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TITLE:

Implantation of Human-Sized Coronary Stents into Rat Abdominal Aorta Using a Trans-Femoral Access

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KEYWORDS:

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SUMMARY:

This protocol describes the implantation of human coronary stents into the abdominal aorta of rats with an apoE$^{-/-}$ background using a trans-femoral access. Compared with other animal models, murine models carry the advantages of high throughput, reproducibility, ease of handling and housing, and a broad availability of molecular markers.

ABSTRACT:

Percutaneous coronary intervention (PCI), combined with the deployment of a coronary stent, represents the gold standard in interventional treatment of coronary artery disease. In-stent restenosis (ISR) is determined by an excessive proliferation of neointimal tissue within the stent and limits the long-term success of stents. A variety of animal models have been used to elucidate pathophysiological processes underlying in-stent restenosis (ISR), with the porcine coronary and the rabbit iliac artery models being the most frequently used. Murine models provide the advantages of high throughput, ease of handling and housing, reproducibility, and a broad

availability of molecular markers. The apolipoprotein E deficient (apoE$^{-/-}$) mouse model has been widely used to study cardiovascular diseases. However, stents must be miniaturized to be implanted into mice, involving important changes of their mechanical and (potentially) biological properties. The use of apoE$^{-/-}$ rats can overcome these shortcomings as apoE$^{-/-}$ rats allow for the evaluation of human-sized coronary stents while at the same time providing an atherogenic phenotype. This makes them an excellent and reliable model to investigate ISR after stent implantation. Here, we describe, in detail, the implantation of commercially available human coronary stents into the abdominal aorta of rats with an apoE$^{-/-}$ background using a trans-femoral access.

INTRODUCTION:

Percutaneous coronary intervention (PCI), combined with the deployment of a coronary stent, represents the gold standard in interventional treatment of coronary artery disease[1]. The long-term success of stents, however, can be limited by the occurrence of in-stent restenosis (ISR) that is determined by an excessive proliferation of neointimal tissue within the stent[2,3]. ISR may require a re-intervention either with coronary artery bypass or re-PCI. A variety of animal models have been suggested for the study of ISR, each of them featuring advantages and shortcomings. The major drawbacks of the most commonly used porcine coronary and rabbit iliac artery models, albeit developing lesions markedly similar to humans after stent implantation[4,5], are large animal and housing costs which brings up logistical difficulties especially in long-term studies, as well as limitations in handling and equipment. Furthermore, availability of antibodies to cellular proteins of swine and rabbits is limited. On the other hand, murine models provide the major advantages of high throughput and reproducibility, as well as ease of handling, housing, and therefore cost-effectiveness. Furthermore, a higher number of antibodies are available. However, while apolipoprotein E-deficient (apoE$^{-/-}$) mice have been broadly used for the study of atherosclerosis[6-8], they are unsuitable for the study of ISR as stents have to be miniaturized to be implanted into mice, potentially changing the stents' mechanical properties. Moreover, the aortic wall of mice measures between 50 µm in young mice and 85 µm in old mice[9], and stents have to be deployed using pressure levels as low as 2 atm, which might lead to malapposition of the stent[10]. Rats, however, allow for the implantation of commercially available human coronary stents, and demonstrate a vascular healing course similar to larger animals after aortic stent implantation, first reported by Langeveld et al.[11]. This technique originally required a trans-abdominal access, which necessitated a physical constriction of the aorta to achieve a temporary interruption of blood flow. To avoid the potentially associated vessel injury and inflammatory reactions, the technique was later refined by the introduction of a trans-iliac access, which additionally resulted in a higher survival rate of the animals[12].

Because wildtype rats do not develop atherosclerotic lesions[13], apoE$^{-/-}$ rats have been generated using nuclease techniques such as Transcription Activator-Like Effector Nuclease (TALEN)[14], Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR/Cas9)[15], and Zinc Finger (ZF)[16]. ApoE$^{-/-}$ rats have been commercially available since 2011. Providing an atherogenic background, apoE$^{-/-}$ rats allow for a more realistic evaluation of human-sized coronary stents, especially with regards to ISR.

Herein, we describe the method via the transfemoral access route and using a commercially available thin-strut cobalt-chromium drug-eluting stent (DES), however, it can also be applied for the study of other stent types, such as bare metal stents (BMS) or biodegradable stents.

PROTOCOL:

The experiments were performed in accordance with the German animal welfare law (TSchG) and Directive 2010/63/EU pertaining to the protection of animals used for scientific purposes. The official approval for this study was granted by the Governmental Animal Care and Use Committee (Protocol No.: AZ 87-51.04.2010.A065; Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, Recklinghausen, Germany). The study protocol complied with the Guide for the Care and Use of Laboratory Animals. Postoperative pain treatment is based on the recommendations of the German Society for Laboratory Animal Science (GV-SOLAS) as well as Initiative Veterinary Pain Therapy.

1. Basic techniques and common procedures

1.1. Use homozygous apoE$^{-/-}$ Sprague-Dawley rats. Identify the genotype of each animal by using standard methods[17].

1.2. Keep the animals under identical conditions (21 °C ± 2 °C, 60% ± 5% humidity, and a 12 h light/dark cycle) and ensure free access to water and food.

1.3. Carry out all procedures under clean but nonsterile conditions.

1.4. Once the rat is anesthetized, perform all procedures under a surgical microscope at a magnification of 16x.

1.5. Use cotton swabs for compression hemostasis. Gauze swabs (5 cm x 5 cm) soaked with lactated Ringer solution are helpful to keep the groin moist.

1.6. Follow waste disposal regulations to dispose used materials.

2. Preparations before surgery

2.1. Prepare the veterinary drugs before starting the operation. Keep all solutions at room temperature, unless otherwise indicated.

2.2. Anesthetize the rat with an intraperitoneal injection of 100 mg/kg body weight (BW) (S)-ketamine and 8 mg/kg BW xylazine.

2.3. Assess the rat's weight using a weighing scale.

2.4. Place the rat on a heating pad and fix the upper and lower limbs using medical tape. Position the rat with its left hind limb fully extended and as much in line with its spine as possible so as to

create a straight line between femoral artery and aorta. This will facilitate advancing the balloon-mounted stent through the aortic bifurcation.

2.5. Maintain anesthesia with inhalation of 1.5 vol% isoflurane in 97.5% oxygen at a flow rate of 2 L/min.

NOTE: Allow the rat to breathe spontaneously, without intubation.

2.6. Apply eye ointment to prevent eye damage during unconsciousness.

2.7. Shave the fur from the groin and lower abdomen area of the rat and sterilize the corresponding skin with a povidone-iodine solution.

2.8. Before starting the surgery, verify adequate depth of anesthesia by pinching the tail tip and the interdigital tissue. In case of inadequate analgesia, administer buprenorphine (0.05 mg/kg) subcutaneously as analgesic.

3. Surgery

3.1. Using sharp micro scissors, make a medial incision of ~0.5–1 cm in the left groin to open the skin and the underlying fascia.

3.2. Bluntly dissect and probe in the depths until the pulsating left femoral artery can be identified.

3.3. Using very fine forceps, prepare the femoral artery by gently removing the surrounding connective tissue. Be careful to harm neither the femoral nerve nor the femoral vein, which is medial to the artery.

3.4. Prepare about 1 cm of the femoral artery. Carefully put the tip of the forceps under the vessel to gently lift it.

3.5. Thread pieces of 4-0 silk suture under the distal and proximal parts of the artery and form slings. Clamp the ends of each of the two thread slings between the branches of a surgical clamp. Use the surgical clamps to control the artery. Gently stretch and lift the slings in order to temporarily interrupt blood flow.

NOTE: Work fast to avoid a prolonged tourniquet which may lead to tissue damage.

3.6. Using sharp micro scissors, perform an arteriotomy in the middle of the femoral artery.

3.7. Introduce a guide wire through the arteriotomy. When reaching the proximal thread sling, release the tension of the thread by moving the surgical clamp and advance the guide wire further towards the abdominal aorta.

NOTE: Cut the guide wire using a wire cutter to facilitate handling.

3.8. Place the proximal end of the guide wire between the diaphragm and the renal arteries.

NOTE: Advancing the guide wire too far bears the risk of aortic or cardiac injury. We recommend opening the abdomen to ensure adequate positioning of the guide wire and the stent at least for the first several animals.

3.9. Introduce a crimped and balloon-mounted coronary stent measuring 2.25 mm x 8 mm (max. 2.5 mm x 8 mm) over the guide wire into the femoral artery and advance it to the abdominal aorta.

3.10. Place the stent just above the aortic bifurcation but below the renal arteries. Deploy the stent by inflating the balloon catheter to 12 atm for 15 s by using an inflation syringe system.

3.11. Deflate the balloon catheter and maintain negative pressure according to the manufacturer's recommendations for the stent in use.

3.12. Slowly withdraw the deflated catheter while leaving the stent in place.

3.13. Just before taking out the catheter, create tension on the thread loop above the incision with the surgical clamp to interrupt blood flow again. Then remove the balloon catheter and directly ligate the vessel proximally.

3.14. Tie the proximal and the distal thread loops to ligate the femoral artery and confirm adequate hemostasis of the arteriotomy. Collateral arteries will ensure further perfusion to the limb.

3.15. Close the muscle overlying the artery, as well as the skin incision by using 10-0 non-resorbable sutures.

4. Animal care after stent implantation

4.1. Immediately after the operation, allow the rat to recover for 60 min in a special intensive care unit cage with warmed air (30–35 °C) and an oxygen supply.

4.2. Watch the animals carefully until fully recovered. Afterwards, move the rats into a normal cage. Provide ad libitum access to water and food.

4.3. Have the food mixed with clopidogrel (15 mg/kg) to avoid thrombosis of the implanted stent.

4.4. To enhance hypercholesterolemic conditions and plaque formation, start western diet feeding at 6–8 weeks after birth and continue until euthanasia. If desired, a cohort of animals fed normal rat chow can serve as control.

5. Tissue collection and processing

5.1. Before starting the tissue explantation at the designated time point, euthanize the animal according to IACUC guidelines. Harvest the stented aorta for histological analysis at the end of the observation period.

5.2. Open the abdomen by a midline incision and remove the stented segment of the aorta as well as adjacent non-stented parts of the aorta, measuring 0.5 cm each.

5.3. Place the tissue into a solution of 4% buffered formalin for 24 h for fixation.

5.4. Embed the stented arterial tissue in plastic and perform histological and immunohistochemical staining according to standard protocols[18,19].

6. Histomorphometric analysis

6.1. Perform histomorphometric analysis of sequential sections of the proximal, middle, and distal part of the stented aorta by means of a microscope linked to a computer with an appropriate image analysis software.

6.2. Trace the contours of the external elastic lamina (EEL, between adventitia and media), internal elastic lamina (IEL, between media and neointima), and lumen with a graphic drawing tablet. From these values, calculate EEL area, IEL area, and lumen area with the software.

6.3. Calculate the percent cross-sectional area in-stent restenosis (ISR):

$$\mathrm{ISR} = 100 \times \left(1 - \left[\frac{\text{Lumen area}}{\text{IEL area}}\right]\right)$$

6.4. Calculate the total neointimal area (A_i):

$$\mathrm{A_i} = \frac{\text{IEL area}}{\text{Lumen area}}$$

6.5. Measure the neointimal thickness (NIT) over each stent strut as the distance between strut and lumen. Measure the NIT between the stent struts as the distance between IEL and lumen.

NOTE: Alternatively, calculate NIT as

$$\mathrm{NIT} = \frac{(2 \times \mathrm{A_i})}{(\mathrm{P_L} + \mathrm{P_{IEL}})}$$

where P_L and P_{IEL} are the lumen and internal elastic lamina perimeter, respectively[20].

6.6. Perform additional analyses according to the requirements of the study.

REPRESENTATIVE RESULTS:

This protocol describes stent implantation in the abdominal aorta of rats using a trans-femoral access route (**Figure 1**). The first central point of this animal model is that it allows for the deployment of human-sized coronary stents. A commercially available crimped and balloon-mounted coronary stent can be placed into the abdominal aorta of rats. Thus, in addition, the same principle of stent deployment as in humans can be applied. Another advantage of the use of rats is the availability of genetically modified strains, such as apoE$^{-/-}$ rats, which are commercially available.

We recently employed this method to evaluate whether apolipoprotein E-deficient rats are more prone to develop ISR as compared to wildtype rats[21]. From a total of 42 male rats undergoing stent implantation, 36 rats completed the study protocol after 28 days (survival rate = 85.71%). Two rats each died from vessel closure failure, internal hemorrhage, and stent thrombosis. Stents from three animals could not be analyzed because the tissue was seriously damaged or disrupted due to processing failures. Most likely, this happened during the sawing procedure. We recommend training to perform this technique several times before the start of the study.

In the remaining 33 rats, human-sized coronary stents were successfully deployed with no sign of malapposition or vessel injury (**Table 1**). Body weight was similar in wildtype apoE$^{+/+}$ and apoE$^{-/-}$ rats (530.1 ± 15.94 g versus 513.6 ± 16.45 g). Homozygous apoE$^{-/-}$ rats developed markedly elevated neointimal hyperplasia and ISR as compared to wildtype apoE$^{+/+}$ rats (**Figure 2**). Although an apoE$^{-/-}$ background renders animals more susceptible for atherosclerosis, especially when fed western diet, we did not observe any antecedent atherosclerotic plaques in our rats, most likely because a western diet was not started until surgery and the subsequent observation period of four weeks was too short for atherosclerotic lesion development.

FIGURE AND TABLE LEGENDS:

Figure 1: Schema of the stent implantation into the abdominal aorta of rats using a trans-femoral access. (**a**) After interruption of the blood flow, a guide wire is introduced through a medial arteriotomy. (**b**) A crimped and balloon-mounted coronary stent is introduced over the guide wire into the femoral artery. (**c**) The balloon-mounted stent is advanced to the abdominal aorta, where it is deployed by balloon inflation. The stent should be placed above the bifurcation and below the renal arteries.

Figure 2: Representative photomicrographs of Giemsa-stained abdominal aorta at 28 days after stent implantation in western-diet-fed. (**a**) Wildtype *apoE*$^{+/+}$ rats and (**b**) Homozygous *apoE*$^{-/-}$ rats. High power images: NI = neointima, St = stent strut, M = tunica media, L = lumen. Figure has been reproduced with modifications from Cornelissen, A. et al.[21].

Table 1: Outcome of stent implantation in rat abdominal aorta using a trans-femoral access.

DISCUSSION:

This protocol describes the implantation of human-sized coronary stents into the abdominal aorta of apoE$^{-/-}$ rats. Several technical points are worth emphasizing. First, a mismatch between the stent size and the size of the aorta should be avoided. Placing too small a stent can lead to stent malapposition, whereas implantation of a stent that is too large for the aorta can cause overstretch, tearing, and injury of the vessel. Therefore, we recommend using stents between 2.0 and 2.5 mm in diameter, and to keep implantation pressure within the recommended range without overstretching the stent. The most suitable implantation pressure is usually given by the stent manufacturer. Excess injury of the femoral vein and subsequently the vena cava should be avoided because the vessel walls are extremely thin and very easy to injure, resulting in bleeding that is hard to stop. The femoral artery is distinguishable from the femoral vein by pulsation, which should be carefully observed. Another pitfall is the possibility of arterial injury and dissection when introducing the guidewire and / or the balloon catheter. Arterial dissection can be minimized by controlling and stretching the femoral artery distally with slings using silk ties while introducing the balloon catheter. It is imperative to immediately stop advancing the device when resistance is encountered. In this case, small movements between thumb and index finger will help change the direction of the device. In our experience, this is most frequently the case just below the inguinal ligament and further up, when the common iliac artery approaches the bifurcation, as it descends deeper into the retroperitoneal space here. There will certainly be a learning curve for the operator before survival rates are stable and with some experience, the average surgical time is about 20 min.

In humans, stents are usually implanted into severely narrowed atherosclerotic arteries. Although apoE deficiency in general renders animals more susceptible for the development of atherosclerotic lesions, we did not observe any plaque formation in our rats, most likely because western diet feeding was not started until stent implantation. If stent implantation in atherosclerotic lesions is desired, western diet should start at 6–8 weeks after birth and continue until sacrifice. Atherosclerotic lesions in susceptible strains will develop after 7–14 weeks on the high fat diet[22]. So far, only limited data on apoE$^{-/-}$ rats have been published. However, no study reported spontaneous lesion development before the age of 20 weeks[23]. Zhao et al. observed typical atherosclerosis in apoE$^{-/-}$ rats after at least 24 weeks with a continuous increase in plaque burden and lesion severity until sacrifice at 72 weeks[15]. Thus, according to the literature, it is improbable that rats would develop spontaneous atherosclerosis at 14–16 weeks of age. Therefore, we recommend using older rats and to start western diet as early as possible if stent implantation in pre-formed atherosclerotic lesions is desired for the study.

Six animals did not survive the surgery. Two animals died from stent thrombosis in spite of administration of clopidogrel. To reduce stent thrombosis, animals can be pre-treated for 48 h with aspirin or receive an intraperitoneal injection of enoxaparin post-operatively. Introducing clopidogrel one day before surgery might also reduce the risk of thrombosis, but any intensification of the anti-thrombotic therapy at the same time increases the risk of hemorrhage. Stent thrombosis is a common complication of PCI[24-26] and can have several reasons. Potentially, in our study, stent thrombosis fatalities resulted from insufficient balloon inflation and concurrent stent malapposition. In contrast to stent implantation in humans, stent deployment

in the rat abdominal aorta was not controlled by angiography. Therefore, ineffective balloon inflation cannot be detected and corrected during surgery. Similarly, stent deployment might lead to an unintentional occlusion of a branching vessel. Considering that it is not practicable to perform the surgery which requires the use of a surgical microscope under fluoroscopic control, we recommend opening the abdomen to confirm the precise deployment of the stent, at least for the first several procedures. Other potential causes for stent thrombosis might be inflammatory reactions, severe injury, or dissections of the vessel wall. The surgeon must be aware of any clinical signs indicating these complications, and the animals must be inspected each day throughout the observation period.

The rat abdominal aorta measures between 1.8 mm and 3.0 mm in diameter, depending on the animal's weight[27,28]. Advancing a bulky stent through the even smaller femoral and iliac arteries may cause intimal tear and damage to the vessel wall. Therefore, this technique is limited to the implantation of smaller stents (between 2.0 and 2.5 mm in diameter) to avoid overstretching or injury of the vessel wall of the aorta.

Another limitation is the necessity of ligating the femoral artery in order to achieve hemostasis after the procedure, potentially bearing the risk of lower limb ischemia. However, previous studies showed that collateral arteries as well as adaptions of the microvasculature distal to the occlusion are able to maintain lower limb perfusion after ligation of the femoral artery in rats[29], and none of our rats exhibited clinical signs of lower limb ischemia during the observation period. Still, the investigators should be aware of this potential risk, as limb ischemia not only represents a potential cause of post-operative death, but can also potentially induce a systemic inflammatory reaction, potentially biasing the results.

While rats in general are a cost-effective animal model, the use of genetically modified apoE$^{-/-}$ rats increases the cost. Another limitation is that it takes a comparatively long time until atherosclerotic plaques have developed in rats. Furthermore, there are some important hemodynamic differences between the aorta and the coronary arteries that deserve closer attention. Shear stress is higher in the aorta as compared to the coronaries, and bifurcations causing turbulent blood flow are absent. This diminishes the development of intimal hyperplasia and the extent of restenosis.

Restenosis is one of the major factors limiting the long-term success of coronary stents. A variety of animal models have been used to study the pathophysiology of restenosis, each featuring their own advantages and shortcomings. In comparison with other animal models, rats hold the advantage of a high throughput, an ease of handling and housing, reproducibility, as well as cost-effectiveness, while at the same time allowing for the implantation of human-sized coronary stents. The first protocol of abdominal aorta stenting in rats was reported by Langeveld et al.[11]. This model, however, requires a trans-abdominal access to introduce the stent, which is associated with a physical constriction of the aorta to achieve a temporary interruption of blood flow. The resulting manipulation and vessel injury might potentially cause inflammatory reactions, which might not only lead to complications, but also to pronounced ISR[12]. Later, Oyamada et al. modified the protocol by introducing the stent through the common iliac artery[12].

They compared the survival rate between the two different approaches (trans-aorta versus trans-iliac artery) and found a significantly higher mortality rate in animals with trans-abdominally deployed stents (57% versus 11%, $p < 0.05$). Rats most commonly died from thrombosis at the incision / suture site, which is catastrophic when occurring in the abdominal aorta[12]. Further reducing trauma and mimicking the implantation technique in humans more closely, we used a trans-femoral access to introduce the stent and reported a mortality rate of 14%. Two rats each died from vessel closure failure, internal hemorrhage, and stent thrombosis. More recent studies, however, reported mortality rates as low as 6% after stent implantation in the rat abdominal aorta even with the trans-aortic access[30,31]. Still, the combined morbidity and mortality rate was 13.4%, in a study by Nevzati et al. after implantation of magnesium stents into the rat aorta[30]. While neither vessel closure failure nor internal hemorrhage were reported in their series, stent thrombosis was evident in 10.5% of rats[30]. On the other hand, Aquarius et al. did not report any stent thrombosis after treating sidewall aneurysms with flow diverters, however, this study used thinner stent strut devices, and dual antiplatelet therapy was administered to the rats[31]. We tried to strike a balance between the risk of stent thrombosis and bleeding risk and administered clopidogrel and heparin in our study. While this might have reduced the risk of stent thrombosis, which occurred in 4.76% of rats, it also might have been the reason for the comparatively higher risk of bleeding (9.52% of rats), either because of internal hemorrhage or vessel closure failure.

Here, we demonstrated the implantation of a drug-eluting stent into the rat abdominal aorta, but likewise this method can be used for the evaluation of other, similarly sized stent devices, for example bare metal stents or bioresorbable vascular scaffolds.

In summary, abdominal aorta stenting of apolipoprotein E-deficient rats is a reliable and reproducible model to investigate ISR after stent implantation. The model can be extended to the use of older rats, which are more likely to develop atherosclerotic lesions spontaneously, and by testing other devices used for human coronary intervention.

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DISCLOSURES:
The authors have nothing to disclose.

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31 Aquarius, R., Smits, D., Gounis, M. J., Leenders, W. P. J., de Vries, J. Flow diverter implantation in a rat model of sidewall aneurysm: a feasibility study. *Journal of NeuroInterventional Surgery.* **10** (1), 88-92, (2018).

Figure 1

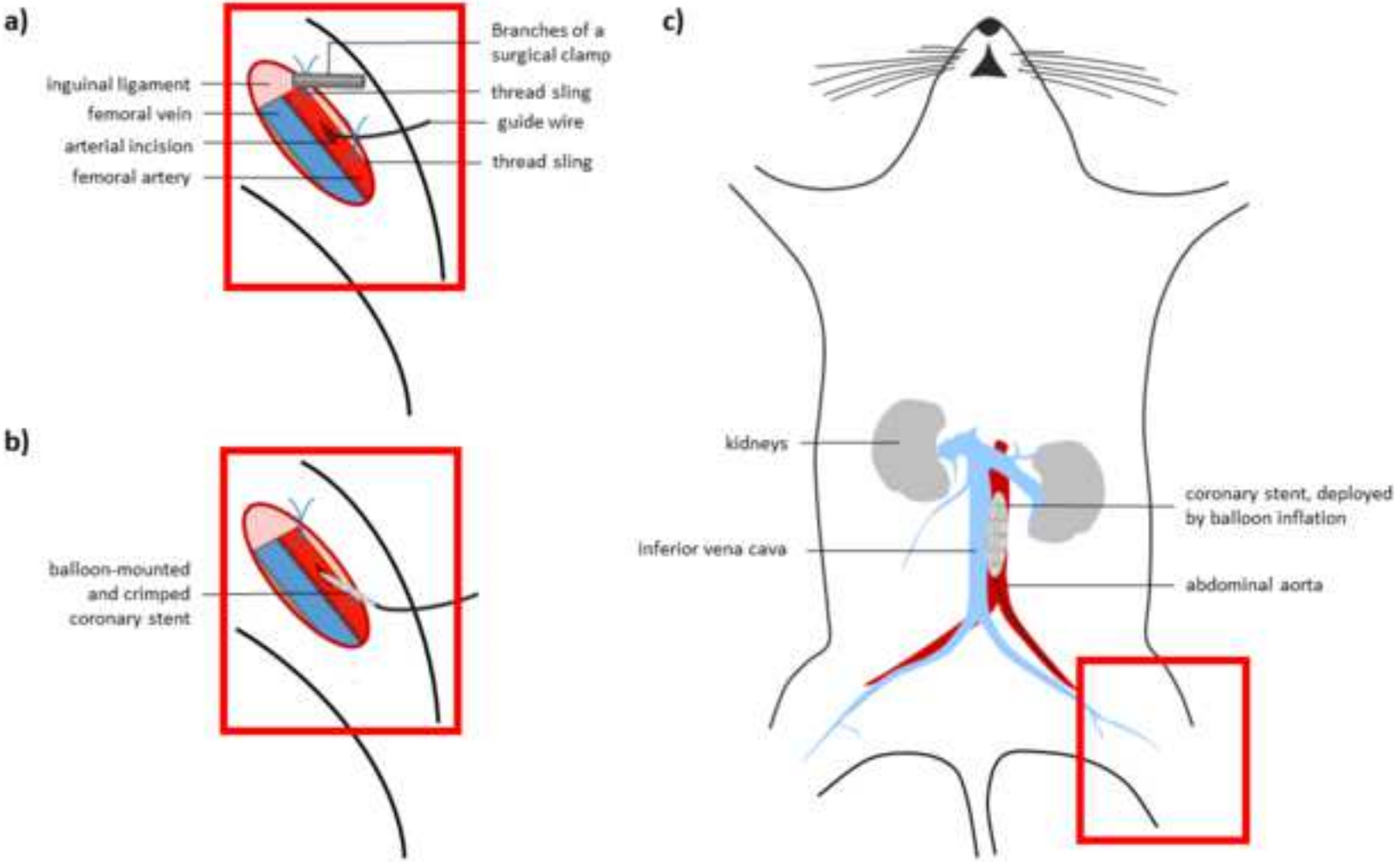
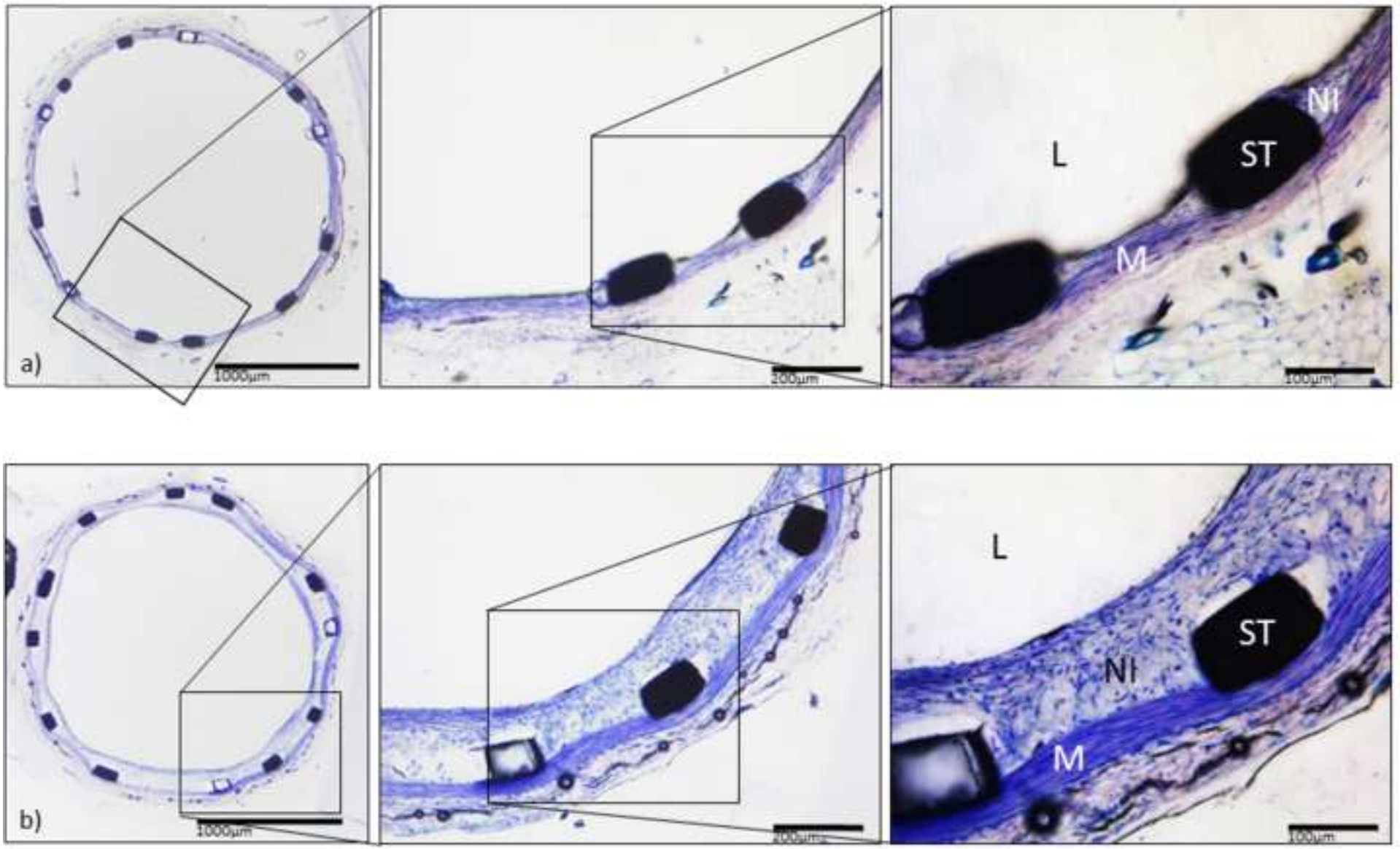


Figure 2

Click here to access/download;Figure;Figure 2.TIF



	number of rats
vessel closure failure	2
internal hemorrhage	2
stent thrombosis	2
tissue processing failure	3
successful completion of the protocol	33

Table of Materials

Name of Material/ Equipment	Company	Catalog Number
Diet		
SNIFF High Fat diet + Clopidogrel (15 mg/kg)	SNIFF Spezialdiäten GmbH, Soest	custom prepared
Drugs and Anesthetics		
Buprenorphine	Essex Pharma	997.00.00
ISOFLO (Isoflurane Vapor) vaporiser	Eickemeyer	4802885
Isoflurane	Forene Abbott	B 506
Isotonic (0.9%) NaCl solution	DeltaSelect GmbH	PZN 00765145
Ringer's lactate solution	Baxter Deutschland GmbH	3775380
(S)-ketamine	CEVA Germany	
Xylazine	Medistar Germany	
Consumable supplies		
10 mL syringes	BD Plastipak	4606108V
2 mL syringes	BD Plastipak	4606027V
6-0 prolene suture	ETHICON	N-2719K
4-0 silk suture	Seraflex	IC 158000
Bepanthen Eye and Nose Ointment	Bayer Vital GmbH	6029009.00.00
Cotton Gauze swabs	Fuhrmann GmbH	32014
Durapore silk tape	3M	1538-1
Poly-Alcohol Skin Desinfection Solution	Antiseptica GmbH	72PAH200
Sterican needle 18 G	B. Braun	304622
Sterican needle 27 3/4 G	B.Braun	4657705
Tissue Paper	commercially available	
Surgical instruments		
Graefe forceps curved x1	Fine Science Tools Inc.	11151-10
Graefe forceps straight	Fine Science Tools Inc.	11050-10

Needle holder Mathieu	Fine Science Tools Inc.	12010-14
Scissors	Fine Science Tools Inc.	14074-11
Semken forceps	Fine Science Tools Inc.	11008-13
Small surgical scissors curved	Fine Science Tools Inc.	14029-10
Small surgical scissors straight	Fine Science Tools Inc.	14028-10
Standard pattern forceps	Fine Science Tools Inc.	11000-12
Vannas spring scissors	Fine Science Tools Inc.	15000-08

Equipment

Dissecting microscope	Leica MZ9	
Temperature controlled heating pad	Sygonix	26857617

Equipment for stent implantation

Drug-eluting stent Xience 2,25mm x 8mm	Abbott Vascular USA	1009544-18
Guide wire Fielder XT PTCA guide wire: 0.014" x 300cm	ASAHI INTECC CO., LTD Japan	AGP140302
Inflation syringe system	Abbott 20/30 Priority Pack	1000186

Tissue processing and analysis

30% H_2O_2	Roth	9681
Ethanol	Roth	K928.1
Giemsas Azur-Eosin-Methylenblau	Merck	109204
Graphic Drawing Tablet	WACOM Europe GmbH	CTL-6100WLK-S
Roti Histofix, Formaldehyd 4% buffered	Roth	P087
Technovit 9100	Morphisto	12225.K1000

	Comments/Description
Western Diet	

Histology

Histology

Histology

Histology

Histology

Editorial comments:

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 thoroughly and all spelling and grammar issues have been corrected.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points.

The manuscript was formatted according to the above mentioned requirements.

3. Please reword lines: 89-92, 93-95, 277-279, as it matches with previously published literature.

Lines 89-95 were reworded:

"Because wildtype rats do not develop atherosclerotic lesions[13], apoE$^{-/-}$ rats have been generated using nuclease techniques such as Transcription Activator-Like Effector Nuclease (TALEN)[14], Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR/Cas9)[15], and Zinc Finger (ZF)[16]. ApoE$^{-/-}$ rats have been commercially available since 2011. Providing an atherogenic background, apoE$^{-/-}$ rats allow for a more realistic evaluation of human-sized coronary stents, especially with regards to ISR."

Lines 277-279 were reworded:

"This model, however, requires a trans-abdominal access to introduce the stent, which is associated with a physical constriction of the aorta to achieve a temporary interruption of blood flow."

4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (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."

The imperative tense was used throughout the protocol, and complete sentences were used.

5. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, alphabets, or dashes.

The numbering of the protocol was adjusted accordingly, and any bullets, alphabets, or dashes were removed.

6. The Protocol should contain only action items that direct the reader to do something.

We ensured that the protocol contains only action items that direct the reader to do something.

7. Please use insert equation function for the all the equations.

The "insert equation function" was applied for equations.

8. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step.

We ensured that each step contains 2-3 actions sentences only.

9. Please use complete sentences throughout.

Any incomplete sentences were changed to complete sentences.

10. Please ensure you answer the "how" question, i.e., how is the step performed?

We took care that the description of each step can answer the "how" question.

11. 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.

1.75 pages identifying the essential steps of the protocol were highlighted for the video.

12. Please ensure the results are described with respect to your experiment, you performed

an experiment, how did it help you to conclude what you wanted to and how is it in line with the title.

The results are described with respect to the experiment and are in line with the title. We applied the protocol to implant human-sized coronary stents into the rat abdominal aorta of apoE$^{-/-}$ rats and present the results of the study in the "representative results" section.

13. Please make a table for 2nd-3rd paragraph of the result section.

We included a table to show the outcome of the study (Table 1).

14. 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]."

Explicit copyright permission was obtained. The figure was cited appropriately. http://creativecommons.org/licenses/by/4.0/

15. As we are a methods journal, 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

The discussion was revised according to the suggested structure above.

16. Please do not abbreviate the journal-title in the reference section.

The full journal title was given in the reference section.

17. Please sort the materials table in alphabetical order.

The materials table was sorted in alphabetical order.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This protocol describes coronary stent insertion through the femoral artery in rats, avoiding aortic surgical trauma. This protocol is really interesting, addressing many drawbacks on animal stent implantation for research.

Major Concerns:

* **One of the main concern is about femoral artery closure. Reading the protocol, it seems that hemostasis is obtained by manual compression of the arteriotomy. It is very likely, regarding the potential intimal damages, that the femoral artery is occluded after hemostasis is obtained, even though the lower limb seems correctly perfused. Moreover, the authors do not report if they checked femoral artery patency before harvesting the legs. In addition to be a potential cause of post-operative death, limb ischemia can induce a systemic inflammatory reaction, and a potential bias.

We thank the reviewer for his/her comment and provided a more detailed description of the temporary tourniquet during the procedure as well as the closure of the arteriotomy. During the surgery, we placed thread loops proximally and distally to the arteriotomy. The end of the threads were clamped into the branches of surgical clamps, and by moving the clamp to the side, we created tension to the threads, and the blood flow was temporarily interrupted. After the procedure, we closed the proximal thread sling, so the femoral artery was ligated. We know that this technique brings the risk of limb ischemia, however, none of the rats showed clinical signs of lower limb ischemia during the observation period. We agree that this is still a drawback of the protocol and discussed this point in the limitations section (lines 500-507).

Minor Concerns:

From a surgical view, some points remain unclear after reading the manuscript:

* I understood that the femoral access was used for stent insertion. The femoral artery has a very small diameter (around 750microMeter), therefore, implying limitations:

The stent is made to be inserted in humans through a 4Fr introducer (internal diameter: 1,32mm). Therefore, stent insertion is very likely to induce important intimal damages.

There is also a high risk of wall tear when the proximal edge of the stent goes through the arteriotomy

These points should be discussed.

We are sorry about the confusion. Unlike human stent implantation, the stent was not introduced though a sheath introducer. However, we agree with the reviewer that both the guidewire and the proximal edge of the stent itself can cause damage to the intima, which cannot be avoided and is an issue with any implantation of intravascular devices, both in animals and humans (e.g. Stathopoulos et al. Guidewire-induced coronary artery perforation and tamponade during PCI: in-hospital outcomes and impact on long-term survival. J Invasive Cardiol. 2014 Aug; 26(8): 371-6). Therefore, we recommend to immediately stop further advancing the device when feeling resistance. We also emphasized that this protocol is limited to smaller stent devices (lines 497-499).

* Stent position is very approximate with deployment about 4-4,5cm after the arteriotomy. Opening the abdomen to precisely deploy the device would not be that time consuming, and would avoid potential misdeployment

We agree with the reviewer that opening the abdomen to ensure precise deployment of the stent is helpful, especially for the first several animal surgeries. To our experience, the surgeon gets more familiar with the technique when he/she has performed the protocol several times, and considering the potential risk of infection that is associated with laparotomy, it is probably not useful to open the abdominal cavity of each single animal. However, we added the advice to open the abdomen at least for the first several animal procedures (lines 215-217). To avoid confusion, the very approximate direction to advance the stent for 4-4.5 cm was removed.

* The authors does not report whether they use a wire cutter to decrease the risk of aortic injury with guidewires about 1.8m long…As long as stents are crimped on coaxial shafts, this is not a problem.

We thank the reviewer for his/her comment and added the advice to cut the wire to facilitate handling (line 211).

* The authors should also emphasize the risk of aortic/cardiaque injury if the guidewire tips goes too far in the aorta

We agree with the reviewer and made the reader aware of the risk of aortic / cardiac injury if the guidewire tip was advanced too far in the aorta (line 215).

* 2 rats died from stent thrombosis. The authors report introducing clopidogrel the day of the procedure. Introducing it one day before could reduce this risk, increasing the risk of per procedure hemorrhage though ! This specific point could be discussed.

We took the reviewer's comment into consideration in the discussion section:

"To reduce stent thrombosis, animals can be pre-treated for 48 hours with aspirin or receive an intraperitoneal injection of enoxaparin post-operatively. Introducing clopidogrel one day before surgery might also reduce the risk of thrombosis, but any intensification of the anti-thrombotic therapy at the same time increases the risk of hemorrhage." (lines 477-480)

Reviewer #2:

Manuscript Summary:

The authors describe an interesting animal model for the implantation of human-sized coronary stents into rat abdominal aorta using a transfemoral access. The surgical procedure has been applied on 42 animals with a mortality rate of 14%, as previously shown by the same group in a different study (PMID: 31796798). The protocol is detailed and seems to provide the necessary information to reproduce the procedure in experienced hands.

However, several concerns arise in this study.

Major Concerns:

1. Please explain how the arteriotomy at the stent insertion site is closed. You mention in the protocol to confirm adequate hemostasis following removal of the balloon catheter, but there is no explanation how you achieve it. This point needs a more detailed explanation, as bleeding at the stent insertion site accounted for a mortality rate of almost 5% in your series.

We agree with the reviewer that this point needs a more detailed explanation. During surgery, we had thread loops placed proximally and distally of the arteriotomy. The ends of the threads were clamped into the branches of vascular clamps, and moving the clamp created tension on the thread, temporarily interrupting blood flow. After the procedure, the thread loops were tied, so the femoral artery was ligated. Knowingly accepting a potential risk of lower limb ischemia, none of our rats showed clinical signs of ischemia. However, as this is a potential drawback of the protocol, we discussed this point in the limitations section (lines 500-507).

2. It would be interesting for the reader to know how long this surgical procedure lasts. Please provide the surgical time, if recorded.

Of course the surgical time depends on the surgeon's expertise and will require more time at the beginning of the study. With some experience, the average surgical time is 20 minutes (line 459-460).

3. Please avoid the term: "the animal has to be killed…." (row 195).

The sentence was changed to "Before starting the tissue explantation at the designated time point, euthanize the animal according to IACUC guidelines." (lines 324-326)

4. In the discussion section you mention how the trans-iliac stent application is associated with significant higher survival rates when compared to the trans-aortal approach (11% vs. 57%) (PMID: 21195423). However, more recent studies demonstrate a low mortality rate (<10%) with the trans-aorta approach, even in combination with more complex vessel surgery (PMID: 28179543; PMID: 28994804). Please consider this information in the discussion.

We thank the reviewer for his/her suggestion. We discussed the mortality rates more thoroughly and added the following passage:

"More recent studies, however, reported mortality rates as low as 6% after stent implantation in the rat abdominal aorta even with the trans-aortic access. Still, the combined morbidity and mortality rate was 13.4%, in a study by Nevzati et al. after implantation of magnesium stents into the rat aorta. While neither vessel closure failure nor internal hemorrhage were reported in their series, stent thrombosis was evident in 10.5% of rats. On the other hand, Aquarius et al. did not report any stent thrombosis after treating sidewall aneurysms with flow diverters, however, this study used thinner stent strut devices, and dual antiplatelet therapy was administered to the rats. We tried to strike a balance between the risk of stent thrombosis and bleeding risk and administered clopidogrel and heparin in our study. While this might have reduced the risk of stent thrombosis, which occurred in 4.76% of rats, it also might have been the reason for the comparatively higher risk of bleeding (9.52% of rats), either because of internal hemorrhage or vessel closure failure." (lines 534ff.)

The above-mentioned studies were cited.

5. The non-visualized deployment of a 2,5x8mm stent might lead to an unintentional occlusion of a branching vessel, especially when considering anatomical variations. This point would be worthwhile to discuss in more detail.

We agree with the reviewer and advised the reader to confirm adequate stent deployment by laparotomy. However, as there is still a risk of side branch occlusion which is also present in human coronary stent implantation, we highlighted this eventuality in the discussion section (lines 485 - 487)

Reviewer #3:

Manuscript Summary:

The authors describe in adequate detail the implantation of human coronary stents into the abdominal aorta of rats using a trans-femoral access. In general, this is a well-written manuscript which adds a nice dimension to existing literature on stent implantation protocols in murine models. The authors outline the advantages of murine models compared with other animal models and demonstrate the efficacy of their protocol which is thoroughly discussed in an unbiased manner.

Major Concerns:

None.

Minor Concerns:

The following suggestions are offered to further improve the manuscript:

* Line 130, (Protocol 2.4): Position the rat with its left hind limb fully extended and as in line with its spine as possible so as to create a straight line between femoral artery and aorta. This will facilitate advancing the balloon-mounted stent through the aortic bifurcation.

We thank the reviewer very much for his/her suggestion. The sentences were added to point 2.4 of the protocol.

* Lines 187-190 (Protocol 4.4), 249-250 (Results) & 317-326 (Discussion): Western type diet should start at 6-8 weeks after birth and continue until sacrifice. Atherosclerotic lesions in susceptible strains will develop after 7-14 weeks on the high fat diet. (https://doi.org/10.1016/B978-0-12-382008-2.00024-6).

Protocol point 4.4. was changed accordingly:

"4.4 To enhance hypercholesterolemic conditions and plaque formation, start western diet feeding at 6-8 weeks after birth and continue until sacrifice."

We also changed the above-mentioned passage in the results part:

"Although an apoE-/- background renders animals more susceptible for atherosclerosis, especially when fed western diet, we did not observe any antecedent atherosclerotic plaques in our rats, most likely because western diet was not started until surgery and the subsequent observation period of four weeks was too short for atherosclerotic lesion development." (lines 404 - 408)

Furthermore, we added the following to the discussion:

"If stent implantation in atherosclerotic lesions is desired, western diet should start at 6-8 weeks after birth and continue until sacrifice. Atherosclerotic lesions in susceptible strains will develop after 7-14 weeks on the high fat diet." (lines 466 ff)

* Line 185 (Protocol 4.3) & 288-289 (Discussion): To reduce stent thrombosis, animals can be pre-treated for 48 h with aspirin or receive intraperitoneal injection of CLEXANE (Enoxaparin) post-operatively

The reviewer's suggestion was added to the discussion part (lines 477 - 480), albeit with the caveat that this might increase the risk of hemorrhage.

* Line 196 (Protocol 5.2): PBS washing and body-perfusion with 4% paraformaldehyde (PFA) solution should be performed via intracardiac puncture before removing the stented segment

We appreciate this suggestion. However, to our experience, body-perfusion with 4% PFA caused artifacts in histology and was therefore not performed.

* Line 305-306 (Discussion): Arterial dissection can be minimized by controlling (and stretching will introducing the balloon catheter) the femoral artery distally with slings using 5/0 or 6/0 silk ties

We thank the reviewer for this advice and added the sentence to the discussion (lines 452-454).

Reviewer #4:

Manuscript Summary:

Stent implantation in rodents is one of the most technically demanded models in the field of vascular biology. This protocol describes a reproducible model to study the mechanism underlying in stent restenosis. Herein, a coronary stent was implanted in the rat aorta. The stent in the descending aorta of the ApoE KO will simulate the restenosis compromising PCI in patients with CAD.

Major Concerns:

1) Implanting the stent without fluoroscopy requires of missing tips in the manuscript to the operator to notice the place for stent deployment.

We agree with the reviewer that some advice how to position the stent correctly is warranted and recommended opening the abdomen to verify accurate positioning at least for the first several rats (lines 215-217).

2) It is unclear whether the stent was deployed on the top of plaque built-up. The vascular reaction to injury will depend on whether the artery had preexisting disease.

We put more emphasis on the lack of preexisting atherosclerotic plaque build-up (lines 404 ff. and 464), and advised the reader to start western diet as early as possible and to continue it for at least 7 weeks if atherosclerotic lesion development is desired before stent implantation (lines 466-469)

3) The use of ApoE null rats increases the cost of the model. The development of atherosclerosis in these animals requires of a long period of HFD feeding. This limitation should be discussed.

We thank the reviewer for his/her comment. Both limitations were added (lines 508-510).

4) The hemodynamics in the descending aorta differs from that in the coronary plexus. The development of intimal hyperplasia is diminished in that area due to high shear stress and the absence of bifurcations.

We thank the reviewer for bringing up this important limitation. We added these considerations to the discussion (lines 510-514).

5) This reviewer doubts that this technique is successfully performed after three animals only.

We agree that the performance also depends on previous experience with small animal surgery. The according sentence was deleted.

6) The histology of stented vessels is quite challenging. The authors need to describe better histological procedures to ensure reproducibility.

We believe that this might be beyond the scope of the protocol. However, adequate sources and protocols were cited.

Minor Concerns:
1) How was the animal weight at the time of surgery?

We added the information on body weight:

"Body weight was similar in wildtype apoE+/+ and apoE-/- rats (530.1 ± 15.94g vs. 513.6 ± 16.45g)." (lines 402-403)

2) Were both female and male used in the study?

Only male rats were used in the study, and this information was added (line 395).