Bee Honey as a Safer Alternative for Routine Formalin Fixation

Srii R,[1] Peter CD,[2] Haragannavar VC,[3] Shashidara R,[4] Sridhara SU,[4] Srivatsava S[5]

[1]Department Oral Pathology and Microbiology, Kathmandu University School of Medical Sciences, Dhulikhel, Kavre, Nepal.

[2]Department of Oral Pathology and Microbiology, Educare Institute of Dental Sciences, Malappuram, Kerala, India.

[3]Department of Oral Pathology and Microbiology, M S Ramaiah Dental college, Bangalore, Karnataka, India.

[4]Department of Oral Pathology and Microbiology, Coorg Institute of Dental Sciences, Virajpet, Karnataka, India.

[5]Department of Public Health Dentistry MGM Dental College and Hospital, Mumbai, India.

Corresponding Author

Ritesh Srii

Department of Oral Pathology and Microbiology,

Kathmandu University School of Medical Sciences,

Dhulikhel, Kavre, Nepal.

E-mail: ritesh3741@gmail.com

Citation

Srii R, Peter CD, Haragannavar VC, Shashidara R, Sridhara SU, Srivatsava S. Bee Honey as a Safer Alternative for Routine Formalin Fixation. *Kathmandu Univ Med J.* 2017;60(4):308-12.

ABSTRACT

Background

Formaldehyde (10% buffered formalin) is still in use as the gold standard fixative in the field of biology however, as reported by Occupational Safety and Health Administration (OSHA) the use of formalin causes health hazards due to its toxicity. Hence, we considered to substitute formalin with natural Bee-Honey to achieve a formalin free laboratory for preservation of the biological specimens.

Objective

To assess the efficacy of honey as a fixative agent for the preservation of the tissue specimens and to study their cellular and structural characteristics by using routine stains, special stains and Immunohistochemistry (IHC) and compare its effectiveness with the currently, universally accepted formalin fixation.

Method

Our study contained sample size of 10 tissue specimens. All samples were fixed in two different solutions one in honey and other in conventionally used formalin solution for 24 hrs in room temperature and then were routinely processed, sectioned and stained using routine, special stains and with immuno-histochemical markers. The slides were viewed by two independent examiners and the entire procedure was blind folded.

Result

We obtained good comparable results with bee honey for Hematoxylin and Eosin, special stains including immunohistochemistry when compared to formalin fixed tissues.

Conclusion

Based on the observations of this study, it can be suggested that natural bee honey could be a safer alternative to formalin as a fixative, considering the health hazards of formalin.

KEY WORDS

Fixation, Formalin, Honey

INTRODUCTION

Fixation of the biological specimen in their natural state remains an at most priority for all histological and cytological evaluation. This process involves a series of chemical events which prevents the cells and tissues from decay, thereby preventing autolysis.[1,2]

Fixation alters the tissue at a molecular level, resulting in preservation of structure, and also renders the tissue hard which helps in ease for sectioning and staining.[3,4]

Significant changes hasn't occurred in the field of tissue fixation over the past century. Formaldehyde, (10% buffered formalin), still remains the gold standard fixative used in diagnostic laboratories for both routine histopathology and immunohistochemistry. It is easily available, easy to use and applicable across a wide range of tissues, economical, internationally accepted and the preparation requires less time. Conversely, formalin is considered harmful due to its toxicity which affects the health professionals, and has gained the attention of two agencies like U.S. Environmental Protection Agency 1987 (EPA) and the International Agency for Research on Cancer (IARC) who have classified formalin as a probable human carcinogen.[5]

Attempts have been made to find non-toxic, safer alternatives, by substituting formaldehyde with less risky substances. For years bee honey has been proved to exhibit antibacterial properties and potential to preserve compounds without causing any harmful effects on its users. In ancient Rome, honey was used to preserve meat for several days.[6-8]

Thus, considering these properties of honey, we aimed to use honey as a fixative for the preservation of the tissue specimens to study their cellular characteristics by using various stains and compare its efficacy with the currently used formalin.

METHODS

The study was conducted in the Department of Pathology, Dhulikhel Hospital, Dhulikhel, Kavre, Nepal and Department of Oral Pathology, Coorg Institute of Dental Sciences, Virajpet, Karnataka, India. The study was done between November 2016 to May 2017.

Our study consisted 10 tissue specimens biopsied from gingiva (tissues were obtained from patient undergoing gingival surgeries for aesthetic purposes). Ethical approval was obtained from the institutional review committee prior conducting the study. Immediately after obtaining the biopsy samples, the tissue specimens were cut into two halves, each half placed in two separate groups, group A (working solution) - where the tissue specimens were fixed with diluted form of honey and group B - where tissue specimens were fixed using 10% formalin.

Commercially available Agmark graded pure Coorg bee honey was used in this study as shown in figure 1. Coorg honey is obtained from apiaries where 4 species of honey bee namely Apisdorsata, A. ceranaindica, A. florea and Apismellifera are kept. Fresh working solution was prepared just before the biopsy procedure. Working solution was prepared by using honey and water in the ratio of 1:9.

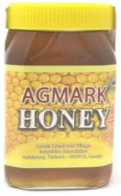

Figure 1. Agmark honey.

Tissue specimens were immersed in both the solutions (Group A and Group B) for 24 hours at 25-27° C. After the tissues were fixed it was taken for routine tissue processing and paraffin embedded blocks were prepared.

The tissues were cut at 5 µm thickness and stained using haematoxylin and eosin (H & E), as well as with specials stains like Masson's trichrome and Van Gieson. Immunohistochemistry for the markers vimentin and pan cytokeratin was also done. The stained tissues were examined by two oral pathologists under compound light microscope (Olympus CX-22) and the procedure was blinded. The histomorphological criteria examined are enlisted in Table 1. Inter-observer variability was determined using Kappa statistics. Further data analysis was done using Statistical Package for Social Sciences (SPSS) software (version 20.0).

Table 1. The histomorphological criteria and evaluation are detailed below.

Histomorphological criteria	Rate on a scale of 1-4
For H&E stain	
Cellular outline	Poor Satisfactory Good Excellent
Nuclear detail	
Staining quality	
Overall morphology	
Specificity of the stain	
Staining intensity	
For Special Stain (MT & VG)	
Specificity of the stain	Poor Satisfactory Good Excellent
Staining intensity	
For IHC	
Specificity of the stain	Poor Satisfactory Good Excellent
Staining intensity	

H&E- Hematoxylin & Eosin
MT- Masson's Trichrome
VG- Van Gieson
IHC- Immuno histochemistry
Scores:
Poor- 1
Satisfactory- 2
Good- 3
Excellent- 4

Preparation of working solution:

Ten percent of concentrated honey- 10 ml of pure concentrated form of honey was mixed with 100 ml of hot water. This prepared solution was then allowed to cool and pH was maintained at 4.5-5.0 and the tissues were immediately immersed in it following removal.[9]

RESULTS

Two independent examiners observed the obtained biopsied samples under compound light microscope and the procedure was blinded. Kappa statistics was used to compare the inter-observer variability and a value of 0.839 suggested a high agreement between the observers hence, the scores given by the first observer were considered.

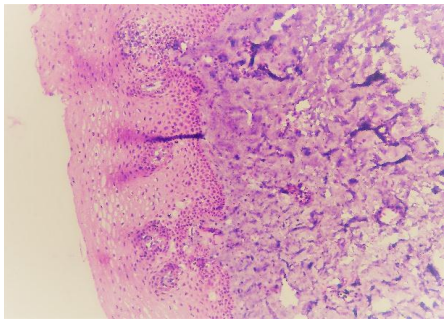

Figure 2. Photomicrograph of H & E stained tissue sections fixed in honey at magnification of 10x showing good cellular morphology with proper nuclear and cytoplasmic details

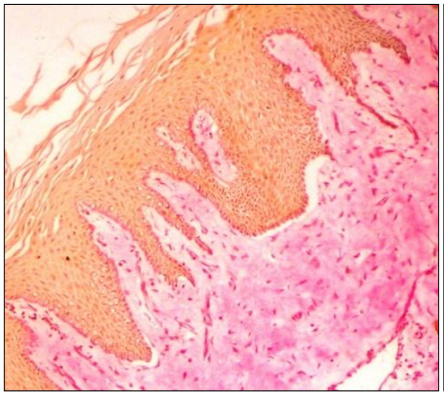

Figure 3. Photomicrograph of tissue sections fixed in honey, stained with Van Gieson stain.

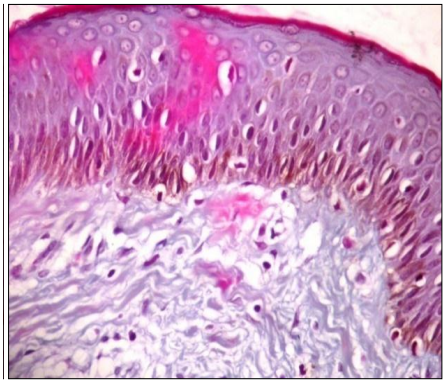

Figure 4. Photomicrograph of tissue sections fixed in honey, stained with Masson's trichrome.

Comparable results were obtained with the tissues fixed in bee honey, maintaining the nuclear and cellular structures, for both haematoxylin and eosin staining as well as for special stains including IHC. The general stain uptake and the preservation of tissue architecture including the nuclear and cytoplasm sizes, was comparable to that of the formalin fixed tissues. Honey fixed tissues exhibited a more hyalinised appearance of the collagen fibres in both H & E staining and special stains when compared to that of formalin as shown in Fig. 2 to 4. Since all the sections were from gingival tissues, staining of muscle fibres could not be evaluated. Immuno-histochemical staining with Pancytokeratin and Vimentin also showed comparable results with tissues fixed in formalin as shown in Fig. 5 and 6. The mean values of the scores given for each parameter were compared using the Independent 't' test and Chi square test; and there were no significant differences ($p<0.05$ considered to be significant) noted for all the variables.

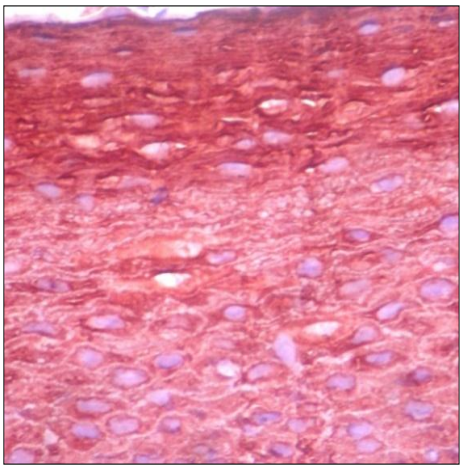

Figure 5. Photomicrograph of tissue sections fixed in honey stained with Pan cytokeratin.

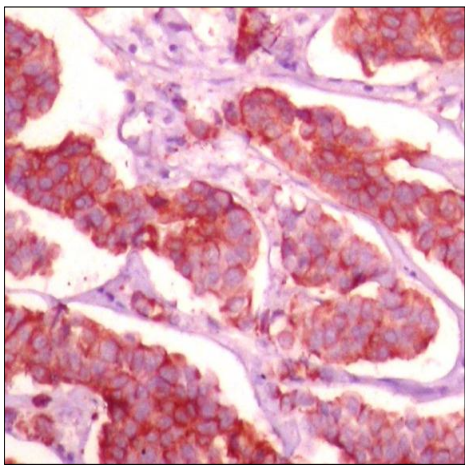

Figure 6. Photomicrograph of tissue sections fixed in honey stained with Vimentin.

Table 2. **Independent 't' test for comparing the scores between honey and formalin for H & E stain.**

		Honey	Formalin	Total	P- value
Cellular Outline	Good	2	0	2	
	Excellent	8	10	18	0.474
	Total	10	10	20	
Nuclear Details	Good	4	2	6	
	Excellent	6	8	14	0.628
	Total	10	10	20	
Staining Quality	Good	2	1	3	
	Excellent	8	9	17	1.000
	Total	10	10	20	
Overall Morphology	Good	3	2	5	
	Excellent	7	8	15	1.000
	Total	10	10	20	
Stain Specificity	Good	3	2	5	
	Excellent	7	8	15	1.000
	Total	10	10	20	
Staining Intensity	Good	2	1	3	
	Excellent	8	9	17	1.000
	Total	10	10	20	

Table 3. **CHI square test for comparing the scores between honey and formalin for Masson's Trichrome stain.**

		Honey	Formalin	Total	P value
Stain Specificity	Satisfactory	1	0	1	0.451
	Good	6	5	11	
	Excellent	3	5	8	
	Total	10	10	20	
Staining Intensity		Honey	Formalin	Total	P value
	Good	5	4	9	0.451
	Excellent	5	6	11	
	Total	10	10	20	

DISCUSSION

Since more than a millennium honey has been used as a medicine for its antibacterial and healing properties. The components in honey like, hydrogen peroxide, low water content and inhibine formed by enzymatic reaction and phytochemical factors are chiefly responsible for its antibacterial and antioxidative properties that have been high lightened to inhibit the growth of a wide range of bacteria, fungi, protozoa and viruses.[8,10,11]

Honey has also been found to prevent decaying of tissues immersed in it for even up to 30 days without showing any signs of autolysis, hence the ancient Roman people used honey to preserve meats for a longer duration of time. Also, human mummy confection was a legendary medicinal substance created by steeping a human cadaver in honey. Honey also renders tissue hard, making its action similar to other fixatives which acts by cross linking of proteins.[12,13]

Table 4. **CHI square test for comparing the scores between honey and formalin for Vangieson stain.**

		Honey	Formalin	Total	P value
Stain Specificity	Good	3	3	6	
	Excellent	7	7	14	1.000
	Total	10	10	20	
		Honey	Formalin	Total	P value
Staining Intensity	Satisfactory	1	0	1	
	Good	4	2	6	
	Excellent	5	8	13	0.307
	Total	10	10	20	

Table 5. **CHI square test for comparing the scores between honey and formalin for Pancytokeratin marker.**

		Honey	Formalin	Total	P value
Stain Specificity	Good	3	3	6	
	Excellent	7	7	14	1.000
	Total	10	10	20	
		Honey	Formalin	Total	P value
Staining Intensity	Good	2	2	4	
	Excellent	8	8	16	1.000
	Total	10	10	20	

Table 6. **CHI square test for comparing the scores between honey and formalin for Vimentin marker.**

		Honey	Formalin	Total	P value
Stain Specificity	Good	3	3	6	
	Excellent	7	7	14	1.000
	Total	10	10	20	

The tissue fixation in honey at low concentrations in the ratio 1:10 at room temperature with pH maintained at 4.5-5.0 gave comparable results to the tissues fixed in formalin.

Dilution of honey results in oxidation of glucose to gluconic acid and hydrogen peroxide in the presence of glucose oxidase enzyme. Conversion of carbohydrates in honey to gluconic acid is thought to be a plausible mechanism for its fixation properties. Gluconic acid is an organic acid which is mildly acidic, non- corrosive, less irritating, non-odorous, nontoxic, easily biodegradable, and non-volatile acid. Gluconic acid is also widely used as a preservative in food and pharmaceutical industry as it prevents the putrefaction of the food and exhibit antiseptic properties.[14]

Another hypothesis is that, glucose/ fructose at low pH levels in honey is broken down to form aldehydes results in cross-linking with amino acids present in the tissue (a reaction like that of formaldehyde) resulting in fixation of tissues. This mechanism is somewhat similar to the Hepes-glutamic acid buffer-mediated organic solvent protection effect (HOPE) technique. HOPE solution is hyperosmolar mixture comprising of amino acids adjusted at pH 5.8 to 6.4 which works like an immersion fixative.[14-16]

In our study, satisfactory results were obtained with no statistically significant differences observed with tissue fixed in honey, in comparison with the formalin fixed tissue and, the staining duration for special stains were less compared to the formalin fixed tissue. Honey contains various artefacts in it, including viable spores such as clostridia which may result in false positive reactions. In the present study Agmark certified pure Coorg honey was used, which did not result in any such artefacts.

The use of formaldehyde is almost banned in the developed countries due to its potential toxicity, also formalin has been classified as a probable human carcinogen as reported by two known agencies like U.S. EPA and IARC. In developing countries, aspiration devices are rarely used and a safe disposal method of toxic waste may be inexistent, hence when combined with the known health hazards the use of formaldehyde should be terminated. Considering the above facts, it is now time to think for a natural substitute like bee honey, which is available easily, easy to prepare, eco-friendly and non-toxic with action similar to that of formalin and giving comparable results thus aiding in the extermination of formalin in near future.[17]

Shortcomings of this study being, few sample size and biopsy specimens involving only gingival tissues. Extensive research, involving a larger sample size and specimens involving other tissues from the oral cavity, would be better to compare the efficacy of honey with the routinely used formalin and eradicate the use of formalin in the near future. As of now this study highlights the properties of honey and encourages its use as a substitute to formalin.

CONCLUSION

For centuries formalin has taken over the field of diagnostic histopathology as a fixative. Now it's an ideal time for a natural substitute like bee honey, which can be a boon to the pathologists and the technicians working in the diagnostic histopathology laboratories when health hazards of formalin are considered. Considering the novel qualities of bee honey it could be concluded that at low concentrations it could help to decrease and abolish the use of formalin in the near future.

REFERENCES

1. Kumar GL, Kieman JA, editors. Fixation and tissue processing. Dako technologies. Carpinteria, California: 2010. 141p.
2. Bancroft JD. Theory and Practice of Histological Techniques. Edinburg, Scotland: Churchill Livingstone; 2001. 63p.
3. Wood MF, Vurgun N, Wallenburg MA, Vitkin IA. Effects of formalin fixation on tissue optical polarization properties. *Phys Med Biol*. 2011; 56(8):115-22.
4. Bosetti C, McLaughlin JK, Tarone RE, Pira E, La Vecchia C. Formaldehyde and cancer risk: a quantitative review of cohort studies through 2006. *Ann Oncol.* 2007; 19(1): 29-43.
5. Zanini C, Gerbaudo E, Ercole E, Vendramin A, Forni M. Evaluation of two commercial and three homemade fixatives for the substitution of formalin: a formaldehyde–free laboratory is possible. *Environmental Health.* 2012; 11:59.
6. Mariani-Costantini R, Awadelkarim KD, Barberis M, Clemente C, de Blasio P, de Gioacchino M, et al. Building Sustainable Capacity for Disease Diagnosis in Sub-Saharan Africa: Case Studies of Cooperation in Diagnostic Pathology. In New Knowledge in a New Era of Globalization. *Im Tech.* 2011; 243–66.
7. Kwakman HSP and Zaat SAJ. Critical Review Antibacterial Components of Honey. *IUBMB Life.* 2012; 64(1): 48–55.
8. Rahma A, Bryant P. The Effectiveness of Honey as a Substitute for Formalin in the Histological Fixation of Tissue. *The Journal of Histotechnology.* 2006; 29(3): 173-6.
9. Lalwani V, Surekha R, Vanishree M, Koneru A, Hunasgi S, Ravikumar S. Honey as an alternative fixative for oral tissue: An evaluation of processed and unprocessed honey. *J Oral Maxillofac Pathol.* 2015; 19(3): 342–7.
10. White JW, Subers MH, Schepartz AI. The identification of inhibine, the antibacterial factor in honey, as hydrogen peroxide and its origin in a honey glucose oxidase system. *Biochem Biophys Acta.* 1963;73:57-70.
11. Molan PC. The antibacterial activity of honey. The nature of the antibacterial activity. *Bee world.* 1992; 73:15-28.
12. Molan PC: A brief review of the use of honey as a clinical dressing. *Austr J Wound Manage.* 1998; 6:148-58.
13. Ramachandran S, Fontanille P, Pandey A, Larroche C. Gluconic Acid. Gluconic Acid: A Review, Food Technol. *Biotechnol.* 2006;44(2)185–95.
14. Mandy G, Philip B. Immunohistochemical evaluation of ductal carcinoma in breast after preservation in honey. *The J Histotechnol.* 2009; 32(2): 54-9.
15. Srinivasan M, Sedmak D, and Jewell S. Effect of Fixatives and Tissue Processing on the Content and Integrity of Nucleic Acids. *American Journal of Pathology.* 2002; 161(6).
16. Vollmer E, Galle J, Lang DS, Loeschke S, Schultz H, Goldmann T. The HOPE technique opens up a multitude of new possibilities in pathology. *Romanian J of Morphology and Embryology.* 2006; 47(1): 15-9.
17. U.S. Environmental Protection Agency, Office of Air and Radiation. Report to Congress on Indoor Air Quality, Volume II Chapter 4: Assessment and Control of Indoor Air Pollution. 1989;12–27.