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Protocol to Create Chronic Wounds in Diabetic Mice

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November 27, 2017

Nandita Singh, Ph.D.
Senior Science Editor
JoVE

Dear Dr. Singh,

Enclosed please find our manuscript entitled "Protocol to Create Chronic Wounds in Diabetic Mice" by J. H. Kim and M. Martins-Green that we wish to submit to the JoVE. This manuscript describes in great detail the steps needed to create chronic wounds in an *in vivo* diabetic mouse model. Chronic wounds are wounds that do not heal despite conventional antibiotic therapy and standard wound care management. The presence of bacterial biofilms significantly hinders physicians' efforts to heal these already complicated wounds. These wounds affect millions of Americans and cost the US healthcare billions. Diabetics are especially at great risk for developing chronic wounds and even greater risk of lower limb amputation if bioburden cannot be controlled. Our model was designed specifically to understand the pathophysiology of these wounds.

The manuscript instructs the use of 6 month old *db/db*^{-/-} mice, both male and female, that are both diabetic and obese. The 7mm full-thickness skin excision is treated with inhibitors for antioxidant enzymes to increase levels of oxidative stress in the wounds. After treatment, the wound has chronic inflammation, high oxidative stress levels, poor angiogenesis and damaged vasculature, similar characteristics found in human chronic wounds. The bacteria in the skin microbiome also begin to form biofilm as early as day 3 after wounding, causing extra damage to host tissue. These wounds are fully chronic in 20 days and can remain open with continual bioburden past 60 days, reflecting the long duration of open wounds experienced by humans. The manuscript also describes key instructions that make the chronic wound a reliable model for studying the fundamental wound healing processes that when impaired lead to the initiation of chronicity.

In addition to the manuscript and suggested potential reviewers and editors for review purposes, an amendment for video recording the surgery procedure has been approved by our Institutional Animal Care and Use Committee (IACUC).

JoVE is our journal of choice for publication because it is highly regarded, and the videos published provides visual instruction that is more intuitive and helpful in teaching the community how to successfully reproduce the model.

Please do not hesitate to contact me if you need any additional information. I look forward to hearing from you.

Sincerely,

A handwritten signature in cursive script that reads "Manuela Martins - Green".

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SHORT ABSTRACT:

Chronic wounds are developed from acute wounds on a diabetic mouse model by inducing high levels of oxidative stress after a full-thickness cutaneous wound. The wound is treated with inhibitors for catalase and glutathione peroxidase, resulting in impaired healing and biofilm development by bacteria present in the skin microbiome.

LONG ABSTRACT:

Chronic wounds develop as a result of defective regulation in one or more complex cellular and molecular processes involved in proper healing. They impact ~6.5M people and cost ~\$40B/year in the US alone. Although a significant effort has been invested in understanding how chronic wounds develop in humans, fundamental questions remain unanswered. Recently, we developed a novel mouse model for diabetic chronic wounds that have many characteristics of human chronic wounds. Using *db/db*^{-/-} mice, we can generate chronic wounds by inducing high levels of oxidative stress (OS) in the wound tissue immediately after wounding, using a one-time treatment with inhibitors specific to the antioxidant enzymes catalase and glutathione peroxidase. These wounds have high levels of OS, develop biofilm naturally, become fully chronic within 20 days after treatment and can remain open more for more than 60 days. This novel model has many features of diabetic chronic wounds in humans and therefore can contribute significantly to advancing fundamental understanding of how wounds become chronic. This is a major breakthrough because chronic wounds in humans cause significant pain and distress to patients and result in amputation if unresolved. Moreover, these wounds are very expensive and time-consuming to treat, and lead to significant loss of personal income to patients. Advancements in this field of study through the use of our chronic wound model can significantly improve health care for millions who suffer under this debilitating condition. In this

protocol, we describe in great detail the procedure to cause acute wounds to become chronic, which has not been done before.

INTRODUCTION:

Wound healing involves complex cellular and molecular processes that are temporally and spatially regulated, organized in sequential and overlapping stages that involve many different cell types including but not limited to the immune response and the vascular system¹.

Immediately after the skin sustains an injury, factors and blood cells aggregate to the wound site and initiate the coagulation cascade to form a clot. After homeostasis is achieved, the blood vessels dilate to let into the wound site oxygen, nutrients, enzymes, antibodies and chemotactic factors that chemoattract polymorphonucleocytes to clear the wound bed of foreign debris and secrete proteolytic enzymes². Activated platelets secrete a variety of growth factors to stimulate the keratinocytes at the wound edge to re-epithelialize the wounded area. Monocytes recruited to the wound site differentiate into macrophages which phagocytose bacteria and dead neutrophils and secrete additional factors to maintain keratinocyte proliferative and pro-migratory signals. In the proliferation phase, while re-epithelialization continues, new granulation tissue composed of fibroblasts, monocytes/macrophages, lymphocytes, and endothelial cells continue the rebuilding process². Angiogenesis is stimulated by promoting endothelial cell proliferation and migration, resulting in new vessel development. Epithelialization and remodeling of the extracellular matrix construct a barrier against the environment. As the wound heals and granulation tissue evolves into a scar, apoptosis eliminates inflammatory cells, fibroblasts, and endothelial cells without causing additional tissue damage. The tensile strength of the tissue is enhanced by fibroblasts remodeling various components of the extracellular matrix, like collagen, so that the newly formed tissue is almost as strong and flexible as unwounded skin².

Any deviation from this highly concerted progression towards wound closure leads to impaired and/or chronic wounds³. Chronic wounds are characterized by increased oxidative stress, chronic inflammation, damaged microvasculature, and abnormal collagen matrix in the wound⁴. Oxidative stress, especially in the wound, can delay wound closure^{2,5}. When, in the first stage of wound healing, the inflammatory phase becomes unregulated, the host tissue assumes extensive damage due to a continuous influx of inflammatory cells⁵ that release cytotoxic enzymes, an increase in free oxygen radicals, and unregulated inflammatory mediators, resulting in cell death^{6,7}.

In this destructive microenvironment, biofilm-forming bacteria take advantage of host nutrients and contribute to the damage of the host tissue². These biofilms are difficult to control and remove because the hydrated extracellular polymeric substances composed of proteins, DNA, RNA, and polysaccharides allows bacteria harbored within to be tolerant to conventional antibiotic therapies and evade the host's innate and adaptive immune response^{2,8,9}.

Studying chronic wounds is crucial because they impact ~6.5 million people and cost ~\$40 billion per year in the US alone¹⁰. Patients with diabetes have increased risks for developing chronic wounds that require amputation in order to contain the spread of infection. These

patients have a 50% mortality risk within 5 years of amputation that is attributed to the pathophysiology mechanism of diabetes¹¹. The relationship between the host's immune system and the microbiome in wound healing is a vital topic of ongoing research because consequences of chronic wounds, if unresolved, include amputation and death¹².

Although a significant effort has been invested in understanding how chronic wounds develop in humans, it is still unclear how and why chronic wounds form. Experiments to study the mechanisms of impaired healing is difficult to conduct in humans, and wound healing specialists only see patients with chronic wounds that have already reached chronicity for weeks to months. Thus, specialists are unable to study what processes went wrong that lead the wound to develop to become chronic². There is a lack of animal models that recapitulate the complexity of human chronic wounds because animals naturally do not develop chronic wounds. Until our model was developed, no model for chronic wound studies existed.

The chronic wound model was developed in mice that have a mutation in the leptin receptor (*db/db*^{-/-})¹³. These mice are obese, diabetic, and have impaired healing but do not develop chronic wounds¹⁴. Blood glucose levels average around 200 mg/dL, but can be as high as 400 mg/dL¹⁵. When high levels of oxidative stress (OS) in the wound tissue are induced immediately after wounding, the wound becomes chronic¹⁶. The *db/db*^{-/-} wounds are considered chronic by 20 days and remain open for 60 days or more. Biofilm produced by bacteria can be seen developing beginning three days after wounding; a mature biofilm can be seen 20 days after wounding and persists until either wound closure. The biofilm-forming bacteria we find in these mice are also found in human diabetic chronic wounds.

Oxidative stress is induced by treating the wounds with two inhibitors of antioxidant enzymes, catalase and glutathione peroxidase, two enzymes with the capacity to break down hydrogen peroxide. Hydrogen peroxide is a reactive oxygen species and can cause cellular damage through the oxidation of proteins, lipids, and DNA. Catalase catalyzes the decomposition of hydrogen peroxide into less harmful chemicals oxygen and water. 3-Amino-1,2,4-triazole (ATZ) inhibits catalase by binding specifically and covalently to the active center of the enzyme, inactivating it¹⁷⁻¹⁹. ATZ has been used to study the effects of oxidative stress both *in vitro* and *in vivo* through the inhibition of catalase²⁰⁻²⁴. Glutathione peroxidase catalyzes the reduction of hydrogen peroxide through the antioxidant, glutathione, and is an important enzyme that protects the cell against oxidative stress²⁵. Mercaptosuccinic acid (MSA) inhibits glutathione peroxidase by binding to the selenocysteine active site of the enzyme with thiol, inactivating it²⁶. MSA has been used to study the effects of oxidative stress *in vitro* and *in vivo* as well^{20,27,28}.

This novel model of chronic wounds is a powerful model to study because it shares many of the same features observed in human diabetic chronic wounds, including prolonged inflammation from increased OS and natural biofilm formation from skin microbiome. The wounds have impaired dermal-epidermal interaction, abnormal matrix deposition, poor angiogenesis and damaged vasculature. Chronic wounds will develop in both male and female mice, so both sexes can be used to study chronic wounds. Therefore, the chronic wound model can contribute significantly to advance fundamental understanding of how such wounds begin.

Using this chronic wound model can provide answers to fundamental questions about how chronicity is initiated/achieved through contributions from the physiology of impaired wound healing and the microbiome of the host.

PROTOCOL:

All experiments were completed in accordance and compliance with federal regulations and University of California policy and procedures have been approved by the University of California, Riverside IACUC.

1. Animal

1.1. Use diabetic and obese B6.BKS(D)-*Lepr*^{db}/J mice for the chronic wound model. Purchasing options include either heterozygotes for breeding or homozygotes directly for experiments.

1.2. Breed heterozygote males and females to produce offspring. Only a quarter of the litter, statistically, will grow to become diabetic and obese (*db/db*^{-/-}).

1.3. Wean and house *db/db*^{-/-} mice together with their littermates 3 weeks after birth. Separate *db/db*^{-/-} mice from their littermates 5 weeks after birth and house with other *db/db*^{-/-} mice until they are 5-6 months old and can be used for the chronic wound model. During this time, a mature and complex skin microbiome can develop.

Note: The *db/db*^{-/-} mice are easily identified visually from littermates between 3-5 weeks of age. The *db/db*^{-/-} mice are obese, are diabetic, and will be significantly larger and rounder than the wild type and heterozygous littermates. Their abdomen may appear slightly pinker and their hips larger. Increased weight should be confirmed prior to surgery. Also, it is possible to measure high levels of glucose in the blood and genotype the mouse to confirm the mutation in the leptin receptor.

2. Vivarium and Husbandry

2.1. House *db/db*^{-/-} mice in a conventional vivarium (not a barrier/specific pathogen free facility) so that a microflora can be established on the skin of *db/db*^{-/-} mice. To specifically model humans who suffer from chronic wounds, do not take special precautions to prevent exposure to pathogens.

2.2. Protect the cages with micro isolator tops to minimize spread of infection within the vivarium. Change cages twice a week with new bedding and feed the mice with regular vivarium chow. Do not autoclave either the bedding or the food.

2.3. Set room temperature to range between 21 and 24 °C, with minor fluctuations depending on the time of year. The humidity, reflective of the climate and location, will range between 19 and 70%.

3. Requirements for the Development of Chronic Wounds

3.1. Use only male and female mice that are phenotypically obese, diabetic, and at least 5-6 months of age for the development of chronic wounds. The weight of these mice should vary between 40-80 g, averaging around 60 g.

3.2. Do not use mice that are considered obese but weigh less than 50 g.

Note: All mice referred to in this protocol pass the qualifications described here unless otherwise noted.

4. Shaving and Application of Depilatory Lotion

Note: Remove unwanted hair on the dorsum of the mouse before wounding. The following procedure is done on live *db/db^{-/-}* mice that are not under anesthesia the day before surgery. Take precautions to prevent stress and harm to the animal.

4.1. Shaving

4.1.1. Place the mouse on a clean surface and grip the base of the tail of the mouse with the thumb and second finger to secure the position of the mouse. The mouse may jump or make sudden movements; if it does, quickly respond and pull back the clippers to prevent injury.

4.1.2. Shave the hair of the mouse with a hair clipper (**Figures 1A, 1B**). Position the blade parallel to the skin of the mouse and shave the whole back from the neck to the tail, including all around the tail, to allow for a large enough surface area to place a transparent film dressing (see **Table of Materials**). Lightly run the blade against the direction of hair growth for the most efficient cut. (**Figure 1B**).

4.1.2.1. Do not press the blade deeply into the skin, since it may damage the skin by bruising or cutting.

4.2. Application of depilatory lotion

Note: Use depilatory lotion to obtain a very smooth skin so that the transparent dressing can adhere firmly.

4.2.1. Soak the skin of the mouse with water to prevent chemical burns by lightly pressing a drenched paper wipe against the shaved skin with enough pressure to wet the already cut hair down against the skin.

4.2.2. While the skin is wet, lightly rub the skin of the mouse with a small dollop of depilatory lotion (see **Table of Materials**) for 15-20 s (**Figures 1C, 1D**). Spread the lotion completely over wherever the hair has been cut short. Use more lotion if the mouse is larger.

4.2.3. Do not apply lotion on the ears of the mouse, tail, or anywhere near the face. If the lotion gets on the ears or tail, simply wipe with a wet paper wipe until rinsing. If the lotion gets on the face of the mouse, immediately wash the mouse under running tap or deionized water to prevent damage to the eyes, nose, and mouth.

4.2.4. Leave the lotion to react with the hair for an additional 20-45 s after application.

4.2.5. Before rinsing, check the completion of the depilatory reaction by lightly wiping away the lotion from the skin at various locations (**Figure 1E**) with a gloved finger or a thin metal spatula. The reaction is complete if the skin is pink without the presence of black hair. It is best to quickly check that the hair has actually been removed before rinsing than rinsing prematurely and then having to apply lotion again.

4.3. Rinsing

Note: Once the completion of the hair removal reaction has been verified, wash the mouse with running tap or deionized water to remove the depilatory lotion and prevent chemical burns.

4.3.1. Place the mouse on the left gloved hand and press the base of the tail against the palm with the left thumb to prevent the mouse from moving around. Close and straighten the rest of the left fingers to prevent the mouse from biting.

4.3.2. Position the mouse so the nose is up and away from the stream of water and the stream of water can fall behind the head and only on the back. Quickly but gently rub the back of the mouse with the right gloved hand to wash away the lotion.

4.3.3. Once the skin of the mouse is free of the lotion, quickly wipe the mouse with a paper towel to absorb most of the water (**Figure 1F**).

4.4. Care after shaving and hair removal

4.4.1. Check and clean any residual depilatory lotion that may remain on the ears and tail with a wet paper wipe.

4.4.2. Place the mouse back in its individual cage and place the cage on a heating pad (40-45 °C) for about 30 min. The mouse should return to normal behavior, scurry around, and groom itself within a few min.

4.4.3. House each mouse in a separate cage throughout the duration of the experiment. The skin of the mice is no longer protected and can be easily scratched and bitten by other mice.

4.4.4. Since the depilatory lotion can have slight irritating effects that may interfere with or alter the wound healing process, wait 18-24 h before surgery to allow the mouse skin to calm down and the mouse to adjust to the lack of hair on its back.

4.5. Removing Hair from Skin with Dark Pigmentation

Note: Some *db/db*^{-/-} mice will potentially have dark patches in the skin that will have hair growing back faster and stronger than skin without these patches. (**Figure 2A**). These dark areas of the skin appear darker in color due to mid-late anagen stage hair follicles and/or pigment incontinence^{29,30}.

4.5.1. If dark patches are noticed, apply depilatory lotion again, but only in these areas, and repeat step 4.2 and 4.3 (**Figures 2B, 2C**).

Note: The dark patches of skin will grow hair faster throughout the duration of the experiment, so clip the hair short every 3-5 days, when necessary. A patch or two away from the desired location of the wound is acceptable. If the back of the mouse is significantly covered by these dark patches, do not use this mouse for the chronic wound model.

5. Reagent Setup

Note: The development of chronic wounds in *db/db*^{-/-} mice is accomplished by treatment with specific inhibitors for catalase and glutathione peroxidase, 3-amino-1,2,4-triazole (ATZ) and mercaptosuccinic acid (MSA), respectively¹⁶. The following procedure details the dose and administration of the analgesia and inhibitors based on the weight of the mouse.

5.1. Inject the pain reliever Buprenex intraperitoneally at 0.05 mg/kg mouse in sterile PBS. Inject a volume of 120 µL for a 60 g mouse approximately 30 min before surgery. Administer another dose 6 h after surgery. An extra dose may be given as needed.

5.2. Inject ATZ intraperitoneally at 1 g/kg mouse in sterile PBS. Inject a volume of 480 µL for a 60 g mouse approximately 20 min before surgery. Inject half of the volume on the left side of the abdomen and the other half on the right side.

5.3. Deposit MSA topically onto the wound between the transparent dressing and the wound tissue at 150 mg/kg mouse in sterile PBS. Administer a volume of 60 µL for a 60 g mouse within 10 min after surgery.

6. Surgery

Note: The success of the chronic wound model relies on non-sterile conditions. These mice are not germ-free and are housed in a conventional vivarium. The bacteria microbiome that resides in the skin is crucial for the subsequent initiation and development of chronic wounds upon treatment with inhibitors of anti-oxidant enzymes. Therefore, traditional pre-surgical preparation of the site is contra-indicated.

6.1. Treatment and Anesthesia

6.1.1. Administer Buprenex 30 min and ATZ 20 min before surgery, as described in Steps 5.1 and 5.2.

6.1.2. Place the mouse in a small plastic container on top of a warm heating pad as shown in **Figure 3A** for approximately 15-20 min. Providing heat support prior to the procedure protects against potential mortality.

6.1.3. Place a paper wipe or paper towel over the top of the small container to better contain the heat. The mouse should calm down as it warms. **Figure 3B** shows materials necessary for surgery.

6.1.4. Place the mouse in an enclosed container that is hooked up to the isoflurane vaporizer in the chemical hood if using an open system.

6.1.5. In an open system, administer 5% isoflurane for 1.5-2 min at a flow rate of 3-3.5 L/min. Continuously monitor the status of the mouse.

6.1.5.1. Once the mouse is unconscious or no longer moving, place the mouse on a white surgical pad and fit the head with a nose cone that is secured to the vaporizer to allow continuous administration of isoflurane during surgery.

6.1.6. In an open system, administer 2-3% isoflurane at the same flow rate during surgery and adjust to the flow of isoflurane to maintain depth of anesthesia.

6.1.7. Minimize both the concentration and duration of isoflurane administered during surgery. *db/db^{-/-}* mice are very sensitive to anesthesia so it is best to keep isoflurane exposure to a minimum. If the mouse is still responsive after 2 min at 5% isoflurane, secure the nose cone and administer 3-5% isoflurane for 15-30 s before starting surgery.

6.1.7.1. Confirm proper anesthetization before wounding. Depth of anesthesia is confirmed by lack of response to physical stimuli such as strong toe pinch. The length of time of the mouse under inhaled anesthesia is under 5 min so no vet ointment is applied to the eyes.

6.2. Wounding

6.2.1. Spray a paper wipe with 70% ethanol or a clinical ethanol towelette and wipe the back of the mouse to clean the area of the wound site. Clean the surface of the skin so the transparent dressing can adhere firmly, since dust from the bedding, food, or skin can prevent the dressing from sticking properly (**Figure 4A**). Do not wipe excessively, or there will be a risk of killing the bacteria present on the skin.

6.2.2. Determine the position of the wound site. The best place to perform the wound is on the dorsal side of the mouse, centered and away from patches of skin with higher pigmentation.

Note: We have determined by experience that these mice can only withstand the burden of one wound.

6.2.3. Create a wound within 30-45 s using a 7 mm skin biopsy punch, tweezers and surgical scissors. Lightly press the biopsy punch onto the desired wound site and twist the punch around just deep enough to leave a slight impression of the punch (**Figure 4B**). Excise the outlined skin by pulling up the center of the punch with tweezers and cutting along the outline with surgical scissors (**Figures 4C-4E**).

6.2.4. Cover the wound firmly with half of a piece of transparent film dressing (6 cm x 3.5 cm).

6.2.5. Stop administration of 2% isoflurane to the mouse (**Figure 4F**).

7. Post-Surgery Treatment and Recovery

7.1. Administer MSA treatment after surgery is complete and the transparent dressing is applied as described in Step 5.3. Deposit MSA onto the wound area under the dressing.

7.2. Place the mouse back in the small container on the heating pad for 30 min to help with recovery. Once the mouse has warmed up, put the mouse back in its cage. The effect of the isoflurane is temporary, and the mouse should be moving around shortly thereafter.

7.3. Do not leave mice unattended nor return them to the vivarium until the mice have regained sufficient consciousness to maintain sternal recumbency.

Note: The anesthesia of choice when working with these mice is isoflurane precisely due to its quick induction and subsequent emergence from anesthesia.

7.3.1. House mice that have undergone surgery individually to avoid one mouse interfering with the chronic wound of another. As stated above, they do not return to the vivarium until fully recovered.

7.4. Administer the second dose of Buprenex 6 h after surgery.

7.5. Observe the mice carefully for the first 48 h after surgery.

Note: The surgery, coupled with the inhibitors to create the chronic wound, is very stressful on the animal that is already both diabetic and obese. Mice that survive the first couple days after surgery will usually survive the duration of the experiment.

8. Data Collection, Survival Strategies, Handling the Mice After Wounding, and Additional Tips

8.1. Data collection

8.1.1. Take pictures as early as immediately after surgery. Biofilms are observed anywhere between 5-10 days after wounding, and as early as 3 days.

8.1.2. If bacteria are the focus of analysis, roll a sterile swap with light pressure around the wound for 10-15 s. Store the swab, in an appropriate freezer media for culturing or dry without any media for culture-independent sequencing analysis, at -80 °C. Collect extracellular polymeric substances via a sterile metal spatula into a microcentrifuge tube and store at -80 °C before analysis.

8.1.3. Do not administer anesthesia to the mouse during handling for biofilm collection or picture taking. During these procedures, place a piece of food in front of the mouse to calm the mouse down and prevent it from running off. Most mice will climb on top of the food, sit on it, and not move.

8.2. As secondary infections and unintended chronic wounds or ulcers can develop if the mouse is not properly moving or if the dressing is not applied correctly, periodically check the activity of the mouse and the ventral side for sores. Friction between the skin and wet bedding (*db/db*^{-/-} mice are polyuric) can disturb the skin if the cages are not changed frequently.

Note: Fluid buildup under the dressing can cause the adhesive to lose its stickiness and allow the fluid to leak. Dead skin cell, bedding, and fecal matter can then stick onto the skin and harden. These dried patches and aggregates on the skin should be cleared promptly to prevent a secondary infection.

8.3. If the mice stand or sit on their hind legs, move these mice a cage where access to food and water is much lower. If the position of food and water in the cage is high, the mouse may stand or sit on its hind legs to reach it. Most mice will not have a problem eating and drinking if they could do so before surgery, though there is a possibility that some mice might flip over onto their backs. These “flippers” may have great difficulty turning over so they will need assistance and further monitoring.

8.4. Leave the transparent dressing on the skin for up to 20 days if the skin is clear of debris, flakey skin and hair. If any of these occur on the skin, remove the old dressing and apply a new piece.

8.4.1. To take off the dressing, lightly pinch the skin directly behind the head, then pull the dressing away from the head in one smooth motion.

8.4.2. To place a piece of dressing on securely, have the mouse stay as still as possible. It is important that the skin is clean and free from flaky dead skin cells, dust from the bedding, and pieces of food. Place and then press the dressing into the skin around the wound to secure.

8.4.3. If placing a piece of food in front of the mouse does not limit its movements, then place the mouse on top of the wire cage. Once the mouse has gripped a rail on the cage, hold the tail as close to the body as possible and pull with minimal force. As the mouse pulls forward the back will stretch and straighten for easy application; place the dressing securely at this point.

8.4.4. Never re-use an old dressing. Always apply new dressing to the back for the greatest adhesion.

REPRESENTATIVE RESULTS:

Figure 5 depicts an example of a wound without treatment of inhibitors progressing towards wound closure and a wound with treatment of inhibitors progressing towards chronicity. The transparent dressing has been left in place on the chronic wound so that biofilm and fluid accumulation can be seen.

Chronic wound initiation takes place in less than 6 hours and the wound margin is visibly altered from oxidative stress. Histological evidence of this wound margin reveals that the tissue is necrotic and will not participate in wound healing. The biofilm-forming bacteria in the wound can later use this necrotic tissue as a source of nutrients and structural components to produce biofilm. A chronic wound is a wound that remains open, enlarged comparing to the initial wound, contains biofilm (EPS plus pathogenic bacterial found in human chronic wounds) and takes month or years to heal depending on the amount and content of the biofilm that prevents the wound from resolving normally. In the chronic wound model, full chronicity is set to > 20 days after surgery because the wounds not treated with the inhibitors will close by this time (**Figure 5**). Healing typically takes > 60 days and the time depends on the primary pathogenic bacteria present in the wound. Sometimes the mice die early, in particular when more aggressive biofilm-forming bacteria, such as *Pseudomonas*, are predominate in the wound. Thus, a chronic wound is defined as a wound that will not close within 20 days, takes more than 60 days to heal, and has biofilm present on the wound.

Gender differences have been found in various diabetes models, including the *db/db*^{-/-} mouse model^{32,33}. While such differences exist, we have observed gender to not be a significant factor in the development of chronic wounds. Chronic wounds in male and female mice develop to a similar extent, so both sexes can be used to study chronic wounds. Thus, utilizing this model is advantageous since human chronic wounds can be found on both male and female diabetic patients.

Figure Legends:

Figure 1. Shaving and depilatory process. (A) The mouse before shaving. (B) The skin of the mouse is shaved to remove most of the hair on the back. (C) A dollop of depilatory lotion on the tip of a finger. More is used if the mouse is bigger. (D) The back of the mouse is covered with depilatory lotion and left to react. (E) A spatula is used to scrape off some of the lotion to see if the hair has been removed. Bright pink skin without any presence of hair is indicative that the hair removal is complete. (F) The lotion on the back is removed with running water. The skin of the mouse should be slightly pink. This figure has been modified from Kim and Martins-Green³¹.

Figure 2. Removing hair from smaller patches of dark skin. (A) The mouse has already been treated once and the skin is wet again to prevent burns. (B) Depilatory lotion is only applied onto the patch of skin that is dark and has dense hair. (C) The lotion is washed off after the reaction to reveal the dark patch of skin without hair. This figure has been modified from Kim and Martins-Green³¹.

Figure 3. Pre-surgery set up. (A) The mice that are to be wounded are placed in small plastic containers on top of a heating pad. (B) Some of the materials used in surgery are shown. The surgical scissors need to be sharp to ensure that the skin is not crushed when cut. The transparent dressing is cut in half. This figure has been modified from Kim and Martins-Green³¹.

Figure 4. Making the excision wound. (A) After the mouse is under anesthesia, the back of the mouse is wiped with 70% ethanol once. (B) The skin biopsy punch is placed on the back of the mouse and pressed hard enough to leave an impression. The biopsy punch can be rotated to make a shallow incision. (C) The middle of the outlined area is pinched with tweezers and a sharp surgical scissor is used to make the initial incision. (D) The surgical scissors are maneuvered to cut along the outline made by the biopsy punch. (E) A region of skin outlined by the biopsy pump is successfully excised. (F) The transparent dressing is positioned on the back of the mouse and secured. This figure has been modified from Kim and Martins-Green³¹.

Figure 5. Pictures of wounds. (A) The wound on a mouse at successive times after surgery as it progresses into chronicity starting on the day of surgery. Biofilm can be seen as early as day 5 and detected as early as day 3. The wound is fully chronic with strong biofilm on Day 20. (B) Examples of human chronic wounds, specifically diabetic foot ulcers. This figure has been modified from Kim and Martins-Green³¹.

DISCUSSION:

Once chronic wounds are created on the mice, the model can be used to study impaired wound healing processes involved in the initiation of chronicity. The model can also be used to test the efficacy of a wide range of chemicals and drugs that can reverse chronic wound development and impaired healing and lead to wound closure and healing. Different time points after the onset of chronicity can be studied: *e.g.*, days 1-5 after wounding for early onset of chronicity and days 20 and beyond for full strength chronic wounds.

The chronic wound model is also a powerful model to study various aspects of wound healing and complications such as bioburden and cachexia. Bioburden is just one of many facets of chronic wounds that can be studied in this model, as it also affects human chronic wounds. Our procedures and Animal Use Protocol clearly identify symptomology to be monitored, along with a defined monitoring schedule. Animals identified as morbid, based on IACUC approved criteria, are euthanized in order to avoid significant suffering. In addition, the Campus Veterinarian and Animal Health Technician are consulted when certain symptomologies arise and provide supportive guidance in assessment of criteria.

Critical steps within the protocol include housing the *db/db*^{-/-} mice in a conventional vivarium, removing hair with depilatory lotion, and heating before isoflurane administration. The development of chronic wounds in the chronic wound model relies on non-sterile conditions and practices. These mice are not germ-free and do not grow in very clean vivariums. The microflora that resides in the skin is crucial for the subsequent initiation and development of chronic wounds upon treatment with inhibitors for anti-oxidant enzymes. These *db/db*^{-/-} mice must be exposed to an environment that contains bacteria, both commensal and pathogenic. If the skin is cleaned and dis-infected with iodine or other antiseptic methods prior to surgery in order to “sterilize” the skin, the wound may not become chronic. The bacteria in the skin microbiome are necessary for the formation and development of biofilm, and delay healing and wound closure. In the clinic, the presence of biofilm in a wound further complicates the wound healing processes and increases the risk of amputation in humans if the infection in the wound is not controlled.

Removing hair with depilatory lotion is an important step to remove excess hair and allow a smooth and clean surface for the transparent dressing to adhere firmly. The depilatory lotion used in this protocol is a chemical depilatory with added aloe for minimizing burns. This product is minimal in altering the morphology of the skin as well as preserving the skin microbiome. The product listed in the **Table of Materials** is recommended over other hair removing products, including physical and mechanical depilatories (waxes and commercial epilators) that can further burn and tug at the skin and/or kill the skin microbiome. Even though this chemical depilatory is physically gentle, it can still irritate the skin slightly, an effect that could alter the wound healing processes. Thus, it is most effective to wait 18-24 hours before surgery to allow the skin to recover from the procedure and ensure the skin and wound are not affected by it.

These mice are extremely docile and non-responsive to stressors. They are very easy to handle without anesthesia. They are calm enough to place on a palm or on top of a bench without running away. If the base of the tail of the mouse is secured with the thumb and second finger, the mouse will not be able to run away or turn back to bite due to their large belly size. Importantly, in our experience, these mice are extremely sensitive to anesthesia, especially as it relates to loss of homeostasis. Thus, in consultation with our IACUC, it was determined that the better option is to limit anesthetic administration.

Previously, injectable anesthesia such as ketamine and xylazine, were used; however, they proved difficult to use with *db/db*^{-/-} mice. With a total operation time of less than 5 minutes,

the long induction and recovery time was not necessary for the purposes of the experiment. Isoflurane was determined to be the better choice of anesthesia for this procedure due to easy administration and titration, rapid onset and recovery, and adequate anesthetic depth. Also, isoflurane causes minimal cardiac depression and maintains BP very well³⁴. So, a major change in the procedure for the chronic wound model was to use isoflurane as the preferred anesthesia.

The heating before surgery is important for preventing mortality in the 2-3 days following surgery. These mice have significantly lower core body temperature³⁵ and are not able to control their core body temperature effectively due to their genetic manipulation, so an external heating source is provided before the surgery to protect against the additional drop in core body temperature induced by anesthesia. We have assessed the need for a heating pad before, during, and after surgery. It is important to note that *db/db*^{-/-} mice have unusual physiological responses. Empirically, we have found these mice to be most effectively protected with pre- and post-surgical heat support. By substituting isoflurane as the anesthetic, we measured and determined that the core body temperature did not drop significantly during the less than five minutes of surgery. While we have found the pre- and post- surgical heat support to be critical for these mice, we have not found surgical heat support to have an effect. It should be noted that the IACUC Chair provided guidance and monitored our tests related to temperature and anesthetic effects. As we are communicating this method, we find it important to indicate what is necessary for the success of the procedure.

A limitation of utilizing this method to study biofilm development is the fact that the bacteria present on the wound and producing the biofilm are not controlled. If a specific biofilm-forming bacterium is to be studied, this model may be useful if the native microbiome can be abolished before wounding either through iodine or other antiseptic methods. In response to excessive levels of oxidative stress, key pathogenic bacteria in the skin microbiome are stimulated to initiate biofilm formation. In our chronic wounds, biofilm-forming bacteria include, but are not limited to, *Pseudomonas aeruginosa*³⁶⁻³⁹, *Enterobacter cloacae*³⁷⁻³⁹, and various *Staphylococcus*^{40,41} and *Corynebacterium* species⁴¹⁻⁴⁵, all of which can be found in human chronic wounds. Several chronic wound microbiome studies have been conducted on human chronic wounds for the bacteria^{39-41,43-45}, and fungi^{46,47} community, including longitudinal surveys associated with poor healing^{48,49}. Longitudinal studies of this nature can also be followed through with the chronic wound model.

It is important to acknowledge that different results may be obtained due to differences in vivaria conditions, supplies, and equipment, vendors, and source colonies for *db/db*^{-/-} mice. To minimize such differences, provided in the protocol is the exact mice variety and source that is used for the chronic wound experiment. For the husbandry of these mice, the exact bedding and food brands have been provided in the **Table of Materials** to limit variability. In our experiments, we find that high oxidative stress is necessary and sufficient to create chronic wounds in these mice, as long as the mice are housed in a conventional vivarium and exposed to bacteria. Bacteria populations and communities may differ with vivaria; however, as long as

a germ-free facility is not used to house the mice, they should have enough bacteria, both commensal and pathogenic, to reside on the hair and skin.

This method of creating chronic wounds in this protocol is significant to study chronic wounds and impaired wound healing because only oxidative stress levels are significantly altered experimentally. Oxidative stress is required for normal wound healing processes². It is important for timely regulation and a crucial component in the functionality of cells necessary for wound healing⁵. However, when the levels of oxidative stress are not controlled, reactive oxygen species can damage endothelial cells, inhibit keratinocyte functionality, and delay wound closure^{2,5}. Human chronic wounds have high levels of oxidative stress⁵⁰. The mouse model has high blood glucose and already increased levels of oxidative stress due to its morbidity. These characteristics are shared with humans living with diabetes and provide a microenvironment conducive for chronicity after sustaining injury⁵⁰. Humans also host diverse and complex microbiota in many locations on the body, including the skin, so a complex, but natural, microbiome is allowed to develop on the skin of the mouse.

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DISCLOSURES:

The authors declare that they have no competing financial interests.

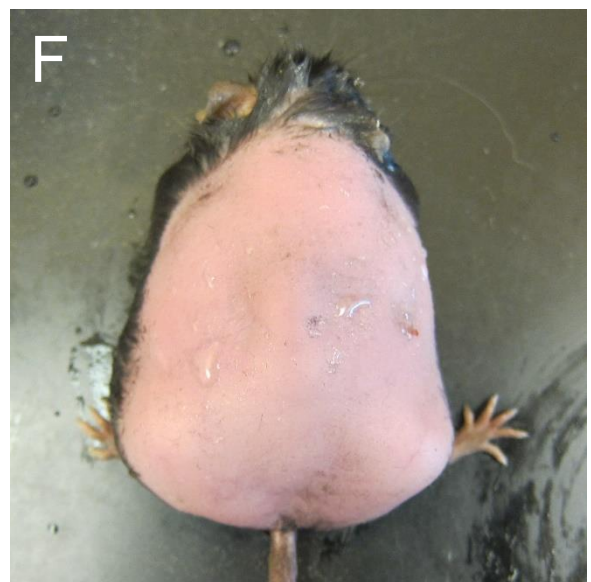
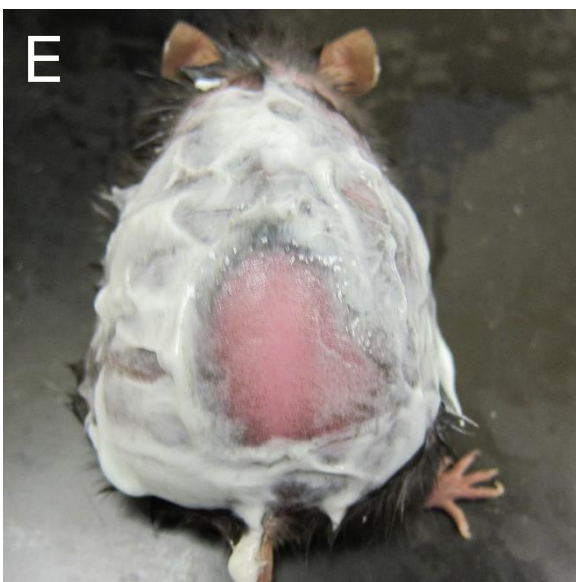
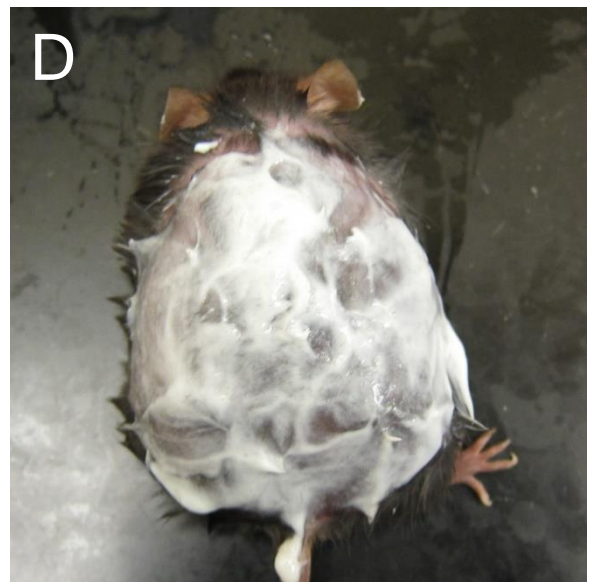
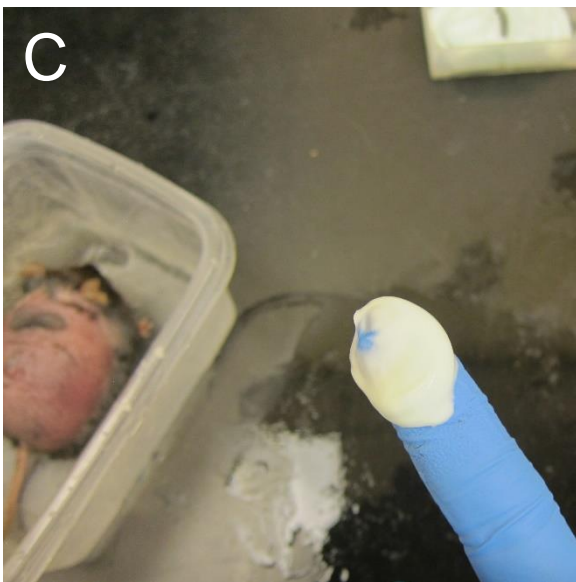
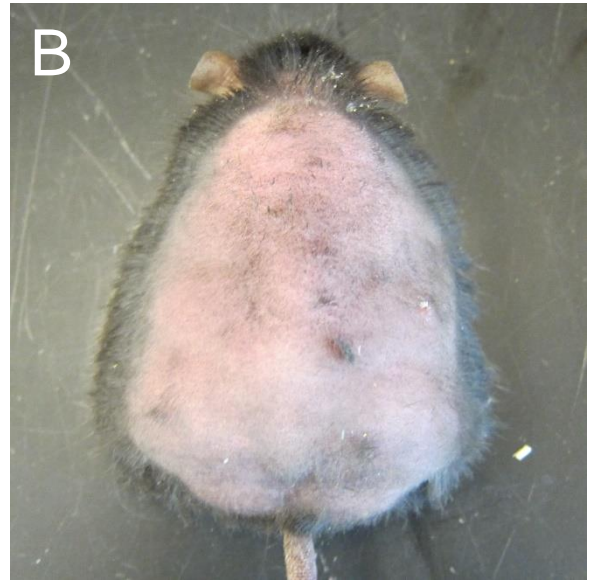
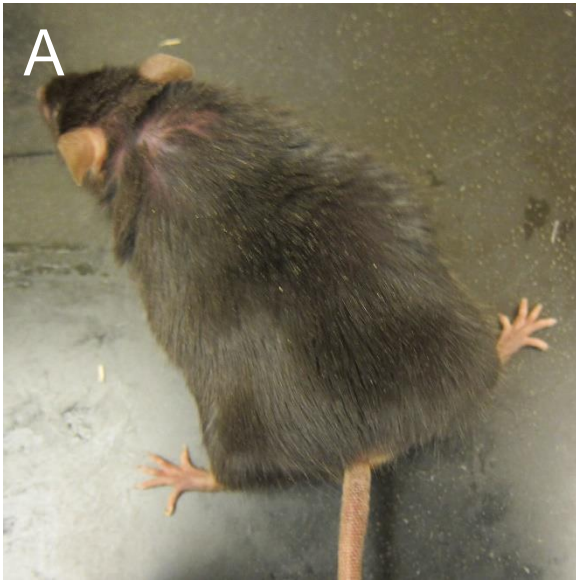
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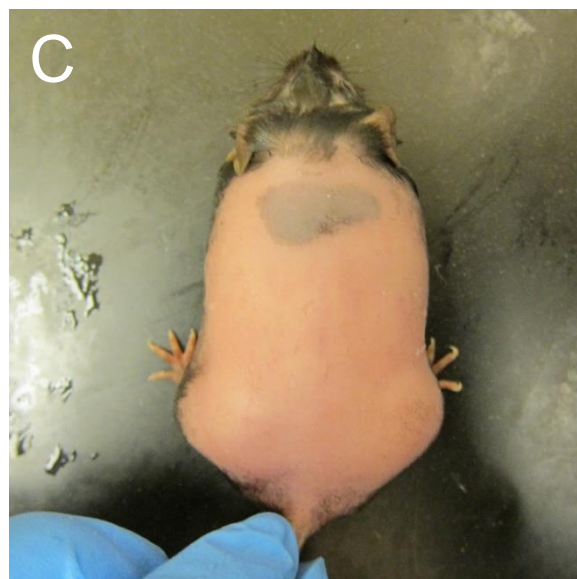
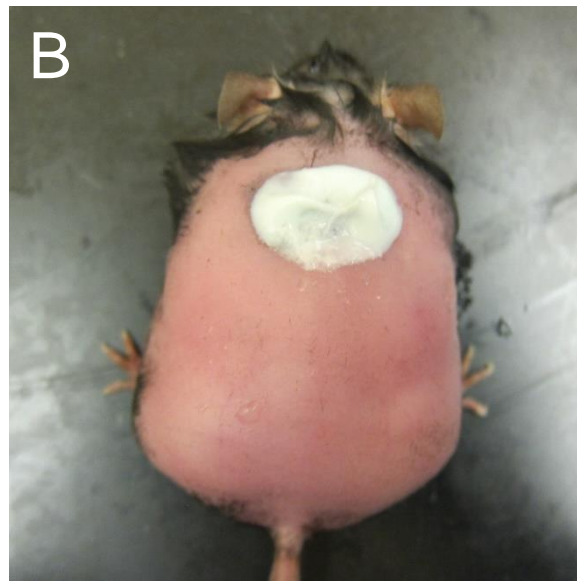
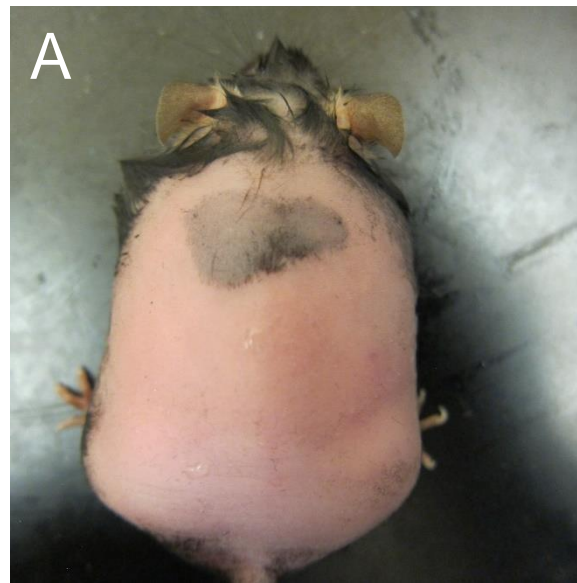
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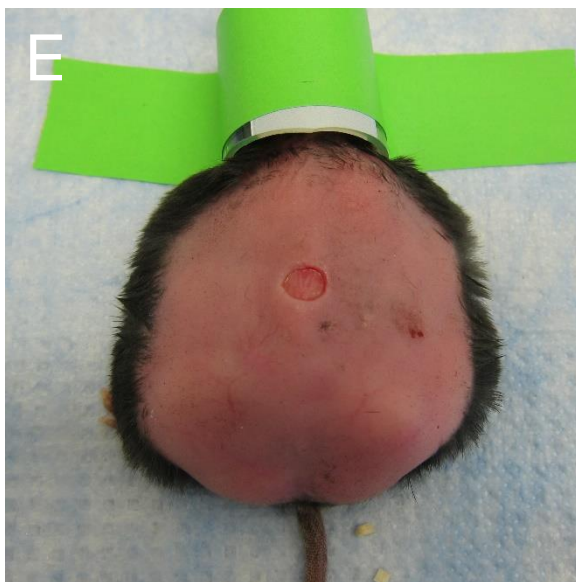
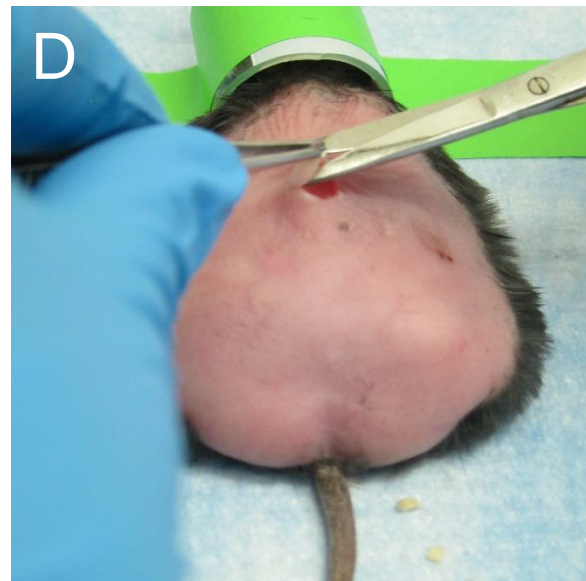
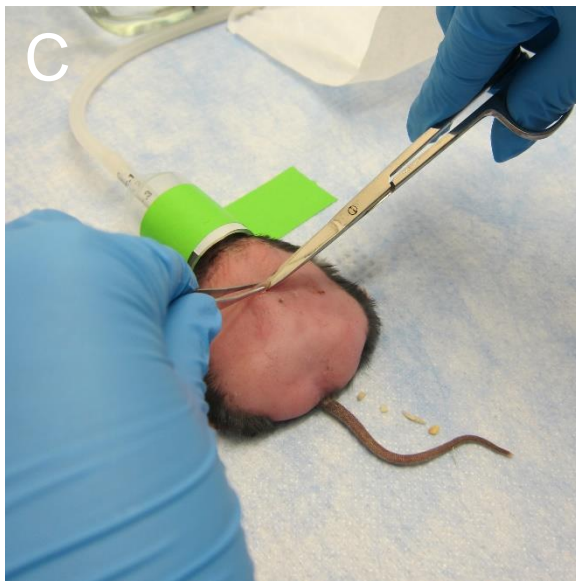
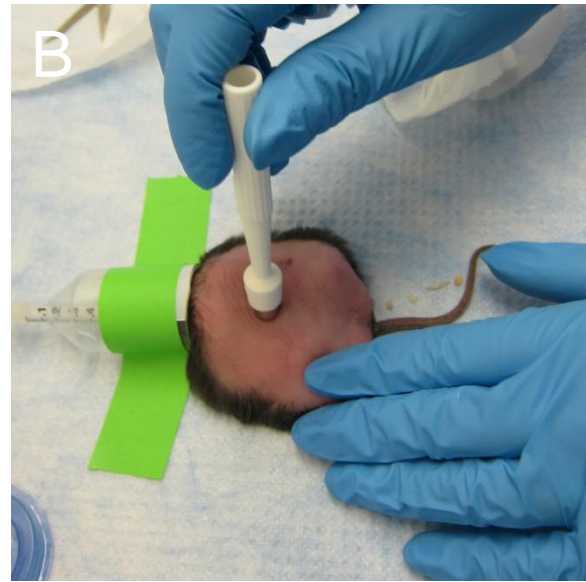
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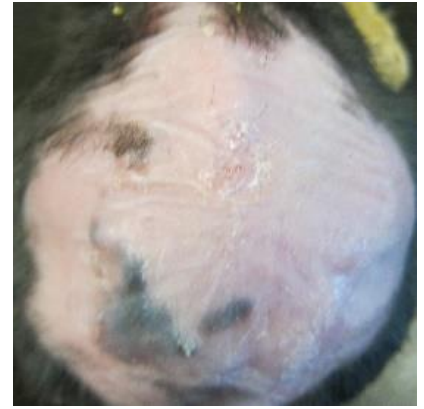






A

Nonchronic Wound



Chronic Wound



Day 0

Day 5

Day 20

B

Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
B6.BKS(D)- <i>Lepr</i> ^{db} /J	The Jackson Laboratory	00697	Homozygotes and heterozygotes available
Nair Hair Remover Lotion with Soothing Aloe and Lanolin	Nair		a chemical depilatory
Buprenex (buprenorphine HCl)	Henry Stein Animal Health	059122	0.3 mg/ml, Class 3
3-Amino-1,2,4-triazole (ATZ)	TCI	A0432	
Mercaptosuccinic acid (MSA)	Aldrich	88460	
Phosphate buffer solution (PBS)			autoclave sterilized
Isoflurane	Henry Schein Animal Health	029405	NDC 11695-6776-2
Oxygen			Tank must be compatible with vaporizing system
Isoflurane vaporizer	JA Baulch & Associates		
Wahl hair clipper	Wahl		Lithium Ion Pro
Acu Punch 7mm skin biopsy punches	Acuderm Inc.	P750	
Tegaderm	3M	Ref: 1624W	Transparent film dressing (6 cm x 7 cm)
Heating pad	Conair		Moist Dry Heating Pad
Insulin syringes	BD	329461	0.35 mm (28G) x 12.7 mm (1/2")
70% ethanol			
Kimwipes			
Tweezers			
Sharp surgical scissors			
Thin metal spatula			
Tubing			
Mouse nose cone			
Gloves			
small plastic containers			



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Article Title:

Protocol to Create Chronic Wounds in Diabetic Mice

Signature:

Manuela Martins-Green

Date:

11/27/17

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Editorial comments:**General:**

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have taken this opportunity to thorough proofread the manuscript and make sure that the manuscript strictly follows the guidelines.

2. Please ensure all margins (including top and bottom) are 1 inch.

All margins of the manuscript have been set to 1 inch.

3. Please ensure all references appear as numbered superscripts (see line 154).

All references now appear as numbered superscripts in the manuscript.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (TM), registered symbols ([®]), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: PJ Murphy Sani Chip, LabDiet, Buprenex, Tegaderm, Wahl, Nair, Kimwipe

The commercial names for the supplies and materials used in the protocol has been limited to the Table of Materials and Reagents. In the protocol, generic terms were used.

Previously published work:

1. JoVE requires that all text be unique. Please ensure that there is no text overlap between this work and your previous work in Nature Protocol Exchange.

Nature Protocol Exchange is a repository, effectively a pre-print server. It is not a problem to re-use content from it and publish with JoVE. See attached email from them

Introduction:

1. Please include more citations in the 1st paragraph of the Introduction

More citations are included in the 1st paragraph.

Protocol:

1. Please add a space between all protocol steps/substeps and align all steps to the left margin, to ensure the length of the protocol can be properly measured.

There is a space between all protocol steps/substeps and all steps are aligned to the left margin to ensure that the length of the protocol can be properly measured.

2. Please ensure that all text in the protocol section is written in the imperative tense.

All the text in the protocol has been written in the imperative tense. Text that follows some of the steps are not written in the imperative tense to provide clarification or explanations.

3. Please split up some of your longer protocol steps so that each step/substep has 2-3 actions and no more than 4 sentences.

The longer sections within the protocol that have more than 2-3 steps have been reorganized to fit the guideline.

4. Being a video based journal, JoVE authors must be very specific when it comes to the humane treatment of animals. Regarding animal treatment in the protocol, please add the following information to the text:

a) Please mention how proper anesthetization is confirmed.

Depth of anesthesia is confirmed by lack of response to physical stimuli such as strong toe pinch [line 276].

b) Please specify the use of vet ointment on eyes to prevent dryness while under anesthesia.

The length of time of the mouse under inhaled anesthesia is under 5 minutes so no vet ointment is applied to the eyes [line 278].

c) For survival strategies, discuss post-surgical treatment of animal, including recovery conditions and treatment for post-surgical pain.

Post-surgical treatment of animals, including recovery conditions and treatment for post-surgical pain have been addressed in section 7 and 8. These section

headers have been reworded to clarify the purpose of these sections [line 295 and 313].

d) Discuss maintenance of sterile conditions during survival surgery.

The success of the chronic wound model relies on non-sterile conditions. These mice are not germ-free and are housed in a conventional vivarium. The bacteria microbiome that resides in the skin is crucial for the subsequent initiation and development of chronic wounds upon treatment with inhibitors of anti-oxidant enzymes. Therefore, traditional pre-surgical preparation of the site is contraindicated. We wipe the skin with only ethanol to ensure presence of bacteria. [line 250 and 431]

e) Please specify that the animal is not left unattended until it has regained sufficient consciousness to maintain sternal recumbency.

The anesthesia of choice when working with these mice is isoflurane precisely due to its quick induction and subsequent emergence from anesthesia. The mice are not left unattended and do not return to the vivarium until they have regained consciousness so that they maintain sternal recumbency. [line 301]

f) Please specify that the animal that has undergone surgery is not returned to the company of other animals until fully recovered.

Mice that have undergone surgery are housed individually to avoid one mouse interfering with the chronic wound of another. As stated above, they do not return to the vivarium until fully recovered. [line 305]

g) Please do not highlight any steps describing anesthesia or euthanasia.

Sections describing anesthesia or euthanasia are not highlighted.

5. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

More details to the protocol steps were added where possible.

Specific Protocol steps:

1. 1.4: Please describe how you will identify db/db^{-/-} mice.

The db/db^{-/-} mice are identified from littermates between 3-5 weeks of age. The db/db^{-/-} mice are visually larger and rounder than the wild type and heterozygous littermates. Their abdomen is slightly pinker and their hips are larger. They are significantly heavier than their heterozygous littermates. Also, we can measure high levels of glucose in the blood and genotype the mouse to confirm the mutation in the leptin receptor. [line 149]

2. 3.1: How will you identify 'phenotypically' obese and diabetic mice?

Obese and diabetic mice are easily identified visually. These mice are significantly larger than their heterozygous littermates and weigh significantly more. However, weight is confirmed prior to surgery. The diabetic mice have high level of circulating glucose. [line 149]

3. 4: This section should be moved to before the surgery.

Section 4 has been moved before the surgery section. [line 233]

4. 6.3.1: How is the wound site determined?

We have determined by experience that these mice can only withstand the burden of one wound. The best place to perform the wound is on the dorsal side of the mouse, centered and away from patches of skin with higher melanin pigmentation. [line 285]

5. 7/8: How long does it typically take for a wound to develop?

Chronic wound initiation takes place less than 6 hours and the wound margin is visibly altered from oxidative stress. Full chronicity is present 20 days after surgery. [line 390]

6. 8.1: How long after surgery are biofilms collected and pictures taken?

Pictures can be taken as early as immediately after surgery. Biofilm collection has been taken as early as 24hrs and then on for at least 20 days. [line 410]

7. 8.1: How are biofilms collected?

Biofilm is composed of bacteria and extracellular polymeric substances. If the bacteria is the focus of analysis, the bacteria can be collected with a sterile swab, rolled with light

pressure around the wound for 10-15 seconds. The swab can then be stored in an appropriate freezer media for culturing or stored dry without any media for culture-independent sequencing analysis at -80 °C. Extracellular polymeric substances can be collected via sterile metal spatula into an Eppendorf tube and stored at -80 °C before analysis. [line 410]

Highlighting:

1. There is a 10 page limit for the Protocol, but there is a 2.75 page limit for filmable content. 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.

Less than 2.75 pages of the protocol was highlighted for filming purposes. It does not include steps of anesthesia.

Representative Results:

1. Please expand on the discussion of the results here; e.g, statistics on how long the wounds take to develop and further similarities with human wounds.

The discussion describing the results has been expanded.

Figures and Figure Legends:

1. Please remove the titles from the uploaded figures.

The titles from the uploaded figures have been removed.

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We have obtained such permission and are including here the email from Nature protocols.

3. Figure 4: The important parts of this figure are unclear-could the actual connection be more prominent, or highlighted in another panel?

Figure 4 was added to show placement of the mouse in a chemical hood when utilizing an isoflurane vaporizer. Upon re-evaluation, such a figure may not be necessary and can be described in the protocol. However, it is up to each university's IACUC to establish the proper protocol used on their respective campuses.

4. Figure 6b: Please ensure you have permission to reuse these, or provide images of your own. Also, please remove the URLs from the figure itself. If you do wind up using the same images, please obtain explicit copyright permission. Please upload this information as to your Editorial Manager account, and cite appropriately, as well.

The URLs have been removed from the pictures and copyright permission will be obtained.

Discussion:

1. Please include more citations in the Discussion.

More citations were included in the discussion and the discussion was expanded.

2. Please include any modifications and troubleshooting of the technique in the Discussion.

Modifications including the use of isoflurane over ketamine/xylazine and the use of heating pads have been described in the discussion section of the manuscript. [line 460]

References:

1. Please ensure references are of consistent format, and remove the extraneous numbers from the beginning

The references are now in a consistent format. The extraneous numbers at the beginning have been removed.

Table of essential supplies:

1. Please ensure that all necessary materials and equipment, especially those explicitly mentioned in the Protocol, are in the Table of Materials.

All necessary materials and equipment that are mentioned in the protocol have been added to the Table of Materials.

Reviewers' comments:

Reviewer #1:

Moderate Concerns:

There is a problem with the reference numbering and no mention of the other paper from Dhall (25313558). It would be excellent to highlight the refinements that appear to have been made to the model in the progression from these 2014 papers to this study.

Previously, injectable anesthesia such as ketamine and xylazine, were used; however, they proved difficult to use with *dbdb*^{-/-} mice. With a total operation time of less than 5 minutes, the long anesthetic induction and recovery time was not necessary for the purposes of the experiment. So a major change in the procedure for the chronic wound model was to use isoflurane as the anesthesia. [line 460]

Minor Concerns:

Line 41 & 105: "fully chronic" is not defined. Presumably it means the wound has features of a chronic wound and not that 20 days is definition of chronic.

Fully chronic is a term used to describe a wound which still open, enlarged comparing to the initial wound, contains biofilm (EPS plus pathogenic bacterial found in human chronic wounds) and takes > 60 days to heal depending on the amount and content of the biofilm that prevents the wound from resolving normally. [line 390]

Line 166: Shaving and Nairing the mice, then rinsing under running water, will unavoidably cause significant stress to the mouse. It could increase the chance of injury to the mouse or person performing the procedure. Any comments/feedback from IACUC that would be useful from an animal welfare aspect. Why not anesthetize the animal? Maybe there is a reason, but it should be addressed.

These mice are extremely docile and non-responsive to stressors. They are very easy to handle without anesthesia. They are calm enough to place on a palm or on top of a bench without worry they would run away. If the base of the tail of the mouse is secured with the thumb and second finger, the mouse will not be able to run away or turn back to bite due to their large belly size. **Importantly**, in our experience, these mice are extremely sensitive to anesthesia, especially as relates to loss of homeostasis. Thus, in consultation with our IACUC, it was determined the better option is to limit anesthetic administration to surgery. In addition, we wait between 18-24 hours after the nairing to allow the skin to recover from the procedure. [line 453]

Line 323 and Fig6: DFU have a hyperkeratotic rim that becomes macerated and give the "white halo". While these mice have a halo, it appears due to the exudate and biofilm and not the same hyperkeratosis and maceration that DFUs have.

We have verified histologically that the white halo that surrounds the tissue is dead tissue. The inhibitor of glutathione peroxidase, causes local oxidative stress levels in the tissue to be very high. Exudate and biofilm are present but reside above the wound and have a yellowish milky consistency. [line 390]

Figure 2 and Section 5.6 (line 222): The dark spots are actually due to the stage of the hair cycle that a particular patch of skin is in. While there is some pigment incontinence that leads to the appearance of skin pigmentation, it is primarily the mid-late anagen hair follicles that account for the color. The fact that these areas are in anagen (growth phase of hair) accounts for the color and the more rapid regrowth of hair in the area.

The “dark spots” are reworded for more specificity. The dark spots are “areas of the skin in mid-late anagen stage hair follicles that can lead the skin to appear more darker in color” [line 224].

Reviewer #2:

Manuscript Summary:

This manuscript described methods used to create chronic wounds in diabetic mice using inhibitors of antioxidant enzymes to increase oxidative stress levels in the wounds. This could be an important model, as currently available "chronic wound" mouse models usually exhibit some healing delays but do not accurately reflect the chronic wounds experienced by patients. Overall, the manuscript contains the details needed for other scientists to replicate the model, but there are several concerns/recommendations that the authors could address to improve the manuscript.

Major Concerns:

It is recommended that the authors provide a more detailed discussion (with more references) on the existing evidence in the literature that supports a role for oxidative stress in chronic diabetic wounds in humans. This would provide a stronger rationale that the model described here accurately reflects conditions associated with human chronic wounds.

The discussion has been expanded to include more the importance of oxidative stress in chronic wounds, especially in combination with humans with diabetes.

Minor Concerns:

1) In multiple places, the authors suggest that studies on chronic wound development cannot be done in humans (abstract, page 4). While it is difficult, these studies can and are being done. It is recommended that the wording be changes in these sections - the authors can highlight how difficult these studies are to perform in humans and how

animal models would be beneficial, but to state that the human studies cannot be done may not be entirely true.

The word choice in the abstract and page 4 has been modified and clarified to state that chronic wounds can be studied in humans but are difficult. This is when the use of an animal model is beneficial, especially during the earlier time points when chronicity is established. [line 33 and 93]

2) There are several recommended changes on page 5: line 133 "can development" should be corrected; line ~148 please specify the blood glucose range for mice used (to be considered diabetic); line 164 "inject a volume..." is misleading, as it appears the drug is being delivered topically. [

"Can development" in line 133 has been corrected to "can develop" -line 148]. The blood glucose range of the mice has been provided for clarity [line 103]. The phrase "inject a volume" has been modified to prevent confusion, however it is true that MSA should be applied topically. [line 244]

3) Please specify in the protocol what the recovery time is between using nair to remove the hair and the time of surgery/wounding.

The time of recovery between the use of nair and surgery is 18-24 hours. [line 220 and 449]

4) MSA treatment should be included in the wounding protocol on page 8.

The MSA treatment is explained in the wound protocol in section 6 and is again mentioned later for timing of injection. [line 244 and 296]

5) It is unclear whether the authors have received the appropriate permissions to reprint the images of human wounds in Figure 6.

The permissions of the images of human wounds in figure 5 will be obtained.

6) Table 1 would be much easier to read in gridlines were included, all text was consistently aligned in each column/cell, and the table was fit to 1 page so that the last column is not on a separate page.

Gridlines were added to the table and the contents of each cell aligned for easier viewing. The table should now fit in 1 page.

Reviewer #3:**Manuscript Summary:**

The authors describe a cutaneous chronic wound healing model in mouse with relevance to human disease i.e., diabetic wound healing.

Major Concerns:

*Line 106-107 states the biofilm persists "until wound closure or death by bioburden and cachexia..." What percentage of mice experience closure and therefore resolution of the chronic wound? What is different about the mice that experience closure? Ethical impact of letting mice die from the wounds due to bioburden and cachexia should be clarified. Shouldn't mice be euthanized rather than death as the endpoint?

We realize that wording in line 106-107 is not clear and may raise ethical questions. The chronic wound model is a powerful model to study various aspects of wound healing and complications such as bioburden and cachexia. Bioburden is just one of many facets of chronic wounds that can be studied in this model as it also affects human chronic wounds. However, our procedures and Animal Use Protocol clearly identify symptomology to be monitored, along with a defined monitoring schedule. Animals identified as morbid, based on IACUC approved criteria, are euthanized in order to avoid significant suffering. In addition, the Campus Veterinarian and Animal Health Technician are consulted when certain symptomology arise, and provide supportive guidance in assessment of criteria. [line 423]

*Inhibitors of catalase and GPx: Line 153 states ATZ and MSA - these chemicals need to be spelled out in full and their respective mechanism of action (and hence, specificity for inhibiting catalase and GPx) clearly explained in a dedicated item under 4.1 Reagent Setup. Were these treatments previously confirmed to increase oxidative stress in vivo?

The full name of ATZ and MSA have been provided and their mode of action has been briefly described under their respective sections. These reagents have been previously confirmed to increase oxidative stress both in vitro and in vivo. [line 114]

*Lines 163-165 - please clarify how the MSA is topically applied and if it is injected into the wound bed or not. Further, clarify timing of MSA administration during the protocol described in Section 6 (starting line 231).

After the full-thickness wound is created, a tegaderm is applied. The MSA is administered topically, under the tegaderm and directed on top of the wound bed. MSA is given after surgery is complete and the tegaderm is applied. [line 293]

*Lines 166-230 _ why is the hair removal not performed under anesthesia? Wouldn't this be less stressful for the animals and eliminate risk of cutting the skin or getting Nair on ears and tail etc? The procedure could also be performed mostly on a heat pad and mice could proceed direct to surgery. Similarly, Lines 259-278 - the mouse could remain on a heat pad while the wound is introduced as a further measure to prevent potential hypothermia.

These mice are extremely docile and non-responsive to stressors. They are very easy to handle without anesthesia. They are calm enough to place on a palm or on top of a bench without worry they would run away. If the base of the tail of the mouse is secured with the thumb and second finger, the mouse will not be able to run away or turn back to bite due to their large figure. **Importantly**, in our experience, these mice are extremely sensitive to anesthesia, especially as relates to loss of homeostasis. Thus, in consultation with our IACUC, it was determined the better option is to limit anesthetic administration. [line 453]

The Nair used to remove the hair can irritate the skin slightly, an effect that could alter the wound healing processes. We have found it most effective to wait 18-24 hours to ensure the skin and wound are not affected by it.

We have assessed the need for a heating pad before, during and after surgery. It is important to note that db/db^{-/-} mice have unusual physiological responses. Empirically, we have found these mice to be most effectively protected with pre- and post-surgical heat support. By substituting isoflurane as the anesthetic, we measured and determined that the core body temperature did not drop significantly during the less than five minutes of surgery. While we have found the pre- and post- surgical heat support to be critical for these mice, we have not found surgical heat support to have an effect. It should be noted that the IACUC Chair provided guidance and monitored our tests related to temperature and anesthetic effects. As we are communicating this method, we find it important to indicate what is necessary to success of the procedure. [line 467]

*What is the frequency of Tegaderm removal and re-application?

The Tegaderm can stay on the skin effectively for up to 20 days if the skin is clear of debris, flakey skin and hair. If any of these occur on the skin, then a new piece of tegaderm must be applied. [line 331]

*Line 323 - presumably "human diabetic foot ulcers" is meant. Please amend and elaborate on significance/physiological changes of the "white halo".

Line 323 has been corrected to “human diabetic foot ulcers” for clarity. The significance of the “white halo” has also been amended and provided. [line 390]

*Lines 398-400 - the authors clearly highlight the limitation with this model i.e., the bacteria producing the biofilm are not controlled. It could be further highlighted that different results may be obtained between vivaria and source colonies for db/db mice.

Yes, different results may be obtained between vivaria and source colonies for db/db^{-/-} mice. However, we have provided the exact mice variety that is used for the chronic wound experiment. For the husbandry of these mice, the exact bedding and food brands have been provided in the table of materials to limit variability. In our experiments, we find that high oxidative stress is necessary and sufficient to create chronic wounds in these mice. Bacteria populations and communities may differ with vivaria; however, as long as a germ-free facility or a clean vivarium is not used to house the mice, they should have enough bacteria, both commensal and pathogenic, to reside on the hair and skin. [line 493]

Minor Concerns:

*The authors could highlight in introduction that older male and female diabetic mice are used, which is a strength of the model. Did males and females develop a similar extent of chronicity?

Gender differences have been found in various diabetes models, including the db/db^{-/-} mouse model. While such differences exist, we have observed gender not to be a significant factor in the development of chronic wounds. Chronic wounds in male and female mice develop to a similar extent, so both sexes can be used to study chronic wounds. Thus, utilizing this model is advantageous since chronic wounds can be found on both male and female human diabetic patients. [line 404]

*Please clarify inconsistency: Line 32-33 states "... cost ~\$25B/year in US alone" but Line 87-88 states "... cost ~\$40 billion per year in the US alone"

The inconsistency in line 32-33 and line 87-88 have been corrected to “\$40 billion”. [line 32 and 86]

*Line 133 "development" should read "developed"

“Development” in line 133 is changed to “develop” in section 1.3 [line 148]

*Line 143 states "70-75 °C". Presumably authors mean 70-75 Fahrenheit. Please convert 70-75 to degrees Celsius equivalent.

The degrees in line 143 is now in Celsius, "21-24 °C" in section 2.3. [line 162]

*Line 146 states "fully diabetic". How this is confirmed? Please state method in text.

Fully chronic is a term used to describe a wound that will not close in 20-30 days. Healing typically takes > 60 days and the time depends on the primary pathogenic bacteria present in the wound. Sometimes the mice die early in particular when more aggressive biofilm forming bacteria, such as *Pseudomonas*, are predominate in the wound. [line 390]

*References (starting Line 413) all have an incorrect format, with random numbers preceding the first author surname. Also, the Dhall et al, 2014 reference is not correctly formatted within the protocol text (Line 154)

"Dhall et al. 2014" in line 154 has been corrected to a superscript. All other references have also been corrected to fit guidelines.



Jane Kim <jkim193@ucr.edu>

Copyright permission for a protocol

Dekker, Bronwen <B.Dekker@nature.com>

Mon, Feb 26, 2018 at 3:08 AM

To: "Exchange, Protocol" <Protocol.Exchange@springernature.com>, "jkim193@ucr.edu" <jkim193@ucr.edu>

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It might be helpful to tell the editor that the Protocol Exchange is effectively a pre-print server. And that it is not a problem to re-use content from it.

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