**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 (™), 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 contra-indicated. 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 exerience 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.

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

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 initital wound, contains biofilm (EPS plus pathogenc 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 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 inhititor 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 anlagen (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 chachexia 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 is 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 the their respective sections. These reagent have been previous 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.