**Response to the comments**

**Editorial comments:**

*The manuscript has been modified by the Science Editor to comply with the JoVE formatting standard. Please maintain the current formatting throughout the manuscript. The updated manuscript (54571\_R0\_020816.docx) is located in your Editorial Manager account. In the revised PDF submission, there is a hyperlink for downloading the .docx file. Please download the .docx file and use this updated version for any future revisions.*

We made all the changes in the downloaded file.

*Changes to be made by the Author(s):*

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

The manuscript was proofread again by an English native speaker.

*2. Please abbreviate all journal titles.*

We have abbreviated all title journals

*3. Please include volume, issue numbers, and DOIs for all references.*

We have now included the volume, issue number and doi in the references.

*4. Formatting:*

*-Please include the mice in the materials table.*

We include at the end of the table the Apolipoprotein E (*apoe*-/-) mice with C57/Bl6 background purchased from Charles River

*-All figure legends should have a distinct title and a short description.*

We have now introduce title in the Figure legend: Figure1. Schematic representation of operative procedure.; Figure 2. Analysis of restenosis plaque.

*-References – Please abbreviate all journal titles and include DOI when available.*

All the references were completed as requested (with volume, issue number, DOI) and the title journal was abbreviated.

*5. Grammar: Please copyedit the manuscript for numerous grammatical errors. A subset of such errors is indicated below. Editing is required prior to acceptance.*-Short abstract – “in a convenient the human condition”; “implied” is also not used correctly.  
*-Please remove any instances of “you” or “your”.  
-Introduction – “multimple” typo; “The major disadvantage are…”  
-All paragraphs should contain at least two sentences (see introduction).  
-4.3 – “Place one…sutures”  
-4.8 – “accompanied in addition to”  
-4.9 – “around common and internal branch”  
-5.2 – “measurements will be perform.”*All suggested grammar errors were corrected.

*6. Additional detail is required:  
-Polishing the guide-wire should be described somewhere in the protocol as a step.*

The polishing the guide-wire is not part of the protocol. Depending of what the operator want to use, this probably is already polished or can be done ones. Then the guide-wire will be re-use and no polishing is necessary. We specified now the protocol that the guide-wire need to be polished.

*-4.7 – How is the incision made?*

We specified now: Perform a small incision (arteriotomy, half of the vessel diameter) distal in the external carotid artery, between the two loops using the small scissors

*-5.3 – What is sufficient consciousness?*

We reformulate the phrase and shortened the paragraph.

*-6.2, 6.3, 6.7 – Please provide citations.*

The required citations were provided.

*-6.4 – How does one explant the carotid artery?*

We added into the manuscript: “*explant carefully the carotid artery including the bifurcation as proximal to the aortic arch using curved forceps and small scissors”*.

*-6.5 – How is embedding performed? How are sections cut? How are they collected? Are they placed on a slide?*

We added to 6.5: “*Embed the carotid artery in the paraffin block using usual embedding protocols. To perform transversal sectioning, place the carotid artery upright on bifurcation. Cut serial 5 µm thick serial sections starting with the bifurcation and collect them all on coated slides*”.

*-6.6 – How is measuring performed? Is this done in software? If so, is a microscope used? There is insufficient detail here. Please include a citation for the staining.*

We compete the information about the measurements analysis: “*Stain every 10th section using Movat staining for highlighting of laminas2,4,11. After taken microscopic pictures of all vessels (usually using 10x objective), measure the lumen, internal and external lamina for each section, using special designed software (DISKUS, Hilgers, Germany)2,4,11, as shown in* ***Figure 2B*** *and calculate the intimal growth and media of the vessels*”.

*7. Results:*

*-All microscopy images should include a scale bar (both Figures 1 & 2).*

We put the scale bars in the Figure 2. In the Figure 1 we obtain the picture from a stereomicroscope and we did not use any scale at that time. Therefore, we should repeat the operation and renew all the figures, which will take more time, since at the moment we do not have in plan more operations. There will be an option to repeat the images during the filming, if you think that will be needed.

*-Please point out the plaques described in Figure 2B with arrows.*

We have pointed out the described areas with arrows.

*-Please describe how Figure 2C demonstrates the re-endothelialization process in more detail in the results section.*

We have explained now that “*The re-endothelialization can be measured after staining with an endothelial marker, and calculating the circumference stained as percent from the all circumference of the lumen*.”

*8. Discussion: Please discuss the limitations of the technique, as well as any modifications/troubleshooting that can be performed.*

We have introduced now in the discussions a separate section about the limitations and troubleshooting.

**Reviewers' comments:**

**Reviewer #1:**

*Manuscript Summary:*

*The article presented here describes a method which is a well-established and -accepted strategy to investigate neointima formation, including the acute inflammatory and the proliferative phase, after mechanical, wire-induced, arterial injury of the carotid artery, the so-called wire-injury model, in atherosclerosis-prone mice mimicking conditions in humans after clinical coronary interventional therapies such as balloon angioplasty with or without stent-implantation. Although limited, this animal model provides a platform to define the outcome of potential novel drugs and the underlying molecular mechanisms by the engagement of a variety of transgenic and knock-out mouse strains. Many articles describe in detail this model and compare it with other existing arterial injury models, also highlighting the benefits and the disadvantages. The present article would be a helpful tool for teaching scientists and technicians with less experience in animal surgeries. The present article is acceptable, but requires minor revision as suggested in the detailed comments.*

We thank very much for the appreciation of our manuscript. We have made now the required changes and respond to all the concerns.

*Major Concerns:*

*The authors should include in the introduction part beside the benefits of the murine model also some limitations which do not rely only on the small size of the arteries.*

We have now added into the introduction and discussions a separate part regarding the limitations of the method.

*Minor Concerns:*

*1.The method herein is used for many years in different labs to analyze underlying molecular mechanisms of (re)stenosis and the effect of potential novel therapies after revascularizing procedures. In the introduction part the authors should include also references from the very beginning of this method (especially Lindner et al., 1993, line 73).*

We thank for pointed this out, we added now this important reference to our citations.

*2. Some included references match inflammatory response, but are not directly related to acute arterial injury. More relevant references would be desirable (e.g. line 79).*

We have now removed this from the introduction and we refer only in the results part. Since the acute mechanical injury of the arterial wall is milder in this model, the researches focused on the inflammatory studies. We have pointed now this aspect in the manuscript.

*3. The authors should mention why strains with affected lipid-metabolism (ApoE-/- or LDL-R-/--deficient mice) are used and explain briefly why the atherogenic diet starting one week before surgery is necessary. Gender is not restricted to female. The weight of the animals is missing, which correlates more directly to the vessel-diameter (line 118ff).*

We thank very much to point this out. We have explained now in the manuscript that the ApoE-/- creat a high plasma cholesterol level, which create the pro-inflammatory environment necessary to compensate the fact that the denudation is performed in a healthy vessel. We also added the weight of the mice (18-20g) and mentioned that the female gender is optional.

*4.Since the paper is thought to be a step-by-step manual, here are some suggestions:*

*a)line 131: alcohol allowed? Instead betadine?*

Thank you for the suggestion, we replace now alcohol with betadine, which is preferred in more countries.

*b)line 136: step blunt-dissection of the muscle layer is missing*

We added now to the step 3.4: “*If present, perform the blunt-dissection of the thin muscle layer covering the carotid artery*”.

*c)line 168: What is meant with: Repeat this movement in each mouse to increase the reproducibility.*

We have replace this phrase with: “*It is important to maintain the same amplitude of rotational movement in each mouse to increase the reproducibility*”

*d)line 180: missing step: disinfection of the skin with betadine.*

We have added now the disinfection with betadine of the skin.

*e)line 268: concerning "not effectively discontinued", here you will not have troubles just at the timepoint of inserting the wire, the bleeding will already occur during the arteriotomy.*

Thank you to pointed this out, we reformulate now the statement accordingly: “*The second critical step, is the high risk of bleeding during arteriotomy and insertion of the wire if blood flow is not effectively discontinued*”.

*f)line 271: guide-wire should also be wet/not-dry, droplet of sodium chloride, could be mentioned in the procedure part. How is the wire disinfected/cleaned before next use?*

We are grateful for this observation. We have now introduce an additional step regarding the preparation of the wire: “*4.8) Prepare a 0.14-inch polished flexible guide-wire by disinfection with alcohol and moisten it in a droplet of sodium chloride to assure proper sliding into the vessel*”.

*g) line 276: which other differences between mouse to mouse should be taken into account? (gender, age, weight…)*

We thank for the suggestion, we have now added in the discussions: “*Since the wire size did not change, it is important to consider and eliminate all the possible differences between the mice by choosing the same gender, age and weight for all mice included in a study*”.

*h) line 285: concerning the discussion about benefits of mice compared to rabbits/rats because of lack of trained personnel…there are also benefits by use of rabbits/rats, e.g. no need for miniaturized equipment (use of devices for humans). Mouse balloons are available, but not widespread and expensive. Instead, wire-injury is accepted, mimics in-stent stenosis. Balloons would have benefits, e.g. adjustment to vessel-diameter possible as in clinical practice which has strong influence on outcome. The high number of preclinical models reflects that none of the models fulfills all the criteria necessary to uncover the whole cellular and molecular mechanisms leading to the pathophysiology in human. This part of the discussion should be expanded on models concerning the arterial injury, as mentioned in the introduction part, also compared to the (diet-induced) native atherosclerosis model, maybe as a table with benefits/disadvantages.*

We are grateful for the suggestions; we have now expanded our discussions and included all aspects mentioned by the referee. We have also added a table with the benefits/disadvantages of each method, as suggested (see Table 2).

*5. line 234: it is important to mention: the wire-size is not changing and therefore not "adapting" to the vessel-size, the procedure with rotational movements by inserting to injure the vessel should be adapted in a way, that the whole luminal endothelium is completely denudated. Mice should not vary significantly in size/weight, vessel-diameter…*

We thank very much for this observation, we have now added in the discussion an advise to increase the reproducibility and to control the efficiency of the denudation: “*Since the wire size did not change, it is important to consider and eliminate all the possible differences between the mice by choosing the same gender, age and weight for all mice included in a study. Therefore, Evans-Blue staining after learning the method will help the operator to appreciate the efficiency of the denudation*”.

*6. Proofreading is mandatory (missing words, typographical errors or grammar, especially in the discussion part), e.g. lines 45, 66, 69, 97, 99 with the help of a wire/by mechanical denudation, 102, 101ff (qualification-> experience), 115, 146(2-> two, each 1.5cm long), 150 (suture), 156/157 (blood flow; forcep), 167 (in addition to -> by), 168 (procedure for a total of three times), 171 (blood flow in the carotid), 179, 182 (infrared), 199ff (with… analyses of interest: paraffin embedding…), 209, 214 (sections), 224, 226, 231, 234, 237-242 (The developed plaque resembles…, should be -> is), 244 (plaque growth during), 247, 248, 255, 266 (reserve-> remaining? residual?), 267 (rupturing-> cutting the vessel), effectively-> efficiently), 298, 306; in general: personnel with less experience in animal surgeries. So far, there is no published model to study in-stent restenosis, just in-stent stenosis. mouse-table -> operating table. Dose of anesthetics in mg/kg bodyweight. operator-> surgeon.*

We thank very much for these corrections, we have now performed all changes required.

*7. Figure 1: in B, please indicate where the nerve and the vein are located; in D, indicate rotational movement; in B+C, a ruler/scale bar would be beneficial. Would be helpful to include a picture before exposing the carotid artery to see the position of the vagus nerve and jugular vein.*

We do not have a picture exposing the position of vagus nerve before the preparation of carotid artery, however, we have now indicated where the nerve and vain are located in Figure 1B. In the Figure 1 we obtain the picture from a stereomicroscope and we did not use any scale at that time. Therefore, we should repeat the operation and renew all the figures, which will take more time, since at the moment we do not have in plan more operations. There will be an option to repeat the images during the filming, if you think that will be needed. The rotation movement is now indicated in Figure 1D.

*8. Figure 2: in B+C, scale bars are missing; all pictures in C should be shown at same magnification, maybe in higher magnification as insets.*

We have now added the scale bars in the images. However, the endothelial-specific staining is not really good at lower magnification; therefore, we choose a higher magnification only for this image.

*9. Table: Ultrasound system is listed, but not relevant to run the method. Should be deleted from the list. Please include the wound closure clips (suture clips) and the Clip Applying Forcep.*

We removed from the list the ultrasound system and added the clips and the alips applying forceps as suggested.

*Additional Comments to Authors:*

1.It would be nice to have a table (time/days after wire-injury, issue to analyze, re-endothelialization [% of whole vessel lumen], neointima formation [µm2], media thickness [µm2]; acute inflammatory phase (day 1, no endothelium, thrombus formation, inflammatory cells, day 7+14, plaque growth, progression re-endothelialization, inflammatory cells, SMCs) and chronic endpoint (day 28, re-endothelialization completed, maximum size of plaque growth).

We are grateful for this suggestion; we have now added a table with the time-dependent Plaque characteristics (see Table 1).

**Reviewer #2:**

*Manuscript Summary:*

*Curaj et al described a minimal invasive procedure of endothelial denudation, that is of interest for readers of JoVE. In the study, also known as the wire-injury model, an in vivo procedure is introduced that is feasible and clinically important to be reproducible under laboratory conditions. Although the manuscript is well organized, the text is sometimes hard to read and to follow, due to the complicated style, numerous errors and over-statements throughout the manuscript. The conclusions are mainly supported by the experimental/descriptive, but are repetitive between results and introduction sections.*

We thanks for this suggestions, we have removed now the redundant phrase from the introduction and added more limitation of the method.

*Major Concerns:*

*The English style throughout the text needs improvement and checking by a native English speaker.*The manuscript was proofread again by an English native speaker.

*Minor Concerns:*

*N/A*  
**Reviewer #3:**

*Manuscript Summary:*

*The manuscript of Curaj et al describes a mouse model of endothelial denudation, which is highly representative for human condition of neointima formation. The procedure is very well described, offering plenty of details so that it can be reproduced by any scientist. However, this reviewer does not really believe the intention of authors is to teach untrained personnel, as an adequate background in animal surgery and qualification in animal work remain important requests for interested students/scientists to be able to do this procedure. Therefore, encouraging students with "low or average qualification in animal work" to perform this procedure may be hazardous and caution is recommended in using such expressions to avoid unnecessary suffering to laboratory animals.*

We thank this referee for pointed this out. To avoid any confusion, we rephrase this and stated that the manuscript presents “*a step-by-step guideline and suggestions to ease the introduction for interested personnel*”.

*There are several errors that need to be corrected.*

*1. "Restenosis refers to the narrowing of the vessel lumen" instead of "Restenosis refers to the narrowing of the vessel wall" (Introduction section, paragraph 2).*

The paragraph was removed from the introduction.

*2. The authors said in the long abstract they were proposing a "minimal invasive procedure of endothelial denudation". However, under Introduction section, the authors said that the described procedure was used "to induce invasively an atherosclerotic plaque". The aspect of invasiveness of the procedure should be clearly established and keep constant throughout the manuscript.*

We correct the formulation into “*an invasive procedure* of endothelial denudation”.

*3. The authors said: "If needed, position the mouse-table with the mouse-head towards the operator". Does it mean that such positioning is recommended for going further with the procedure or it is optional?*

We thank very much for this observation, we rephrase now: “*Position the mouse-table with the mouse-head towards the operator to assure proper position for the guide-wire during the denudation*”.

*4. The state: "…and continue the diet until the end of the experiment" is unclear. It should be better replaced with "continue the diet until the atherosclerotic plaque analysis is to be performed"*

Thank you for this suggestion, we have made the changes in the manuscript.

*5. The phrase "Repeat this movement in each mouse to increase the reproducibility" should be better replaced to "It is important to maintain the same amplitude of rotational movement in each mouse to increase the reproducibility".*

Thank you for this suggestion, we have made the changes in the manuscript.

*Besides, other several minor grammatical errors should be corrected, such as:*

*1. "The major disadvantage are the small size…"(Introduction section, paragraph 3).*

*2. "The surgery takes place under the microscope" (Introduction section, paragraph 4).*

*3. "The method can be learned and used for both accelerated neointima formation.." (Introduction section, paragraph 5).*

We have made all the suggested corrections.

**Reviewer #4:**

*Manuscript Summary:*

*This is a useful manuscript on inducing an accelerated form of atherosclerosis using the apoE-/- mice and wire injury. The methods are described in sufficient detail in most cases so that the reader can easily replicate the procedure. However, there are a few issues that should be addressed, as described below.*

We thanks very much for the suggestions, I have responded now to all remained concerns.

*1. One of the most important and difficult to perform parts of the procedure is the opening and closing of the arteriotomy, the incision in the external carotid artery. This procedure is not described very explicitly and should be written in more detail.*

We have now decribed more in detail this step and provide also a troubleshooting alternative for the operator.

*2. ApoE-/- given the high fat diet as described will have plasma cholesterol levels that will be extremely high. The authors should describe how this might pose as a limitation in using the model proposed.*

We thank very much for this observation. Since the procedure is performed in healthy vessels, the high level of plasma cholesterol represents an advantage for the method, because the thus the pro-inflammatory environment needed for the neo-intima formation is obtained. This aspect is now pointed out in the manuscript.

*3. There are numerous places in the manuscript where the English sounds awkward. It is recommended that the manuscript be reviewed by a native English speaker.*

The manuscript was proofread again by an English native speaker.