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The Effect of 10 % Buffered Formalin Fixation Duration on The Quality Of Hematoxylin Eosin (HE) Staining in Tissue Suspected of Breast Cancer

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Abstract

Breast cancer is a malignant condition that is a result of atypical cell division in breast tissue. Histopathological examination plays an important role in providing an accurate diagnosis. Fixation is the single most important factor in the initial procedure of histopathologic examination. This study aimed to determine the optimal fixation duration of 10% buffered formalin against suspected breast cancer tissue. This study used an analytical descriptive design, tissue samples suspected of breast cancer were fixed for 1 day, 3 days and 7 days. The research was conducted at the Anatomical Pathology Laboratory of Dr. Saiful Anwar Hospital, East Java Province, in May 2024 with 9 samples and 2 repetitions. Hematoxylin Eosin staining was carried out automatically using a Gemini ASS Automated Slide Stainer. The positive control used appendix tissue samples stained with Hematoxylin Eosin. The staining of quality assessment data was tested with the Kruskal Wallis test shows sig. value of 0.000 (< 0.05) was obtained, indicating a significant difference between 1 day and 3 days and between 1 day and 7 days. The 3-day and 7-day fixation treatments show the best results for staining.

INTRODUCTION

Breast cancer is a malignant condition defined by abnormal cell division in the breast tissue, which can stem from the ductal or lobular epithelium. This malignancy can arise from the epithelial cells lining the ducts (ductal) or lobules (lobular) within the breast. Breast cancer can develop without exhibiting obvious symptoms and is often identified through routine screening (Ningrum and Rahayu, 2021). The emergence of cancer cells can be triggered by genetic mutations resulting from DNA damage in otherwise normal cells. The BRCA genes, integral components of the DNA, are crucial for regulating cellular growth to maintain normal physiological processes. However, under specific circumstances, these BRCA genes can mutate into BRCA1 and BRCA2 variants. Such mutations impair their functionality in regulating cell growth, which may result in uncontrolled cellular proliferation. Consequently, a woman who inherits a mutated BRCA gene experiences a markedly elevated risk of developing breast cancer (Suryani, 2020).

Data from the Global Burden of Cancer (GLOBOCAN) shows that in 2020, the prevalence of breast cancer in Indonesia reached 396,914 cases. A study on the incidence of breast cancer conducted from 2018 to 2020 at the Anatomical Pathology Department of Saiful Anwar Hospital in Malang recorded 542 cases of breast cancer. There were 243 cases (45%) in 2018, 270 cases (50%) in 2019, and 29 cases (5%) from January to March 2020 (Ervina et al., 2021).

Histopathological examination is an essential component in providing precise diagnoses for breast cancer cases. It enables doctors to take appropriate medical action and administer the correct treatment. This examination is routinely conducted on all tissue samples sent to the anatomical pathology laboratory. Proper tissue handling ensures the production of high-quality slides, facilitating thorough pathological assessment (Khristian and Inderiati, 2017).

In histopathological examination, the hematoxylin and eosin (HE) staining method is widely utilized as a histological stain. It is known for being user-friendly, easily automated, and adept at distinguishing various tissue structures. Hematoxylin imparts a blue-black color to the cell nucleus, thereby emphasizing clear intranuclear details, while eosin stains the cell cytoplasm and most connective tissue fibers in varying shades of pink, orange, and red (K. S. Suvarna et al., 2018).

One of the critical factors that affect the quality of tissue processing slides is the process of fixation, tissue processing, and tissue staining. Fixation, which is the initial stage of tissue processing, is crucial for producing histopathological slides that can be properly analyzed (K. S. Suvarna et al., 2018). The fixation solution commonly used in anatomical pathology laboratories is 10% buffered formalin. This solution is the primary standard in pathology labs because it is readily available, simple to use, and has a pH level that is close to neutral. The advantages of this fixation solution include its near-neutral pH, the ability to be stored in large quantities, and its long shelf life. The downside of this solution is its long fixation time, which ranges from 12 to 24 hours (Agustin, 2021).

Currently, some healthcare facilities must send anatomical pathology specimens to referral hospitals. Due to distance and shipping time, surgical tissue samples need to be fixed first using 10% buffered formalin. The process of collection and transportation causes variability in the fixation duration between different healthcare facilities. This variation in fixation time leads to differences in the quality of hematoxylin and eosin staining. This issue affects the overall histopathological examination process. One type of sample affected is for histopathological examination of breast cancer. Therefore, it is necessary to conduct research on the fixation duration in the process of using 10% buffered formalin. The aim of this study was to analyze the effect of varying fixation durations using 10% buffered formalin on the quality of histopathological slides of breast cancer tissue stained with hematoxylin and eosin (HE).

METHOD

This study uses a descriptive analytical design to describe and determine the effect of fixation duration with 10% buffered formalin on the quality of Hematoxylin and Eosin staining. This is done after breast cancer suspected tissue samples are fixed for 1 day, 3 days, and 7 days (Hikmawati, 2020).

The research was carried out in the Anatomical Pathology Department of RSUD Dr. Saiful Anwar, located in East Java Province. The study received ethical approval for sample collection from the same institution, reference No: 400/043/K.3/102.7/2024. Tissue specimens suspected of containing breast cancer were analyzed utilizing Hematoxylin and Eosin (H&E) staining techniques.

The samples were obtained using purposive random sampling, in which tissue samples diagnosed with breast cancer were randomly selected from cases that met the inclusion criteria. A total of nine samples, each subjected to various fixation durations, were included in this investigation. Each procedure was conducted in duplicate, and observations were made across three distinct fields of view.

The inclusion criteria for the tissue samples mandated that they exhibited characteristics of solid, elastic tissues with well-defined boundaries, as determined by an initial diagnosis from a physician. H&E staining was automated via the Gemini Automated Slide Stainer (Gemini ASS), which required the setup of several components, including staining chambers, xylene, 80% and 96% ethanol solutions, cotton, filter paper, glass slides, cover slips, hematoxylin dye, eosin dye, Canada balsam, and labeling materials.

The preparation method involved the following steps: slides were subjected to a three-step deparaffinization process in xylene, each step lasting 10–15 minutes. Subsequently, samples were immersed twice in 96% ethanol for 5 minutes per immersion, followed by thorough washing with running water to eliminate any residual alcohol. The specimens were then treated with hematoxylin dye for a duration of 7–10 minutes, followed by washing with running water until the excess dye was no longer visible. Following this, the slides underwent decolorization with hydrochloric acid (HCl) twice. They were subsequently rinsed with water for 3–5 minutes and then immersed in eosin dye. After staining, the slides were washed again with running water, followed by immersion in 80% and 96% ethanol solutions for 2 minutes each. Finally, the slides were subjected to a rinse in running water and dried using tissue.

For positive controls, appendix tissue samples were used, which were also stained with H&E. The criteria for assessing H&E staining are outlined in Table 1. The data obtained from the staining assessments were analyzed using the Kruskal-Wallis statistical test with SPSS version 26.0, maintaining a confidence level of 95%.

Table 1. Table of staining assessment criteria

No	Structure	Description	Nominal Scale
1	Nucleus	The nucleus cannot be identified	1
		Unclear cell nucleus	2
		Poorly defined cell nucleus	3
		Well-defined cell nucleus	4
2	Cytoplasm	The cytoplasm cannot be identified	1
		Unclear cytoplasm	2
		Poorly defined cytoplasm	3
		Well-defined cytoplasm	4
3	Uniformity of colour	Uniformity of colour cannot be identified	1
		The colour on the slides is not uniform.	2
		The uniformity of colour on the slides is inadequate	3
		The colour on the slides is uniform.	4

RESULTS AND DISCUSSION

Based on Table 2, the characteristics of patients with suspected breast cancer tissue used in the study are classified according to gender, age, and histopathological grading.

Table 2. Table of characteristics by age and histopathological grading

Variable	Number (n)	Precentage (%)
Gender		
Male	0	0
Female	9	9
Age (year)		
30-39	1	11
40-49	4	44
50-59	3	33
60-70	1	11
Histopathological Grading		
Grade 1	1	11
Grade 2	3	33
Grade 3	5	56

Table 2, the majority of breast cancer tissue samples, accounting for 44%, are derived from patients within the age group of 40 to 49 years. Furthermore, a significant proportion of these samples, specifically 56%, are classified as grade 3. This grading is performed to evaluate the morphological characteristics of cells suspected to be associated with the tumour tissue. The histological grading of breast cancer is correlated with the tumour's aggressiveness and its overall prognosis (Agustin, 2021). Histological evaluation of breast cancer includes the examination of three standardized morphological characteristics: the count of mitotic figures, the degree of nuclear pleomorphism, and the presence of tubule formation. These three characteristics collectively underpin the Nottingham Grading System, which aids in the characterization and prognosis of breast cancer (Jaroensri et al., 2022). The macroscopic image of the tissue of suspected breast cancer used as a research sample, has a solid greyish-white criterion with a firm boundary (Figure 1).

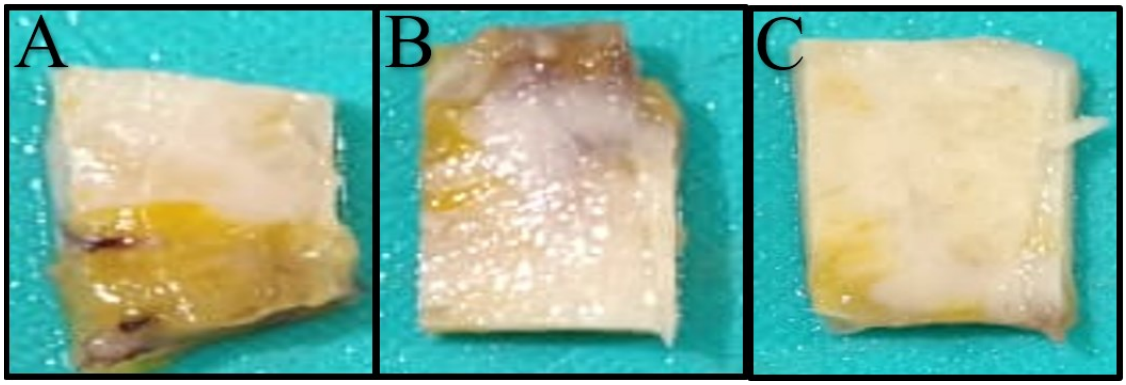

Figure 1. Macroscopic observation of the tissue after fixation for 1 day (A), 3 days (B), and 7 days (C).

The microscopic examination of suspected breast cancer tissue specimens, which were fixed in 10% buffered formalin for durations of 1, 3, and 7 days, was performed using Hematoxylin and Eosin staining. The staining process was carried out utilizing the Automated Slide Stainer Gemini (ASS Gemini). Observations were conducted at a magnification of 40x, systematically analyzing three distinct fields of view across a total of nine tissue specimens. The findings were interpreted by an expert in Anatomical Pathology and are illustrated in Figure 2

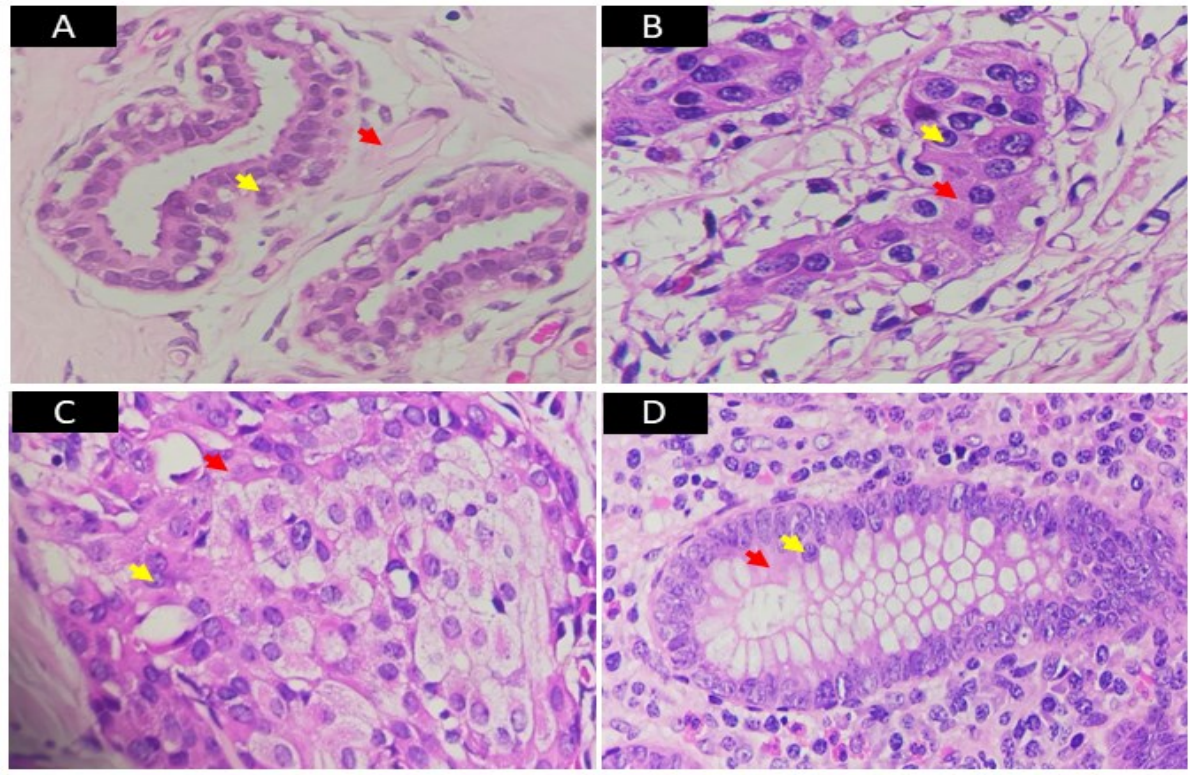

Figure 2. Microscopic analysis of suspected breast cancer tissue with different 10% buferred formalin fixation time treatment. 1 day (A); 3 days (B); 7 days (C); positive control (D) Using HE 400x staining, and qualitative analysis. Nucleus and cytoplasmic depictions (yellow arrows); connective tissue (red arrow).

Figure 2 indicate that the quality of specimens observed under a 40x objective lens for suspected breast cancer tissue, preserved in a 10% buffered formalin fixation solution, improved significantly with fixation durations of 3 and 7 days. Specifically, samples subjected to 3 and 7 days of fixation exhibited well-defined cellular characteristics: the nuclei were distinctly stained blue, the cytoplasm displayed a red hue, and the connective tissue structures were clearly visible, demonstrating enhanced color uniformity. In contrast, specimens fixed for only 1 day showed poorly defined staining in the cell nuclei, cytoplasm, and connective

tissue. Thus, the 3-day and 7-day fixation periods resulted in superior color consistency compared to the shorter 1-day fixation period.

Table 3. Evaluation results of H&E staining quality under varying treatment conditions

Treatment (days)	Mean ± Standard Deviation		
	Nucleus	**Cytoplasm**	**Uniformity of colour**
Control	4± 0,00a	4 ± 0,00a	4± 0,00a
1	1,2± 0,44b	1,2 ± 0,44b	1,2 ± 0,44b
3	3,8± 0,44a	3,4± 0,52a	3,4± 0,52a
7	3,9 ± 0,33a	3,9 ± 0,33a	3,9± 0,33a

The results of the observation of the assessment of the quality of tissue preparations for suspected breast cancer that were fixed using a 10% buffered formalin with a fixation duration of 1, 3 and 7 days showed an excellent microscopic picture at the fixation duration of 3 and 7 days, where the cell nucleus was blue, the cytoplasm was red and the cell colour uniformity was clear. This is shown by the results of the assessment on the average cell nucleus staining of 3.8, the average cytoplasmic staining of 3.4 and the average colour uniformity of 3.4. Good staining results were obtained at a fixation duration of 3 days to 7 days because the absorption process of 10% buffered formalin into the cell was optimal so that the absorption of Hematoxyslin and Eosin dyes became adequate (Suvarna et al., 2018).

Fixative solutions commonly used in histopathological examinations, such as 10% buffered formalin, act through a well-defined mechanism. The initial phase of fixation involves the infiltration of formalin into the cellular architecture, where it forms methylene hydrate within approximately 6 hours. This hydrate subsequently reacts with cellular proteins, forming hydroxymethyl cross-links that stabilize tissue morphology (Suvarna et al., 2018). Complete fixation is achieved within approximately 24 hours, and further stabilization occurs with prolonged exposure. This mechanism explains why fixation durations of 3 to 7 days result in better staining quality: adequate cross-linking ensures optimal preservation of nuclear and cytoplasmic components, allowing for precise uptake of hematoxylin (which targets acidic nuclear structures) and eosin (which stains basic cytoplasmic structures).

However, excessively prolonged fixation may lead to over-hardening of tissues due to excessive cross-linking, complicating microtome sectioning and potentially reducing dye penetration (van Seijen et al., 2019). Thus, a fixation window of 3 to 7 days is ideal for balancing tissue preservation and staining efficiency. In addition to fixation duration, several pre-analytical factors significantly influence histological outcomes. These include the thickness of paraffin ribbons, which affects light penetration and image resolution, as well as proper deparaffinization to remove residual wax prior to staining. Inadequate deparaffinization or excessive dehydration with alcohol can result in uneven staining. The pH of the staining solutions is also crucial: hematoxylin, a basic dye, selectively binds to acidic nuclear material, while eosin, an acidic dye, stains basic cytoplasmic and connective tissue components (Musyarifah et al., 2018).

The use of positive control tissue, such as the appendix, is essential for quality assurance in H&E staining. Appendix tissue is favored because it contains a variety of structures—including epithelial cells, smooth muscle fibers, and lymphoid tissue—allowing for comprehensive evaluation of staining consistency and reactivity (Garrido et al., 2021; Suvarna et al., 2018).

Overall, these findings emphasize the importance of standardized fixation protocols in histopathology. Fixation durations of 3 to 7 days in 10% buffered formalin are recommended to ensure optimal H&E staining results in breast cancer tissue specimens, thus enhancing diagnostic accuracy and supporting better clinical decision-making.

CONCLUSION

Fixation duration significantly affects the quality of hematoxylin and eosin (H&E) staining in breast cancer tissue. Fixation with 10% buffered formalin for 3 to 7 days produced optimal slides with clear nuclei, distinct cytoplasm, and uniform color, while 1-day fixation resulted in poor staining. A fixation period of 3–7 days is recommended to ensure consistent staining quality and improve the accuracy of histopathological diagnosis.

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