Minimum Formalin Fixation Time for Consistent Estrogen Receptor Immunohistochemical Staining of Invasive Breast Carcinoma

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Abstract

To identify the minimum time necessary for consistent immunohistochemical estrogen receptor (ER) results in our laboratory, we evaluated results in timed fixation blocks and cases with disparate and similar needle core biopsy and partial mastectomy specimens. Tissue sections of 24 ER-positive, invasive breast carcinomas were fixed for 3, 6, 8, and 12 hours and 1, 2, and 7 days. ER values were quantified using the Q score (0-7).

In timed fixation blocks, the mean Q score per block was 2.46 for blocks fixed for 3 hours, 5.75 for blocks fixed for 6 hours, and 6.70 for blocks fixed for 8 hours (P < .001). The difference between the case maximum and mean block Q scores was a plateau of almost 0 at 6 to 8 hours of formalin fixation. For needle core biopsy specimen fixation times, the means for specimens with ER-disparate and ER-similar results were 1.2 and 6.3 hours, respectively (P = .01). The minimum formalin fixation time for reliable immunohistochemical ER results is 6 to 8 hours in our laboratory, regardless of the type or size of specimen.

Immunohistochemical analysis is the standard detection method for evaluating estrogen receptor (ER) expression levels in invasive breast carcinoma cells. Consistent ER results are important because they are integral in clinical therapeutic decisions.1-3 The application of heat antigen retrieval pretreatment improves ER staining.3-5 A threshold in the amount of heat energy applied during this step can reduce or eliminate staining variations caused by irregular formalin fixation.4-7 Most studies have focused on issues pertaining to antigen retrieval of carcinomas that were fixed for 24 hours or longer.8,9

Prolonged formalin fixation is rarely a problem in our laboratory. Rapid turnaround times requested by clinicians result in minimal fixation. Most specimens were processed the same day they were excised, allowing only several hours of fixation. Several cases of disparate ER results between needle core specimens received late in the afternoon and the subsequent partial mastectomy specimen prompted us to question whether a threshold of insufficient fixation had been crossed.

We studied relationships between short formalin fixation times and ER results and identified the minimum formalin fixation time to yield consistent ER results in our laboratory.

Materials and Methods

The study had 2 parts.

Part 1: Timed Fixation Tissue Blocks

In part 1 of the study, we evaluated ER levels using timed fixation tissue blocks from 24 large carcinomas that had strongly ER-positive results (stained nuclear area, >90%...
Q score, 6 or 7) in corresponding needle core biopsy specimens. We retrieved the carcinomas from the operating room immediately when they were removed from the patients. Tissue sections from the periphery of the carcinoma that included adjacent normal breast parenchyma were placed simultaneously in 10% neutral-buffered formalin. None of the patients had undergone neoadjuvant therapy. All carcinomas were index neoplasms and from the initial resection; none were recurrences. Seventeen neoplasms were ductal (not otherwise specified) carcinomas, 6 were lobular carcinomas, and 1 was a tubular carcinoma.

The tissue sections were removed after fixing for precisely 3, 6, 8, 10, and 12 hours and 1, 2, and 7 days. The tissue sections were temporally stored in 100% cold ethanol until transfer to the automated tissue processor instruments. The blocks were fixed for an additional 90 minutes in heated (40°C) 20% formalin on the tissue processor before being placed in dehydration solutions. Tissue sections 3 to 4 µm thick were placed on charged slides and stored in a refrigerator until they were stained immunohistochemically.

ER staining was assessed semiquantitatively by using the Q score method. This method incorporates intensity and distribution of reactivity. Intensity was scored as follows: 0, negative (no staining of any nuclei at high magnification); 1, weak (staining visible only at high magnification); 2, moderate (staining readily visible at low magnification); or 3, strong (staining strikingly positive at low magnification). The proportion of stained cells was scored as follows: +0, 0%; +1, 1% to 25%; +2, 26% to 50%; +3, 51% to 75%; or +4, >75%. Intensity and proportion of stained cells were added for the Q score, which had a range of 0 to 7.

Part 2: Needle Core Biopsy Specimens

The second part of the study evaluated the clinical impact of the shortened fixation time for needle core biopsy specimens on immunohistochemical ER results. Needle core biopsy specimens from 9 patients with disparate results (ER-negative needle core biopsy specimens and ER-positive partial mastectomy specimens) were identified retrospectively from the files of William Beaumont Hospital, Royal Oak, MI, for the period July 1, 1999, through December 30, 2001. The control group was 36 randomly selected, needle core biopsy specimens with ER values that were similar in the needle core biopsy and resection specimens. Twelve of the 36 (33%) control group needle core biopsy specimens had invasive carcinoma with a stained nuclear area of less than 10% (ER-negative), 12 (33%) had invasive carcinoma with a stained nuclear area of 20% to 50% (ER-positive, low), and 12 (33%) had invasive carcinomas with a stained nuclear area of more than 80% (ER-positive, high).

All needle core biopsy specimens were unfixed when they arrived in surgical pathology, where they were immediately accessioned and placed into formalin. An approximate fixation time for the needle core biopsy specimens was recorded in each case by using the accession time and the tissue processor start time. A CAS 200 Image Analyzer (Becton Dickinson, Elmhurst, IL) was used to quantify the stained nuclear area of invasive carcinoma cells compared with the total nuclear area.

Immunohistochemical Analysis

Slides from every tissue block were stained immunohistochemically using 2 procedures that differed only in the length of time for antigen retrieval. The standard procedure used 40 minutes of antigen retrieval with a 0.001-mol/L concentration of EDTA buffer (pH 8) in a 95°C water bath. The second immunohistochemical staining procedure used 25 minutes, instead of 40, for antigen retrieval. The second staining procedure was used to amplify any possible signal alterations induced by the length of formalin fixation.

After cooling for 20 minutes on the counter, the slides were removed from the antigen retrieval containers, washed with tap water, and incubated with 3% hydrogen peroxide for 15 minutes. The slides were transferred to an autostainer (DAKO, Carpinteria, CA) in which the primary ER antibody (clone 1D5, 1:50 dilution; DAKO) was incubated over the slides for 1 hour. After washing, the chromogenic components of the DAKO Envision system were used with diaminobenzidine. The slides were counterstained with methyl green, dehydrated, and coverslipped using synthetic mounting media.

Statistical analysis was performed using the Systat computer program (version 10, SPSS, Chicago, IL). Mean values were compared by using the paired t test. Analysis of variance was used to compare differences in the mean Q score values of the 2 protocols. Linear regression was used to compare the difference in immunohistochemical ER staining in needle core biopsy specimens and resection specimens with the fixation period for the needle core biopsy specimens.

Results

Timed Fixation Tissue Sections

The maximum Q score in each case was 7 in 20 (83%) of 24 cases and 6 in 4 cases (17%). The mean Q score with 40 minutes of antigen retrieval was 2.46 in sections fixed for 3 hours, 5.75 in sections fixed for 6 hours, and 6.70 in sections fixed for 8 hours. The differences in the mean Q scores for sections fixed for 3, 6, and 8 hours were significantly different (P < .001). The mean Q scores obtained with 25 minutes of antigen retrieval were lower but showed trends...
Differences between case maximum and individual timed block Q scores were plotted for each timed tissue section. A plateau, or the case maximum Q score, was reached after 6 to 8 hours of fixation.

**Needle Core Biopsy Specimens**

The mean values for carcinomas in needle core biopsy specimens and the associated resection specimens were 61.3% (range, 2%-98%; SD, 29.6%) and 49.7% (range, 1%-96%; SD, 36.5%), respectively. The mean difference in ER values between the 2 specimens was 16 (range, 1%-55%; SD, 14.8%).

The mean fixation time for needle core biopsy specimens was 5.1 hours (range, 0.8-9.7 hours; SD, 2.8 hours). There was a significantly larger difference in ER results between needle core biopsy specimens and associated resection specimens with shorter fixation periods for needle core biopsy specimens. The mean fixation time for the 9 needle core biopsy specimens with disparate results for ER was 1.2 hours; in the 36 needle core biopsy specimens with ER results similar to those for the resection specimens, it was 6.3 hours ($P = .01$).

**Discussion**

We found that consistent ER results were obtained from tissue sections that had been fixed for 6 to 8 hours. This result is similar to the optimal formalin fixation time of 8 to 36 hours for adequate histochemical and immunohistochemical results reported by authors. One group found that

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**Table 1**

Formalin Fixation Times and Estrogen Receptor Staining With Standard, 40 Minutes of Antigen Retrieval Pretreatment

<table>
<thead>
<tr>
<th>Formalin Fixation Time</th>
<th>Mean Q Score (Range)</th>
<th>Mean Difference in Q Score (Range) $^*$</th>
<th>$P^†$</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 h</td>
<td>2.46 (0-6)</td>
<td>4.36 (1-7)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>6 h</td>
<td>5.75 (2-7)</td>
<td>1.14 (0-4)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>8 h</td>
<td>6.70 (5-7)</td>
<td>0.04 (0-1)</td>
<td>.791</td>
</tr>
<tr>
<td>10 h</td>
<td>6.70 (5-7)</td>
<td>0.08 (0-1)</td>
<td>.791</td>
</tr>
<tr>
<td>12 h</td>
<td>6.70 (5-7)</td>
<td>0.04 (0-1)</td>
<td>1.000</td>
</tr>
<tr>
<td>1 d</td>
<td>6.70 (5-7)</td>
<td>0.04 (0-1)</td>
<td>1.000</td>
</tr>
<tr>
<td>2 d</td>
<td>6.70 (5-7)</td>
<td>0.04 (0-1)</td>
<td>.625</td>
</tr>
<tr>
<td>7 d</td>
<td>6.60 (5-7)</td>
<td>0.12 (0-1)</td>
<td>—</td>
</tr>
</tbody>
</table>

$^*$ Case maximum minus block.

$^†$ Compared with adjacent block fixed for a longer period.

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**Image 1** Fixation, 3 h; antigen retrieval, 40 min.

**Image 2** Fixation, 6 h; antigen retrieval, 40 min.

**Image 3** Fixation, 8 h; antigen retrieval, 40 min.
tissue sections fixed for less than 6 hours yielded immunohistochemical results that resembled ethanol-fixed rather than formalin-fixed tissue.25

Fixatives halt the temporal changes of autolysis, microorganism-induced alterations, and soluble-molecule diffusion in tissues. Aqueous, completely dissolved formaldehyde, termed formalin, fixes tissues by numerous immediate and prolonged chemical reactions. Formaldehyde immediately produces reactive hydroxymethyl groups on amino acids that cross-link proteins and large molecules.10,26,27 The rate of formaldehyde tissue fixation is slow. Plots of carbon 14–formaldehyde binding using 1.6-µm-thick fresh tissue sections show that the subtotal binding plateau occurs at approximately 24 hours and saturation at approximately 36 hours of fixation.12,28 The rate of tissue penetration by formaldehyde is relatively fast, approximately 1 mm/h, owing to its diffusion coefficient of 0.79.12,29

Permeation is not fixation. A common misconception is that permeation is equivalent to fixation. Formaldehyde is hydrated rapidly to form methylene glycol when dissolved in water.12,29 The equilibrium between methylene glycol and aqueous formaldehyde is strongly in the direction of methylene glycol. The covalent chemical reaction of formaldehyde and tissue molecules removes aldehydes from solution and permits additional formaldehyde to form from the dissociation of methylene glycol.12 Conversion of methylene glycol to formaldehyde occurs at a pace that is measured in hours, referred to as a clock reaction.12,30-32 The conversion of methylene glycol to formaldehyde is the rate-limiting step of formaldehyde fixation. For a sufficient number of aldehydes to be made available to initiate the cross-linking chemical reaction and complete the fixation process, 24 hours is necessary, regardless of the specimen size, tissue section thickness, or formaldehyde volume.10,12,24,33 Higher temperatures increase the aldehyde-mediated fixation reaction rate and secondarily cause a minimal shift in the methylene glycol–formaldehyde equilibrium reaction. However, the time gained from higher temperatures is marginal given the slow pace of the equilibrium clock reaction. These chemical reaction rates and minimum formalin fixation times equally affect larger breast specimens and needle core biopsy specimens.

Many laboratories, including our own, predominantly use needle core biopsy specimens to assess the level of ER expression by invasive carcinoma. The smaller dimension of needle core biopsy specimens causes them to harden more quickly than resection specimen tissue sections. The fast pace of tissue hardening in small specimens is caused by alcohol-mediated dehydration-type fixation.34 The rate of equilibrium at which methylene glycol is converted to formaldehyde is constant, regardless of the specimen size. Needle core biopsy specimens fix at a pace identical to that for tissue sections from partial mastectomy specimens. Review of the cases of disparate ER results between needle core biopsy specimens (ER-negative) and resection specimens (ER-positive) at our institution revealed that all of the needle core biopsy specimens had been fixed for less than 3 hours before being loaded onto the tissue processors. Many laboratories, including our own, use needle core biopsy specimens to assess immunohistochemical ER expression of invasive carcinoma. The smaller dimension of the needle core biopsy specimens may cause them to harden quickly owing to alcohol-induced dehydration-type fixation. The needle core biopsy specimens in this study with ER results disparate from those for the mastectomy specimens constituted fewer than 1% of the needle core biopsy specimens evaluated at our institution, which is similar to the high degree of correlation reported by other authors.35-41
The importance of minimum formalin fixation times and properly fixed tissue are not novel topics. Authors repeatedly have made these points in the context of good histologic sections, consistent and accurate immunohistochemical results, and optimum immunohistochemical image ER values.

Almost all of the tissue sections in the timed fixation part of the study had ER staining levels above the clinically relevant “positive” immunoreactivity threshold, regardless of the quantifying method or the scoring system used. This could be interpreted as suggesting that increased attention to tissue section fixation times is unwarranted because the gain in ER staining was of no or minimal clinical importance. However, this conclusion would be erroneous. ER staining was well above the threshold for a positive result, including in the minimally fixed tissue sections because of the bias of our case selection. We used only strongly and diffusely ER-positive cases so we could evaluate the amount of ER staining lost owing to shorter fixation times. We did not evaluate cases of borderline ER-positive or ER-negative carcinomas. The clinical impact of too short a fixation time was demonstrated in the second part of the study in which cases with discordant ER-negative needle core biopsy specimens and ER-positive resection specimens were studied.

We found that tissue sections need to be fixed for 6 to 8 hours before being loaded onto tissue processors for consistent and reproducible ER stains in our laboratory, regardless of the length of antigen retrieval pretreatment for immunohistochemical analysis or specimen size. The minimum fixation time for accurate immunohistochemical analysis of ER expression probably is longer than the time needed to harden tissues for cutting. These results should be evaluated in the context of the daily workflow and the practices of individual pathology laboratories.

References


