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Efficacy of Papain-based Wound Cleanser in Promoting Wound Regeneration

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Abstract: A new invention, papain-based wound cleanser is formulated by incorporating papain, a proteolytic enzyme extracted from *Carica papaya* into the formulation. This cleanser is invented to simplify the methods in wound management by combining wound cleansing and wound debridement using a single formulation. This study describes the preparation and preclinical study of papain-based wound cleanser in accelerating wound healing. In this study, papain-based wound cleanser was used to treat wound incision on Sprague-Dawley rats while distilled water and Betadine® were used as negative and positive control. Twenty-seven clinically healthy white rats were randomly divided into three groups and treated accordingly until the 21st day post-incision. Wound reduction rates and histological analysis were obtained to asses the healing pattern. Rats treated with papain-based wound cleanser showed a progressive wound healing based on the wound reduction rates and histological analysis when compared with rats treated with distilled water and Betadine®. Better collagen deposition and presence of skin organelles in rats treated with papain-based wound cleanser demonstrated its efficacy in promoting wound healing. In addition to its wound healing effect, papain-based wound cleanser is also integrated with antibacterial properties which make it a complete package for wound management. However, further studies should be carried out to ensure its safety for human usage.

Key words: Papain, wound cleanser, regeneration, wound healing

INTRODUCTION

The field of wound care is in constant evolution. Wound management has seen progresses in medications, materials and methods that are used in wound repair and regeneration. Wound is defined as discontinuation of skin defect that leads to implications in term of illness and lesions which can cause death when it is not properly managed. Wounds are generally classified as wounds without tissue loss (e.g., in surgery), wounds with tissue loss (burn wounds), wounds caused as a result of trauma, abrasions or as secondary events in chronic ailments (venous stasis and diabetic ulcers) and iatrogenic wounds (skin graft donour) and derma abrasions (Paul and Sharma, 2004).

Injury to the skin triggered a homeostatic response with the purpose to protect the skin and restore the epidermis which involves edema formation and inflammation, eschar formation, cell proliferation and migration, production and remodeling of the extracellular matrix, angiogenesis and re-epithelialization (Coulombe, 1997; Singer and Clark, 1999). For the first 48 h, an inflammatory phase takes place which was characterized by cell proliferation and migration; preceded by a proliferative phase dominated by collagen deposition and angiogenesis; and lastly a maturation phase involving resolution of inflammation and scar maturation (Greenhalgh and Staley, 1993; Clark, 1996). Failure to properly treat wound at its beginning will later cause problems to the individual which could be avoided through adequate wound management.

Inadequate wound management could lead to poor healing and increased cost in terms of nursing time, product use, cost of treatment and patient suffering. To avoid this, proper wound bed preparation is recommended before wound treatment. Conventional wound management includes wound cleansing using saline water or wound cleanser, treatment of infection, the debridement process, compression and wound dressing by occlusive dressings (Falanga, 2003). The purpose of wound

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cleansing in the management of wound is to prevent infection and to promote healing of the injured tissues while wound dressing acts to ensure moisture at wound bed.

Hebda *et al.* (1998) conducted an investigative study on the performance of papain-based wound debriding ointments which was found to be effective in debriding three types of eschar while promoted wound healing. Wright and Shi (2003) published their findings on Accuzyme®, a white, hydrophilic ointment containing papain and urea which effectively digested the eschar proteins. However, these debriding ointments needs two adjunctive products which are wound cleanser and wound debrider to achieve greater wound repair. In addition, the gel-like substance has no function as rinse. According to Mulder (1996), the spray-like function enables a complete flushing of wound site to soften and rinse away wound debris hence minimizing physical scrubbing of wound debris. Mulder (1996) also patented a formulation of wound cleanser in a spray form which aims to facilitate cleansing and bodily healing process yet this formulation have no debriding effect. Therefore, additional debriding agent is required to achieve adequate wound management.

Papain-based wound cleanser is formulated to embrace wound management paradigm via a single formulation. It introduced a new concept in wound care where it has dual function effects; wound cleanser and wound debrider. It features the necessary characteristics in a wound cleanser; including effective separation of devitalized tissue, bacteria and blood from wound surface, active microbial agent that reduces bioburden and ability to retain moisture at wound bed (Witkowski and Parish, 1996). More importantly, this cleanser contains skin cells regeneration properties and the breakthrough ingredient, papain to incorporate debriding action into the cleanser. Papain, is an enzyme derived from the fruit of the papaya tree (*Carica papaya*). Papain is a nonspecific cysteine proteinase (an enzyme that contains a cysteine residue at the active site) that is capable of breaking down a wide variety of necrotic tissue substrates over a wide pH range (3.0 to 12.0) (Zucker *et al.*, 1985).

The aim of the present study was to investigate the efficacy of papain-based wound cleanser in promoting wound regeneration on Sprague Dawley rats.

MATERIALS AND METHODS

Formulation of papain-based wound cleanser: For the formulation of papain-based wound cleanser, 5 g of papain is constituted in 79.68 mL deionized water along with 3.6 g mannitol, 0.72 g 8-hydroxyquinoline, 0.5 g sorbic acid, 5 mL glycerin, 5 mL cocoamidopropyl betaine and 0.5 mL alpha tocopherol. This solution was mixed vigorously to obtain a uniform product.

Animals: Healthy inbred Sprague Dawley rats of female sex, weighing between 200 and 250 g were obtained from the animal house of the School of Medicine, Universiti Sains Malaysia in 2009. The animals were housed in separate cages on normal food and water *ad libitum*. They were allowed to acclimatize in the research laboratory for 5 days before the commencement of the study.

Animals grouping: A total of 27 rats were divided into three groups (A, B and C) with nine animals in each group; Group A: Negative control (distilled water), group B: treatment using papain-based wound cleanser and group C: positive control (Betadine®). The experiment was performed at three intervals which were 7, 14 and 21 days of post wounding with 3 rats in each group.

Excision wound model: The rats were anaesthetized by inhalation of diethyl ether and then their paws were tied to boards. Their fur was clipped from the dorsal region and thorough antisepsis was carried out with 70% ethanol prior to inflicting the experimental wounds. An 8 mm wound was created using sterile wound bio-puncher. Each animal was wounded with two circular excision wounds on dorsal individually. The medication was applied to the wound of each animal according to the animal's assigned group.

Macroscopic assessment: All animals were photographed immediately postoperatively. The excision wound margin was traced after wound creation using planimetry method. The animals were again photographed every day until the 21st day. Distances, lenses and magnifications were kept constant. Wound contraction was measured every day until complete heal and was expressed in percentage of healed wound area.

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

A total of three rats from each group (group A, B and C) were sacrificed at different time point intervals at day 7, 14 and 21 of post wounding.

Microscopic assessment: Skin specimens from each group were collected and fixed in 10% buffered formalin. After usual processing, 4 μm thick sections were cut and

stained with Masson's trichrome stain and were assessed under the light microscope to evaluate the fibroblast proliferation, collagen formation and re-epithelialization. The stained slides were examined using a software image analyzer (Video Test Image Masters) and measurements were made of the intensity of the colour which represents the collagen density.

RESULTS

Figure 1 shows the average percentage of wound closure, based on the original wound area. Data was taken everyday on each day of the experimental treatment with distilled water (group A), papain-based wound cleanser (group B) and a commercial cleanser, Betadine® (group C).

Rats in group B achieved complete skin epithelialization by day 14, followed by rats in group A which was 100% healed at day 16 and rats in group C was completely healed after 18th post-incision day. Overall, the results which were determined by the reduction of the wound size showed treatment using papain-based wound cleanser provided faster healing process compared to distilled water and Betadine®.

Figure 2 shows *in vivo* wounds photographed on day 1, day 4 , day 7, day 14 and day 21. Physically, there was no significant difference in the wound appearances of all groups. However, group B which was treated using papain-based wound cleanser

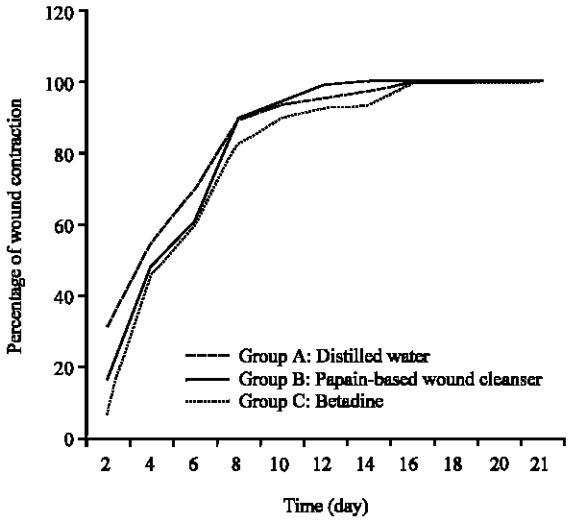

Fig. 1: Percentage healing of *in vivo* wounds

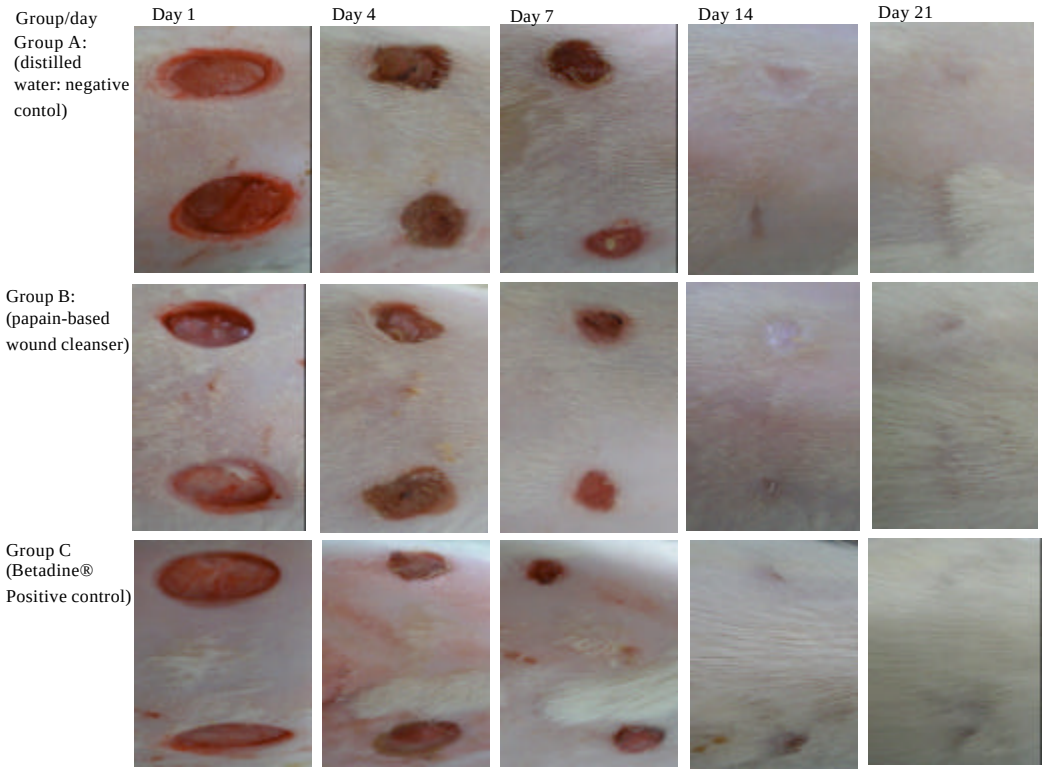

Fig. 2: Gross development of wound healing process of group A, B and C

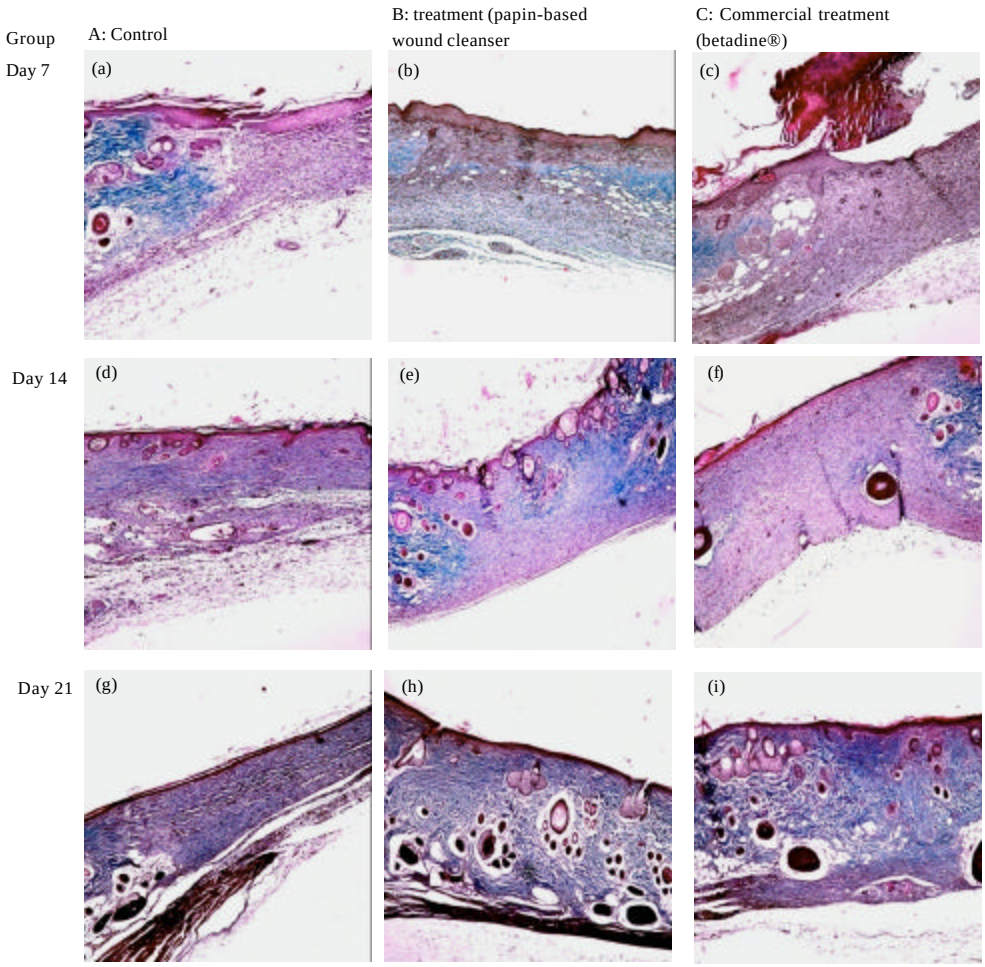

Fig. 3: Photomicrographs of the cross-sectioned skins stained with Masson's trichrome. (a) inflammatory phase, (b) early proliferation pahse, (c) early inflammatory phase, (d) early proliferation pahse, (e) early remodeling phase, (f) inflammatory phase, (g) early remodeling phase, (h) complete remodeling phase and (i) remodeling phase

showed less scar formation when compared with group A and C (Refer figure at day 4 and day 7).

Constriction percentage of the wound area was faster in group B but rats in group C showed faster hair growth compared to other groups. The wounds treated with papain-based wound cleanser showed the least fluid exudation and the greatest epithelialization.

This may be due to the cleanser's efficacy of eliminating fluid and bacteria from wound, hence accelerating the epithelialization rate. Furthermore, papain-based wound cleanser is constituted of ingredients that are vital for enhancing wound repair.

After 7 days injury, photomicrographs of skin in group A clearly had shown the infiltration of polymorphonuclear leucocytes (Fig. 3a). The skin was undergoing the inflammatory phase which can be seen through the infiltration of polymorphonuclear neutrophils (PMNLs) shown in Fig. 3. Neutrophils and macrophages were dominating the wound site. These leucocytes helped in sterilizing the wound as well as secreting growth factors vital for wound repair (Clark, 1996). Collagen deposition was not evident during the first week after injury while epithelial migration was noted in the wounded skin in this figure. Wound healing in group A progressed through the orderly development of a mature granulation tissue formation via proliferating inflammatory cells and fibroblasts.

Wound healing in group B progressed in an advanced manner when compared with group A and C. The skins in group B had already undergone the proliferation phase after seven days post-wounding while group A and C were still in inflammatory phase. The

proliferative phase is characterized by angiogenesis, collagen deposition, granulation tissue formation, epithelialization and wound contraction which were demonstrated in Fig. 3b. It was noted that the bridging off fibroblast matrix was evident in group B. Furthermore, the complete scar formation and continuous epidermis was noted under papain-based wound cleanser treatment while the granulation tissue formation in group B was also more solid.

After seven days of wounding, Masson trichrome's stained skin showed open wounds, clot formation and acute inflammation (Fig. 3c). Inflammation is the first stage of wound healing. Wound repair in group C progressed in a slower basis compared to group A and group G. The epidermal migration has begun but slightly hindered due to the presence of blood clot. Conversion of fibrinogen to fibrin resulted in the formation of blood clot. It can be seen from the photomicrographs that the infiltration of polymorphonuclear leucocytes was obvious after 7 days post-injury. From Lorenze and Longaker (2003), the presences of leucocytes or more specifically neutrophils scavenge debris, secrete cytokines for monocyte and lymphocyte activation. There was no evidence displayed on migration of fibroblasts into wound site.

By day 14, the bridging of fibroblast matrix had started in group A which was clearly shown in Fig. 3d with the help from Masson trichrome staining. The staining distinguished collagen as blue entity. The fibroblast is the connective tissue responsible for collagen deposition required to repair tissue injury (Goss, 1969). Hence, when tissues are destroyed due to injury, collagen is required to repair and restore normal structure and function. During this stage, wounds in group A were in early proliferation phase which were demonstrated by fibroplasia. Fibroplasia is where fibroblast migrate into the wound using the newly deposited fibrin and fibronectin matrix as a scaffold (Lorenze and Longaker, 2003).

After fourteen days, skins in group B showed complete establishment of new layer of epidermis with presence of new hair follicle and sebaceous glands (Refer Fig. 3e). The collagen deposition was more prominent during this phase but scar was still evident. Scar is defined as the lack of tissue organization compared to surrounding normal tissue architecture and is characterized by disorganized collagen deposition (Lorenze and Longaker, 2003). However, the continuous secretion of new collagen fibers by fibroblasts helped to overcome the scar problem. During this phase, group B also displayed advanced wound healing compared with group A and C.

From the photomicrographs, the progress in healing seems to be lacking in group C since they were still in late inflammatory phase or early proliferation phase after fourteen days injury (Fig. 3f). Re-epithelialization has begun during this phase resulting in the continuity of epidermis. After cell division and proliferation, fibroblasts began the synthesis and secretion of extracellular matrix products. The collagen deposition has started in group C which can be seen clearly on the Masson trichrome's stained skins but was not very prominent as compared with group B.

After 21st day post-incision, skin in group A had undergo remodeling but still in its initial phase (Fig. 3g). Epithelialization of the surface occurred by proliferation of epithelium at the edges of the skin defect in order to restore the epidermal continuity. Immature collagenous tissue formed scar which interrupts normal collagen of the dermis on either side.

At 21 days after injury, wounds in group B were well-healed with complete re-epithelialization as well as a decrease in overall cellularity and vascularization which is the characteristic of wound maturation (Fig. 3h). During remodeling phase, wound gradually became stronger with time. Good construction of tissue was proven with the presence of skin appendages such as hair follicle, sebaceous glands and new blood vessels. Less plastic tissue formation was seen in group B when compared to group A. There is an increased collagen deposition and better organization of collagen fibers was noted in group B when compared with group C hence explained the lesser presence of scar.

After 21days of wounding, the skins in group C were well-healed with complete epithelialization, presence of skin structure such as hair follicle and new blood vessels (Fig. 3i). Less plastic tissue formation was seen in both group B and C when compared to group A. However, increased collagen deposition and better organization of collagen fibers was noted in group B when compared with group C.

On the whole, group B showed progressive wound healing. Wounds in group B started off healing with a remarkably advanced process compared to group A and group C and achieved remodeling phase evidenced by the presence of skin organelles and fully-deposited collagen.

Histologic assessment enables the viewing of collagen with Masson trichrome's staining which stain leaves collagen green or blue (Sanders *et al*., 1999). In this study, with the help of a software image analyzer, measurements were made on the intensity of the colour, which represents the collagen density. Collagen is produced by fibroblasts and helps the wound gain tensile strength during repair (McFarlin *et al*., 2006). This may be

Table 1: Average collagen density values in the wound and normal dermis and percentage collagen ratio. Colour intensity values (absolute number)

	Day 7		Day 14		Day 21	
Groups	Intensity values	Ratio (%)	Intensity values	Ratio (%)	Intensity values	Ratio (%)
A-Negative control-distilled water	100.03	56.77	124.93	70.85	129.63	73.51
B- Papain-based wound cleanser	122.25	69.33	153.40	86.88	171.73	97.39
C-Positive control-Betadine®	136.64	77.49	149.62	84.85	161.29	91.47
Normal skin	176.34					

due to the presence of alpha tocopherol in papain- based wound cleanser formulation which has direct effect on inflammation, blood cell regulation and connective tissue growth.

Compared with the normal dermis, the collagen density measured in the centre of the wound at day 7 was greatest in group C (77.49%) followed by group B (69.33%) and group A (56.77%). At day 14, the greatest collagen density belongs to group B (86.88%) followed in decreasing order by group C (84.85%) and group A (70.85%). By day 21, group B produced the highest collagen density under the wound (97.39%) followed in decreasing order by group C (91.47%) and group A (75.31%) (Table 1).

DISCUSSION

Wound closure in humans and animals takes place by contraction of wound margins and neoepithelization. Very rapid contraction occurs in animals. Both regeneration and scarring occurs simultaneously in excisional wounds (Stocum, 2006). Dermal connective tissue heals by fibrosis while the epidermis and the vascularate regenerate, eventhough the epidermis may lack structures such as hair follicles and sebaceous glands (Yannas, 2001). Wound healing process involve three main processes which are inflammatory phase, proliferation phase and finally the remodeling phase which are often overlapped (Clark, 1996).

In this study, wound size was created using an 8 mm sterile bio-puncher. Two identical wounds were created on the dorsal region of the animals to represent replicate. The wounds were treated with their specific treatment modalities at a daily basis and physical examination of wounds and wound tracing was also conducted once daily. The progression of wound healing can be judged by the periodic assessment of the contraction of excision wounds. Suguna *et al.* (2002) defines wound contraction as the centripetal movement of the edges of a full-thickness wound to assist in closure of the wound site. Referring to Kyriakides *et al.* (2009), wound reepithelialization was estimated in a blind fashion from H and E-stained sections and assumed to be complete when the entire wound surface was covered with a new epithelial layer.

According to Starley *et al.* (1999) and Pieper and Caliri (2003), papain has been exploited traditionally in treating wounds due to its debridement properties. In this study, effect of debridement was not investigated thoroughly because the focus was only on the cleanser's ability to hasten wound healing. On the whole, papain-based wound cleanser on the wounds appeared to be effective in aiding wound healing. However, the results of wound tracing had been proven statistically that there was no significant difference between the control group (GA), treatment group (GB) and commercial treatment group (GC). Through visible inspection, the wounds treated with papain-based wound cleanser does not differ much in terms period of epithelialization and wound appearances from other treatment groups but the Masson's trichrome staining proved the greatest wound regeneration was achieved in group treated with papain-based wound cleanser (GB).

The histological assessment revealed the greatest number of the new blood vessels formation as well as the increase in the collagen deposition under the wound treated with papain-based wound cleanser. In addition, the photomicrographs of the wounds treated with papain-based wound cleanser showed advanced wound healing when compared with the two groups treated with distilled water and Betadine®. As can be seen on Day 7, wounds in group A (distilled water) and group C (Betadine®) were still in inflammatory phase meanwhile rats in group B (papain-based wound cleanser) had already proceeded to the early proliferation phase. At the end of the study, skin treated with papain-based restored almost similar to the normal structure and function.

From physical inspection, it was noted that wounds treated with papain-based had less eschar or scab formation when compared with the wounds treated with water or Betadine®. This may be due to the incorporation of papain into the cleanser's formulation which debriding action is expected. The debridement property of papain has been studied religiously by many other researchers including Hebda *et al.* (1998) and Wright and Shi (2003). However, the wound type used in this study was an open, acute wound hence the debridement activity was not clearly exercised. It is recommended for further observation to be conducted using papain-based on the evolution of wounds with necrotic tissue. According to Brenda *et al.* (1995) in wounds with tissues displaying good vitality, papain does not appear useful.

The fibroblast is the connective tissue cell responsible for collagen deposition required to repair the tissue injury (Goss, 1969). Collagen accounts for 30% of the total protein in the human body (Prockop and Kivirikko, 1995). In normal tissues, collagen provides strength, integrity and structure. When tissues are disrupted following injury, collagen is required to repair and restore normal structure and function. Collagens are a key component of all phases of wound healing. Increased wound contraction in treated rats might be a result of the enhanced activity of fibroblasts in the treated rats (Kumar *et al*., 2006). Wound contraction is mediated by specialized myofibroblasts found in the granulated tissue (Moulin et al., 2000). The high rate of collagen synthesis within a wound generally returns to normal tissue levels by 6 to 12 months (Mutsaers *et al*., 1997). However, these collagen fibres never regain the original strength of normal unwounded skin and only a maximum of 80% unwounded skin strength can be achieved (Stocum, 2006). Nevertheless, in our study, treatment using papain-based wound cleanser increased the percentage of collagen on wounded area which contradicted with the theory. Hence, this result proved that papain-based wound cleanser indeed aided wound regeneration through promoting collagen formation.

Histologic assessment enables the viewing of collagen with Masson trichrome's staining which stain leaves collagen green or blue (Sanders *et al*., 1999). In this study, with the help of a software image analyzer, measurements were made on the intensity of the colour, which represents the collagen density. Collagen is produced by fibroblasts and helps the wound gain tensile strength during repair (McFarlin *et al*., 2006). Comparisons were made with normal dermis and results showed the greatest collagen density belongs to group B (papain-based wound cleanser) which accounts to 97.39% collagen at the end of the study. This may be due to the presence of alpha tocopherol in papain- based wound cleanser formulation which has direct effect on inflammation, blood cell regulation and connective tissue growth.

Papain-based wound cleanser displayed antimicrobial effects against gram-positive and gram-negative bacteria due to the biocidal agent, 8-hydroxyquinoline constituted in the formulation. This antimicrobial property assisted the healing progress as presence of microorganisms which inhibited wound contraction and impaired healing (Kumar *et al*., 2006). It might shed the idea on why the progress of healing in control rats was slightly hindered.

CONCLUSIONS

From the results obtained in the study, papain-based wound cleanser displayed a promising potential in promoting wound regeneration. Of the treatments used, papain-based accelerated the speed of the healing process of wounds in rats which achieved complete re-epithelialization. It also promoted the greatest collagen deposition under the wound which totaled up 97.39% when compared with dermis of normal, untreated skin. In order to investigate the debridement effects of papain-based wound cleanser, further preclinical studies should be carried out in multiple wounds of three types: full-thickness excisions, partial thickness burns and partial thickness excision with chemical ablation (Hebda *et al*., 1998). Collectively, this study has proven that papain-based wound cleanser is a potential item to be included in wound care protocols nevertheless results from clinical trials are needed to support its effectiveness.

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