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Induction of accelerated atherosclerosis in mice: the "wire-injury" model

--Manuscript Draft--

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Corresponding Author:	Elisa A. Liehn, M.D., Ph.D. Institute for Molecular Cardiovascular Research Aachen, NRW GERMANY
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	eliehn@ukaachen.de
Corresponding Author's Institution:	Institute for Molecular Cardiovascular Research
Corresponding Author's Secondary Institution:	
First Author:	Adelina Curaj
First Author Secondary Information:	
Other Authors:	Adelina Curaj
	Zhuojun Wu
	Mareike Staudt
Order of Authors Secondary Information:	
Abstract:	Atherosclerosis is a proliferative fibro-inflammatory disease developing in the arterial wall, inducing a deficient blood flow or a lack of blood flow. Moreover, by rupture of the defect vascular wall, atherosclerosis induces an occlusive thrombus formation, which represents the main cause of myocardial infarction or stroke and the most frequent cause of death. Despite the advances in the cardiovascular field, many questions remain unanswered, and additional basic research is essential to improve our understanding of the molecular mechanisms during atherosclerosis and its effects. Due to limited clinical studies, there is a need for representative animal models recreating atherosclerotic conditions such as neointima formation after stent implantation, balloon angioplasty or endarterectomy. Since the mouse presents many advantages and is the most frequently used model for studying molecular processes, the current study proposes an minimal invasive procedure of endothelial denudation, also known as the wire-injury model, which is representative for the human condition of neointima formation in arteries after revascularization procedures.
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	Experiments and no any potential conflict of interest exists. We hope that the manuscript is now suitable to be considered for publication in Journal of Visualized Experiments and we look forward to hearing from you. Sincerely yours, Elisa A. Liehn, MD, PhD
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TITLE:
Induction of accelerated atherosclerosis in mice: the "wire-injury" model

AUTHORS:
Adelina Curaj
Institute for Molecular Cardiovascular Research
RWTH Aachen University
Aachen, Germany
acuraj@ukaachen.de

Wu Zhuojun
Institute for Molecular Cardiovascular Research
RWTH Aachen University
Aachen, Germany
zwu@ukaachen.de

Mareike Staudt
Institute for Molecular Cardiovascular Research
RWTH Aachen University
Aachen, Germany
mstaudt@ukaachen.de

Elisa A. Liehn
Institute for Molecular Cardiovascular Research
RWTH Aachen University
Aachen, Germany
and
Human Genetic Laboratory
University of Medicine and Pharmacy
Craiova, Romania
eliehn@ukaachen.de

CORRESPONDING AUTHOR:
Elisa A. Liehn
eliehn@ukaachen.de
Tel. +49 241 80 35983
Fax. +49 241 80 82122

KEYWORDS:
Atherosclerosis, mouse model, neointima formation, stabile atherosclerotic plaque

SHORT ABSTRACT:
This study describes an invasive procedure for the induction of accelerated atherosclerosis in mice. In comparison to other methods using electric- or cryo-induced injury, mechanical-

induced injury mimics the human condition of restenosis after revascularization therapies and is ideal for the study of the molecular mechanisms involved.

LONG ABSTRACT:
Atherosclerosis is a proliferative fibro-inflammatory disease developing in the arterial wall, inducing a deficient blood flow or a lack of blood flow. Moreover, by rupture of the defective vascular wall, atherosclerosis induces occlusive thrombus formation, which represents the main cause of myocardial infarction or stroke and the most frequent cause of death. Despite the advances in the cardiovascular field, many questions remain unanswered, and additional basic research is essential to improve our understanding of the molecular mechanisms during atherosclerosis and its effects. Due to limited clinical studies, there is a need for representative animal models recreating atherosclerotic conditions such as neointima formation after stent implantation, balloon angioplasty, or endarterectomy. Since the mouse presents many advantages and is the most frequently used model for studying molecular processes, the current study proposes an invasive procedure of endothelial denudation, also known as the wire-injury model, which is representative of the human condition of neointima formation in arteries after revascularization procedures.

INTRODUCTION:
Atherosclerosis is the main pathology underlying cardiovascular events such as myocardial infarction or stroke. The main mechanisms triggering acute cardiovascular syndromes are plaque rupture, superficial erosion, and thrombus formation. There are multiple clinical situations connected to the plaque development: native atherosclerotic plaque, restenosis after endarterectomy, and restenosis after balloon angioplasty with/without stent implantation[1]. After arterial injury, suppression of the inflammatory processes[2,3] and the recovery of the endothelial compartment are essential to prevent further complications[1]. Clinical research is limited to tissue and blood samples due to ethical considerations, costs, and a lack of knowledge in basic mechanisms. For these reasons, there is a need to study molecular mechanisms in animal models[4-6], which can recreate the clinical conditions. Our model of accelerated neointima formation in the context of atherosclerosis is the result of many years of experience in the implementation of these models in small animals[7-11]. The mouse model is the most attractive model for research, due to its ease of handling, the ability to have large animal groups due to low costs related to animal purchase and care, and the availability of various transgenic and knockout strains.

The major disadvantage of the mouse model is the small size of the main arteries subjected to atherosclerotic disease (the carotid artery, the aorta, and the femoral artery), which requires qualified surgical expertise and skills to manipulate the vessels and to invasively induce an atherosclerotic plaque. Therefore, the model of accelerated neointima formation, in the context of restenosis after endarterectomy or stent implantation, proposed in this paper is presented with a step-by-step guideline and suggestions to ease the introduction for interested personnel. Another disadvantage is that the denudation is made on the normal arterial wall, and therefore, the neo-intima formation will be moderate compared with the clinical situation. The high level of plasma cholesterol reached in apolipoprotein E knockout (*Apoe*$^{-/-}$) mice fed

with a high fat-diet creates a proper pro-inflammatory environment needed for the neo-intima formation.

The surgery is performed under a stereomicroscope. The carotid artery is exposed by a median incision in the ventral cervical area. Anatomical structures on top of and surrounding the carotid artery are minimally manipulated to reduce post-surgical inflammation. The carotid artery bifurcation is exposed. To induce accelerated neointima formation, internal and external carotid arteries are prepared for blood flow cessation and subsequent common carotid artery denudation. In conclusion, the method can be learned by personnel with minimal experience in animal surgeries.

PROTOCOL:

Experiments presented in this paper are performed according to the German law and to the European animal care guidelines. The animals are bred in the animal facility of the Institute for Laboratory Animal Science, University Hospital Aachen, Germany, under supervision of Prof. Dr. R. Tolba and Dr. A. Teubner (animal welfare officer).

1) Animal care

1.1) Keep the mice in a specialized care unit, ensuring proper access to food and specialized veterinary control and treatment. If the animals are moved or purchased from third parties, please ensure a one week accommodation period before undergoing the procedure.

2) Hyperlipidemia inducement

2.1) Feed 6-8 week old, 18-20 g, female (optionally) ApoE$^{-/-}$ mice with an atherogenic diet (21% fat, 0.15% cholesterol, 19.5% casein, wt/wt) one week prior to the surgical procedure and continue the diet until the atherosclerotic plaque analysis is to be performed.

3) Surgical preparation

3.1) Anesthetize the mice using an intraperitoneal injection of 100 mg/kg ketamine by bodyweight and 10 mg/kg xylazine by bodyweight. Confirm proper anesthetization prior to surgery by the lack of reflexes and whisker movement. Cover the mouse's eyes with a film of eye cream to prevent dryness and blindness during surgery.

3.2) Ensure the maintenance of sterile conditions to avoid infections during surgery by using sterile materials and instruments.

3.3) Shave the mice in the ventral neck region. Disinfect the skin with betadine prior to incision. Make a 1 cm skin incision in the median region of the neck area, on top of the trachea.

3.4) Separate the two fat bodies to ensure a proper view over the tracheal region. Use retractors to hold the muscle layer and expose the carotid artery. If present, perform the blunt-dissection of the thin muscle layer covering the carotid artery.

3.5) Use sharp curved forceps to separate the carotid artery from the vagus nerve and jugular vein. Thus, the bifurcation area with the internal and external carotid artery should be visible. Use 0.9% NaCl in order to avoid tissue dryness during the surgical procedure.

4) Wire-injury

4.1) Place a 7 cm long 0/5 silk suture under the carotid artery, proximal to the aortic arch. Make an open loop, ready to be closed at any time.

4.2) Place two 0/7 silk sutures (each 1.5 cm long) around the external carotid artery: one loop close to the bifurcation point, and one loop as distal as possible. Prepare them as an open loop, ready to be closed at any time.

4.3) Place one 0/7 silk suture (1.5 cm long) under the internal carotid artery. Prepare it as an open loop, ready to be closed at any time.

4.4) Position the mouse table with the mouse head towards the operator to ensure proper positioning for the guide-wire insertion during the denudation (**Figure 1A**).

4.5) Under the microscopic view, stop the blood flow through the common carotid artery by holding and pulling the ends of the 0/5 silk suture with hemostat forceps.

4.6) Immediately after the common carotid artery ligature, close the suture loops placed on the internal carotid artery and the distal suture on the external carotid artery tightly (**Figure 1B**).

4.7) Perform a small incision (arteriotomy, half of the vessel diameter) distal to the external carotid artery, between the two loops, using small scissors (**Figure 1C**). If the incision is too big, please follow the troubleshooting instructions (see the Discussion).

4.8) Use commercially polished guide wires or use in-house specialized personnel to polish the guide wires. Disinfect the 14 inch polished flexible guide-wire with alcohol and moisten it in a droplet of 0.9% NaCl to ensure proper sliding into the vessel.

4.9) Insert the guide-wire into the common carotid artery via the transverse arteriotomy of the external carotid artery (**Figure 1D**). Obtain endothelial denudation by passing the guide wire along the vessel while rotating. Repeat this procedure three times. Maintain the same amplitude of rotational movement in each mouse to increase the reproducibility.

4.10) Close the proximal loop on the external carotid artery tightly. Restore the blood flow in the carotid artery by cutting the suture around common artery and the suture around the internal carotid artery.

5) Suture and recovery

5.1) Remove the retractors and return the muscle layer and the two fat bodies to the physiological position.

5.2) Close the skin with three separated sutures 0/6, if echocardiographic measurements are needed. If no imaging is needed, use metallic clips to close the skin.

5.3) Lay the mouse down on its left side under the infrared light until it wakes up. Do not leave an animal unattended nor in the company of other animals until fully recovered.

5.4) For future identification, mark the mouse using the local system. Ask the animal welfare officer from the local institution.

6) Analysis of the atherosclerotic plaque

6.1) Anesthetize the mice at the end-time point using an intraperitoneal injection of 100 mg/kg ketamine by bodyweight and 10 mg/kg xylazine by bodyweight. Confirm proper anesthetization by the lack of reflexes and whisker movement.

6.2) Perform exsanguination by retro-orbital or cardiac puncture and collect the blood for further analysis[2].

6.3) Disinfect the skin with betadine. Open the thoracic cavity and remove the right auriculum of the heart. Perfuse phosphate buffered solution through left ventricle to remove the remaining blood from the vessel and then perfuse 4% PFA to fix the tissue.

6.3.1) If no fixation is required, explant the carotid artery immediately after washing[2,4,11]. Perform standard protocols with analyses of interest: paraffin embedding, cryosection, mRNA or protein analysis, etc.

6.4) For morphometrical measurements, carefully explant the carotid artery including the bifurcation, with minimal manipulation, as proximal to the aortic arch using curved forceps and small scissors.

6.5) Embed the carotid artery in the paraffin block using standard embedding protocols. To perform transversal sectioning, place the carotid artery upright on bifurcation. Cut 5 µm thick serial sections starting with the bifurcation and collect them all on coated histological slides (**Figure 2A**).

6.6) Stain every 10th section using Movat staining to highlight the laminas[2,4,11]. After collecting microscopic pictures of all vessels (using a 10x objective), measure the lumen, as well as the internal and external lamina for each section, using special designed software[2,4,11], as shown in **Figure 2B**. Calculate the intimal growth and media of the vessels.

6.7) Analyze smooth muscle cells and macrophage content, or endothelial recovery in serial sections, using usual immunohistological staining[2] (**Figure 2C**).

REPRESENTATIVE RESULTS:

The atherosclerotic plaque induction procedure takes 15-20 minutes and shows a minimal mortality rate, mostly due to the bleeding occurring during the procedure. After surgery, the mice recover from anesthesia within 20-25 minutes. No physical impairment, such as paralysis, or feeding disturbance was observed after the surgery.

The wire-injury induces a de-endothelialization, mimicking vascular lesions after balloon denudation or stent-implantation. Immediately after injury, the denuded vascular wall will be covered with a layer of thrombocytes, which mediates and favors the adhesion of the monocytes[12]. Activated smooth muscle cells from the media will proliferate and migrate into the intimal spaces, forming the neointima. Other progenitors for smooth muscle cells will migrate from the blood (estimated to be 40%) and contribute to the neointima growth. The plaque formation will end after the complete re-endothelialization, usually 4 weeks after the wire-injury.

The neointima formation can be assessed using Movat staining. The plaque size is calculated for each slide using software as shown in **Figure 2B**. The total plaque size (left carotid artery) can vary between 70 000 – 100 000 µm², while the control vessel size (right carotid artery) can vary between 7 000 – 8 000 µm². These values depend largely on the surgeon. Therefore, we strongly recommend using the same surgeon during the experiments for the same study.

The developed plaque resembles *in stent* restenosis, which predominately consists of proliferated and migrated smooth muscle cells from the media. The cellular composition determined by immunological staining procedures shows that the smooth muscle cell content is approximately 30-40%, while macrophages are found in 15-25% of the neointima of the injured vessel. The re-endothelialization can be measured after staining for an endothelial marker, and calculated as the percentage of circumference stained over the entire circumference of the lumen. Usually the re-endothelialization reaches 80-90% after 3 weeks, and should almost be complete after 4 weeks (**Figure 2C**). To track the plaque growth during its development, the same analysis can be repeated for every time-point after the wire-injury, depending on the interest and the subject studied (see Table 1).

Figure 1. Schematic representation of operative procedure.
(A) The positioning of the operation table toward the operator during the wire-injury procedure (**B)** Enlarged view of the common carotid artery and its branches, as it appears under the microscope at 10X magnification (**C)** The size of the incision in the external carotid artery under the microscope at 10X magnification (**D)** Schematic representation of wire-injury procedure using the 14 inch guide wire.

Figure 2. Analysis of restenosis plaque.
(A) Schematic representation of plaque analysis in the common carotid artery, 4 weeks after wire-injury induction (**B)** Neointima formation 4 weeks after the wire-injury and schematic representation of main parameters used for analysis. Intima (green area) is the difference between the lumen (red) and the lamina interna (green line). Media (yellow area) is the difference between the lamina externa (yellow line) and interna (green line). Scale bar 100 µm

(**C)** Representative images of the staining of the main cell types involved in neointima formation. Smooth muscle cells (smooth muscle actin –red, scale bar 100 μm), macrophages (Mac 2- green, scale bar 100 μm) and endothelial cells (CD31- red, arrows, scale bar 50 μm).

Table 1. Time-dependent Plaque's development.

Table 2. Advantages and disadvantages of existent models of arterial injury.

DISCUSSION:

In this paper, we provide useful tips to perform the wire-injury procedure even by personnel with minimal experience in animal surgeries. There are two critical steps in performing this procedure: the incision of the external carotid artery and the insertion of the wire. The incision in the external carotid artery needs to be performed as far as possible from the bifurcation, in order to ensure enough remaining material (**Figure 1C**). The incision should not be too large, due to the risk of cutting the entire vessel. The second critical step is the high risk of bleeding during the arteriotomy and the insertion of the guide wire if blood flow is not efficiently discontinued. Moreover, endothelial denudation might not take place or arterial rupture is possible if the guide wire is not properly introduced in the lumen vessel. To avoid this, the surface of the guide wire must be carefully polished before the operation.

To optimize the protocol, the position of the operating-table with the mouse-head towards the surgeon ensures a better view, accessibility and control for the proper guide wire manipulation. Moreover, to increase the reproducibility, use the same guide wire in all of the studies. Since the wire size does not change, it is important to consider and eliminate all the possible differences between the mice by using the same gender, age and weight for all mice included in a study. Thereafter, Evans-Blue staining will help the surgeon determine the efficiency of the denudation. The existence of appropriate equipment is a prerequisite for the success of the procedure. A 10X stereomicroscope is essential for performing this procedure. The proper preparation of the guide-wire (for example polishing it) is crucial. Therefore, we strongly recommend that the guide wire preparation be performed by specialized technical personnel where available.

There are many troubleshooting steps in this protocol. If incising the external carotid artery near the bifurcation, carefully bind the externa, near the bifurcation, so no bleeding occurs. During cutting, the external carotid artery cannot be seen. Therefore, consider the bifurcation at the level of silk suture. Collect sections when the silk suture disappears. If the incision in the external carotid artery is too large and the vessel is ruptured, ensure that the blood flow into the carotid communis and internal carotid artery is effectively interrupted and try to find the opening of the vessel using forceps. After introducing the guide wire and performing the denudation, bind the vessel near the bifurcation. During cutting, start to collect when the silk from the suture starts to disappear. If arterial rupture occurs during the denudation with the guide wire, check under the microscope if the guide wire is properly polished.

Despite the similarity of the wire-injury model to clinical situations, many groups are focused on native atherosclerosis in mice, or they choose invasive atherosclerosis inductions, such as balloon angioplasty in rats or rabbits, because of the lack of trained personnel who can perform small animal surgeries. Despite the benefits of using rabbits/rats, e.g. no need for miniaturized equipment, neither rat models nor rabbit models offer a variety of different knock-out strains, in terms of studying molecular mechanisms involved in neointima growth and in-stent thrombosis.

The existing models for studying in-stent restenosis in mice are difficult, require high surgical skills, and have high risks of complications such as bleeding or paralysis. For example, the mechanical injury or stent-implantation into the thoracic aorta via femoral artery is accompanied by a high mortality rate (35%) due to hind leg paralysis or bleeding[13-15]. We also describe stent implantation in the carotid artery of a mouse[16]. The procedure is similar; however, the tissue processing for analysis is complicated and is not available to all laboratories[16]. The carotid artery is directly accessible, not only for operation procedures, but also for existing imaging methods such as ultrasound imaging. Other injury inductions in the carotid arteries in mice can be done using electrical devices[17]. This method is easy to perform and ensures high reproducibility. However, it induces injury in all vessel layers, which is not identical with mechanical injury. Balloon applications have benefits, e.g. the adjustment to the vessel diameter in line with the clinical practice and has strong influence on the pathological outcome. Even though mouse balloons are available, they are very expensive and therefore, not widely used. Instead, the wire-injury is the established method, mimicking in-stent stenosis.

The denudation is performed on the normal arterial wall, though with an atherosclerotic background. Therefore, the neointima formation will be moderate compared to the clinical situation. The high number of preclinical models demonstrates that none of the models fulfill all of the criteria necessary to uncover the entirety of the cellular and molecular mechanisms leading to the pathophysiology in humans (see Table 2).

After performing the wire-injury procedure, other biological and molecular analysis can be performed to identify cells, proteins, mRNAs, microRNAs, genes or other biomarkers, which can be used as therapeutic targets to develop new treatment strategies for atherosclerosis, and in particular for neointima formation after vascular injury. If available, the plaque growth can be monitored using high frequency ultrasound or other high-resolution imaging techniques. Moreover, mastering this technique would give the operator the opportunity to adapt the protocol to other invasive atherosclerosis inducement models, such as collar placement, partial ligation or even stent implantation.

DISCLOSURES:
There are no disclosures by the authors.

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Figure 1

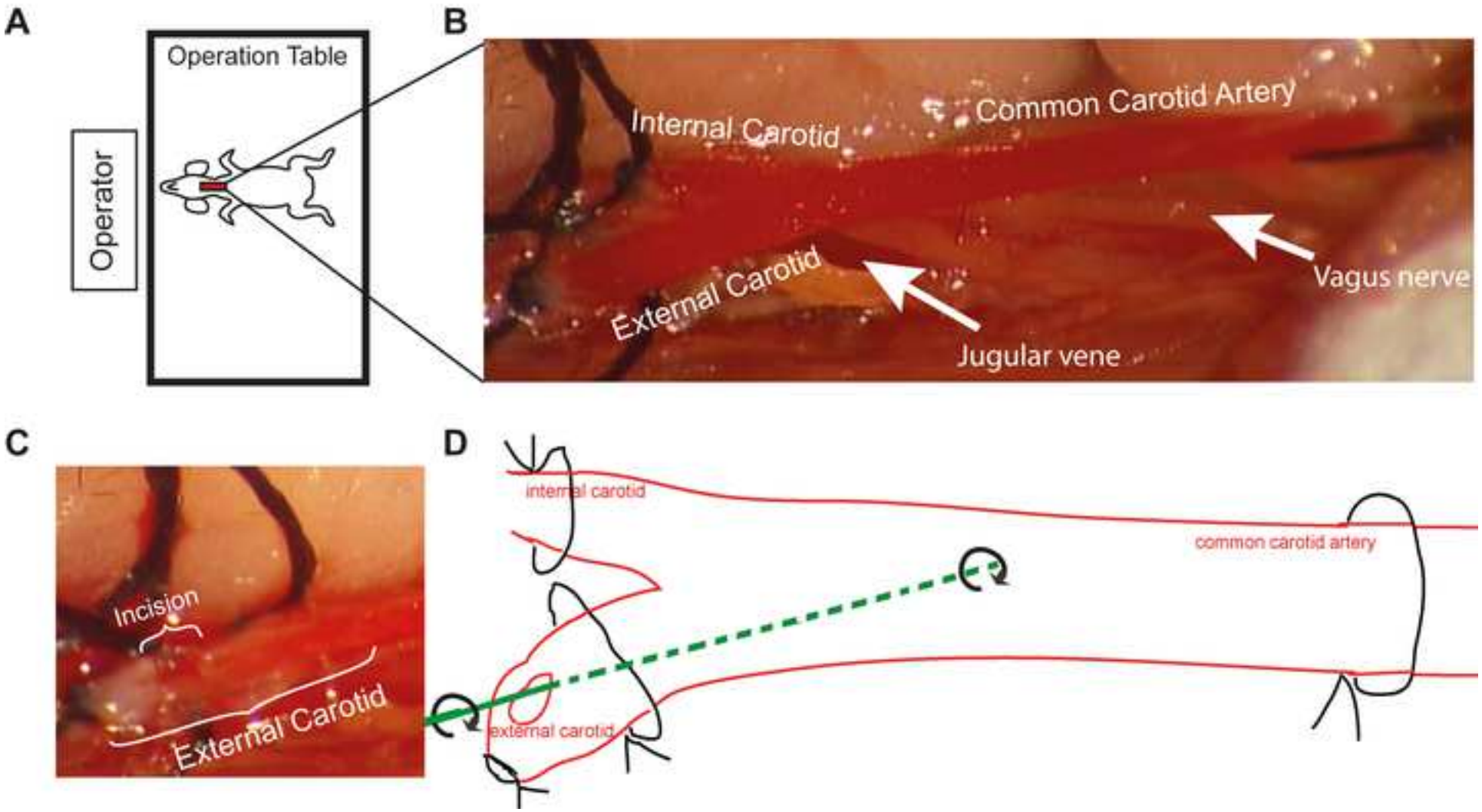

Figure 2

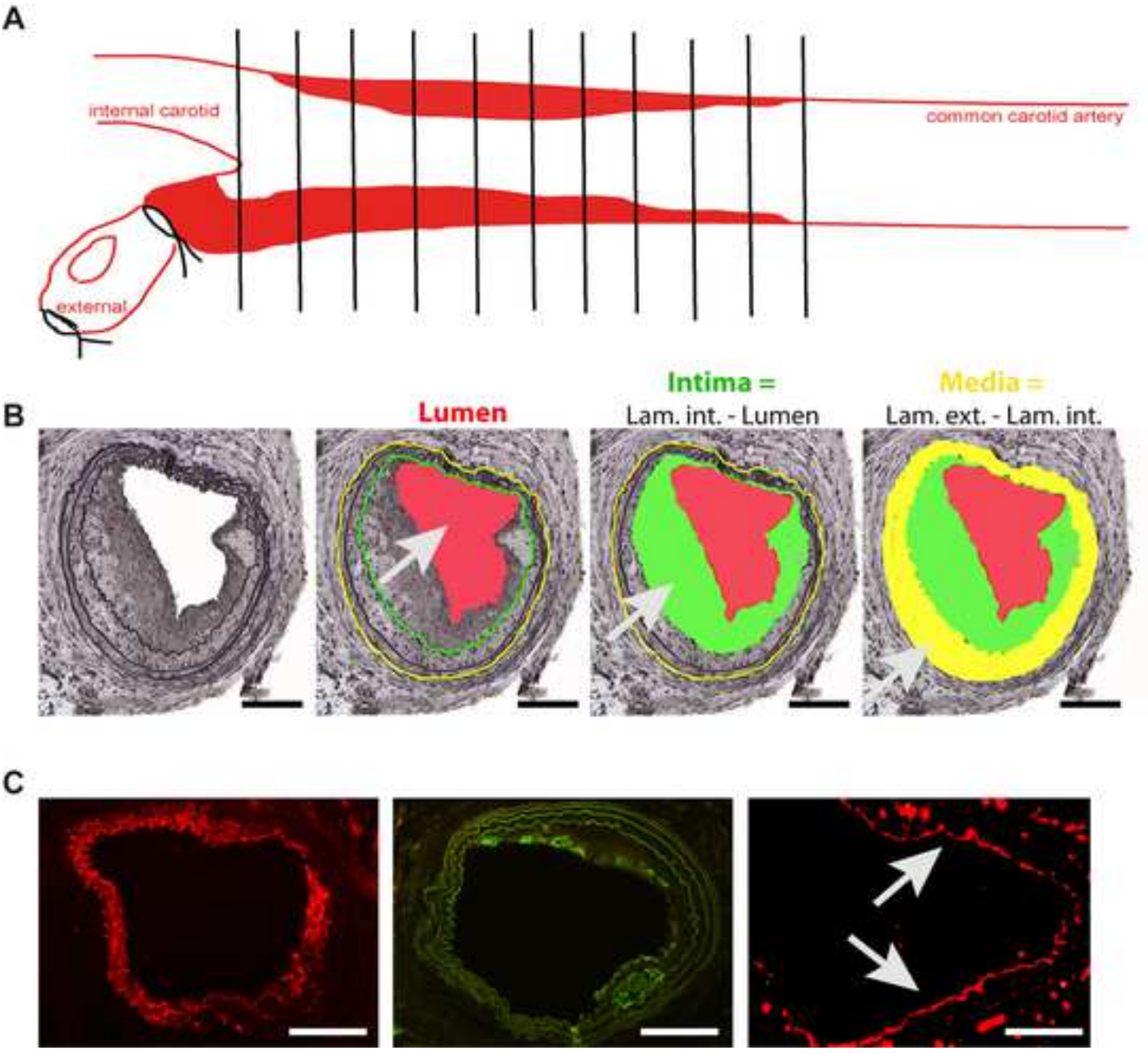

Table 1. Time-dependent Plaque's development

Time	Trombus	Plaque (µm²)	Macrophages (% from Plaque)	Smooth muscle cells (% from Plaque)	Re-endothelialization (% lumen circumference)
1 day	present	0	0	0	0
1 week	-	< 30 000	> 10	< 50	< 50
2 weeks	-	< 50 000	> 10	< 50	> 50
3 weeks	-	< 70 000	15-25	30-40	80-90
4 weeks	-	70 000 – 100 000	15-25	30-40	complete

Table 2: Advantages and disadvantages of existent models of arterial injury.

Model	Animals	Advantages	Disanvantages
Diet-induced native atherosclerosis	Small	- mimics the atherosclerosis pathology - ease of handling - no surgery - no stress for the animals - low costs related to animal purchase and care - availability of various transgenic and knockout strains	- low reproductibility - high variance - increased animal's number required - increased waiting time
	Big	- mimics the atherosclerosis pathology - ease of handling - no surgery - no stress for the animals	- low reproductibility - high variance - increased animal's number required
Balloon dilatation	Small	- mimics restenosis after balloon angioplasty - low costs related to animal purchase and care - availability of various transgenic and knockout strains	- small size of the main arteries - requires qualified surgical expertise - balloons very expensive - denudation is made on the normal arterial wall - existence of appropriate equipment - risks of complications as bleeding or paralysis
	Big	- mimics restenosis after balloon angioplasty - ease of handling - use of devices for humans	- denudation is made on the normal arterial wall
Wire Injury	Small	- mimics restenosis after balloon angioplasty - ease of handling - minimal mortality rate - low costs related to animal purchase and care - availability of various transgenic and knockout strains - no physical impairment	- small size of the main arteries - requires less qualified surgical expertise - denudation is made on the normal arterial wall - existence of appropriate equipment
Stent implantation	Small	- mimics restenosis and thrombosis after stent implantation - low costs related to animal purchase and care - availability of various transgenic and knockout strains	- small size of the main arteries - requires qualified surgical expertise - small stents not available - denudation is made on the normal arterial wall - increased mortality - existence of appropriate equipment - risks of complications as bleeding or paralysis
	Big	- mimics restenosis	- denudation is made on the

		and thrombosis after stent implantation - ease of handling - use of devices for humans	normal arterial wall

Excel Spreadsheet- Table of Materials/Equipment

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Name	Company	Catalogue number	Comments (optional)
Stereomicroscope	Olympus	SZ/X9	-
Forceps	FST, Germany	91197-00	standard tip curved 0,17 mm
Hemostat forceps	FST, Germany	13007-12	curved
Scissors	FST, Germany	91460-11	Straight
Vannas scissor	Aesculap, Germany	OC 498 R	-
Retractors	FST, Germany	18200-10	2.5mm wide
Retractors	FST, Germany	18200-11	5mm wide
Ketamine 10%	CEVA, Germany	-	-
Xylazine 2%	Medistar, Germany	-	-
Bepanthene eye and nose cream	Bayer, Germany	-	-
Silicon tube	IFK Isofluor, Germany	custom-made product	diameter 500µm, section thickness 100 µm, polytetrafluorethylene catheter
PROLENE Suture 6/0	ETHICON	8707H	polypropylene monofilament suture, unresorbable, needle CC-1, 13mm, 3/8 Circle
7/0 Silk	Seraflex	IC 1005171Z	-
Michel Suture Clips	FST, Germany	12040-01	-
Clip Applying Forcep	FST, Germany	12018-12	-
14”Wire for Catheter	Abbot	1000462H	Use 10 cm from stiff part and equalize the ends
Mice	Charles River	Apolipoprotein E -/- mice with C57/Bl6 background	-

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CORRESPONDING AUTHOR:

Name: Liehn, Elisa A.

Department: Institute for Molecular Cardiovascular Research

Institution: RWTH Aachen

Article Title: Induction of accelerated atherosclerosis in mice: the "wire injury" model

Signature: [signature]

Date: 3.02.2016

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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 laminas*[2,4,11]*. 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.