

# Full-Thickness Thermal Injury Delays Wound Closure in a Murine Model

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**Objective**: The contemporary treatment of a full-thickness burn consists of early eschar excision followed by immediate closure of the open wound using autologous skin. However, most animal models study burn wound healing with the persistence of the burn eschar. Our goal is to characterize a murine model of burn eschar excision to study wound closure kinetics.

**Approach**: C57BL/6 male mice were divided into three groups: contact burn, scald burn, or unburned control. Mice were burned at 80°C for 5, 10, or 20 s. After 2 days, the eschar was excised and wound closure was documented until postexcision day 13. Biopsies were examined for structural morphology and  $\alpha$ -smooth muscle actin. In a subsequent interval-excision experiment (80°C scald for 10 s), the burn eschar was excised after 5 or 10 days postburn to determine the effect of a prolonged inflammatory focus.

**Results**: Histology of both contact and scald burns revealed characteristics of a full-thickness injury marked by collagen coagulation and tissue necrosis. Excision at 2 days after a 20-s burn from either scald or contact showed significant delay in wound closure. Interval excision of the eschar, 5 or 10 days postburn, also showed significant delay in wound closure. Both interval-excision groups showed prolonged inflammation and increased myofibroblasts. **Innovation and Conclusions**: We have described the kinetics of wound closure in a murine model of a full-thickness burn excision. Both contact and scald full-thickness burn resulted in significantly delayed wound closure. In addition, prolonged interval-excision of the eschar appeared to increase and prolong inflammation.

### INTRODUCTION

BURN INJURY IS MOST commonly classified by the depth of skin penetration. Superficial partial thickness burns (*i.e.*, those that involve only the superficial part of the dermis) are predicted to heal on their own, usually within 2 weeks without any effect on the overall structure and appearance of the injured skin. Full-thickness burns are those that damage all layers of the skin, including epidermis and dermis, and do not heal properly without intervention, typically resulting in significant distortions to adjacent structures. This is important for all areas of the body but especially in mobile or aesthetic regions, such as the face, neck, and extremities. Despite early excision of the dead skin and subsequent grafting, the outcomes for deep partial and full-thickness burns may still be suboptimal, including altered aesthetics and functional impairments.

Necrotic tissue acts as an inflammatory nidus, activating the complement cascade and releasing



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Standard Form 298 (Rev. 8-98) Prescribed by ANSI Std Z39-18 pro-inflammatory and chemotactic cytokines to attract neutrophils.<sup>1,2</sup> Wound contraction and scarring are initiated by platelet-derived growth factor- and transforming growth factor beta (TGF- $\beta$ )induced transdifferentiation of fibroblasts into myofibroblasts.<sup>3-5</sup> Myofibroblasts organize their actin filaments along the lines where skin tension is the highest and adhere to other myofibroblasts and fibronectin-rich wound beds, initiating contraction of the granulation tissue via the sliding mechanism of actin and myosin filaments.<sup>6</sup> Studies have shown that pro-inflammatory cytokines, particularly tumor necrosis factor alpha, can suppress TGF- $\beta$ 1 signaling, thereby decreasing TGF- $\beta$ 1-stimulated functions, such as myofibroblast differentiation and the production of type I collagen, fibronectin, and periostin during the remodeling phase of tissue repair.<sup>7–10</sup>

### CLINICAL PROBLEM ADDRESSED

Current strategies to optimize the outcome after deep partial or full-thickness burns require early excision of the eschar and immediate wound closure. When there is insufficient donor skin, skin replacement is achieved using autologous skin graft or skin substitute graft. While this strategy of early excision and grafting, first proposed in the 1960s,<sup>11</sup> has become the standard of care for the treatment of full-thickness burns, a few animal models reflect this important clinical strategy. Instead, most models allow the full-thickness or deep partial thickness eschar to slough spontaneously, a strategy rarely employed in the current treatment of full-thickness burn injury.<sup>12–14</sup> Although spontaneous sloughing may be relevant for investigating therapies that decrease burn progression or burn depth, it is less ideal in the study of a woundhealing micro-environment which has been altered by the presence of a burn eschar. While several investigators have utilized the more appropriate model of burn eschar excision followed by spontaneous wound closure or by skin graft or skin substitute graft application 15-17 the kinetics and morphology of healing in an excisional burn wound model has not been well described.<sup>15,16,18</sup>

The aim of this study was to characterize the healing kinetics of an excisional burn wound in a murine model of excised scald and contact burns, without the application of a skin graft or a skin substitute graft. Furthermore, the effect of interval burn eschar excision on wound closure is also reported. We hypothesized that burn injury would impair normal wound healing kinetics that can be measured both quantitatively and qualitatively. In addition, delaying burn eschar excision would further impair the healing process.

### MATERIALS AND METHODS Animals

C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) aged 8-20 weeks and weighing 20-40 g were used for all experiments. This study was reviewed and approved by Institutional Animal Care and Use Committees (IACUC) at the United States Army Institute of Surgical Research (USAISR, Fort Sam Houston, TX). All animals received care in strict compliance with the 2011 Guide for the Care and Use of Laboratory Animals by the National Research Council and were maintained in an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC, Int.)-accredited facility at the USAISR. Animals were assigned to contact burn, scald burn, or control (unburned) groups. The animals were further assigned to subgroups with a burn duration of 0, 5, 10, or 20 s (n = 6 - 10 per subgroup). In the delayed excision experiment, the animals were divided into two groups: excision at 5 days postburn and excision at 10 days postburn (n = 17 - 20 per group).

### **Thermal injury**

The mice were anesthetized with an intraperitoneal injection of ketamine (120 mg/kg) and xylazine (10 mg/kg). The dorsal surface of the trunk of all mice used was shaved.

Contact burn. A 2 cm diameter burn was created on the dorsal surface of the mice over the midline by applying a heated ( $80^{\circ}$ C) brass disc of the same size, attached to a soldering unit, for -5, 10, or 20 s.

Scald burn. Animals were secured in a plastic container with a 2 cm-diameter circle cut out. The dorsum of the animals was then submerged in an 80°C water bath for 5, 10, or 20 s, and the seal was maintained by the junction of the mouse skin and container plastic. After injury, mice were allowed to recover, and Buprenorphine (0.05 mg/kg) was given subcutaneously every 12 h for the first 48 h. Two days after burn, the burn eschars were excised.

Control. Instead of burning, mice were subjected to a full-thickness excision of an equal size on the same day as the burn eschar excision. Tegaderm was applied over the wound to maintain a moist and sterile environment. Serial planimetry was performed on postexcision day (postoperating day [POD]) 3, 8, and 13 to quantify wound closure. In the delayed excision experiment, animals were subjected to 10 s scald burns. Burn eschar was excised either 5 or 10 days after burn, and the rest of the postexcision assessments and tissue harvest were the same as previously described. The 10 s scald group from the previous experiment (eschar excised after 2 days) will serve as the control for this delayed excision experiment. At the experiment conclusion, animals were euthanized with Fatal-Plus<sup>®</sup> under deep anesthesia and wound tissue was biopsied.

### Wound contraction by planimetry analysis

Photographs collected throughout the animal experiment were analyzed by ImageJ software (National Institute of Health, Bethesda, MD) to quantify wound area. Wound closure was expressed as a percentage of the initial wound closed.

#### Histology

The biopsied tissues were fixed in 10% neutralbuffered formalin, dehydrated, and embedded in paraffin for histological analysis. Hematoxylin & eosin (H&E) stain was used to observe granulation tissue formation. Verhoeff's Elastic-Masson's Trichrome and picrosirius red stain were used to examine collagen maturation, structure, and density.

#### Immunohistochemistry

Paraffin embedded tissues were cut into 5  $\mu$ m sections. After deparaffinization and hydration, sodium citrate (pH 6.0) antigen retrieval was performed and sections were blocked with 10% normal goat serum for 1 h at room temperature. Sections were then treated overnight at 4°C with primary antibodies, rabbit monoclonal antibody (mAb)  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), rabbit mAb cytokeratin 10 (K10), or rabbit polyclonal antibody

(pAb) leukocyte common antigen (CD45) (Abcam, Cambridge, MA). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide. Primary antibody was detected with horse-raddish peroxidase-conjugated secondary antibody (goat anti-rabbit) (Bio-Rad, Hercules, CA) followed by 3'3'-diaminobenzidine development (Vector Lab, Burlingame, CA). For negative controls, primary antibodies were replaced with 10% normal goat serum. Slides were observed under a Nikon Eclipse 55i light microscope, and photographs were taken with a Nikon DS-Fi1 camera.

### Statistical analysis

Statistical differences were determined by twoway repeated measures ANOVA with pairwise comparison and Tukey–Kramer adjustment, using JMP statistics software (SAS, Cary, NC). Results were presented as mean±standard error of the mean.  $p \leq 0.05$  was considered significant.

### RESULTS

### Collagen stain confirmed full-thickness burn injury

Masson's Trichrome stain of heat-denatured collagen was used to assess burn depth. Both contact and scald burned groups showed dark purple to red staining from the epidermis to the hypodermis, indicating a full-thickness burn injury (Fig. 1). By contrast, normal, unburned skin revealed loose, wavy, normal appearing collagen fibers. This is consistent with published reports of collagen in burned skin taking on a red or purple color instead of the normal blue.<sup>19</sup> Based on this change in collagen staining, we concluded that the conditions of scald and contact burn resulted in full-thickness thermal injury.



**Figure 1.** Verhoeff's Elastic-Masson's Trichrome (VEMT) staining of burned and unburned normal mouse skin (control) sections on postoperating day (POD) 0. The control represents normal collagen architectures on POD 0, while both contact and scald burn groups demonstrated full-thickness burn marked by collagen coagulation (*green arrows*) and necrosis down to the skeletal muscle (*orange arrows*) on POD 0. (100×).



**Figure 2.** (A) Percent wound closure over 13 days after burn eschar excision. Contact: On POD 13, the 20-s group showed a significant difference in wound closure compared with the 10-s group (73.18% vs. 88.55%, p < 0.01) and the unburned control (73.18% vs. 87.07%, p < 0.05). Scald: Significant difference in wound closure was detected between the 5-s group and control (-10.64% vs. 11.88%, p < 0.01) on POD 3. Wound closure between the 20-s and control groups was found to be significantly different on POD 8 (40.05% vs. 63.49%, p < 0.05) and on POD 13 (69.04% vs. 87.07%, p < 0.001), while there was a near significance between the 20- and 5-s group on POD 13 (69.04% vs. 79.27%, p = 0.0509). Error bars are ± standard error of the mean (SEM), \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, and \*\*\*\*p = 0.0509. (B) Representative images of wound closure as a result of contact or scald burn of different duration on POD 13. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

### Full-thickness burn injury (from scald or contact) delayed wound closure

Compared with the unburned control, both contact and scald burn excision groups showed delayed wound closure. By POD 13, with the exception of the 20-s burn groups, most wounds had achieved complete closure (Fig. 2). Whether contact or scald, the mechanism of burn did not significantly alter the rate of wound closure.

On POD 13 after contact burn, compared with the 10-s group and the nonburn control, the 20-s group showed a significant delay in wound closure (73.18% vs. 88.55% and 87.07%, respectively, p < 0.01 and p < 0.05) (Fig. 2). Similarly, on POD 13 after a scald burn, compared with the nonburn control, the 20-s group also showed a significant delay in wound closure (69.04% vs. 87.07%, respectively, p < 0.001).

### Interval burn eschar excision significantly delayed wound closure

The effect of interval eschar excision (5 or 10 days post-10 s scald burn) was compared with 2 day excision control. On POD 8 and 13, both interval excision groups (5 or 10 day) showed delayed wound closure, although not significant, compared with the 2 day, early excision control group (POD 13: 67.89% delayed interval excision vs. 76.78% early interval excision, p = 0.08) (Fig. 3).

### Burn injury retards wound re-epithelialization, closure, and remodeling

Wound histology, histochemistry, and immunohistochemistry were used to determine epithelialization,



**Figure 3.** Percent wound closure over 13 days after interval burn eschar excision. Ten second scald burns were applied to all groups. On POD 3, the 10-day interval group is significantly different than the 5-day interval and the excision-only control (-7.35% vs. 3.50% and 11.88%). Both the 5- and 10-day interval groups showed significant difference compared with the excision-only control on POD 8 (34.79% and 39.05% vs. 63.49%) and on POD 13 (67.72% and 68.08% vs. 87.07%). Error bars are  $\pm$  SEM, \*p < 0.05. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound



**Figure 4.** Histological and immunohistochemical comparison of burned versus nonburned (control) wounds on POD 13. (**A**) The nonburn group showed reepithelialization of the entire wound, while the 20-s group clearly showed delayed re-epithelialization demonstrated by the widened epithelial gap between positive K10 staining. The *insets* depict the edge of the epithelium on higher magnification. (**B**) The 20-s burn group also has less mature collagen deposition (less blue on VEMT and more green on picrosirius red) and lower myofibroblast presence (less brown staining on  $\alpha$ -smooth muscle actin [ $\alpha$ -SMA]). Magnification: K10 20×, 200×(*insets*), VEMT 40×, picrosirius red 100×,  $\alpha$ -SMA 100×.

qualitative collagen deposition, and relative presence of myofibroblasts. Based on histologically and immunohistochemically (K10) observed migration of the epithelial edge, the 20-s burn groups showed that reepithelialization was significantly delayed (Fig. 4A). Consistent with a deficiency in the wound repair mechanism, comparison of nonburn control with the 20-s burn groups by picrosirius red staining demonstrated delays in both deposition and maturation of collagen (Fig. 4B).

### Interval delayed excision of the burn eschar increases inflammation and promotes scar collagen proliferation and myofibroblast content

Both the 5- and 10-day interval excision groups showed a thick layer of densely packed light bluestaining fibers laden with abundant dark staining nuclei and positive CD45 staining, indicating increased granulation tissue and leukocyte infiltrate. Compared with the early 2-day excision group, both 5- and 10-day delayed groups have denser collagen fibers (largely yellow) and higher numbers of myofibroblasts (more intense  $\alpha$ -SMA staining), consistent with scar-like collagen proliferation (Fig. 5).

### DISCUSSION

Full-thickness burns are known to result in adverse consequences for wound healing.<sup>20,21</sup> In this study, we describe the healing kinetics of an excisional murine burn model. Although we recognize that mice are not the ideal model for a wound-

healing study, mice were chosen for our burn wound-healing study for several reasons: availability, low cost, the ability to test large numbers of animals with reproducible results, and the potential to test a variety of genetic knockout animals. Unlike in humans, mice heal primarily by contraction due to the presence of panniculus carnosus muscle in the subcutaneous tissue.<sup>22</sup> To inhibit contraction after burn eschar excision in future studies, we can splint the wound open.<sup>23</sup>

This model differs importantly from traditional mouse burn models in that it mimics more closely



**Figure 5.** Histological comparison between 5- and 10-day interval delayed excision groups and 2-day early excision group. Both the 5- and 10-day delayed group demonstrated increased granulation tissue and leukocyte infiltrate (thick layer of blue staining nuclei on hematoxylin & eosin [H&E] and VEMT and positive CD45 staining). Both interval excision groups also showed increased collagen proliferation and maturation (yellow birefringence on picrosirius red). In addition, both delayed interval excision groups have increased myofibroblast presence (greater  $\alpha$ -SMA staining). Magnification: H&E 20×, VEMT 40×, picrosirius red 100×,  $\alpha$ -SMA 100×, CD45 200×.

the current clinical strategy for the treatment of deep partial or full-thickness burns: The burn eschar is excised timely instead of being allowed to slough spontaneously. In the burn literature, a few burn models utilize this strategy, and the few that do so have not characterized its healing kinetics. In this study, we found that with either contact or scald, full-thickness burn resulted in a significant delay in wound closure. In addition, prolonged interval excision of the burn eschar also led to delayed wound closure and prolonged inflammatory response.

There are several reasons that wound closure after excision of a full-thickness burn eschar may be delayed. Ample evidence suggests that burn injury results in dysfunctional keratinocytes and fibroblasts,<sup>24</sup> thereby impairing the normal wound repair mechanisms, decreasing re-epithelialization, granulation tissue formation, and collagen deposition. Despite removal of the burn eschar, the surrounding cellular and biochemical mechanisms for wound repair and closure are still impaired. This explains why it can be particularly difficult to achieve wound closure after burn injury. Indeed, reconstitution or repair of dysfunctional cells through the addition of autologous cells has demonstrated early promise in improving wound closure after burn injury.<sup>25–28</sup>

Several other variables may also affect the kinetics of wound closure in this model, including the mechanism of burn and the timing of eschar removal. Regarding the mechanism of burn, we found that burns resulting from either scald or contact demonstrated no significant differences in wound closure characteristics, which supports the notion that either method transfers heat in a similarly efficient manner. We have found experimentally, however, that scalding can achieve a more even burn. By contrast, depending on the degree of contact between the burn device and the surface of the animal, contact burn with a brass block may result in areas of unevenness. This finding could be the reason that there is a slight difference in wound closure on POD 13 between contact and scald burn (Fig. 2). For that reason, in our subsequent prolonged interval excision experiments, scald was chosen as the preferred method of heat transfer.

The interval excision experiments revealed more granulation tissue, scar-like collagen proliferation, inflammatory cells, and myofibroblasts, demonstrating that persistence of the burn eschar not only resulted in delayed wound closure but also increased inflammation. In excisional burn wounds, leaving the inflammatory nidus in place for an additional 3–8 days resulted in increased contraction. This is consistent with our clinical experience, as well as with published findings, that the initial excisional preparation is delayed or inflammation is prolonged or aberrant greater burn scar contracture is observed.<sup>29</sup>

It was initially unclear why prolonged persistence of the burn eschar would delay wound closure in spite of the greater number of myofibroblasts. For this reason, it is important to draw the distinction between wound closure and scar contraction (Fig. 6). Wound closure refers to a specific point in time when an interruption in the skin is restored by a continuous epithelial layer. Wound closure



Figure 6. Differences between wound closure and scar contraction. In this model, wound closure is the closure of an open wound by secondary intention that takes place between 1 and 2 weeks postwounding. Scar contraction takes place after wound closure, and it occurs between 1 and 6 months postwounding and frequently results in secondary deformities. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

may be achieved by two mechanisms: (1) healing by primary intention with surgical closure or the application of a skin graft or skin substitute graft, or (2) healing by secondary intention through a combination of wound contraction and/or epithelialization. An intact wound repair mechanism is a prerequisite for wound closure. Scar contraction of burns, on the other hand, is a late effect. It refers specifically to a previously closed burn wound that develops secondary deformities, such as decreases in size and pliability. Scar contraction is generally thought to be mediated through the action of myofibroblasts and prolific scar-like collagen deposition. Temporally, wound closure is achieved in the first weeks after injury, while scar contraction is an active process that takes place over months to years. In this model, delayed wound closure and increased late contraction may coexist in a wound that has both dysfunctional wound repair mechanisms and increased inflammation.

While early eschar removal is most ideal, there are many examples of why it may be delayed: (1) Intoxicated and mentally altered patients are known to delay reporting of their injuries<sup>30,31</sup>; (2) in rural settings or when returning from overseas combat, patients injured remote from medical care may not receive their initial excision for approximately 10 days<sup>32</sup>; and (3) for patients with large surface area burns in whom the priority of lifesaving treatment is paramount, late treatment face and hand burns is typical.<sup>33</sup> When early removal of the burn eschar is not possible, investigations into anti-inflammatory strategies may be beneficial, particularly if they can reverse the contractile potential of the wound.

Published studies<sup>2,34,35</sup> of reperfusion and burn injuries have shown that complement is an early trigger of inflammation. The complement cascade can be activated via three distinct pathways: classical, alternative, and mannose-binding lectin. Genetically altered animals deficient in one or all of these pathways may be used to elucidate their contribution to burn wound closure. Alternatively, strategies that involve topical application of anti-complement peptide or antibody after burn eschar excision in our mouse model might lead to diminished inflammation and enhanced wound closure.

In this study, we specifically omitted the coverage of the open wound using a skin graft or a skin substitute graft. Although the standard of burn care includes earliest coverage of the wound after eschar excision, in this study, we were interested in how the temporary presence of a burn eschar affected the native burn wound micro-environment.

### **KEY FINDINGS**

- We compared the methods and duration of burn injury and their effect on wound closure.
- We varied the time to burn eschar excision and examined its effect on wound closure.
- Longer duration burn resulted in delayed wound closure regardless of the burn method.
- Prolonging the time to eschar excision further delayed wound closure and increased inflammation.
- We present a murine model of full-thickness burn excision to describe wound closure kinetics.

In the future, to determine the quality of healing after skin replacement therapy, the coverage of the open wound using a skin graft or skin substitute may be studied. After burns to the face and hands, numerous strategies could be considered important components to our overall goal of improved functional outcomes.

### INNOVATION

Regardless of the method used to transfer thermal energy, full-thickness burn injury significantly delays wound closure with impairments in both epithelialization and collagen deposition. In addition to delaying wound closure, delayed removal of the burn eschar from 2 days to 10 days also increases inflammation and propensity for late contraction. This model can serve as a useful framework for future studies involving (1) antiinflammatory strategies to elucidate more detailed mechanistic knowledge about inflammation and wound closure; and (2) skin replacement therapy to improve skin functions and quality after burn injury.

### DISCLAIMER

This study has been conducted in compliance with the Animal Welfare Act, the implementing Animal Welfare Regulations, and the principles of the Guide for the Care and Use of Laboratory Animals. The opinions or assertions contained here are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

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#### Abbreviations and Acronyms

- $\alpha\text{-SMA} = \text{alpha-smooth muscle actin}$ 
  - ${\rm H\&E}\,{=}\,{\rm hematoxylin}\,\,{\&}\,\,{\rm eosin}$
  - mAb = monoclonal antibody pAb = polyclonal antibody
  - POD = postoperating day
- SEM = standard error of the mean
- $TGF-\beta = transforming growth factor beta$
- VEMT = Verhoeff's Elastic-Masson's Trichrome