Immunocytochemical Evaluation of Ductal Carcinoma in Breast after Preservation in Honey

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Abstract

In histology, formalin is the fixative of choice for tissues embedded in paraffin wax. However, formalin fixes tissues by cross-linking proteins, thereby masking certain tissue antigens. As a result, immunostaining of these tissues requires antigen retrieval so that the tissues can be free to combine with their specific antibodies. In this limited study, honey was substituted for formalin for the preservation of breast tumor samples. These samples were then assessed by the use of selected antibodies with and without antigen retrieval. Results showed good levels of staining without antigen retrieval for common leukocyte antigen, cytokeratin AE1/AE3, and epithelial membrane antigen in breast tumor samples treated with honey. Although localization of some of the antigens was less satisfactory when compared with formalin-fixed tissues, the results compared favorably with control tissues that had been fixed in formalin and stained after retrieval with protease at room temperature and citrate buffer pH 6.0 at high temperature. Staining for cytokeratin 5 without antigen retrieval in breast tumor treated with honey was poor when compared with formalin-fixed tissue using high temperature retrieval in ethylene diamine tetraacetic acid buffer pH 9.0. Also, immunostaining of control tissue pretreated with distilled water was equally satisfactory for breast tumor samples preserved in honey. These results indicate that the preservation of tissues in honey may not influence immunostaining as previously thought and suggest that the alcohol dehydrant may play an important role during tissue processing. (*The J Histotechnol* 32(2):54–59, 2009)

Submitted November 18, 2008; accepted with revisions March 24, 2009

Key words: antigen retrieval, formalin substitute, honey, immunochemistry

Introduction

Tissue fixation is a complex series of chemical events that help to preserve tissues by preventing autolysis and putrefaction. Formalin, the most widely used fixative in histopathology, is regarded as the gold standard by providing good quality histomorphology for tissues stained with hematoxylin and eosin and special stains (1). During formalin fixation, certain antigenic sites are less resistant and consequently require antigen retrieval to improve their immunoreaction. However, methods for unmasking these antigens require the use of reagents of varying pH and temperature, which may influence immunostaining by producing false staining patterns (2). Also, formalin remains a potential carcinogen for laboratory workers and, as a result, many studies have been conducted to find safer alternatives (3–5).

The use of honey as a reliable substitute for formalin has previously shown that tissues preserved in low concentrations afford results comparable with those obtained when tissues are stained with the use of hematoxylin and eosin and special stains after fixation in formalin (6,7). Honey is able to preserve and protect tissues by several means, most notably by its osmolarity and antiseptic powers that are provided by the hydrogen peroxide and phenol content (7). To date, no documentary evidence exists that considers the effect that honey preservation may have on antigen retrieval and subsequent immunostaining of tissue sections.

This current study was designed to examine these effects by the use of different honey types, breast tumor samples, and the mouse monoclonal antibodies epithelial membrane antigen, common leukocyte antigen (CD45), cytokeratin 5, and the cytokeratin cocktail AE1/AE3 using the Bond Max immunostainer (Leica Microsystems, Milton Keynes, UK). These antibodies were selected because they were readily available and required different methods of retrieval when used with formalin-fixed tissues using our current laboratory protocols. Immunostaining was performed with and without antigen retrieval by the use of protease digestion at room temperature and citrate buffer pH 6.0 and ethylene diamine tetraacetic acid (EDTA) buffer pH 9.0 at high temperature. The selection of breast tumor was made on the basis that it was readily available from the surgical room as fresh tissue and, therefore, different preservative agents could be applied. Also, the application in this laboratory of immunohistochemistry on paraffin sections of breast tumor is frequently applied for diagnostic purposes. Subsequently, it was thought that use of the small panel of antibodies with their respective but different modes of retrieval was sufficient to test the hypothesis described.

The work was supported and performed at the Department of Pathology, Princess of Wales Hospital, Bridgend, Wales, UK.

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Materials and Methods

Fresh mastectomy specimens from three anonymous patients were collected and brought immediately to the laboratory. The specimens were dissected, and five samples of tumor up to 5 mm in length were obtained from each breast by the use of normal laboratory protocols. The samples were placed into prelabeled processing cassettes and immersed in preservatives A–E diluted with distilled water as shown in Table 1. The commercial honey was Gale's pure honey, a dark amber honey obtained from a supermarket; the local honey was a pale amber honey obtained from a local apiary, and the manuka honey was in the form of Manukacare 18+, a dark amber, high potency, sterilized New Zealand honey.

All samples were preserved for 24 h and processed manually by use of the protocol described in Table 2. After processing, the tissues were embedded in paraffin wax, and sections were cut at 4 microns and mounted onto adhesive-coated microscope slides. All sections were placed in the oven at 60°C for 1 h to achieve maximum adhesion. One section from each of the formalin-fixed samples was stained with hematoxylin and eosin as previously described for histological diagnosis (6).

Antigen retrieval and immunostaining was conducted on the Bond Max immunostainer (Leica Microsystems) using routine laboratory protocols. Before staining, all slides were dewaxed at 60°C with Bond De-wax. The sections were then treated with absolute alcohol for 3 min followed by three washes of water for 3 min before antigen retrieval (see Table 3). For antigen retrieval with the use of citrate buffer pH 6 (Bond ER1) or EDTA pH 9 (Bond ER2), slides were incubated for 20 min at 100°C. For antigen retrieval with the use of proteolytic enzyme, slides were incubated for 10 min at room temperature in protease (17 mg/mL) and washed three times with water as described previously. After retrieval, all slides were washed in three washes of water for 1 min each. Immunostaining using the mouse monoclonal antibodies (Table 3) was then performed with Bond (Leica Microsystems) reagents (Table 4). After each step, three washes of 1 min each were performed with phosphate-buffered saline (PBS), pH 7.4.

Sections were stained with common leukocyte antigen diluted 1:50 with Bond primary antibody diluent containing 0.35% Proclin. Cytokeratin 5, cytokeratin AE1/A3 cocktail, and epithelial membrane antigen were diluted 1:200. The sections were stained with and without antigen retrieval, as shown in Table 3. After staining, all slides were randomly numbered to mask them before microscopic examination by a state registered biomedical scientist, a specialist biomedical scientist, and a consultant histopathologist. Intensity and selectivity of staining of the cells that were expected to stain were assessed microscopically on a scale of 1–6 by use of the following guidelines:

1: poor staining of the cells
2: very weak staining of the cells
3: weak demonstration of the cells
4: good demonstration of most of the cells
5: very good demonstration of most of the cells
6: excellent demonstration of all the cells

Although these guidelines were applied generally, marks also were deducted for uneven and excessive background staining, poor morphology, and any other factor that affected the interpretation of the samples.

Table 1. Labeling and pretreatment of breast tumor samples

Label	*Preservative*
A	10% Formalin
B	Distilled water
C	10% Manuka honey
D	10% Commercial honey
E	10% Local honey

Table 2. Processing schedule for the breast tumor samples

Stage	*Reagent*	*Duration*
1	Absolute alcohol	30 min
2	Absolute alcohol	30 min
3	Absolute alcohol	30 min
4	Absolute alcohol	30 min
5	Absolute alcohol	30 min
6	Xylene	30 min
7	Xylene	30 min
8	Xylene	30 min
9	Paraffin wax 60°C	1 h
10	Paraffin wax 60°C	1 h

Results

The breast tumor samples were histologically diagnosed previously as invasive, ductal carcinomas by the use of hematoxylin and eosin–stained sections. The immunostaining was performed as previously, and the results and mean of the three assessment scores are shown in Table 5 and visualized graphically in Figures 1–4. The highest mean score

Table 3. Immunomarker characteristics and methods of retrieval

Immunomarker (isotype/clone)	*Method of retrieval*
Common leukocyte antigen (RP2/18, Clone RP2/22)	Citrate buffer pH.6.0 (ER1) No retrieval
Cytokeratin 5 (XM26)	EDTA buffer pH 9.0 (ER2) No retrieval
Cytokeratin AE1/AE3 (AE1, Clone AE3)	Protease enzyme concentrate (17 mg/mL) No retrieval
Epithelial membrane antigen (Clone GP1.4)	No retrieval

Table 4. The Vision BioSystems Bond X immunostaining protocol

Stage	*Reagent*	*Length of time (min)*
1	Peroxide block	5
2	Primary marker/antibody	15
3	Post primary	8
4	Polymer	8
5	DAB	10
6	Bond DAB enhancer	5
7	Hematoxylin	7

Table 5. Immunochemical assessment of the tissue sections

Antibody	*Fixative/preservative*	*Antigen retrieval*	*Mean*
Common leukocyte antigen	10% Formalin	Citrate buffer pH 6.0	4.2
Common leukocyte antigen	10% Formalin	None	3.7
Common leukocyte antigen	Commercial honey	Citrate buffer pH 6.0	3.9
Common leukocyte antigen	Commercial honey	None	4.1
Common leukocyte antigen	Local honey	Citrate buffer pH 6.0	5.0
Common leukocyte antigen	Local honey	None	4.9
Common leukocyte antigen	Manuka honey	Citrate buffer pH 6.0	3.7
Common leukocyte antigen	Manuka honey	None	4.3
Common leukocyte antigen	Water	Citrate buffer pH 6.0	3.2
Common leukocyte antigen	Water	None	3.9
Cytokeratin 5	10% Formalin	EDTA buffer pH 9.0	3.4
Cytokeratin 5	10% Formalin	None	2.3
Cytokeratin 5	Commercial honey	EDTA buffer pH 9.0	3.9
Cytokeratin 5	Commercial honey	None	2.4
Cytokeratin 5	Local honey	EDTA buffer pH 9.0	3.9
Cytokeratin 5	Local honey	None	2.8
Cytokeratin 5	Manuka honey	EDTA buffer pH 9.0	3.9
Cytokeratin 5	Manuka honey	None	2.3
Cytokeratin 5	Water	EDTA buffer pH 9.0	4.1
Cytokeratin 5	Water	None	4.6
Cytokeratin AE1/AE3	10% Formalin	Protease enzyme	5.0
Cytokeratin AE1/AE3	10% Formalin	None	2.9
Cytokeratin AE1/AE3	Commercial honey	Protease enzyme	2.2
Cytokeratin AE1/AE3	Commercial honey	None	3.4
Cytokeratin AE1/AE3	Local honey	Protease enzyme	2.0
Cytokeratin AE1/AE3	Local honey	None	4.1
Cytokeratin AE1/AE3	Manuka honey	Protease enzyme	2.9
Cytokeratin AE1/AE3	Manuka honey	None	3.8
Cytokeratin AE1/AE3	Water	Protease enzyme	2.0
Cytokeratin AE1/AE3	Water	None	4.8
Epithelial membrane antigen	10% Formalin	None	5.2
Epithelial membrane antigen	Commercial honey	None	4.1
Epithelial membrane antigen	Local honey	None	4.2
Epithelial membrane antigen	Manuka honey	None	4.3
Epithelial membrane antigen	Water	None	4.1

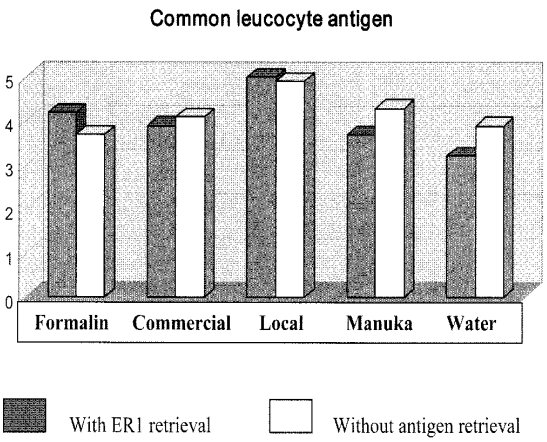

Figure 1. Assessment of results for ductal carcinoma of breast with the use of common leukocyte antigen with and without citrate buffer pH 6.0 (ER1) antigen retrieval.

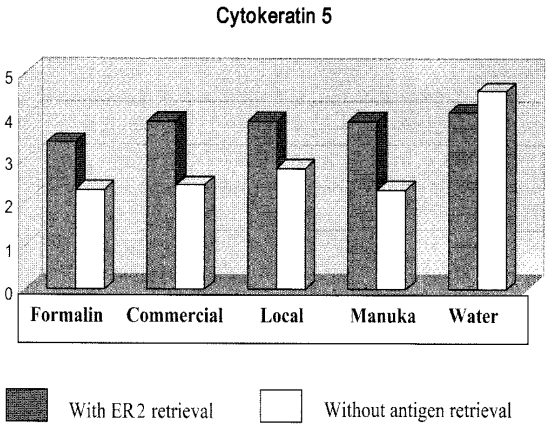

Figure 2. Assessment of results for ductal carcinoma of breast with the use of cytokeratin 5 with and without EDTA pH 9.0 (ER2) antigen retrieval.

recorded (5.2) was obtained with formalin-fixed tissue sections that were stained with epithelial membrane antigen without antigen retrieval. The lowest mean score (2.0) was obtained after enzyme retrieval and staining for cytokeratin AE1/AE3 in tissues treated with local honey and water (Table 5). When common leukocyte antigen was used without antigen retrieval, staining was comparable with the results obtained when conventional antigen retrieval using citrate buffer pH 6.0 (ER1) was performed (Figure 1). In contrast, the staining of cytokeratin 5 with EDTA buffer pH 9.0 (ER2) was superior to those obtained when no antigen retrieval was performed (Figure 2).

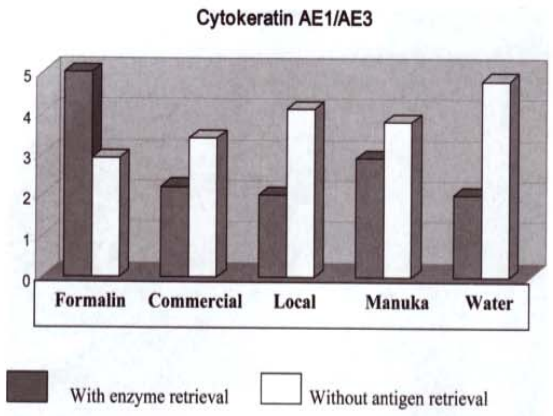

Figure 3. Assessment of results for ductal carcinoma of breast with the use of cytokeratin AE1/AE3 with and without protease enzyme antigen retrieval.

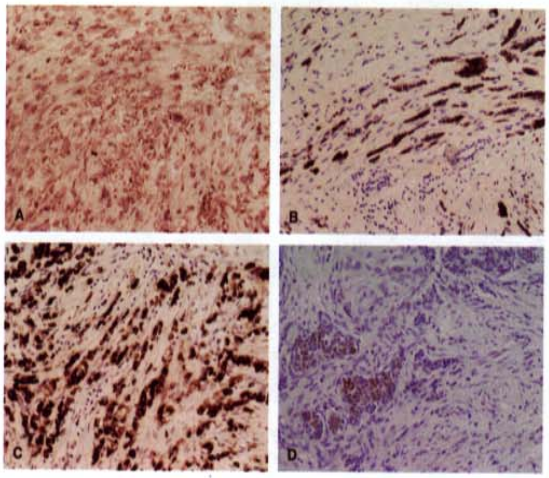

Figure 4. Immunostaining of ductal carcinoma of breast with cytokeratin AE1/AE3. (A–C) Magnification ×200; (D) magnification ×100.

Assessment of tissues preserved in honey and stained with cytokeratin AE1/AE3 without enzyme retrieval with protease showed significant improvement when compared with those when antigen retrieval was performed (Figure 3). Enzyme retrieval of tissues preserved in local honey showed poor localization of cytokeratin AE1/AE3 with prominent over digestion of the tissue section (see Figure 4A). However, when no enzyme retrieval was conducted, an improved level of staining was obtained (Figure 4B). In contrast, when formalin fixation was conducted, enzyme retrieval and staining with cytokeratin AE1/AE3 produced superior results to those sections that were stained without using antigen retrieval (Figures 4C and 4D).

The immunomarker epithelial membrane antigen is routinely used in this laboratory without antigen retrieval. When all the stained slides were assessed, it was shown that although formalin fixation gave acceptable results, all the other preservatives performed equally well (Figure 5). Sections of breast tumor preserved in local honey (Figure 6A) showed good localization of epithelial membrane antigen but with significantly more background when compared to sections of breast tumor that had been fixed in 10% formalin (Figure 6B). Overall, it was found that the morphology of tissues preserved in honey was compromised when compared with control tissues that had been fixed in formalin.

Discussion

In cellular pathology, formalin is the fixative of choice for preventing autolysis and putrefaction and stabilizing cellular and tissue constituents so that they are able to withstand the subsequent stages of tissue processing (2,8). However, because formalin fixation can conceal tissue antigens by cross-linking proteins, their immunocytochemical localization may require retrieval to make them accessible for reaction with their specific antibodies (9). Although this problem may be overcome by treating formalin-fixed sections with the reversible protein cross-linking agent citraconic anhydride (10), the most important factor for retrieval of antigens masked by formalin fixation is high-temperature heating (11). Because the universal application of antigen retrieval methods with constant conditions for all antigens cannot be achieved, variations in the type of solution, pH, and temperature are essential. In this work, heat-mediated retrieval was performed for the demonstration of common leukocyte antigen and cytokeratin 5 with citrate buffer pH6.0 and EDTA buffer pH 9.0, respectively, whereas retrieval of cytokeratin AE1/AE3 was achieved with proteolytic enzyme digestion at room temperature. Epithelial membrane antigen did not routinely require antigen retrieval before immunostaining.

Because of the hazardous properties of formalin, there have been many attempts to find safer substitutes for laboratory use (3,4,12). Commercial, formalin-free substitutes are readily available, but they have not always been adequate for this purpose (13,14). The use of honey as an alternative to formalin has previously been described and has been used successfully in the production of hematoxylin and eosin–stained sections and in the use of special stains for the demonstration of connective tissue components (6,7). The presence of antioxidants in honey both inhibits oxidation and neutralizes the effects of damaging free radicals, thereby helping to protect cells and tissues against damage (15). Properties of honey, such as high osmolarity, low pH, and the presence of components such as hydrogen peroxide and the phenol inhibine, all contribute to its antioxidative and antibacterial powers (16–20).

Honey is produced in many different forms and, therefore, accurate predictions on the preservative properties of generic honey can be unreliable. The degree of activity of honey depends both on the plant source and geographical location, and laboratories may benefit from experimenting with available products beforehand. However, this study and others have shown that when routine laboratory protocols are used, results appear largely unaffected by honey type (6,7). The effect of honey preservation on the immunolocalization of antigens had not previously been documented, and this work was designed to examine the hypothesis that immunostaining could be performed successfully without the need for antigen retrieval. This hypothesis was largely based on the assumption that, unlike formalin, preservation in honey probably does not form protein cross-links with the aldehyde hydroxymethyl furfural as was previously thought (7). Consequently, antigenic sites would be readily available for reaction with their specific antibodies.

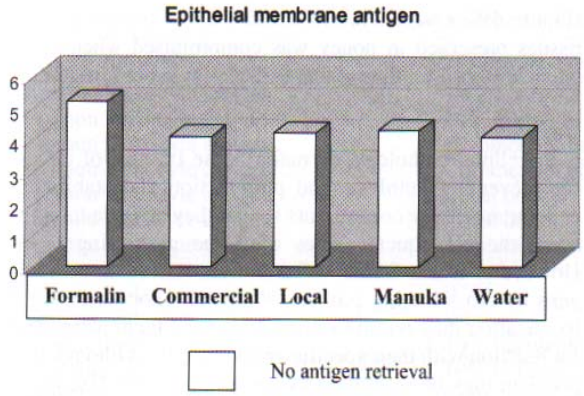

Figure 5. Assessment of results for ductal carcinoma of breast with the use of epithelial membrane antigen without antigen retrieval.

When immunostaining was performed for common leukocyte antigen with high temperature retrieval with citrate buffer pH 6.0, the results corresponded with those that were obtained in the absence of antigen retrieval (Figure 1). This finding was probably attributable to the fact that both the 10% honey and the citrate buffer used for retrieval had a similar pH (6). Conversely, when immunostaining for cytokeratin 5 was performed, superior results were obtained for tissues treated with formalin and 10% honey solutions when high temperature antigen retrieval using EDTA buffer pH 9.0 was performed (Figure 2). The poor results obtained in the absence of antigen retrieval were probably a consequence of the inability of the acidic honey solutions to adequately retrieve cytokeratin 5 because alkaline retrieval was a prerequisite when formalin-fixed tissue was used. In this laboratory, room-temperature retrieval with the use of protease enzyme is recommended for the demonstration of cytokeratin AE1/AE3 in formalin-fixed tissue, which is supported in this study (Figures 3 and 4C). However, enzyme digestion of tissue sections preserved in 10% honey solutions consistently gave suboptimal results, with overdigestion occurring in some areas of the tissues (Figure 4A). In complete contrast, 10% honey solutions consistently gave superior results for cytokeratin AE1/AE3 (Figures 3 and 4B) when compared with those obtained with formalin fixation in the absence of antigen retrieval (Figures 3 and 4D). Also, this study has shown that optimal staining of epithelial membrane antigen is achievable without retrieval when tissues are treated with 10% solutions of honey or formalin (Figures 5 and 6).

After pretreatment of tissues with water, immunostaining of all antigens performed equally well when no retrieval was carried out. Because water does not have preservative properties, it is reasonable to assume that the alcohol used as the tissue dehydrant is responsible. When tissues are dehydrated in alcohol, the hydrophobic bonds that contribute to the maintenance of the tertiary structure of proteins are disrupted (21). Because hydrogen bonds are more stable in alcohol than in water, alcohol will efficiently remove both interstitial and bound water (22). The precipitation and aggregation of proteins during alcohol fixation is a very different process from the cross-linking that occurs with formalin. Consequently, antigenic sites are readily available, with methods

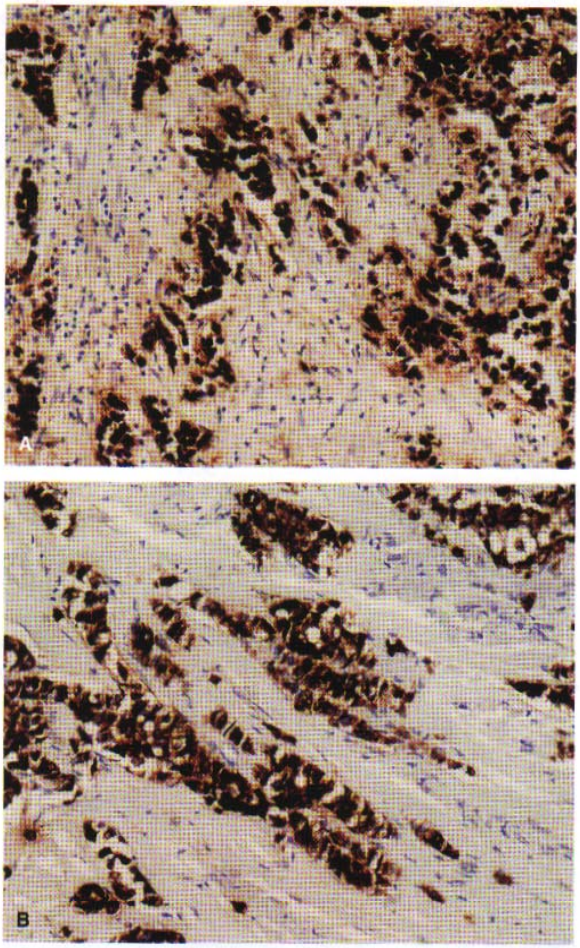

Figure 6. Immunostaining of ductal carcinoma of breast with epithelial membrane antigen. Magnification ×200.

of retrieval being rendered unnecessary. This is also true when tissues are preserved in honey, and this study showed that the source of the honey was not a significant factor. However, what cannot be ignored is the fact that equally good results were obtained when control tissues were treated with distilled water for 24 h. This finding suggests that the alcohol used as the dehydrating agent may play an important role and appears to contradict previous work in which suboptimal concentrations of honey compromised staining of connective tissue components after conventional processing (7).

There is a definite need for a safe alternative to formalin, and it is envisaged that honey can reliably fulfill that role. Honey from different sources and manufacturers does have different characteristics, a phenomenon which has been described elsewhere (23). Available data, however, have demonstrated that preservation in honey can be used for staining tissues with hematoxylin and eosin and special stains despite its source (6,7). In addition, this study has shown favorable results during immunostaining with a reduction in turnaround time for certain antigens by the omission of antigen retrieval. However, this work was performed using the Bond Max immunostainer with on-line retrieval using our current laboratory protocols. Studies that use other honey varieties with both manual and automated systems using off-line retrieval need to be completed so that

the feasibility of using honey in this way can be fully assessed. These studies should include a varied tissue selection and a broader spectrum of antibodies so that the benefits and pitfalls of using honey in this way can be realized.

Acknowledgments

We would like to thank our colleagues in the cellular pathology laboratory at the Princess of Wales Hospital, Bridgend, for their help and support during the preparation of this work.

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