Original Article

Standardization of deep partial-thickness scald burns in C57BL/6 mice

Jorge L Medina¹, Andrea B Fourcaudot¹, Eliza A Sebastian¹, Ravi Shankar¹, Ammon W Brown², Kai P Leung¹

¹Dental and Craniofacial Trauma Research and Tissue Regeneration Directorate, ²Comparative Pathology Branch, United States Army Institute of Surgical Research, Fort Sam Houston, 78234, Texas, USA

Received March 8, 2018; Accepted March 23, 2018; Epub April 5, 2018; Published April 15, 2018

Abstract: Mouse burn models are used to understand the wound healing process and having a reproducible model is important. The different protocols used by researchers can lead to differences in depth of partial-thickness burn wounds. Additionally, standardizing a protocol for mouse burns in the laboratory for one strain may result in substantially different results in other strains. In our current study we describe the model development of a deep partial-thickness burn in C57BL/6 mice using hot water scalding as the source of thermal injury. As part of our model development we designed a template with specifications to allow for even contact of bare mouse skin (2×3 cm) with hot water while protecting the rest of the mouse. Burn depth was evaluated with H&E, Masson’s trichrome, and TUNEL staining. Final results were validated with pathology analysis. A water temperature of 54°C with a scalding time of 20 seconds produced consistent deep partial-thickness burns with available equipment described. Other than temperature and time, factors such as template materials and cooling steps after the burn could affect the uniformity of the burns. These findings are useful to burn research by providing some key parameters essential for researchers to simplify the development of their own mouse burn models.

Keywords: Partial-thickness burn model, mice, wound healing, scalds

Introduction

Burn wounds are caused by a thermal insult and the acute inflammatory response that follows soon after. Burns are induced by different sources of thermal injury including: electrical, friction, fire, and scalds. The sources of thermal damage cause injuries with different depths. The depth of injury is very important because it determines how the wounds are treated [1]. Superficial burns affect the upper layer of the dermis. These burns are painful, but will heal spontaneously with minimal scarring. Deep partial-thickness burns are typically minimally painful and are some of the most difficult to treat due to the inability in accurately determining depth of burn and the potential to progress to a full-thickness burn. Patients with partial-thickness burns need to be evaluated periodically to assess progression of the burn [2]. Full-thickness burns are not painful and require surgical intervention to heal, including debridement and skin grafts [3, 4].

Creating consistent experimentally induced burns in animal models is very important in studying the burn wound healing process and evaluating experimental treatment options. Choosing the method of burn induction is important as this impacts the burn outcome. Scalding with constant water temperature has the benefit of uniformly covering the entire skin exposed to the water regardless of surface variations [5].

There are a variety of animal models used to study full- and deep partial-thickness burn wounds and their healing. These animal models include pigs, rabbits, guinea pigs, rats and mice [6-12]. Rodents are some of the more commonly used animals in burn research.

There are benefits and downsides of using rodents in biomedical research. The benefits of using mice for burn wound studies include the ability to use large numbers of animals to achieve statistical significance, the availability of a wide range of mouse specific reagents and the availability of genetically modified mice [6]. Downsides include the overall thinness of the mouse skin compared to pigs and humans, making it difficult to control the depth of the
burn wound. Wound healing in particular is an area where mice stray significantly from human wound healing. Mice heal predominantly from contracture instead of reepithelization of the entire area [6, 13]. This is due in part to the panniculus carnosus, a subcutaneous muscle layer, that is not found in humans except at the platysma of the neck [13]. Nevertheless, many aspects of mammalian burn wound healing are similar across species.

Here, we sought to establish a deep partial-thickness burn model in C57BL/6 mice. Our laboratory already had an established full-thickness burn model. However, we found that a reproducible deep partial-thickness burn model in mice is lacking [11] for understanding the healing process and evaluation of treatment effects. The model development information provided can help bridge the gap in knowledge of the underlying mechanisms in wound healing in partial-thickness burns.

Materials and methods

Burn template

A mouse burn template was fabricated from a single block of polypropylene. The thickness of the walls was 5 mm with a slight curvature machined into the bottom of the template. An opening of 2×3 cm was cut into the bottom of the template. The 2×3 cm area corresponds to an approximate percent of total body surface area (TBSA) of 6.6% for a 28 g mouse using the Meeh formula [14]. The bottom of the template was lined with rubber pipe insulation tape (50.8 mm wide and 3.18 mm thick) (Thermwell Products Co., Inc., Mahwah, NJ). The template was maintained in a 54°C water bath with just the bottom of the template touching the water to prevent any large temperature shifts when the template is placed in scalding water bath with the mouse.

Mouse scald procedure

This study was conducted in compliance with the Animal Welfare Act, the implementation of the Animal Welfare Regulations, and the principles of the Guide for the Care and Use of Laboratory Animals. Male C57BL/6 mice 15 weeks old between 24-35 g (Jackson Laboratory, Bar Harbor, ME), were allowed to acclimate in individual housing for one week prior to initiation of burn procedures, with 5-12 mice per group. Mice were shaved with BravaMini (Wahl, Sterling, IL) small animal clippers and depilated with Nair chemical depilatory (Church & Dwight Co. Inc., Ewing, NJ) the day before the scald under 3-5% isoflurane controlled with an RC2 Rodent Circuit Controller anesthesia machine (VetEquip Inc., Livermore, CA). The chemical depilatory was allowed to stay on the mouse’s dorsal side for 1 minute and 40 seconds. The excess depilatory was wiped away gently twice with dry gauze. Afterwards, gauze soaked with water was used to wipe the skin area clean 4 times to ensure there was no depilatory left behind that could cause a chemical burn. Mice were allowed to recover before returning to the holding area.

Mice were injected subcutaneously at the scruff of their neck with 1.0 mg/kg of Buprenorphine SR Lab (Zoopharm, Windsor, CO). The mice were allowed to rest for at least 30 minutes to allow the buprenorphine to start taking effect. Mice were then anesthetized with 50-100 mg/kg ketamine (VetOne, Boise, ID) and 5-10 mg/kg xylazine (Akorn, Inc., Lake Forest, IL) injected intramuscularly. An adequate anesthetic plane was determined by toe pinch. After the appropriate anesthetic plane was reached mice were photographed prior to scald. Next, mice were placed in a pre-warmed template, exposing the dorsal area below the shoulders over the opening in the template. The corners of the exposed dorsal skin were marked to accurately define the burn area. A plastic shield fashioned from a 50 mL conical tube was placed lengthwise across the ventral surface of the mouse between the legs to evenly distribute the minimal downward pressure placed on the mouse. At this point water temperature is confirmed and lid is lifted to allow placement of the template into the 54°C water that is being circulated with a stir bar at 140 rpm. The mouse inside the template is immediately placed in the scalding water at an angle to prevent bubble trapping, and then laid flat. Time is started immediately upon the template hitting the water by an assistant. Twenty seconds after placement into the scalding water, the mouse is transferred into a container layered with room temperature soaked paper towels. The burn area on the mouse dorsum is dabbed with the wet paper towels to remove excess hot water and dabbed dry with gauze. One-half to 1 mL of pre-warmed (36°C) lactated ringers are injected intraperitoneally to aid
in resuscitation and prevent dehydration. The burn area is photographed. Using the corners pre-marked with the Sharpie® as a guide, the mouse is tattooed with tattoo paste (Ketchum Manufacturing Inc., Brockville, Canada) using lancets (Medipoint, Inc., Mineola, NY) to mark the corners and sides of the burn wounds. Mice were allowed to recover in either an incubator or warming pad under a cage to help them to maintain their body temperature. The mice were returned to their cages once they were awake and ambulatory. Hydrogel food supplements (Clear H₂O, Westbrook, ME) and other nutritional supplements were placed in the cage to reduce dehydration and weight loss. Pain assessments were performed twice daily for 72 hours and any additional fluids were administered as needed based on assessments. Fluids and pain medication were administered after the 72 hours as needed but generally were not required. Control mice were taken through entire process of shaving, depilation and sham burned in room temperature water.

Biopsies and other samples were collected at 3 days after burn. Mice were anesthetized with 50-100 mg/Kg ketamine and 5-10 mg/Kg xylazine, photographed, and then euthanized with 40-50 mg/Kg Fatal-Plus® (Vortech, Dearborn, MI) by intracardiac puncture. Strips of skin were collected from the middle of wound that included adjacent unburned skin.

**Histology**

Skin biopsies were fixed in 10% neutral buffered formalin for 48 hours. Samples were processed using the Leica Tissue Processor ASP-6025 (Leica Biosystems, Buffalo Grove, IL).

Using Leica Biosystems reagents (Leica Biosystems, Buffalo Grove, IL), hematoxylin and eosin (H&E) staining were done according to the standard protocol provided in a Leica Autostainer XL (Leica Biosystems, Buffalo Grove, IL). Masson’s trichrome stains were done manually using a standard protocol with reagents obtained from Polyscientific R&D Research Corp (Bayshore, NY). TUNEL stains were performed using the Clik-iT TUNEL kit (Thermo Fisher Scientific, Waltham, MA) based on the manufacturer’s protocol. H&E and Masson’s trichrome stain were scanned using the Zeiss Axio Scan Z1 slide scanner. TUNEL stains were photographed with Zeiss Axio Scope and Zeiss Zen software.

**Pathology**

A scoring standard was developed in-house by a board certified veterinary pathologist, using H&E and Masson’s trichrome stained sections to determine the burn depths. Skin samples were assigned a score based on the visual norm of the tissue section. The scoring parameters are as follows: 0 = normal skin; 1 = epithelial necrosis within the epidermis, but basement membrane remains intact; 2 = epithelial necrosis extends beyond the basement membrane, but hair bulbs remain intact; 3 = hair bulbs are necrotic, but necrosis of other cells such as fibroblasts and endothelial cells as well as collagen denaturation does not extend past the deep border of the dermis; and 4 = necrosis of fibroblasts and endothelial cells as well as collagen denaturation extend beyond the deep border of the dermis.

**Results**

**Materials and equipment impact burn uniformity**

Initially, as we progressed in developing the model, we found that using a template from a single piece of polypropylene that had a slight curvature at the bottom with added rubber pipe insulation worked best in preventing water leakage into the chamber (Figure 1A). The use of the plastic shield fashioned from a 50mL
conical tube ensured correct seating of the mouse in the template by providing even pressure and reduced pressure points around the opening of the template that could impact the burn uniformity on the mouse skin. Additionally, using a circulating bath was found to be necessary to insure burn uniformity.

**The most important factors in partial-thickness burn induction in C57BL/6 mice are time and temperature**

We initially followed a previously described deep partial-thickness burn model by Cribbs et al. Using C57BL/6 mice, we induced deep partial-thickness burns using parameters established by Cribbs et al. by scalding mice at 60 °C for 45 seconds followed by a cooling step at 4 °C for 45 seconds. These parameters (temperature and burn duration) which were originally established for FVB mice induced 4th degree burns with clear damage present in the skeletal muscle of C57BL/6 mice. We reassessed our model and dropped the temperature to 54 °C and evaluated times from 10 to 22 seconds. Based on our equipment and materials used, we determined that 20 seconds at 54 °C was optimal to produce more uniform burn depth although...
Partial-thickness burns in C57BL/6 mice

Some surface variation and contraction at 3 days after burn were still observed (Figure 1B). Burn depth was verified by histology, TUNEL, and pathology analysis.

Skin samples taken from the wound center bordered by the tattoo markings were evaluated by histology to validate burn depth. Histology stains showed intact skin without cellular infiltration and rare TUNEL positive staining cells in control sham burns (Figure 2A-C). H&E sections showed the cellular infiltration due to the burn-associated inflammation along with damage of the epidermis in deep partial-thickness burns (Figure 2D). Masson’s trichrome highlighted the denatured constitutive proteins of the epidermis and upper dermal layers (red stain; Figure 2E). Finally, positive TUNEL staining (green fluorescent stain) shows DNA fragmentation indicating dying cells deeper into the dermis (Figure 2F). Full-thickness burns were marked by denatured dermal collagen past the border of hypodermis seen in H&E and Masson’s trichrome stains (Figure 2G, 2H). Dying cells were highlighted with positive TUNEL staining throughout the dermis and into the hypodermis (Figure 2I).

The histological sections were analyzed for cellular pathologies. H&E slides were evaluated for burn depth based on the overall visual norm. Based on the visual norm we, including a board certified pathologist, developed a scoring system from 0-4 to evaluate the biopsy samples from the middle of the wound to determine burn depth histologically (Figure 3A-F). Using the pathology scoring system we developed, mice scalded for 18 seconds at 54°C consistently developed superficial partial-thickness burns with a score of 2 (Figure 4A). The majority of mice scalded for 20 seconds at 54°C had a visual norm pathology score of 2 or greater, indicative of a deep partial-thickness burn in mice (Figure 4B). We did observe that there were some mice with either deeper or shallower burns, but the majority fell within the range of a deep partial-thickness burn. The mice scalded for 22 seconds were consistently full-thickness burns with a score of 4 (Figure 4C).

Discussion

Burn wounds are significant sources of morbidity and mortality around the world. Burns affect a wide swath of society including service men...
Partial-thickness burns in C57BL/6 mice

and women returning from the war theater. Burns accounted for up to 5% of all battlefield injuries in Operation Iraqi Freedom and Operation Enduring Freedom [15]. Treatment of burn wounds can be challenging for a variety of reasons including infections, underlying comorbidities and the development of hypertrophic scars. Understanding of the underlying mechanisms of wound healing in the context of thermal injury is incomplete and requires further study. Animal models are important to this understanding and involve a variety of species including pigs, rats and mice. Pigs are the closest to humans for replicating the burn pathology [16]. However, pigs are limiting because of cost of housing, space availability in vivariums, and lack of availability of genetically modified animals. Mice are chosen because they are the least sentient for burn research. Additionally, they are easy to handle, readily available, and genetically alterable. Moreover there is an abundance of reagents compatible with mouse research. There are a variety of burn models in mice including full- and partial-thickness burns. However, most provide incomplete details, which make them more difficult to reproduce. In the current study we detail the standardization of deep partial-thickness burns induced by scalds in mice.

After trying different materials and equipment we found that they had a significant impact on the uniformity of the burn. As part of the initial trials we used a wire mesh across the 2×3 cm opening in the template to prevent excessive protruding of the mouse body through the opening. We discovered that the combination of the low water temperature and the wire mesh could significantly reduce the water temperature at the opening of the template and affect the uniformity of the burn. Additionally, using non-circulating water baths could lead to hot spots and affect the burn uniformity. To address these issues, the wire mesh was removed and we used a hot-plate with a stir bar to maintain uniform water temperature in the water bath. A similar burn induction method was used to evaluate the impact of mast cells on burns of a smaller TBSA [17]. They induced superficial epidermal partial-thickness burns at 54°C, using a time of 25 or 35 seconds depending on conditions. Cribbs et al. published a report detailing a method for scalding mice with an area of 6 cm² [18]. Their method of scalding mice at 60°C for 45 seconds allowed them to achieve a deep partial-thickness burn. Numerous factors that may account for the large discrepancies in time and temperature can influence the induction of partial-thickness burns in mice. While there may be differences in strains of mice used, other factors could be in play. We found that both equipment and materials used to induce the scald burns were essential in producing burn wounds of consistent burn depth.

Cribbs et al. provided the most detailed study on developing a mouse model of partial-thickness burns. As such we developed our model with modifications based on their study. In their study they used a cooling step of 4°C to stop the burn progression. During the initial steps of validation we cooled the burn area immediately after burn by placing the template directly into 4°C water to halt the burn. In order to get water to 4°C, ice was added to our water bath. Subsequently, we found that ice has a deleterious effect on reepithelization as shown in some

Figure 4. A pathology scoring standard for evaluation of the burn depth. A: A burn wound from a mouse scalded for 18 seconds. Black arrowheads point at the areas of denatured collagen. The biopsy had a pathology score range of 1-2 and a visual norm of 2. B: A burn wound from a mouse scalded for 20 seconds. Black arrowheads point at areas of denatured collagen. The biopsy had a consistent burn, with a visual norm of 2 across the wound. C: A burn wound from a mouse scalded for 22 seconds. Black arrowheads point at areas of denatured collagen at the border of the dermis. The biopsy had a pathology score range of 2-4 and a visual norm of 4. n = 5-12 mice per group.
porcine studies [19]. This may explain the uneven burns as seen in our histology using these burning and cooling parameters. We found that we achieved more uniform burns by simply blotting the excess hot water from the burn area by gently rolling the mouse dorsum back and forth for 5 seconds on room temperature water soaked paper towels. This improved the gross and histological appearance of the burn.

Besides the equipment or materials used, size of wound, or strain of mouse, the most important factors that will impact the burn are time and temperature based on the researchers' technical arrangement. In their protocol, Cribbs et al. used a temperature of 60°C for 45 seconds with a 4°C for 45 seconds cooling step to produce the deep partial-thickness skin thermal injury [18]. Additionally, other researchers used 65°C for 45 seconds in C57BL/6 mice to induce a deep partial-thickness burn [20]. Yet others using 65°C for 20 seconds reported that those conditions elicit a full-thickness burn [21]. The discrepancies can be confusing and cause researchers to waste time in trying to optimize their burn models.

Researchers used different temperatures to induce partial-thickness burns starting at 54°C and as high as 65°C in some studies [6, 17, 18, 20, 21]. We chose 54°C because this gave us the optimal control in the induction of the scald given the thinness of the mouse skin. At a temperature of 54°C one can see the narrow window between a partial-thickness and a full-thickness burn of only 2 seconds as we described above. The use of higher temperatures is less forgiving on exact scald time than inducing burns at lower temperatures with longer times.

Our experimentally induced mouse partial-thickness burns showed undulations of burn depth in some wound areas as seen in clinical burns. These areas could be a result of differential burn wound progression that can occur for 2-4 days after a burn [2]. In a clinical setting most large area burns are a mixture of burn depths [22]. For experimentally induced partial-thickness burns it is important to establish a protocol that will most consistently and reproducibly yield the desired burn depth uniformity in the majority of the burn wounds with the expectation that some areas will fall outside the expected depth range.

In the current study, we detail the standardization of a mouse deep partial-thickness burn protocol. Many other studies used partial-thickness burn models in mice. Unfortunately, the lack of detail needed to reproduce the exact outcome is common. It is our intention that these findings can help other investigators to establish a mouse partial-thickness burn model at their own sites with greater ease.

Acknowledgements

This research was supported in part by an appointment to the Postgraduate Research Participation Program at the U.S. Army Institute of Surgical Research administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and USAMRMC. This work was supported in part through the Congressionally Directed Medical Research Programs, U.S. Army Medical Research and Material Command W81XWH-15-2-0083 and the Naval Medical Research Center’s Advanced Medical Development program (MIPR N3239815MHX040).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Kai P Leung, Dental and Craniofacial Trauma Research and Tissue Regeneration Directorate, United States Army Institute of Surgical Research, 3650 Chambers Pass, STE B, Fort Sam Houston, 78234, Texas, USA. Tel: 210-539-3803; Fax: 210-539-9190; E-mail: kai.p.leung.civ@mail.mil

References

Partial-thickness burns in C57BL/6 mice


