



Title: Qualitative Assessment Of Processed Honey In Comparison With Formalin As An Alternative Fixative

Subtitle : Honey as a fixative auxiliary to formalin

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Abstract: Fixation is an initial and important step in tissue processing which preserves the tissues in a life-like state, prevents bacterial putrefaction, prevents autolysis, and increases the refractive index of the tissue. This study has been done to evaluate the fixation ability of honey in oral tissues and to compare the fixative efficacy of honey and formalin. The study group comprised of 21 tissue samples. Each tissue was cut into 2 segments and one was immediately fixed in 10% Formalin and the other segment in Processed Honey (in the ratio of 70:30- honey and distilled water), for 24 hours at room temperature. The sections were assessed for cytoplasmic, nuclear details & staining quality under light microscope and evaluated accordingly by two observers. Results were analysed by Chi-square test. Inter-observer variability was determined by Kappa statistics. When all the stained sections were assessed for the parameters, there was no statistically significance difference between tissues fixed in processed honey compared to formalin ($P > 0.05$). Kappa score is 0.755, it falls within the "substantial agreement" range, indicating that the observers have a high level of agreement. Our results suggest that processed honey can be used as a safe alternative for formalin. It can be successfully adopted in routine histopathology laboratories in place of formalin. Hence honey can be considered as an equally effective formalin substitute.

Key words - Formalin, Processed honey, Cellular outline, Cytoplasmic preservation, Blood capillary preservation.

INTRODUCTION:

The basic purpose of fixation, which forms the basis of pathology, is to stop tissue autolysis and deterioration so that sections of the tissue can be examined both anatomically and microscopically after

sectioning¹. Several fixatives have been around for decades or even more, but formaldehyde has been used for, over a century, while others were developed within the last ten years. Formalin has been considered as the gold standard in tissue fixation after its discovery in 1859². Ferdinand Blum was the first person to use formaldehyde as a tissue fixative³. The primary aim of fixation is to preserve the tissues in a life-like state, prevent bacterial putrefaction, autolysis, and increase the refractive index of the tissue¹.

The International Agency for Research on Cancer (IARC) classified formaldehyde as “carcinogenic to humans” and therefore represents a risk to anyone handling the solution⁴. It was found that formaldehyde inhalation at 6 ppm and above causes nasal squamous cell carcinoma in rats⁵. In some studies, it has been found that there are genotoxic and cytotoxic modes of action for the carcinogenesis of inhaled formaldehyde in respiratory nasal epithelium⁶. Sometimes the chemical action of formalin binds severely to DNA, RNA, and proteins which makes them difficult to extract in a useful form for molecular tests. The side effects of formalin harm skin and mucous membranes, respiratory system, gastrointestinal tract, cardiovascular system, central nervous system, and eye. Irritation, nausea, vomiting, diarrhoea, loss of appetite, burns and ulceration, abdominal pain, gastrointestinal haemorrhage, pharyngeal congestion, and chronic pharyngitis are a few signs and symptoms of formalin vapours⁶.

With the increasing concerns about the potential carcinogenicity of Formaldehyde, an attempt to find safer alternatives is necessary. Bee honey has been shown to preserve tissue morphology similar to that of Formalin. It also possesses Antimicrobial, Antiviral, and Antimutagenic properties. Many studies have stated that honey possesses the property of dehydration⁷ and preservation.

In line with the Codex Alimentarius, "Honey is the naturally occurring sweet substance produced by honeybees from plant nectar, plant secretions, or plant-sucking insect excretions on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store, and leave in honeycombs to ripen and mature."⁸ Honey is a concoction of several substances and sugars. It contains tiny amounts of several compounds including chrysin, pinobanksin, vitamin C, catalase, and pinocembrin⁹. The various properties of honey include antioxidant, antimicrobial, and anti-autolytic effects. It can stop autolysis, putrefaction and can penetrate the deepest tissue. It has been discovered that honey inhibits autolysis since tissues fixed in it for up to 30 days did not exhibit any putrefaction or autolysis. The tissue hardening property makes it similar in action to fixatives which act by hardening the tissues.

A search for a formalin substitute started since formalin is known to cause cancer and does not guarantee full recovery of messenger RNA (mRNA) or DNA. It has been demonstrated that bee honey preserves tissue morphology that resembles formalin. Sugar and jaggery may also be able to protect tissues because their composition is closest to that of honey¹⁰. Therefore, the rationale of this study is to know if honey has any fixative properties in comparison with Neutral Buffer Formalin.

The study aims to evaluate the efficacy of natural fixative honey compared to the standard fixative-formalin.

MATERIALS& METHODS:

This is an invitro experimental study carried out in the Department of Oral & Maxillofacial Pathology and Oral Microbiology. Ethical clearance for this study was obtained (134/ IEC/ XXX/2023) from the Institutional Ethics Committee.

Patients with pulp polyp, gingival polyp, and gingival enlargement were chosen as the study subjects and the tissues with inadequate size were excluded. The study group comprised of cases reported during July 2023- Sept 2023 (n = 21). These tissues include oral epithelium, gingival polyp, and pulp polyp that were taken from the Department of Oral Surgery and Periodontology. Each tissue was cut into two segments and was immediately fixed in a bottle a bottle containing 10% NBF (Group A), and containing 70% honey (Group B) for 24 h at room temperature.

Preparation of fixatives: Two groups were taken where group A is 10% neutral buffered formalin (pH 7.2-7.4) and group B is 70% honey (pH: 3.6) which is made by mixing 70ml of honey with 30ml of distilled water.¹¹

After fixation, tissues were processed using the routine standard processing protocol; followed by Haematoxylin and Eosin staining (H and E).

All the stained slides were blinded (labeled only with a number) and were evaluated independently by two observers and data was entered into data sheets for statistical analysis.

Histomorphology Criteria¹¹;

CRITERIA	VALUE
Nuclear Detail	Good – 1 Bad – 2
Cytoplasmic Detail	
Tissue Preservation	
Cellular Outline	
Staining Quality	
Blood Capillary Preservation	

RESULTS:

The values obtained were compiled and analyzed using the Chi-square test and Kappa statistics. The association between the observer's data was measured using the Chi-Square test and the interobserver reliability was measured using Kappa statistics. $P < 0.001$ & was considered statistically significant. The average values of the examiners were tabulated. (Table 1)

Chi-square test was performed between both the observers for the tissues fixed in honey and formalin, the p value for cellular outline is (0.00), tissue preservation is (0.00), cytoplasmic detail is (0.01), nuclear detail is (0.09), staining quality is (0.00), blood capillary preservation is (0.00). There was no significant

difference in cellular outline and staining quality, nuclear and cytoplasmic detail among the two fixatives. When the blood capillaries were evaluated, there was no significant difference between tissues fixed in formalin and honey. [Table 1]

There were no noticeable differences in sectioning, formation of ribbons, and floating on the water bath in both the groups.

When the interobserver variability was measured using kappa statistics,

$$\text{Kappa Score} = (0.950 - 0.796) / (1 - 0.796) = 0.755$$

It falls within the "SUBSTANTIAL AGREEMENT" range, indicating that the observers have a high level of agreement beyond what would be expected by chance.

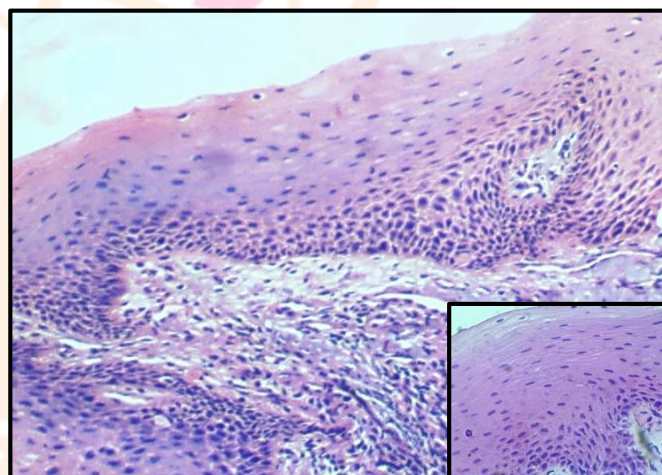
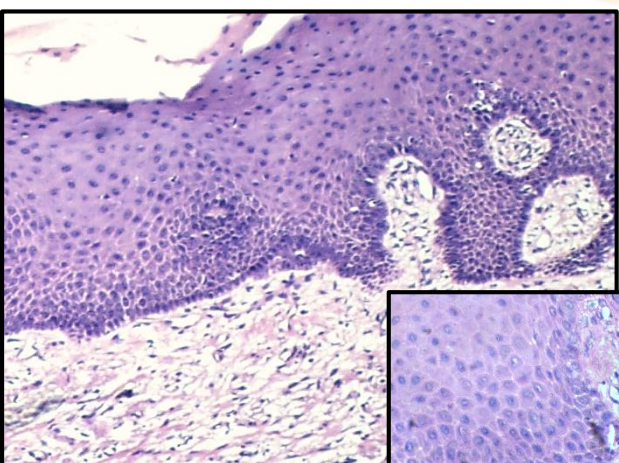


Figure 1: 10x and 40x view of the photomicrograph showing epithelium fixed in formalin.

Figure 2: 10x and 40x view of the photomicrograph showing epithelium fixed in honey.

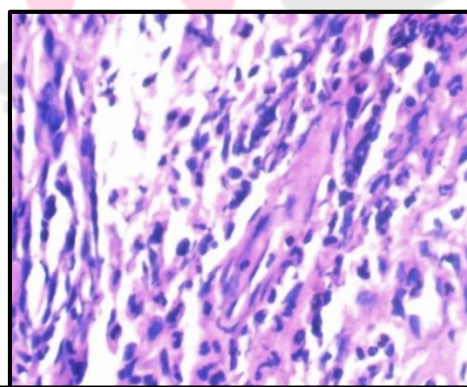
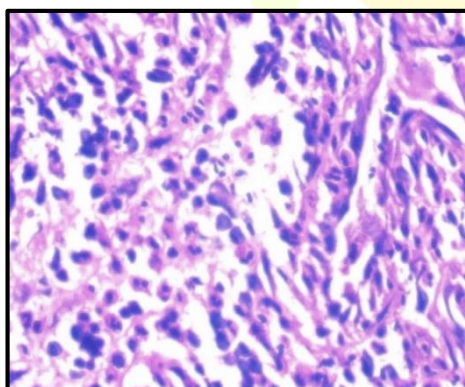


Figure 3: 40x view of the photomicrograph showing connective tissue fixed in formalin.

Figure 4: 40x view of the photomicrograph showing connective tissue fixed in honey.

DISCUSSION:

Fixation is an initial and important step in tissue processing for the microscopical examination of tissues. The main goals of fixation are to maintain the life-like quality of the tissues, prevent bacterial putrefaction and autolysis, and raise the refractive index.

Many approaches to fixation and types of fixatives have been developed and tested throughout the years. Formalin has been established as a fixative for routine surgical pathology, and most clinicians are aware of its pungent odour.

Though formalin is a well-established fixative, it has been shown to produce deleterious effects on humans. The well-known disadvantages of formalin are highly toxic and formaldehyde reacts with hydrochloric acid in the presence of water to form bis-chloromethyl ether, which is a known carcinogen for humans.

Fixatives without formaldehyde are available and have generated interest recently because of their lesser likelihood of affecting health. The naturally occurring sweet material known as honey is made by honeybees from flower nectar.

Modern medicine ignored the health benefits of honey until the advent of multi-resistant bacteria, which led to a rediscovery of the substance's antibacterial, wound-healing, and tissue-preserving properties. Many different types of bacteria, fungi, protozoa, and viruses have been demonstrated to be inhibited in growth by honey.

The osmotic impact (high sugar and low water content), acidity, hydrogen peroxide produced by an enzyme reaction, and phytochemical components all influence the antibacterial properties of honey. The majority of raw honey releases hydrogen peroxide when it is diluted gradually. This is because the enzyme glucose oxidase is activated, converting glucose to gluconic acid and hydrogen peroxide.

The presence of acid accounts for honey's low pH (between 3 and 4). Usually, the fixatives that contain acids or have low pH do not favour the preservation of cytoplasmic constituents; however, they act as good nuclear fixatives⁶.

In the present study, cellular outline and quality of staining of honey-fixed tissues were similar to that of formalin-fixed tissues. **Lalwani v et al (2015)**; also confirmed that tissue morphology and staining adequacy for diagnosis in honey-fixed tissues were similar to formalin-fixed tissues.¹¹

Current study states that tissues were well preserved in the honey, although few tissue sections fixed in honey showed compromised architecture. Studies by **Al-Maaini et al (2008)** and his coworkers showed better staining of connective tissue with honey fixation⁷. This is due to aldehyde hydroxymethyl furfural that forms cross-links with amino acids, similar to formalin.

Nuclear detail in the tissue sections fixed in both formalin and honey shows no significant difference. **Sabarinath B et al (2014)**¹² and **Chittemsetti S et al (2018)**¹³ stated that the nuclear details of the tissues

fixed with formalin and honey were similar with no difference in staining as well as in the microscopical morphology.

The nuclear detail and cytoplasmic detail were preserved in our study so that the tissues can be used for immunohistochemical staining, **Al-Maaini et al (2008)⁷** and **Sabarinath B et al (2014)¹²** used markers for detecting antigens to demonstrate the cytoplasmic and nuclear details. The result of immunostaining was positive, demonstrating the optimal dilution of honey.

In rural areas where screening programs, medical camps were conducted, doctors generally see large number of patients. Patients with suspicious lesions are advised for immediate biopsy. In situations where formalin is not readily available, the biopsied tissues will be discarded or get damaged if left out for drying which poses difficulty in diagnosis. In such cases natural substitutes like honey which are readily available can be used. Over time, the natural substances are liable to develop molds; so, thymol crystals can be used as an antimicrobial agent.

CONCLUSION:

Thus, the above discussion concludes that honey, which is easily available with no known toxicity, can be used as an alternative to formalin. Natural fixative- Honey can be used in place of the hazardous formalin with equal competence. We conclude that natural fixatives that are beneficial to the environment possess all the unique characteristics needed to substitute formalin. To get more definite conclusions, more research with larger sample sizes is needed.

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Table 1: This table shows the comparison of Histomorphometric criteria between the observers.

	Formalin		Honey		P value
	Observer 1	Observer 2	Observer 1	Observer 2	
Cellular outline					
Good	21	20	21	13	0.00
poor	0	1	0	8	
Tissue preservation					
Good	21	16	12	6	0.00
poor	0	5	9	15	
Cytoplasmic detail					
Good	21	19	12	13	0.01
Poor	0	2	9	8	
Nuclear detail					
Good	21	20	17	20	0.09
poor	0	1	4	1	
Staining quality					
Good	19	20	12	18	0.00
poor	2	1	9	3	
Blood capillary preservation					
Good	21	19	12	13	0.00
poor	0	2	9	8	

Chi-square test, *P<0.05=significant