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STUDY OF WOUND HEALING IN RATS TREATED WITH SKIN OF POISONOUS FROG, *ODORRANA HOSII*

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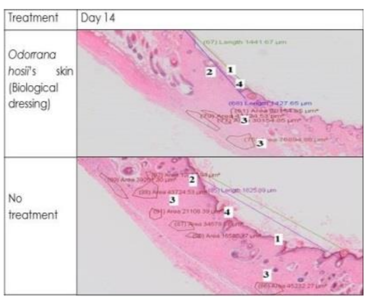
Graphical abstract



Abstract

A grafting techniques or using various synthetic and biological dressing also widely used to protect the wound area. *There are 8 peptides with differential antimicrobial activities contained in Odorrana hosii's skin secretion*. However, to our best knowledge no study has been scientifically conducted to reveal the value off this species on wound healing. Primarily, the aim of this study was to look at the potential use of *O. hosii*'s skin as a biological dressing in wound healing management. This study assessed the wound healing in rat compared between wound grafted with *O. hosii's skin* and wound treated with normal saline dressing. Histological examination was done to assess the wound healing activities after 14 days. The result shown, both wounds which were treated with *O. hosii*'s skin and untreated wound heal completely on day 14 as the epidermis and dermis completely close. Histologically, the percentage of neutrophils, macrophages and fibroblasts, were reduced on day 14. However, wounded skin, which was treated with *O. hosii*'s skin, had better healing quality as more new tissues and hair follicle regrowth compared with the untreated wound. It is suggested that poison gland in the *O. hosii*'s skin did not harm the wounded rat skin, instead, poison that act as defensive mechanism can help the species to fight the pathogen on the wound.

Keywords: Wound, biological dressing, histological analysis, frog skin, *Odorrana hosii*

1.0 INTRODUCTION

A wound is defined as any break in the normal continuation of the cellular and molecular structure of tissue or body [1]. The wound can also be classified as partial-thickness, or fully thickness depends on the depth of the injury. A partial thickness wounds only involve the loss of epidermis and extends into but not through, the dermis, meanwhile, full thickness wound involved the total loss of the epidermis, dermis and subcutaneous fat tissue [2; 3].

The wound can be healed spontaneously, which starts from the various chemical signals in the body, and finally facilitate the restoration of the anatomical structure and function of the tissue. A partial thickness wound heals by mere epithelialization, and the full thickness wound heals through the dermis, which involves more complex and well regulated biological events [1]. The wound healing process in human adult can be distinguished by 3 distinct phases which is the inflammatory phase, the proliferative phase, and the remodeling phase. It is involves a series of cellular, physiological and biochemical processes [4].

Inflammatory phase is where the immediate body response to the injury and the blood vessels in the wound bed contract and form a clot. The predominant cells that are important in this phase are the phagocytic cells; neutrophils and macrophages. These cells will phagocytes and auto-lysing any devitalized slough tissue. The neutrophils migrates from wound margin and move towards the fibrin clotting. Proliferative phase is the second phase of wound healing where it is characterized by the re-epithelisation, angiogenesis, fibroblasia and wound contraction [5]. The granulation tissues will progressively invade the wound space and collagen fibril will gradually create a bridge at the wound during this phase. The remodeling phase starts with the continuous accumulation of collagen and proliferation of fibroblasts followed by reduction of leukocyte infiltration and edema. This phase involves the synthesis of collagen fibers, and leads to increase the tensile strength of the skin [6].

The wound is usually treated with dressings which contained antimicrobial agent where it can control the infection and speeding wound recovery. The ideal dressing needs to keep moist, which can insulate the wound and protect it from contamination. The normal saline is a normal dressing that is frequently used for open wound [7]. A grafting techniques or using various synthetic and biological dressing also widely used to protect the wound area [8]. Anurans skin has been used for wound healing since the ancient time [9]. The anurans thus have become the biological dressing for wound healing treatment and have been used for many years ago. The study indicated animal fat such as lipids of frog skin, whether applied topically or injected can accelerate the healing of wounds [4].

Odorrana hosii is known as poisonous rock frog or Hose's frog. It is a slender body frog with long and strong body limbs as shown in Figure 1. The dorsal skin is finely pebbled with a weak fold of skin on each side. The lateral sides are usually brown; the upper surface of the legs light brown with dark crossbars and the belly is greyish or silvery white [10]. *O. hosii* is listed as least concern in the International Union for Conservation of Nature because of its widely distributed, with a large population tolerance of a degree of habitat modification and it is unlikely declining fast enough to qualify for more threatened category [11]. This species can be found throughout Borneo in appropriate environments and it also occurs in Peninsular Malaysia and Sumatra [10].

There are 8 peptides with differential antimicrobial activities contained in *O. hosii*'s skin secretion [12]. The presence of this antimicrobial peptide lead to many efforts in the search for new antimicrobial agents as lead compounds for new antibiotics [13]. However, no study has been scientifically conducted to reveal the medical value of this species in wound healing management. Therefore, we investigate the potential of *O. hosii* as wound healing management. The skin of *O. hosii* contains poisonous secretion and other properties include the antimicrobial peptide, lipids, and mucus that's stored in this frog skin may help to facilitate the recovery of the rat wound.

Figure 1 *Odorrana hosii* (Photographed by Nur Amirah Md Sungif)

2.0 RESEARCH METHODOLOGY

Frogs were captured according to protocols approved by Sarawak Forestry and under process license number NCCD.907.4.4(jlid.8)-46 and Park permit number 313/2012. The frogs sampling were conducted at Batang Ai National Park in Lubok Antu and Gunung Gading National Park in Lundu, Sarawak to harvest the frog samples for our research study. The location was chosen because Batang Ai National Park and Gunung Gading National Park consist of suitable stream habitats for the species and was also based on reports from previous collections.

All experiments with live animals were approved by the Animal Research Ethics committee of Universiti Malaysia Sarawak and were carried out by authorized investigators. The dorsal frog skins were peeled off and split longitudinally from the frog's dorsal body. The dissecting of frog skin was started from the anus and cut into further sub lot and kept after harvesting.

The rat was anaesthetized by Intra-Peritoreal (IP) Inoculation technique using KTX (Zoletine, Ketamine, Xylazine) dilution. Two excision wounds that were approximately 5mm (diameter) up to the level of subcutaneous, adipose tissues were made over the dorsal of rats by using the 5mm punch biopsy tool. After the wounding process, the rat was kept in a sterilized cage and given food and water.

One wound was grafted with the *O. hosii* skin (5 mm diameter) sutured to the wound surface and the other wound was left untreated. The wounded skin was taken after 14 days of post-wounding to observe the healing process through histological analysis. The skin was placed in 10% formalin (fixative solution) for 24 hours.

The skin was dehydrated in a six times graded series of ethanol, embedded in paraffin and cut into sections. The sections were then stained with haematoxylin-eosin to identify granulation tissue, inflammatory cells and tissue structure at the wounded area [14]. Finally the skin samples were observed under Olympus image capture microscope.

The wounded area was examined by analyzing the repairing tissue structure under the skin as a guide to examine the duration of the healing process [15]. Modified method of Braiman-Wiksman *et al.* was used to quantify wound healing through histology [16]. The histological assessment was carried out on the widest and deepest part which is the most completely disrupted part of the wound was considered in the assessment. The overview of the wounded area which consists of granulation tissue, inflammatory cell and other tissue structures was shown in Figure 2.

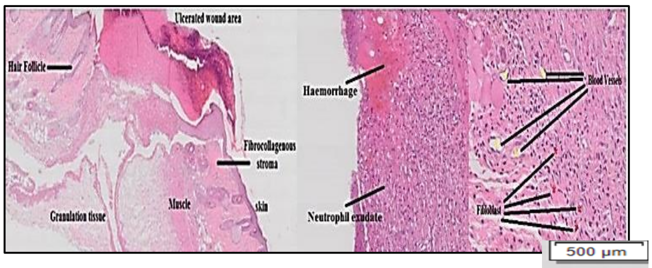


Figure 2 Overview of a wound of rat skin

There are four parameters that were modified from Braiman-Wiksman *et al*. [16] and Gal *et al*. [17] which consist of (1) epidermal closure, (2) dermal closure, (3)infiltration of granulation tissues and (4) inflammation rate. The dermal closure was measured by taking a percentage of contraction and new dermis formation in the dermal layer. The infiltration of granulation tissues in the wound was measured by taking percentage of the number of fibroblast per wound area. The inflammation rate is determined by the abundance of neutrophils, and macrophages in the wound area. Semi quantitative method was used to estimate the rate of inflammation modified from Gal P *et al*. [17] .The classification of inflammation is very subjective. If the inflammatory cells are absent, the inflammatory scale is 0, however, if the scattered inflammatory cells were usually mild, scale is 1; cells that were densely aggregated were classified as severe, scale as 3 and anything between are classified as moderate, scale 2 .

3.0 RESULTS AND DISCUSSION

Figure 3 displays the condition of the wounded skin surface of rat treated by *O. hosii*'s skin and control on day 0 and day 14 post wounding assessment. Both wounds were completely re-epithelised. We observed that the scab formation in skin treated with *O. hosii*'s skin was completely disappeared, meanwhile for the untreated skin; the scab was not completely disappeared. Hair was started to cover the scar in both treatment, however more hair has covered the scar area on the skin treated with *O. hosii*'s skin when compared to the untreated wound.

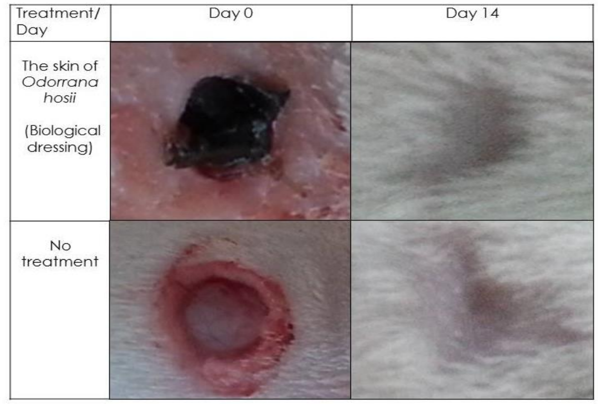


Figure 3 Rat's skin surface on day 0 and day 14 after treatment

As shown in Figure 4 and Table 1, the result and the analysis of the wounded area were shown that all wounds were 100 % fully re-epithelized by new epidermis and dermis for both treated and untreated wound. No wound gap appeared in both treatments. It was observed that the new epidermis and dermis started to be occupied with hair follicles and other new tissues. The formation of collagen, and fiber were abundant in this healing phase to increase the tensile strength of a new skin. This finding was in agreement with study by [6].

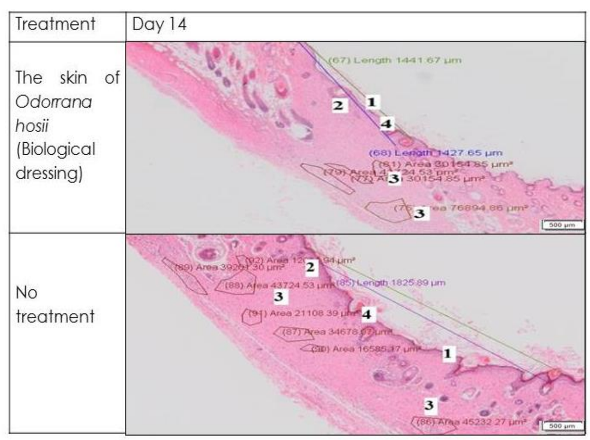


Figure 4 The skin structure view under Olympus Image Capture Microscope (1: epidermal closure; 2: dermal closure; 3: Granulation tissue; 4: Inflammation)

The granulation tissues were still presence in both wounds, with more prominent in an untreated wound. The treated wound showed 3% granulation of tissue whereas the untreated wound had 5% tissue granulation. In addition, less area was occupied by new tissues and hair follicles in untreated wound areas as compared to the treated wound even though on day 14. In addition, the inflammation rates for both wounds were scaled 1 as the inflammatory cells were scattered in certain part of the wounded area and was considered as mild inflammation rate. The inflammation is essential in wound healing process because within acceptable levels of inflammation rate, it helps to facilitate the response to fight with bacteria that may presence in the wound surface area. Figure 3 showed that the wound treated with *O. hosii*'s skin was well remodeled, while the untreated wound was less remodeling.

Granulation tissue is an intermediary replacement for normal dermis, which matures into scar during the remodeling phase of wound healing [18]. The presence of this tissue is a sign of the healing process is taking place and it appeared as a red lump within the normal paler pink matrix. The new tissue actually will act as wound protection from bacteria colonization. This tissue demonstrates an elevated cell density, incorporating fibroblasts and macrophages and collagen fibers [19].There were presence of granulation tissues in both wounds at day 14. This indicates that the wounds were well recovered.

Poisons are often served as a chemical defense for organisms such as anuran. Such poisons either are produced from the synthesis of complex molecules from simple molecules or sequestered from their dietary sources or symbiotic organisms [20]. *O. hosii* is known as poisonous frogs and the secretion of the skin may kill small animals such as other frogs that get too much contact with this frog [10]. However, from this study wound that treated with this frog skin healed better than the untreated wound. This might suggests that the role of poison may acts as a defensive mechanism that might help in fighting the pathogen at the wound area. Study done by Daly showed that the glanular gland in frog and toad skins acted as a storage that contained high level of amines, vasoactive peptides, which have high activity as antimicrobials [20].

Table 1 The analysis of wound using 4 parameters modified from Braiman-Wiksman *et al*. [16] and Gal *et al*. [17]

Wound Treatment	Epidermal closure(%)	Dermal closure(%)	Granulation tissue (%)	Inflammation rate
Odorrana hosii's skin	100	100	3	1, Mild
No treatment	100	100	5	1, Mild

*The epidermal closure and dermal closure were determined by the percentage of the wound that have been re-epithelised by a new epidermis and dermis tissue. The percentage of granulation tissue was determined by the presence of fibroblast and blood vessel that accumulate at the wounded part. The rate of inflammation was scaled by the presence of inflammatory cell (0, absent; 1, mild; 2, moderate; 3, severe)

The antimicrobial presence in the skin may help to attack the bacteria that accumulate on the wound surface, hence prevent infection. This may prolong the healing process. However, there is no further information about the response of frog poison towards the pathogen yet; new drugs were discovered that was extracted from the toxic skin of Ecuador rare frog and the poisonous South Africa frog. [21].

The successful preparation of a wound is very crucial because it can affect the whole result of the study [22]. In this study, we used 5 mm punch biopsy to create the wound so that all wounds will be in uniform standardized size. In contrast all studies involving wound excision model used scalpel that produce unrounded wound area [1, 6].By using the biopsy, it did not severely traumatize the animal and it is easier to create a well-rounded wound edge and depth of the wound. This tool is normally used in dermatology and surgical field to remove the skin tissue from human body.

Histopathology study of frog skin is needed to be further explored. Understanding the skin properties and their function, it may help determine the properties that may have potential in accelerating the complete wound healing process.

4.0 CONCLUSION

Our research finding suggested that wound grafted with *O. hosii* skin has fully completed the wound healing process within 14 days as indicated by regrowth of hair follicles and rapid regeneration of new tissues. The findings suggested that the *O. hosii* skin may have the potential in wound healing. Further study should be done to further validate this finding.

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