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Utilization of 1% of Methylene Blue in Staining Histopathological Preparations at Anatomic Pathology Laboratory

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Abstract

Tissue staining using hematoxylin-eosin (HE) is a standard method of histopathological staining. The tissue staining is hampered when there is no hematoxylin reagent in laboratory. Therefore, other reagents are needed that can replace the use of hematoxylin. Methylene blue is a basic dyes that interact with cell nuclei which has a negative ionic charge of the tissue. It can be used as an alternative nuclei staining. This study aims to evaluate the use of 1% of methylene blue in cell nuclei staining in histopathological preparations. The research sample were 15 pathology preparations which were randomly selected including breast cancer, cervical cancer and ovarian cancer in the bank of sampel at anatomical pathology laboratory of RSUD Dr. Slamet Garut, Indonesia. The experiment showed that the methylene blue dyes yielded "worth" result (40%) and "poorly" result (60%). Further research can be carried out by modifying the pH of 1% of methylene blue reagent so that it can maximize the staining preparations results as good as those using hematoxylin.

Keywords
Cancer, hematoxylin, histopathology, methylene blue

INTRODUCTION

Cancer prevalence is increasing in the world every year. According to the International Agency for Research on Cancer (IARC), the number of cancer patients worldwide increases since 2012. More than14 million new cases of cancer, of which 8 million or more cases, died from cancer. Based on the Ministry of Health of the Republic of Indonesia data in 2018, the prevalence of tumors or cancer in Indonesia shows an increase from 1.4 per thousand populations in 2013 to 1.7 per thousand populations in 2018 (1). In Garut Regency, especially in the anatomic pathology laboratory of RSUD dr. Slamet Garut, there were 189 cancer patients (44.9% of breast cancer patients, 10.5% of cervical cancer, 8.9% of lymph node cancer, 7.4% of colon cancer, 5.8% of ovarian cancer, 4.7% of

thyroid cancer, 3.7% of bone cancer, 2.1% of prostate cancer, 1.05% of parotid cancer (salivary glands), 1.05% of molar pregnancy, 1.05% of cancer of body fluids, and other types of cancers which count up to 9.5%).

Precancerous and cancer is characterized by excessive cell proliferation due to the increase in mitotic cells (2). The increased and abnormal mitosis indicates a damaged tissue of an important feature in precancerous and cancer tests. The identification and quantification of mitotic cells is used in histological examination to support the diagnosis (3). Histological staining has been widely used in pathology (4). Examination of the development of cancer can be conducted microscopically through histopathology examination.

Important stages of histopathological examination starts from fixation, processing, embedding, cutting and staining (5). Tissue staining using the hematoxylin-eosin (HE) is a standard method of histopathological staining. Hematoxylin is a basic dye that is commonly used for staining cell nuclei and provides a bluish tint while eosin yields a pink dye (2). The principle of such coloration is chemical interactions binding between tissue and dyes (4). The tissues with negative or anionic charges are more easily stained with a blackish-blue color with an alkaline staining called basophilic using hematoxylin dyes. Meanwhile, tissue components with a positive or cationic charge (cytoplasm) are more easily colored into pink with an acidic staining called acidophilic using a substance eosin color (7).

The services in the anatomic pathology laboratory are often hampered when the hematoxylin reagent runs out and it takes a long time for the reagent to arrive since the availability of hematoxylin is quite rare. Thus, the process of hematoxylin maturation requires a long time (8). Nevertheless, the examination service in the anatomic pathology laboratory must be done continuously. Therefore, other reagents are needed that can replace the use of hematoxylin in histopathological preparations staining.

The other reagents that can be applied for staining are crystals violet (2, 3, 9), toluidine blue, neutral red, methylene green, and methylene blue (4, 10, 11). Methylene blue is a cationic dye that has a positive ionic charge so that it will interact with cell nuclei that have a negative ionic charge of the tissue (4) Löffler's alkaline of methylene blue is used in histopathologically conjunctival epithelial cells and blue-colored neutrophils and more intense colored nuclei (12). Previous stain with 1% of methylene blue detect oral dysplasia and carcinoma resulted in good validity (11). However, the utilization of 1% of methylene blue for histopathological staining cancer mitosis has not been reported. Therefore, the author aims to apply methylene blue compound in the process of

histopathological preparations as a substitute for hematoxylin in the hematoxylin-eosin staining.

MATERIALS AND METHODS

The tools used in this study were microtome knife, pencil, camera, tissue, label paper, oven, cover glass, object-glass, beaker glass, microscope, microtome, water bath, cassette, tweezers, mold, refrigerator, pipette, and staining jar. The materials used include tissue cuts, 10% of buffered formalin, absolute alcohol, 96% of alcohol, 80% of alcohol, 70% of alcohol, xylol, paraffin, lithium carbonate, Mayer hematoxylin, eosin, blue methylene, tap water, distilled water, and entelan.

This research was conducted in the sitohistotechnology laboratory from July to August 2019. Sample were obtained from 15 pathology preparations and were randomly selected, including breast cancer, cervical cancer and ovarian cancer in the bank of sampel at anatomical pathology laboratory of RSUD Dr. Slamet Garut, Indonesia. The quality assessment of the preparation is carried out by an anatomist pathologist.

The sample was fixed in the second stage with a 10% of formalin buffer solution for 24 hours. The tissue preparation consisted of several stages, including dehydration, clearing, impregnation, embedding and tissue cutting. The dehydration process used alcohol in an oven at a temperature of 65°C–70°C for 45 minutes, starting from 70%, 80% and 95%. The clearing step used xylol I and xylol II, respectively, for 45 minutes in the oven with a temperature of 65°C–70°C. The impregnation step used liquid paraffin for 45 minutes in an oven with a temperature of 65°C–70°C. Furthermore, the embedding step was done to plant the tissue into paraffin molds then it was stored in the refrigerator for 2 hours until it was solidified completely. Lastly, a gross cut with a thickness of 4 microns was done to obtain a network band. The tissue tape was affixed to the glass object and was followed by mounting in a water bath with a temperature of 40°C to obtain histopathological preparations that were ready to be coloured.

Histopathological preparations for the control group used the Mayer hematoxylin-eosin staining method while the experimental group used methylene blue-eosin staining. The blue methylene used 1% of distilled water. The results of staining was analysed under a microscope with a magnification of 400x. An optimal-quality staining as nuclei exhibiting ‘blue hematoxylin’ with chromatin patterns, permit the differentiation of distinct cell type (13). A sub-optimal staining, the nuclei were weakly stained and appear pale (14). The data were categorized based on the quality of the stained nuclei (Chapman category modified), it is classified as “good” if the nucleus was clearly stained and the chromatin was clearly visible;

"worth" if the nucleus was stained but less clear and the chromatin was lacking clearly visible, and it was classified as "poorly" if the cell nucleus is not colored.

RESULTS

The quality of staining cell nuclei using hematoxylin-eosin in five breast cancer preparations has good quality (the cell nuclei were visible with coarse chromatin). The results of the staining showed the presence of mitotic cell nuclei (observed cell nuclei that were three lobes in one cell), as shown in figure 1 (a). The results of staining the cell nucleus with methylene blue-eosin in a breast cancer preparation showed poor staining quality that was characterized by colorless cell nuclei, mitotic cell nuclei and chromatin were not observed, as shown in Figure 1 (b). Meanwhile, the four breast cancer preparations have a pretty good staining quality, the cell nucleus was colored but it was not seen and the chromatin was less clear, as shown in figure 1 (c).

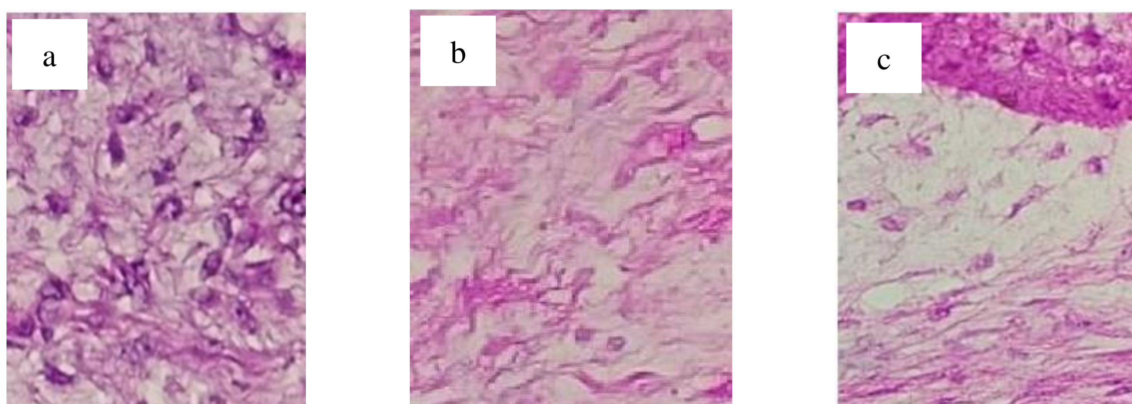

Fig 1. Staining cell nuclei in breast cancer preparations with 400x magnification. (a) Hematoxylin, (b–c) methylene blue

The quality of staining of cell nuclei with hematoxylin-eosin in four cervical cancer preparations showed good staining quality with clear observations of cell nuclei. The appearance of mitotic cell nuclei is shown in figure 2 (a). The results of cell nucleus staining with methylene blue-eosin in a cervical cancer preparation showed a quite good quality. The cell nucleus was stained but it was not seen and chromatin was less clear so that there should be a cell nucleus, as in figure 2 (b). Meanwhile, the four cervical cancer preparations showed poor quality colour which means that the cell nucleus was not stained and chromatin was not observed, as shown in Figure 2 (c).

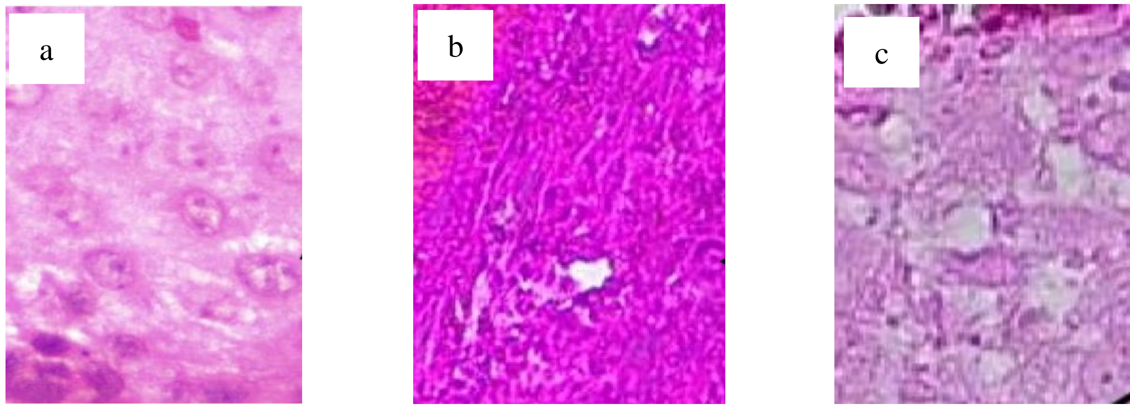

Fig 2. Staining cell nuclei in cervical cancer preparations with 400x magnification. (a) Hematoxylin, (b–c) methylene blue

The results of cell nucleus staining with hematoxylin-eosin in five ovarian cancer preparations showed good staining quality, dye the cell nucleus was stained and observed with coarse chromatin as shown in Figure 3 (a). The results of nucleus cell staining with methylene blue-eosin in four ovarian cancer preparations showed poor quality meaning that the cell nucleus was not stained with chromatin which was not observed, as shown in Figure 3 (b). Meanwhile, the four ovarian cancer preparations showed quite good quality of staining. It means that the cell nucleus was colored but it was not seen, it only looked like a small dot from the cytoplasmic outline so that it could not be seen, as well as the cell nucleus and chromatin nuclei were also not observed, as shown in Figure 3 (c).

The results of the breast cancer, cervical and ovarian cancer staining preparations using hematoxylin-eosin method and methylene blue-eosin in the control group can be seen in Table 2.

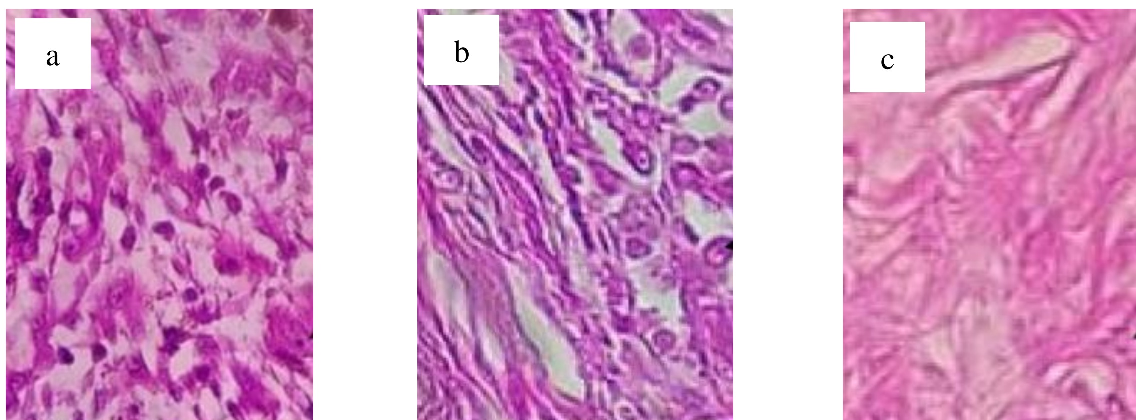

Fig 3. Staining cell nuclei in ovarian cancer preparations with 400x magnification. (a) Hematoxylin, (b-c) methylene blue

Table 2. Results of staining cell nuclei of breast, cervical and ovarian cancer

Dye	Cancer preparation	Quantity (Percentage%)		
		good	worth	poorly
Hematoxyilin	Breast	5 (100%)	0	0
	Servical	4 (80%)	1 (20%)	0
	Ovarian	5 (20%)	0	0
Total		**14 (93%)**	**1(7%)**	**0**
Methylen blue	Breast	0	4 (80%)	1 (20%)
	Servical	0	1 (20%)	4 (80%)
	Ovarian	0	1 (20%)	4 (80%)
Total		**0**	**6 (40%)**	**9 (60%)**

DISCUSSION

Methylene blue is a cationic dye which is also known as basic dyes. It has a positive ionic charge so it will interact with cell nuclei that have a negative ionic charge of the tissue (4). This dye can react with anionic groups as phosphate groups of nucleic acids (DNA and RNA) which is commonly used as nuclear stains. It is a basophilic dye which will bind to acidic tissue. This relates to the concept of tissue staining, where acidic tissue is more easily stained with base or basic dyes (7). Bonding staining to tissue is not different from chemical bonds and the mechanism is similar in other organic bonding components. The bonding of tissue coloring involves ion interactions. It is a combined form and it can bind to tissues and dyes as long as two different ion poles interact properly (4).

1% of methylene blue dye in previous research can detect the dysplasia and oral carcinoma. The validity of methylene blue to detect the dysplasia and carcinoma was increase (11). In this study, 1% of methylene blue dyes in coloring the cell nucleus from histopathological preparations resulted in poor quality cell nuclei with as much as 60% of the cell nucleus was not successfully stained. While, the other experiment has a pretty good staining quality (40%) where the nucleus cells were colored but the mitosis could not be seen from the cell nucleus itself. Meanwhile, by using hematoxylin-eosin staining, 93% of the preparations were well colored and 7% were quite well colored. The nucleus staining in the control group underwent mitosis with three lobes, whereas in the cell nucleus experiment, the shape was visible from the methylene blue staining. It was also visible from outside the cytoplasm so that there should be a cell nucleus division with three lobes yet it was only visible in one lobe. The extracellular pH environment has more acidic pH than normal cells (the pH of normal cells was 7.4 and pH of of tumor cells was 6.5) (15). The charge variation of the breast cancer cells and fibroblasts was good at pH of 2.5–9 (16). Therefore, the interaction between methylene blue dye is greater with breast cancer tissue, so that the quality of the

staining is better compared to cervical cancer and ovarian cancer.

The lack of staining quality by using methylene blue is affected by the composition of the dye and the acidity or pH which is different from hematoxylin. Hematoxylin is mixed with potassium or ammonium alum, sodium iodate, and chloral hydrate (8). Meanwhile, methylene blue was not added with other strong alkaline materials to increase the pH. In acidic condition, the pH of methylene blue will produce a different color that will color the cell nuclei but the protein remains colorless. Cell nuclei that are not successfully stained by methylene blue are also caused by changing in pH because the ionic bonds in dyes are very sensitive to pH. To be conclude, there was no interaction between tissue ions and dye ions. Based on previous research, maximum coloration obtained by methylene blue with loffer formula produced best result, which used potassium hydroxide to increase the pH to color all protein components and nucleic acids. Ultimately, the tissue will appear blue (12).

CONCLUSIONS

Staining preparation with 1% of methylene blue can color the cell nucleus quite good. From the results of this study, it is necessary to modify the pH of the 1% of methylene blue reagent in order to maximize the staining preparations to get good results as well as those using hematoxylin.

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CONFLICT OF INTEREST

There are no conflicts of interest.

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