What is the Best Procedure to Remove Formalin Pigment from Formaldehyde-Acetic Acid-Alcohol Fixed Tissues?

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Abstract
There has been some disagreement as to whether formalin pigment is bleached by potassium permanganate/oxalic acid (PPO). This study analyzed the bleaching characteristics of PPO on tissues fixed in formaldehyde-acetic acid-alcohol and compared PPO with other bleaching agents and conventional formalin pigment removal methods. It was found that PPO removed formalin pigment. Other bleaching methods (hydrogen peroxide, periodic and chromic acids) also removed the pigment to some extent. Garvey’s pigment removal procedure was more effective than alcoholic picric acid for the routine removal of formaldehyde-acetic acid-alcohol associated formalin pigment. (The J Histotechnol 33(3):109–111, 2010)

Introduction
Several authors have reported that bleaching with potassium permanganate/oxalic acid (PPO) removes formalin pigment (1,2). Culling (3), however, has recorded that formalin pigment (acid formaldehyde hematin) is not bleached. Bleaching is commonly used to remove melanin from heavily melanocytic lesions (4). The aim of this study was to ascertain whether PPO removes the pigment present in formaldehyde-acetic acid-alcohol (FAA)-fixed tissues.

Methods
Four-micrometer paraffin sections from placental specimens fixed in FAA were used. FAA fixative was prepared as follows:

- absolute ethanol, 1680 mL;
- glacial acetic acid, 128 mL;
- concentrated formalin (37%), 200 mL; and
- distilled water, 2000 mL.

Four-micrometer paraffin sections from tissues containing a range of pigments fixed in neutral-buffered formalin also were examined. These included a lymph node fixed in B5 (mercury pigment), liver with bile pigment, liver with hemochromatosis (iron), lymph node containing a metastatic pigmented melanoma (melanin), substantia nigra (neuromelanin), and lateral geniculate body (lipofuscin) (5).

Techniques for formalin pigment removal included:
- 1.8% picric acid in absolute alcohol (15 min), followed by a 15-min wash in water.
- Garvey’s formalin pigment removal technique (6). Sections were placed in alkylphenol ethoxylate (APE) (phenol 5 mL, HCl 5 mL, absolute ethanol 90 mL) for 5 min followed by washing in water for 5 min.
- PPO: sections treated with 5% potassium permanganate for 2 min, washed in water 2 min, and decolorized in 5% oxalic acid, 2 min (1,2).

For heavily pigmented cases, treatment times were extended as follows: picric acid 1 hr, APE 30 min, and PPO 10 min in KMnO₄. A few cases also were treated with concentrated (30%) hydrogen peroxide for 10 min, 1% periodic acid (15 min), or 5% chromic acid (10 min), followed by 2% sodium bisulfite.

All treated slides, including nontreated (controls), were stained with hematoxylin and eosin (H&E). Duplicate treated sections of bile-containing liver was stained with Fouchet’s stain for bile, liver with hemochromatosis was stained with Perl’s stain (7), and lateral geniculate body was stained with periodic acid-Schiff (after diastase) for lipofuscin (8).

Results
Results are summarized in Tables 1 and 2. PPO was effective in removing the formalin pigment present in FAA-fixed tissues (Figures 1 and 2). When used for the recommended times, PPO performed better than alcoholic picric acid, although it was not as efficient as Garvey’s APE (Figure 3).
Removal of Formalin Pigment from Formaldehyde-Acetic Acid-Alcohol Fixed Tissues / Henwood

For heavily pigmented sections, PPO performed better than Garvey’s APE and alcoholic picric acid when treatment times were extended.

PPO removed formalin pigment, bile, dermal, and neuromelanin. Iron, demonstrated with the Perl’s stain, was partially removed. Lipofuscin, as demonstrated by periodic acid-Schiff reaction (after diastase), was partially removed. PPO caused an increased basophilia of the tissues and diminished affinity for the eosin counterstain (Figure 2).

Garvey’s APE removal technique was by far the most efficient method for the removal of formalin pigment. Alcoholic picric acid was not as efficient and sections often required prolonged washing to remove the yellow coloration. Both Garvey’s APE and alcoholic picric acid removed formalin pigment, whereas mercury, bile, iron, lipofuscin, dermal, and neuromelanin were unaffected.

Thirty percent hydrogen peroxide treatment for 10 min removed most of the formalin pigment in most cases but the procedure often needed to be extended to 30 min to be as effective as Garvey’s or PPO treatment. Much pigment was also removed by periodic acid and chromic acid, but the efficiency of removal was not as good as Garvey’s or PPO treatment.

**Discussion**

Formalin pigment, also known as acid hematin pigment, is commonly associated with the use of nonbuffered or acidic formalin fixatives (9,10). The extracellular pigment is often seen associated with congested tissues and especially close to blood vessels (10). The pigment appears as microcrystalline dark brown birefringent needles or rhomboids (1,11). A similar pigment occurs in congested blood vessels and interstitial hemorrhages in Carnoy-fixed tissues, which have not been exposed to formaldehyde (10).

Several authors have reported that bleaching with potassium permanganate/oxalic acid removes formalin pigment

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**Table 1. FAA fixed tissues (5+ = heavily pigmented)**

<table>
<thead>
<tr>
<th>Case</th>
<th>Untreated</th>
<th>Alcoholic picric acid</th>
<th>Garvey’s APE</th>
<th>PPO</th>
</tr>
</thead>
<tbody>
<tr>
<td>3829</td>
<td>1+</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5529</td>
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<td>4+</td>
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</tr>
<tr>
<td>3502</td>
<td>5+</td>
<td>5+</td>
<td>3+</td>
<td>4+</td>
</tr>
<tr>
<td>3502 E</td>
<td>5+ (1 hr)</td>
<td>1+ (30 min)</td>
<td>0 (10 min)</td>
<td></td>
</tr>
</tbody>
</table>

**E, extended times.**

**Table 2. Nonformalin pigments**

<table>
<thead>
<tr>
<th>Pigment</th>
<th>Alcoholic picric acid</th>
<th>Garvey’s APE</th>
<th>PPO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mercury</td>
<td>2+</td>
<td>2+</td>
<td>2+</td>
</tr>
<tr>
<td>Bile</td>
<td>2+</td>
<td>2+</td>
<td>0</td>
</tr>
<tr>
<td>Iron</td>
<td>2+</td>
<td>2+</td>
<td>1+</td>
</tr>
<tr>
<td>Dermal melanin</td>
<td>2+</td>
<td>2+</td>
<td>0</td>
</tr>
<tr>
<td>Neuromelanin</td>
<td>2+</td>
<td>2+</td>
<td>0</td>
</tr>
<tr>
<td>Lipofuscin</td>
<td>2+</td>
<td>2+</td>
<td>1+</td>
</tr>
</tbody>
</table>

2+, pigment unaffected; 1+, pigment partially removed; 0, pigment removed.

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**Figure 1.** Placenta fixed in FAA, stained with hematoxylin and eosin (H&E). Dark brown extracellular pigment granules are present.

**Figure 2.** Placenta fixed in FAA, stained with H&E after bleaching with PPO. The pigment has been removed, but the resultant basophilia is not acceptable.

**Figure 3.** Placenta fixed in FAA, stained with H&E after treatment with Garvey’s APE. The pigment present in Figure 1 has been removed.
Culling (3), however, has recorded that formalin pigment is not bleached. Our results indicate that the formalin pigment present in FAA-fixed tissues is bleached by PPO and prolonged hydrogen peroxide bleaching. Periodic acid and chromic acid will also remove significant amounts of formalin pigment. PPO treatment will adversely affect H&E staining, causing increased basophilia and decreased eosinophilia.

Of the techniques available for formalin pigment removal, Garvey’s APE was by far the most efficient. Alcoholic picric acid was disappointing. This finding is at odds with other investigators recommending alcoholic picric acid as the removal method of choice (1,3,12). Waldrop and coworkers (10) found that the critical alcohol concentration of the saturated alcoholic picric acid solution for complete removal of formalin pigment lies between 90% and 95%. It is feasible that picric acid treatment would be improved if the recommendations of Waldrop and coworkers (10) are followed.

Ammoniacal ethanol solutions have also been successfully used to remove formalin pigment (2,11), and Tseng (13) recommended 30 min of treatment in a solution of 30 mL of acetone and 9 mL of 28% ammonium hydroxide.

A matter of concern is the number of studies that use PPO as a definitive procedure for the identification of melanin (14). The current study shows that, apart from formalin pigment, bile, neural, and dermal melanins are also removed. PPO will also remove, to some degree, lipofuscin and iron. It is important to include Garvey’s APE or alcoholic picric acid treatment to exclude formalin (or acid hematin) pigment when considering the nature of any pigment since, apart from malarial pigment, no other known pigments are removed.

Conclusions

The formalin pigment resulting from FAA fixation can be bleached with PPO. Garvey’s APE is recommended for the routine removal of this pigment.