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Original Article

A new histological score grade for deep partial-thickness burn wound healing process

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Abstract: Burns are injuries on the skin or other tissues. Burns are divided into superficial, partial, and full-thickness, characterized by the depth of the affected tissues. Histological analysis is critical to assess the burn wound healing process. Thus, a systematic evaluation system is imperative for burn research. In the present study, a total of thirty Sprague-Dawley rats were randomly divided into five groups. Deep partial-thickness burn wound was induced on the dorsal part of the rats. Six animals from each group were sacrificed on the 3rd, 7th, 11th, 14th and 21st day post-burn, respectively. Half of the wound tissue was immediately fixed in buffered neutral formalin for hematoxylin & eosin staining. The healing of the epidermis was evaluated with scores ranging from 0 to 7 based on the state of crust on wound surface, the degree of epithelialization as well as the formation of rete ridges. Meanwhile, healing of the dermis was also evaluated with scores ranging from 0 to 7 according to the proportion of adipose cells, inflammatory cells and fibroblasts, the state of collagen deposition as well as the formation of hair follicles. Furthermore, temporal changes of histological score of epidermis and dermis in the skin tissue with deep partial-thickness burn was evaluated. In conclusion, a new comprehensive system for assessing microscopic changes in the healing process of deep partial-thickness burn wound in hematoxylin & eosin staining slides was established, which simplified the scoring process and helped to obtain reproducible and accurate results in the burn study.

Keywords: Burn wound, histological evaluation, partial-thickness burn, score grade

Introduction

Burns are injuries happen on the skin or other tissues caused by heat or other acute trauma such as hot liquids, hot solids and flames [1]. Radiation, electricity or exposure to chemical substances may also cause burns [2]. Burns are characterized by depth of the affected tissues. Injuries that affect only the epidermis are known as superficial or first-degree burns. It is congested, dry and painful. If burns are kept clean, healing can be achieved within five days without the formation of scars [3]. When the lesions penetrate into the dermis, it is referred to as partial-thickness or second-degree burn, further classified as superficial or deep partial-thickness burn [4]. The superficial partial-thickness burn involves the epidermis and half of the dermis. The blister will be present with pain; however, the hair is still intact. Usually, the damage will heal within one to three weeks (no surgery). The deep partial-thickness burn can cause deep dermis damage. Burns appears pale than red. Skin is drier and the sensation of the skin tends to weaken and hair will easily fall off. Deep partial-thickness burns heal slowly and are accompanied by scar formation and potential functional loss [5]. In full-thickness or third-degree burns, the damage extends to all dermis. The skin is dry, leathery, and may be white or charred, usually requires skin grafts [6].

Burn is an acute wound [7]. The wound healing process is similar to any other type of wound and is divided into four overlapping stages:

hemostasis, inflammation, proliferation, and tissue re-modeling [8]. Histological analysis is critical to assess the burn wound healing process where a systematic evaluation system is needed in research. Histological results were usually shown either in the form of images or histological scores in the literature. In the first case, different histological staining images are displayed for comparison. However, the study is only qualitative, and to be worse, the individual differences in the same group are not taken into account. In the second case, wound healing is assessed with histological scores that represent different grades of wound healing, which is quantitative analysis. In this case, the criteria for these scores become rather important in the histological evaluation. To the best of our knowledge, the criterion to judge the stage of healing in the literature is very limited. In the present study, a new set of histological scoring system was designed to evaluate deep partial-thickness burn wound healing process in the rat model.

Materials and methods

Animals

Male Sprague-Dawley (SD) rats (n = 30, 250 ± 50 g) were purchased from breeder, A Sapphire Enterprise (Seri Kembangan, Selangor, Malaysia). They were all kept in a temperature-controlled environment (25 ± 1°C), fed *ad libitum* with standard rat chow and water daily. Wood shavings were used as bedding and changed twice a week. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee, Universiti Putra Malaysia (UPM), Serdang, Malaysia (Ref: AUP/R095/2014).

Burn wound model

The experiment was conducted in the Animal House of Faculty of Medicine and Health Sciences, Universiti Putra Malaysia. Burn injury was induced and the depth of burn wound was confirmed according to the method described in our previous study [9]. First, the rats were acclimatized for one week before the subsequent experiment. Animals were then weighed and anesthetized by using 15 mg/kg Xylazine® (Troy Laboratories, Australia) and 75 mg/kg Ketamine® (Troy Laboratories, Australia) injections by intramuscular route. Then, the animal's dorsum was shaved by electric clippers where commercial depilatory cream (VEET™) was used for depilation. Burn injuries were initiated by a temperature-regulated aluminum head 20 mm in diameter with a contact temperature of 70°C. It was applied for 10 s to produce a deep partial-thickness burn. The whole pressure exerted on the rat dorsal skin was 300 g. Wounds were created at 5-min intervals to allow the aluminum head to recover to 70°C. The burn depth was then confirmed from samples that were collected on day three post-burn by hematoxylin and eosin (H&E) staining.

Treatment of wounds

Animals were housed in individual cages after receiving experimental burn wounds. After five minutes, induced wounds were irrigated by spraying a sterile saline solution and dried using sterile gauze. During the whole experimental period, the experimental wounds were kept uncovered.

Sample collection

After inducing the burn wound, animals were randomly divided into 5 groups (n=6 in each group) based on the dynamic burn healing process. Animals in all the groups were treated with the same procedure but anesthetized and sacrificed on a different day, which were days three, seven, 11, 14, and 21 post-burn, respectively. The whole damaged skin tissue was cut off and washed thoroughly with physiological saline (4°C). Subsequently, half of the tissue was fixed in 10% formaldehyde for histological studies according to a previous study [10].

Histological assessment

This experiment was carried out according to the methods of our previous studies [9, 10]. After the animals were sacrificed, blocks of half of the damaged skin tissue were cut off and fixed in 10% formaldehyde. The samples were then processed through graded alcohol, chloroform, infiltrated with molten wax and embedded in paraffin in Paraffin Embedding Station (Leica EG1600, Germany). Sections were cut at 5 μm thickness with Rotary Microtome (Leica RM 2135, Germany) and dried at room temperature for at least one day. Prior to dyeing, the slides were placed in an oven (Venticell, UK) at 60°C for an hour to melt the paraffin. The H&E

Table 1. Histological score range for the epidermis

Score	Crust	Epithelialization	Rete ridge
0	Loosely attached	no	no
1	Tightly attached	minimal	no
2	Tightly attached	mild	no
3	Tightly attached	moderate	no
4	No crust attached	moderate	no
5	No crust attached	severe	no
6	No crust attached	complete	no
7	No crust attached	complete	yes

staining was carried out in an Autostainer (Prisma E2S, Japan). Histological changes in the stained sections were observed using an optical microscope (Leica Microsystems AG, Germany). The number of adipose and inflammatory cells and fibroblasts were observed and counted under the optical microscope with a magnification of 400.

Statistical analysis

In each experiment, the average value of the repetitions was obtained for statistical analysis. All data were presented as mean ± S.E.M. One-way ANOVA where Bonferroni's multiple comparisons test was used as a post-hoc test for comparison between groups. All analysis was completed with Statistical Package for the Social Sciences (SPSS) V22 software (SPSS Inc., Chicago, IL, USA). $P<0.05$ was considered to be statistically significant.

Results

Histological score grade for the epidermis

Cutaneous wound healing includes epidermal epithelialization and dermal healing. According to the observation of different healing stages of wound epidermis and dermis, a new histological score grade of epidermis and dermis was set respectively. The healing of the epidermis was evaluated with scores ranging from 0 to 7 based on the state of crust on the wound surface, the degree of epithelialization, and the formation of rete ridges (**Table 1** and **Figure 1**). Burn injury is a dynamic process where the depth of the wound evolves over time, and usually, the necrosis will stabilize on the third day after-burn. In the present study, a deep-partial thickness burn wound was induced. By day three post-burn, the crust was barely observed in the histological staining slides although it was obvious macroscopically, which was due to its loose attachment to the wound resulting in its disappearance in the staining process. The entire epidermis was absent. This stage was scored as 0. With the healing of epidermis proceeds, the crust was tightly attached to the wound where minimal, mild as well as moderate epithelialization was observed on both sides of the wound, which was scored as 1, 2 and 3, respectively. Finally, the crust was detached from the wound with the ongoing moderate and severe epithelialization, which was scored as 4 and 5. By the time the epidermis was completely covered, it was scored as 6; until the rete ridge was well-developed, it was scored as 7, which was the final stage of epidermal healing.

Histological score grade for the dermis

The healing of the dermis was evaluated based on the proportion of adipose cells, inflammatory cells, fibroblasts, the state of collagen deposition, and the formation of hair follicles, with scores ranging from 0 to 7 (**Table 2** and **Figure 2**). Starting from day three post-burn, the dominant cells in the dermis were adipose cells and mild inflammatory cells that were characterized as score 0. With the healing of the dermis proceeds, the proportion of the adipose cells decreased while the inflammatory cells become more prominent, which was scored as 1. While the proportion of inflammatory cells was still prominent and only few adipose cells, this feature was marked as score 2. Score 3 observed increased in the proportion of inflammatory cells and was score 4 when infiltration of collagen in the dermis, Score 5 was characterized when complete disappearance of the adipose cells, moderate inflammatory cells, severe fibroblasts and coalescing of collagen deposition. A score of 6 represented by mild inflammatory cells and severe fibroblasts in the dermis. When collagen deposition become profound together with emerging of immature hair follicles it was scored as 7 which was the final stage of dermal healing.

Temporal changes of the histological score of epidermis and dermis in the skin tissue with deep partial-thickness burn

Histological changes of skin tissue with deep partial-thickness burns were assessed on the 3rd, 7th, 11th, 14th, and 21st day post-burn accord-

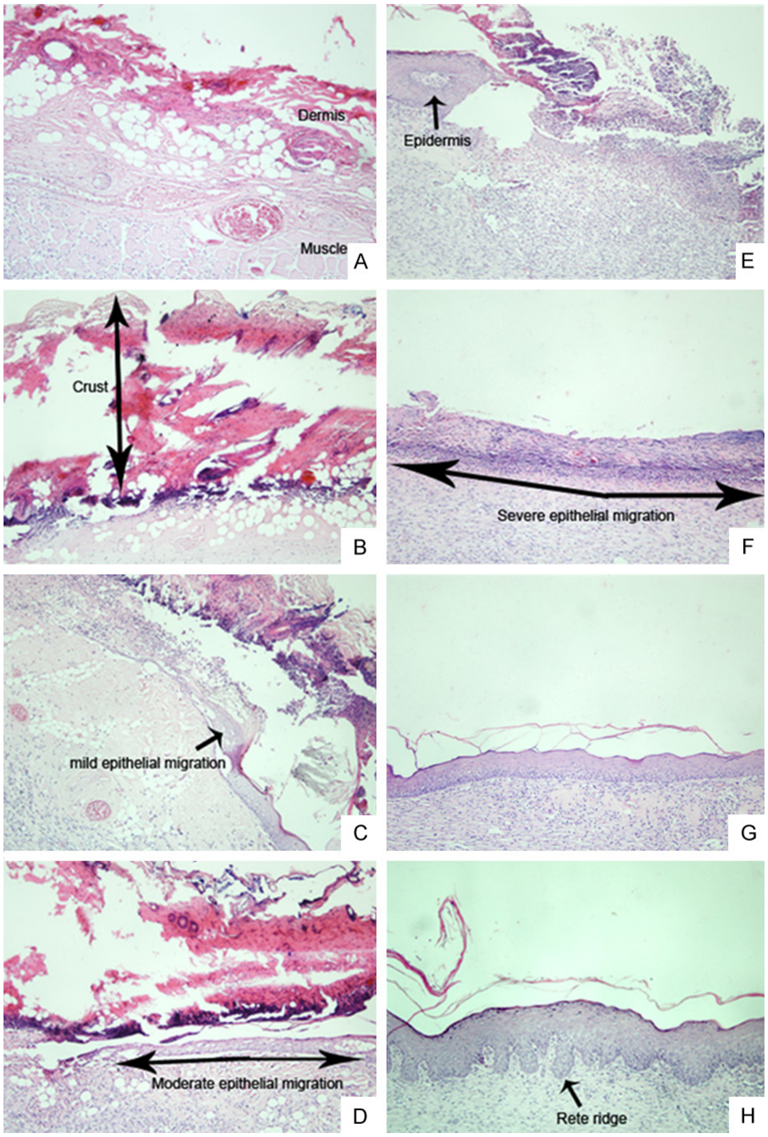

Figure 1. The representative histological images of deep-partial thickness burn wound healing process of the epidermis (400×). A. Represents score 0 for the epidermis, with loosely attached crust (easily lost in the staining process) and absent epidermis; B. Represents score 1 for the epidermis, with tightly attached crust and minimal epithelialization; C. Represents score 2 for the epidermis, with tightly attached crust and mild epithelialization; D. Represents score 3 for the epidermis, with tightly attached crust and moderate epithelialization; E. Represents score 4 for the epidermis, with no crust attached and moderate epithelialization; F. Represents score 5 for the epidermis, with no crust attached and severe epithelialization; G. Represents score 6 for the epidermis, with no crust attached and complete epithelialization; H. Represents score 7 for the epidermis, with complete epithelialization and obvious rete ridge.

ing to the epidermal and dermal histological scoring criteria described above. The results were shown in **Table 3.** On the third day post-burn, the epidermis score was 0, which means that the epidermis was completely destroyed without epithelialization. In addition, the score of the dermis was also 0, meaning the dermis was occupied by severe adipose cells. By day seven, the scores of the epidermis and dermis were both 1, indicating that minimal epithelialization was observed. In the dermis, the dominant cells were adipose cells and inflammatory cells. On the 11th day post-burn, the score of the epidermis was 2.50 ± 0.5, indicating mild to moderate epithelialization was found. Similarly, the dermis also scored 2.50 ± 0.50, signifying the decrease of adipose cells, an increase of inflammatory cells, and the presence of fibroblasts in the dermis. On day 14, the histological score of the epidermis was 3.67 ± 0.42. The moderate epithelial formation was observed. In addition, the crusts in some animals were detached from the wound. The score of the dermis was 4.00 ± 0.37, indicating mild collagen deposition was present in the wound site. By day 21, the crust was totally detached from the wound, and moderate to severe epithelialization was observed. Thus, the epidermis had a score of 4.75 ± 0.25. In the dermis, the collagen deposition was apparent but still not well organized. Therefore, the score was 5.25 ± 0.25. Incomplete healing was specified by the absence of skin appendages although the healing process has reached on day 21.

Discussion

Histological analysis is critical to assess the wound healing process, which is usually shown in two forms: image and histological scores,

Table 2. Histological score range for the dermis

score	Adipose cell	Inflammatory cell	Fibroblast	Collagen deposition	Hair follicle
0	+++	+	-	-	-
1	++	++	+	-	-
2	+	++	+	-	-
3	+	+++	+	-	-
4	+	+++	++	+	-
5	-	++	+++	++	-
6	-	+	+++	++	-
7	-	+	++	+++	+

Note: -: absent +: mild ++: moderate +++: severe.

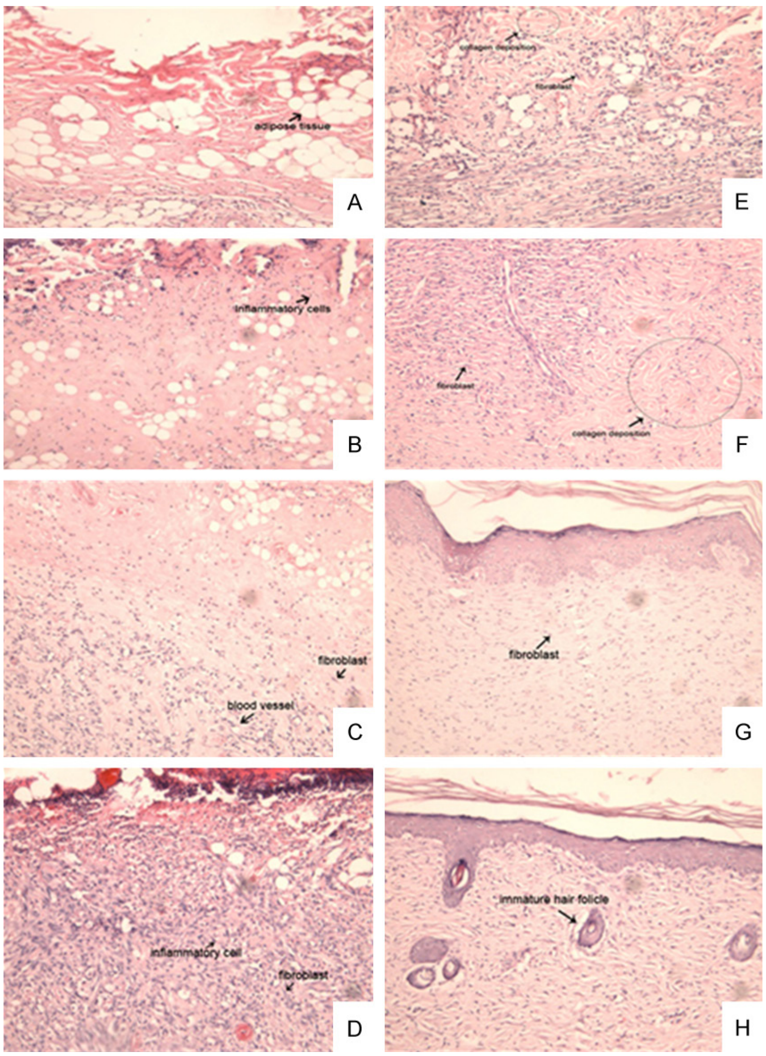

Figure 2. The representative histological images of deep-partial thickness burn wound healing process of the dermis (400×). A. Represents score 0 for the dermis, with severe adipose cells and mild inflammatory cells with absent of fibroblasts; B. Represents score 1 for the dermis, with moderate adipose cells, moderate inflammatory cells, and mild fibroblasts; C. Represents score 2 for the dermis. Mild adipose cells, moderate inflammatory cells and mild fibroblasts; D. Represents score 3 for the dermis, with mild adipose cells, severe inflammatory cells, and mild fibroblasts; E. Represents score 4 for the dermis, with mild adipose cells, severe inflammatory cells, moderate fibroblasts, and mild collagen deposition; F. Represents score 5 for the dermis, with moderate inflammatory cells, severe fibroblasts and moderate collagen deposition; G. Represents score 6 for the dermis, with mild inflammatory cells, severe fibroblasts and moderate collagen deposition; H. Represents score 7 for the dermis, with mild inflammatory cells, moderate fibroblasts, severe collagen deposition, and immature hair follicle.

representing qualitative and quantitative analysis, respectively. In the case of histological scores, the criteria for these scores become important in the histological evaluation. However, the description of the meaning of each criterion in the literature is not clear. For example, it was reported that histological score was set in the range of 0-4 to evaluate granulation tissues, with a criterion described as none, minimal, mild, and evident [11], which is hard to understand and follow in practice. Other investigators subdivided the histological scores from 1 to 12 [12]. However, these 12 marks were divided into four ranges of 1-3, 4-6, 7-9, and 10-12. Within each range, it is difficult to match the corresponding features in the slide with its histological scores. Due to the above reasons, a new set of the histological scoring system was designed to evaluate the deep partial-thickness burn wound healing process.

One of the salient features of the histological scoring system designed in this study is to estimate the healing state

Table 3. Temporal changes of the histological score of the epidermis and dermis in the skin tissue with deep partial-thickness burn

Structure of skin	Histological score				
	Day 3	Day 7	Day 11	Day 14	Day 21
Epidermis	0.00 ± 0.00	1.00 ± 0.00	2.50 ± 0.50^{a}	3.67 ± 0.42^{a,b}	4.75 ± 0.25^{a,b,c}
Dermis	0.00 ± 0.00	1.00 ± 0.58	2.50 ± 0.50^{a,b}	4.00 ± 0.37^{a,b}	5.25 ± 0.25^{a,b,c,d}

Note: Data were expressed as Mean ± S.E.M. $^{a}P<0.05$, $^{b}P<0.05$, $^{c}P<0.05$, $^{d}P<0.05$ compared to those recorded on day three, seven, 11, 14, and 21, respectively.

of the epidermis and dermis, respectively. This is based on the different structures of the epidermis and dermis. The epidermis of the normal skin consists of four layers including basal, spinous, granular, and stratum layer [13]. Some parts of the epidermis are projected into the dermis and form a wavy shape called the rete ridge. The dermis is the toughest fibrous layer of the skin, mainly consisting of collagen fibers, fibroblasts, blood vessels, nerves, and immune cells [14]. Due to the structural differences, the healing processes of the epidermis and dermis are evaluated using different evaluation systems.

Another notable feature of the evaluation system was to score according to the progress of healing processes observed by the H&E stained slides. Based on the observations in the present study, the healing process of the epidermis was relatively simple compared to those of the dermis. After the burn, the epidermis was completely destroyed. The epidermal cells then migrated from both sides of the wound and finally covered it. It was reported that the migrating epidermal cells were mainly produced from the existing basal layer [15, 16]. The epidermis then continued to grow up to the required thickness and eventually projected into the dermis, forming the rete ridge.

In contrast, the healing process of the dermis was found to be much more complicated. It required the coordinated completion of a variety of cells and events [17, 18]. In the present study, the score of the dermis was completed according to the proportion of adipose cells, inflammatory cells, and fibroblasts, the degree of collagen deposition and the development of hair follicles. Adipose cells were the most abundant cells observed in the wound on the 3rd day post-burn. With the healing of the wound, the number of adipose cells gradually decreased and eventually disappeared. The role of adipose cells in the wound healing was still not clear. Inflammatory cells were the first that enters the wound. They appeared in the wound area to scavenge bacteria and other foreign particles to avoid wound infection. Upon performing its function, their numbers declined [19]. Fibroblasts usually appeared in the wound on the 4th day post-burn. Once within the wound, fibroblasts proliferated and produced new extracellular proteins such as fibronectin, hyaluronan, collagen and proteoglycans [7]. In addition, fibroblasts undergone phenotypic changes and differentiated into myofibroblasts to form very tight adhesions to the surrounding granulation and provided force for wound contraction [20]. Collagen deposition often occurred at the late stage of wound healing. The collagen fibers were initially evident on both sides of the wound, then expanded to the central area, and finally arranged regularly in the entire dermis. In addition, the recovery of skin appendages took a longer time [21]. Even on the 21st day post-burn, only the initial stage of follicles formation was observed in this study. In short, the coordinated efforts of various cells led to the dermal healing and the scoring system developed in this study completely followed the established physiological changes in the healing process.

With this precise histological score technique, the healing state of the epidermis and dermis was easily quantified, making the evaluation process more systematic and reproducible. Moreover, the current system could be useful to make the evaluation quantitative instead of qualitative, which could be proceeded for statistical analysis.

In conclusion, a new comprehensive system for assessing microscopic changes in the healing process of deep partial-thickness burn wound in H&E slides was established. The evaluation system simplified the scoring process and helped to obtain reproducible and accurate results.

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Disclosure of conflict of interest

None.

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