

4.3 MATERIALS AND METHODS

4.3.1 MATERIALS

4.3.1.1 EXPERIMENTAL ANIMAL

Sprague-Dawley rats as described in Chapter 3.3.1.1.

4.3.1.2 WOUND DRESSING

Gelam honey and Nenas honey, Intrasite Gel and normal saline as described in Chapter 3.3.1.2.

4.3.1.3 CHEMICALS AND REAGENTS

All chemicals and reagents used in this part of study were analytical graded. Fixative solutions (Bouin's solution and 10% buffered formaldehyde solution, pH7.0) were used for tissue fixation in histological process.

The chemicals for the three staining protocol were prepared. In Harris Hematoxylin and Eosin staining (H&E). chemicals such as Ferum III Chloride ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), Weigert Iron Hematoxylin and Xylene were needed. Chemicals and reagent were well prepared in the staining kit of SIGMA ACCUSTAIN TRICHROME STAIN KIT (Catalog#HT15). The chemicals needed for this staining method include bierbrich scarlet-acid fuchsin Solution, Phosphotungstic Acid solution, phosphomolybdic acid solution and aniline blue solution. In the SIGMA ACCUSTAIN ELASTIC STAIN (Catalog#HT25), chemicals such as alcoholic hematoxylin solution, ferric chloride solution, weigerts iodine solution and van Gieson's solution were included.

Other than that, three concentrations of alcohol solutions were used in histological process: ethyl alcohol 70% (v/v), ethyl alcohol 95% and ethyl alcohol

absolute 99.8% (v/v) (v/v). Different mounting solutions were prepared for mounting the slide in different staining. These mounting solution include canada balsam and DPX Mount.

All chemicals such as fixative solutions and stains needed for the histological process were prepared according to the intended ratios (APPENDIX A) while at the same time practicing the safety precautions.

4.3.1.4 INSTRUMENTS AND APPARATUS

Special instruments for histological process were used in this study such as automated tissue processing mechine (Lieca) used for tissue processing, embedding station (Leica) for the embedding process, microtrome (Lieca) for tissue sectioning, water bath (Lieca), slide warmer (Lieca), fume cupboard and image analyser and light microscope (Olympus) and light microscope (Nikon).

4.3.2 METHODS

4.3.2.1 ETHICS

Ethics No. PM/27/01/2010/KMY (R) was obtained. Ethical protocol as described in Chapter 3.3.2.1.

4.3.2.2 EXPERIMENTAL WOUND MODEL

Excisional wounds as described in Chapter 3.3.2.2.

4.3.2.3 WOUNDING PROCEDURES

The wounding procedure of this animal study was as described in Chapter 3.3.2.3.

4.3.2.4 TISSUE SAMPLE PREPARATION AND HISTOLOGICAL TISSUE PROCESSING

4.3.2.4.1 TISSUE ABSTRACTION AND SAMPLING

The rats were sacrificed accordingly on Day 1, 5, 10 and 15 days of treatments as described in Chapter 3 (Refer to Chapter 3.3.2.4). Before dissection, the rats were given overdose of diethyl ether. The entire part of the wound area (up to the depth above the muscle layer) for each newly sacrificed rat was carefully removed to minimize the damage of the tissue. This step was done quickly by using a surgical blade and a pair of surgical scissors.

Each wound specimens was cut vertically through the skin into the adipose tissue that is normally found underneath the skin to ensure that it included the entire thickness of the corium and part of the subcutaneous tissue. The wound tissue specimen was then trimmed and adhered immediately to a piece of cold paper before placing it into the specimen cassette. This step was done to prevent the tissues from curling.

4.3.2.4.2 FIXATION

The abstracted tissue specimens were immersed immediately into Bouin's solution to prevent tissue degeneration and damage. After 24 hours, Bouin's solution was changed with another fixative solution, the 10 % buffered formaldehyde solution to prevent tissue hardening or shrinkage. Each specimen bottle was filled with fixative solution up to at least three quarter of its volume to ensure enough fixative solution could fully penetrate the whole tissue specimen. These specimens were allowed to remain in the 10% buffered formaldehyde solution for not more than 4 days to prevent specimens to become brittle. They were then washed under the running tap water until

the yellow color of Bouin's solution fade off. This step was done to prevent the contamination of chemicals during the dehydration step. Due to the carcinogenic nature of the fixative solution, extra care such as wearing protective mask and gloves were taken when handling these chemicals.

4.3.2.4.3 TISSUE PROCESSING

Tissue specimens were put into the automated tissue processing machine for histological processing steps. This time consuming procedure included dehydration, clearing and infiltration.



FIGURE 4.1: Automated tissue processing machine.

In the steps of dehydration, tissue samples were passed through a series of alcohol solution with increasing concentration for 2 hours each to remove water from the tissue specimens. Each specimen was transferred into a succession of 70% alcohol solution, 95% alcohol (I) solution, 95% alcohol (II) solution, absolute alcohol (I) solution, absolute alcohol (II) solution and a mixture solution of 1:1 of ethanol: xylene. These steps were necessary because complete paraffin infiltration and embedding

processes could only take place without the presence of water, since paraffin is immiscible with water.

After the dehydration steps, the specimens were transferred into xylene (I) solution and xylene (II) solution for clearing process. This was done to remove the remaining alcohol in the tissue specimens. Xylene acted as an intermediate fluid that was miscible to both paraffin and alcohol.

After the clearing process, the tissue specimens were moved into a mixture of xylene and paraffin solution (1:1). After this change, the tissue specimens were transferred sequentially to three changes of pure paraffin for 2 hours each. This was to make sure that paraffin would completely infiltrate into the tissue specimens. After infiltration was done, the specimens were then embedded carefully in the paraffin mold. This was an important step as the skin tissue must be aligned properly in the paraffin block to get ideal sections and also to make sure the cutting process would be easy. Since skin tissue is hard and elastic, it required special attention during embedding. The epidermal surface of each tissue specimen was identified and the specimen was placed to stand 90 ° into the mold. The blocks were then left to cool on the cool plate until it was ready for use.



FIGURE 4.2: Embedding station.

4.3.2.4.4 SECTIONING

The paraffin block which was ready to use was placed on ice prior to the sectioning process. After cooling and hardening, each paraffin blocks was placed into the microtome and was cut into a long ribbon of sections with the thickness of 5 μm .



FIGURE 4.3: Microtome used for sectioning of paraffin blocks.

The ribbons were then placed into the water bath at 42 ° Celsius. Few sections were chosen from the long ribbon and fished onto the cleaned slides. Then the slides with the sections were placed on the slide warmer at 50° Celsius for the sections to expand. This step was done to prevent wrinkles and folding of the sections. It also helped to adhere the paraffin sections onto the slides. After the sections were fully expanded and adhered onto the slides, the slides were stored in the oven at 50 ° Celsius. During the whole process it was important to prevent air bubbles from getting trapped in between the sections and the glass slides.



FIGURE 4.4: Water bath (left) and hot plate (right).

4.3.2.4.5 DEPARAFFINATION AND HYDRATION

Slides had to undergo deparaffination and hydration before staining. Deparaffinization involved two changes of xylene for at least 4 minutes each. The sections then underwent the hydration step in a decreasing concentration of alcohol solutions to remove the xylene before going into the staining protocol. These hydration steps involved two changes of 95% of alcohol and one change of 70% alcohol for at least 4 minutes each.

4.3.2.4.6 HISTOLOGICAL STAINING

There were three staining methods used in this study. All of these staining methods were done in the fume cupboard. These methods were (i) Hematoxylin and Eosin (H&E) staining technique, (ii) Masson's Trichrome staining technique and (iii) Verhoeff's Elastic Staining technique. Alternates slides of each specimen were submitted to each staining protocol.

i) HEMATOXYLIN AND EOSIN (H&E) STAINING TECHNIQUE

The sections fixed in Bouin's Solution took more than 4 minutes in 70 % alcohol solution for the slide to decolourize before H&E staining was carried out.

The slides that had gone through the deparaffinization and hydration steps were then transferred into distilled water before immersion into Harris' alum Haematoxylin for about 20-25 seconds. After that, slides were transferred into running tap water to wash away the excess stain of hematoxylin. The slides were then dipped into 0.2 % hydrochloric acid (HCl) for 3 seconds for decolourization and to avoid over stained. The slides were then washed again under running tap water before treated with 0.2% sodium bicarbonate solution (NaHCO_3). This solution was used to neutralize the acid and stop the decolourization before the slides were again washed under running tap water.

The slides were next rinsed in distilled water before the eosin step. Slides were left into eosin solution for 1 to 2 minutes and the excess stained of Eosin were blotted dry with paper towel before slides being transferred into 95% alcohol solution. Two changes of 95% alcohol solution were carried out before the slides were transferred into two changes of absolute alcohol solution for 3 minutes each step.

After that, the slides were transferred into two changes of xylene solution (three minutes each). It was stored in the xylene solution until the mounting steps. The stained slides were mounted carefully using Canada Balsam to avoid air bubbles. The slides were kept in the oven for at least 48 hours for drying purpose.

TABLE 4.2: Result of the performance characteristic of H&E staining.

STRUCTURES	COLOR
Nuclei	Blue/Black
Cytoplasm	Pink to Red
Collagen, Elastin, Erythrocytes	Pink to Red

ii) MASSON'S TRICHOME STAINING PROTOCOL

The Masson's Trichrome staining was done by using protocol of the Accustain Trichrome Stain (Masson) - HT15 [Sigma-Aldrich, Inc.] with modification. Normal precautions exercised in handling laboratory reagents were followed. This procedure was based on the work of Masson as modified by Lillie, using aniline blue as a collagen stain instead of green dye.

After the slides underwent deparaffinization and hydrated in the distilled water, they were placed into Bouin's solution at 56 °C for 15 minutes. This step could also be replaced by placing the slides into Bouin's solution at room temperature overnight. Next, the slides were then taken out and left to cool at room temperature. It was then placed under running tap water to remove the yellow colour from the sections. The slides were then rinse in distilled water before staining in working solution of Weigert's Iron Hematoxylin solution for 5 minutes and followed by a rinse with distilled water.

These slides were then placed under running tap water for 5 minutes to wash out the excessive color of the Weigert's Iron hematoxylin. They were then transferred into Biebrich scarlet-acid fuchsin for 5 minutes followed by rinse in distilled water before immersing in working phosphotungstic/ phosphomolybdic acid solution for 5 minutes. The excess solution was dried with a paper towel before the slides were immersed in aniline blue solution for 5 minutes.

The slides were then transferred into 1% acetic acid for 2 minutes. This solution was discarded after each use. After the usual rinsing in distilled water, the slides underwent the dehydration process through alcohol series starting from two changes of 95% alcohol solution and two changes absolute alcohol solution for 3 minutes each. Lastly, the slides were placed in two changes of xylene solution for clearing purposes. The slides were then stored in the xylene solution ready for cover slip mounting.

The stained slides were mounted carefully with cover slips using DPX to avoid the air bubbles. The slides were kept in the oven at room temperature for at least 48 hours before slide analysis.

TABLE 4.3: Results of the performance characteristic of Masson’s Trichrome staining.

STRUCTURES	COLOR
Nuclei	Black
Cytoplasm, Keratin	Red
Muscles Fibers, Erythrocytes	Red
Collagen, Mucus	Blue

iii) VERHOEFF’S ELASTIC STAIN

The Verhoeff’s elastic staining was done by using staining kit and following the protocol of Accustain Elastic stains- HT25 [Sigma-Aldrich]. Normal precautions exercised in handling the laboratory reagents used in this protocol were followed. Van Gieson solution was used as counterstain for collagen fibres. Specimens were fixed in 10% neutral buffered formaldehyde solution.

After the slides went through the deparaffinization, the slides were then placed into the Verhoeff’s working solution for 15-30 minutes. Then they were rinsed with distilled water and placed into the differentiating fluid for 1-2 minutes until the elastic tissue appear on a grey background. After the differentiation, the slides were put under the running tap water and then checked microscopically. If the slides were over differentiated, the slides were returned into the working elastic stain solution.

The slides were then rinsed in 95% alcohol solution to remove any staining due to iodine and continued rinsing with distilled water before they were counter stained with Van Gieson solution for 1-3 minutes. After that, the slides were rinsed using 95% alcohol and dehydrated before immersion in xylene solution and mounting of cover slips using permanent mounting medium (DPX).

TABLE 4.4: The performance characteristic of Verhoeff's elastic stain.

STRUCTURE	COLOR
Elastic Fibers	Blue to black
Nuclei	Blue to black
Collagen	Red
Muscle/Cytoplasm	Yellow

4.3.2.4.7 MICROSCOPIC EVALUATION OF WOUND HEALING

The interpretation of the histological slides was important in this study. Visualizing of the slide was done by using light microscope under magnification power of 20x, 40x, 100x and 400x. The slides were first seen by using the low power objective of light microscope and switched to higher power of magnification for examining certain areas in details.

Several histological parameters such as macrophages, fibrosis, angiogenesis, epithelization and collagen level could be used to assess the progression of wound healing from inflammatory stage to mature stage (Nisbel et al., 2010). Histological scoring system was adapted from Nisbel et al. (2010), and it was modified from Abramov's and Greenhalgh's scoring system. This scoring system assessed each parameter independently with a score of 0-3.

TABLE 4.5: Histological parameter adapted from Nisbel et al. (2010).

SCORING	0	1	2	3	4
Collagen	None	Little	Scant	Moderate	Abundant
Epithelization	None	Partial (thin)	Partial (thick)	Complete, Immature/thin	Complete, Mature/Thick
Angiogenesis	None	<5vessels/HPF	6-10vessels/HPF	>10vessels/HPF	>20vessels/HPF
Fibroblast	None	Few fibroblasts	Moderate fibroblast	More fibroblast	Predominantly presence
Macrophages	None	Few	Scant	Moderate	Abundant

4.3.3 STATISTICAL ANALYSIS

Data obtained were statistically examined and analysed by using Statistical Package for Social Science (SPSS). The values were expressed as Mean \pm Standard Error Mean (S.E.M) for different parameters. Repeated measurements of Analysis of Variance (ANOVA) tests were done to compare the differences of data between and within the groups. Level of statistical significance was set at <0.05 .