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COMPARISON CYTOLOGICAL STAINING RESULTS OF PLEURAL EFFUSION AFTER DELAYED TIME WITHOUT FIXATIVE AND WITH ALCOHOL FIXATIVE

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ABSTRACT

The delay in the examination of pleural effusion fluid may lead to damage to the specimens. To minimize this damage, the addition of fixative solutions is often employed, one of which is 50% alcohol. Therefore, this study aims to determine the effectiveness of using 50% alcohol in fixing smears of pleural effusion fluid that are delayed in examination, as well as to assess the morphological quality of cells in effusion smears stored with and without fixative solutions. The research method is experimental, utilizing a pretest-posttest design. Sampling was conducted using accidental sampling, with specimens categorized into four treatment groups (immediate examination, immediate examination with 50% alcohol fixative, delayed examination without fixative, and delayed examination with 50% alcohol fixative). Smears were stained using the Diff-Quik method. The assessment results were analyzed descriptively and with a paired T-test. In the comparison between immediate without and delayed without, the T-test results showed a significant difference ($p = 0.002$, <0.05). Conversely, in the comparison between immediate and delayed with fixative, no significant difference was found ($p = 0.707$, >0.05). The comparison between immediate without and immediate with fixative showed a significant change ($p = 0.005$, <0.05), while the comparison between delayed without fixative and delayed with fixative was not significant ($p = 0.775$, >0.05). The alternative hypothesis was rejected based on the paired T-test, as no significant difference was found in the delayed samples compared to the samples that were delayed with the addition of 50% alcohol fixative.

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Introduction

Pleural fluid is a common type of fluid for cytological examination. This fluid comes from a membrane called the pleura. This membrane covers the lungs, and between the pleural membrane and the lungs is the pleural cavity, which normally contains 10–20 milliliters of fluid that acts as a lubricant to allow the lungs to move freely during breathing. Cytological analysis of pleural fluid in the Anatomic Pathology laboratory is crucial for early detection of malignancy. The most common cytological analysis method is to prepare conventional pleural fluid smears immediately after the sample arrives (D. Putri 2022; R. G. P. Putri 2022; Saldi, Wahid, and Jumriati 2022)

Cytological examination is used to observe the morphology of body fluids through fixation and staining, followed by reading the results with the aid of a microscope. Cytological analysis of pleural fluid is the first early test performed after pleural effusion is detected, providing a diagnostic yield of 60% (Dila 2023; Hayuningrum 2020). Pleural fluid cytology examination is currently the most specific routine procedure to distinguish malignant and non-malignant pleural effusions(Inderiati and Pratiwi 2021; Oktaviana 2021).Specimen examination is generally performed as soon as possible, on the other hand, delays in processing specimens may occur in the field. Cytology specimens are fixed to prevent drying and changes in cell shape caused by external factors (Erick and Dewi 2017; Ramkita 2022). Other literature states that errors in the pre-analytical stage are as high as 68%, while errors in the analytical stage are around 13%, and errors in the post-analytical stage are around 19%(Syafaat and Safari 2024).The fixation stage is the initial stage of the pre-analytical phase. Delayed fixation can affect cell morphology, which has been proven by the variation in fixation time speed affecting cell morphology in peripheral blood smear preparations (Anita, Santosa, and Farug 2018)

Various fixatives are mentioned in the book Cytohistotechnology (Erick and Dewi 2017), including; Formalin, Denatured alcohol, propanol, isopropanol, 95% alcohol ether, absolute methanol, and also 95-96% alcohol. Most cytology laboratories recommend a 95-95% alcohol solution as the ideal fixative. This fixation produces ideal nuclear properties. This 95-96% alcohol solution is a dehydrated solution that can cause cell shrinkage because it will replace the water in the cells. Using absolute ethanol is also possible, but it is more expensive. According to another theory, adding 95–96% alcohol will make the cells adhere to the slide glass more strongly than placing wet specimens in lower concentrations (Erick and Dewi 2017). Most cytology laboratories recommend a 95-95% alcohol solution as the ideal fixative. This fixation results in ideal core properties (Erick and Dewi 2017).

Cytology examination looks at the morphology of body fluid cells through the process of fixation and staining, then readings with the help of a microscope (Dila 2023). Diff Quick staining is a modified of Romanowsky staining method used to differentiate cells in pathological

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preparations. One of the rapid dyeing techniques for air-dried cytology smears is the diff-quick staining principle, which is used to look at tumor cells and diagnose cell samples (Astuti 2017; Mizan, Damayanti, and Nuroini n.d.).

This study was conducted to determine the comparison of cytological staining results of pleural effusion with and without alcohol fixative delay time at the Anatomic Pathology Laboratory of Karsa Husada Regional General Hospital - Batu. Based on this background, the problem was formulated regarding the effectiveness of using 50% alcohol in fixing pleural effusion fluid smear preparations that delayed examination, as well as the morphological quality of cells in pleural effusion smears stored with and without fixative solution. The aim was to determine the morphological quality of cells in pleural effusion smears fixed with and without fixative solution.

Materials and Methods

Location and Time of Research

This research was conducted at the Anatomic Pathology Laboratory of Karsa Husada Regional General Hospital, Batu City, East Java, from April 26 to May 26, 2025.

Tools and Materials

The instrument used in this study included a microscope, glass slides, cover slips, a centrifuge, Pasteur pipettes, serological tubes, and urine containers. The materials used are: Diff-Quick stain (methanol, eosin, and methylene blue), 50% alcohol, and pleural fluid specimens.

Data Collection Techniques

The sampling technique used accidental sampling where sampling techniques were not planned and carried out as a matter of course(Sudirman et al. 2023).Population in the form of patients diagnosed with pleural effusion with pleural fluid samples sent to the laboratory during the 1-month study period After the specimen enters the analytical phase, it is evaluated by a specialist anatomical pathologist based on agreed-upon criteria. Data was collected through microscopic morphological evaluation of preparations using specific criteria (cell nucleus, cell cytoplasm, background clarity, cell shape, cell type, and red blood cell count). The assessment results are in the form of a master sheet on a point scale and are averaged for each specimen to obtain a representative value for each sample and treatment.

Reasearch Methods

This research is an experimental study using a pretest-posttest design. The measurement results from the pretest will be compared as a control with the measurement results from the posttest to evaluate the effect of the treatment. This design procedure, known as the experimental laboratory study using a pretest-postest design, provides a clear picture of the changes that occur as a result of the treatment administered (Rinaldi and Mujianto 2017)

Data Analysis

The average points obtained for each sample in each treatment were analyzed using the Shapiro-Wilk normality test and the paired T-test to examine the significance of differences between pre-test (control) and post-test (test) treatments. This Shapiro-Wilk test is used on data with fewer than 50 samples (N<50). The Shapiro-Wilk test is performed if the data meets the following criteria: (1) interval or

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ratio scale (quantitative), (2) single data or not yet included in a frequency distribution table, and (3) data comes from a random sample (Nuryadi et al. 2017)

The paired t-test is one of the hypothesis testing techniques that uses non-independent (paired) data. The most common characteristic of paired cases is that one person (the research subject) is given two different types of treatment (Nuryadi et al. 2017)). Paired t-tests were conducted to determine the factors of delay time and the use of alcohol fixative, so the test was performed 2x with different test pairs.

Results and Discussion

Result

After treatment, the samples were then processed in the analytical stage and observed microscopically, with the following results:

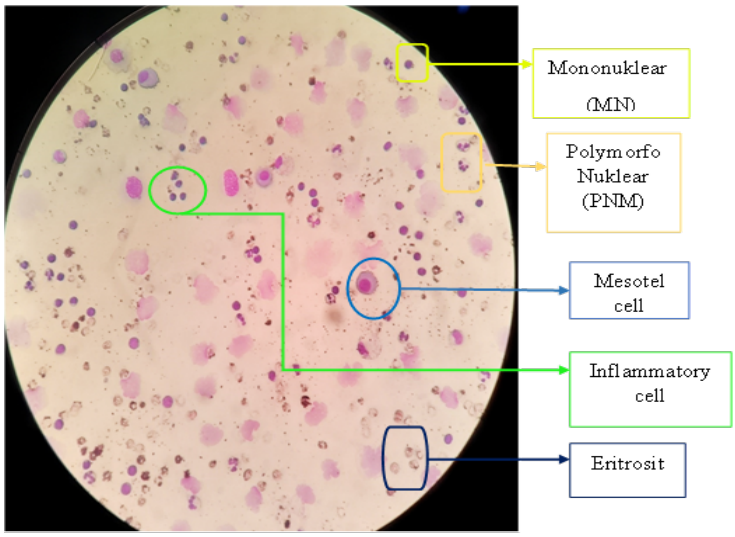

Figure 1 Description of cells in Pleural Effusion with good category, magnification of 400

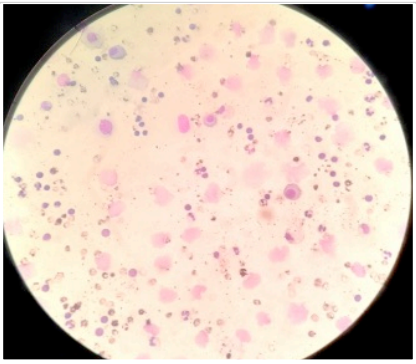

Figure 2 Description of Pleural Effusion Staining Results Good Category

Description of pleural effusion staining results is a good criterion with a clear and bright nucleus and cytoplasm, a clear background and normal cells of shape and size. (Magnification 400×)

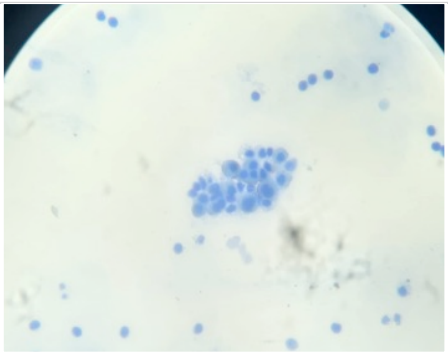

Figure 3 Description of Pleural Effusion Staining Results Good Category

Description The results of pleural effusion staining are good criteria with a bright picture of the nucleus and cytoplasm as well as a clear and observed background of normal cells. (Magnification 400×)

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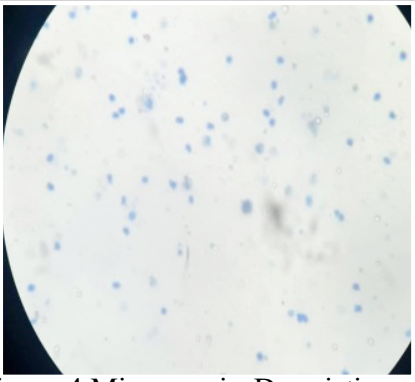

Figure 4 Microscopic Description of Pleural Effusion Category Sufficient

The microscopic picture of pleural effusion preparations is sufficient criteria with the nucleus still quite clear and the nucleus boundary is less observable, the clarity of the background with sufficient values. (Magnification 400×)

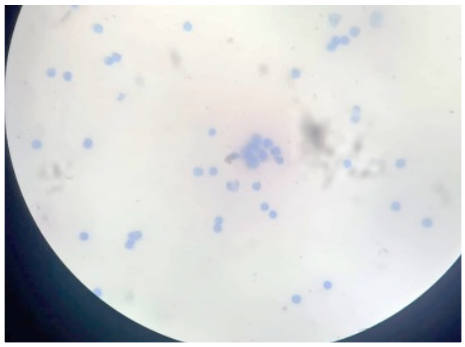

Figure 5 Microscopic Description of Pleural Effusion with Sufficient Background Clarity

The microscopic picture of pleural effusion criteria is sufficient, the background clarity is sufficient, the cytoplasm and cell nucleus are less contrasting but still quite clearly observed than normal cell types. (Magnification 400×)

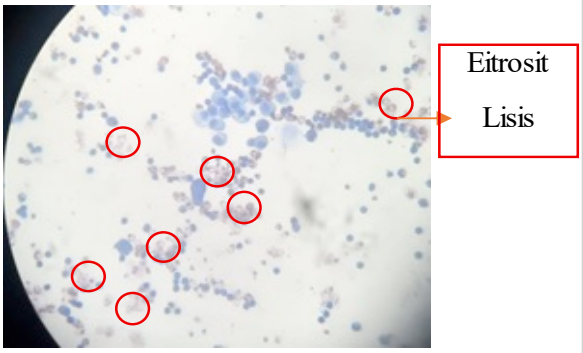

Figure 6 Microscopic image of erythrocyte lysis in pleural effusion with 400 condensing alcohol addition treatment×

The nucleus can still be easily observed with the cytoplasm of the cell appearing smaller but still quite bright and the shape of the cell is still very clear. Lysis occurs quite a lot and erythrocyte cells can no longer be counted in number.

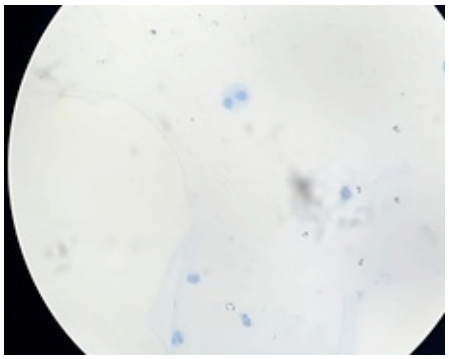

Figure 7 Microscopic Image of 400 Magnification × Pleural Effusion Preparations with a Slightly Cloudy Background on Preparations. U1III.2

Description of delayed staining results without the addition of alcohol, the nucleus and cytoplasm are bright but the limits of the cytoplasm are bright on average, The clarity of the background is also slightly decreased

Statistical Tested

This study evaluated the effect of delay time and the use of alcohol fixative on the quality of cytological staining of pleural effusion fluid using the Diff-Quik method. A total of 12 pleural fluid specimens obtained from the Anatomic Pathology Laboratory of Karsa Husada General Hospital during the period of April 25–May 24, 2025, were divided into four treatment groups: immediate examination without fixative (K1), immediate with fixative (K2), delayed without fixative (U1), and delayed with fixative (U2). Each specimen was duplicated, resulting in a total of 96 slides that were evaluated by a specialist anatomical pathologist. The assessment results for each criterion (on a point scale)

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listed in the staining quality assessment are summed and averaged to obtain a more representative value for each specimen in the specimens where slide preparation was repeated. The results of these calculations are presented in the table below:

Table 1. Results of Pleural Effusion Specimen Staining Assessment with Diff-Quik Stain for Each Treatment

NO SAMPLE	K1	K2	U1	U2
I	19	14	14	12
II	15	15.5	11	12
III	15	14	11	12
IV	19.5	13.5	15	17
V	17	13	13	17.5
VI	15.5	14	17.5	11
VII	16.5	17.5	17	14
VIII	15.5	14	15.5	14.5
IX	18	15	15	16
X	16.5	16	14.5	16
XI	17	14	13.5	15
XII	17	15	12	15

Note. K1: Immediate Examination Without Fixative. K2: Immediate Examination With Fixative. U1: Delayed Examination Without Fixative. U2: Delayed Examination With Fixative

Testing was conducted using the JASP (Jeffreys's Amazing Statistics Program) software version 0.19.3.0 as an aid. The Shapiro-Wilk normality test showed that the data were normally distributed, so the paired T-test was performed. The calculations were performed by pairing the data according to the treatment time factor (immediate and delayed) to determine the influence of the time factor on the examination results, and calculations were also performed by pairing the fixative factor (with and without fixative).

Table 2. Paired T-Test Table for the Effect of Delay Time on Pleural Effusion Fluid Examination, Examined Immediately and Delayed

Measure 1		Measure 2	t	df	p
K1	-	U1	4.026	11	0.002
K2	-	U2	0.386	11	0.707

Note. K1: Immediate Examination Without Fixative. K2: Immediate Examination With Fixative. U1: Delayed Examination Without Fixative. U2: Delayed Examination With Fixative

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Based on the paired T-test, as shown in the table above (Table 4.2 Table of Delayed Time Effect Test), there is a significant difference between the results of cytological staining of pleural effusion fluid that was not fixed in the examination performed immediately and that was delayed for 8 hours without the addition of fixative (p=0.002 <0.05). However, there was no significant difference between the immediate and delayed (8-hour delay) examinations with the addition of fixative (p =0.707 > 0.05).

Table 3. Paired T-Test Table of Fixative Use Effect on Pleural Effusion Fluid

Measure 1		Measure 2	t	df	p
K1	-	K2	3.532	11	0.005
U1	-	U2	-0.293	11	0.775

Note. K1: Immediate Examination Without Fixative. K2: Immediate Examination With Fixative. U1: Delayed Examination Without Fixative. U2: Delayed Examination With Fixative

The results of the paired T-test in the table above (Table 5.5, Table of Fixative Use Effect Test) show a significant difference in samples examined immediately, where K1 (samples examined immediately without fixative) is compared to K2 (samples examined immediately with added fixative), indicating a p-value < 0.05 (p = 0.005 < 0.05). However, in the examination of delayed samples without the addition of fixative (U1), no significant difference was observed, where U1 compared to U2 (delayed examination with added fixative) had a p-value > 0.05 (p = 0.775, t = 0.347).

Discussion

The research findings indicate that delay time has a significant impact on staining quality, particularly for specimens that do not use a fixative. Immediate staining without a fixative provides optimal cell morphology quality, as evidenced by sharp nuclei, bright cytoplasm, and a clear background, The results of this treatment are in line with (Tarigan 2023). This result aligns with the recommendations in the literature "Collection and Processing of Effusion Fluid For Cytopathologic Evaluation," which states that cooling to 4°C is sufficient to maintain the integrity of cytological specimens for several days without the need for fixative (Vinod B Shidam 2022)

The use of 50% alcohol fixative in immediate examination showed good staining quality but was accompanied by the side effect of erythrocyte lysis, which could interfere with the clarity of the slide background. This finding is supported by studies by Prasetyani (2017) and (Vinod B Shidam 2022) which state that alcohol can disrupt cell morphology and cause protein degradation, thereby affecting cytological interpretation.

Delaying examination for 8 hours without fixative still yields preparations suitable for cytological evaluation.

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However, the background clarity parameter shows a decrease, and the cytoplasmic image tends to be more blurred. This indicates that although the core morphology can still be observed, the process of cellular autolysis has begun and may increase over time. Conversely, adding fixative to the suspended samples did not significantly improve quality and even led to further lysis of erythrocytes.

Adding 50% alcohol fixative to delayed specimens did not significantly improve quality. This is due to dehydration from excessive alcohol exposure for too long. Longer contact times also cause reversible bonds, preventing the fixative fluid from detaching from the cells, which leads to shrinkage or crenation (Tasry 2018).As a comparison, a scientific journal titled "Preanalytical Phase in Pleural Fluid Analysis" (2021) mentions that in cases of delayed cytological examination, pleural fluid can remain viable for 48 hours if stored in a refrigerator. However, for cell counts with a 24-hour delay, an anticoagulant like EDTA is necessary, and the sample should be stored at 4°C (Kopcinovic and Culej 2021)

Analysis shows a significant difference between immediate examination without fixative (K1) and delayed examination without fixative (U1) with a p-value of 0.002, indicating that the timing of the examination influences the results. Conversely, no significant difference was found between immediate examination with fixative (K2) and delayed examination with fixative (U2), with a p-value of 0.707, suggesting that the use of fixative does not impact the results within that timeframe. Additionally, there was a significant difference between K1 and K2 ($p = 0.005$), indicating that the presence of fixative affects the outcomes of the immediate examination. However, the comparison between U1 and U2 did not show a significant difference ($p = 0.775$), indicating that the timing of the examination, whether with or without fixative, does not significantly influence the results.

Conclusion

Based on the research findings from 12 pleural effusion fluid specimens, the hypothesis analysis can be summarized as follows:

1. There is a significant difference between specimens examined immediately with fixative and those delayed without fixative, although the overall use of alcohol fixative is considered positive but not significant.
2. Cell morphology is well preserved when using a 50% fixative solution.
3. The best staining quality was found in immediate examination without fixative.
4. Delayed examination with fixative showed results equivalent to immediate examination with fixative.
5. Comparison between immediate examination without fixative and delayed examination without fixative showed a significant difference ($p = 0.002$), while comparison with fixative did not show a significant difference ($p = 0.707$). Additionally, there was a significant change between

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immediate without fixative and immediate with fixative (p = 0.005), while delayed without fixative and delayed with fixative did not show a significant difference (p = 0.775).

Accordingly, alcohol fixsation is effective in maintaning the morphological integrity off cells in pleural effusion smears,especially under delayed examination conditions.

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