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TITLE:

Severe Burn Injury in a Swine Model for Clinical Dressing Assessment

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SUMMARY

To closely mimic the mode of burn injuries requires the interplay between clinical observation and studies in animal models. In this study, a swine model of severe burn injury was established to assess an experimental dressing in physiological and pathophysiological settings.

ABSTRACT

Wound healing is a dynamic repair process and is the most complex biological process in human life. In response to burn injury, alterations in biological pathways impair the inflammation response, resulting in delayed wound healing. Impaired wound healing frequently occurs in patients with diabetes leading to unfavorable outcomes such as amputation. Hence, dressings having beneficial effect in promoting burn wound repair are needed. However, studies on burn wound treatment are limited due to lack of proper animal models. Our previous study demonstrated wound-healing performance in rat and swine models using a minimally invasive surgical technique. This study aimed to demonstrate a swine model of severe burn injury that eliminates wound contraction and more closely approximates the human processes of re-epithelialization and new tissue formation. This protocol provides a detailed procedure for creating consistent burn wounds and examining the wound-healing performance under the treatment of an experimental dressing in a swine model. Six burn wounds were created symmetrically on the dorsum, which were covered with a clinical dressing composed of four layers: an inner contact layer of experimental materials, an inner intermediate layer of waterproof film, an outer intermediate layer of gauze, and an outer layer of adhesive plaster. Upon the completion of experiments, wound closure, wound area, and Vancouver Scar Scale score were examined. The samples of skin resected from each animal post-sacrifice were histologically prepared and stained using hematoxylin and eosin staining. Antibacterial activity of each dressing in the context of wound healing was also examined. The application of the clinical dressing to the wounds in swine model mimics the biological processes of human wound healing with respect to the processes of epithelialization, cellular proliferation, and angiogenesis. Therefore, this swine model provides an easy-to-learn, cost-effective, and robust method to assess the effect of clinical dressings in severe burn injury.

INTRODUCTION

A burn injury initiates the inflammatory process and induces complex pathological effects, which influence numerous body functions immediately after an accident, resulting in negative impact on patients' quality of life. Impaired wound healing causes significant morbidity and mortality among patients with diabetes mellitus[1,2]. Most patients with burn injuries experience pain during burn wound debridement, which is known as an excruciating process despite the use of powerful opioid analgesics[3].

In contrast to other mammals, swine share several anatomic and physiologic characteristics with humans regarding the process of epithelialization, cellular proliferation, and angiogenesis. This makes swine a potentially better model for certain procedures and studies, and they are often used in subsequent studies that demonstrated promising results in mice. These features have led to the increasing use of swine as a major species in preclinical testing. Recently, a rapid increase in biomedical research with respect to cardiovascular, urinary, integumentary, and digestive systems has been observed[4,5,6]. This study aims to demonstrate a swine model of severe burn injury that eliminates wound contraction and provides a closer approximation of the human wound healing processes and the formation of new tissue. Six burn wounds were created symmetrically on the dorsum, three on each side of the spine of the swine. Next, an experimental dressing was examined in a swine model of severe burn injury, which can be adapted to replicate human wound healing (**Figure 1**). The wounds were covered with a clinical dressing, which is composed of four layers: an inner contact layer of experimental materials, an inner

intermediate layer of waterproof film, an outer intermediate layer of gauze, and an outer layer of adhesive plaster. A waterproof film keeps the wound environment moist while preventing bacterial infection and allowing gases to permeate the dressing. The outer intermediate layer of gauze was applied on the waterproof films and secured by an outer layer of adhesive plaster. At the completion of experiments, wound closure, wound area, and Vancouver Scar Scale (VSS) score were examined. The samples of skin resected from each animal post-sacrifice were histologically prepared and stained using hematoxylin and eosin (HE) staining. Antibacterial activity of each dressing in the context of wound healing was also examined in this model. Our previous study has demonstrated wound-healing performance in rat and swine model using a minimally invasive surgical technique[7]. Since there were six burn wounds on the dorsum within each swine, each experimental dressing was tested and evaluated in all positions to minimize bias related to wound healing process in different spots on the swine dorsum. Therefore, the swine model of severe burn injury established in this study provides a new approach for the evaluation of clinical dressings and facilitates the development of a novel treatment for burn injury. This study provides crucial tools to uncover the pathophysiology of burn wound healing.

PROTOCOL

Procedures involving animal subjects have been approved by the Animal Care Committee at National Defense Medical Center, Taiwan (R.O.C). This study was conducted in the Laboratory Animal Center at the National Defense Medical Center. Swine weighing between 20 and 25 kg has been successfully instrumented using this protocol.

1. Adaptation of the Animals to Human Handling

1.1. After arrival in the facility, house the animals solitarily but let them interact with each other.

1.2. Provide the animals *ad libitum* access to food and water.

1.3. Acclimate swine to human handling and transportation from the animal facility to the experimental laboratory by handling the animal at least once a day for one week.

1.4. Fast the animal for at least 12 hours before surgery to prevent nausea, vomiting, and aspiration of stomach fluids.

2. Sedation

2.1. Before the burn wound creation, sedate animals *via* an intravenous injection with Zoletil 50 (25 mg/kg).

3. Intubation and Ventilation

3.1. Place the animal on a table and/or trolley in standing position.

3.2. Open the mouth of the animal with an oral spreader.

3.3. In case of insufficient relaxation of the jaws or presence of swallowing reflexes, which hinder intubation, mask the swine with isoflurane to induce sedation.

3.4. Monitor blood pressure, heart rate, and body temperature by physiological signal monitor during the surgery to prevent potential complications.

4. Anesthesia

4.1. Induce and maintain anesthesia; preferably anesthetize the animal *via* an intramuscular injection of ketamine (5 mg/kg), sternal (cazaporonum, 20 mg/kg), and atropine (5 mg/kg).

4.2. Intubate the animal with the endotracheal tube when muscle relaxation, characterized by loss of jaw tone and palpebral reflexed, was observed.

4.3. Maintain all pigs in an anesthetic state at a vaporizer setting of 0.5–2.5% (v/v) isoflurane until the end of the surgery.

4.4. Examine the depth of anesthesia by testing pain reflexes with a hind leg toe pinch before surgery. When necessary, add additional anesthesia or wait for a few minutes. Check pain reflexes regularly throughout the surgery.

5. Sterilization of the Surgical Site

5.1. Shave and clean the skin of the animal over an area of approximately 25 cm width from the vertebral column all the way to the axilla on both sides.

5.2. Scrub the moisturized skin with povidone-iodine scrub (75 mg/mL) for approximately 5 min.

5.3. Remove the povidone-iodine soap from the skin using wet sterile gauzes.

5.4. Sterilize the skin with povidone-iodine lotion (100 mg/mL).

5.5. Cover the animal with sterile surgical drapes to reduce bacterial transfer and subsequent contamination of the surgical site.

6. Burn Wound Creation

6.1. Use a surgical marking pen to mark the center of six burn wounds symmetrically on the dorsum of the pig. Ensure that the distance between each burn wound is at least greater than the radius of the wound (**Figure 1A**).

6.2. Fill a modified soldering iron with 50 mL of glycerin and insert an electronic thermometer into it to monitor the temperature. The hot iron possesses a flat area of approximately 9 cm^2 (**Figure 1B**).

6.3. Heat up the iron to 137–139 °C with a hot plate (**Figure 1C**).

6.4. Create six uniform burn wounds by placing the iron on the marked area without applying any force for 30 seconds (**Figure 1C**).

6.5. Wash the burn wounds with 0.9% saline solution (**Figure 1D**).

6.6. Measure wound dimensions and record the wound by photomicrography (**Figure 1E**).

7. Preparation of Dressings

7.1. Cover each wound with the inner contact layer of a four-layer clinical dressing through direct contact. For this layer, use CAPS-containing dressing or alternative materials (**Figure 1F**).

7.2. Apply a waterproof film onto the clinical dressing to serve as a barrier against bacterial penetration (**Figure 1F**).

7.3. Cover each wound with a gauze (0.5 cm thick) and fix with paper tape to serve as the mid-layer of the dressing (**Figure 1G**).

7.4. Secure the gauze with an outer layer of adhesive plaster. Extend this layer to the torso to avoid the displacement of the dressing (**Figures 1H-1J**).

8. Post-burn Care and Measurement

8.1. Inject the swine with buprenorphine (0.1 mg/kg, IM) for pain management for one week to reduce potential pain.

8.2. Allow the pig free access to feed and water.

8.3. Change the clinical dressings every 2 days for the first 10 days and then twice a week for the 6-week study.

8.4. Clean and measure wounds before reapplying clinical dressings. Administer anesthetics during dressing changes.

8.5. Record the wound by photomicrography for comparison of wound healing rate every 2 days for the first 10 days and then twice a week for the 6-week study.

8.6. Calculate the wound re-epithelialization or contraction as the percentage of the original wound size according to a previously described method. The analysis of wound closure was conducted in a double-blinded manner.

8.7. Measure the burn scar using VSS, which consists of four variables: vascularity, height (thickness), pliability, and pigmentation on post-burn days 0, 7, 21, and 42. Each variable has four to six possible scores. The total score ranges from 0 to 14, whereby a score of 0 reflects normal skin.

9. Bacterial Growth Experiments of Post-burn Tissues

9.1. Swab the wound for antibacterial testing on post-burn days 0, 7, 21, and 42.

9.2. Place the swab into 100 mL of 0.9% sterile saline solution and gently vortex to achieve a homogenous suspension.

9.3. Serially dilute (10^{-1}–10^{-5}) the homogenate and plate 100 µL of each dilution in selective and nonselective media, respectively.

9.4. Incubate all dilutions under aerobic conditions at 37 °C for 24–72 hours.

9.5. Plate triplicate aliquots of 10 µL each from all dilutions onto blood agar plate supplemented with 5% sheep blood to isolate aerobic Gram-positive organisms.

9.6. Incubate the sample by spreading or pouring the sample uniformly on the surface of an agar plate overnight for determining the number of colony-forming units (CFUs).

9.7. Read the plates after overnight incubation. Invert the sheep blood agar plate and divide the bottom of the dish into four equal quadrants using a marker and small ruler.

9.8. Place the plate onto the stage of a dissection microscope and count the colonies on each plate. By definition, a colony must have a minimum of 300 CFU to be enumerated.

9.9. Count the bacterial colonies in each of the three replicates. Calculate the average value of the three replicates. Determine the CFU per plate by multiplying the average value by the final dilution factor.

10. Euthanasia and Tissue Fixation

10.1. Intraperitoneally inject an overdose of sodium pentobarbital euthanasia solution (80–120 mg/kg).

10.2. Perform en bloc excision of burn wound tissue to include the underlying musculature and surrounding unwounded tissue.

10.3. Fix tissues with 10% neutral buffered formalin.

10.3.1. Mix 10 mL of formaldehyde (37%) in 90 mL of phosphate buffered solution (PBS) and store in 4 °C.

10.3.2. Transfer tissues to fixative and swirl the container to ensure all tissues are completely immersed in fixative. The volume of fixative must be 30 times the tissue volume.

10.3.3. Fix tissues overnight at 4 ˚C.

10.4. Dehydrate tissues with ethanol and embed into paraffin blocks. Perform the following steps at 4 ˚C on a shaker.

10.4.1. Wash twice with PBS for 30 minutes.

10.4.2. Dehydrate tissues with 70% ethanol for 8 hours, 80% ethanol overnight, 95% ethanol for 8 hours, and then in 100% ethanol overnight.

10.4.3. Incubate tissues in 100% ethanol for a further 8 hours.

10.4.4. Incubate tissues in three changes of xylene each for 30 minutes.

10.4.5. Replace the xylene with freshly melted (52 °C) wax, and incubate at 52 °C in an oven for 1 hour.

10.4.6. Replace the wax with fresh wax and incubate at 52 °C in an oven for 3 hours, and then replace once more and incubate at 52 °C overnight.

10.4.7. Incubate tissues with two more changes of wax each for 1 hour, and then embed the tissue and store at 4 °C.

10.5. Cut and stain the paraffin-embedded sections with HE, and visualize *via* a light microscope with 100× magnification.

10.5.1. Create paraffin sections using a rotary microtome.

10.5.2. Dewax sections with three changes of xylene each for 3 minutes.

10.5.3. Rehydrate tissues with 100%, 95%, 80%, and 70% ethanol each for 3 minutes, and then immerse in distilled water.

10.5.4. Stain with hematoxylin for 10 minutes, and then rinse in running tap water.

10.5.5. Differentiate with 0.1% hydrochloric acid ethanol for 5 minutes, and rinse in tap water.

10.5.6. Stain with 0.5% eosin for 1 minute.

10.5.7. Dehydrate tissues with 70%, 80%, 95%, and 100% ethanol each for 2 minutes.

10.5.8. Clear the staining with xylene, and dry in fume hood.

REPRESENTATIVE RESULTS

Burn duration of 30 seconds by hot iron resulted in wounds that were circular with a well-defined margin and uniformly pale with a rim of erythema (**Figure 1D**). Within each animal, there were six burn wounds on the dorsum. The arrangement of burn wounds was depicted in **Figure 1K**. Burn wounds were completely covered with CAPS-containing dressing and used to evaluate the depth of scar formation on post-burn days 0, 7, 21, and 42 and re-epithelialization, as determined by gross inspection. Burn wounds were re-epithelialized based on gross inspection on post-burn day 42. Wound size in these animals was evaluated to determine the wound healing rate (**Figure 2A**). Wound areas were 9, 10, 8, and 3 cm^2 on post-burn days 0, 7, 21, and 42, respectively. A significant reduction in wound area was observed on post-burn day 42 compared with day 0. The healing rate was defined as the greatest average wound margin distance from the wound center divided by the time to complete wound closure. Wounds treated with CAPS-containing dressing showed a 73.43±6.33% wound closure on post-burn day 42 (**Figure 2B**).

Figure 3 shows the VSS scores with respect to scar vascularity, pliability, pigmentation, and height on post-burn days 0, 7, 21, and 42. The VSS score peaked at 7.4±0.5 on post-burn day 21 and decreased to 3.33±0.58 on post-burn day 42.

All animals were sacrificed on post-burn day 42. Samples of skin resected from each animal post-sacrifice were histologically prepared and stained by H&E. Histologic examination of the samples confirmed that full-thickness burns were achieved, and the wounds appeared fully healed (**Figure 4**). Necrosis resulting from burns could be observed in the epidermis, dermis, and dermal components of the wound without significantly affecting the underlying muscle (**Figure 4**). The dermis thickness beneath the experimental dressing was 5.4 mm (**Figure 4A**). In addition, the sloughing of the dermis and lymphocytic infiltration are observed in the H&E staining, as indicated by the red arrow in **Figure 4B**.

The antibacterial property of the experimental dressing was determined using a CFU assay on post-burn days 0, 7, 21, and 42. The result showed a slight increase in bacterial cell numbers observed between post-burn days 0 and 21 followed by a significant increase on post-burn day 42 (**Figure 5**). This result suggests that the swine model of severe burn injury established in this study could be used to monitor the clinical performance of experimental dressings, including antibacterial property.

Figure 1. Burn wound creation and application of a CAPS-containing clinical dressing. Following hair removal and preparation of the skin with iodine and alcohol, **(A)** surgical marking pen was used to outline six circles on the dorsum, either side of the midline. **(B)** The modified iron was filled with glycerin and an electronic thermometer inserted into glycerin to show the temperature. **(C)** The iron is heated to 137–139 °C with a hot plate, and six uniform burn wounds were created on the marks of the skin. To create burn wounds, place the hot iron with no external force on the swine's back skin (30 seconds) to create two full-thickness wounds. **(D)** After all the wounds were created, the wounds were washed with 0.9% saline solution. **(E)** The scales were placed next to the wounds for taking pictures. **(F)** The first layer is covered with a test dressing and the second layer with a waterproof film. **(G)** The third layer is covered with the gauze of about 0.5 cm thickness and fixed with paper tape. **(H-J)** Secure the gauze with an outer layer of adhesive

plaster. This layer extends to the torso to avoid the displacement of the dressing. **(K)** Schematic diagram of the burn wound distribution.

Figure 2. Change of wound size in a swine model. (A) The rate of wound closure was determined as a percentage of the original wound on day 0. Wounds had almost completely contracted on post-burn 42 day, and **(B)** changes of the wound area were observed between 0 to 42 days of a post-burn animal model. It shows the greatest decrease on post-burn day 42, exhibiting 90 ± 4% wound area closure.

Figure 3. The average total scores of Vancouver Scar Scale (VSS) using a double-blind experimental design on post-burn days in a swine model. Scar assessment includes pigmentation, vascularity, pliability, and height of scar. A lower score indicates the scar on the condition that closely approximates normal skin (P = 0.0005).

Figure 4. Hematoxylin and eosin staining on post-burn day 42. (A, B) The black inverted triangle indicates the repaired burn tissue. The morphology of the full-thickness wound was smooth and continuous, and the papillary layer resembled features of hypertrophic scarring. Note the presence of neo-epidermis covering the wound surface. **(B)** The red arrow indicates the dermal connective tissue-infiltrated inflammatory cells in the burn wound eschar above the viable dermis below. Original magnification, ×10.

Figure 5. A bar diagram showing bacterial counting of wounds at different time points. Antibacterial activity of post-burn wound animal model at 0, 7, 21, and 42 days. Antibacterial activity was evaluated by colony-forming units (CFU) assay in three independent experiments.

DISCUSSION

The present study established a swine model of severe burn injury and examined the model using a CAPS-containing dressing. Our results suggest that this swine model can be used for monitoring the clinical performance of experimental dressings, including antibacterial property. Wound healing rate, wound closure, and antibacterial activity were also analyzed using VSS, H&E staining, and antibacterial test. The use of animal burn models has been developed as a valuable tool to review the pathophysiology of burn injury. There are certain biological benefits of using rats as experimental subjects, including the high number of rat-specific reagents and the practical aspects of obtaining transgenic rats, to discovering the molecular signaling pathways active in the recovery process. However, the major disadvantage of using rats in an animal burn model is its failure to completely approximate the human wound healing process. Re-epithelialization is the main healing approach observed in humans[8], while wound healing in rats strongly depends on wound contraction in a rapid manner[9]. In addition, the non-vulnerability of rats to hypertrophic or keloid scar formation confirmed the dissimilarity of their wound recovery process from that of humans. On the other hand, swine have recently become the subject of researcher attention because their skin architecture is similar to that of human skin. Additionally, the wound healing process in swine and humans occurs through physiologically similar phases such as inflammation, proliferation, re-epithelialization, and remodeling. Generally, burns in swine heal by 21 days, with re-epithelialization occurring between 7 and 14 days post-wound infliction[10,11], which fit to the timelines observed in humans. Swine also show greater

morbidity compare to the rats due to their body size that makes more resistant to wound infection. Mice are usually the first model used to evaluate burn wound conditions under different treatments. Additionally, in contrast to other mammals, swine share a number of anatomic and physiologic characteristics with humans regarding the process of epithelialization, cellular proliferation, and angiogenesis. This makes swine potentially a better model, and they are often used in subsequent studies that demonstrated promising results in mice [12,13,14,15]. Moreover, the distribution of dermal collagen and elastic content, cutaneous blood supply, the sequence of events in wound healing, and re-epithelialization have made the swine a standard model of wound healing and reconstructive surgical treatments[15,16,17,18]. Within each animal, there were six burn wounds located on the upper, middle, and lower back of the swine and distributed symmetrically to the spine. Burn wounds can be created on the similar locations but on different swine. Therefore, each experimental dressing can be tested and evaluated in different locations to minimize bias related to wound healing process in different spots on the swine dorsum. In addition, this swine model of severe burn injury established in this study also benefits from ensuring that each swine serves as its own control with one wound receiving novel treatment and the other as vehicle control, leading to a significant reduction in the number of animals.

The management of burn injuries is one of the major complications in wound healing. The human healing process is composed of four biological processes: hemostasis, inflammation, proliferation, and maturation. Once the healing process is disturbed or retarded, it promotes a fibroblastic proliferation that results in a hypertrophic scar, which is visible and tends to be raised above the surrounding skin[16]. To ensure reproducibility and accurate quantification, adequate adhesion using adhesive plaster is required immediately after the injury to minimize the delay in time. Cutaneous irritation is a common reaction from adhesive plaster that results in a rash around the clinical dressing. Thus, caution is recommended in the use of currently available adhesive plaster to prevent itchiness. Following aseptic techniques and thoroughly disinfecting all equipment (*e.g.*, caliper) to avoid cross-contamination between swines are imperative.

Burn wound healing is an intricate process. A certain number of factors have been identified to have a negative influence on the wound healing process such as pain, itching, and bacterial contamination. Pain has a negative impact on animal's diet. Without sufficient nutrient uptake, delayed wound healing is inevitable. Therefore, each swine was injected with buprenorphine (0.1 mg/kg, IM) daily for 7 consecutive days to reduce pain. Food intake was monitored one week after the burn wound creation. Once a decrease in food intake was observed on day 7, buprenorphine (0.1 mg/kg, IM) administration was continued until day 14. Bacterial infection, which is a potential risk for wound healing, may trigger inflammation and further dampen the healing process. Therefore, clinical dressings were covered with waterproof films to prevent and minimize potential contamination. Itching is an uncomfortable part of the wound healing process. Swine may scratch their own back on the ground to reduce itch, which can be unfavorable for wound healing process. A good dressing possesses the properties of accelerating burn wound healing with an acceptable scar, preventing infection and inflammation, alleviating pain, and permitting easy and early mobilization[19,20]. The dressing can be removed when required for further topical applications and/or measurement of the wound area[21,22]. Although applying a four-layer clinical dressing to a swine is a more complicated and time-consuming process than clinical,

swine model allows closer approximation to a human biological response than mouse model. The many potential advantages of using swine model make it a better platform for preclinical testing than other species.

The clinical efficacy in terms of wound closure efficiency and wound area reduction was monitored using a CAPS-containing dressing that revealed a 73.43±6.33% wound closure on post-burn day 42. Moreover, the VSS score, a validated subjective scale score, was also used to assess wounds in swine in this study[17]. It is important to monitor hypertrophic scar formation during treatment with experimental dressings, particularly in clinical trials[18]. Application of CAPS in the treatment of burn wounds resulted in a significant change in VSS scores on post-burn day 42 compared with the scores on day 21. A lower score indicates the injured area attempts to restore to a condition that closely approximate normal skin color pigmentation, vascularity, pliability, and height. In addition, the results of H&E staining indicated an intact stratum corneum layer in the CAPS-treated area. This result suggests that the swine model of a severe burn injury can be used reliably to evaluate the wound area, wound closure, VSS score, and healing progress.

This protocol represents the numerous detailed steps for demonstrating the use of an experimental dressing on a swine model of severe burn injury and can be applied to the evaluation of any types of clinical dressing. A modified soldering iron filled with 50 mL of glycerin is capable of maintaining temperature within 137 to 139°C during the wound creation process. However, this method has limitations such as inability to absolutely mimic the real burn temperature and the narrowness of the burn created area due to the soldering iron scope. However, the main advantages of the method overcome those limitations. This modified iron can be used to create consistent wounds with accuracy and ease compared to the traditional methods. Moreover, this model provides a sensitive measure of the physiological and pathophysiological response of a thermal burn injury under the treatment of various clinical dressings. The advantage of the proposed model, besides the economic advantages, is that it is easy to be handled by those with relatively little surgical experience. The application of the clinical dressing to the wounds in the swine model mimics the biological processes of human wound healing. Therefore, the swine model of severe burn injury established and tested with clinical dressing in this study facilitates the development of novel treatment for burn injury. This study provides crucial tools to uncover the pathophysiology of burn wound healing. In conclusion, this swine model provides an easy-to-learn, cost-effective, and robust method to assess the effect of clinical dressings in severe burn injury.

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DISCLOSURES

The authors have nothing to disclose.

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Figure 1

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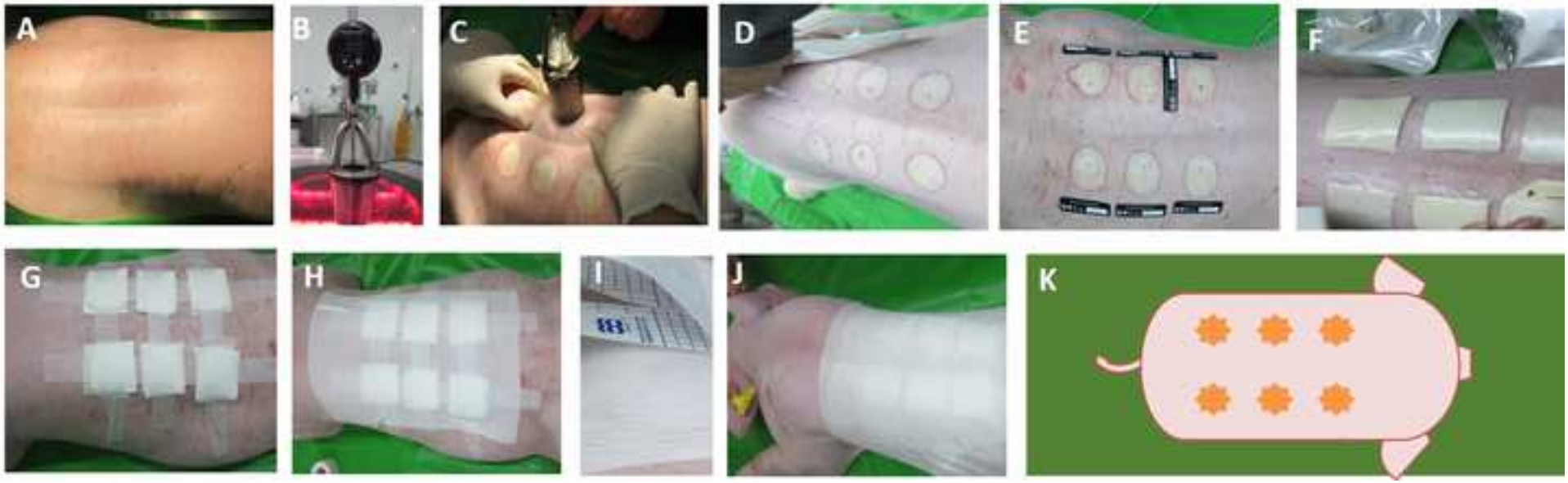

Figure 2

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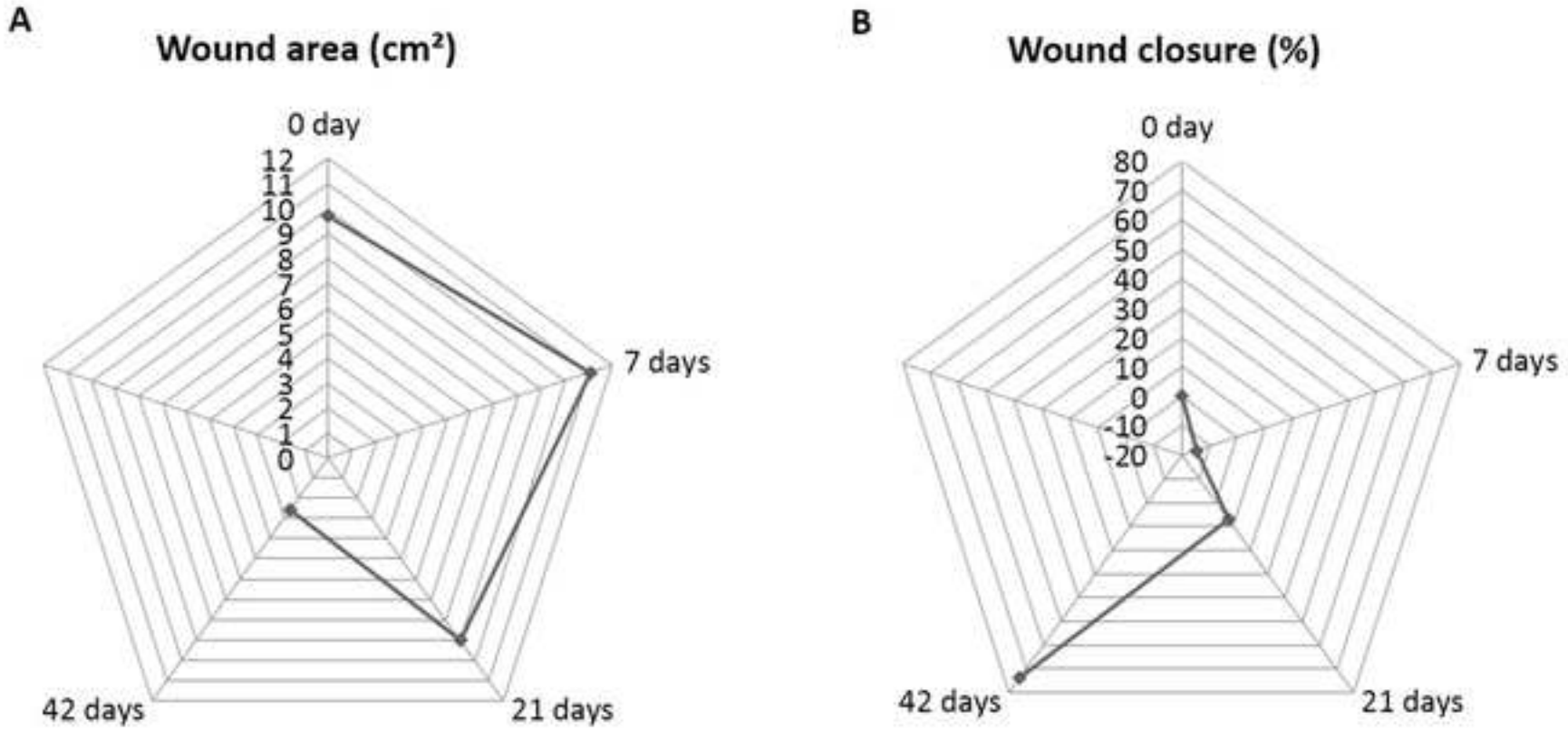

Figure 3

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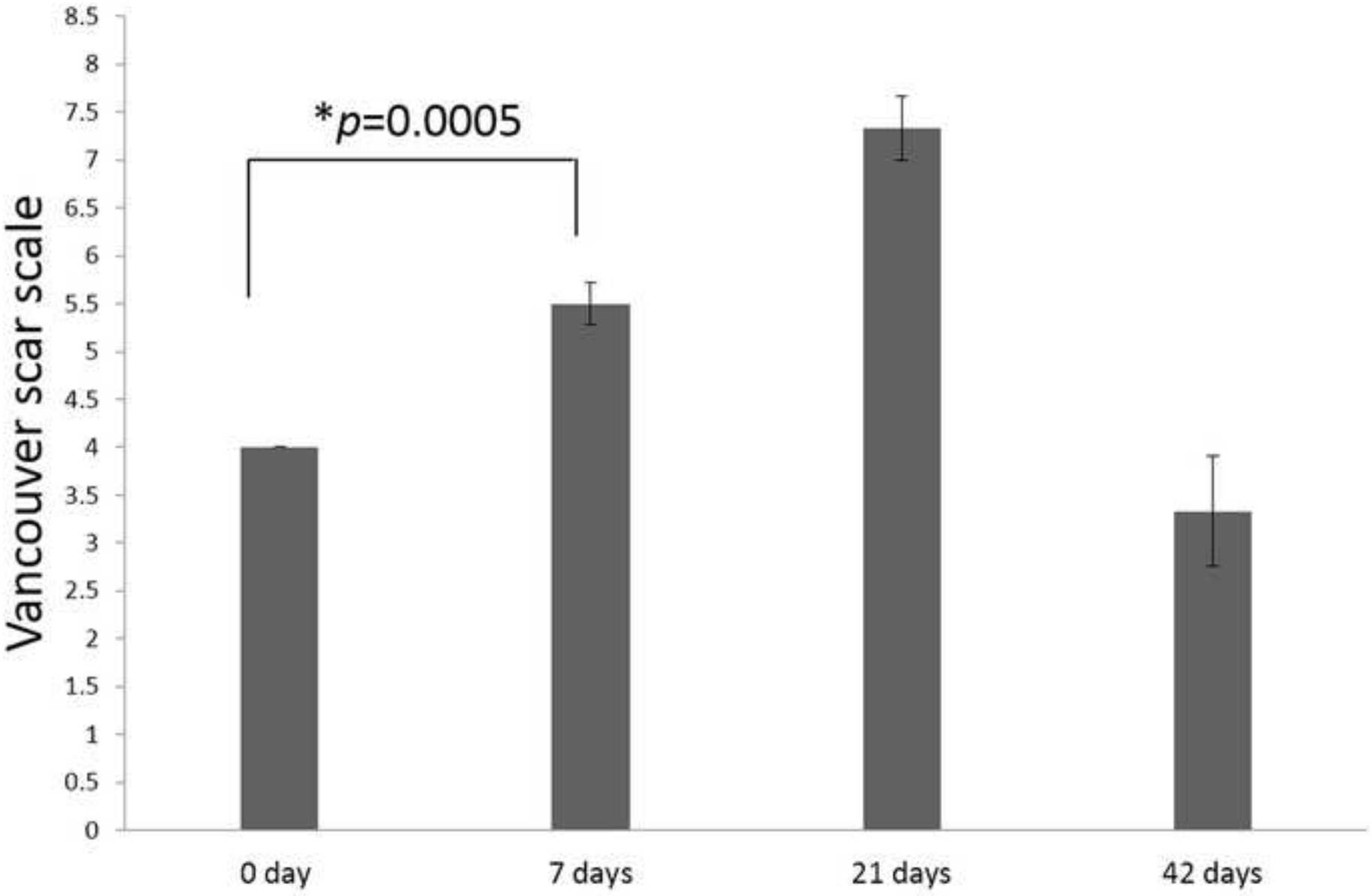

Figure 4

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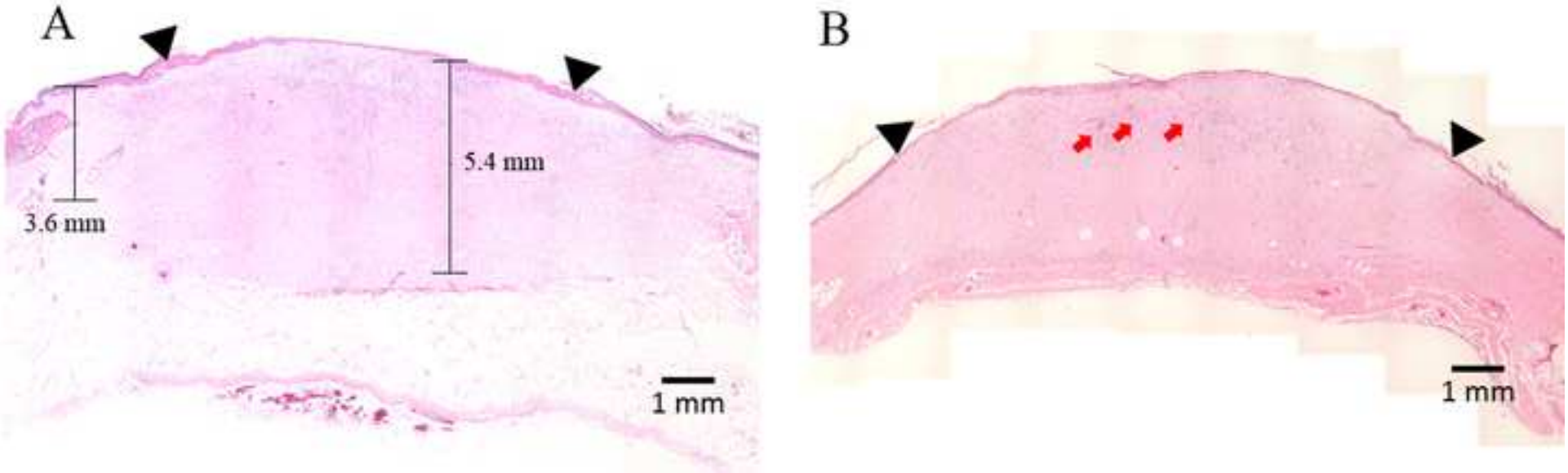

Figure 5

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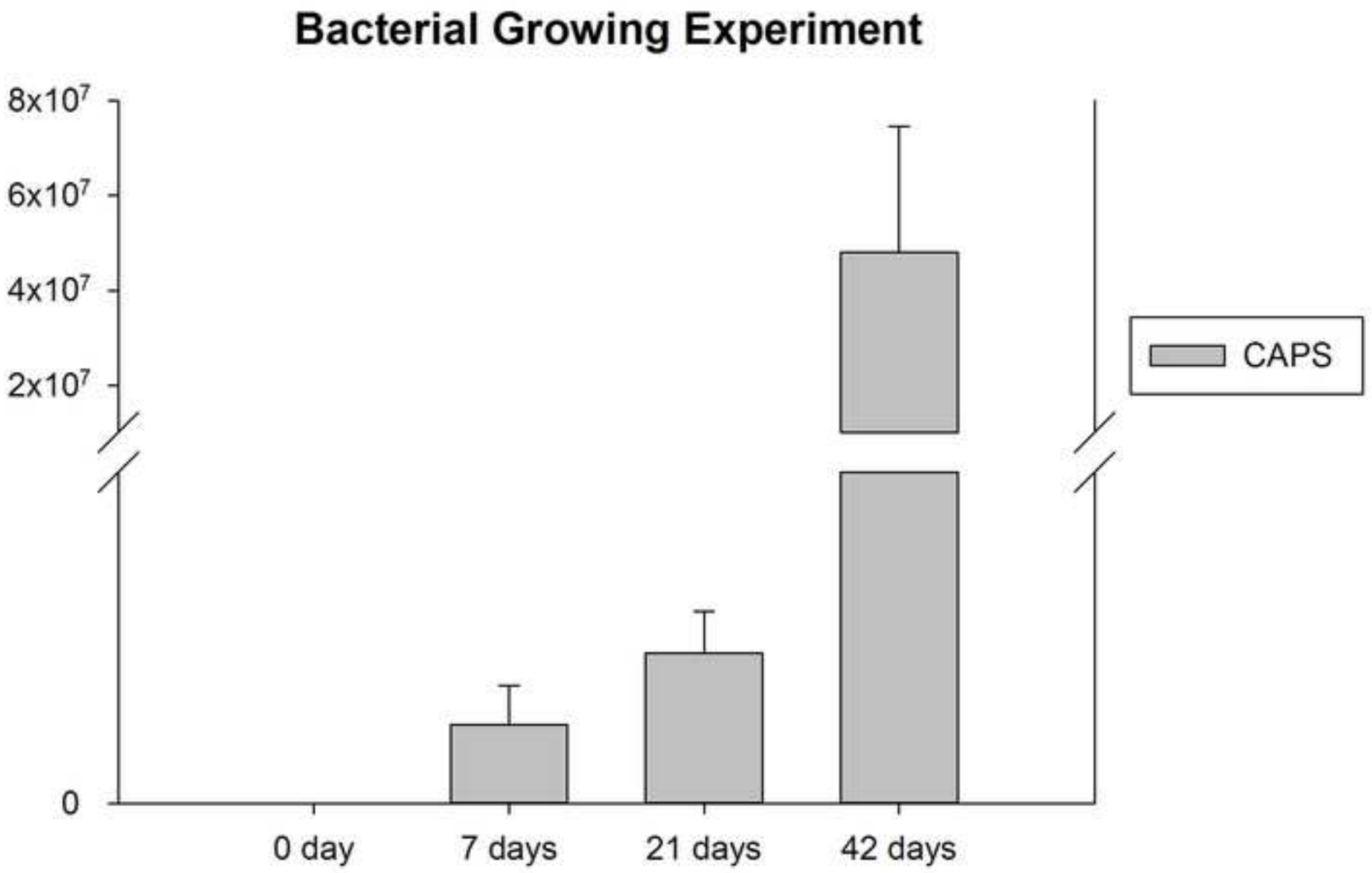

Table of Materials

Click here to download Table of Materials Materials table-0614.xlsx

Item	Company	Catalog Number	Comments
Sedation:			
ketoprofen	Astar	1406232	2 mL vial
azaperone	China chemical & pharmaceutical	47W406	100 mL vial
atropine	Oriental chemical works	IN120802	1 mL vial
Anesthetic:			
Zoletil 50	Virbac	BC91	5 mL vial
isoflurane	Baxter	N002A225	100 mL vial
Surgery:			
hair clippers	Moser	-	-
povidone iodine scrub solution	Ever star	HA161202	4 L barrel
modified iron	-	-	-
electronic thermometer	Dogger	A9SA-ST9215C	- 50~300℃
0.9% saline solution	CHI SHENG	KC130	500 mL vial
gauze	China Surgical Dressings Center	MO15900080	10 x10 cm
CAPS	CoreLeader Biotech Co., Ltd, Taipei, Taiwan	-	-
paper tape	3M	NDC-8333-1530-01	2.5 cm x 9.1m
waterproof film	3M	NDC-8333-1600-40	10 cm x 10 m
adhesive plaster	Young chemical	BH1426015	10 cm x 10 m
Dissection:			
Pair of standard sharp/blunt straight scissors (14 cm)	Shinetec instruments	ST-S114	-
Halstead-Mosquito (12.5 cm)	Shinetec instruments	ST-H012	-
Handle(# 4)	Shinetec instruments	ST-H004	-
Surgical Blade(#21)	Shinetec instruments	ST-B021	-
Post-Fixation & Storage:			
50 ml Plastic centrifuge tube	Falcon	352070	-
10% neutral buffered formalin,	Leica	3800604EG	-
Bacterial Growth Experiments			
Blood agar plate (BAP) (TSA with 5% sheep blood)	CMP	-	90 mm Mono

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Title of Articles: Sever Burn Injury Swine Model for Clinical Dressing Assessment

Author (S): **Gang-Yi Fan, MS, Juin-Hong Cherng, Ph.D, Shu-Jen Chang, Ph.D, Yao-Horng Wang, Ph.D, Chih-Hsin Wang, MD, Raju Poongodi, Ph.D, Yi-Wen Wang, Ph.D, Cheng-Che Liu, Ph.D En Meng, Ph.D.**

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CORRESPONDING AUTHOR:

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Department:	Department of Surgery
Institution:	Tri-Service General Hospital, National Defense Medical Center
Article Title:	Sever Burn Injury Swine Model for Clinical Dressing Assessment

Signature: En Meng Date: 2018-01-31

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2018.04.09

Dear Editor,

Sincerest thanks for your response and reviewers' comments on our manuscript "JoVE57942-Sever Burn Injury Swine Model for Clinical Dressing Assessment". We sincerely apologise for the great time it has taken us to respond to these comments, and hope that a revised version of the manuscript will still be considered to accept by JoVE. We have modified the paper in response to the extensive and insightful reviewer comments. We have rewritten sections of the manuscript and we hope that this comply with the referee's remarks. We hope that the revised manuscript may suitable for the publication in JoVE. We are looking forward to hear from you.

Thanking You

Sincerely,
En Meng

…….
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Editorial comments: Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Corrected.

2. Figure 2A: Please provide units on the scale bar.

Corrected.

3. Please be consistent with panel labeling for the Figures. Figure 1 has no parentheses but the other Figures have parentheses for the panel labels (A), etc.

Corrected.

4. Please combine all panels of one figure into a single image file.

All panels have been combined in a single image file.

5. Please mention how proper anesthetization is confirmed.

Please refer to “4. Anesthesia”.

6. 6.3: 137 degrees Celsius? Where are the 6 wounds made? What order?

It should be 137 degree Celsius. Wound creation please refer to “6. Burn Wound Creation”.

7. 9.2: What temperature for growth?

Plates were incubated at 37°C. Please refer to “9. Bacterial Growth Experiments of Post Burn Tissues”

8. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Corrected

9. Please specify the composition of all solutions/media used.

The composition of all solutions/media are shown in “MATERIALS”.

10. Please specify the euthanasia method.

Please refer to “10. Euthanasia and Tissue Fixation”.

11. Please move step 11 to the Discussion.

Step 11 has been incorporated into Discussion.

12. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Highlighted in yellow. Please refer to “Protocol”.

13. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

Reviewer #1:

Manuscript Summary:

The manuscript describes a method to create burn wounds in a pig model. The authors are evaluating whether a CAPS wound dressing vs. traditional dressings (designated "CMC", Aquacel) will improve wound healing (re-epithelialization and scarring).

Major Concerns: Burn depth and lack of clinical translation: Burns of >100C for 30 seconds will be full thickness wounds (and acknowledged by the author on line 255). Clinical practice would be to excise the necrotic tissue and apply a graft (if donor sites are available) or an allograft/skin substitute. The proposed model does not mimic clinical practice and therefore makes it less desirable.

In this study, we are seeking treatments which are differ from clinical practice. Rat, mouse, and rabbit have been used in dermatologic toxicology. However, swine share a number of anatomic and physiologic characteristics with humans in terms of the process of epithelialisation, cellular proliferation, and angiogenesis that make swine potentially a better model for certain procedures and studies comparing with other species. Although there is still a slightly different in burn conditions between human and swine model, this model still facilitates the development of novel treatment for burn injury.

Analgesia concerns: The authors use ketoprofen as analgesia daily for at least 1 week but it is an NSAID. It is widely known that NSAIDS delay wound healing. A more appropriate analgesic for swine would be buprenorphine SR-LAB which requires administration every 72 hours and will not impact wound healing.

Ketoprofen should be corrected as transdermal fentanyl patch (50 μg/hour).

Comparison between treatments: The authors are comparing CAPS vs. "CMC" (Aquacel); however, Figures 2-4 do not show any of the results from the Aquacel group. In the corresponding text of the results section and the figure legends, again only CAPS data is provided. If the authors want to state CAPS is superior to traditional dressings (lines 315- 317), the appropriate data needs to be shown with significantly statistical results.

Comparison between different dressings was not our intension. Related paragraphs have been rewritten.

Bacterial quantification: Swabs only collect bacteria on the surface of the wound. Non debrided burn wounds will get infected and the most prevalent bacterial species (e.g> Pseudomonas aeruginosa) can penetrate the eschar which may not all be collected with swabs. A more common approach would be to collect biopsies and report as CFUs/g of tissue.

Although collection of biopsies is a better method to evaluate the anti-bacterial activity, it significantly changes the wound condition to create other factors which may further affect the study. Besides, the collection of biopsies in different time points in different conditions may increase the number of animals in this study, which is impractical.

VSS scores: What is the point of performing VSS analysis on days 0, 7, 21? The necrotic eschar wasn't removed which delays the wound healing. Until the wounds are completely re-epithelialized which signifies a transition to the remodeling phase of wound healing, it is pointless to perform any type of scar assessment. Extending the study to 60, 90, or 120 days to allow remodeling to actually occur will provide more insight into scarring.

The scar scale reflects the progress of wound closure. Rapid wound closure implies a short inflammatory phase, suggesting the dressing facilitate the healing process.

Minor Concerns: Timeline: Lines 164, 168, and 176 do not coincide. "The dressings were changed every 2 days for 10 days and then twice a week for 6 weeks." The entire experiment was only 42 days. Also how was a swab collected on days 0 and 7 when the dressings were changed every 2 days for 10 days?

The dressings were changed every 2 days for 10 days and then twice a week until week 6. We did collect samples on day 0 and 7. The dressings were removed for measurements and put back immediately without renewing.

Wound size: Line 147 states the hot iron creates wounds of 20cm2 but Figure 2 shows a ruler with the diameter of the wounds to be 3 cm which results in a wound size of ~7cm2. This is further confirmed in Figure 3 with a starting wound area depicted of 9cm2. Please reconcile. Image quality: Figure 1 and 4 are too low quality to see details.

Wound area should be 9 cm^2.

Terminology issues: Exercise, darning, adhesive plaster, epithelial gap - These terms were used and need further clarification. Please elaborate on their meaning or change the terminology.

Corrected.

Pain: line 96-97 states "the present study provide new insights into how a CAPS containing dressing affects pain". No data was provided to show a difference in pain. How can pain be measured in a pig and differentiated between wounds? Was CAPS and Aquacel both used on the same animal?

Any types of dressing can be applied to the swine model. However, comparison between CAPS and Aquacel was not our intension. Related paragraphs have been rewritten.

Reviewer #2:

1. There is no novelty in the protocol of experiment to publish in Journal of Visualized experiments. Similar experiments have been carried out in rats and mice "Flexible, micro-porous chitosan-gelatin hydrogel/nanofibrin composite bandages for treating burn wounds" published in RSC advances in 2014. "Honey based hydrogel: In vitro and comparative In vivo evaluation for burn wound healing" published in Nature: Scientific reports in 2017.

This model may benefit from ensuring that each swine serves as its own control with one wound receiving treatment and the other vehicle control, thereby reducing animal numbers.

2. Check for spelling, punctuation marks, grammatical errors and sentence formation throughout the article. Eg: In Abstract, line no. 3 "exercise", line no:8 "complementary" In article, line no:49 "composite", line no:57 "scar", line no:69 "trigger-off" are some of the irrelevant words to be replaced.

Corrected

3. No reference for protocol supporting material preparation. Try to include reference of your previously published paper.

Included. Please refer to Ref. 7.

4. Check for the standard protocol for antibacterial activity. Lack of data supporting antibacterial activity of your scaffold. No error bar and statistical significant data in bacterial growth experiments though you have mentioned in the line 246 as CFU assay was performed in triplicates.

Corrected.

Editorial comments:

1. Please employ professional copy-editing services as the language in the manuscript is not publication grade. The language significantly detracts from the scientific value of the manuscript.
Response: We have had the manuscript edited by a professional editing service.

2. Please revise the following lines of the manuscript to avoid previously published text: 60-61, 65-72, 78-80, 246-247, 267-270, 327-333, 335-336, 343-349, 381-384,
Response: Thank you for pointing this out. All the above sentences have been edited

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5. Please include at least 6 keywords. Please use MeSH terms whenever possible.
Response: We have included the following keywords in the manuscript
Swine; Polysaccharides; Burn; Surgical; Experimental animal models; Scarring

6. Please reduce the Short Abstract to be 50 words or less.
Response: The short abstract is within 50 words now

7. Revisions are required for the protocol:
Why are there 10 panels of Figure 1 that are not referenced? Please reference these panels or remove Figure 1.
Response: Each panel has been referenced with a corresponding step.

How many animals are used? Where are they housed and in what conditions?
Response: We created 6 burns on each of the 3 pigs; for a total of 18 burns, with 3 replicates dressed with CMC and CAPS. This study was conducted in the Laboratory Animal Center at the National Defense Medical Center. Three domestic pigs were used in this study. The animals were provided a standard diet ad lib several days before the investigation and were fasted overnight before any procedure. The animals were housed in individual pens upon their arrival and allowed to acclimatize for at least 7 days.

What volume of glycerin?
Response: We used 50 mL of glycerin

Streak on what?
Response: The samples were collected on post-burn days 0, 7, 21, and 42; they were placed in sterile tubes containing 0.9 % normal saline.

What dilution? How many Petri dishes?
Response: We estimated microbial concentration from measured counts on a single agar plate and used serial dilutions. We used 10 small sterile test tubes, labeled the tubes 1 through 10, and added 4.5 mL of 0.9 % normal saline to each test tube. The dilution ratios ranged from 10^{-1} to 10^{-5}.

Employ the agar how? What is actually done?
Response: Blood agar plate (BAP) (TSA with 5% Sheep Blood) was used in routine laboratory procedures. Each sample was inoculated with 10 µL onto BAP.

How is each step explicitly done? This is unclear.
Response: Each step has been described in detail.

8. Figures: Please define the error bars in Figure 3 and 5 (SD, SEM, etc.). Please reference Figure 5 in the manuscript.
Response: These errors have been corrected

9. Please do no abbreviate journal titles.
Response: This has been corrected

Reviewers' comments:
Reviewer #2:
Authors duly revised the manuscript. Accept.

Reviewer #3:
Major Concerns:
Why do you use antiseptics before creating the burn wounds? Not similar to "normal" burn wounds.
Three layer clinical dressing? Why this? That´s not adapted to the amount of wound exudate. Why gauze on top and then paper tape?
So in sum you have the test-material, film dressing, gauze and paper-tape? Why gauze and paper-tape? Test-material and film dressing is absolutely enough at low exudate level? With the gauze and the paper-tape you reduce the MVTR of the film dressing.
Response: This model was not established to mimic clinical practice but to provide a robust platform to compare the clinical efficacy of different experimental dressings. Thus, the use of antiseptics before creating the burn wounds provides a consistent environment for all dressings.
Due to the constant movement of the animal (standing and lying down, running around, scratching against enclosure, and rubbing the wall), we needed waterproof film, gauze, and an outer layer of adhesive plaster to fix the dressing, as per our experience. Additionally, the gauze absorbed fluids from the wound, which made it easy for us to observe the healing process.

Swab on day0? After antiseptic and burn this result is worthless.
Response: The swab on day 0 served as a control to ensure that the disinfection process was executed thoroughly.

Reviewer #4:
Manuscript Summary:

The manuscript describes a method to create burn wounds in a pig model. The authors are evaluating whether a CAPS wound dressing will improve wound healing.

Major Concerns:

Line 86 and 333: "Currently, there are no swine models that examine the six burn wound simultaneously."

This statement is blatantly false. A number of groups work on burn wounds in pigs and can simultaneously monitor wound healing. Drs. Singer, Sen, Herndon/Finnerty, Christy, Shupp, Tredget, Davis/Tomic-Canic, etc…

Response: We apologize for the misleading information. The content has been rewritten.

Burn depth and lack of clinical translation: Burns of >100C for 30 seconds will be full thickness wounds (and acknowledged by the author on line 266). Clinical practice would be to excise the necrotic tissue and apply a graft (if donor sites are available) or an allograft/skin substitute. The proposed model does not mimic clinical practice and therefore makes it less desirable.

Issue is still not addressed

Response: Although the swine model of severe burn injury proposed in this study is not identical to clinical practice, it is an easy-to-learn, cost-effective, and robust method to assess the effect of clinical dressings at several time points. Besides, there is still a slight difference in burn conditions between humans and the swine model. This model was not designed to mimic clinical practice but to establish a reliable platform to facilitate the development of novel treatments for burn injuries.

Image quality: Figure 1 and 4 are too low quality to see details. The authors are attempting to establish this model in their laboratory. It would be helpful to see the burn depth generated by this method. I agree this is a full thickness burn without muscle damage but the histology provided is not sufficient.

5.4mm of granulation tissue within the wound bed is significant. The dermal thickness for ~50 kg Yorkshire pig is only 2-3mm. This is an artificial outcome generated by not performing the standard of care of debriding the burns and treating with a graft/skin substitute.

Response: The thickness of normal skin next to the black triangle on Figure 4A is 3.6 mm. When the wound starts to fill with granulation tissue, the new skin begins to form over the tissue. This structure is not scar tissue and will keep remodeling, resulting in a smooth area. The skin thickness of swine varies with foster and individual differences, we try to show the difference between normal tissues and burned wound in the figure at the same time. Regards to provide macroscopic view of large area and continuous skin which may be sacrificed the resolution of the graph under our limitation of tools.

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Comparison between treatments: The authors are comparing CAPS vs. "CMC" (Aquacel); however, Figures 2-4 do not show any of the results from the Aquacel group. In the corresponding text of the results section and the figure legends, again only CAPS data is provided.

Figure 5 (bacterial activity) is the only comparison to the CMC that is reported. Obviously wounds were covered with the CMC and all other data would have been collected. Did the CAPS and CMC heal similarly?

Response: In this study, we did not intend to compare the clinical efficacy of CMC and CAPS. Thus, all the comparison between these dressings has been removed.

Bacterial quantification: Swabs only collect bacteria on the surface of the wound. Non-debrided burn wounds will get infected and the most prevalent bacterial species (e.g> Pseudomonas aeruginosa) can penetrate the eschar which will not be collected with swabs. A most common approach would be to collect biopsies and report as CFUs/g of tissue.

Issue is still not addressed

Figure 5 legend doesn't match the figure. No values below the cutoff are shown on the y axis of the figure so can't make any sense of the CFU's at day 7 or 21. The clinical definition of an infection is 1 x 105, which the day 42 values exceeds. The new figure removed the CMC data completely.
Response: We agree that collection of biopsies is a better method to evaluate the anti-bacterial activity as it significantly changes the wound condition to eliminate other factors that may further affect the study. Additionally, collecting biopsies at different time points in different conditions would have increased the number of animals in this study, which is impractical. As mentioned above, the comparison between CAPS and CMC is confusing and diverts attention from the purpose of this study. Thus, in the new figure, we have removed the CMC data completely.

VSS scores: What is the point of performing VSS analysis on days 0, 7, 21? The necrotic eschar wasn't removed which delays the wound healing. Until the wounds are completely re-epithelialized which signifies a transition to the remodeling phase of wound healing, it is pointless to perform any type of scar assessment. Extending the study to 60, 90, or 120 days to allow remodeling to actually occur will provide more insight into scarring.
Not addressed.
Response: The scar scale reflects the progress of wound closure. Rapid wound closure implies a short inflammatory phase, suggesting that the dressing facilitated the healing process. This protocol provides a tool to compare the clinical efficacy of clinical dressings in a limited time.

Also, given how delicate any newly formed epidermis wound be, how was the pliability measured without damaging this newly formed epidermis? No score was given for "normal" skin. The day 0 states it is post-burn with the figure only showing significance from 0 to 7 but the discussion states differences from 21 to 42 (line 371). It is not surprising to see the scores increase from 0 to 7 or 21. Nor is it surprising to see scores decrease by day 42 as the wounds heal. In line 334 you state "This model may also benefit from ensuring that each swine serves as its own control with one wound receiving treatment and the other vehicle control, thereby reducing animal numbers."
And then on line 372 "A lower score indicates the injured area attempts to restore to a condition that closely approximate normal skin color pigmentation, vascularity, pliability and
height."
But then don't show VSS scores for this comparison or even to normal skin.
Response: In this study, we provide a comprehensive protocol to evaluate experimental dressings on a swine model of severe burn injury. CAPS-containing dressing was only used as an example. We did not intend to compare the efficacy of any clinical dressings in this study. The results of wound closure, VSS score, and antibacterial activity collected from this model can be analyzed to compare the clinical efficacy of experimental dressings within 42 days.

Minor Concerns:
Timeline Issues are not resolved: "The dressings were changed every 2 days for 10 days and then twice a week for 6 weeks." The entire experiment was only 42 days.
Change to state "….then twice a week for the 6 week study."
Reconcile the dressing changes with VSS and bacterial swabs and figure legends:
Line 194 and 200: "every 2 days for the first 10 days and then twice a week for 6 weeks."
But on line 208 and 214: "on post-burn days 0 and 3 and week 1, 2, 3, 4, 5
215 and 6"
Then line "298 Figure 2. Change of wound size in a swine model. (A) The burns were photographed immediately after the burn injury and every three days afterwards,"
What was the actual time line?
The grammar has significantly improved as far as grammar/copy editing. Some errors still persist.
Lines 86, 369 - incorrect references - I would suggest checking all of them for accuracy.
Response: All these errors have been corrected.

Wound closure/Contraction: Figure 2 shows wound contraction and wound closure

Are the authors taking into account the growth of the animals over the 42 day study? We have seen the animals increase in body weight significantly during these longer studies. We actually tattoo around the wounds to make it easier to monitor the contraction over time and also tattoo a normal non-injured area of the same size to account for any increase in body weight/surface area. Generally speaking, for a 42day study, we may see an increase in surface area of 10-20% which needs to be account for the calculations.

Response: Thank you for pointing this out. We did not consider the growth of the animals in this study. However, six wounds were created on one animal, which allow several experimental dressings to be evaluated simultaneously to further reduce the impact of animal growth on the evaluation of clinical efficacy.