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The Use of Modified Masson's Trichrome Stain to Recognize the Stratum Corneum in the Epidermis of the Skin and Measure It

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ABSTRACT

Many types of research have stained the skin to determine its layers, especially the epidermis because it is an important and large body barrier against foreign materials. The most popular method was the routine stain that gives the tissues two colors depending on the acidophilic and basophilic to eosin and hematoxylin when the epidermis stain the trichrome stain the layers appear in two colors: pink color in the cytoplasm of the stratum germanitivium and the stratum spinusium and the stratum corneum, the nuclear stain with violet to gray color, in this paper we attempt to modify the Masson's trichrome to stain the stratum corneum in a different color to recognize it and measure it by using Bouin's solution with Carnoy's solution as fixative material. The result of this modification was the stratum corneum appeared in yellow color and the other layers stayed in the same color as the general trichrome method and compared it with routine stain and measured the dead layer in the three methods. This research concludes indicates that the modified method can identify dead layers of the epidermis and becomes more accurate when measured with modern measurements.

INTRODUCTION

The layers of the skin consist of two areas: the upper part is the epidermis, and the lower part is the dermis, and under the skin there is a subcutaneous layer, the epidermis in vertebrates has three layers from the inner to the upper layer: stratum germanitivium which have clearly nuclear and are very active in mitotic, and then stratum spinosum which seems like spine shape, and last the died and scaly upper layer stratum corneum (Yousef *et al*., 2022).

Lizards have three layers and the stratum corneum seems large and clear because of the environment that lives in it (Ahmed *et al*., 2016).

Masson's trichrome is a classical method of visualization with several variants. The nuclear dye can be Masson's hemalum or several hematoxylin solutions. The cytoplasmic dye is fuchsine. An aniline-derived dye allows the differentiation of collagen fibers. This staining method yields good results for morphological studies because the different parts of the tissue are very well contrasted (Exbrayat, 2016).

MATERIALS AND METHODS

Masson's Trichrome Stain

In the protocol staining with Masson's trichrome stain according to Luna (1968), it used Weigert's iron hematoxylin stain the nuclei in black, the acid fuchsin stains cytoplasm and muscle fibers in red then treated with phosphmolybdic acid, the aniline blue stain the collagen in blue.

In routine embedding of skin, after dissection the selective region was fixative in formalin 10% to about 18-24 hrs., then washed in tape and distilled water each for 30 minutes, to dehydrate the sample used ascending of ethanol alcohol (70,80,90,95%) for 30 minutes, the sample then immersed in xylol to purify it for 30 minutes. Paraffin wax was used for the infiltration, molting it at 58–60°C for 30 minutes, embedding it in the oven, and then blocking it in a stainless steel template for sectioning with a rotary microtome at a thickness of 5 microns.

Staining Procedure

1-Preparation

Solutions and Reagents

1- Bouin's Solution

Picric acid (saturated) ---------- 75 ml
Formaldehyde (37-40%) ---------- 25 ml
Glacial acetic acid ---------- 5 ml
Mix well. This solution will improve Masson's Trichrome staining quality

2-Weigert's Iron Hematoxylin Solution

The use of trichrome by immersion of the fixative sample in Weigert's iron hematoxylin then in three different solutions (A,B,C):

Stock Solution A

Hematoxylin ---------- 1 g
95% Alcohol ---------- 100 ml

Stock Solution B

29% Ferric chloride in water --------- 4 ml
Distilled water ---------- 95 ml
Hydrochloric acid, concentrated ---- 1ml

Weigert's Iron Hematoxylin Working Solution

Mix equal parts of stock solution A and B. This working solution is stable for 3 months (not good after 4 months)

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3-Biebrich Scarlet-Acid Fuchsin Solution
Biebrich scarlet, 1% aqueous --------- 90 ml
Acid fuchsin, 1% aqueous ----------10 ml
Acetic acid, glacial ---------- 1 ml

4-Phosphomolybdic-Phosphotungstic Acid Solution
5% Phosphomolybdic acid ---------- 25 ml
5% Phosphotungstic acid ---------- 25 ml

5-Aniline Blue Solution
Aniline blue ---------- 2.5 g
Acetic acid, glacial ---------- 2 ml
Distilled water ---------- 100 ml

1% 6-Acetic Acid Solution
Acetic acid, glacial ---------- 1 ml
Distilled water ---------- 99 ml

2- Staining

a. The method modified by Kiernan (2008) where samples are placed after putting them on the slides in a Coplin jar and immersed in three stages of xylol for about 4 minutes, then putting them in descending concentrations of ethanol 100%,96%, 90%, 80%, 70% for 4 minutes per once.

b. Immerse slides in Bouin's solution at 60°C for 45 minutes and wash them in tap water until the yellowish color of Bouin's solution disappears but this step is not necessary if the samples are not fixed in formalin.

c. To recognize the nuclei, immerse the slides in Weigert's hematoxylin for 8 minutes and wash them in tap water for 2 minutes.

d. Acid fuchsin is used to stain the cytoplasm and erythrocytes for 5 minutes then washed in tap water for 2 minutes.

e. Put the slides in Phosphomolybdic acid for about 10 minutes to moderate the stain.

f. To stain the collagen and fibroblast immerse the slides in methyl blue for 5 minutes.

g. Wash the slides in tap water for 2 minutes.

h. At last, put the slides in 1% glacial acetic acid for 1 minute.

i. Immerse it in ascending ethanol 70%, 80%, 90% 95%, 100% for 1 minute each concentration.

j. Put the slides in xylol for 1 minute.

k. Mount the coverslips by DPX on the slides and examine them with a light microscope.

Modified Masson's Trichrome

1-Preparation

The normal formation of the prepared tissues to be stained with Masson's trichrome is formalin as a fixative solution in 10% concentration, but in this modified method the fixative solution is Carnoy's which is used in fixative of neural tissues and glycogen.

The Carnoy's solution is prepared as follows:
Glacial acetic acid 10 ml
Chloroform 30 ml
Absolute ethanol alcohol 60 ml

The Fixation period is about 30 minutes to 1 hour, tt will shrink if it is placed more than this time. The preparation steps will be carried out after fixation normally and the sample is sectioned with a thickness of 5 microns.

2-The Staining

1-The methods of staining are done normally. First, the slides are put in Coplin jar and immersed in three stages of xylol for about 4 minutes and then they are placed in descending concentrations of ethanol 100%,96%, 90%, 80%, 70% for 4 minutes per once.

2- The slides were immersed in Bouin's solution at 60°C for 45 minutes and washed in tap water until the yellowish color of Bouin's solution disappeared (but this step is not necessary if the samples are not fixed in formalin). In this modified method we used Bouin's solution although formalin was not used.

3- The steps of staining were then done as normal Masson's trichrome stain.

RESULTS AND DISCUSSION

The epidermis of lizards stained with Masson's trichrome showed three colors, the stratum germanitivium appeared with blue to gray nuclei and red cytoplasm, the middle region of the epidermis the stratum spinosum is seen red in the upper region the stratum corneum, in normal Masson's trichrome seen red, the upper two layers are not recognizable, and the Bouin's solution colored the corneum in yellowish color, This result makes the Bouin's solution color the stratum corneum with yellow color and can be identified and measured easily compared to the hematoxylin and eosin stain method and the normal Masson's trichrome method (fig 1-A,B,C).

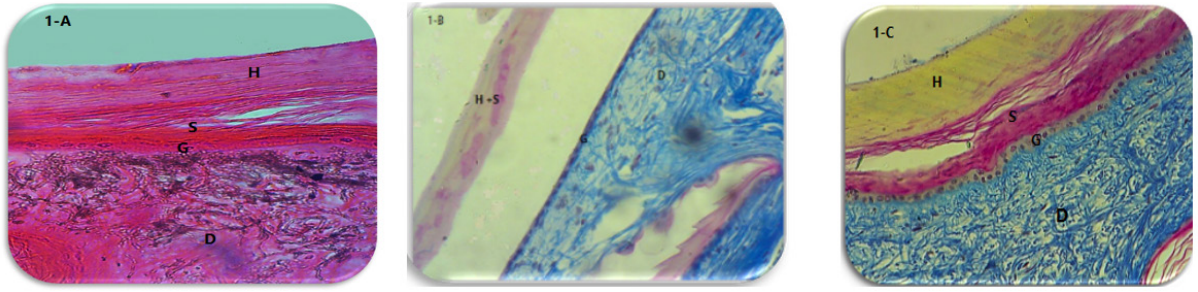

Figure 1: Shows the skin, the epidermis (G) stratum germanitivium, (S) stratum spinusum, (H) stratum corneum, (D) dermis, in (1-A) the skin stain with (H&E), (1-B) stain with Masson's trichrome, (1-C) skin stain with modified Masson's trichrome (40X).

Table 1: The measure of stratum corneum in µm

Length	Median	Angle	StdDev	Area	Figure
124.236	133.222	-85.236	15.157	325.988	1-A
106.716	149.843	-14.036	9.121	254.671	1-B
112.253	128.5	-56.976	4.6	271.28	1-C

The measuring of the corneum is seen as very difficult in routine stain and Massons stain because there is no border between stratum spinusum and corneum, but in modified Masson's trichrome it can be measured as the table (1) that shows the measured of the stratum corneum in the three methods.

The results agree with Jamie (2010) who refers to the bouin's solution containing 10% formaldehyde, acetic acid, picric acid, and water. Picric acid coagulating proteins and dyes tissue yellow.

Also, the finding agrees with Singhal *et al.* (2016), that explains fixation with carnoy's fluid was a fast fixation with good results and is used in urgent diseases and it takes a maximum time of 6 hours, while 10% buffered formalin and bouin's solution take 18 hours in minimum.

The finding agrees with Lihui *et al.* (2011) and Bultitude *et al.* (2011) in the contain of Bouin's fluid of picric acid that gives the fixative samples suitable for staining by trichrome stains, the picric acid slow penetrating that precipitates proteins and forming salts (picrate) with proteins and it associated with yellow staining to bright.

This study uses of carnoy's fixative because the formalin if fixed in the specimens for a long time causes the formation of formic acid and precipitate formalin pigments in the histological section and it agrees with Thavarajah *et al.* (2012).

CONCLUSIONS

The use of bouin's fluid in this study assisted in determining the stratum corneum from other regions by staining it yellow despite washing it in tap water and alcohol and it is the novel of this research.

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