THE USE OF MODIFIED MASSON'S TRICHROME STAINING IN COLLAGEN EVALUATION IN WOUND HEALING STUDY

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ABSTRACT. A number of studies have measured collagen fibers and collagen deposition in wound healing process with advances imaging techniques. However, these are performed by complicated methods and need specific tools. In search of the easier ways in routine histopathological laboratory, collagen measurement and staining pattern of wound healing process were observed in wounded skin of Sprague Dawley's rat by using two different stains which are standard haematoxylin and eosin (H&E) and modified Masson's trichrome staining (MT). The comparison between these staining in wounded tissues was made to evaluate the advantages and disadvantages of both staining in wound healing study for 21 days post-wounding. Tissues which stained with MT staining was then evaluated its collagen re-organization and density by using polarized light microscope with the aid of image analyzer software. Results showed that tissues stained with standard H&E could not be used to measure and differentiate the collagen deposition which is contradictory to MT staining. Wounded tissue stained with MT staining has showed a clear view of collagen fibers deposition and re-organisation compared to H&E staining. This finding could validate the using of modified MT staining which leads to accurate histopathological analysis and observation in wound healing study.

Keywords: wound healing, hematoxylin and eosin staining, Masson's trichrome staining, collagen

INTRODUCTION

Histopathological study of wound healing process is normally used for evaluating the efficacy of pharmacological products which promote and accelerate dermal skin substitutes. The study is usually related to phases of cutaneous wound repairing which can be categorised into four phases such as homeostasis, inflammation (early and late), proliferation and remodeling phases. In histopathological study of wound healing, a number of criteria are considered to determine the level of histopathological change such as the depth and length of healed wound, epithelial stratification, leucocytes and macrophage infiltration, fibroblast, extent of elastin formation and

the most important is collagen fiber as it plays a dominant role in maintaining the structural integrity of wound healing (Ukong *et al*., 2008).

However, by using a conventional staining method such as haematoxylin and eosin (H&E), the study of wound healing becomes more challenging as the stain is not able to differentiate important histopathological change in the wound healing process such as collagen deposition and scab formation which could later lead to misinterpretation in histopathological observations. Many studies have attempted to quantify the amount of collagen change and orientation in any stage of wound healing such as by using the epipolarisation microscope with picrosirius red-stained (Noorlander *et al*., 2002), computer vision analysis of collagen fiber bundles (Elbischger *et al.,* 2005), Fourier transform infrared (FTIR) spectral imaging (Potter *et al*., 2001), and laser scanning confocal microscopy (Taylor *et al*., 2002). There was also a study made by Dallon *et al*., (2006) which used a mathematical approach and equation model to evaluate the alignment and arrangement of collagen fibers in wound healing process; however this method is difficult to be understood and interpreted by non-mathematicians. Conversely, all these methods and protocols are complicated steps which require some special technicians or equipment in the routine of histopathological laboratory.

To overcome this problem, an alternative staining such as modified Masson's trichrome staining (MT) can be used in the histopathological study of wound healing. Differing from H&E staining the MT staining is able to differentiate clearly the important morphological keys for wound healing assessment such as keratin, haemoglobin, and muscle fiber (red colour), cytoplasm and adipose cells (light red or pink), cell nuclei (dark brown to black) and collagen fiber which stained blue in colour and later could be measured by using imaging analysis software. Clear differentiation of morphological and anatomical structure in the stained skin tissue are advantageous and provide further understanding in histopathological study of wound healing in future.

MATERIALS AND METHODS

Animals

Eighteen clinically healthy Sprague Dawley's female rats weighing between 200 to 250 g were obtained from the Animal Laboratory of Universiti Sains Malaysia, Kubang Kerian, Kelantan for wound healing study. All animals were housed in standard environmental conditions with temperature of 25 ± 1°C with 12 hours light and 12 hours dark cycle. They were acclimatised to a hygienic laboratory condition for 7 days before the start of experiment and observed for any clinical sign such as diarrhoea, food and water intake, behavior and blood in urine (Tuffery, 1995). Animals were fed with standard commercial pellet diet (10% of

their body weight) and distilled water ad libitum.

Experimental design for wound healing study

A total of 18 clinically healthy male white rats were used in the wound healing study and divided into three groups with six rats per group for different interval days of 7, 14 and 21 of post-wounding. All animals in each group was anaesthetised with light ether prior to the wound creation and 70% of alcohol was applied as topical disinfection on a shaved area at the dorsal thoracic region. An 8 mm diameter of full thickness wound was created by using sterile wound biopsy punch. Each animal was wounded on the dorsal part individually to represent a duplicate. At the end of the interval days, samples of the skin were harvested and processed for histology examination with three samples of wounded skin from each group was subjected to Masson's trichrome staining and another three rats were subjected to standard haematoxylin and eosin staining. The wounds were measured based on the percentage according to the healed wound area. The epitelisation time was measured from initial day (Singhai *et al.*, 2006).

$$\text{Percentage of wound contraction} = \frac{\text{Healed area (mm}^2\text{)}}{\text{Total wound area (mm}^2\text{)}} \times 100$$

Histopathological studies

Skin specimens for Masson's trichrome staining was fixed in Bouin's solution, meanwhile for standard haematoxylin and eosin staining the skin specimens was fixed in normal 10% buffered formalin. All the epithelial tissues which subjected to these staining were assessed under light microscope to evaluate fibroblast proliferation, collagen formation and re-ephithelization and wound healing processes (Reddy *et al.*, 2007).

Haematoxylin and Eosin (H&E) Staining

Slides were placed in staining jar and deparaffinized by submerging into three series of absolute xylene for 4 minutes followed by 100%, 100%, 95%, 90%, and 70% of ethanol for 4 minutes of each percentage. Next, slides were washed in running tap water for 2 minutes. Then, slides were submerged into Harris Hematoxylin (Sigma-Aldrich, GERMANY) for 2 minutes and then washed in running tap water for 2 minutes. The slides then were submerged into 1% acid alcohol for 3 dips to decolorize it and washed in running tap water for 2 minutes. Next, slides were submerged into 2% potassium acetate for 3 minutes and again washed in running tap water for 2 minutes. After that, slides were submerged into Eosin for 2 minutes followed by washing in running tap water for 2 minutes. Stained slides were dried for 24 hours at 38°C. Before observation,

slides were dipped into absolute xylene for 1 minute and finally mounted with cover slip using DPX mounting.

Collagen Special Stain (Modified Masson's Trichrome Staining)

Method was modified from Kiernan (2008). Granulation skin tissue slides were placed in staining jar and deparaffinised by submerging into three series of absolute xylene for 4 minutes each followed by 100%, 95%, 90%, 80% and 70% of ethanol for 4 minutes in each percentage. The slides then were submerged in warmed Bouin's solution at 60°C for 45 minutes. Next, the slides were washed in running tap water until yellow colour in samples disappeared. To differentiate nuclei, slides then were immersed in modified Weigert's haematoxylin for 8 minutes, after that washed in running water for 2 minutes. In order to stain cytoplasms and erythrocytes, slides were submerged in anionic dyes, acid fuschin (C.I. 42590, Merck, Germany) for 5 minutes; then again slides were washed with running tap water for 2 minutes. Next, slides were treated with phosphomolybidic acid solution for another 10 minutes as a mordant and immediately slides were submerged into methyl blue (C.I. 42780, Merck, Germany) solution for 5 minutes in order to stain fibroblast and collagen. After that, slides were washed in running water for 2 minutes and lastly treated with 1% acetic acid solution for 1 minute. Slides then were dehydrated into a series of alcohol of 70%, 80%, 95% and 100% for 1 minutes each percentage. Before observation, slides were dipped into absolute xylene for 1 minute and finally mounted with cover slip using DPX mounting.

Collagen Density Evaluation

Method was modified from Elizabeth *et al.,* (1995) and Ukong *et al*., (2008). The slides stained with Masson's trichrome stain were examined using polarised light microscope (Leica, Germany) and with the aid of a software image analyser (Video Test-Master 4.0 software), measurements were made at the intensity of blue colour which represent the collagen density. Collagen density was measured under the wound area compared to normal dermis at 100× magnification. The mean of the collagen values obtained for the normal dermis was accepted as the equivalent of 100. For each group, the mean of the collagen density under wound area was expressed in the ratio of percentage compared to collagen density of normal dermis during the post-wounding day.

$$\text{Ratio} = \frac{\text{Average collagen intensity under wound}}{\text{Average collagen intensity of normal dermis}} \times 100$$

RESULTS

Gross observation and wound contraction

The normal healing of full thickness wound has showed that at day1 of post wounding (Figure 1), blood was accumulated at the wound area and followed by swelling which showed the inflammatory phase. At day 7, the blood became dry and turned into scab which initiates the proliferative phase of normal wound healing. The scab was detached from the skin on day 11 as the wound contraction become higher. Scar was formed on day 21 of normal wound healing which lead to remodeling phase.

Histological Analysis

Histological analysis showed the wounded skin stained with standard haematoxylin

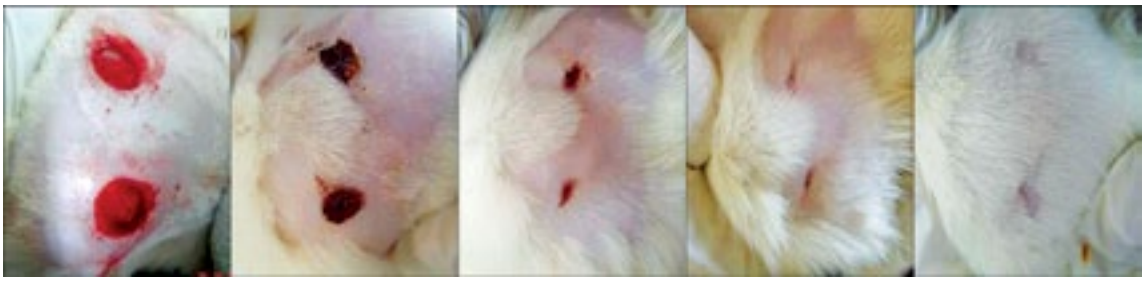

Figure 1. Normal healing of full thickness wound from day 1 to day 21 post-wounding in rats.

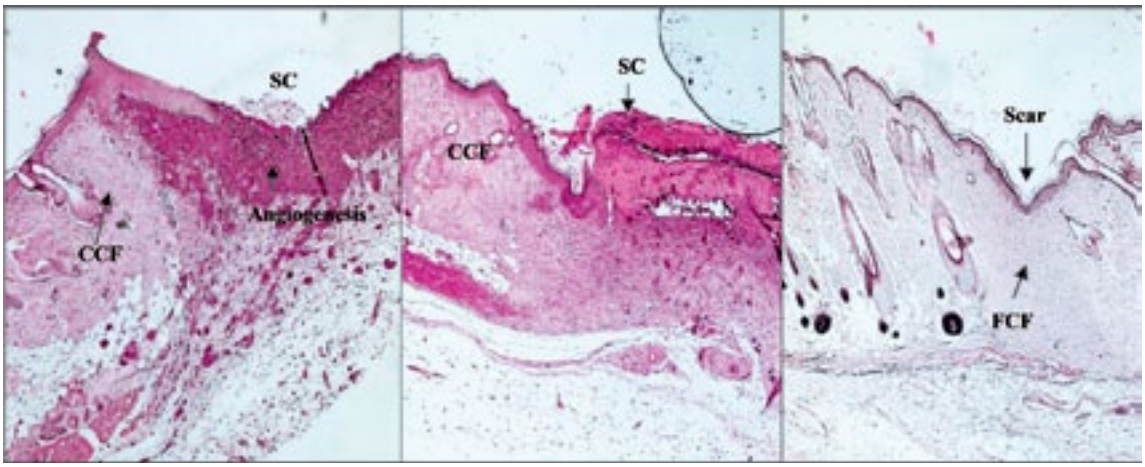

Figure 2. Photomicrograph of the normal wounded skin tissue stained with standard haematoxylin and eosin, 40×. Note that poor visible of fine and coarse collagen fiber and its arrangement throughout the wound healing processes. **CCF**: Coarse Collagen Fibers, **FCF**: Fine Collagen Fibers, **HF**: Hair Follicles, **SC**: Scab

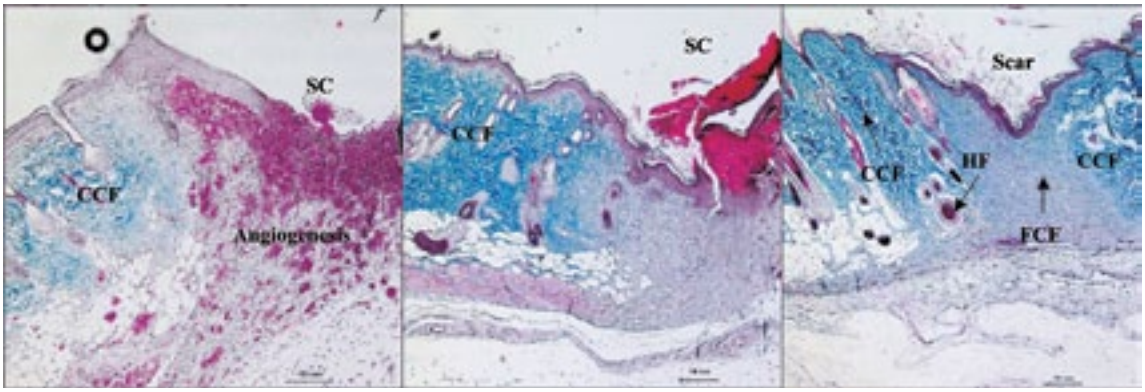

Figure 3. Photomicrograph of the normal wounded skin tissue stained with modified Masson's Trichrome staining, 40×. Note that clear visible and differentiation of fine and coarse collagen fiber and its arrangement, scab formation and angiogenesis throughout the wound healing processes. **CCF**: Coarse Collagen Fibers, **FCF**: Fine Collagen Fibers, **HF**: Hair Follicles, **SC**: Scab

and eosin has poor tissue reconstruction and collagen deposition toward the wound area. Skin stained with H&E only showed the angiogenesis and scab formation with less visible of other organelles and structure such as fine and coarse collagen fibers (Figure 2). However, by using the modified Masson's trichrome staining, it showed clear visible fine and coarse collagen deposition and its arrangement in the wounded skin together with visible differentiation of angiogenesis, scab formation, collagen fibers, adipose tissue and hair follicle (Figure 3). Even though the gross observation showed complete epithelisation at day 17, the collagen was still less deposited at the wound area even at day 21 post-wounding, a sign of the formation of fine collagen fiber (Figure 3).

Collagen Density Evaluation in Wound

Computerised quantification of collagen deposition in normal wounded tissue stained with Masson's trichrome staining evaluated that collagen deposition and density was significantly ($p<0.05$) different for every week of post-wounding as presented in Table 1. Even though the gross observation showed complete epithelisation at day 17 (Figure 1), the collagen was less deposited (97.82%) in the wounded skin even at day 21 post-wounding as presented in Table 1.

DISCUSSION

In histopathological study of wound healing, various characteristics are considered to determine the stage of histopathological change such as the depth and length of healed wound, epithelialisation period, white blood cells

Table 1. Average collagen density values under the wounded area compared to normal dermis percentage collagen ratio. Colour intensity values (absolute number). Treatment group has higher collagen density compared to normal on every post wounding day.

Group	Absolute Average Values			Normal Dermis Collagen Intensity
	Day 7	Day 14	Day 21	
Control	202.64 ± 27.8 (80.44%)*	244.96 ± 2.04 (97.24%)*	246.41 ± 8.6 (97.82%)*	251.89 ± 1.59 (100%)

n=18 values are expressed in means ± S.D. *p<0.05 statistically difference in comparison between each day post-wounding.

infiltration, elastin formation, fibroblast aggregation and the most important is collagen fiber as it plays a dominant role in preserving the anatomic integrity of wound healing. When tissues are disrupted following injury, collagen is needed to repair the defect in order to restore anatomic structure and function. This unique protein with three alpha chains that intertwine into a triple helix is very important in all stages of the wound healing process as it provides strength and integrity to all tissues (Mayer & Willemsteijin, 2008). For example, during the proliferative phase of the wound healing mechanism, secretion of collagen subtype within the injury site increases to replace necrotic tissue (Kondo, 2007). Meanwhile in the remodeling phase of the wound healing process, the collagen is cross-linked into a more organised structure to produce greater wound tensile strength (Enoch and Leaper, 2007).

Fibroblast fibers is a connective tissue cell which is responsible for production and synthesis of the collagen protein in skin (Diegelmann and Evans, 2004). It would be advantageous if the collagen fibers could be evaluated or measured in order to deeply understand how the collagen is synthesised and re-organised in the wound healing process. As mentioned, plenty of studies has been done to measure the collagen in wound healing, but somehow all the methods used were not practically applied to some of histopathological laboratory as it needed special equipment and protocols. The most used method by researchers all around the world in histopathological study of wound healing process is by using the standard haematoxylin and eosin (H&E) staining. This standard staining could only give a basic study of anatomical and morphological changes in the wounded tissues but not for the collagen re-organisation and deposition.

This study attempted to develop and modify the usage of a known special stain that is the Masson's trichrome (MT) staining in wound healing. The MT staining is widely used in medical pathology laboratories to differentiate between collagen and smooth muscle in tumours, determine the increase of collagen in disease such as cirrhosis and it is also a routine stain for liver and kidney (Sheehan and Hrapcahk, 1980). However, there has been not many studies of using MT staining in wound healing. The results

of this study showed that wounded skin stained with MT provides an understanding of the wound healing process as it illustrates the migration and re-organisation of collagen fibers in the healed skin at every post-wound stage (Figure 3). By using the MT staining we can differentiate the fine and coarse collagen fiber (Figure 3.) which usually appear in the remodeling phase of wound healing that influence scar formation. Collagen remodeling and degradation occurs simultaneously to provide the tensile strength and reduce scar formation (Enoch and Leaper, 2007). Other than collagen, MT staining also helps histopathologists differentiate other anatomical structures and organelles in the healed skin such as scab, fine and coarse collagen fibers, hair follicle and adipose tissue (Noorlander *et al.,* 2002).

The difference between H&E and MT staining were more related to the steps and dyes used in the staining preparation. Standard H&E staining involves two types of dyes which are haematoxylin and eosin Y. This staining method involves application of haemalum, which is a complex formed from aluminium ions and oxidised haematoxylin. The dye stains nucleus of cells and a few other objects, such as keratohyalin granules dark blue or purple in colour. The nuclear staining is followed by counterstaining with an aqueous or alcoholic solution of eosin Y, which colours eosinophilic and other structures in various shades of red, pink and orange (Junqueira and Carneiro, 2007).

However, the modified MT staining involves three colours of staining dyes, as the name implies, "trichrome". The principle of trichrome staining is that the less porous tissues are coloured by the smallest dye molecule and followed by the larger molecule (Sheehan and Hrapcahk, 1980). Theoretically, most of the stains are based on the attraction of the opposite charges in the tissues to the dyes applied. In MT staining the sections are first stained with an acidic dye such as acid Fucshin, C.I. 42590. In this step, all acidophilic tissue elements such as cytoplasm, muscle and collagen will bind to the acid dyes. The section is then treated with phosophomolybidic acid to decolourise the collagen but not to the cytoplasm. The decolorised collagen is then stained with fiber stain such as methyl blue, anilline blue or fast green dye (Masson, 1929). The blue colour of collagen can be enhanced by preliminary treatment of the section in hot Bouin's solution which is absent in the H&E staining protocol. The blue colour intensity of the collagen can be measured by a computerised imaging analyser with the aid of software. Several studies used this computerised method to measure collagen content in order to quantify dermal wound recovering for application to pharmacological products such as toxicity and efficacy tests in wound healing (Truong *et al*., 2005; Bae *et al.,* 2005 and Rao *et al*., 2007).

The abilities of modified Masson's trichrome (MT) staining to differentiate the collagen fibers in skin tissue gives an

alternative method for histopathologists to have a better understanding of the wound healing process as it could give clearly visible differentiations of morphological and anatomical structures of the skin. Moreover, by using this special staining, collagen fibers which were not differentiable using haematoxylin and eosin (H&E) staining could be clearly seen using MT staining represented by its blue colour.

CONCLUSION

Collagen measurement and arrangement is very essential in wound healing studies as the collagen restores the integrity of the skin. Alternative staining methods such as the modified Masson's trichrome staining and the use of computerised software to measure the collagen colour intensity helps histopathologists make accurate interpretations and have a better understanding of the histopatohological study of wound healing which is absent in standard haematoxylin and eosin staining.

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