

Honey as an Alternative to Formalin in the Demonstration of Connective Tissue Components

Rahma Al-Maaini¹ and Philip Bryant²

¹ Institute of Health Sciences, Khoula Hospital, Sultanate of Oman

² Department of Pathology, Princess of Wales Hospital, Bridgend, Wales, UK

Abstract

The preservative and antimicrobial powers of honey have been known for centuries. Recently, it has been shown that tissues preserved in low concentrations of honey and stained with hematoxylin and eosin yield results comparable with tissues that have been conventionally fixed in formalin. This work set out to establish that honey could be used as an alternative to formalin in the demonstration of connective tissue components using special staining techniques. In this study, fresh goat tissues were preserved for 24 h in concentrations of honey ranging from 1% to 20% diluted with distilled water. The tissues were processed to paraffin wax, sectioned, and connective tissues demonstrated using conventional textbook staining techniques. Results obtained from this work showed that tissues treated in 5%, 10%, and 20% honey at room temperature gave excellent demonstration of connective tissue components by all staining methods and were comparable to those obtained with formalin-fixed control tissues. However, tissues preserved in 1% honey gave inferior levels of staining, with results ranging from weak to unsatisfactory. These results support the use of 10% honey as an alternative to formalin in the histological demonstration of connective tissues without the need for amendments to existing laboratory protocols. (*The J Histotechnol* 31:67, 2008)

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Introduction

The function of fixation in cellular pathology is to preserve tissues in a life-like condition by preventing both au-

tolysis and putrefaction (1). Formalin is the most widely used fixative because of its ease of use, low cost, and speed of fixation (2). However, because of increasing concerns about its effect as a potential carcinogen, attempts have been made to find safer alternatives (3,4). In honey, for example, the presence of antioxidants conveys tissue protection against cellular damage, primarily as a result of the slow release in diluted honey of low concentrations of hydrogen peroxide, a byproduct of the action of the enzyme glucose oxidase that is present in honey (5). Also, because of the high sugar concentration and acid pH, honey is not a suitable medium for bacterial growth (6–8).

Previous work has shown that tissues preserved in low concentrations of honey and stained with hematoxylin and eosin provided results that favorably compared with those that were prepared with formalin-fixed control tissues (9). Histological staining processes make use of dyes that are generally chosen for their ability to stain various cellular components. Many stains are referred to as special stains because they are used in specific situations according to their diagnostic need (1). This work describes the use of such stains in the histological demonstration of connective tissue components on tissues that have been preserved in low concentrations of honey.

Materials and Methods

Fresh goat tissues were obtained from the Institute of Health Sciences, Sultanate of Oman. Tissues comprising liver, kidney, brain, lung, heart, bowel, stomach, spleen, and tongue were excised and dissected into small segments (3–5 mm) with the use of normal laboratory protocols. The samples were placed into labeled processing cassettes and transferred immediately into sample pots containing honey dilutions of 1%, 5%, 10%, and 20% in distilled water for 24 h at room temperature. The type of honey used in this work was obtained from the Al-Qabel desert in the Sultanate of Oman. Control tissue samples were placed into 10% neutral-buffered formalin for the same duration. After fixation, the tissues were processed conventionally with an auto-

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Address reprint requests to Philip Bryant, Department of Pathology, Princess of Wales Hospital, Bridgend, Wales, UK.
E-mail: philip.bryant@bromor-tr.wales.nhs.uk

Table 1. Tissue-processing schedule

Stage	Reagent	Duration
1	70% alcohol	2 h
2	80% alcohol	2 h
3	95% alcohol	2 h
4	Absolute alcohol	2 h
5	Absolute alcohol	2 h
6	Toluene	2.5 h
7	Toluene	2.5 h
8	Toluene	2.5 h
9	Paraffin wax at 60°C	3 h
10	Paraffin wax at 60°C	3 h

mated tissue processor following the routine departmental schedule in Table 1.

Once tissues were processed, they were embedded in molten paraffin wax at 60°C, and sections cut at 4 microns on a rotary microtome. All sections were dried for 30 min at 60°C in readiness for staining. The staining methods used were van Gieson for collagen, Miller's stain for elastin, Gordon and Sweet's silver impregnation method for reticulin, and the Masson trichrome, with use of the textbook protocols (1).

Results

After staining, we assessed all the slides on a scale from 0 to 4 where 0 = poor and 4 = excellent (see Table 2). When paraffin sections of tissue preserved in 1% honey were stained with the van Gieson method, the collagen was poorly demonstrated (Figure 1) when compared with similar tissue that had been preserved in 10% honey (Figure 2) or

fixed in 10% formalin (Figure 3). However, preservation of background, cellular, and nuclear detail was not compromised. Similar results were found when silver impregnation was conducted on tissue sections preserved in 1% honey (Figure 4), 10% honey (Figure 5), and 10% formalin (Figure 6) for the demonstration of reticulin fibers using Gordon and Sweet's method.

When 1% honey was used, reticulin fibers failed to demonstrate, yet strong silver impregnation was observed when concentrations of honey greater than 5% were used for tissue preservation (Table 2). The demonstration of elastic using Miller's method showed dramatic improvement in the intensity of staining in all the tissues tested when concentrations of honey above 5% were used. Weak 1% honey showed poor to weak demonstration of elastic using Miller's method (Figure 7) when compared with the same tissue that had been preserved in 10% honey (Figure 8) and 10% formalin (Figure 9). When sections of skin were stained using the Masson trichrome method, staining of collagen and keratin occurred when 1% honey was used (Figure 10). However, when 10% honey was used, staining intensity improved (Figure 11) which compared favorably with the formalin control section (Figure 12).

Discussion and Conclusion

The requirement for a safe alternative to formaldehyde has been the subject of previous work (3,4). Although a recent study has indicated the use of low concentrations of honey as a tissue preservative for histological examination (9), the significance and effect of honey on cellular structures and components has not yet been described. In tissues, one of the most important collections of cells are those that comprise the supporting stroma. Commonly termed connective tissues, they occur in many forms, have diverse physi-

Table 2. Staining assessment of the tissue samples

Staining method	Formalin control	1% honey	5% honey	10% honey	20% honey
Van Gieson stain	3	0	4	4	3
Gordon & Sweet's reticulin	4	0	4	4	3
Miller's elastic stain	3	1	4	4	3
Masson trichrome	4	3	4	4	4

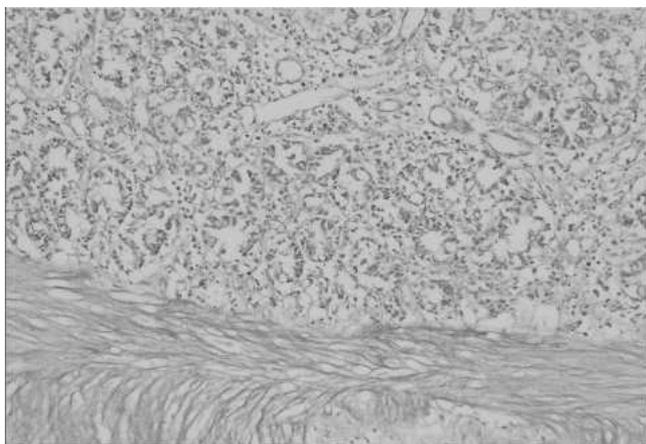


Figure 1. van Gieson stain showing bands of collagen in a section of large bowel treated with 1% honey (×200).

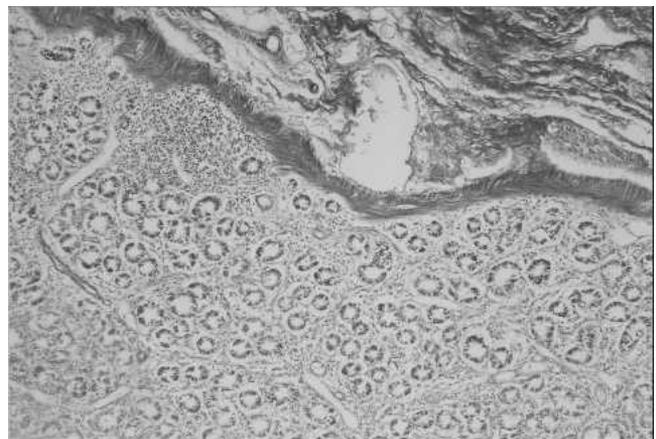


Figure 2. van Gieson stain showing bands of collagen in a section of large bowel treated with 10% honey (×200).

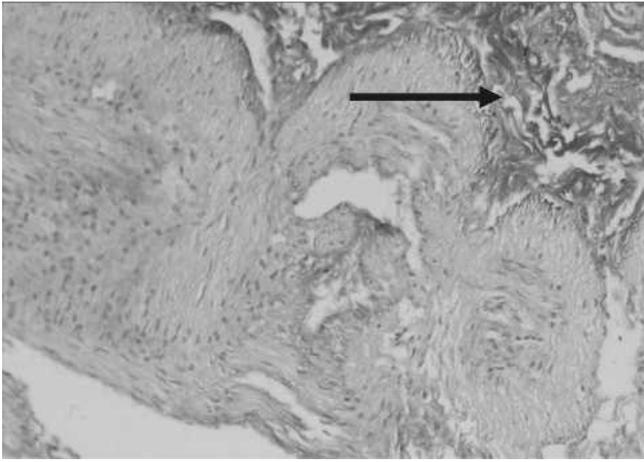


Figure 3. van Gieson stain showing bands of collagen (arrowed) in a section of large bowel fixed in 10% formalin ($\times 200$).

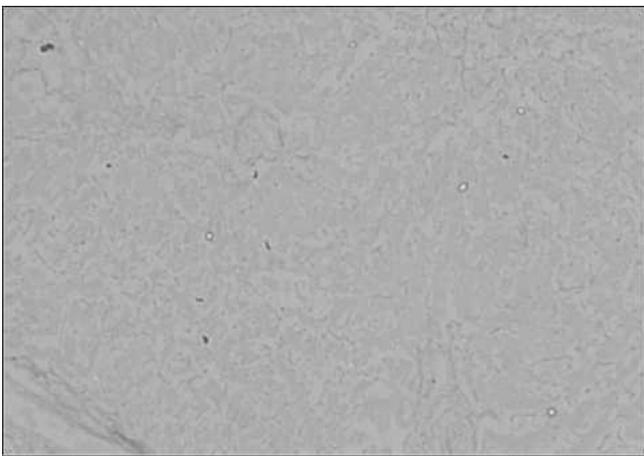


Figure 4. Gordon and Sweet's silver impregnation showing failure to demonstrate reticulin fibers in a section of liver treated with 1% honey ($\times 200$).

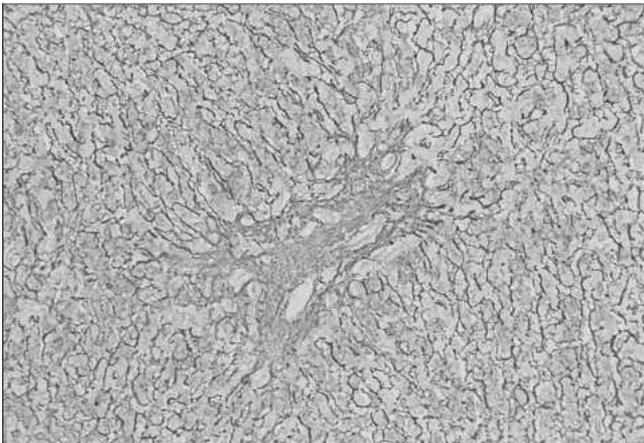


Figure 5. Gordon and Sweet's silver impregnation showing demonstration of reticulin fibers in a section of liver treated with 10% honey ($\times 200$).

cal properties and provide support for internal organs and other structures of the body. Connective tissue consists of bundles of elastin and collagen fibrils in a matrix of ground substance. Loose connective tissue is an admixture of adi-

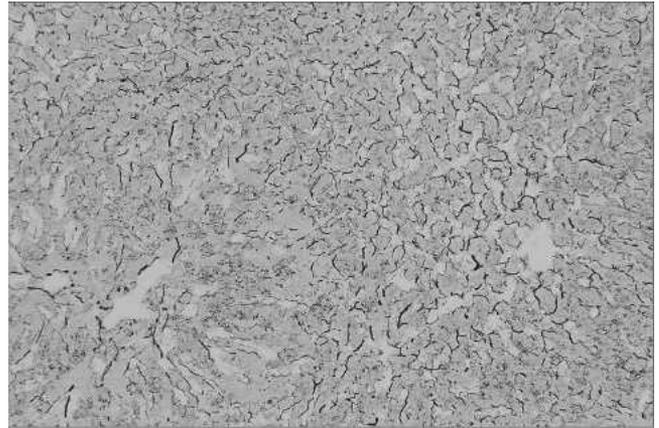


Figure 6. Gordon and Sweet's silver impregnation showing demonstration of reticulin fibers in a section of liver fixed in 10% formalin ($\times 200$).

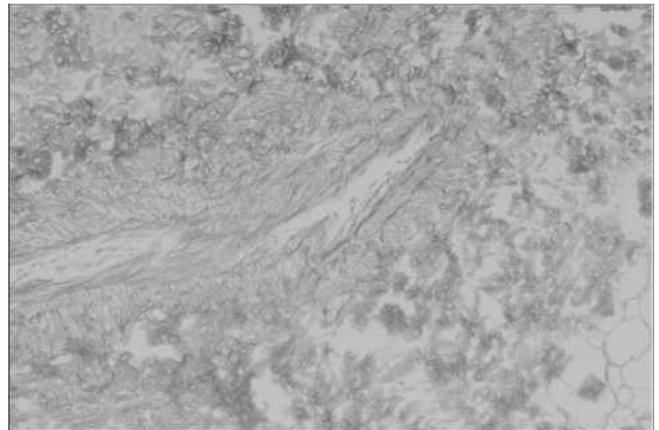


Figure 7. Miller's method showing weak demonstration of elastic fibers in a section of lung treated with 1% honey ($\times 200$).

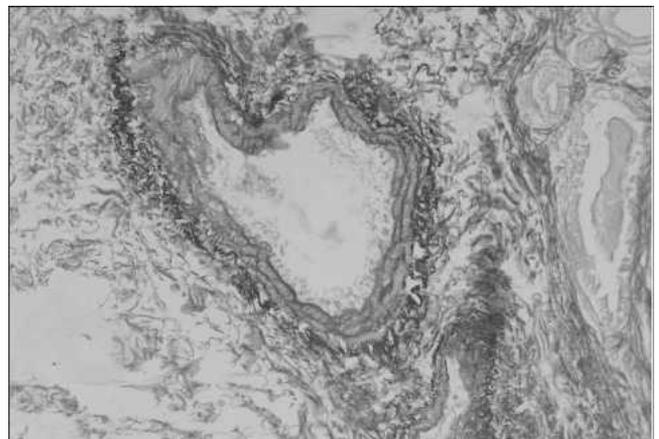


Figure 8. Miller's method showing good demonstration of elastic fibers in a section of lung treated with 10% honey ($\times 200$).

pose that helps to protect internal structures whilst dense connective tissue (which is primarily collagen) provides more solid support and strength (1). Although many types of collagen have been identified, only types designated I-VI have been characterized in detail (10). In the histology laboratory, collagen fibers are routinely demonstrated in tissue sections using the van Gieson method. This stain

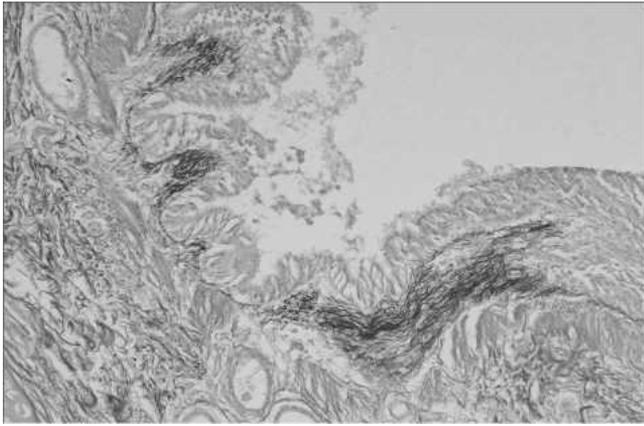


Figure 9. Miller's method showing good demonstration of elastic fibers in a section of lung fixed in 10% formalin ($\times 200$).

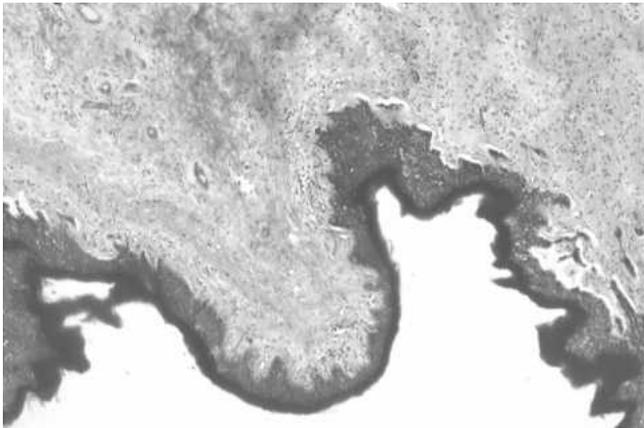


Figure 10. Masson trichrome method showing pale demonstration of connective tissues in a section of skin treated with 1% honey ($\times 200$).

consists of a small, rapidly penetrating dye molecule having a high tissue affinity (picric acid) and a larger, slower penetrating dye molecule with a lower tissue affinity (acid fuchsin). The van Gieson stain is rate limited with denser structures such as muscle staining yellow and more loosely constructed tissue such as collagen staining red (11). This work has shown that preservation of tissue in concentrations of honey above 5% is effective for the demonstration of collagen using the van Gieson method when compared with tissues that have been fixed in formalin (Figures 2 and 3).

Similar observations were found in tissues after staining for reticulin fibers using silver impregnation. Reticulin is comprised of slender bundles of collagen fibrils that are often combined with carbohydrates such as glycoproteins and proteoglycans. They are positive with the periodic acid Schiff and are able to react with silver that is normally provided in the form of an ammoniacal silver solution. It is from this solution that metallic silver readily precipitates in the presence of a reducing agent such as formalin. Not only is this achievable for paraffin sections of tissues that have been fixed conventionally in formalin (Figure 6), it is also effected in sections treated with diluted honey (Figure 5).

Connective tissue elastin is an extracellular, insoluble



Figure 11. Masson trichrome method showing demonstration of connective tissues in a section of skin treated with 10% honey ($\times 200$).

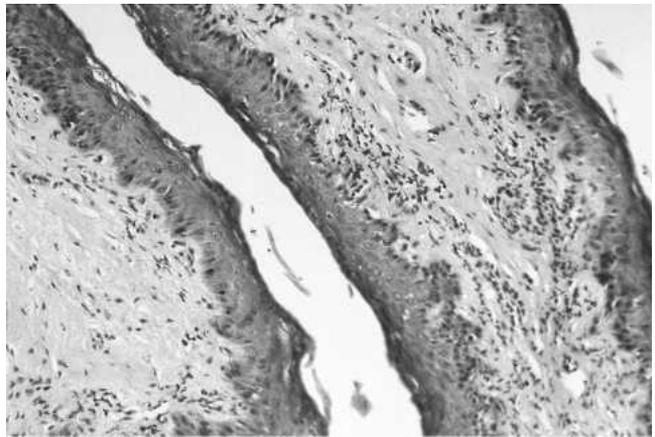


Figure 12. Masson trichrome method showing demonstration of connective tissues in a section of skin treated with 10% formalin ($\times 200$).

protein that can be demonstrated using several methods although why this occurs is not completely understood. However, it is thought that aldehyde residues are involved when the staining solution is between pH 9 and pH 10 because when these groups are blocked, the staining is impaired (1,10). This may help to understand why tissues treated in honey are able to demonstrate elastic fibers using the resorcin-fuchsin method of Miller because honey is known to contain hydroxymethylfurfural, an aldehyde (12).

Trichrome methods also work equally well with dilute honey (see Figures 10–12). However, the intensity of staining was less compromised as the honey became more dilute (see Table 2). There is no reason to suggest that the use of other special staining methods would produce dissimilar results to those above when 10% honey is used as tissue preservative. What this work has shown is that honey has proved to be an acceptable substitute for formalin in the histology laboratory, allowing the application of special staining methods that are able to complement the production of hematoxylin and eosin stained sections (9).

If honey were to be a genuine contender as a substitute for formalin in the histology laboratory, then a deeper understanding of theoretical issues is required. The ability of honey to protect tissues and inhibit bacterial growth is not

exclusively attributable to the osmolarity of the solution but also to the slow release of hydrogen peroxide at low concentration, to the presence of the antiseptic phenol, and to metabolites of nitric oxide, which have been shown to have a potent biological effect (9,12,13). In addition, the acidity of honey contributes to the antibacterial properties where studies have shown that contrary to expectation, the more dilute the honey, the greater the activity (14,15). Research has shown that there are many pathological organisms that are susceptible to concentrations of honey, including mycobacteria (16), the fungus candida albicans (17), and the herpes virus (18). However, because honey is a natural substance, potency is dependent upon its origin and processing. Subsequently, its antimicrobial powers are variable and are restricted not only to sugar content, but to hydrogen peroxide production (peroxide honey) and additional antibacterial activity which is independent of the generation of hydrogen peroxide (nonperoxide honey) (15).

The presence in honey of the aldehyde hydroxymethylfurfural is caused by the breakdown of fructose at low pH. The amount of aldehyde present is variable, but because the rate of reaction of hydroxymethylfurfural is accelerated by heat and storage, it can be measured and used as an indicator of quality (12,19). Because honey contains aldehydes, there is the distinct possibility that honey may form cross links with tissue amino acids in the same way that formaldehyde does (19). If the tissue changes seen in this work are not a direct result of honey preservation, it suggests that they are either a consequence of the alcohol dehydrant used during tissue processing or a combination of both. Although these are contentious issues, the concepts would apply equally to any fixative (including that of formalin) when processing is carried out using these conventional methods. If that were the case, then one would have expected similar results with the staining for all the honey dilutions used in this work. However, what this study has shown is that when sub optimal dilutions of honey are used, the demonstration of connective tissue components is compromised.

One of the disadvantages of using honey for the preservation of tissue is the production of mold. Moisture, warmth, and food are prerequisites for the growth of mold, and all of these are met during room temperature storage in dilute honey. Also, at this temperature, fermentation can occur and this may cause expansion of plastic sample containers with the air that is produced during the fermentation process, causing them to burst. Both fermentation and mold formation, however, can be prevented by cold storage (19).

The effect of histological fixatives on laboratory health and safety has been the subject of scrutiny for many years (3,20). Despite the fact that commercial substitutes such as Glyo-Fixx, STF-Streck, Omnifix, Histochoice, and Histofix are readily available, many of them have been found to be inadequate for critical histological studies (21,22). The use of honey as a safe alternative to formalin is an interesting concept especially because many of the commercial products are alcohol-based, flammable and toxic. Whether sucrose alone would produce similar effects is disputed because the antibacterial activity of honey is not always attributed solely to the sugar content (14).

There is no doubt, however, that the results of this work using honey as a preservative for tissue biopsies are commendable, although the hypotheses on how these are achieved are controversial. Much work still needs to be performed to assess the feasibility of using honey as a complete replacement for formalin and studies using immunocytochemistry are currently in progress. This work has proved invaluable even though uncertainties remain around the viability of using honey for the preservation of large tissue samples and resections and as a vehicle for the long-term storage of tissues.

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