

## CHAPTER 5: GENERAL DISCUSSIONS

### 5.1 DISCUSSIONS

Wound healing process has had become one of the concerns among researchers due to the complications caused by modern wound dressings and also the resistance of bacteria (e.g. MRSA) to antibiotics. Thus, in the past decade, there has been an increase of interest among the scientist in the use of traditional and complementary medicine. A number of natural substances or products such as honey had attracted the interest of scientists as having potential to be used for wound treatments.

Honey has been used for its medicinal properties since ancient time in many cultures. Although it is a commonly used as folk remedies in treating human diseases especially in wound management, its scientific and medical reports are still comparatively very few (Jeffrey and Echazarreta, 1996). Thus, honey was selected as wound dressing for acute wound treatments in this study; specifically two Malaysian pure honey, Gelam honey and Nenas honey. The efficacy of Gelam honey and Nenas honey on wound healing was not been fully studied in all aspects. To complete the previous study which was done using the biochemical approach (Aljady, 2003), this study was carried out to study the efficacy of both Gelam honey and Nenas honey by using both macroscopic and microscopic approaches. Besides that, there was no previous research done to compare the efficacy between this two selected honey.

Gelam honey is one of the most abundant and widely consumed Malaysian pure honey collected from the forest (Aljady, 2000). This honey was chosen because it showed a higher antibacterial in previous study. High antioxidant and high growth promoting activity of this honey was already determined in previous study (Aljady, 2003). There were also other studies reported that the topical application of Gelam

honey could stimulate the rate of burn wound healing by increasing the contraction rate (Rozaini et al., 2004; Rozaini et al., 2005).

As for Nenas honey, it is another commonly consumed Malaysian pure honey, which was collected from pineapple plantation. Thus, the supply of this honey is more convenient compared to the Gelam honey. Other than that, it was selected as another honey wound dressing in this research since no other work had been done on it. As honey is well known in its wound healing properties, the data from this study contributed to fingerprinting the efficacy of the two honey in wound healing.

There were different types of antiseptic agents that are commonly used in treating the wound by fighting the wound microbes. However, some studies had reported the controversy of using these antiseptics on treating wounds. It might possess certain level of toxicity due to its antibacterial properties. There are reports that showed antiseptic such as providine-iodine are toxic to fibroblast and epithelial cells and should not be used on open wound (Kaye, 2000). Thus, hydrogel was chosen as the dressing in this study. The reason for choosing the Intrasite gel as the positive control dressing in this study was that it is a commonly used wound dressing prescribed by physicians in treating acute and chronic partial or full thickness wounds. This study demonstrated that the gel can be directly applied to the wound once a day. Intrasite gel good for early stage of wound healing process. It provided moist healing environment to the wound, as it was non-adherent to the skin surrounding, thus making the dressing not harmful to the process of wound healing (William, 1994).

Normal saline with the concentration of 0.9 % was chosen as the negative control treatment in this study. It is a widely recommended irrigating and wound dressing solution that is compatible to the human tissue. It is the standard moist dressing for wounds and causes no damage to new tissue. This dressing would not

affect the function of the fibroblasts and keratinocytes in the healing wounds (Salami et al., 2006).

Generally, due to the different experimental approaches and models, different researches sometimes produced different results. Hence, a standardized and reproducible model is very important in wound research for better understanding in wound healing process which could indirectly improve the medical technologies.

Standardized model is very important in wound research with regards to the comparability of the results between studies and possibility of transferring data to human clinical study. Some matters to consider when choosing are whether it is *in vivo* or *in vitro*, factors being investigate (e.g. dressing efficacy) and outcome parameters (e.g. size, collagen deposition). Animal model is defined as a living organism that mimics the human body (Aljady, 2003). Different animal models have been used in wound research, although animal study of specific phase of wound healing process is almost not possible (Su, et al., 2010). Rats have been widely used as the experimental animal in the study of skin wound healing and the efficacy of different wound treatment. There is review show that male *Sprague-Dawley* rats weighing in 250-300g were the most preferred animal model among researcher (Dorsett-Martin, 2004). Thus, male *Sprague-Dawley* rats were selected as the experimental animal for this wound healing study.

Only male rats were selected for this wound research to reduce the variability which could be caused by estrogen on various phases of cutaneous wound healing (Melissa, 2000). Oestrogen hormone has effects in maintaining the skin quality in women and also the healing of wounds. There are lots of literatures available on the physiological effects of oestrogen on various phases of wound healing such as inflammation, proliferation and remodeling (Clark, 1996; Melissa, 2000). In addition,

there are some contradictory findings on the effects of ovarian hormones in the healing process, which could lead to the difficulties in making a valid conclusion in wound healing research (Melissa, 2000). Thus, usage of different gender experimental animals in wound healing research and the diversity of methods for assessing healing were avoided in this study; only male animal were used for the purpose of standardization.

Establishment of a simple and reproducible wound model is very important in wound research. Literatures reported that the commonly used wound model in the skin wound research were incisions, excisions and burns (Dorsett-Martin, 2004). Work using *in vivo* experimental wound models were widely used in wound research because it is accurately mimicking the common injury in human body (Tomlinson and Ferguson, 2003). This *in vivo* model allows quantitative and qualitative evaluation of treatments at all stage of wound repair including granulation and re-epithelialization (Tomlinson and Ferguson, 2003). Incisional and excisional wounds are the two main models used in wound research for determination of the wound healing phases.

The healing pattern studied in *in vivo* wound model involves three major phases namely, inflammation phase, proliferation phase and maturation phase (Kerstein et al., 1998; Baie and sheikh, 2000, Sussman and Bates-Jansen, 2001, and Aljady, 2003). This model was suitable for the investigation of the efficacy of different types of wound dressing (Gottrup, 2001). The efficacy of both Gelam and Nenas honey were evaluated from the histological aspect in this study. Excisional wound model contains significantly broader morphological changes, which occur during the healing process compared to the incisional wound (Gal et al., 2008). However, there were also disadvantages for using this model. The main disadvantage of using this model was that most of the wound healing measurements in this model were indirect (Gottrup, 2001).

Wound healing process involved healing by primary healing wound closure, secondary wound closure or delayed primary closure. The experimental wound model used in this study involved the loss of tissues. Thus, wound used could not be closed. Therefore, secondary wound closure took place. Wound was left open and allowed to heal by granulation which caused it to heal at much longer time than primary closure. More care was then needed for wound management. Other than that, this type of healing formed more scar tissue because the wound area was larger. Thus, secondary wound closure is not as good as the primary closure in cosmetics and functional results. However, it means that the model is suitable to use as a method to compare the efficacy of the treatment in scar minimizing.

Majority of wound healing studies used the dorsum area of the rat as the wound location (Dorsett Martin, 2004). The experimental wound was created on the dorsum area for the purpose of minimizing the differences in wound gape and interferences caused by animal biting, scratching or rubbing against the caging. All the animals were caged singly one week prior to wounding in order to prevent any skin lesion caused from fighting between the rats, which might affect the results for the evaluation of wound healing (Tomlinson and Ferguson, 2003).

Ketamine and Xylazine were injected intramuscularly for the general anesthesia while subcutaneous injection of lignocaine was for local area anesthesia. The rubber stamp was used as a wound mold to create a standardized wound area. Parameters involving measurements wound then be of comparable values. The application of treatment was based on the way normally practiced in wound treatment. Hence, the most convenient way of application of wound treatments topical application of dressings to the open wound was done.

This wound model could produce standardized and reproducible wound healing experiments. It could also produce homogenous groups. To attain the statistical significance groups for each time line or healing parameter, each group included at least six animals (n=6). The animals were selected randomly to ensure an even distribution of various factors such as weight and age. The six animals of each group of the same time point were all wounded on the same day. Specific established control groups were carried out together with the experimental groups to test the effects of treatments (vehicle-treated group).

Based on the results of previous study (Aljady, 2003), the amount of dressing which was used in this study was fixed at 0.25ml/cm<sup>2</sup>. There were studies showing that low dose of topical application resulted in less effective treatment of wound healing process while high dose resulted in over growth or dehydration of the newly formed granulation tissue and also the surrounding tissue (Aljady et al., 2000 and Aljady, 2003). However, the dose of honey used in this study was the same as in any other modern treatment on wound.

The dose of honey depends on the types and the healing activities of the selected honey (Aljady, 2003). According to the previous study, there were no side effects found in this dosage of Gelam honey application. Thus, the dosage of treatments for all the other treatments such as Intrasite gel and Nenas honey were also set at 0.25ml/cm<sup>2</sup>. The quantity and times of application also depend on the severity of the wounds. Therefore, this dose used may not be the standard dose for all types of honey treatments.

Many studies indicated that the efficacy of honey on wounds and burns showed better results when systemic administration (intraperitoneal way) were carried as compared to the topical application (Suguna et al, 1992; Efem, 1993). However,

intraperitoneal administration of wound treatment with honey is not convenient for patients in daily treatments. Thus, the topical application of wound treatment is selected as the way of administrations for all experimental wounds.

Three types of staining were done in this study to observe the specific parameter of the wound healing process, namely H&E staining, Masson's staining and Verhoeff's Elastic Staining. The wound specimens were fixed in Bouin's solution for 24 hours because skin tissue is very hard and elastic. Bouin's solution softened the skin tissue for easy cutting. It was changed to 10 % Neutral Buffered formaldehyde to prevent tissue from becoming brittle or shrink. There are studies showing that the staining intensity of the tissue in H&E stain and Masson's Trichrome were less apparent in the specimens that were fixed in 10% NBF compared to the Bouin's solution. There is also no fixative effect found on the architecture of the tissue but it affects the color intensity of the samples because of the enhancement properties (Ukong et al., 2008). Thus, Bouin's solution was selected as the fixative in this study due to its enhancement properties on the color intensity.

After applying the treatments, the wound was left open in this animal study. The wound was left uncovered to prevent the stripping of the newly formed epithelial layer. Topical treatment for wound is only a small part of the healing process. There are still many other factors that could lead to the impairments such as malnutrition, edema, and bacterial contamination (Moon and Crabtree, 2003). The method of application of wound dressing in this study would be equivalent to normal and most convenient ways of daily application among patients.

Intrasite Gel was chosen because it is a commercialized wound dressing, which is commonly used by physician for topical acute wound treatment. It is good in minimizing wound exudates in dermabrasion, superficial burns and chronic ulcers

(Moon and Crabtree, 2003). The results obtained in this study showed that topical application of Intrasite gel could accelerate the wound healing duration in all phases of wound healing. It dried up the wound exudates and helped scab formation. However, since the scab was difficult to detach from the wound, this indirectly resulted in the delay of wound healing.

Honey is a common household product that is nonirritant, nontoxic, easily available and comparatively not expensive. It has been studied extensively in wound healing but the effects in clinical practice are not fully established. Topical application of honey (Gelam honey and Nenas honey) in this study had significantly accelerated the rates of wound healing compared to the untreated and saline treated groups. Both honey took about 13 days in wound healing. Common properties of both honeys include the hygroscopic, antibacterial, antioxidant properties, nutritional and also the production of hydrogen peroxide responsible for the positive wound healing effect (Aljady, 2003 and Chepulis, 2008). All of these properties of honey provide a suitable environment for wound and indirectly accelerate the duration of wound healing. There are also reports indicating that honey help in clearing infection and protecting the wound from being further infected, hence help to shorten the duration of healing (Norimah et al., 2007). Other than that, application of honey dressing could also stimulate the healing process by stimulation of tissue regulation, reduction of inflammation and non-adhesiveness of tissue dressing (Medhi et al., 2008).

Sample collections were done on Day 1, 5, 10 and 15 of treatments. These days were selected because in this time frame, it was possible to investigate all the three phases of the wound healing. According to Gal et al. (2008), two days post-surgery allow the evaluation of the acute inflammatory reaction and also beginning of the reepithelialization process. Day 2 of post-injury is equivalent to the Day 1 of treatment in this study. Day 6 of post injury was suitable to evaluate the proliferation phase and



also reepithelialization phase. While Day 14 of post injury equivalent to Day 15 of treatments in this study was suitable to evaluate the maturation and scar formation.

Since there is a concern in the phenomenon of dishonest act of honey adulteration and also the presence of synthetic honey, a number of analytical methods have been developed to verify the purity of honey all over the world. Honey quality standard varies according to countries. Malaysia has own standard of honey quality tests which can be done easily in the laboratory. The samples were identified as pure when they meet the characteristics of the pure Malaysian Honey Standard from the Department of Standards Malaysia as showed in Chapter 2.

In this study, the methods chosen for honey purity test were mainly due to the low cost of the experimental procedures and also less time consuming. In addition they are accurate and repeatable as proven from the previous study. However, the honey purity could not be determined by solely referring to any specific tests. The purity of both samples of Gelam honey and Nenas honey were based on the combination of the result of the different tests.

The macroscopic observation in the present study showed that topical application of all the wound dressings accelerated the wound healing process. Both types of honey treatments gave similar results as Intrasite gel treatment in the efficacy of wound healing process. They provided moist environment which contributed to wound healing process. Topical application of honey also reduced the amount of exudates and scab formation. In addition, honey treatment also promoted the detachment of scab. The scabs formed in both honey treated groups were thin and moist compared to the one in Intrasite gel group. The results in the present study showed that topical application of Nenas honey had slightly better efficacy in wound healing in enhancing the wound contraction.

Macroscopic and microscopic observations indicated that the application of both Gelam honey and Nenas honey influenced the various phases of wound healing leading acceleration of healing process. The Untreated and Saline treated groups were seen to be slow in the healing progress. Lesser wounds contraction was also seen in wound of both groups. The acceleration of wound healing was also demonstrated through the histological parameters observed in the present study.

Nenas honey may contain bromelain that could have also contributed to the wound healing process. Bromelain is a proteolytic enzyme derived from *Ananas cosmosus*. There are studies showing that oral administration of bromelain help in reduction of edema, bruise, pain, and healing time (Mackay and Miller, 2003). Thus, Nenas honey gave better results in the control of inflammatory phase compared to the Gelam honey.

The antibacterial activity of honey also contributed to the wound healing process. Low pH level and the ability of honey to absorb moisture were among the factors that favored the wound healing process. These two characteristics of honey form a protective layer on the wound that prevented the penetration and colonization of bacterial on the wound (Lotfi, 2008). The results of the present study were in agreement with the previous study that showed that honey sterilized the wound within 7-10 days of treatment and promoted the healthy growth of granulation tissue (Jerry and Echazarreta, 1996).

Honey is a high concentrated sugar solution that facilitates a strong interaction between sugar and water molecules. The high osmolarity of the honey helps in bringing the lymph into the wound site. This helps in the supply of the nutrient to the wound surface (Molan, 2006; Salmi et al., 2011). It indirectly reduces the swelling (inflammation) and cleans the wound surfaces (Lofti, 2008). This is important in

promoting the wound healing process because honey might also provide the energy source for the surrounding cells and a protection for the protein layer of the wound surface (Subrahmanyam, 1999). These characteristics of honey leave the wound surface with very few water molecules and thus minimize the growth of microorganisms (Salmi et al., 2011).

One of the antibacterial elements in honey is hydrogen peroxide. The low level and slow release of hydrogen peroxide in honey contribute to the antibacterial properties of honey. Hence, the result from this study which demonstrated topical application of honey prevented infection and reduced the inflammatory phase.

Honey dressing enhanced angiogenesis during the wound healing process. Angiogenesis in granulation tissues improved the blood circulation to the wound site (Abdulla et al., 2010). Honey might stimulate the growth factor in the wound site helping in the enhancement of the development of new blood vessels. Thus, it provides the wound area with the nutrients needed for wound healing. In addition, it also enhances the supply of the oxygen which is essential for the healing process such as reepithelialization (Szabo et al., 1995).

Antioxidant has been reported to be an important compound in wound healing process. There are reports that topical application of antioxidant compounds on animal models has significantly improved the wound healing process. It also protects the tissue from oxidative damage, thus accelerated the healing process. (Shukla et al, 1999). Honey contained a number of antioxidant compounds. The present study showed that wound healing properties of honey might also be due to the contribution of its antioxidant level. Previous study done by Aljady and Kamaruddin (2002) reported that the antioxidant activity of honey and its phenolic content had high correlation. The phenolic content in honey could vary due to their floral sources. For example, Gelam

honey contains gallic, ferullic, caffeic, benzoic, and cinamic acids (Aljady and Kamaruddin, 2002).

The antioxidant properties in honey help in neutralizing the free radicals produced by hydrogen peroxide (Frankel et al., 1998). Bacterial growth has been shown to be inhibited by 0.02-0.05 mmol/l of hydrogen peroxide. Hydrogen peroxide produced in honey is activated by dilution of typically 1 mmol/l and it is about 1000 times less than the solution used as antiseptic. This concentration of hydrogen peroxide produced does not damage the fibroblast cells (Salmi et al., 2011). Other than that, growth factors also stimulated the epithelial cell proliferation. Thus, this might be important in wound healing activity (Abdulla et al., 2009).

In addition, the wound healing evaluation in this study was done by observing the collagen level in the wound. Collagen deposition was greater in the wounds that received treatment. Gelam honey treated group showed better results in collagen deposition compared to the other groups. Several studies reported collagen content for wound evaluation (Ukong et al., 2008). Collagen fibers were clearly observed using light microscope in the Masson Trichrome stained slides. The collagen visibly stained in the sections showed that the intensity in the wound area was less compared to the normal skin. Collagen is very important in wound healing. It provides structural framework for the regenerating tissue (Abdulla et al., 2009).

## 5.2 SUGGESTION FOR FURTHER STUDIES

More research has to be done on the characteristic and chemical properties of both types of honey used in this study to support the data of the efficacy of each one. Other types of Malaysian pure honey should also be investigated and compared with these two types of honey for the efficacy in wound healing since floral source of honey sample affects the efficacy of wound healing. Further study can also be done on the mechanism of wound healing in honey treatments from various sources. Research can also be done on application of Gelam honey and Nenas honey to other different wound types, such as ulcers, burns and surgical incisions.

More parameters should be done to observe the efficacy of wound healing such as the inflammatory cells in the wound healing process. Immunohistochemistry methods could be used for better results.

Previous studies have showed that collagen fiber is one of the important wound characteristics during the healing process (Goly et al., 1997; Rao et al., 2007). Quantification of the wound healing characteristics, especially the changes of collagen in wound healing, were done using special instruments which are not available in routine histology laboratory unit. These instruments included epipolarization microscope (Noorlander et al., 2002) and laser scanning confocal microscopy (Taylor et al., 2002). Result of current study could be further confirmed using these instruments. Other than that, how innervation of affected by wounds and whether honey could assist the returned of nerved function should also be study.

Stem cell research in producing different specific cells needed for wound healing is also an avenue to be explored. Effects of honey or other natural products in facilitating the production of such cells could also be considered.