectomy

patients, cut into four pieces and placed in 10% formalin, 20% sugar syrup, 30% jaggery syrup and distilled water, 24 hours tissue fixation was attained at room temperature followed by conventional processing and staining. Tissue sections were assessed for cytoplasmic, nuclear details and staining quality under light microscopy. Each criteria was rated on a scale of 1 - 4 (1 for poor and 4 for excellent) and whole procedure was blinded. Results were analysed by Kruskal Wallis ANNOVA test. The histomorphological criteria examined are elaborated below.



Figure 1: - Photograph showing 10% Formalin, 20% Sugar syrup, 30% Jaggery syrup and Distilled water



Figure 2: - Photograph of gross tissue specimens fixed in: F - Formalin; S - Sugar syrup; J - Jaggery syrup; D - Distilled water

HISTOMORPHOLOGICAL CRITERIA

Histomorphologic Criteria is based on following details.

1. Cellular outline
2. Nuclear detail
3. Staining quality
4. Overall morphology

Each histomorphologic criteria was rated on scale of 1- 4

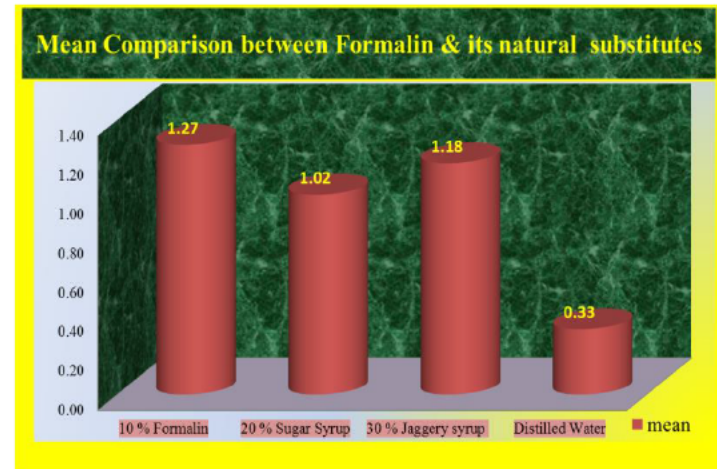
1. Poor
2. Satisfactory
3. Good
4. Excellent

Results

A combined comparison was carried out between the mean value of Formalin and its natural substitutes (Jaggery syrup, Sugar syrup and Distilled water) by Kruskal Wallis ANOVA non parameter test. The highest mean score of 1.27 was obtained for tissue section fixed with 10%

buffered formalin which acted as the positive control (Figure 2F, Graph 1). Followed by jaggery syrup with mean value 1.18 and showed good overall morphology, good nuclear details and staining quality. The cellular outlines were clearly discernible. (Figure 2J, Graph 1)

Sugar and jaggery syrup with mean value were closed to formalin. With sugar syrup fixation, the tissue section showed a fair cytoplasmic details, with mean score of 1.02 (Figure 2S, Graph 1). The lowest mean score of 0.33 was obtained for tissue section fixed with distilled water. It showed significant cellular swelling and poor staining with H & E (autolysis) (Figure 2D, Graph 1). Thus out of 3 natural substitutes, jaggery was superior.



Graph 1: - Bar Chart with mean bars showing the comparison between Formalin and its natural substitutes (Sugar syrup, Jaggery and Distilled water).

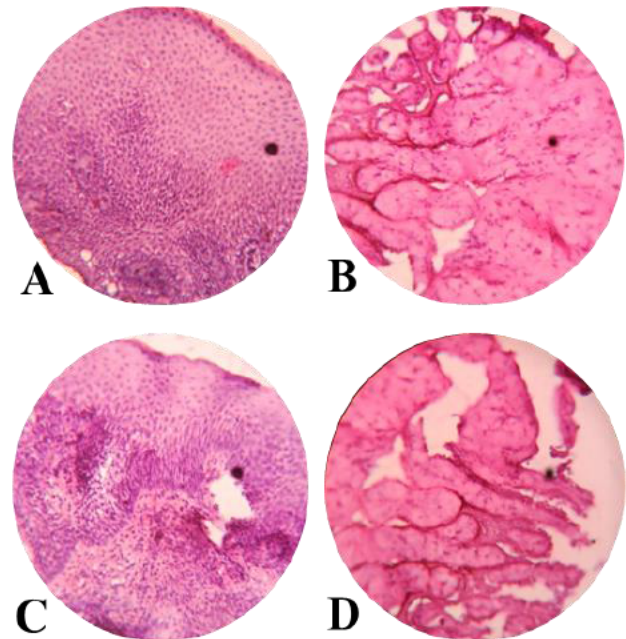


Figure 3: - Histopathologic picture of the tissues fixed in A - Formalin; B - Sugar; C - Jaggery; D - Distilled water (H & E 40X)

Discussion

Formalin has long been universal standard fixative in histopathology & clinical routine worldwide. The fundamental advantage stems from its continuous and almost universal use for over 100 years and all the accumulated scientific knowledge on it. Also, formalin is readily available, economical, fairly convenient to store, allows long-term storage, preserves lipids well, and has been accepted as the closest thing there is to the perfect fixative, with no clear “all-purpose” alternative found to date.^{3,4}

On the other hand, formalin has two well-known disadvantages. Firstly, formalin is highly toxic. The International Agency for Research on Cancer (IARC) classifies formaldehyde as a human carcinogen that can cause nasopharyngeal cancer. Lu *et al* found strong evidence that can support a genotoxic and cytotoxic mode of action for the carcinogenesis of inhaled formaldehyde in respiratory nasal epithelium. The various health hazards of formalin are collated in Table 1. Guidelines for ambient formaldehyde levels in living spaces have been set in several countries in the range of 0.05 to 0.4 ppm, with a preference to 0.1 ppm. Secondly, the chemical action of formalin binds severely to DNA, RNA and proteins, which makes them difficult or impossible to extract in a useful form for molecular tests.³

ORGAN SYSTEM	ADVERSE EFFECTS
Skin and mucous membrane	Irritation of the mucous membranes of the mouth and upper respiratory tract, allergic contact dermatitis
Respiratory system	Sneezing, coughing, laryngospasm, pulmonary edema, temporary reversible decrease in lung function, degenerative diseases, inflammatory and hyperplastic changes of the nasal mucosa, asthma, chronic rhinitis, loss of olfactory functioning
Eye	Irritation, lacrimation, conjunctivitis
Gastrointestinal tract	Irritation, nausea, vomiting, diarrhoea, loss of appetite, burns and ulceration, abdominal pain, gastrointestinal haemorrhage, pharyngeal congestion, chronic pharyngitis
Cardiovascular system	Tachypnoea, nodal tachycardia
Central nervous system	Dizziness, depression, headaches, sleep disorders, memory loss, convulsions and coma
Haematopoietic	High serum alanine-amino

system	transferase (ALT), Lower RBC, WBC, platelet and haemoglobin counts
Renal system	Renal failure
Reproductive system	Menstrual disorder, dysmenorrhea and spontaneous abortion

Table 1: Health hazards of formalin.^{3,5,6}

For centuries, honey has been shown to be a successful antibacterial agent having the potential to preserve compounds without any harmful effects on users. Many studies were conducted to compare honey with formalin which gave results comparable to formalin fixed control tissues. Properties of honey such as high osmolarity, low pH and the presence of components such as hydrogen peroxide and phenol inhibine, all contribute to its anti-oxidative and antibacterial effects.³ Later, several studies proved that honey can be a safe alternative to formalin in conventional histochemical and immunohistochemical staining methods.^{7,8,9}

Similarly Sugar and jaggery are derivatives of sugarcane juice and are well known for their preservative properties.^{10,11,12} The use of sugar for wound healing is one of the earliest known methods. According to Herszage and associates, treatment with sugar destroys bacteria non-specifically by creating an environment of low water activity which inhibits bacterial growth.¹³

Jaggery is a widely used traditional Indian sweetener. Of the total world production, more than 70% of jaggery is produced in India.¹⁴ A study by M.A. Harish Nayaka *et al*, proved the presence of cyto-protective and antioxidant activity in jaggery.¹¹ On the other hand, molasses which is also a cane derivative; is used widely in US, UK, Denmark, Russia, Africa and Asia. Table 2 elaborates the properties of molasses and jaggery and Figure 4 depicts the two products.

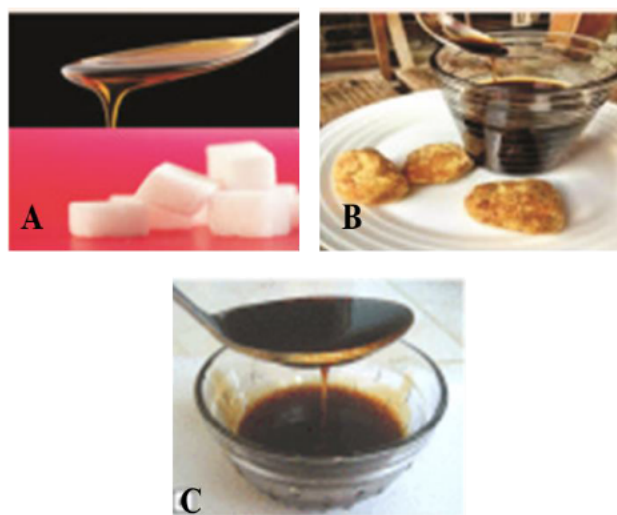


Figure 4:- A. Sugar Syrup; B. Jaggery Syrup; C. Molasses

FEATURES	MOLASSES	JAGGERY
Description	Thick, dark brown, uncrystallized juice obtained from raw sugar during the refining process.	If pure clarified sugarcane juice is boiled, what is left as solid is jaggery.
Total sucrose content	>46 %	65- 85 %
Water content	20 %	10- 12%
Usage	Livestock and poultry feeds, fertilizer, fuel	Traditional Indian sweetener
Availability	US, UK, Denmark, Russia, Africa, Asia	India, Pakistan, Mexico, South America, Burma, African countries, Srilanka, Thailand

Table 2 : Properties of Molasses and Jaggery^{15,16}

Since honey is not universally available and it is impractical to use honey in large scale due to its high cost, sugar and jaggery have started being chosen as a substitute for formalin (although very few in literature only a single study yet) as their composition is similar to that of honey. A study was done in 2013 by S. Patil to compare preservation properties of formalin with that of natural products like honey, sugar syrup and jaggery syrup, and found that out of the three natural products tissue fixation ability was good in jaggery syrup followed by honey and sugar syrup.³ So in our study we choose a concentration of 20% sugar and 30% jaggery syrup for tissue fixation comparable to formalin instead of using honey and found that both sugar syrup & jaggery syrup gave good results according to the histomorphological criteria, out of which jaggery syrup gave excellent results i.e. very close to formalin to fix the tissue. Our study too gave the similar results as the above mentioned study.

The possible mechanism of fixation by sugar & jaggery requires an acidic pH similar to that of honey. The pH ranged between 4.5 to 5.5 for the concentrations of sugar and jaggery that was used and was in favour of this concept (Table 4).

In our study all the two natural substances: sugar & jaggery gave promising results. But jaggery gave excellent results. Several advantages and drawbacks of using sugar & jaggery for tissue fixation

Advantages

1. They are non- hazardous, compatible with routine processing, staining and do not require additional equipments.
2. Jaggery, in addition is easily available and highly economical when compared to honey. It costs about

1/6th the price of honey. The natural substitutes can be used where formalin may not be available on time of biopsy and also in large scale, as in screening camps.

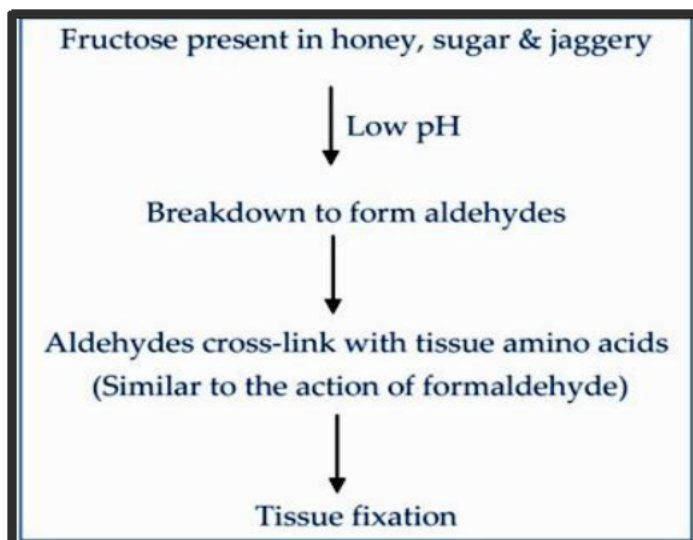


Table 4: The possible mechanism of fixation by Sugar and Jaggery

Drawbacks

1. It is advisable to use thymol crystals as an antimicrobial agent as these natural substances are liable to develop molds over time.
2. Jaggery fixed specimen showed brownish discoloration. (Figure 2) Although, there was no interference with subsequent staining.
3. Some of the problems faced with usage of these natural fixatives during tissue handling and their remedy are enlisted in Table 5

FIXATIVES PROBLEM	PROBLEM	REMEDY
<ul style="list-style-type: none"> • Sugar syrup • Jaggery syrup 	Breach in continuity of sections	<ul style="list-style-type: none"> • Re-impregnate the tissue for another hour • Use new blades • Handle the sections carefully
<ul style="list-style-type: none"> • Sugar syrup 	Intense staining with eosin	<ul style="list-style-type: none"> • Minimize the staining time with eosin
<ul style="list-style-type: none"> • Sugar syrup 	Folding of the tissue sections	<ul style="list-style-type: none"> • Difficult to avoid • Careful microtomy and floatation techniques

Table 5: Problems encountered with different fixatives and their remedies

Conclusion

Many approaches to fixation and type of fixatives have been developed and tested. Since 19th century formalin has taken over the field of fixation.^{1,2}

Natural substitutes like sugar and jaggery which are easily available with no known toxicity are a boon when health hazards of formalin are considered. Fixation of tissue by Sugar syrup and jaggery syrup is actually an innovative attempt. Among the two natural fixatives investigated, the jaggery syrup has really all novel qualities to be an excellent substitute for formalin in tissue fixation.

In our study we concluded that, when the preservation of tissue by sugar and jaggery was compared to that of formalin, jiggery syrup showed better preservative result over a period of 24 hours than sugar syrup. Thus, jaggery syrup is a better substitute for formalin. It is eco-friendly natural and healthy fixative. Natural fixatives are the arena which requires further exploration and large scale implementation.

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