

Response to Reviewers' Comments

Editorial comments:

Changes to be made by the author(s) regarding the manuscript:

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

Response: We have reread the manuscript thoroughly and corrected any grammatical errors found.

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]."

Response: We have obtained copyright permission for reusing an illustration in one of our figure and have modified the legend to include a reference to the source.

3. Please provide an email address for each author.

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4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Ziploc, Medrad, etc.

Response: We have removed commercial language from the body of the paper.

5. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

Response: An ethics statement has been added at the end of the introduction.

6. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

Response: All steps have been switched to the imperative tense.

7. 1.4, 1.5, 1.7, 3.11, 7.1, etc.: The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary. Please move the discussion about the protocol to the Discussion.

Response: The steps have been shortened or split up to make directions that are more clear and discrete.

8. As Figure 4 shows immunostaining results, please consider moving details in Appendix A in the protocol (after step 9.13). Please note that some of the shorter Protocol steps can be combined so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

Response: One of the reviewers suggested that we remove this protocol from the paper. However, we feel it is an important protocol to have with the paper because it took considerable effort to find antibodies/methods that work for staining blood vessels in rabbits. We also feel that it would be distracting to have all of the steps within the main protocol. Thus, having the protocol in the appendix seems a good compromise for these issues.

9. Please also consider moving details in Appendix B in the protocol; for instance, step 1.1 that mentions the cholesterol chow may be appropriate.

Response: We also feel this protocol would be best to be included as an appendix. The reason is that it is not essential and high cholesterol food can be purchased commercially. However, for many labs trying to reduce costs this protocol would be very useful and we did not know of another paper that describes this method.

10. After you have made all the recommended changes to your protocol (listed above), 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.

Response: We have highlighted essential the steps that would be included in the video.

11. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.

Response: We have now highlighted complete steps that are imperative commands.

12. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Response: We have highlighted all sub-steps of highlight steps.

13. Figure 1: Please explain the difference between the upper and bottom panels.

Response: We have now included an explanation of the upper and lower panels for this figure in the figure legend.

14. Figure 4: Please describe what the different panels are.

Response: we have now described the different panels in the figure legend.

15. JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

- a) Critical steps within the protocol
- b) Any modifications and troubleshooting of the technique
- c) Any limitations of the technique
- d) The significance with respect to existing methods
- e) Any future applications of the technique

Response: We have revised the discussion extensively to roughly follow this outline.

16. References: Please do not abbreviate journal titles.

Response: We have removed the abbreviations from the references.

Reviewers' comments

Reviewer #1

Manuscript Summary:

In this interesting manuscript, Sligar and Howe et al show that hind limb ischemia model in rabbits with diabetes and hyperlipidemia may be a useful preclinical model. Although the research method was described, there are some information that need to be added in order to aid in the understanding of the reader:

Major Concerns:

1. If possible, could you add angiograms of non-diabetic model in Figure 1?

Response: We have added an angiogram of a non-diabetic rabbit to the figure.

2. Is there a significant difference to the blood pressure at 8 weeks in Figure 3?

Response: We did not perform statistics on these samples as the data was from a single animal for each group as an illustration of the typical response of each model.

Minor Concerns:

3. What does "Control" mean in Figure 4? Non-diabetic rabbit, diabetic rabbit no ischemia?

Response: We have added the details of the control to the legend of figure 4.

Reviewer #2**Manuscript Summary**

In this nice paper the authors have perfected a new angiogenesis model. This model has reduced collateral formation and blood pressure recovery in comparison to a model with a higher cholesterol diet. Thus, the model may provide better correlation with human patients with compromised angiogenesis from the common co-morbidities that accompany peripheral vascular disease.

Major Concerns:

- a laser doppler additional evaluation could be useful to make the model more available.

Response: We have added a sentence to refer the reader to references to other imaging methods that can be used to assess recover from ischemia including Doppler ultrasound, laser Doppler imaging, infrared thermography, microsphere determined perfusion, computed tomography (CT) imaging, and magnetic resonance imaging (MRI). These modifications can be found on page 13 of the revised manuscript.

Minor Concerns:

- none.

Reviewer #3:

Manuscript Summary:

This manuscript describes in an excellent way the induction of hind limb ischemia in a clinical highly relevant animal model with comorbidities frequently seen in patients and therefore is a welcome contribution the field, namely a rabbit model with diabetes. The procedure is clearly described and raises just a few remarks.

Minor Concerns:

The restoration of the blood flow after ligation of the femoral artery mainly depends on collateral formation, and to a much lesser extent on angiogenesis in the distal ischemic tissue. Therefore, the authors should speak about therapeutic neovascularization instead of therapeutic angiogenesis since this term covers both arteriogenesis and angiogenesis.

Response: We have modified the text to refer to therapeutic neovascularization rather than therapeutic angiogenesis.

Section 1.4 line 2 : change ' major factor the alters ' to ' major factor that alters' .

Response: We have corrected this sentence in the revised manuscript.

Comment

Section 4.6: Ligate all of the above arteries with 4.0 silk sutures: it is unclear what is meant with "all of the above arteries" since in the section above two different sets of arteries are described that need to be isolated:

'all branches of the femoral artery, including the inferior epigastric, deep femoral, lateral circumflex, and superficial epigastric arteries (Fig. 2A). Dissect further along the popliteal and saphenous arteries as well as the external iliac artery (Fig. 2A)'

Response: We have modified the statement in 4.6 to refer to the diagram that shows the specific locations for ligation.

Comment

9.1 how is the end point day determined or decided upon?

Response: The time point after an extensive review of the literature for studies that could observe recovery of the animals after induction of ischemia. The appropriateness of the time point was also supported by the presence of significant ischemia in the diabetic group at the end of the study.

Comment

The appendices on the immunohistochemistry protocol and on chow preparation are redundant and can be left out.

Response: In our own experience, we have had difficulty staining for blood vessel in formalin fixed paraffin sections on samples from rabbit muscle tissue. We screened through many primary antibodies to create the current protocol and felt it would save other groups time and money if we include our optimized protocol.

For the chow preparation, we also felt this could provide considerable cost savings and did not know of a published protocol that described this method of creating high fat rabbit chow.