3.3 MATERIALS AND METHODS

3.3.1 MATERIALS

3.3.1.1 EXPERIMENTAL ANIMAL

A total of 150 clinically healthy adult male *Sprague-Dawley* rats, 8 weeks old weighing between 180g and 300 g were used in this study (Figure 3.1). These animals were obtained from Animal House (Breeding Centre), Faculty of Medicine, University of Malaya. They were housed in an environmentally controlled facilitiy at Animal Care Unit, Animal House, Faculty of Medicine, University of Malaya for at least one week prior to the experiment for acclimatization purpose.

The rats were caged separately and singlyin sterile individual wire-bottom cage. There were subjected to standard 12/12 light/dark cycle with controlled room temperature in Animal Care Unit throughout the experiment. They were fed with standard laboratory rat pellet diet daily and had accessed to tap water *ad libitum*. Care was taken in order to avoid unwanted stress and discomfort for the rats throughout the experiment. Cages and the air-ventilated room were cleaned daily to prevent unwanted infection to the wounds.

FIGURE 3.1: *Sprague-Dawley* rats weighing 180-300g were the experimental animal for this study.

3.3.1.2 WOUND DRESSING

Three types of wound dressing were used in this study. For honey, two selected local Malaysian honey were used. A standard positive control and negative control were also applied on the wounds as treatments in this study.

Honey Samples: The two local pure monofloral honey (Gelam honey and Nenas honey) produced by *Apis mellifera* bees (as described in Chapter 2) were chosen for wound treatment in this part of study. These *Apis mellifera* honey are named according to their flora source; Gelam honey from Gelam (*Melaleura spp*) tree, whereas nenas honey from Nenas (*Ananas Comosus*) plant. Both honey were obtained from an Apiary at Malaysian Agriculture Department. They were obtained by the normal procedure of centrifuging the cut comb in a stainless steel container and filtered once by using a fine muslin cloth. These honey samples used in wound dressing were then sterilized by gamma-irradiation (25kGy) and kept in laboratory temperature (20 ℃) away from direct sunlight in aluminum foil covered glass bottle before used.

Intrasite Gel: Treatment using this gel is the standard positive control for the study. This wound dressing is a trademark for Smith & Nephew Ltd., UK. This dressing was obtained from the pharmacy of University of Malaya Medical Centre. It is a clear amorphous hydrogel containing modified carboxymethyl cellulose (CMC) polymer, propylene glycol and water (Williams, 1994). This wound dressing promotes natural debriment through autolysis by gently rehydrating necrotic tissue. It also loosens and absorbs slough and exudates. It provides a moist healing environment for wound closure. Its non-adherent to skin surrounding the wounds makes it harmless to all stages of wound healing process (Abdulla et. al, 2010; Williams, 1994).

Normal Saline: Treatment using normal saline is the stardard negative control for the study. 0.9 % normal saline which was used for control was obtained from the pharmacy.

3.3.1.3 CHEMICALS AND REAGENTS

Ketamine and Xylazine from Ilium used for general anesthesia were purchased from Pet Arcade. Lignocaine HCl (2%, 100mg/5ml) which was used for local anesthesia purchased from the Experimental Animal House, Faculty of Medicine, University of Malaya (Delta Veternary Laboratory PTY LTD, NSW 20011). 0.5ml of Lignocaine for was injected subcutaneously each time.

All chemicals and reagents used in this part of study were of analytical graded. Ethyl Alcohol 70 % (v/v), used as washing agent for the surgical apparatus for aseptic purpose in this study. It was swabbed on the dorsum area of the wounding area before the surgery.

3.3.1.3 INSTRUMENTS AND APPARATUS

Electrical Hair Clipper was used to remove the dorsal fur behind the shoulder of each rat. Rubber stamp of 2cm x 2cm square shape (wound mold) and Methylene Blue were used as a standard mold to outline the wound area. Standard instruments for surgical purpose such as surgical scissors, scalpels and scalpels blade no. 24 and no. 21, and forceps were also used in this study. 1 cc/ml, 5cc/ml and 10 cc/ml syringe with needle (TERUMO) were used or injections.

Other instruments and apparatus for data collection such as: transparent plastic paper for tracing the wound area, hand held digital microscope (Dino-Lite) and digital camera (SonyDSC-W290) for wound observation and measurement.

3.3.2 METHODS

3.3.2.1 ETHICS

The experimental protocol for animal study of this part of the project was approved by the Ethics Committee for animal exprimentation in the Faculty of Medicine, University of Malaya with reference number: Ethics No. PM/27/01/2010/KMY(R).

3.3.2.2 EXPERIMENTAL WOUND MODEL

To evaluate normal wound healing activity and the efficacy of the two selected Malaysian honey on wound healing, uninfected, full-thickness, 4cm^2, excisional wound model adapted from Gottrup (2001) and Aljady (2003) were chosen as the cutaneaous wound model in this study.

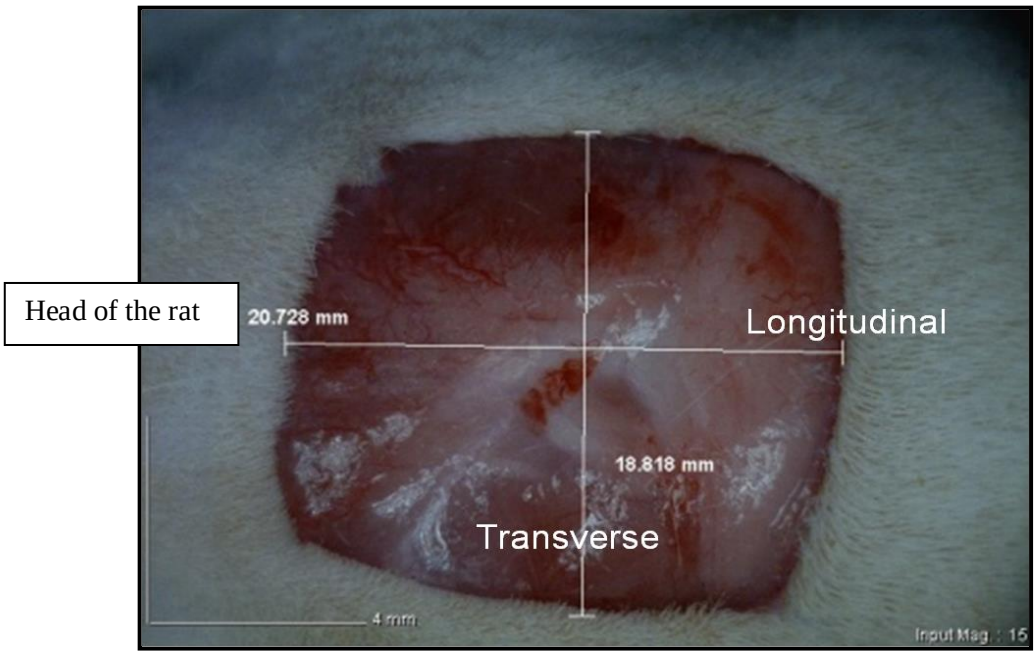

Figure 3.2: Experimental wound model of 4cm ^{2} area used in this study.

3.3.2.3 WOUNDING PROCEDURES

Wounding procedure in this study was adapted and modified from Aljady (2003) and Tomlison and Ferguson (2003). This protocol was carried out in aseptic conditions. The procedure was carried out under general anesthesia by using Ketamine 50mg/kg and Xylazine 5mg/kg (Figure 3.3) in mix in the same syringe. The mixture was injected intramuscularly at the gluteal area of the rats, producing 30 minutes of anesthesia.

Dorsal fur behind the shoulders of each rat was shaved using electrical hair clipper and disinfected with 70 % alcohol. A 4cm^2 excision wound area to be created was outlined on the shaven area by a rubber stamp and methylene blue (Figure 3.4) on the posterior neck area between the shoulders. The wound mold did not contact the wound, since it was only used in marking the border of the wound before the surgery. 0.5ml of Lignocaine HCl (2%, 100ml/5ml) was injected subcutaneously into the marked area 5 minutes before the surgery as local anesthesia (Figure 3.5).

After local anesthetics injection and in aseptic condition, a full thickness excision was then created along the marked area by a pair of surgical pointed end scissors and tooted forceps. The wound mold, surgical scissors and forceps were cleaned with 95 % alcohol before and after the surgery of each rat for aseptic purpose to avoid infections. The underlying muscle was visible and there should be little or no bleeding from the wounds. If excessive bleeding occurred after the surgery, hemorrhage was controlled by sterile cotton balls compresses. These experimental wounds were of full thickness and extending vertically down to the subcutaneous tissue (Figure 3.2). The entire wound was left opened and air exposed to environment. The same procedure was repeated when creating other wounds. The day of wound creation was labeled as Day 0 of treatment.

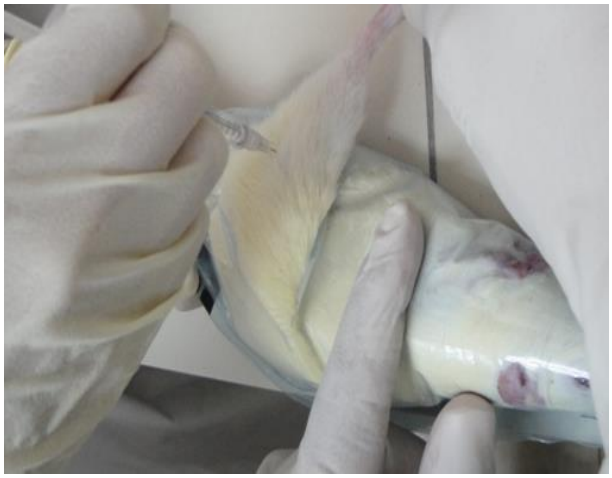

Figure 3.3: Injection of Ketamine and Xylazine at gluteal area of rat.

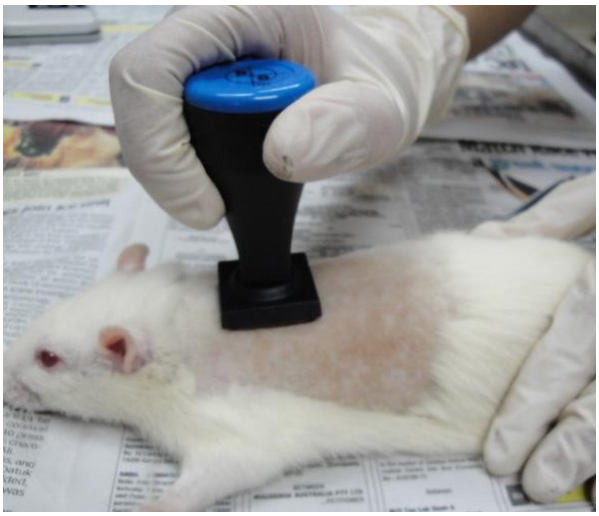

Figure 3.4: Outlined of excision wound made by wound mold.

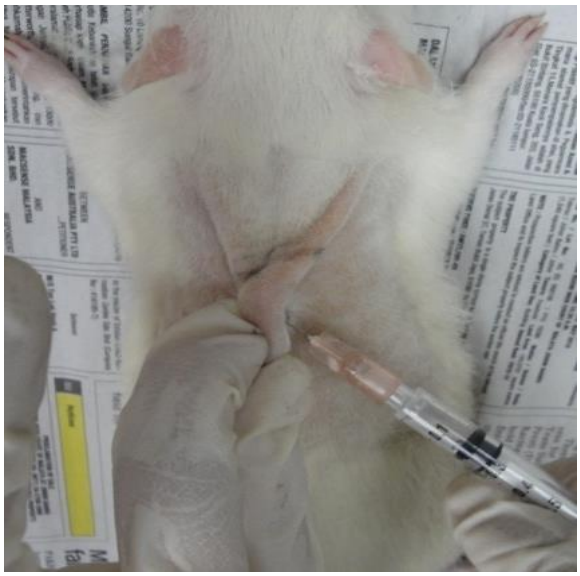

Figure 3.5: Subcutaneous injections of local anesthesia before wound creation.

3.3.2.4 GROUPING AND MODE OF TREATMENTS

The rats were assigned randomly into five groups with 30 rats (N=30) each to receive different treatments. The rats were treated with different types of treatments respectively according to Table 3.1.

Table 3.1: Grouping and treatments used in animal study.

GROUP	GROUP NAME	TYPE OF GROUP	TYPE OF TREATMENT
NO	No dressing	Control	No dressing
SA	Saline	Negative control	Normal saline
IN	Intrasite	Positive control (Reference Standard)	Intrasite gel
GE	Gelam	Experimental	Gelam honey
NE	Nenas	Experimental	Nenas Honey

Experimental wounds were cleaned with normal saline prior to topical application according to each treatment. Quantities of approximately 0.25ml/cm^2 of different wound treatments were applied topically onto the surface of each excisional

wound after wound creation according to the treatment groups. The wounds were air exposed and treatments were applied topically once a day until they were euthanased.

3.3.2.5 SAMPLING

Each rat was closely observed and inspected for the general morphological changes of the wound healing process. Evaluation of the wound healing process was done macroscopically and microscopically. Body weight of each rat and measurements of each wound were done and recorded. Six rats representing each treatment group were sacrificed at Day 1, 5, 10 and 15 of treatments (n=6). These days were chosen as it represent each phases of wound healing process. Wound tissue was carefully removed from each rat and fixed immediately in the Bouin's solution for histological analysis.

All samples were properly labeled before storage. The measurements were done blindly depending only on labels but not the treatment group. This method was used to avoid experimental bias. Samples were made known and assigned to the data respectively after analysis was completed.

3.3.2.6 ASSESSMENT AND MACROSCOPIC EVALUATION OF WOUND HEALING

The body weight of 30 rats for 15 days of treatment was weighted on day 1, 5, 10 and 15 of treatments. Other rats were periodically weighed before and after the experiment. Data were recorded and changes of the body weight along the duration of the study were taken into account.

The duration needed for wound healing process in all treatment groups were observed and recorded prior to the macroscopic and histological evaluation. The assessment of wound healing was done macroscopically as described. Observation was done at Day 1, 5, 10 and 15 of treatments. Wound margins were traced on transparency paper using fine tip permanent marker pen at Day 1, 5, 10 and 15 of treatments. Each traced area was measured by graph paper. The progress of the healing process was recorded and photographs of each wound were taken by digital camera (DSCW290) and the hand held digital microscope (Dino Lite) for analytical purposes. Morphological evaluations were done on wound size (wound diameter and wound area), healed area, wound contraction (contracted wound diameter and wound area) and wound appearance.

Wound size included both wound diameter and wound area. Assessment on wound diameter was adapted and modified from Rozaini et al. (2005). Widest length of the wound diameter were measured longitudinally (length) and transversely (width) across the body length as shown in Figure 3.2. The data obtained were recorded. The wound area was measured using square counting procedure according to Aljady et al. (2003) modified from Schubert (1997) and Richard et al. (2000). The number of squares (0.04 cm^2) that appeared complete (N_c) and partial (N_p) inside the tracing area were counted and the wound area was determined according to the following formula:

$$A_{c+p} = (N_c + 0.4x\ N_p) \times 0.04$$

Healed area is the area that healed after the treatments. It was measured and calculated according to the formula:

Healed Area = Original wound area- Wound area after N days of treatments.

Wound contraction included both contraction of wound diameter and wound area. The rate of wound contraction for wound diameter was determined using the following formula adapted from Rozaini et al. (2005) cited from Baie and Sheikh (2000). Formula of the rate of contraction for wound diameter:

Percentage of wound contraction = (Healed diameter/Total diameter) x 100

As for rate of the contraction for wound area, the calculation was as described by Aljady et al. (2003) by using the following formula.

$$C = (A_1 - A_n) / A_1 \times 100$$

C represents the rate of wound contraction in percent at any day of treatments; A_1 is the original wound area before treatment while A_n is wound area on the selected day of treatments

For the assessment of wound appearance, the experimental wounds were observed and examined daily. Photograph of each wound was taken on Day 1, 5, 10 and 15 of treatments for analytical purposes. All wounds were assessed clinically according to the scoring system modified from the clinical judgement by Bates Jensen (2001) and Khoo et al. (2010) as shown in Table 3.2.

Table 3.2: Macroscopic Wound Evaluation System adapted from Bates Jensen (2001) and Khoo et al. (2010).

WOUND EVALUATION	**0**	**1**	**2**	**3**
Wound bed				
Color	-	Pink	Dark red	White
		(Healthy)	(Unhealthy)	(Infected)
Dryness	-	Dry	Moderate	Wet
Exudates				
Types	-	Serous	Bloody	Purulent
		(Thin,watery)	(Blood)	(Yellow)
Amount	None	Less	Moderate	Heavy
Necrotic tissue				
Types	-	None	Yellow slough	Black eschar
Amount	None	Less	Moderate	More
Scab				
Amount	None	Less	Moderate	A lot
Color	None	Light brown	Dark brown	Bloody
Texture	None	Thin	Moderate	Hard

3.3.3 STATISTICAL ANALYSIS

The data obtained were statistically examined and analysed by using Statistical Package for Social Science (SPSS) software. The values were expressed as Mean $\pm$ Standard Error Mean (SEM) 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 signifinace was set at 0.05.