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The effectiveness of using dye models for small tissue biopsies in the surgical pathology laboratory

Predaporn Nonsiri[1], Potchara Srivilai[2, 3], Supatsorn Onkaew[4], Nontawat Benjakul[4, 5]

[1]Anatomical Pathology Service Unit, Vajira Hospital, Faculty of Medicine Vajira Hospital, Navamindradhiraj University, Bangkok 10300, Thailand

[2]Faculty of Medical Science, Naresuan University, Phitsanulok, 65000, Thailand

[3]Division of Pathology, Thammasat University Hospital, Pathum Thani 12120, Thailand

[4]Department of Anatomical Pathology, Faculty of Medicine Vajira Hospital, Navamindradhiraj University, Bangkok 10300, Thailand

[5]Vajira Pathology-clinical-correlation Target Research Interest Group, Faculty of Medicine Vajira Hospital, Navamindradhiraj University, Bangkok 10300, Thailand

Abstract

Introduction. Losing of small tissues during tissue preparatory steps may seriously affect pathological diagnostic performance. Using an appropriate tissue marking dye could be an alternative solution. Therefore, the aim of the study was to find a suitable tissue marking dye to enhance the observable ability of various types of small-size tissues during several steps of tissue preparation.

Material and methods. Various small-size samples of various organs and tissues (0.2 to 0.3 cm), including breast, endometrial, and cervical tissue, stomach, small and large intestine, lung, and kidney, were marked with different dyes such as merbromin, hematoxylin, eosin, crystal violet, and alcian blue prior to tissue processing step and their colored-observable ability was evaluated by pathology assistants. Moreover, the diagnostic interfering effect of each tissue marking dye was determined by pathologists.

Results. Merbromin, hematoxylin, and alcian blue increased the colored-observable ability of small tissue samples. We suggest using hematoxylin as a tissue marking dye over merbromin and alcian blue because of less toxicity and no interference effect in the step of routine pathological slide examination.

Conclusions. Hematoxylin could be a suitable tissue marking dye for small-size samples and may improve the pre-analytical process of tissue preparation in pathological laboratories. (*Folia Histochemica et Cytobiologica 2023, Vol. 61, No. 2, 123–129*)

Keywords: tissue biopsy; merbromin; hematoxylin; eosin; crystal violet; alcian blue; tissue marking

Introduction

The biopsy procedure generally samples small pieces of tissues, 0.2–0.3 cm in size, from a suspected benign change or malignant tumor and is usually fixed in 10% formalin for a pathological diagnosis [1]. According to the small size of the biopsy, tissue processing becomes an important step in routine procedures. Unfortunately, the mixed up of samples with look-alike tissues and loss of the tissue sample probably happen during the tissue preparation step and may result in pathological misdiagnosis and ineffective treatment in patients [2–4].

Currently, pre-processing protocol basically starts with a gross examination step which determines general tissue characteristics by using the naked eyes of

Correspondence address:
Nontawat Benjakul, MD
Department of Anatomical Pathology,
Faculty of Medicine Vajira Hospital,
Navamindradhiraj University, 681 Samsen Road, Dusit,
Bangkok 10300, Thailand;
e-mails: nontawat.b@nmu.ac.th, nontawat.benjakul@gmail.com,
phone: +662 244 3000, mobile: +668 8761 2702

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a pathologist or pathologist assistant [5, 6]. After that, those tissues will pass through the tissue preparation processes such as tissue fixing and clearing, embedding, sectioning, staining, and slide preparation steps. Moreover, during the tissue processing or clearing step using xylene, a clearing agent, turns the tissue to colorless or less colored. Consequently, the small tissue sample is difficult to be seen after this step, especially for fat-rich tissues such as the breast. To prevent the loss of small tissue samples during these many processing steps, the small samples usually were placed on a paper or background to enhance the ability of tissue recognition. [7, 8]. However, the prevention of loss of tissue and the difficulty of selecting tissue target during section processes still represents the challenging step of pathological diagnosis.

In this study, we aimed to find an appropriate marker for the small-size tissue sample staining prior to tissue processing steps. Such a marker should provide more ability for tissue recognition, prevent the loss of tissue targets and be able to use routinely in pathological diagnosis without interference.

Material and methods

Study design. This experimental-observational study was approved by the institutional review board of the Faculty of Medicine Vajira Hospital (COA 136/2565) prior to the study. We stained leftover tissue samples with five different dyes and observed the ability of colored-observable of those tissues during the tissue preparation by pathologist assistants. Moreover, the interference of staining dyes was evaluated by pathologists.

Tissue samples. Various tissue/organ samples including breast, endometrial, cervical tissue, stomach, small and large intestine, lung, and kidney are included in this study. Inclusion criteria are: 1) leftover specimen without any further use in the pathological diagnosis, 2) tissues underwent the fixing process within 12 to 72 hours, and 3) size of tissue is suitable for staining and processing. Tissues were sampled by forceps and cut into 0.2 cm and 0.3 cm pieces size. Briefly, eight types of tissues (20 samples per type of tissue) were separated into 0.2 cm and 0.3 cm groups (160 pieces in each group) followed by stained (tissue marking) with five different dyes and control (unstained) before the tissue processing. Thus, 20 pieces of tissue were stained with each dye.

Tissue marking. Tissue samples were stained with various dyes including merbromin [The United Drug (1996) Co. Ltd., Bangkok, Thailand], hematoxylin (C.V. Laboratories Co. Ltd., Bangkok, Thailand), eosin (C.V. Laboratories Co. Ltd., Thailand), crystal violet (Sigma-Aldrich Pte. Ltd., St. Louis, MO, USA), and alcian blue (Sigma-Aldrich Pte). All dyes were diluted at a concentration of 1:10 with alcohol or distilled water based on the manufacturer's recommendation prior to the staining step. 1 mL of diluted dyes was individually used for the tissue marking (1 drop of dye, waiting for 3 seconds, and then putting in formalin) before the tissue processing step. After the marking, those samples were wrapped in filter paper and sent to pathologist assistants (pathology scientists/ /technologists) for evaluating the ability of colored-observable tissue. In further steps, those tissues were processed, sectioned (3–5 microns), and stained with hematoxylin and eosin (H&E) for pathological diagnosis.

Colored tissue observation and assessment. Pathologist assistants performed the assessment by counting the colored--observable tissues in each staining dye with bare eyes. They gave 1 or 0 scores for observable and non-observable tissue, respectively. A possible number of observable tissue per group would start from 0 to 20 regarding the number of tissue in each tissue type and each staining dye or control group (not stained samples).

Well-prepared tissue slides which passed whole processes of tissue sectioning and staining for pathological diagnosis were sent randomly to pathologists for the assessment of the interfering effect of staining dye on the pathological diagnosis. Pathologists recorded which dye interfered with the pathological slide evaluation and reported results as score 1 or 0 for interference or non-interference dye, respectively.

Statistical analysis. Descriptive and comparative analyses were carried out by Prism 9 (121) for Windows OS (GraphPad Software Inc., San Diego, CA, USA). The one-way ANOVA and Wilcoxon test were utilized for non-parametric paired sample tests. $P < 0.05$ was considered as statistical significance.

Results

Stained tissue and colored-observable assessment

According to the tissue marking, the stained tissues were assessed during the tissue preparation (before a formalin fixing step). Stained tissues and control tissues from both 0.2 and 0.3 cm-size groups are shown in Fig. 1. Notably, the stained tissues with merbromin, hematoxylin, and alcian blue were easily recognizable by pathologist assistants. Furthermore, the ability of colored-observable dyes was analyzed in different types of organs/tissues (Fig. 2). The colored-observable tissues in 0.2 and 0.3 cm-size groups were plotted individually to show the total counting number of the observable tissues in each dye (Fig. 2A). Particularly, various tissues stained with merbromin, hematoxylin, and alcian blue dyes were easily observable. For merbromin, the number of observable samples of breast tissues from both 0.2 and 0.3 cm-size groups was 19 of 20 tissues or 95% observable while in other tissue types from both size groups were 20 of 20 or 100% observable. For merbromin, 19 out of 20 breast tissues in both the 0.2 cm and 0.3 cm groups were observable, accounting for a 95% observability rate. Similarly, for other tissue types in both groups, all 20 tissues were

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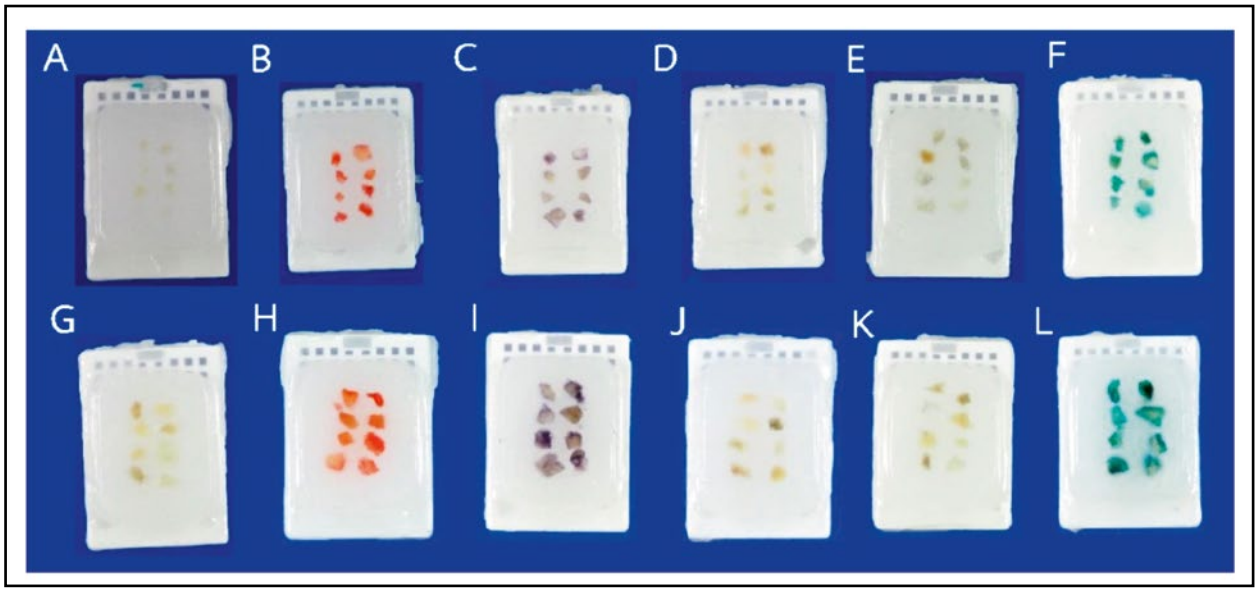

Figure 1. Various tissue samples in both sizes, 0.2 cm, top (**A** to **F**) and 0.3 cm, bottom (**G** to **L**) were not stained with any dye as the unstained control (**A** and **G**) or stained with different dyes (at 1:10 dilution) including merbromin (**B** and **H**), hematoxylin (**C** and **I**), eosin (**D** and **J**), crystal violet (**E** and **F**), and alcian blue (**F** and **L**) prior to tissue processing step.

observable, resulting in a 100% observability rate. With hematoxylin, the total numbers of observable tissues from the 0.2 cm group, *per* tissue type (out of 20 tissues), were as follows: breast tissue (18 or 90% observable), endometrial tissue (17 or 85% observable), cervical tissue (16 or 80% observable), stomach (19 or 95% observable), small intestine (15 or 75% observable), large intestine (15 or 75% observable), lung (17 or 85% observable), and renal tissue kidney (17 or 85% observable). Similarly, the 0.3 cm group also showed a comparable total number of observable tissues, ranging from 14 to 19 or 70% to 95% observability. In the case of alcian blue, the observability of various tissues in both the 0.2 cm and 0.3 cm groups ranged from 19 to 20 or 95% to 100% observability, respectively. In contrast, eosin and crystal violet staining resulted in mostly unobservable tissues, similar to the control tissues. However, a few endometrial tissues (6 out of 20 or 30% observable) from the 0.3 cm group exhibited observability when stained with eosin dye (Fig. 2A). Additionally, the ability of colored-observable dyes was scored from 0 to 1 based on the assessment. The results showed that various tissues stained with merbromin, hematoxylin, and alcian blue had significantly higher scores compared to the control and other dyes (Fig. 2B).

Coloring ability of 0.2 and 0.3 cm-size tissues

The comparisons of staining ability among dyes in 0.2 and 0.3 cm-size tissues were analyzed in this study. When each type of tissue are separated individually, the staining ability of each dye in both 0.2 and 0.3 cm--size groups was not different as shown in the heat map (Fig. 3A). However, it is clearly evident that these stainable tissues were based on the dyes used and did not depend on the size of the tissues. Additionally, when the staining ability was analyzed irrespective of the different dye uses, there was no significant difference observed between the 0.2 cm and 0.3 cm size tissues (Fig. 3B).

Interfering effect of staining dye on the pathological diagnosis

After the whole process of sample processing was performed pathologists performed the assessment of the interfering effect of each staining dye during their pathologic slide reviews. The outcome revealed that only alcian blue can interfere with pathologic slide examination and diagnostic performance (Fig. 3C). Examples of lung samples (0.2 cm-size), stained with H&E after the preceding staining dye marking, are shown in Fig. 3D.

Discussion

The tissue biopsy is one of the critical procedures for the diagnosis of various disease conditions, especially for cancer. Therefore, the processing of tissue and slide preparation becomes an important step of the pre--analytical process. Nowadays, small tissues between the size of 0.2 and 0.3 cm are widely used for pathological diagnosis. However, the small size of the tissues can sometimes make them difficult to see with the naked eye, especially for fat-rich tissue or

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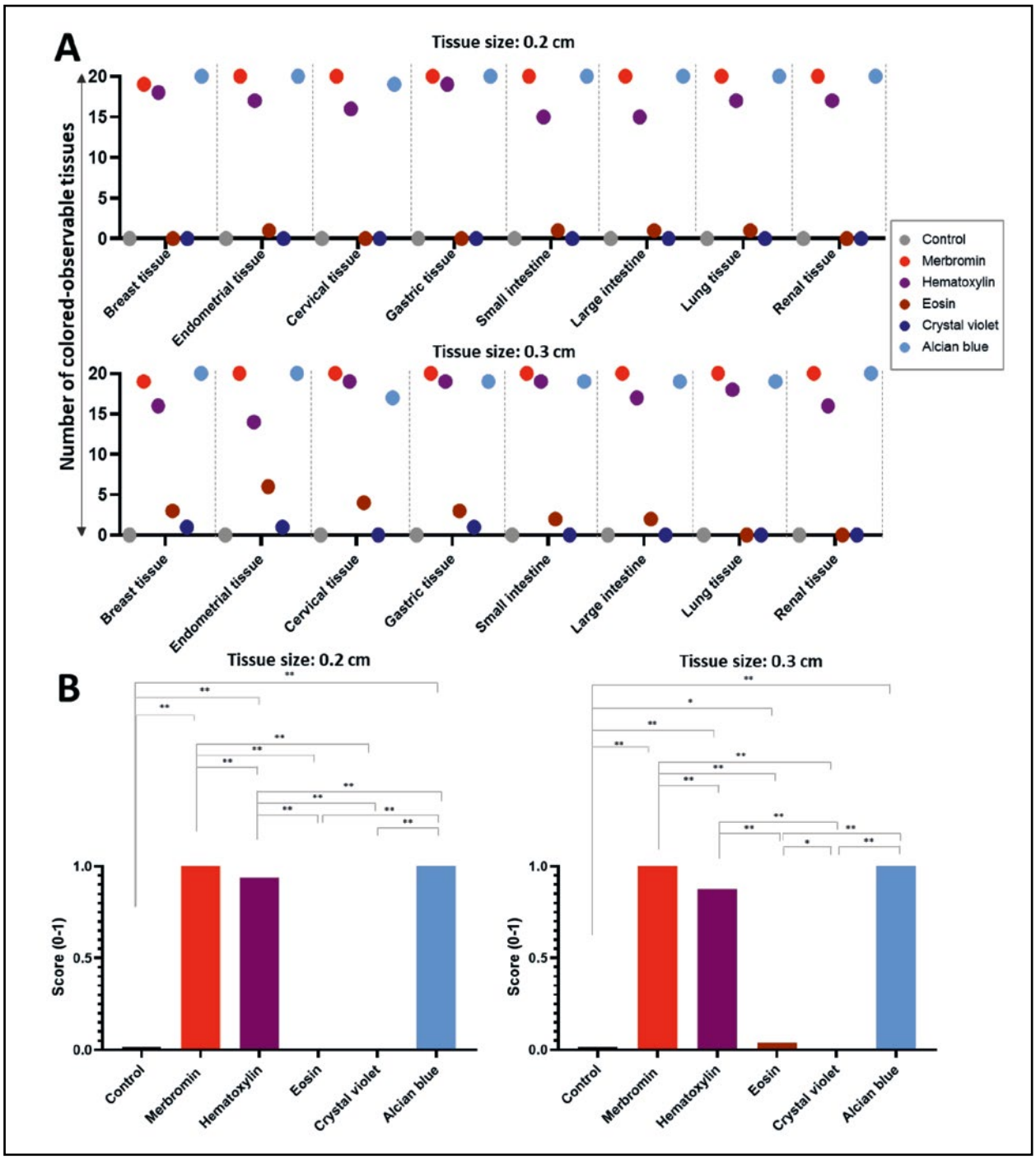

Figure 2. Number of colored-observable tissues (**A**) and overall staining score from 0 to 1 (**B**) for tissue marking in both 0.2 and 0.3 cm-size tissue samples were assessed by pathology assistants (pathology scientist/technologist). Wilcoxon test was used for the non-parametric paired sample test. $^{*}P < 0.05$, $^{**}P < 0.01$, Control = unstained tissue control.

white-to-colorless appearance tissue. This may lead to the loss of those small size tissues during the tissue processing and embedding steps [3, 7, 9].

Accordingly, the marking of the tissue prior to the tissue processing step has been currently used in pathological laboratories. Practical guidelines suggested that using mercurochrome or eosin for tissue marking can improve the ability of tissue to be observable and easy to be seen by pathology lab workers [5, 6]. Unfortunately, mercurochrome or merbromin has toxicity effects, evident especially for mercury poisoning which excludes merbromin from potential marking dyes [10–12].

This study aimed to evaluate various staining dyes including; merbromin, hematoxylin, eosin, crystal violet, and alcian blue, to be used as tissue marking dyes.

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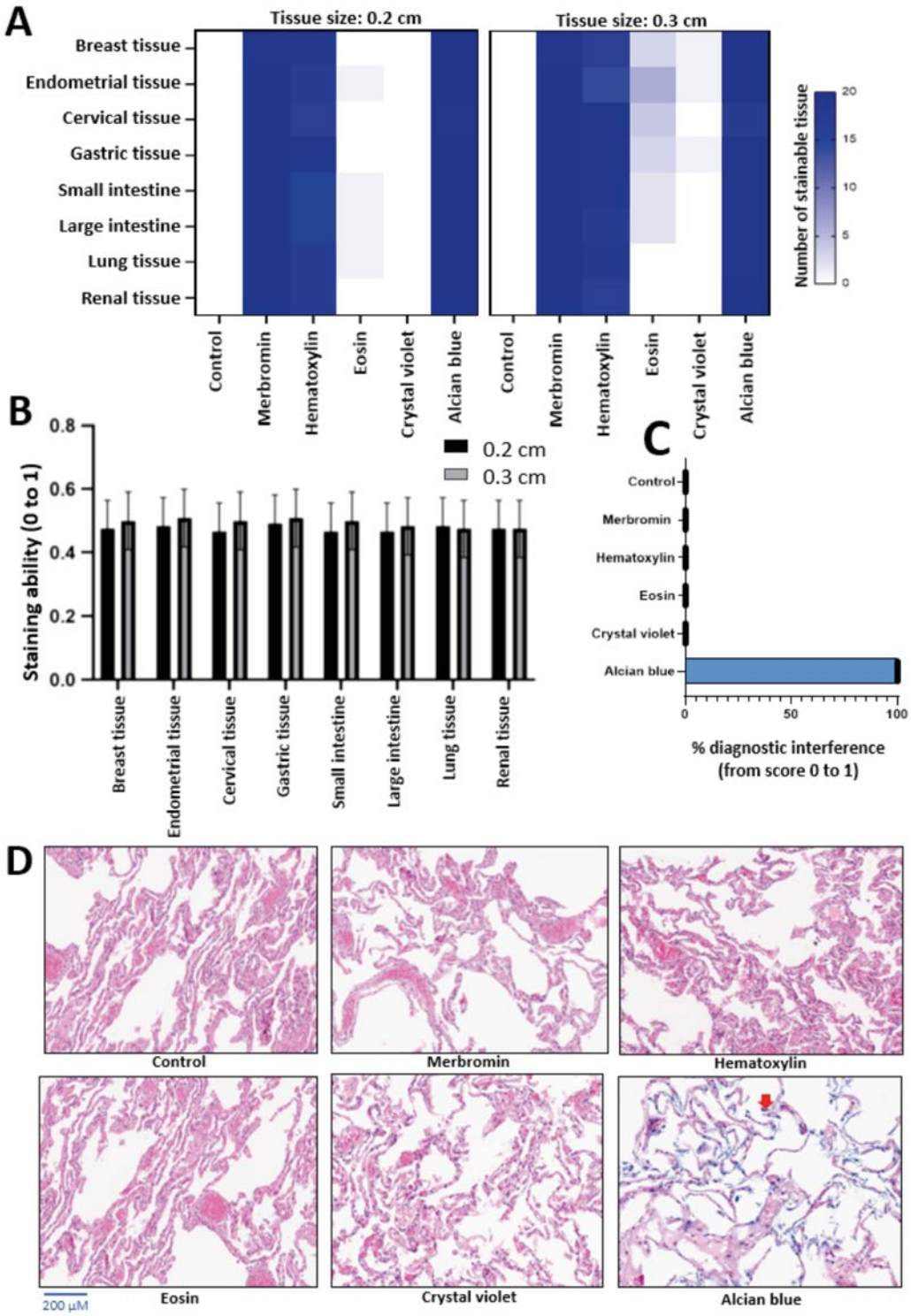

Figure 3. Stainable tissues and the interference effect of tissue marking dyes. **A.** Heat map of stainable tissues from 20 pieces *per* type of various organs/tissues in both 0.2 and 0.3 cm-size groups were plotted in each staining dye. **B.** Overall stainable tissues were represented by scoring 0 to 1 in both 0.2 and 0.3 cm groups. **C.** Percentage of interference in each tissue marking dye was evaluated by pathologists. **D.** Examples of lung tissue stained with hematoxylin and eosin (H&E) after various tissue samples were marked by marking dyes. Red arrow indicates the example of blue colored interfering stains in tissue marked by alcian blue.

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The results showed that merbromin, alcian blue, and hematoxylin were greatly observable staining dyes in both size groups (0.2 and 0.3 cm) of various tissues, whereas eosin and crystal violet were not applicable for tissue marking. For the staining ability of various types of tissues, we demonstrated that most types of tissue can be stained by merbromin and alcian blue. Endometrial tissue, small intestine, and large intestine had fair-acceptable staining ability while other types of tissues had outstanding staining ability when using hematoxylin. In contrast, crystal violet and eosin were ineffective for tissue marking. However, when considering overall staining ability across various tissue types, no significant differences were observed.

In terms of diagnostic interference, only alcian blue had an interfering effect on the pathological slide reviews. Regarding the permanent staining ability of alcian blue, some pathological laboratories also used this dye for tissue marking, especially for mucosubstance or mucin-riched tissues and glycosaminoglycan-riched tissues [9, 13]. However, our report suggested that alcian blue is not an appropriate tissue marking dye and probably interfered with the pathological slide examination. Fortunately, other dyes, including; merbromin, hematoxylin, eosin, and crystal violet, had water-soluble or alcohol-soluble ability [8, 14, 15]. Hence, most dyes were removed from tissues during tissue processing.

It is important to note the limitations of this study. The sample tissues were derived from leftover specimens preserved in 10% neutral buffered formalin for less than 48 hours, which may not precisely represent fresh tissue marking. Additionally, a semi-moisture tissue-chopping board was used, which could potentially affect the staining ability of water-soluble dyes.

Conclusions

In summary, this study suggested that hematoxylin is likely the appropriate staining dye for tissue marking with respect to the observable color capacity, tissue staining ability, non-toxicity, and no interference effect. Merbromine has outstanding performance, however, its toxic effects should be a concerned. Additionally, alcian blue might interfere with the pathologic slide examination.

Ethics statement

The study was approved and was granted an exemption from requiring written informed consent by the institutional review board of the Faculty of Medicine Vajira Hospital (COA 136/2565). The data were obtained and analyzed anonymously.

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Conflict of interest

The authors declare no conflict of interest.

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