

Natural substitutes for formalin: A boon to histopathology!!

Samatha Chittamsetti, Shilpa Nallamala, Taneeru Sravya, Venkateswara Rao Guttikonda, Praveen Kumar Manchikatla, Sudheerkanth Kondamari

Department of Oral and Maxillofacial Pathology, Mamata Dental College, Khammam, Telangana, India

Abstract

Introduction: In routine tissue processing, formalin has been proved as efficient as fixative since inception and hazards associated with it are of major safety and health concern.

Aim: The aim of this study is to compare the efficacy of natural fixatives such as jaggery and Khandsari over formalin.

Materials and Methods: Ninety normal tissue specimen collected during minor oral surgical procedures were included in this study. Thirty specimen each were fixed in 30% jaggery (Group-A), 30% Khandsari (Group-B) and 10% formalin (Group-C). The slides obtained after tissue processing were analyzed for the quality of fixation. The tissue sections were assessed for cellular outline, cytoplasmic details, nuclear details, staining quality and overall morphology. Each criterion was rated on a scale of 1–4. (one for poor and four for excellent). The study was double-blinded and subjected to the Kruskal–Wallis ANOVA and Chi-square test.

Results: The cellular outline is excellent in 90% (Group-C) followed by 36.67% (Group-B) of specimens. With respect to cytoplasmic staining 83.33% (Group-C) of tissues showed excellent results followed by 60% (Group-B), 33.33% (Group-A). Nuclear details are excellent in 86.67% (Group-C) followed by 83.33% (Group-B), 36.67% (Group-A) of specimens. With respect to staining quality 93.33% (Group-C) followed by 50% (Group-B), 26.67% (Group-A). Overall morphology is excellent in 90% (Group-C) followed by 46.67% (Group-B).

Conclusion: In the present study, Khandsari was on par with formalin and our effort of using Khandsari and jaggery for tissue fixation in human oral tissues yielded good results.

Keywords: Fixative, formalin, jaggery, khandsari

Address for correspondence: Dr. Samatha Chittamsetti, Postgraduate, Department of Oral and Maxillofacial Pathology, Mamata Dental College, Khammam, Telangana, India. E-mail: samathachittamsetti@gmail.com

Received: 28.12.2016, **Accepted:** 11.12.2017

INTRODUCTION

Fixation is an initial and essential step in histopathological processing. The fixative preserves the tissue from autolysis, maintains the macromolecular structures and also protects it from bacterial putrefaction. Although various types of fixatives have evolved over the years, formaldehyde in its 10% neutrally buffered form is the most common fixative

used in diagnostic pathology.^[1] Alexander M. Butlerov first discovered formaldehyde in 1859, but it was used as a tissue fixative by Ferdinand Blum.^[2]

Ever since the formalin-fixed tissue stained with hematoxylin and eosin (H and E) is considered as the “gold standard” due to its ease of availability and cost-effectiveness. In spite of these advantages, the health and safety risks are

Access this article online

Quick Response Code:



Website:

www.jomfp.in

DOI:

10.4103/jomfp.JOMFP_53_16

This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

For reprints contact: reprints@medknow.com

How to cite this article: Chittamsetti S, Nallamala S, Sravya T, Guttikonda VR, Manchikatla PK, Kondamari S. Natural substitutes for formalin: A boon to histopathology!! J Oral Maxillofac Pathol 2018;22:143.

of concern and the International Agency for Research on Cancer (IARC) emphasized it as Group-A carcinogen.^[2] Motivated by this, an expedition have been made to find safer alternatives.

The goal of the present study is to implement eco-friendly materials as alternative fixatives. The use of jaggery as tissue preservative^[3] has already been proved, but the use of khandsari is first of its kind, and there is no existing literature as per our knowledge. Hence, the present study was undertaken to evaluate and to compare the efficacy of jaggery and khandsari over formalin as fixatives.

Objectives

- To compare and evaluate efficiency of jaggery and formalin
- To compare and evaluate efficiency of khandsari and formalin
- To compare the efficiency of jaggery, khandsari over formalin as fixatives in hematoxylin and eosin (H and E).

MATERIALS AND METHODS

Ethical clearance was obtained from the Institutional Review Board and 90 tissue specimen were procured during minor oral surgical procedures, with 30 specimen assigned into three groups;

1. Group-A: 30 specimen fixed in 30% jaggery
2. Group-B: 30 specimen fixed in 30% khandsari
3. Group-C: 30 specimen fixed in 10% formalin.

All the specimen were fixed in their respective solutions for 24 h followed by conventional tissue processing and H and E staining. Double-blinded study was conducted and two interpreters independently assessed the quality of fixation from the obtained tissue sections for the histomorphological criteria.^[3] The tissue sections were assessed for cellular outline, cytoplasmic details, nuclear details, staining quality and overall morphology. Each criterion was rated on a scale of 1–4 (1-poor, 2-satisfactory, 3-good and 4-excellent). The obtained data were tabulated and subjected to the Kruskal–Wallis ANOVA and Chi-square test.

RESULTS

In Group-A, the poor cellular outline was noted in 26.67% of sections, satisfactory in 46.67% and good in 26.67% of specimen. With respect to cytoplasmic staining, 13.33% of sections showed satisfactory results, 53.33% good and 33.33% showed excellent results. With respect to nuclear details, 3.33% showed satisfactory, 60% good and 36.67% showed excellent results. With respect to staining quality,

13.33% of specimen showed satisfactory, 60% good and 26.67% showed excellent results. With respect to overall morphology, 33.33% showed satisfactory, 66.67% showed good results were obtained [Figure 1].

In Group-B, with respect to cellular outline, 3.33% of the specimen showed satisfactory results, 60% good and 36.67% showed excellent. With respect to cytoplasmic staining, 40% good, and 60% showed excellent results. With respect to nuclear details, 16.67% good and 83.33% excellent results were seen. With respect to staining quality, 6.67% specimen showed satisfactory, 43.33% good, 50% excellent results. With respect to overall morphology, 53.33% good, 46.67% excellent results were obtained [Figure 2].

In Group-C, with respect to cellular outline, 10% good and 90% showed excellent results. With respect to cytoplasmic staining, 16.67% good, and 83.33% showed excellent results. With respect to nuclear details, 13.33% good, 86.67% excellent results were seen. With respect to staining quality, 6.67% specimen showed good, 93.33% showed excellent results. With respect to overall morphology, 10% good, 90% excellent results were obtained [Figure 3]. Figure 4 illustrates comparison of high power images of jaggery, khandsari and formalin fixed tissues.

The mean and standard deviation with respect to all the histomorphologic criteria in three groups is depicted in Graph 1.

Pairwise comparison of all the parameters between groups was made using the Mann–Whitney test which revealed statistical significance ($P < 0.05$) between Group-A and Group-B, Group-A and Group-C and no statistical significance ($P > 0.05$) between Group-B and Group-C [Tables 1-5].

DISCUSSION

Formalin is a universal fixative and is routinely used as a preservative in museums since years. However, serious issues were raised due to its deleterious effects on different organ systems. However, the main motive for its popularity among pathologists is ease of availability, cost-effectiveness, straightforward laboratory preparation procedure and long-standing traditional usage.^[4] Eight hours of time-weighted average toxic levels of the formalin is 0.75 ppm and beyond which it is considered to cause harmful effects.^[5] The most common adverse effects associated with formalin exposure include irritation of mucosal membranes primarily respiratory epithelium

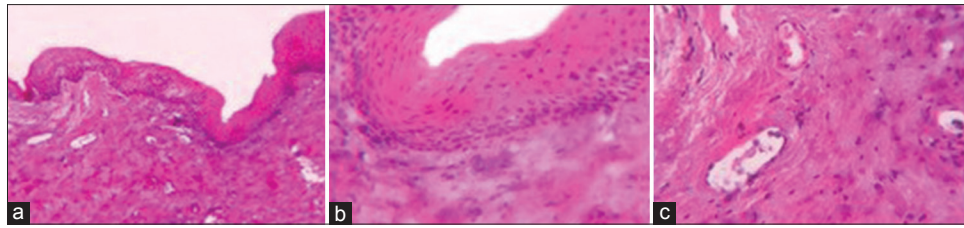


Figure 1: Photomicrograph illustrating (a) Low power magnification jaggery-fixed tissue (H&E, x10). (b and c) homogenization in lamina propria in high power magnification (x40)

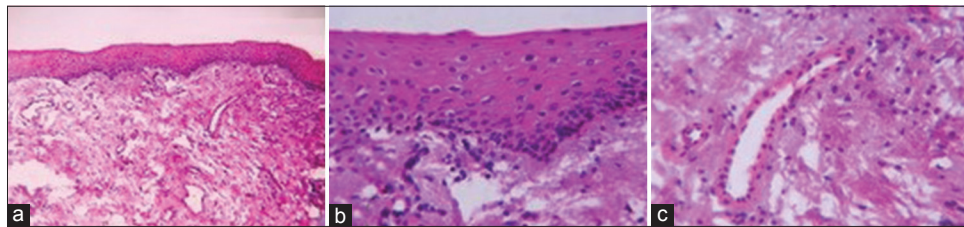


Figure 2: Photomicrograph illustrating (a) Low power magnification khandsari-fixed tissue (H&E, x10). (b and c) adequacy of nuclear and cytoplasmic staining in high power magnification (x40)

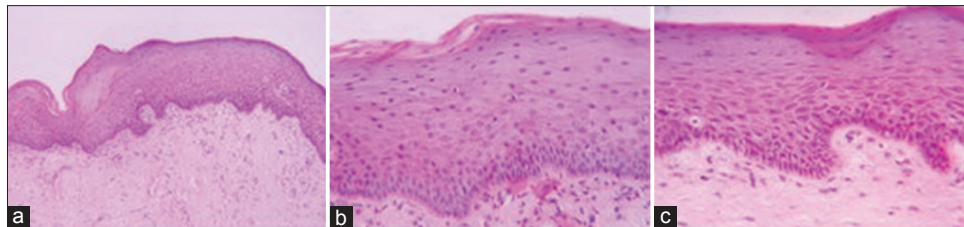


Figure 3: Photomicrograph illustrating (a) Low power magnification formalin-fixed tissue, (H&E, x10). (b and c) adequate details of all the parameters in high power magnification (x40)

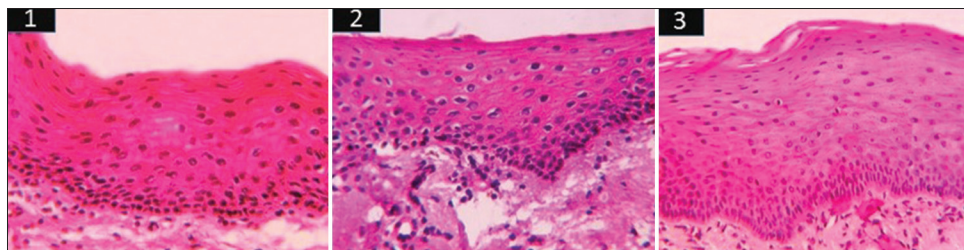


Figure 4: Photomicrograph illustrating high power image (x40) of jaggery-fixed tissue (1), khandsari-fixed tissue (2) and formalin-fixed tissue (3)

leading to cough, wheeze, chest pain, laryngospasm, pulmonary edema and short exposures to high levels of formaldehyde may result in sudden onset of asthmatic symptoms referred to as “Reactive airways dysfunction syndrome.” Other hazardous effects include eye irritation, conjunctivitis, ulcers in the gastrointestinal tract, abdominal pain, nausea, vomiting, diarrhea, neurotoxicity and acute renal failure.^[6] In the presence of water, formaldehyde reacts with hydrochloric acid to form bischloromethyl ether which is a known carcinogen for humans.^[7]

According to the IARC 2006, it causes nasopharyngeal cancer in humans and squamous cell carcinomas in the nasal respiratory epithelium of rats and mice.^[6]

A study by Lu *et al.* also found strong evidence that supports genotoxic and cytotoxic mode of action for the carcinogenesis of inhaled formaldehyde.^[7]

Due to these potential health hazards, an alternate fixative is recommended. Evidence-based studies using natural alternatives such as jaggery, honey, sugar syrup and molasses have proved to be as efficient as formalin.^[3] The present study is an initial attempt to use khandsari as natural fixative along with jaggery. The fixative properties of various solutions are depicted in Table 6.^[3,7,8,9,10,11]

The main motives for choosing these substitutes is due to their ease of availability, eco-friendly nature, nontoxicity,

Table 1: Comparison of three groups (A, B, C) with respect to cellular outline by Kruskal Wallis ANOVA and Pair wise comparison by Mann-Whitney U test

Groups	Mean	SD	Median
Group A	2.0	0.7	2.0
Group B	3.3	0.5	3.0
Group C	3.9	0.3	4.0
p-value		0.00001*	
Group A vs. group B		P=0.00001*	
Group A vs. group C		P=0.00001*	
Group B vs. group C		P=0.0004*	

*P<0.05

Table 2: Comparison of three groups (A, B, C) with respect to Cytoplasmic details by Kruskal Wallis ANOVA and Pair wise comparison by Mann-Whitney U test

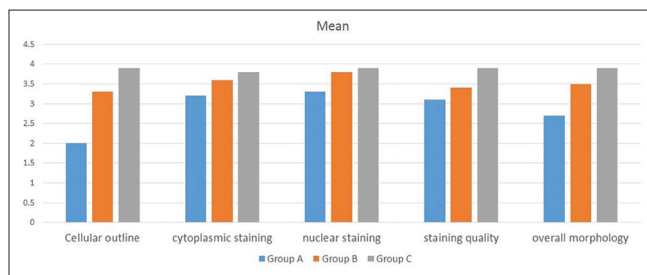
Groups	Mean	SD	Median
Group A	3.2	0.7	3.0
Group B	3.6	0.5	4.0
Group C	3.8	0.4	4.0
p-value		0.0002*	
Group A vs. group B		P=0.0333*	
Group A vs. group C		P=0.0005*	
Group B vs. group C		P=0.1206	

*P<0.05

Table 3: Comparison of three groups (A, B, C) with respect to nuclear details by Kruskal Wallis ANOVA and Pair wise comparison by Mann-Whitney U test

Groups	Mean	SD	Median
Group A	3.3	0.5	3.0
Group B	3.8	0.4	4.0
Group C	3.9	0.3	4.0
p-value		0.0001*	
Group A vs. group B		P=0.0017*	
Group A vs. group C		P=0.0008*	
Group B vs. group C		P=0.8245	

*P<0.05



Graph 1: Mean values of all the three groups

cost-effectiveness and minimum armamentarium required.^[3]

According to Patil *et al.*,^[3] the probable mechanism of tissue fixation by means of sugar substitutes can be explained as follows: in the presence of low pH, fructose present in jaggery lyse down to aldehydes which crosslink with the tissue amino acids resulting in fixation which is similar to that of formalin.

Table 4: Comparison of three groups (A, B, C) with respect to staining quality by Kruskal Wallis ANOVA and Pair wise comparison by Mann-Whitney U test

Groups	Mean	SD	Median
Group A	3.1	0.6	3.0
Group B	3.4	0.6	3.5
Group C	3.9	0.3	4.0
p-value		0.0001*	
Group A vs group B		P=0.0036*	
Group A vs group C		P=0.00001*	
Group B vs group C		P=0.0948	

*P<0.05

Table 5: Comparison of three groups (A, B, C) with respect to overall morphology by Kruskal Wallis ANOVA and Pair wise comparison by Mann-Whitney U test

Groups	Mean	SD	Median
Group A	2.7	0.5	3.0
Group B	3.5	0.5	3.0
Group C	3.9	0.3	4.0
p-value		0.0001*	
Group A vs group B		P=0.00001*	
Group A vs group C		P=0.00001*	
Group B vs group C		P=0.0937	

*P<0.05

Table 6: Properties of various solutions used as fixatives

Natural fixatives	Properties
Khandsari	It is a finely granulated crystalline form of sugar containing 94-98% of sucrose. It has the same constituents as that of jaggery but in lower quantity
Jaggery	It is the solidified product obtained after boiling of clarified sugarcane juice. Jaggery is available as three forms such as solid, liquid and granular. In the present study solid form was used. It has antitoxic, antioxidant, cytoprotective and anticarcinogenic properties. It has sucrose content of 65-85% and the optimal pH for fixation of tissue is 4.5-5.5
Molasses	It is a thick, dark brown, uncrystallized juice obtained from raw sugar during the refining process. It has sucrose content of >46%. Effective fixative at pH
Honey	It is usually produced from flowers and has antibacterial, dehydrative properties, promotes wound healing and prevents autolysis, putrefaction. Due to its acidic pH (3-4) it acts as a good nuclear fixative. Fixative properties are enhanced at low concentration as high concentration causes homogenization and hardening of tissue leading to difficulty of sectioning

Patil *et al.*^[3] conducted a study using buccal mucosa of fresh goat meat to analyse the efficiency of different fixatives such as 20% honey, 20% sugar syrup, 30% jaggery syrup with 10% formalin as a positive control and distilled water as a negative control and concluded that among all the three natural fixatives jaggery syrup excelled. These results were in contrast to our study where khandsari showed superiority over jaggery.

In 2015, Patil *et al.* conducted another study to evaluate the long-term efficiency of jaggery and honey over 6 months duration and compared the results using special stains such as Periodic acid-Schiff and Masson trichrome. All the

three fixatives showed similar results with jaggery being equivalent to formalin.^[9]

Maaini and Bryant conducted a study using honey as a fixative and stated that tissues which are fixed in low concentrations at room temperature yielded results comparable to that of formalin.^[10]

Studies have been conducted using animal mucosa previously, but the pioneer idea of research in human oral tissues was by Sabarinath *et al.* in 2014, where honey and formalin were compared. Results showed no significant difference in nuclear and cytoplasmic details and staining, but a limitation regarding the homogenization in the connective tissue was mentioned.^[11] In the present study, similar limitation was observed when jaggery was used.

Lalwani *et al.* compared the fixative properties of processed and unprocessed honey in oral tissues with formalin and found no statistically significant difference. The tissue morphology and staining adequacy for diagnosis in honey fixed tissue were at par with formalin-fixed tissue.^[7] Patil *et al.*, Rajanikanth *et al.* have insisted on the significance of natural alternatives as transit fixatives during mass screening procedures in case of unavailability of formalin.^[12]

In the present study Cellular outline is excellent in 90% of specimen (Group-C) followed by 36.67% (Group-B) of specimen. With respect to cytoplasmic staining 83.33% (Group-C) of tissues showed excellent results followed by 60% (Group-B), 33.33% (Group-A). Nuclear details are excellent in 86.67% of the specimen (Group-C) followed by 83.33% (Group-B), 36.67% (Group-A) of specimen. With respect to the staining quality 93.33% (Group-C) showed excellent results followed by 50% (Group-B), 26.67% (Group-A). Overall morphology is excellent in 90% (Group-C) followed by 46.67% (Group-B). On fixation with jaggery^[3] and khandsari, tissue folds and difficulty in preparing sections was observed. These were rectified by increasing the impregnation time for another 2 h. Histologically, homogenization of tissue was observed in jaggery-fixed tissues where differentiation among the structures was lost. The above-mentioned tissue artifact was not noticed with respect to khandsari and based on these findings it is considered to be superior over jaggery and is on par with formalin.

CONCLUSION

This pilot study yielded good results in fixing human oral tissues by khandsari and jaggery suggesting their usage as substitutes for the hazardous formalin. This study emphasizes the need to prevent the toxicity of formalin and elicit the novel qualities of natural fixatives to preserve tissues in life like manner. Further, studies with large sample size are recommended to obtain more definitive and conclusive results.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Bancroft JD, Gamble M. Theory and Practice of Histological Techniques. 6th ed. Edinburgh: Churchill Livingstone; 2007.
2. Dimenstein IB. A pragmatic approach to formalin safety in anatomical pathology. *Lab Med* 2009;40:740-6.
3. Patil S, Premalatha B, Rao RS, Ganavi B. Revelation in the field of tissue preservation – A preliminary study on natural formalin substitutes. *J Int Oral Health* 2013;5:31-8.
4. Zanini C, Gerbaudo E, Ercole E, Vendramin A, Forni M. Evaluation of two commercial and three home-made fixatives for the substitution of formalin: A formaldehyde-free laboratory is possible. *Environ Health* 2012;11:59.
5. Buesa RJ. Histology safety: Now and then. *Ann Diagn Pathol* 2007;11:334-9.
6. Lu K, Collins LB, Ru H, Bermudez E, Swenberg JA. Distribution of DNA adducts caused by inhaled formaldehyde is consistent with induction of nasal carcinoma but not leukemia. *Toxicol Sci* 2010;116:441-51.
7. Lalwani V, Surekha R, Vanishree M, Koneru A, Hunasgi S, Ravikumar S, *et al.* Honey as an alternative fixative for oral tissue: An evaluation of processed and unprocessed honey. *J Oral Maxillofac Pathol* 2015;19:342-7.
8. Rao PV, Das M, Das SK. Jaggery – A traditional Indian sweetener. *Indian J Tradit Knowl* 2007;6:95-102.
9. Patil S, Rao RS, Ganavi BS, Majumdar B. Natural sweeteners as fixatives in histopathology: A longitudinal study. *J Nat Sci Biol Med* 2015;6:67-70.
10. Maaini RA, Bryant P. The effectiveness of honey as a substitute for formalin in the histological fixation of tissue. *J Histotechnol* 2006;29:173-6.
11. Sabarinath B, Sivapathasundharam B, Sathyakumar M. Fixative properties of honey in comparison with formalin. *J Histotechnol* 2014;37:21-5.
12. Rajanikanth M, Ravi Prakash A, Sreenath G, Sonia Bai JK, Ndivn S. Transit fixatives: An innovative study. *J Clin Diagn Res* 2015;9:ZM01-3.