Protocol to Create Chronic Wounds in Diabetic Mice

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MATERIALS AND ANIMALS

Animal, Vivarium and Husbandry

The diabetic and obese mice used for the chronic wound are B6.BKS(D)-*Lepr*^{db}/J mice obtained from The Jackson Laboratory (https://www.jax.org/strain/000697). Heterozygote males and females are bred to produce offspring; only a quarter of the litter grows to become diabetic and obese (*db*/*db*$^{-/-}$). At 3 weeks after birth, the *db*/*db*$^{-/-}$ mice and their littermates are weaned and kept together for 2 weeks for the *db*/*db*$^{-/-}$ mice to become significantly larger than their litter mates. At 5 weeks after birth, the *db*/*db*$^{-/-}$ mice are separated from their littermates and housed with other db/db$^{-/-}$ mice until they are 6 months old and can be used for the chronic wound model.

The *db*/*db*$^{-/-}$ mice are housed in a conventional vivarium, therefore the mice are exposed to the normal microbial world. Much like with humans who suffer from chronic wounds, no special precautions are taken to prevent the exposure to pathogens. Cages are changed twice a week with new PJ Murphy Sani Chip bedding and the mice are fed Labdiet Rodent Chow (pellets). Neither the bedding or food are autoclaved. The cages are protected with micro isolator tops to minimize spread of infection. Room temperature ranges between 70-75°C, with minor fluctuations depending on the time of year. Humidity, reflective of the climate and location, ranges between 19-70%.

Requirements for the Development of Chronic Wounds

Only mice, both male and female, that are phenotypically obese and at least 6 months of age are used for the chronic wound model. The weight of these mice varies between 40-80g but on averages they weigh 60g. Mice that are considered obese but weigh less than 50 g are not used for the chronic wound model because they do not survive well the chronic wound burden. All mice referred to in this protocol pass the qualifications described here unless otherwise noted.

Reagents and Analgesia

- Nair Hair Removal Lotion with Soothing Aloe and Lanolin
- Buprenex (buprenorphine)
- 3-amino-1,2,4-triazole (ATZ)
- Mercaptosuccinic acid (MSA)
- 70% ethanol
- Sterile phosphate buffer solution (PBS)
- Isoflurane
- Oxygen

Equipment

- Isoflurane vaporizer
- Wahl hair clipper
- Acuderm Inc. (Acu Punch 7mm skin biopsy punches)
- Kimwipes
- Tweezers
- Sharp surgical scissors

- 3M Tegaderm Film 6 cm x 7 cm Ref: 1624W
- Heating pad
- Insulin syringes
- Thin metal spatula
- Tubing
- Mouse nose cone
- Gloves
- Small plastic containers

Reagent Setup

The development of chronic wounds on the back of *db*/*db*$^{-/-}$ mice is accomplished by treatment with specific inhibitors for catalase (ATZ) and glutathione peroxidase (MSA) (Dhall et al., 2014). The following procedure details the dose and administration of the analgesia and inhibitors based on the weight of the mouse.

1. Buprenex is a pain reliever and is injected intraperitoneally at 0.05mg Buprenex/kg mouse in sterile PBS. The volume injected for a 60g mouse is 120μl approximately 30 mins before surgery. Another dose is given 6 hours after surgery. An extra dose may be given if the mouse is in pain.
2. ATZ is injected intraperitoneally at 1g ATZ/kg mouse in sterile PBS. The volume injected for a 60g mouse is 480μl approximately 20 minutes before surgery. Half of the volume is injected on the left side of the abdomen and the other half on the right side.
3. MSA is administered topically onto the wound between the Tegaderm and the wound tissue at 150mg MSA/kg mouse in sterile PBS. The volume injected for a 60g mouse is 60μl within 10 minutes after surgery.

PROCEDURE

Shaving and Nairing

Before the skin of the mouse can be wounded, the hair must be removed. The following procedure is done on live *db*/*db*$^{-/-}$ mice; this procedure is not performed under anesthesia. All precautions are taken to avoid getting bitten and prevent stress and harm to the animal.

1. The hair of the mouse is shaved with a Wahl clipper (**Figure 1A,B**). Position the blade parallel to the skin of the mouse and shave the whole back from the neck to the very bottom all around the tail to allow for a large enough surface area to place the Tegaderm (**Figure 1B**). Run the blade against the direction of hair growth for the most efficient cut. Pressing the blade too deeply into the skin can damage the skin by bruising or cutting. It is best to hold the mouse by the base of its tail because there is a possibility that the mouse will jump into the clipper and hurt itself. It is thus important to quickly respond and pull back the clippers so the skin is not bruised or cut.
2. At this stage there is still a considerable amount of hair, therefore we need to use Nair to remove the remaining hair and obtain a very smooth skin for the Tegaderm to adhere firmly. The skin of the mouse is soaked with water to prevent chemical burns by the Nair. A drenched Kimwipe is pressed against the shaved skin with enough pressure to lay the remaining hair down against the skin.

3. The skin of the mouse is "Naired" by lightly rubbing a small dollop of Nair onto the back of the mouse for 15-20 seconds (**Figure 1C,D**). It is spread completely over wherever the hair has been cut short. If the mouse is larger, more Nair needs to be used. The Nair should not be applied on the ears of the mouse, tail, or anywhere near the face. If the Nair gets on the ears or tail, a simple wipe with a wet Kimwipe is sufficient until rinsing. If the Nair gets on the face of the mouse, the animal should be immediately bathed under running tap or deionized water to prevent any damage on the eyes, nose, and mouth.
4. The Nair is left to react with the hair for an additional 20-45 seconds after application. The completion of the Nair reaction can be checked by lightly wiping away the Nair from the skin at various locations (**Figure 1E**) with a gloved finger or a thin metal spatula. The Nair reaction is complete if the skin is pink without the presence of black hair. The hairless skin should not be exposed to Nair again. It is best to quickly check that the hair has actually been removed before rinsing than rinsing prematurely and then having to apply Nair again.
5. Once it is verified that the hair has been removed, the mouse is washed with running tap or deionized water to prevent chemical burns. 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 running away. Close and straighten the rest of the left fingers to prevent the mouse from biting. Position the mouse so the nose is up and the stream of water is falling behind the head and only on the back. The mouse will be stressed under running water so quickly but gently rub the back of the mouse with the right gloved hand to wash away the Nair.
6. Once the skin of the mouse is free of the Nair, quickly wipe the mouse with a paper towel to absorb most of the water (**Figure 1F**). The mouse is put on a heating pad (40-45˚C) for about 30 minutes. The mouse should return to normal behavior, scurry around, and groom itself within a few minutes. Be sure to check and clean any residual Nair that may remain on the ears and tail.
7. After shaving and Nairing, the mice need to be housed in separate cages 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.

Removing Hair from Dark Patches on the Skin

Some *db*/*db*$^{-/-}$ mice will have dark patches in the skin of the back have higher density of hair follicles (**Figure 2A**) and the hair will grow back faster and stronger. Nair can be applied again but only in these areas (**Figure 2B,C**). If the back of the mouse is significantly covered by these dark patches, it is recommended that the mouse not be used. A patch or two away from the center of back is acceptable. The dark patches of skin will grow hair faster throughout the duration of the experiment, so clipping the hair short every 5 days is recommended.

Surgery

The surgical procedures described here were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California, Riverside.

1. Buprenex and ATZ are administered 30 and 20 minutes before surgery, respectively. The mouse is then placed in a small plastic box on top of a warm heating pad as shown in **Figure 3A.** A Kimwipe or paper towel can be placed 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.

2. The mouse is then placed in an enclosed container that is hooked up to the isoflurane vaporizer in the chemical hood (**Figure 4**). 5% isoflurane is administered for 1.5-2 minutes at a flow rate of 3.5 L/min. Once the mouse is unconscious or no longer moving, it is placed on a white surgical pad and the head is fitted with a nose cone that is secured to allow continuous administration of isoflurane, this time at 2% isoflurane for the duration of the surgery. It should be noted that the concentration of isoflurane should not be increased; *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 minutes at 5% isoflurane, secure the nose cone and administer 2% isoflurane for 15-30 seconds before starting surgery.
3. A Kimwipe sprayed with 70% ethanol or a clinical ethanol towelette is used to wipe the back to clean the area of the wound site. This also guarantees a clean surface for the Tegaderm to stick well because dust from the bedding, food, or skin can prevent the Tegaderm from sticking properly (**Figure 5A**). A one-time wipe is sufficient. Do not over wipe or you run the risk of killing the bacteria present on the skin.
4. A wound is created within 30-45 seconds using a 7mm skin biopsy punch, tweezers and surgical scissors. The punch is lightly pressed onto the wound site and twisted just deep enough to leave an impression **(Figure 5B)**. The outlined skin is then excised by pulling up the center of the punch with tweezers and cutting along the outline with surgical scissors **(Figure 5 C,D,E)**.
5. At this time, pictures can be taken and the tissue excised can be either fixed and embedded or frozen for further analysis.
6. The wound is covered very well with half of a Tegaderm and the mouse is taken off the isoflurane (**Figure 5F**).
7. The mouse is then placed back in the small container on the heating pad for 30 minutes to help with recovery. These mice have a "normal" body temperature of 35°C on average. After surgery, the temperature drops about 2°C which results in hypothermia. 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.
8. The mouse is administered the second dose of Buprenex 6 hours after surgery.

Post-Surgery and Care

1. The mice after surgery must be observed carefully for the first 48 hours. The surgery, coupled with the inhibitors to create the chronic wound, are very stressful on an 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 although the burden of bacteria and biofilm or "secondary infections" can kill the mouse at a later time. One symptom that usually precedes death is hypothermia. If the mouse is lethargic and inactive, then it should be checked by placing the mouse on a gloved palm. If the mouse feels cold to the touch, it must be placed on the heating pad with easy access to food and water.
2. Secondary infections and unintended chronic wounds or ulcers can develop if the mouse is not properly moving or if the Tegaderm is not applied correctly. Be sure to 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. Fluid buildup under the Tegaderm 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.

Handling the Mice After Wounding and Additional Tips

1. Anesthesia is not administered to the mouse during handling for biofilm collection or picture taking; placing a piece of food in front of the mouse will calm the animal down and prevent it from running off. Most mice will climb on top of the food, sit on it, and not move.
2. 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 it 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. If this is the case, the mice may need to be moved to a cage where access to food and water is much lower.
3. To place a Tegaderm on securely, the mouse should stay as still as possible. The Tegaderm is placed and then pressed into the skin around the wound to secure. It is important that the skin is clean and free from flaky dead skin cells, dust from the bedding, and pieces of food. If placing a piece of food in front of the mouse does not restrict 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. The mouse will pull forward to get away, stretching the back straight. The Tegaderm can then be applied securely. To take off the Tegaderm, the skin directly behind the head is pinched and the Tegaderm is pulled away from the head in one slow motion. Always apply a new Tegaderm to the back for the greatest adhesion.

Outcomes

Figure 6 depicts an example of a wound and how it progresses to chronicity. The Tegaderm has been left in place so that the biofilm and fluid accumulation can be seen. In this figure we also show examples of diabetic foot ulcers showing the white halo around the wound much as in the mouse wounds.

FIGURE LEGENDS

Figure 1. "Shaving and Nairing" 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 Nair on the tip of a finger. More is used if the mouse is bigger. (**D**) The back of the mouse is covered with Nair and left to react. (**E**) A spatula is used to scrape off some of the Nair to see if the hair has been removed. The bright pink skin without any presence of hair is indicative that the hair removal is complete. (**F**) The Nair on the back is removed with running water. The skin of the mouse should be slightly pink.

Figure 2. "Nairing" smaller patches of dark skin: (**A**) The mouse has already been "Naired" once and the skin is wet again to prevent burns. (**B**) The Nair is only applied onto the patch of

skin that is dark and has dense hair. (**C**) The Nair is washed off after the reaction to reveal the dark patch of skin without hair.

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 showcased. The surgical scissors need to be sharp to ensure that the skin is not crushed when cut. The Tegaderm is cut in half.

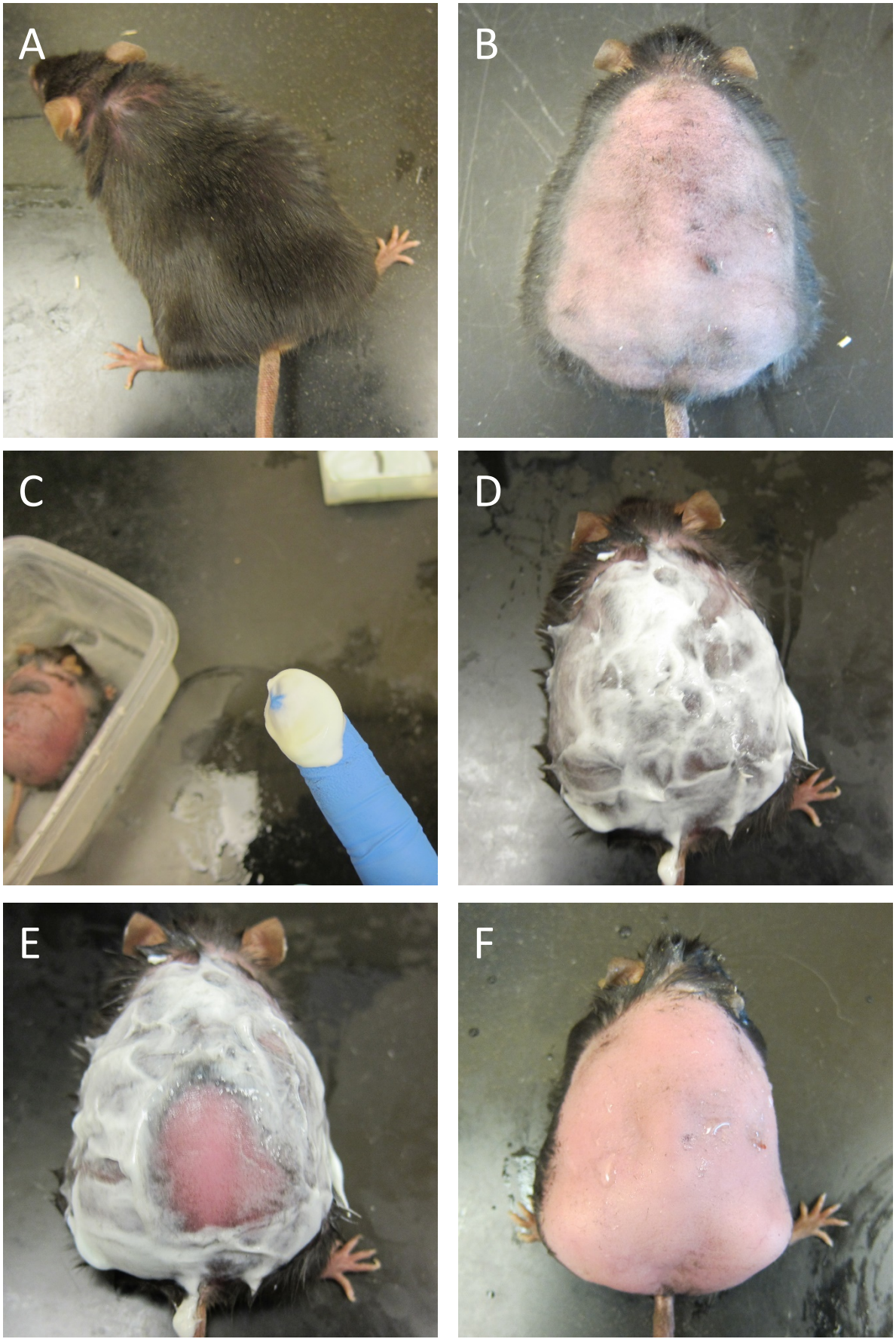
Figure 4. The isoflurane vaporizer: The vaporizer is positioned outside the hood and a tube is used to connect the vaporizer with the plastic container holding a mouse to be wounded.

Figure 5. 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 is 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 Tegaderm is positioned on the back of the mouse and secured.

Figure 6. 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. The wound is fully chronic with strong biofilm on Day 20. (**B**) Humans wounds for comparison.

REFERENCE

Dhall, S., Do, D., Garcia, M., Kim, J., Mirebrahim, S., Lyubovitsky, J., Lonardi, S., Nothnagel, E., Schiller, N. & Martins-Green, M. Generating and Reversing Chronic Wounds in Diabetic Mice by Manipulating Wound Redox Parameters. *Journal of Diabetes Research* 2014, 1-18 (2014).



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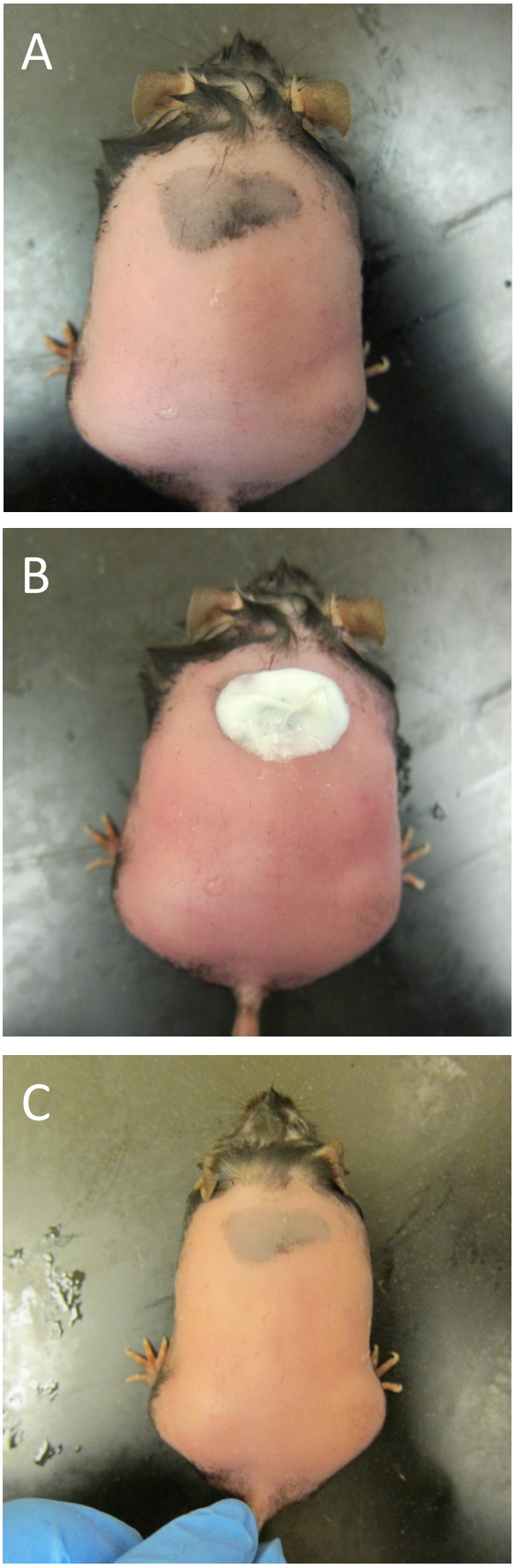

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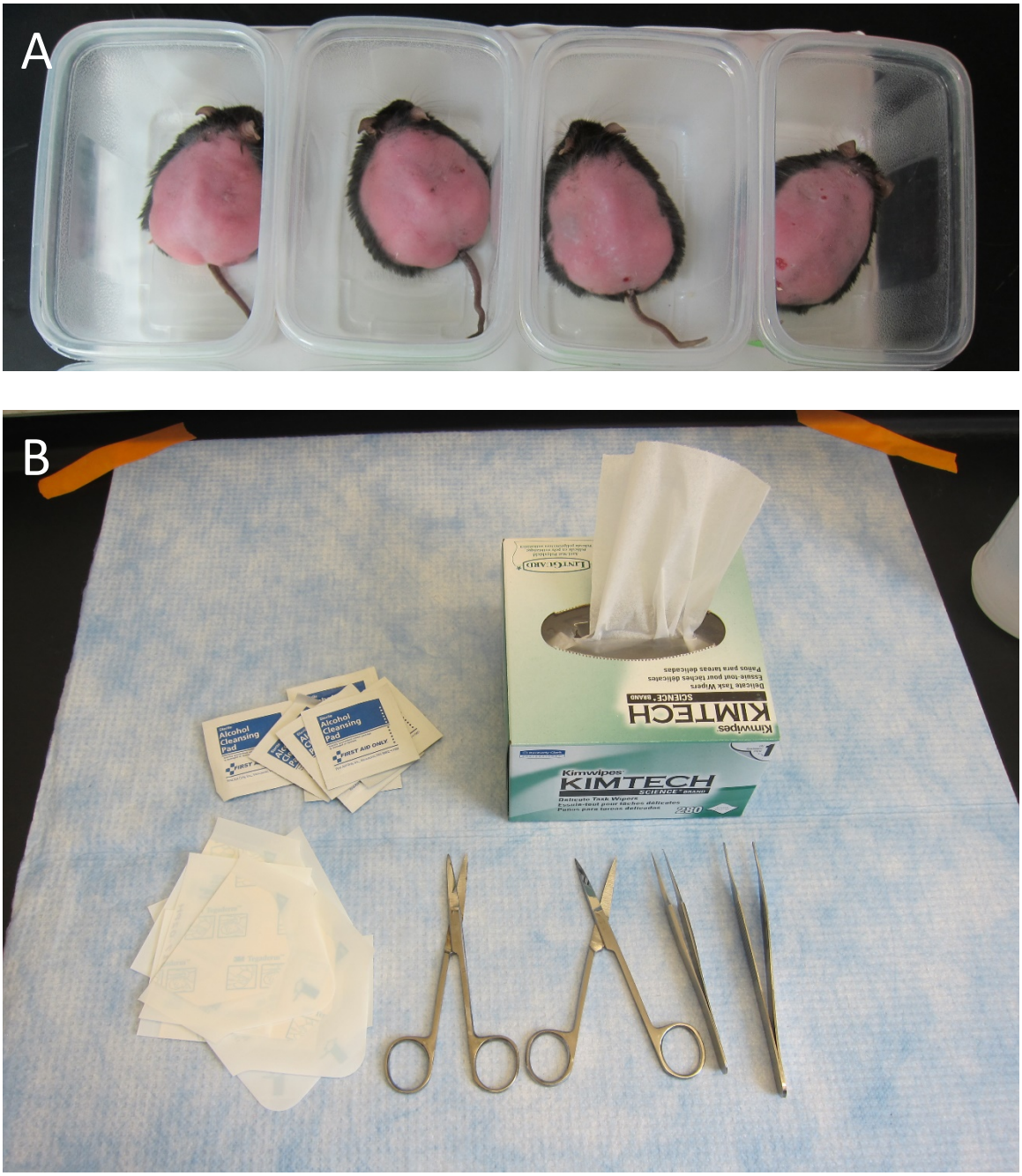
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Kimwipes
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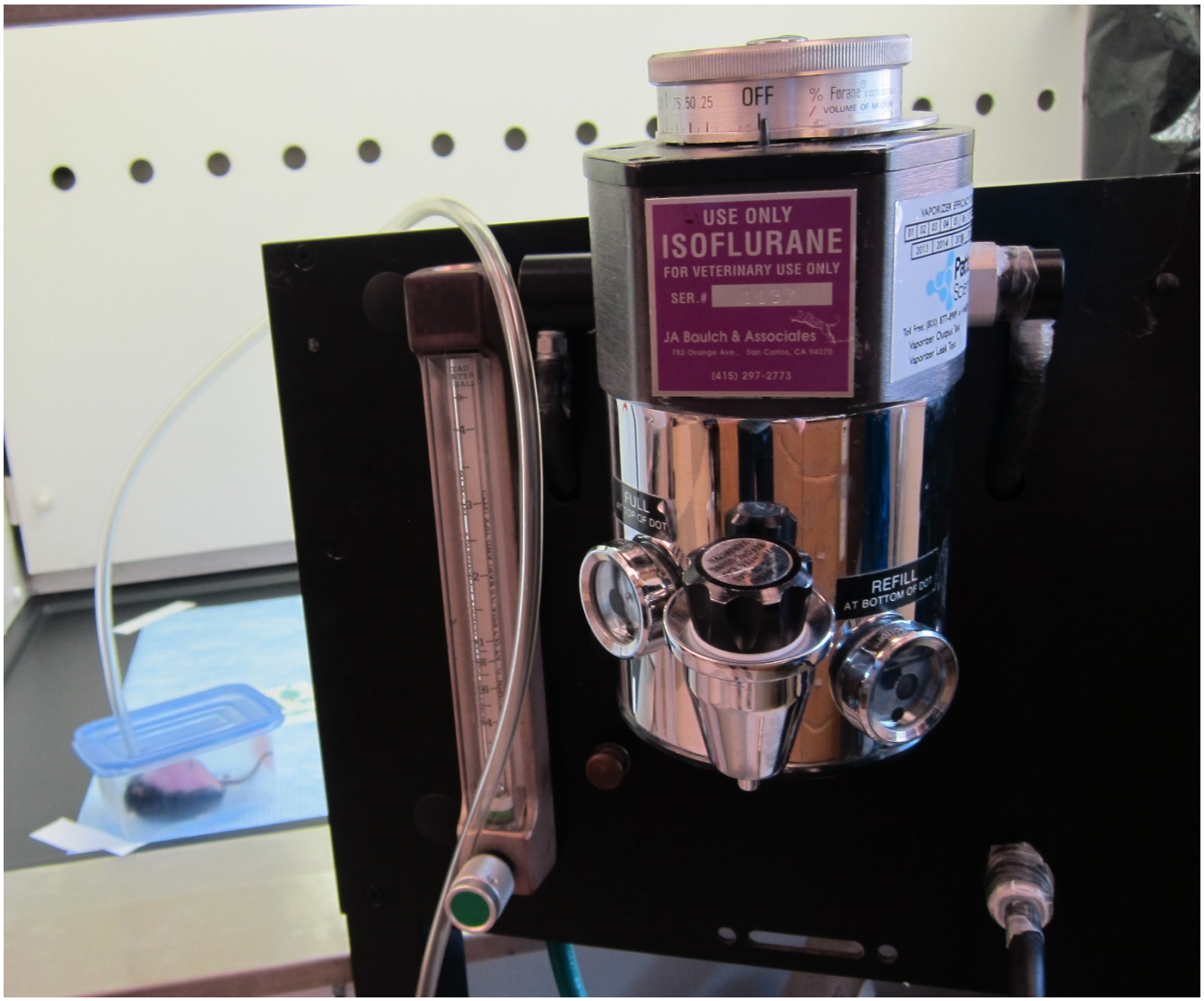
Figure 3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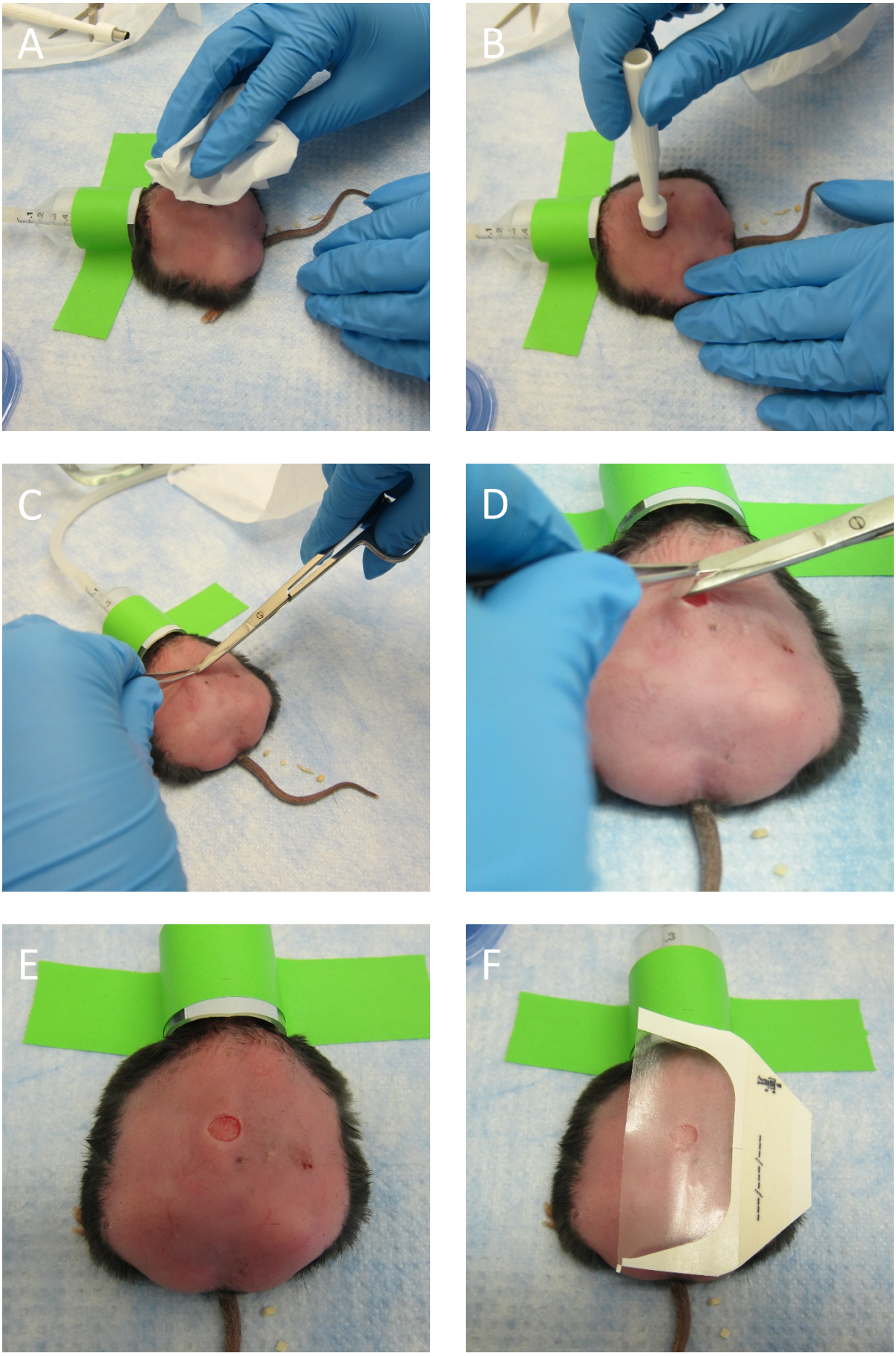

Figure 5

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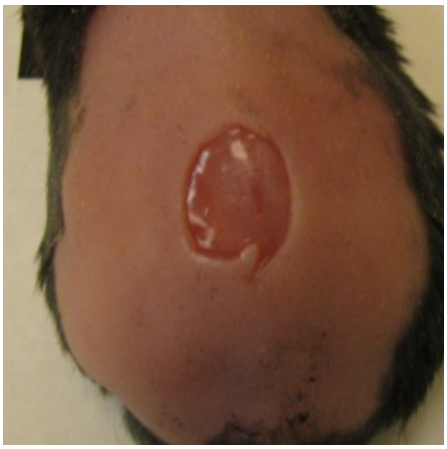
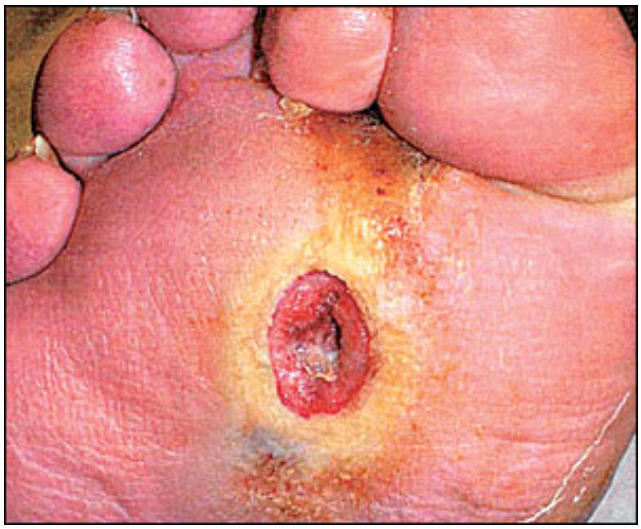


Day 0

Day 5

Day 20

B



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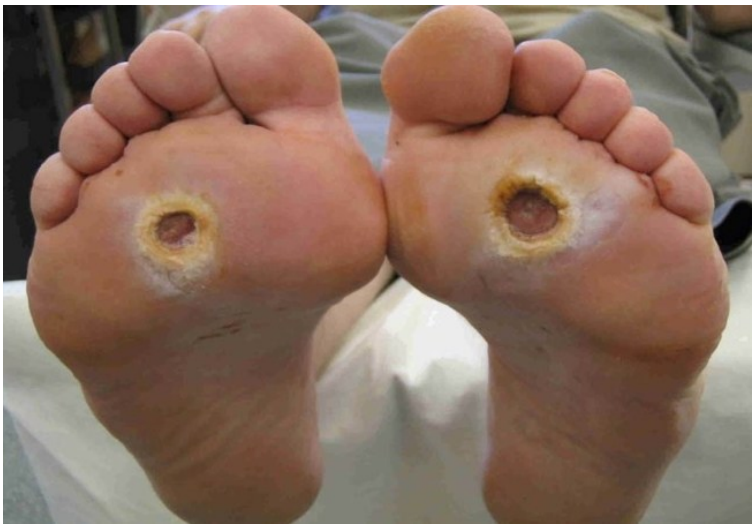

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Figure 6