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Standardization of honey as a tissue fixative for histopathology: A morphometric study

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ABSTRACT

Background: Tissue fixation is a crucial step to preserve the tissues in a life-like state with minimal disruption to its cellular and chemical composition for histopathological examination. The search for an effective alternate tissue fixative to the routinely used formaldehyde has gained interest as constant exposure to formaldehyde has proven to be toxic. Honey, an organic substance with high acidity and hygroscopic nature, exhibits tissue fixative properties and has been used in the present study. The present study aimed to standardize honey as a tissue fixative for histopathology by comparing it with formalin.

Materials and Methods: *In vitro* study Oral tissue samples of goat were fixed in 10% honey and 10% formalin solution, respectively, for 24–48 h, followed by routine tissue processing and microscopic examination of 37 slides per group. 2200 epithelial cells (1100 per group) were selected for the computer-aided morphometric image analysis (Fiji-Image J) by three observers. Cell area (CA), cell perimeter (CP), nuclear area (NA), nuclear perimeter (NP), cytoplasmic area (Cyt A), and nuclear-cytoplasmic ratio were the parameters studied. Mann–Whitney *U*-test (STATA/IC version 16) for inter-group comparison was done and $P < 0.05$ was considered statistically significant.

Results: The probability of epithelial cells in the honey-fixed group to have greater NA, NP, and N/C ratio was about 50%–60%. The probability of epithelial cells in formalin-fixed tissues to have greater CA, CP, and Cyt A was about 70%.

Conclusion: Honey is a better nuclear fixative than formalin. Cytoplasmic shrinkage of epithelial cells should be taken into consideration while fixing tissues with honey.

Key Words: Epithelial cells, fixative, formalin, honey, tissue fixation

INTRODUCTION

Fixation of biopsy tissues is a crucial step before tissue processing for histopathological examinations. Tissue fixation helps to preserve the tissue in a life-like state, prevent autolysis and putrefaction, alter the refractive index, and provide mechanical rigidity to maintain its integrity during consecutive stages of processing.[1] Ideally, fixation should maintain clear and consistent morphological features of the tissue with minimal disruption to its cellular-extra cellular relationships and chemical composition after staining for microscopic examination.[2]

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Since the late 19th century, formalin has been the fixative of choice following the accidental discovery by Ferdinand Blum, that a diluted solution of formaldehyde not only "fixed" tissues but also provided excellent staining with hematoxylin and aniline dyes.[3] Formaldehyde acts by the formation of methylene bridges between the amino acids of the protein molecules present in the tissues, thereby forming crosslinks.[2] The routine fixative (neutral-buffered formalin of pH 7.2–7.4), which is a 10% solution of formalin (approximately 4% formaldehyde solution), is readily available, easy to use, cost-effective, and provides adequate fixation for good quality histology. However, it was classified as a carcinogen by the U.S Environmental Protection Agency when there was prolonged or high exposure, with a permissible level of 0.75 ppm average daily exposure.[4] Formaldehyde was classified as a Class 1 human carcinogen with the potential to cause different neoplasms including nasopharyngeal carcinoma by the International Agency for Research on Cancer (IARC).[5] Hence, the search for safe alternative fixatives has gained interest as pathologists and laboratory technicians are at a high risk of toxicity from constant exposure to formalin. Recent literature shows many studies exploring the possibility of using natural eco-friendly substances such as honey and jaggery to fix tissues.[6-8]

Honey is an organic, naturally sweet substance produced by honeybees by the collection of plant nectar which consists of a mixture of sugars and other compounds such as lysozymes, vitamins, minerals, trace elements, acids, hydrogen peroxide, etc.[9] Literature shows that honey exhibits antibacterial properties due to high acidity and hygroscopic nature and also anti-autolytic and tissue hardening properties thus satisfying the requirements to be a fixative.[9] The staining properties and tissue morphology of histological sections of tissues fixed in honey have been found to be at par with formalin.[8,10]

Many studies are continuously being done to prove the efficiency of honey as a good fixative for cytology and histology. However, the standardization has not been done so far. The present study is aimed to standardize honey as a tissue fixative for histopathology. Cell shrinkage, an inevitable effect of fixation, is an important characteristic when choosing a fixative so that the histomorphometric patterns can be reproduced.

MATERIALS AND METHODS

In vitro study was conducted in the Department of Oral Pathology and Microbiology at a Private Dental College in Chennai, India.

Sample size calculation

The sample size per group was estimated as 29 after setting α error as 5% and the power of the study as 90%.

Tissue preparation

The samples for the *in vitro* study were oral tissues of goat (buccal mucosa), which were remnants from the meat sold commercially for the human consumption at the butcher's shop. The tissues were collected at the time of slaughter of the animal, which was done for the purpose of sourcing meat for the consumption. The fresh samples of 1 cm × 1 cm × 1 cm dimension were fixed straightaway by immersing them in containers with 10% honey and 10% formalin solution (Oxford laboratory, Thane, India), respectively. Ten percentage honey was prepared by mixing 10 mL honey with 90 mL of distilled water. Store-bought processed honey (Dabur honey, Dabur India Limited, Solan, India) was used in this study as unfiltered honey may contain various artifacts and viable spores like clostridia causing false-positive reactions.[9] The tissues were fixed in the respective solutions for 24 h at the room temperature and were subjected to routine processing. The adequately fixed tissues were kept under running water for 10 min to remove the fixative from the tissues. Tissue dehydration was done subsequently by immersing the tissues in ascending concentration grades (70%, 90%, and 100%) of isopropanol (EMPLURA, Merck Life Science Private Limited, Mumbai, India) for 30 min each. Removal of alcohol from the tissues was done by immersing them in the clearing agent xylene (Merck Life Science Private Limited, Mumbai, India) for 1–2 h. Molten paraffin wax (Merck Life Science Private Limited, Mumbai, India) in a wax bath (54–55°C melting point) was used to impregnate the tissues by keeping them in it for a few hours to remove xylene. The wax-infiltrated tissues were then embedded in paraffin wax with the help of L-shaped metal mounts. 4 μm sections were cut from the tissue blocks with a rotary microtome (Leica RM 2155, Ambala Cantt, India). The tissue sections were mounted on glass slides for viewing in a light microscope. Routine staining with hematoxylin (Nice Chemicals Pvt. Ltd, Kochi, India) and eosin (Thermo

Fisher Scientific India Pvt Ltd, Mumbai, India) was done [Figures 1 and 2].

Image analysis

A total of 40 slides were made for the formalin-fixed (F) and honey-fixed (H) groups, respectively. Three slides from each group were excluded based on improper staining. Hence each group consisted of 37 slides. Five fields per slide were examined under ×40 using Accu-Scope EXC-350 with an attached photo micrographic unit [Figure 2]. Six cells per field (30 cells per slide) were chosen for the evaluation. The captured images were transferred to a computer for the morphometric analysis using an open-source image analysis software called Fiji-(Fiji is just) Image J 2.3.051; Java 1.8.0_172 (64 bit) [Figure 3]. A total of 2200 cells with each group containing 1100 cells, respectively, were analyzed using three observers separately. Cell area (CA), cell perimeter (CP), nuclear area (NA), nuclear perimeter (NP), cytoplasmic area (Cyt A), and nuclear-cytoplasmic ratio (N/C) were the parameters included.

Statistical analysis

The statistical analysis was performed using STATA/IC version 16.1 Statistical Software (STATA Corp., College Station, Texas, USA). The morphometric data were analyzed statistically by computing descriptive statistics, i.e., mean, standard deviation, median (P50), and interquartile range (IQR = P25–P75). Shapiro–Wilk test was used to test for the normality of the distribution for the different morphometric variables (CA, CP, NA, NP, Cyt A, and N/C ratio). Based on the results of the Shapiro–Wilk test, the hypothesis for the data to be normally distributed was rejected ($P < 0.0001$) for all the morphometric variables [Table 1]. Intergroup comparison was performed between the honey-fixed and formalin-fixed groups for the different morphometric variables (CA, CP, NA, NP, Cyt A, and N/C ratio) using the Mann–Whitney *U*-test. The results were considered to be statistically significant when the $P < 0.05$. Inter-rater agreement was calculated for all the study variables using the percent agreement and Cohen's kappa coefficient.

RESULTS

The descriptive statistics, i.e., mean, standard deviation, median (P50), and IQR = P25–P75 computed for the different morphometric variables (CA, CP, NA, NP, Cyt A, N/C) for both the honey-fixed and formalin-fixed groups are represented in Tables 2 and 3.

The intergroup comparison for the different morphometric variables was performed using Mann–Whitney *U*-test which showed that there was

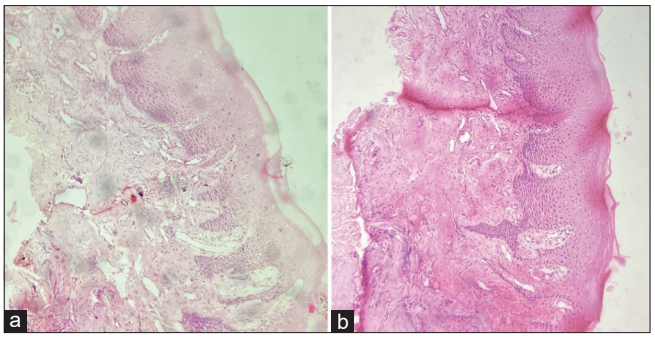

Figure 1: H and E stained histological section of tissue as viewed in x10 light microscope – (a) tissue fixed in formalin and (b) tissue fixed in honey.

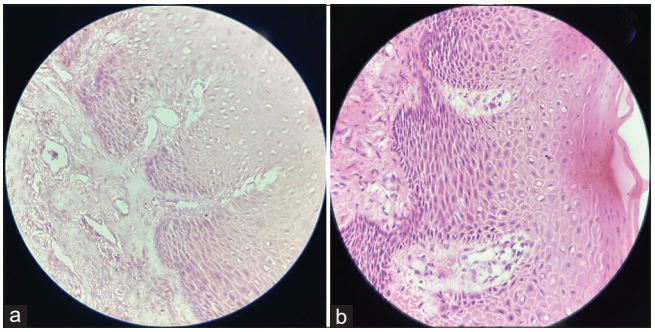

Figure 2: H and E stained histological section of the tissue as viewed in x40 light microscope – (a) tissue fixed in formalin and (b) tissue fixed in honey.

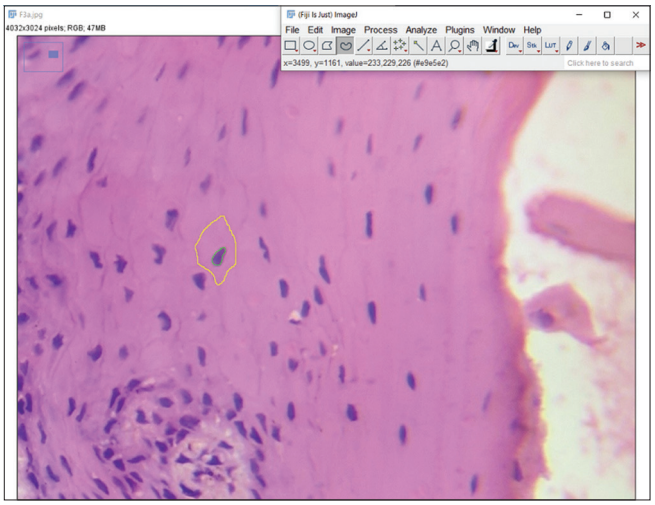

Figure 3: Cell perimeter (yellow line) and nuclear perimeter (green line) marked for measurement in the image analysis software Fiji - (Fiji is just) Image J.

a statistically high significant difference between the groups for the variables: CA ($P < 0.0001$), CP ($P < 0.0001$), NP ($P = 0.0006$), Cyt A ($P < 0.0001$) and N/C ratio ($P < 0.0001$).

Table 1: Shapiro–Wilk's test to check for normality (cell area, cell perimeter, nuclear area, nuclear perimeter, cytoplasmic area, and nuclear cytoplasmic ratio)

Variable	OBS	*W*	*V*	*Z*	*P>Z*
Shapiro–Wilk *W*-test for normal data (honey group)					
CA	1110	0.96436	24.722	7.977	0.00000
CP	1110	0.94544	37.843	9.036	0.00000
NA	1110	0.89670	71.651	10.624	0.00000
NP	1110	0.97215	19.317	7.364	0.00000
CYT A	1110	0.98555	10.021	5.731	0.00000
N/C	1110	0.89055	75.915	10.767	0.00000
Shapiro–Wilk *W*-test for normal data (formalin group)					
CA	1110	0.98595	9.745	5.662	0.00000
CP	1110	0.98112	13.097	6.397	0.00000
NA	1110	0.99076	6.408	4.619	0.00000
NP	1110	0.53139	325.029	14.384	0.00000
CYT A	1110	0.97506	17.299	7.089	0.00000
N/C	1110	0.96863	21.757	7.660	0.00000

CA: Cell area; CP: Cell perimeter; NA: Nuclear area; NP: Nuclear perimeter; CYT A: cytoplasmic area; N/C: Nuclear cytoplasmic ratio

Table 2: Descriptive statistics (cell area, cell perimeter, nuclear area, nuclear perimeter, cytoplasmic area, and nuclear cytoplasmic ratio)

Variables	Fixative honey (mean±SD)	Fixative formalin (mean±SD)
CA	119.7017±16.60088	137.1844±14.61799
CP	218.9394±37.91455	247.8596±27.7726
NA	35.95131±7.838882	36.18755±6.431545
NP	65.35214±12.00889	65.63518±23.92248
CYT A	83.75043±15.12484	100.9968±15.59636
N/C	0.4471249±0.1419194	0.3717045±0.1058719

CA: Cell area; CP: Cell perimeter; NA: Nuclear area; NP: Nuclear perimeter; CYT A: Cytoplasmic area; N/C: Nuclear cytoplasmic ratio; SD: Standard deviation

The probability for the honey-fixed group to have higher values than the formalin-fixed group as elucidated using the Mann–Whitney *U*-test was found to be 0.515 for NA, 0.542 for NP and 0.684 for N/C ratio.

The probability for the formalin-fixed group to have higher values than the honey-fixed group as elucidated using the Mann–Whitney *U*-test was found to be 0.777 for CA, 0.716 for CP and 0.782 for Cyt A.

The data distribution pertaining to the different morphometric variables (CA, CP, NA, NP, Cyt A, and N/C) for the honey-fixed and the formalin-fixed groups are depicted using box and whisker plot [Figure 4].

The inter-rater agreement for the variables CP, NA, and NP was strong and for CA and Cyt A was weak.

DISCUSSION

The growing concern regarding the biohazards associated with regular formalin usage in the laboratories has fueled the search for alternate fixatives that can effectively replace formalin. Literature shows that exogenous formaldehyde exposure may result in irritation in the eyes and upper respiratory tract. The IARC, based on comprehensive results from extensive human and animal studies, concluded that formaldehyde caused nasopharyngeal cancer, leukemia and was positively associated with sinonasal cancer.[11] It is cytotoxic, genotoxic and can cause mutagenic changes and DNA damage.[12] Earlier studies have shown that the formation of monoadducts, DNA-DNA crosslinks, DNA-protein crosslinks while binding to DNA has been related to the genotoxic effects of formaldehyde.[13] Formalin being a cross-linking fixative binds to amino acids and nucleotides by the formation of methylene bridges and blocks the

Table 3: Hypothesis testing for the comparison of variables (cell area, cell perimeter, nuclear area, nuclear perimeter, cytoplasmic area, and nuclear cytoplasmic ratio) between fixative honey and fixative formalin using the Mann–Whitney *U*-test

Variables	Fixative honey (median/IQR)	Fixative formalin (median/IQR)	*P*	Probability (variables [group-fixative honey] > variables [group-fixative formalin])
CA	120.918 (105.477–132.143)	138.089 (128.916–148.279)	0.0000**	0.223
CP	220.769 (184.301–249.834)	249.076 (232.969–268.842)	0.0000**	0.284
NA	37.243 (31.592–41.327)	37.197 (31.592–39.745)	0.2090	0.515
NP	68.172 (55.415–75.017)	64.176 (54.985–71.756)	0.0006**	0.542
CYT A	84.184 (70.826–94.898)	102.929 (91.719–114.139)	0.0000**	0.218
N/C	0.4316474 (0.3699428–0.4910215)	0.3660712 (0.290479–0.4365536)	0.0000**	0.684

*$P<0.05$ statistically significant, **$P<0.005$ statistically highly significant. IQR: Interquartile range; CA: Cell area; CP: Cell perimeter; NA: Nuclear area; NP: Nuclear perimeter; CYT A: Cytoplasmic area; N/C: Nuclear cytoplasmic ratio

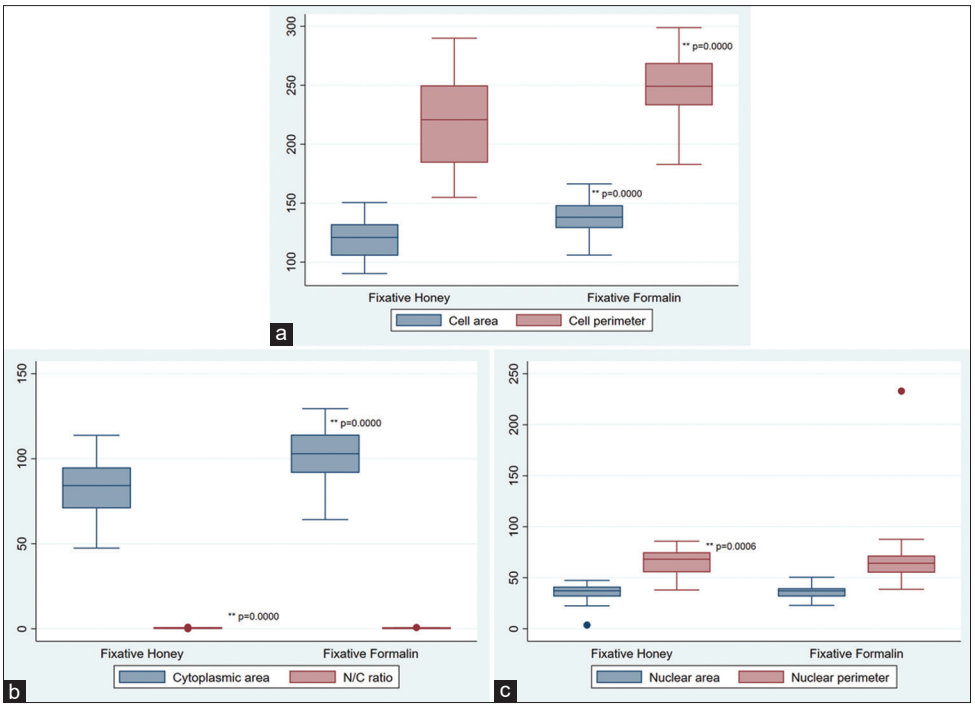

Figure 4: Box and Whisker plot for data distribution of, (a) Cell area and cell perimeter for honey and formalin fixatives, (b) Cytoplasmic area and N/C ratio for honey and formalin fixatives, (c) nuclear area and nuclear perimeter for honey and formalin fixatives.

retrieval of nucleic acids and also degrades the material making them unsuitable for further molecular studies. Similarly, it has deleterious effects on the resolution of proteins in the tissues making it difficult for immunohistochemistry.[14]

Honey, an organic product, has been used for centuries for mummification of human cadavers and also to preserve meat because of its high acidity, antibacterial, and hygroscopic properties.[10] In addition, honey also exhibits anti-autolysis and tissue-hardening properties.[9] Taking these properties into consideration, honey is being used in various studies to see if it is an effective alternative to formalin as a tissue fixative. A pilot study was conducted earlier in the department to compare the fixative properties of honey and formalin qualitatively.[15] The present study was conducted as an attempt to standardize honey as a tissue fixative using the morphometric analysis.

In the present study, the tissues were adequately fixed in formalin as well as processed honey. Processed honey was preferred over the unprocessed one as unprocessed honey has been found to show increased fixation artifacts in tissues as compared to processed honey. Furthermore, greater slit like spaces were also evident in the basement membrane due to epithelial shrinkage with unprocessed honey.[9]

Morphometric analysis done in the present study enabled in the identification of dimensional changes in the epithelial cells when fixed with honey. The probability of epithelial cells fixed with formalin to have greater CA, CP, and Cyt A was 0.777 (77.1%), 0.716 (71.6%), and 0.782 (78.2%), respectively, when compared to those tissues fixed with honey. This was suggestive of shrinkage corresponding to cytoplasm in the tissues fixed in honey. Similarly, the probability of the epithelial cells tending to have greater NA, NP and N/C ratio was 0.515 (51.5%), 0.542 (54.2%), and 0.682 (68.2%), respectively, for tissues fixed in honey. Since the morphometric method was not used for comparison in the pilot study done earlier, only qualitative analysis was possible, and no change was observed visually in the epithelium.[15] The result suggests that honey is a better nuclear fixative than formalin which is in accordance with the pilot study that showed honey is a good nuclear fixative.[15] The acidic component of honey provides for its low pH and usually fixatives with low pH act as good nuclear fixatives as they do not favor preservation of cytoplasmic constituents.[9] Hence, the probability of shrinkage of epithelium during the fixation of tissues in honey should always be taken into account.

The concentration of honey should also be considered while using it as a tissue fixative. The results of the previous studies were based only on qualitative comparison between honey and formalin fixed tissues. Literature has shown that honey at different concentrations modified the various aspects of fixation. At higher concentrations (70%–100%), honey was suitable for long-term gross preservation with moderate tissue morphology and at lower concentrations (20%–50%), it exhibited excellent staining characteristics.[16] When 10% honey was used as a fixative, both the stain uptake and preservation of tissue architecture were at par with tissues fixed in formalin though collagen fibers in the connective tissue gave a hyalinized appearance.[14,17]

A study by Patil *et al*. showed that though tissues fixed in 20% honey over 6-month duration relatively retained their gross morphology, there was a gradual reduction in the cellular and nuclear clarity. Furthermore, evident cellular and nuclear shrinkage was observed in honey-fixed tissues in comparison to formalin on microscopic examination.[18] In the present study, some amount of shrinkage was evident in the cytoplasm of epithelial cells of honey-fixed tissues (24 h) on morphometric analysis. However, the extent of shrinkage in honey over the long periods of fixation is yet to be analyzed. The dimensional changes in the connective tissue were not analyzed in this study due to homogenization observed in honey fixed tissues. However, it is safe to state that honey would effectively satisfy all the properties of a fixative if there were methods to eliminate homogenization.

Although formalin fixation has some undermining effects on the extraction of nucleic acids and antigen retrieval for immunohistochemical staining, it is still being used.[14] Earlier studies have shown that tissues fixed in honey are suitable for IHC as both cytoplasmic and nuclear antigen expressions were effectively demonstrated.[15,19,20] Similarly, although honey has shown effective nuclear fixation, retrieval of nuclear material for downstream applications and molecular studies from tissues fixed in honey requires further analysis.

CONCLUSION

This study has shown that honey is a better nuclear fixative than formalin. The probability of epithelial cells to have greater NA, NP, and N/C ratio is about 50%–60% in honey-fixed tissues. While, the probability of epithelial cells in formalin-fixed tissues tending to have greater CA, CP, and Cyt A is about 70%. Hence, researchers should be mindful of the cytoplasmic shrinkage of epithelial cells in honey-fixed tissues when compared to formalin fixation. By including tissues of other animal species and human tissues with varying concentrations of honey, the study may be expanded in future. This would provide further standardization of honey as a fixative.

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Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or non-financial in this article.

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