"Histological Study of Phases of Wound Healing in Rat"

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Abstract

Wound healing is a physiologic, very complex phenomenon that occurs when skin integrity is lost and consequently also the barrier function of the skin is impaired. This may occur often since the skin is much exposed to external insults and the need to avoid systemic infections drives a rapid defense mechanism.

Objective: To study structural changes in rat cutaneous wound at different phases of wound healing.

Design: Two full thickness linear incisions were established on the back of 18 rats. Rats were sacrificed at day 3, 7, 14 postwounding. Histological study of rat skin was done.

Results: The inflammatory phase was recorded at the first 3 days of incision. The peak of the proliferative phase was dated to day seven. The initiation of the maturation and remodeling phase of the healing process was observed 14 days after wounding. Results of this work can serve as an experimental model for further research using external pharmacological and physical factors (laser light, magnetic field) by which the wound healing can be favourably influenced.

Key Words: Rat Wound, Histology of Wound, Phases of Healing.

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Introduction

Wound healing is a complex physiological process that occurs to restore skin integrity after injury. This process involves the spatial and temporal synchronization of a variety of cell types (1).

Through physiologic healing, the normal status of the skin can be fully recovered although only a maximum of 70% of previous tensile strength is usually achieved. This process progresses in different subsequent steps and usually three successive phases are recognized: a hemostasis, an inflammatory, a proliferative, and a remodeling phase (2).

The hemostasis and inflammatory phase of wound healing begins immediately upon injury and is completed within hours. It is dedicated to hemostasis and the formation of a provisional wound matrix. The normal progression of this initial short phase is critical for the subsequent steps that occur during the ensuing phases of wound healing (3).

The proliferative phase follows up on the subsiding inflammation. The cellular mechanisms predominantly support the repair of the damaged skin tissue (fibroplasia), the reestablishment of the blood vessel network (angiogenesis) and the impermeable barrier (re-epithelialization) (4). This phase is characterized by high cellular activity due to the increased keratinocyte migration and proliferation at the wound edge to cover the wound site, where these cells attach to the basement membrane (5).

The remodeling phase is the maturation of the scar tissue, where immature collagen type III replaces the mature type I collagen (6). Collagen remodeling during the transition from granulation tissue to scar is governed by proteolytic enzymes called matrix metalloproteinases (MMPs), which are secreted by skin-cell-like macrophages, epidermal cells, and endothelial cells, as well as fibroblasts (7).

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Wound remodeling continues for up to 2 years, during which time there is no net increase in the collagen content. On the other hand, the collagen fibers spatially rearrange into a more organized reticular network through cross-linking, coordinated by the local soluble factors, giving the scar its tensile strength (8). Fibroblasts migrate in high abundance to the wound site, predominantly due to their stimulation by TGF-β and PDGF. In addition, they secrete extra cellular matrix (ECM) proteins (hyaluronan, fibronectins and proteoglycans) and subsequently produce collagen and fibronectin (9).

Material and Methods

The experiment was approved by the ethical committee for animal handling for research work in Faculty of medicine, Minia University. According to the international guidelines (Act 1986).

Animals

18 adult male albino rats (12 weeks old, 150-225 gm) were purchased from the animal house of faculty of Agriculture of Minia University. Animals were housed in clean plastic cages and fed a standard laboratory diet with free access to water and diet. They were kept in clean well ventilated room. Rats were maintained at a laboratory temperature ranged from 24-30ºC and exposed to 12 hours light and 12 hours dark cycle. Animals were acclimatized for 2 weeks before the experiment.

Incision Wound Model

Rats were anesthetized by intraperitoneal injection of xylazine hydrochloride (10 mg/kg) and their hair was shaved. Two full thickness linear incision ±2 cm in length, was created in their dorsal surface (10). The wounds were allowed to heal by secondary intention and immediately after incision, the wound bed was injected with 100 µl saline. After fully recovering from anesthesia, rats were placed in individual cages.

**Sample Collection*

Rats were subdivided into 3 groups as follow:

a. **Group I:** rats were sacrificed at 3rd day of incision.
b. **Group II:** rats were sacrificed at 7th day of incision.
c. **Group III:** rats were sacrificed at 14th day of incision.

The dorsal skin was removed from the animals by using a sterile surgical blade to prepare tissue sections for morphological and morphometric studies.

Methods

Histological Study

a) The Paraffin Technique (11)

The skin specimens were fixed in 10% neutral-buffered formalin at room temperature for 24 hours. After proper fixation the samples were dehydrated in a graded alcohol series. Cleared in xylene and embedded in paraffin wax. Then cut by microtome. 5 µm sections were mounted on glass slides for further staining.

b) Staining with Hematoxylin and Eosin (H&E) (11): Technique

Sections which were mounted on glass slides deparaffinized to be stained with H&E. The de-waxed sections were put in hematoxylin stain for 7 minutes, washed well in running tap water. Then put in eosin for 3 minutes and the surplus stain was washed off in water. The sections were dehydrated in ascending grades of alcohol. Cleared by xylene. Then mounted on glass slides to be viewed by the light microscopy for the general histological analysis study.

Photography

Olympus light microscopy (Olympus, Japan) was used for examining and capturing images for the histological sections. Slides were photographed using Olympus digital camera. Images were saved as jpg and processed using Adobe Photoshop 7 to standardize brightness, contrast and background color then printed.

Morphometric Study

The morphometrical evaluation were performed in 6 non overlapping fields (n=6). Image analysis software; Image J was used to assess the epithelialization, granulation tissue, angiogenesis, and mean number of PMNL in H&E stained sections. Images (original) were imported to image J using interactive measurements menu.

Statistical Analysis

Quantitative data was analyzed by SPSS (IBM Corp. Released 2010. Windows, Version 19.0). The mean and standard deviation (SD) was calculated for the parameters of each group. Values were expressed as means ± SD.

Results

Postoperatively, the animals appeared comfortable as evidenced by normal appetite and behavior, all rats showed no mortality through the experimental period.

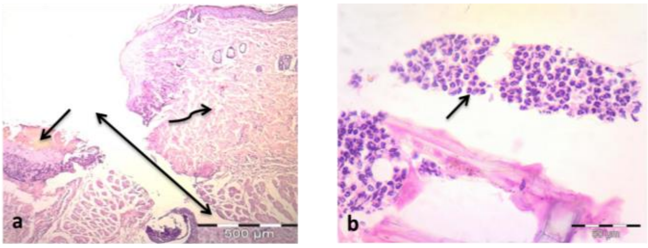
Morphometric Results

Table 1. Morphometric evaluation of histological changes/structures during skin wound healing in rats (values are presented as mean ± standard deviation)

Group	PMNL	Epithelialization	Granulation tissue	Neovascularization
Group I	7.2±0.5	0.0±0.0	0.09±0.03	1.8±0.6
Group II	0.0±0.0	34.6±4.2	2.61±0.37	3.6±0.8
Group III	0.0±0.0	40.7±1.5	0.53±0.1	1.2±0.4

Histological Results

Histological studies of rat skin of group I at the third day of incision showed full thickness skin loss up to the level of muscles. The epidermal edges showed degeneration of keratinocytes and distorted keratin (Fig. a). The dermis showed inflammatory cells infiltration mainly neutrophils (Fig. b).



Representative images of rat skin wound at the 3rd day of incision a) showing full thickness skin loss (double headed arrow), degenerated keratinocytes (arrow), and loss of skin appendages (curved arrow) . b) the rat dermis showing inflammatory cells infiltration mainly PMNL (arrow).

H&E aX40, bX400

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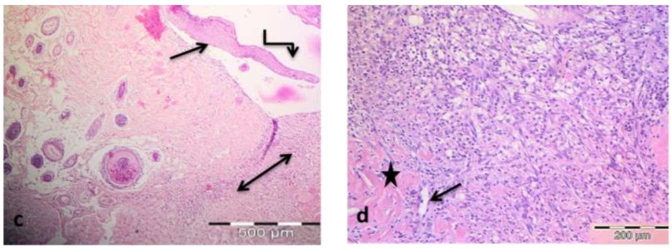
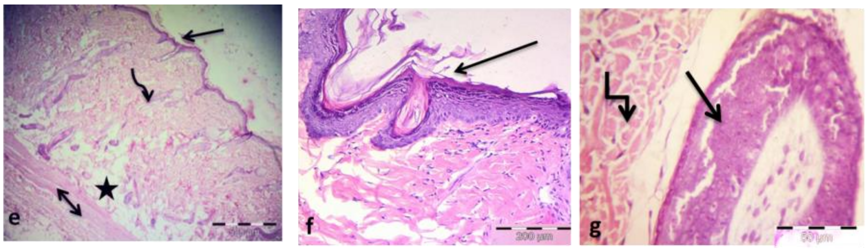
Rat skin of group II at the 7th day of incision showed appearance of the epidermal tongue to cover the wound surface but there was separation between epidermis and dermis was noticed (Fig. c). The dermis showed granulation tissue formation filled the wound gap with new vessels were seen within it. Thin interlacing collagen fibers were formed. Increased cellularity of dermis was noticed, and no skin appendages were formed yet (Fig. d).



Representative images of rat skin wound at the 7th day of incision c) showing re-epithelialization and formation of epidermal tongue (elbow arrow), separation between epidermis and dermis (arrow), formation of granulation tissue but no skin appendages were formed (double headed arrow). d) a higher magnification of the granulation tissue showing new vessels formation (arrow), collagen formation and increased cellularity (asterisk).

H&E cX40 dX100

Rat skin tissue of group III showed that the skin nearly restored its normal structure. The wound site was covered by fully regenerated epidermis with prominent dermal papillae, and regeneration of muscle beneath the skin was noticed (Fig. e). The epidermis showed well developed stratum basale, stratum spinosum, stratum granulosum and stratum corneum (Fig. f). The dermis showed thick disorganized collagen bundles in different directions. Reappearance of skin appendages in the form of newly formed hair follicles (Fig. g).



Representative images of rat skin wound at the 14th day of incision e) showing complete regeneration of skin layers: epidermis (arrow), dermis with appearance of skin appendages (curved arrow), and hypodermis (asterisk). Notice regeneration of muscle (double headed arrow). f) the epidermis showing regeneration of epidermal layers and keratin (arrow). g) the dermis showing regeneration of hair follicle (arrow), and thick irregular collagen bundles (elbow arrow).

H&E eX40, fX100, gX400

Discussion

Wound represents a major health problem, both in terms of morbidity and mortality. The processes involved in wound healing are epithelization, contraction, and connective tissue deposition. The healing process depends, to a large extent, on the regulated biosynthesis and deposition of new collagens and their subsequent maturation (12).

In this study, we used rat model of incisional wound to evaluate the histological changes in different phases.

In the beginning of the healing process, there was a migration of neutrophils to the site of wound at the 3rd day then disappeared at the 7th and 14th day. This finding might be explained by som authors stated that neutrophils, which arrive early after injury, are recruited into the wound from damaged vessels, attracted by chemoattractants, including interleukin 1 (IL-1), tumour necrosis factor-alpha (TNF-α) and bacterial endotoxins, such as lipopolysaccharide (LPS) (13). In the absence of infection, wound neutrophils decline within a few days of injury onset In the absence of infection, wound neutrophils decline within a few days of injury onset (14). Most neutrophils are extruded from the wound site as they adhere to the fibrin scab, while others are removed by innate clearance mechanisms such as macrophage efferocytosis (15). Remaining neutrophils are cleared by apoptosis, necrosis or phagocytosis, or may leave inflamed tissue and return to the circulation through reverse transendothelial migration, as observed in mice (16).

We also found migration of keratinocytes to cover the wound surface at the 7th day and complete epithelialization was achieved at the 14th day. Re-epithelialization could be discussed as keratinocytes are activated by changes in mechanical tension, growth factors and cytokines (17). This activation causes keratinocytes at the wound edge to undergo partial epithelial–mesenchymal transition, where they develop a more invasive and migratory phenotype (18). Front-to-rear polarity replaces top-to-bottom polarity, allowing the leading-edge keratinocytes to migrate laterally across the wound to reform the epidermal layer (19).

Our results revealed formation of minimal granulation tissue at the 3rd day that was increased at the 7th day to fill the wound gap then it was replaced by normal dermis at the 14th day. This finding could be explained by fibroblast which are responsible for replacing the provisional fibrin-rich matrix with a more substantial granulation tissue. Resident and mesenchymally derived fibroblasts respond to a milieu of signalling molecules from platelets, endothelial cells and macrophages, including transforming growth factor (TGF-β) and PDGF. These signals direct fibroblasts to either become pro-fibrotic, laying down ECM proteins, or differentiate into myofibroblasts which drive wound contraction (20). It is important to note that this is again a simplification, as in reality fibroblasts exhibit functional diversity, assisting dermal repair in different ways. Driskell et al. demonstrated that skin fibroblasts originate from two distinct lineages, where the upper lineage aids re-epithelialization while the lower lineage contributes to ECM deposition (21). Recent findings have further challenged conventional understanding of wound fibroblast origin, showing that two-thirds of granulation tissue fibroblasts are actually myeloid derived (5). Fibroblasts degrade the provisional matrix by producing MMPs and replace it with a granulation tissue rich in fibronectin, immature collagens and proteoglycans. This granulation tissue acts as a scaffold for the migration and differentiation of wound cells, supporting both the formation of new blood vessels and the deposition of mature ECM. This granulation tissue acts as a scaffold for the migration and differentiation of wound cells, supporting both the formation of new blood vessels and the deposition of mature ECM (22).

Moreover, we found new vessels formation within the granulation tissue that increased at the 7th day then regressed after that. New blood vessels are created during the process of angiogenesis to meet the metabolic demands of the highly proliferative healing tissue. Angiogenesis is triggered by hypoxia, which in turn drives the expression of hypoxia-inducible factors (HIFs) and cyclooxygenase 2, and subsequent release of VEGF and other factors (23). In response to these changes, microvascular endothelial cells proliferate and migrate into the wound bed, sprouting new vessels that fuse with others to develop stable, tubular networks (24).

The present work revealed deposition of fine collagen fibers at the early phases of wound healing then replaced by coarse collagen fibers at the 14th day. As healing progresses, collagen type III is replaced by collagen type I, directly increasing the tensile strength of the forming scar (25).

Conclusion

It is essential that we continue to strive to more fully understand the mechanisms that underpin both normal and pathological healing. While not without their limitations, emerging wound models provide an unprecedented opportunity to further explore the molecular and cellular features of wound repair. Indeed, the future holds great promise for the development of innovative new therapeutic strategies for advanced wound care.

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