Original Article

Quantitative evaluation of different fixatives for Masson trichrome staining in canine intestinal tissue: An image analysis-based comparative study

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Abstract

Neutral buffer formalin (NBF) has been widely used as a fixative for histological staining for ages. However, for some special staining, such as Masson trichrome staining, it has been proposed that the suitable fixative was Bouin's solution. The purpose of the present study was to compare the different fixatives and post-fixative protocols on the quality of Masson trichrome staining. The canine intestines (n = 10), which were the duodenum and colon, were used as the candidate organs since they comprised of various tissue components. The fixatives and staining protocols were divided into four groups, which were Groups 1 and 2; the tissues were fixed in Bouin's solution; Groups 3 and 4 were fixed in NBF. In addition, Groups 2 and 4 were post-fixed with Bouin's solution before Masson trichrome staining. The quantitative staining was evaluated by using Image analysis software (ImagePro plus 6.0). The results clearly showed the differences between post-fixation protocols rather than fixatives used. The strongest blue intensity was observed in the groups with post-fixation, which was omitted (Groups 1 and 3), while the red area was found to be the highest in the groups with post-fixation (Groups 2 and 4). When comparing different fixatives within the same post-fixation protocol, a higher red area was observed in NBF groups. On the other hand, the blue area and the blue intensity were found in the groups with Bouin's solution as a fixative. In conclusion, the results from this study showed no obvious differences in Masson trichrome staining between different fixatives i.e. NBF and Bouin's solution. Instead, the post-fixation protocol revealed the significant differences between the parameters studied. Therefore, the essential step for better-differentiated staining was the post-fixation process with Bouin's solution, as it can increase the distinction between collagen fibers and the other components.

Keywords: Bouin's solution, canine, colon, duodenum, Masson trichrome, neutral buffer formalin

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Introduction

The tissue fixation is a critical step in tissue processing after tissue removal from the body. It prevents autolysis, putrefaction of tissue as well as tissue component degradation. The appropriate fixative depends on multiple factors, such as the characteristics of the sample, research objectives, and stains in use (Dey, 2018). Although 10% Neutral buffer formalin solution (10%NBF) is the most routinely used solution for the fixation of tissue, Bouin's fixative is considered a good fixative for conserving soft tissue structures due to better preservation for nuclear architecture (Bultitude *et al*., 2011). Additionally, Bouin's solution is less prone to cause artifacts in tissue samples and is particularly effective in preserving delicate structures such as in the testes parenchyma (Roomratanapun *et al*., 2015). Bouin's fixative consisted of picric acid, acetic acid, and formaldehyde, which can preserve the protein of interest by coagulative and cross-linking effects on proteins (Dey, 2018; Suvarna *et al*., 2018). By using Masson trichrome, several gastrointestinal disorders have been diagnosed i.e. chronic intestinal fibrosis, inflammatory bowel diseases (Mao *et al*., 2016; Kawasako *et al*., 2017; Xiao *et al*., 2018; Chen *et al*., 2019) as well as in wound healing study (Liang *et al*., 2012; Suvik and Effendy, 2012; Silva *et al*., 2015) and even in the detection of intestinal protozoa (Agrawal *et al*., 2006). Though the NBF is still the best fixative, Bouin's solution was shown to be an alternative fixative requiring a longer suitable fixation time than NBF (Singhal *et al*., 2016).

Masson trichrome is the technique based on the difference in acid-base levels in the tissues andbased on the difference in acid-base levels in the tissues and the molecular weight of the chemical substance used in the protocol. This staining is used to differentiate between collagen and muscle, and it is used for the diagnostic procedure in several pathological conditions, especially fibrosis deformation in the intestines (Mao *et al*., 2016; Kawasako *et al*., 2017; Bandara *et al*., 2023). The fixation and differentiation, the process of selectively staining specific tissue components to distinguish them clearly, are the most important steps, and the tissues fixed in formalin required the post-fixation in Bouin's or other substitute substances, such as Gram's iodine or Citrate buffer for proper staining (Moore, 1996; Yu and Chapman, 2003). Thus, Bouin's solution was not the standard fixative in most laboratories, and the preparation of this solution is time-consuming compared to formalin. Therefore, the purpose of this study was to compare the fixative between 10%NBF and Bouin's solution on the Masson trichrome staining characteristics of the canine intestinal tissues using image analysis software.

Materials and Methods

Samples: The organs used in this study were canine intestinal tissues (n=10) which died from natural causes and requested for pathological necropsy from Small Animal Hospital, Chulalongkorn University. During necropsy, duodenum and colon were collected and fixed in different fixatives, which were Bouin's solution and 10% Normal buffer formalin solution for 24-36 h at room temperature. Thereafter, they were processed using standard tissue preparation for the histological section. Briefly, the tissue samples were dehydrated through a graded series of alcohol to remove water content. Thereafter, they were immersed in xylene, which replaced the alcohol and rendered the tissue transparent. The subsequent infiltration with paraffin wax at 56-58°C embedded the tissues within a supportive medium, facilitating precise sectioning. The paraffin-infiltrated tissues are then embedded in paraffin blocks and sectioned into 5 µm thick, using a microtome. These sections were mounted on glass slides and kept for staining and further image analysis.

Masson Trichrome staining protocols: The experimental protocols of this study were categorized into four groups. Groups 1 and 2, the specimens were fixed in Bouin's solution for 24 h before being processed histologically and trichrome staining. Groups 3 and 4 were fixed with 10%NBF before further steps as described for Groups 1 and 2. In addition, the tissue samples in Groups 2 and 4 were post-fixed with Bouin's solution for another 24 h before the Masson trichrome staining, while this post-fixation with Bouin's solution was omitted in Groups 1 and 3. The detail of the step-by-step staining protocol was concluded in Table 1. After each paraffin-embedded block was cut into 4 µm thick sections by JUNG Biocut 2035 microtome (Leica biosystems, USA) and kept in the incubator at 65°c for 30 min for drying, the tissue sections were deparaffinized in xylene four times for a span of 20 min. It was then re-hydrated in absolute and degrading alcohol for 5 min per each step of the graded series of alcohol. In the Bouin's solution fixative groups (Groups 1 and 2), the sections were washed in running water until the yellow color was clear from the sections and then briefly rinsed with distilled water to prepare for the next step. In the NBF groups (Groups 3 and 4), the sections were washed until the formalin fixative was completely removed from the sections. This description pertains specifically to the post-fixation process in Bouin's solution for slides in Group 2 and Group 4. The protocol of Masson trichrome staining was modified from Suvarna *et al*. (2018) . In detail, the sections were stained with Weigert's hematoxylin for 10 min and left in running water for 5 min. They were also rinsed briefly in distilled water. Next, the sections were subjected to cytoplasmic staining with Biebrich Scarlet-Acid Fuchsin Solution for 15 min and left in running water for 5 min, rinsed briefly in distilled water followed with phosphotungstic/phosphomolybdic acid solution for 10 min which was subjected to prepare for the uptake of the Aniline Blue Solution for collagen staining for 10 min. The sections were rinsed in distilled water between each staining for 5 min. In the final step, the sections were differentiated by 1% glacial acetic acid for 3 min and processed for dehydration in ascending graded alcohol. All staining sections were mounted with permount (Fisher Chemical™) before evaluation under a light microscope and image analyses.

Image Analysis: All histological slides were stained with Masson trichrome and evaluated by using Image analysis software, Image-Pro Plus 6.0 (Media

Cybernetics). Briefly, a whole tissue section was examined at 4X using an Olympus BX50 microscope annexed with and The QImaging MicroPublisher 5.0 with Real-Time Viewing (RTV). Each sample section was photographed to visualize and evaluate the whole picture. The tissue sections from both the duodenum and colon were divided into three different compartments: the epithelium (Epi), subepithelial connective tissue (Sub), and the smooth muscle (M). For each compartment, the percentage of area stained with predefined colors: Red, Blue, and Light Blue was quantitatively evaluated. In addition to the staining area, the level of the blue coloration in each compartment was quantified by calculating the average blue color intensity.

Statistical analysis: The statistical analyses were performed using R programming language (version 3.6.1) and R studio (version 1.2.1335). The mean area of each staining protocol was calculated and compared regarding several parameters: red staining area, blue staining area, light blue staining area, and the blue staining intensity. The normality of the data was assessed using the Shapiro-Wilk test. Since the data could not be normalized, the Wilcoxon rank-sum test with Bonferroni correction was utilized for analysis. *P*-value <0.05 was defined as significantly different. All data are presented as mean ± standard error of the mean (SEM) and median.

Table 1 The detail of Masson trichrome staining with different protocols

Staining protocol	**Group 1** Fix Bouin's/ no post-fix	**Group 2** Fix Bouin's/ post-fix Bouin's	**Group 3** Fix NBF/ no post-fix	**Group 4** Fix NBF/ post-fix Bouin's
1. Fix in Bouin's solution for 24 h	/	/	x	x
2. Fix in 10%NBF for 24 h	x	x	/	/
3. Tissue Processing for 18 h	/	/	/	/
4. Post-fix with Bouin's solution for 24 h	x	/	x	/
5. Running water for 10 min	x	/	x	/
6. Rinse with distilled water	/	/	/	/
7. Weigert's hematoxylin for 10 min	/	/	/	/
8. Biebrich Scarlet-Acid Fuchsin Solution for 15 min	/	/	/	/
9. Phosphotungstic/Phosphomolybdic Acid Solution for 10 min	/	/	/	/
10. Aniline Blue Solution for 10 min	/	/	/	/
11. Differentiated in Glacial Acetic Acid for 3 min	/	/	/	/
12. Ascending graded alcohol	/	/	/	/
13. Clearing	/	/	/	/
14. Mounted with permount	/	/	/	/

Results

In general, the morphology of all tissue samples was acceptable with different tissue compartments obviously distinguished from the others (Figure 1). All intestinal sections demonstrated black color in the nuclei, red in the cytoplasm and muscle fibers, and blue or light blue in collagen fibers, but with different intensity and proportion. Therefore, the blue staining areas were evaluated separately regarding toing different intensity, which were blue and light blue (Figure 2).

For the quantitative image analysis, the results of Masson trichrome staining were shown in Tables 2 and 3 for the colon and duodenum, respectively. In the epithelia, the highest blue area as well as blue intensity was observed in Group1, while the highest light blue area was observed in Group 3 though it was not significantly different from Group 1. For the connective tissue, no significant difference was observed for blue nor light blue areas in both organs examined. In addition, the highest blue intensity in the connective tissues was found in Group1 compared to the others for both organs. In the muscular layer, the lowest red area was obviously observed in Group 1. When comparing different fixatives with the same post-fixation with Bouin's solution protocol (Group 2 VS Group 4), no difference was observed in any parameter studied in all compartments of both organs.

Table 2 The image analysis results of Masson trichrome staining in canine colon represented as mean + SEM (median). Different letters within the same parameter (tissue type and color) indicates significantly different.

Staining protocol (fixative/ post fixative) (n=10)	**% Area**		
	Red	Blue	Light blue
Epithelium			
Group 1 Fix Bouin's/ none	1.02 ± 0.29 (0.93) ^{A}	74.14 ± 7.54 (82.19) ^{A}	24.84 ± 7.61 (16.51) ^{A}
Group 2 Fix Bouin's/ Bouin's	93.61 ± 1.22 (94.82) ^{B}	3.6 ± 1.00 (2.80) ^{B}	2.8 ± 0.92 (1.11) ^{B}
Group 3 Fix NBF/ none	23.62 ± 5.48 (26.65) ^{A}	13.94 ± 3.36 (12.7) ^{C}	62.43 ± 0.50 (60.78) ^{A}
Group 4 Fix NBF/ Bouin's	58.25 ± 8.21 (56.86) ^{B}	3.7 ± 1.28 (2.92) ^{B}	38.05 ± 7.69 (41.53) ^{B}
Subepithelial Connective Tissue			
Group 1 Fix Bouin's/none	0.14 ± 0.05 (0.11) ^{A}	64.83 ± 5.82 (66.91)	35.03 ± 5.81 (32.98)
Group 2 Fix Bouin's/ Bouin's	7.46 ± 1.51 (6.91) ^{B}	58.78 ± 9.99 (69.91)	33.76 ± 10.37 (20.57)
Group 3 Fix NBF/ none	6.88 ± 1.63 (5.45) ^{B}	47.03 ± 8.77 (54.94)	46.09 ± 9.24 (41.90)
Group 4 Fix NBF/ Bouin's	12.13 ± 2.01 (11.51) ^{B}	52.42 ± 8.62 (65.89)	35.45 ± 8.9 (22.75)
Muscular layer			
Group 1 Fix Bouin's/ none	4.92 ± 3.29 (1.12) ^{A}	77.85 ± 9 (88.22) ^{A}	17.22 ± 8.63 (7.75) ^{AB}
Group 2 Fix Bouin's/ Bouin's	89.11 ± 6.96 (98.43) ^{B}	5.98 ± 3.88 (1.10) ^{BC}	4.91 ± 3.09 (0.85) ^{A}
Group 3 Fix NBF/ none	41.37 ± 10.99 (37.23) ^{C}	17.61 ± 5.69 (12.75) ^{B}	41.02 ± 10.5 (41.79) ^{B}
Group 4 Fix NBF/ Bouin's	89.47 ± 3.92 (93.37) ^{B}	1.76 ± 0.58 (1.58) ^{C}	8.76 ± 3.41 (6.25) ^{AB}

Table 3 The image analysis results of Masson trichrome staining in canine duodenum represented as mean + SEM (median). Different letters within the same parameter (tissue type and color) indicates significantly different.

Staining protocol (fixative/ post fixative) (n=10)	**% Area**		
	Red	Blue	Light blue
Epithelium			
Group 1 Fix Bouin's/ none	2.54 ± 0.66 (2.34) ^{A}	68.25 ± 5.98 (76.41) ^{A}	29.21 ± 5.69 (21.29) ^{ACD}
Group 2 Fix Bouin's/ Bouin's	85.72 ± 3.26 (87.31) ^{B}	8.06 ± 2.6 (5.04) ^{B}	6.22 ± 1.9 (3.59) ^{BD}
Group 3 Fix NBF/ none	29.85 ± 6.69 (24.93) ^{C}	19.97 ± 3.2 (22.04) ^{C}	50.19 ± 6.67 (47.64) ^{C}
Group 4 Fix NBF/ Bouin's	81.98 ± 5.09 (87.34) ^{B}	4.58 ± 1.27 (3.47) ^{B}	13.43 ± 4.18 (11.28) ^{D}
Subepithelial Connective Tissue			
Group 1 Fix Bouin's/ none	0.12 ± 0.06 (0.05) ^{A}	62.89 ± 5.68 (61.15)	36.99 ± 5.71 (38.83)
Group 2 Fix Bouin's/ Bouin's	9.2 ± 1.82 9.71) ^{BC}	57.42 ± 7.71 (66.02)	33.38 ± 8.3 (25.24)
Group 3 Fix NBF/ none	3.94 ± 1.23 (2.65) ^{B}	63.2 ± 6.5 (65.13)	32.86 ± 6.45 (30.40)
Group 4 Fix NBF/ Bouin's	13.92 ± 2.1 (13.01) ^{C}	55.88 ± 7.17 (67.33)	30.2 ± 7.07 (23.44)
Muscular layer			
Group 1 Fix Bouin's/ none	4.13 ± 1.11 (2.87) ^{A}	72.66 ± 9.41 (84.76) ^{A}	23.21 ± 9.48 (10.93) ^{A}
Group 2 Fix Bouin's/ Bouin's	94.59 ± 1.64 (95.14) ^{B}	1.6 ± 0.5 (1.19) ^{B}	3.81 ± 1.64 (2.64) ^{B}
Group 3 Fix NBF/ none	45.34 ± 10.64 (49.74) ^{A}	18.13 ± 3.46 (19.89) ^{C}	36.53 ± 10.42 (22.21) ^{A}
Group 4 Fix NBF/ Bouin's	96.4 ± 0.8 (97.61) ^{B}	0.78 ± 0.34 (0.37) ^{B}	2.82 ± 0.76 (1.77) ^{B}

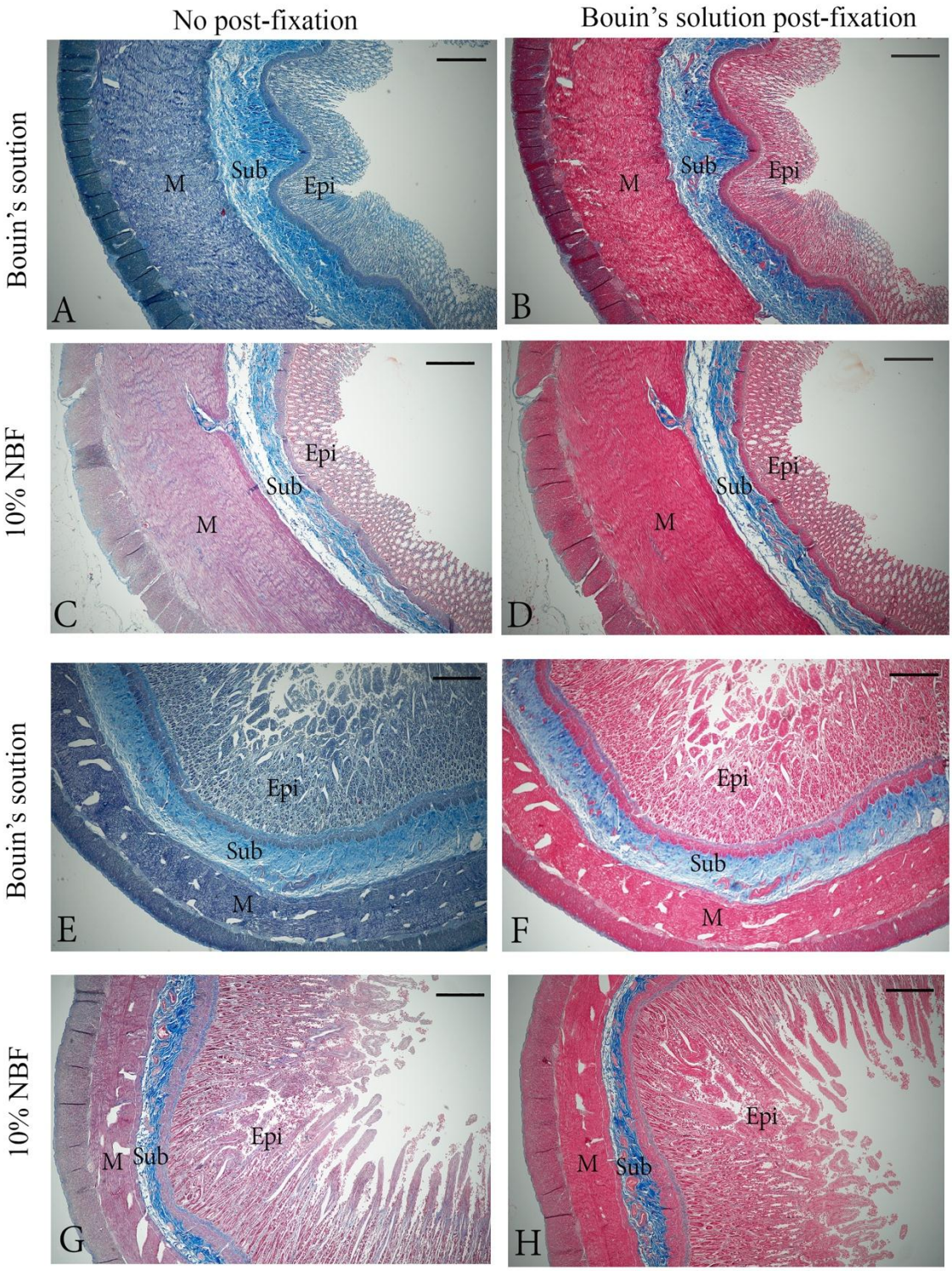
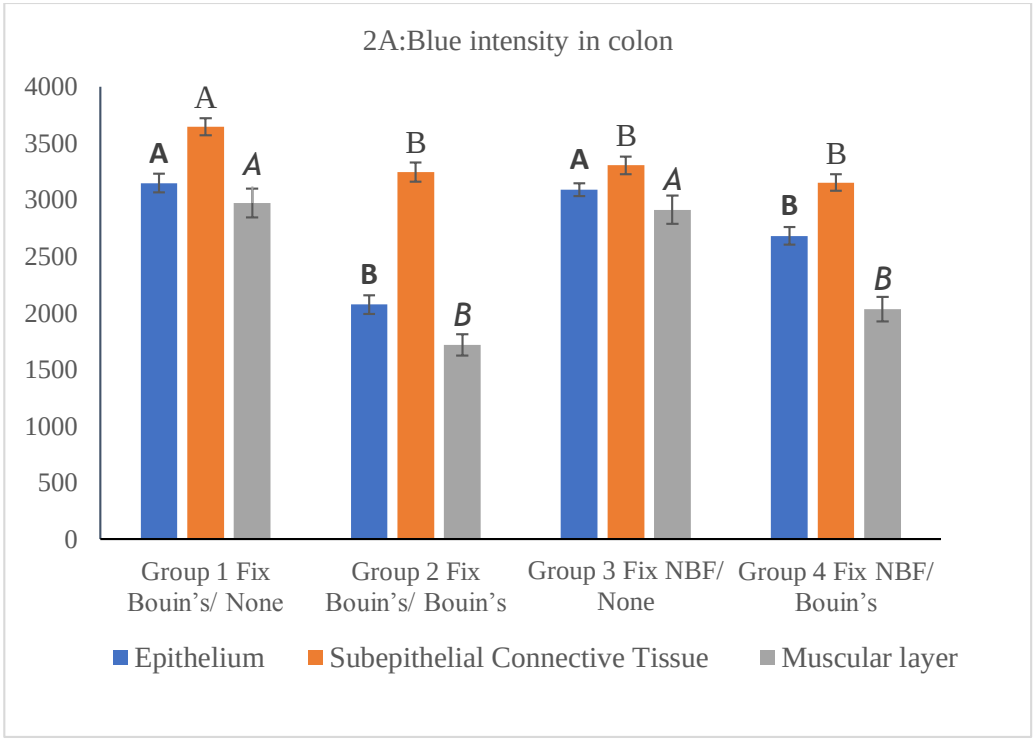


Figure 1 Masson trichrome staining in canine colon (A-D) and duodenum (E-H) with different protocols. A, E Group 1 fixed with Bouin's solution and no post-fixation (Bouin's solution/ no post-fixation). B, F Group 2, fixed with Bouin's solution and post-fixed with Bouin's solution (Bouin's solution/ Bouin's solution). C, G Group 3 fixed with 10% NBF and no post-fixation (10% NBF/ no post-fixation). D, H Group 4 fixed with 10% NBF and post-fixed with Bouin's solution (10% NBF/ Bouin's solution). epi =epithelium, sub =subepithelial connective tissue, M= muscular layer. Nuclei were stained black; muscle fiber and cytoplasm were stained red and collagen fiber was stained blue. Bar represents 50 μm.



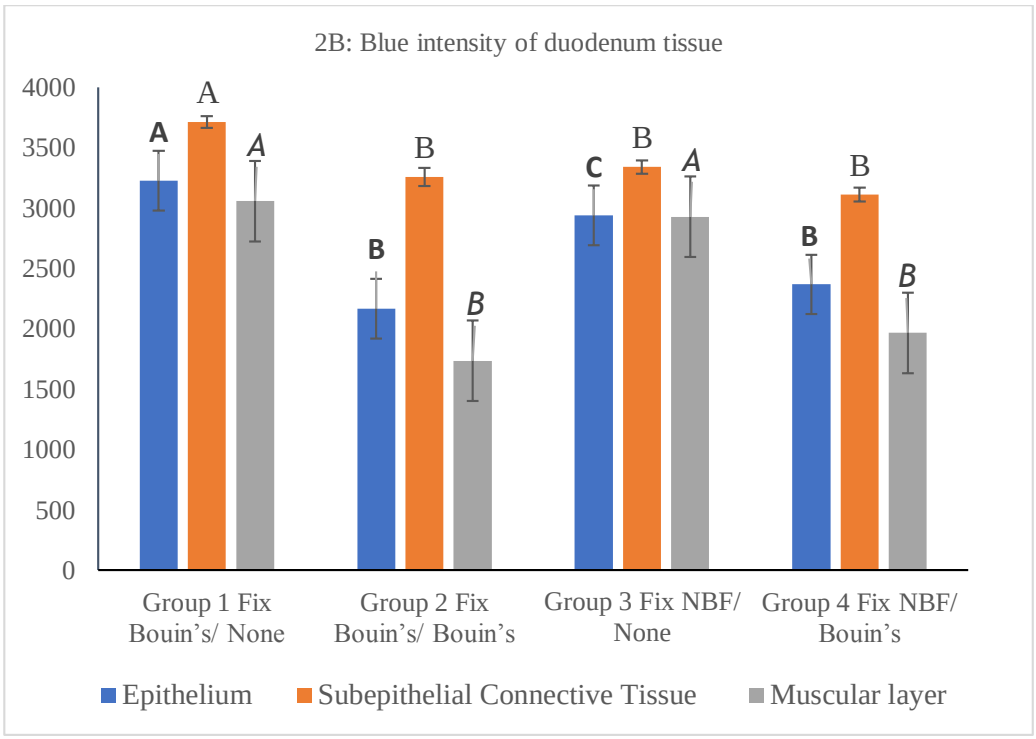


Figure 2 Blue intensity level from Masson trichrome staining in canine intestine (2A in colon, 2B in duodenum). Different letters with specific characteristic (bold, plain and italic letters) between tissue compartments were significantly different (P <0.05). The different letters (A, B, C) above the bars denote statistically significant differences (P < 0.05) between the tissues within each group, with different font styles (bold, plain, and italic) indicating distinct statistical groupings.

Discussion

The principle of Masson trichrome is based on the difference in acid-base levels in the tissues as well as the molecular weight of the chemical substance used in the protocol. The nuclei were generally stained with Wiegert's iron hematoxylin, which is resistant to the acid fuchsin solution. The acid fuchsin is applied to stain cytoplasm, muscle, and collagen, which were adjusted as acidic tissues. In order to de-colorize the acid fuchsin from collagen fiber, the phosphomolybdic acid was used, and finally, the aniline blue was applied to stain collagen in the studied organs (Suvarna *et al.*, 2018). The present study chose the intestine, which were duodenum and colon, since they are composed of various tissue layers, such as epithelium, connective

tissue, and muscle. Therefore, it could be used as the candidate organs to study the Masson trichrome staining in which these three different tissue layers would be stained differently.

Regarding the red staining area in each compartment of the duodenum and colon, a higher red area was observed in groups with post-fixed with Bouin's solution regardless of the fixatives. The higher red area may account for the clarity between smooth muscles and collagen fibers. Therefore, the Bouin's solution post-fixed groups (Groups 2 and 4) demonstrated a clear differentiation between each compartment of the intestines than the group with no post-fixation (Groups 1 and 3). On the other hand, a lower red staining area was observed in NBF fixative groups (Groups 3 and 4) since NBF can block the binding site of acid fuchsin dye. Thus, this could be overcome by post-fixing with Bouin's solution as it Wpost-fixing with Bouin's solution, as confirmed by the higher red area staining results in Group 4 compared to Group 3 in all compartments. Though Bouin's solution is the preferred fixative for Masson trichrome staining, the results from the present study demonstrated that the post-fixative with Bouin's solution obviously accounted for the quantitative staining of the red color rather than the fixatives chosen from the beginning, especially in the organ compartments which comprised of several different tissue types.

No difference in blue and light blue staining area was noticed in the subepithelial connective tissue. As blue-stained collagen fiber was the main substance found in this compartment, the quantitative image analysis results clearly demonstrated similar staining. In contrast to blue and light blue areas, the area of red staining and the blue intensity show variations among different fixatives and post-fixatives. The higher blue intensity was observed in groups with no Bouin's post-fixation (Groups 1 and 3). These results confirmed that Bouin's solution could enhance the acidic staining of acid fuchsin dye. On the contrary, when the post-fixation with Bouin's solution was omitted, the larger molecular weight dye, such as Aniline blue can penetrate and replace the acid fuchsin dye, which resulted in the higher blue intensity in all tissue compartments.

In addition to Aniline blue for collagen fiber staining, the small molecule of acid fuchsin dye can bind to all tissue compartments and yield a red color, which was selectively removed from the collagen fibers by phosphotungstic and phosphomolybdic acids. Therefore, in the muscular layers of the duodenum and colon, the higher red-stained area was observed in groups that post-fixed with Bouin's solution (Groups 2 and 4), while the different fixative did not have an obvious effect on red staining in the muscular layer in all tissues examined. This may because adding the Bouin's post-fixed solution could enhance the tissue's acidic level and increase the difference between muscle fibers and collagen fibers. Further, as the muscle fiber is the main substance in smooth muscle layers of the intestine, the removal of red staining from collagen fiber in the intestinal muscular layer by phosphotungstic and phosphomolybdic acids could hardly occur.

Regarding the post-fixative substance, an earlier study on trichrome staining has demonstrated that using Bouin's solution as a post-fixative could result in clearer differentiated staining between muscle fiber and collagen fiber (Yu and Chapman, 2003). In addition, the results from that study demonstrated that the post-fixation with Bouin's solution could yield a more orange hue in the muscle, cytoplasm, and red blood cells from the combination of red acid staining and yellow staining from the Bouin's solution. However, this was not observed in our study since the yellow color from picric acid in Bouin's solution had been washed away before Masson trichrome staining. An attempt has been made to use other substances to replace the post-fixative with Bouin's solution in Masson trichrome staining. For safety concern, the Gram iodine or citrate buffer solution could be applied to substitute the hazardous picric acid in Bouin's solution (Yu and Chapman, 2003). However, it was recommended from the results of our present study that post-fixed with Bouin's solution was necessary for acidic staining from acid fuchsin rather than the fixatives. As formaldehyde has been widely used in routine histological staining, it is more convenient to use as a routine fixative for histochemical staining, including Masson trichrome. Nevertheless, for better differentiation between tissue types, especially collagen fibers, the post-fixation with Bouin's solution is strongly recommended.

In the final step of Masson trichrome staining, the collagen fiber was stained by aniline blue. Since the phenol in aniline blue has a great affinity to conjugate with phosphorus ion, which binds to collagen fiber in acidic pH, the acetic acid has been added to enhance the aniline staining function (Cima *et al*., 2017). Furthermore, diluted acetic acid in alcohol was suggested for the final rinse in order to reduce the loss of blue staining (Young, 2001). Therefore, in the future perspective study for "Routine to Research" histological staining protocol, the comparison between post-fixation with Bouin's solution and acetic acid addition to Aniline blue, as well as final rinse with acetic acid in alcohol, should be studied to provide the more safety and practical protocol for Masson trichrome staining.

In conclusion, for Masson trichrome staining in the intestinal tissues, no obvious differences were observed between different fixatives i.e. normal buffer formalin and Bouin's solution. The essential step for better-differentiated staining was the post-fixation process with Bouin's solution, as it can increase the difference between collagen fibers and the other components. Moreover, the Bouin's solution should be wiped away before staining protocol to avoid the distorted yellow color from picric acid. The results of Masson trichrome could be developed into a routine histological process for the specific study and evaluation of the tissues are composed of various compartments, as it can demonstrate the more obvious staining of different tissues than routine hematoxylin and eosin staining.

Conflicts of interest: There were no conflicts of interest that may have biased the work reported in this study.

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