Honey and Olive Oil as Bio-Friendly Substitutes For Formalin and Xylene in Routine Histopathology

Abstract

Background: Formalin has long been the standard fixative and xylene has been the clearing agent for routine histopathology and immunohistochemistry worldwide. In recent years, as a result of increasing concerns about the potential carcinogenicity of formaldehyde and xylene, attempts have been made to find safer alternatives. In the present study, we considered honey as better alternative for formalin and olive oil as safer substitute for xylene. Aims: The aim of this study was to know whether honey could be a possible substitute for formalin and olive oil could be a possible substitute for xylene. Materials and Methods: Thirty routine biopsy tissues of 1–2 cm were taken. The study group was divided into Group A and Group B. Group A were subjected to normal processing. Group B were fixed into honey for 24 h after which it was taken through routine processing, and then immersed in olive oil instead of xylene. All the sections will be stained with routine hematoxylin and eosin staining. Compare the sections of both the methods. Results and Conclusion: The preservation of tissue by honey giving superior result when compared to that of formalin. Olive oil was found to be effective clearing agent compared to xylene.

Keywords: Eosin, hematoxylin, honey, olive oil

Introduction

Fixation is an initial and important step in tissue processing for microscopical examination. The primary aim of fixation is to preserve the tissues in a life-like state, prevent bacterial putrefaction, prevent autolysis, and increase the refractive index of the tissue. Formalin is traditionally a popular and widely used fixative for histopathology processing of tissues due to its ease of use, economic viability, fairly fast fixation. Although formalin is the gold standard fixative in routine histopathology, a search for its alternate substance is explored, primarily due to its adverse effects on health. Recently, the potential carcinogenicity of formaldehyde has been emphasized. Repeated exposure or prolonged inhalation of formaldehyde in occupational settings is a causative irritant of the mucous membrane of eyes, nose, mouth, and upper respiratory tract, which has potential health hazards.[1] Non-formalin fixatives do not contain an aldehyde component thereby, avoiding any potential toxic effect. Currently, natural substitutes have better scope due to their desirable effects. Honey has documented antibacterial, acidic, and dehydrative properties. Furthermore, one of the studies highlighted the anti-autolysis as well as tissue hardening property of honey, apart from its wound healing and antibacterial nature. The above properties are part of the requirements of a fixative that present honey in terms of a fixative rather than a preservative.[2]

Following fixation, clearing is an important step in the preparation of histological sections, aiming to remove alcohol and other dehydrants from tissues before infiltration of the embedding material (usually paraffin wax). In the past dozens of years, xylene with excellent compatibility to alcohol and paraffin wax has been widely used as a clearing agent.[3] A large number of animal studies have demonstrated that being excessively exposed to xylene can cause toxicity to multiple tissues such as the nervous system, liver, skin, and lungs. The cell toxicity of xylene has been linked to the induction of mitochondrial uncoupling and oxidative stress. Considering the serious adverse effects of xylene, many attempts have been made to replace this agent with safer alternatives.[4]

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In recent years, numerous xylene substitutes have been commercially developed, among which are aromatic derivatives of terpene and others hydrocarbons. Since their costs and effectiveness are still unsatisfactory, it makes the development of more novel, more effective, and safer substitutes increasingly necessary. Vegetable oils such as olive oil have been documented to have potentials of being used as clearing agents. In general, these essential oils are used widely as natural flavor additives for food as fragrances in perfume and in medicine and alternative medicines such as aromatherapy. To overcome toxic effects of formalin and xylene, the present study, which is the first of its kind, used naturally available substances such as honey and olive oil to substitute for formalin and xylene, respectively.

Materials and Methods

Sample selection

Specimens for this study were routine biopsy specimens. The inclusion criteria were as follows: only soft tissue was considered for this study. The specimen size was 0.5 cm × 1 cm or greater and a thickness of 3–5 mm was taken for processing (for better penetration of the processing fluids).

A total of thirty fresh soft tissue specimens were included in the present study. Each specimen was cut into two equal halves (sixty samples) [Figure 1]. All the samples were divided into two groups: Group A and Group B, each group containing thirty samples. Group A specimens were fixed in formalin for 24 h at room temperature, followed by conventional processing with xylene and staining with hematoxylin and eosin.

Group B specimens were fixed in honey (diluted with distilled water to 1:10) for 24 h at room temperature and then dehydrated in alcohol. Dehydrated tissues were dealcoholized (cleared) using two changes of olive oil for 1 h each. The cleared tissues were infiltrated in two changes of molten paraffin wax for 1 h and 1 h 30 min, respectively. Embedding was done in molten paraffin wax and allowed to solidify before microtomy. All the tissue blocks were sectioned at four micrometers with a rotary microtome; sections were floated in a warm water bath and each section was picked in pairs on albuminized glass slides. Before staining, the sections were dewaxed in olive oil for 5 min, hydrated in 95% alcohol dip, and were stained with hematoxylin and eosin to permit evaluation of histological details.

The tissue bits of both the study Groups A and B were measured before and after fixation to check for shrinkage. After dealcoholization, the specimens were also tested for gross changes after clearing.

Few of the sections from Group B were subjected to periodic acid–Schiff (PAS) and van Gieson’s stain to see whether honey or olive oil was interfering in this routinely used special staining procedure. To check if any consistent change existed between the study groups, the sophisticated technique of computer-assisted morphometry was performed to all the study groups to observe the morphological features such as cell and nuclear perimeters.

Evaluation

Gross tissue specimen

The tissue bits were measured just after fixation, to compare the gross-shrinkage for the two groups. After clearing in two different solvents, the gross tissue features such as translucency (surface translucency when viewed for reflected light), rigidity (palpation with two fingers), change after impregnation (change in the rigidity because of infiltration of wax), and ease in section cutting were noted down for each specimen separately. Scoring was done while comparing the parameters for both the study groups:

- Score 0 - The finding of Group B that was inferior to Group A
- Score 1 - The finding of Group B similar to Group A
- Score 2 - The finding of Group B that was superior to Group A.

Cellular architecture

- For cellular details, distinct architecture and good nuclear-cytoplasmic contrast are considered as score 1 and indistinct/blurred nuclear-cytoplasmic contrast as score 0.
- For nuclear details, distinct chromatin condensation, prominent nuclear membrane, and crisp staining of the nucleus are considered as score 1 and indistinct smudging and pyknosis of the nuclei as score 0.

Quality of staining

The staining of tissues was evaluated as poor, satisfactory, and good.
a. Score 0 (poor) - that the tissue failed to take up the stain adequately, stained unevenly
b. Score 1 (satisfactory) - pointed toward details but not visualized up to the mark
c. Score 2 (good) - designated good contrast between the nucleus and cytoplasm and visibility.

Morphometric analysis

After reviewing, the sections were further subjected to cytomorphic analysis using image analyzer software-Pro insight 8.0 version (Mediacybernetics) to overcome the intra- and inter-observer variability. The images were captured using a three-chip charge coupled device camera attached to a trinocular research microscope with a ×40 objective. The final image captured on the monitor had a magnification of ×400. For each specimen, three most representative fields were selected. The selected fields included representative cells where distinct cellular and nuclear outlines were seen, avoiding overlapping. A total of thirty cells (ten cells in three different fields) were randomly selected and measured for any difference in the Group B specimens and Group A specimens. Histologically identifiable epithelial cells in the parabasal layer along with acini and adipocytes were also subjected to cytomorphic analysis. The cellular and nuclear perimeters were observed in square microns. The obtained data were subjected to statistical analysis.

Results

Gross shrinkage after fixation

There were no significant changes in shrinkage observed in both the study groups [Figure 2].

Gross features after clearing

Most of the Group B specimens (67%) were less rigid and few were (33%) at par with Group A when compared. Translucency was visibly better in all Group B specimens (100%) when compared with Group A. However, there was no difference observed in the tissue bits as far as rigidity after impregnation and ease of sectioning was concerned, in both the study groups [Table 1].

Cellular architecture and staining quality

There was no difference in cellular architecture in both the study groups [Figure 3]. Quality of staining was almost same in both the study groups except for few sections in Group B (17%) which were superior than those of Group A [Table 2].

| Sample                        | Score 0                                                                 | Score 1                                      | Score 2                     |
|-------------------------------|-------------------------------------------------------------------------|----------------------------------------------|-----------------------------|
| Shrinkage (after fixation)    |                                                                         | 100% (no change in shrinkage)                |                             |
| Translucency                  |                                                                         | 100% (more translucent)                      |                             |
| Rigidity                      | 67% (less rigid)                                                        | 33% (rigidity was same)                      |                             |
| Impregnation                  |                                                                         | 100% (no change in impregnation)             |                             |
| Sectioning                    |                                                                         | 100% (no change in sectioning)               |                             |

Cytomorphometric analysis

Cytomorphometrically, there was less cellular and nuclear mean areas of individual cells in Group A sections when compared with Group B suggesting less cell shrinkage in Group B and is statistically significant [Figure 4]. [P = 0.002, 0.001 respectively; Tables 3 and 4]. P <0.05 is considered statistically significant.

Special staining

There was no significant difference in cellular architecture and staining quality in Group B samples when compared to Group A [Figure 5].

Discussion

Considering the toxicity of formalin and xylene, the present study was performed to find best substitutes for the same. Depending on natural availability, economical reasons, nonhazardous nature, and bio-friendly alternatives such as honey and olive oil were used as best substitutes for formalin and xylene, respectively.

For several centuries, honey has been used as a medicine, particularly for the treatment of wounds because of its antibiotic properties. A literature search suggested that apart from these antibiotic properties, honey has been found to prevent autolysis as tissues fixed in it for 30 days did not show any sign of autolysis. The tissue hardening property makes it similar in action to fixatives which acts by hardening tissues.[6] In general, fixatives such as formalin containing acids or those with low pH do not favor preservation of cytoplasmic constituents, but they are good nuclear fixatives. Honey contains several minerals, trace elements, and vitamins as well as carbohydrates and acids principally glucose and fructose and ascorbic acid,
respectively. The latter may account for its low pH of between three and four, hence, by extension a good nuclear fixative. The present study used honey as fixative.

The results of the present study showed that shrinkage of the tissues fixed in formalin and honey were similar macroscopically. This is attributed to the low osmolality and low concentration of honey (1:10 dilutions). Most often, formalin is used as 10% formalin, i.e. dilution of ten times the volume of formalin with water. Hence, the same was used for the present study, and concentrated commercial honey was diluted to the same 10% and used as fixative. The speed of fixation depends on the rate of diffusion of fixative into the tissue and the rate of chemical reactions with various components. In practice, it is assumed that these processes require at least 1 h per millimeter of tissue thickness; but routinely, the tissues are fixed with formalin for 24-48 h. In the present study, fixation by honey took the same duration of 24 h as does formalin.

The results of the present study are in concordance with Sabarinath et al., where they compared formalin fixed tissues with honey fixed tissues, concluded similar results with honey.

After fixation, olive oil was used as clearing agent in the present study. All the Group B tissues (100%) appeared transparent after clearing as compared with Group A tissues [Table 1] indicating that olive oil has similar clearing properties such as xylene. This may be attributed to a number of factors. During clearing, olive oil with a refractive index of 1.467, closer to that of tissue proteins (varying between 1.33 and 1.4) infiltrates the intercellular spaces of tissues leading to the reduction in the light scattering properties and increase in optical clearance of the tissue making them appear transparent. In the present study, 67% of Group B tissues showed less rigidity compared to Group A which may be due to high viscosity of olive oil and slight miscible nature with alcohol.

Andre et al. substituted xylene with a mixture of peanut oil, soybean oil, coconut oil, and cotton oil and concluded that the quality of sections with respect to xylene were better.

Bruun Rasmussen et al. tried a mixture of coconut oil and olive oil as clearing agent and noted incomplete impregnation, leading to problems in the cutting sections, and therefore, they concluded that this mixture was ineffective as a clearing agent. In contrast to their observation, the present study found that olive oil, when

![Figure 3: H and E-stained tissue sections (a) Group I (b) Group II](image)

![Figure 4: Morphometric analysis, (a and a1) Group I (cellular and nuclear measurements, respectively). (b and b1) Group II (cellular and nuclear measurements, respectively)](image)

![Figure 5: Special stains, (a) Periodic acid–Schiff, (b) Van Gieson](image)
used alone, was as effective as xylene, without interfering with further impregnation and cutting.\(^5\)

Buesa used a mixture of ethanol, isopropyl alcohol, and mineral oil as an alternative for xylene and found the mixture to be as efficient as xylene in dealcoholization.\(^9\) Instead, the present study considered the environment-friendly, readily available alternative, olive oil, to avoid chemicals such as ethanol and isopropyl alcohol, which are also hazardous.

In the present study, following fixation and clearing process, normal staining procedure was carried out and cytomorphometric analysis was performed to know the shrinkage of the cells microscopically by Image software Pro insight 8.0 version. Cytomorphometrically, there was less cellular and nuclear mean areas of individuals cells in Group A sections when compared with Group B suggesting less cell shrinkage in Group B, which is statistically significant (\(P = 0.002, 0.001\), respectively).

Few of Group B sections were subjected to special staining procedures where they were stained using PAS and van Gieson’s stains, respectively, and found that even the special staining procedure showed good results proving no interference by honey and olive oil with tissue composition and they just acted as transient media.

Al‑Maaini and Bryant used vimentin and Ki‑67 as markers for detecting antigens in the connective tissue in honey fixed tissues. They concluded that honey can be used as fixative for advanced diagnostic procedures also.\(^{11}\)

The present study showed less shrinkage, good translucency, and better staining qualities attained with the usage of honey and olive oil compared to formalin and xylene. The results were similar during impregnation and section cutting for both the study groups. However, the rigidity was less in the sections treated with olive oil because of its high viscous nature when compared to xylene. The present study is unique and first of its kind as the study involves assessing the efficacy of honey and olive oil as substitutes for formalin and xylene, respectively, in routine histological procedures, where they were measured at different stages such as fixation, processing, impregnation, sectioning, staining, and microscopic evaluation including cytomorphometry and special staining procedures.

**Conclusion**

The results of the present study infer that honey and olive oil can be used as best efficient substitutes for formalin and xylene, respectively, as they are nonhazardous, naturally occurring substances causing less shrinkage of the tissues. Honey can be used as a fixative and olive oil can be used as a clearing agent in the histopathological laboratory without losing the quality of the histological details. Moreover, these naturally available substances are nontoxic and environmentally bio‑friendly substances, so they can be discharged through ordinary waste pipes thus avoiding the cost of disposal. The present study concludes that substituting formalin with honey and xylene with olive oil is highly desirable from the points of view of both the quality of work and safety.

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**Conflicts of interest**

There are no conflicts of interest.

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