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A Rat Carotid Artery Pressure-Controlled Segmental Balloon Injury with Periadventitial Therapeutic Application

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A Rat Carotid Artery Pressure-Controlled Segmental Balloon Injury with Periadventitial Therapeutic Application

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SUMMARY

The rat carotid artery balloon injury mimics the clinical angioplasty procedure performed to restore blood flow in atherosclerotic vessels. This model induces the arterial injury response by distending the arterial wall, and denuding the intimal layer of endothelial cells, ultimately causing remodeling and an intimal hyperplastic response.

ABSTRACT

Cardiovascular disease remains the leading cause of death and disability worldwide, in part due to atherosclerosis. Atherosclerotic plaque narrows the luminal surface area in arteries thereby reducing adequate blood flow to organs and distal tissues. Clinically, revascularization procedures such as balloon angioplasty with or without stent placement aim to restore blood flow. Although these procedures reestablish blood flow by reducing plaque burden, they damage the vessel wall, which initiates the arterial healing response. The prolonged healing response causes arterial restenosis, or re-narrowing, ultimately limiting the long-term success of these revascularization procedures. Therefore, preclinical animal models are integral for analyzing the

pathophysiological mechanisms driving restenosis, and provide the opportunity to test novel therapeutic strategies. Murine models are cheaper and easier to operate on than large animal models. Balloon or wire injury are the two commonly accepted injury modalities used in murine models. Balloon injury models in particular mimic the clinical angioplasty procedure and cause adequate damage to the artery for the development of restenosis. Herein we describe the surgical details for performing and histologically analyzing the modified, pressure-controlled rat carotid artery balloon injury model. Additionally, this protocol highlights how local periadventitial application of therapeutics can be used to inhibit neointimal hyperplasia. Lastly, we present light sheet fluorescence microscopy as a novel approach for imaging and visualizing the arterial injury in three-dimensions.

INTRODUCTION

Cardiovascular disease (CVD) remains the leading cause of death worldwide[1]. Atherosclerosis is the underlying cause of most CVD-related morbidity and mortality. Atherosclerosis is the build-up of plaque inside arteries that results in a narrowed lumen, hindering proper blood perfusion to organs and distal tissues[2]. Clinical interventions for treating severe atherosclerosis include balloon angioplasty with or without stent placement. This intervention involves advancing a balloon catheter to the site of plaque, and inflating the balloon to compress the plaque to the arterial wall, widening the luminal area. This procedure damages the artery, however, initiating the arterial injury response[3]. Prolonged activation of this injury response leads to arterial restenosis, or re-narrowing, secondary to neointimal hyperplasia and vessel remodeling. During angioplasty the intimal layer is denuded of endothelial cells leading to immediate platelet recruitment and local inflammation. Local signaling induces phenotypic changes in vascular smooth muscle cells (VSMC) and adventitial fibroblasts. This leads to the migration and proliferation of VSMC and fibroblasts inwards to the lumen, leading to neointimal hyperplasia[4,5]. Circulating progenitor cells and immune cells also contribute to the overall volume of restenosis[6]. Where applicable, drug-eluting stents (DES) are the current standard for inhibiting restenosis[7]. DES inhibit arterial re-endothelialization, however, thus creating a pro-thrombotic environment that can result in late in-stent thrombosis[8]. Therefore, animal models are integral for both understanding the pathophysiology of restenosis, and for developing better therapeutic strategies to prolong the efficacy of revascularization procedures.

Several large and small animal models[9] are utilized for studying this pathology. These include balloon-injury[3,10] or wire-injury[11] of the luminal side of an artery, as well as partial ligation[12] or cuff placement[13] around the artery. The balloon and wire injury both denude the endothelial layer of the artery, mimicking what occurs clinically after angioplasty. In particular, balloon-injury models utilize similar tools as in the clinical setting (i.e., balloon catheter). The balloon injury is best performed in rat models, as rat arteries are an appropriate size for commercially available balloon catheters. Herein we describe a pressure-controlled segmental arterial injury, a well-established, modified version of the rat carotid artery balloon injury. This pressure-controlled approach closely mimics the clinical angioplasty procedure, and allows for reproducible neointimal hyperplasia formation two weeks after injury[14,15]. Additionally, this pressure-controlled arterial injury results in complete endothelial layer restoration by 2 weeks after

surgery[16]. This directly contrasts the original balloon injury model, described by Clowes, where the endothelial layer never returns to full coverage[3].

After surgery, therapeutics may be applied to or directed towards the injured artery through several approaches. The method described herein uses periadventitial application of a small molecule embedded in a Pluronic gel solution. Specifically, we apply a solution of 100 µM cinnamic aldehyde in 25% Pluronic-F127 gel to the artery immediately after injury to inhibit neointimal hyperplasia formation[15]. Pluronic-F127 is a non-toxic, thermo-reversible gel able to deliver drugs locally in a controlled manner[17]. Meanwhile, arterial injury is local, hence local administration allows for testing an active principle while minimizing off-target effects. Nevertheless, effective delivery of a therapeutic using this method will depend on the chemistry of the small molecule or biologic used.

PROTOCOL

All methods described here have been approved by the Institutional Animal Care and Use Committee (IACUC) of the University of North Carolina at Chapel Hill.

1. Preoperative procedures

1.1. Sterilize surgical instruments. Autoclave all surgical instruments before surgery. If performing multiple surgeries on the same day, sterilize instruments between surgeries using a dry bead sterilizer.

1.2. Prepare therapeutic in 25% Pluronic-127 gel (diluted in sterile distilled water).

1.3. Set up a 2F Fogarty balloon catheter to the insufflator and place the balloon end of the catheter in a 1 mL syringe filled with saline solution.

1.4. Induce anesthesia by placing the rat in a chamber with 5% isoflurane.

1.4.1. Remove the rat from the chamber and record the rat's weight. Use hair clippers to shave fur on the ventral neck rtegion.

1.4.2. Place the rat back into the chamber with 5% isoflurane to ensure induction of anesthesia.

1.5. Place the rat supine on a surgical platform, inserting the face into the nose cone so that the rat face is toward the surgeon.

1.5.1. Reduce inhalational anesthesia to 1.5% isoflurane. Verify the depth of anesthesia by a toe-pinch reflex on all four feet.

1.5.2. Tape all four legs down to the surgical platform.

1.6. Turn on the heat lamp.

1.7. Inject Atropine (0.01 mg/kg) subcutaneously to reduce airway secretions.

1.8. Inject Carprofen (5 mg/kg) subcutaneously for pain management.

1.9. Apply lubricant eye ointment to both eyes using a sterile cotton swab to prevent corneas from drying during surgery.

1.10. Swab the neck three times in a circular motion alternating between 70% ethyl alcohol followed by Betadine from the center of the shaved region outwards to sterilize the incision site.

1.11. Put on sterile surgical gloves before handling sterile surgical instruments and supplies.

1.12. Lay out all autoclaved surgical instruments on a sterile surgical sheet.

1.13. Cut three independent 1 inch strands of sterile 7-0 Prolene suture.

1.14. Place cotton swabs and gauze on surgical sheet.

1.15. Drape the rat with a sterile surgical sheet that only exposes the sterilized neck region.

1.16. Cut an additional small opening in sheet that exposes part of the nose cone. This will be the site for taping down the balloon catheter during injury.

2. Operative procedures

2.1. During the entire surgical procedure, assess depth of anesthesia by monitoring the respiratory rate (rate should be consistent and deemed normal) as well by toe pinch every 15 min. If respiratory rate increases or there is a response to the toe pinch, then pause surgical manipulation and increase isoflurane up to 2.5%.

2.2. Expose the common carotid artery (CCA).

2.2.1. Make a superficial, straight, longitudinal neckline incision between the jaw bones of the rat. Incision will be approximately 1.5-2 cm in length.

2.2.2. Make an incision through the connective tissue under the skin until the muscle layer is exposed. Displace the salivary glands underneath the skin to access the muscle tissue.

2.2.3. Bluntly separate the connective tissue from the muscle by inserting closed scissors between the muscle layer and connective tissue and gently opening the scissor while pulling the skin upward.

2.3. Dissect the two visible muscles (sternohyoid and sternomastoid) longitudinally along the left side of the trachea until a third muscle (omohyoid) that runs perpendicular to the two superficial muscles is observed.

2.4. Use forceps to create a window separating this perpendicular muscle (omohyoid) from the longitudinal muscle (sternohyoid) running atop the trachea. Gently perform this separation to prevent blunt trauma to the trachea.

2.5. Reach forceps underneath the perpendicular muscle and cut to separate the two longitudinal muscles and expose the CCA.

2.6. Dissect the CCA.

2.6.1. Dissect the CCA near the bifurcation until the internal carotid artery (ICA) and external carotid artery (ECA) are exposed.

2.6.2. Dissect the superior thyroid artery (STA), which branches from the ECA.

2.6.3. Using the pre-cut Prolene sutures, ligate the STA and the ECA near their respective bifurcation. Leave the majority of the suture to one side of the knot and grab each suture with a curved hemostat.

2.6.4. Finish dissecting around the ICA, reach forceps underneath and around the ICA, and use a non-crushing vascular clamp to achieve distal control. Clamp the occipital artery together with the ICA.

2.6.5. Dissect the CCA proximal to the bifurcation, ensuring to separate the vagus nerve from the CCA.

2.6.6. Reach forceps underneath and around the CCA and use a non-crushing vascular clamp to achieve proximal control. Place clamp at least 5 mm from the bifurcation.

2.7. Perform balloon injury.

2.7.1. Maneuver the curved hemostats holding each ligated artery branch to expose the bifurcation between the ECA and superior branch.

2.7.2. Gently dissect tissue at the bifurcation and then make an arteriotomy incision between the ECA and superior branch using microdissection scissors.

2.7.3. Use a cotton swab to push all blood out of the CCA and clean up the arteriotomy site.

2.7.4. Insert the uninflated balloon catheter through the arteriotomy and advance into the CCA until the proximal end of the balloon is past the bifurcation.

2.7.5. Tape catheter to the nose cone so the balloon does not slip out of the artery during inflation.

2.7.6. Slowly inflate the balloon to 5 atmospheres of pressure and leave in the CCA for 5 min to induce arterial injury. Ensure that the pressure stays constant for the entire 5 min.

2.7.7. After 5 min, deflate balloon and gently remove from the CCA through the arteriotomy.

2.7.8. Flush the CCA by gently squeezing on the clamp at the CCA. Do not remove the clamp.

2.7.9. Ligate the ECA proximal to the arteriotomy and then remove the clamps from the CCA and ICA to restore blood flow through the CCA to the ICA. Ensure there is no visible bleeding around the arteriotomy and that the CCA is pulsating.

2.8. Apply 100 µL of therapeutic or Pluronic gel vehicle alone periadventitially along the injured CCA. Do so by applying 50 µL to the left side of the CCA and then 50 µL to the right side of the CCA to ensure even coating of the injured artery.

2.9. Close the wound site.

2.10. Cut excess Prolene sutures.

2.11. Close the wound using interrupted 4-0 or 6-0 vicryl layers along the connective tissue.

2.12. Finish closing the wound using running 4-0 nylon suture along the skin.

3. **Postoperative procedures**

3.1. Place the rat alone in a clean cage with half the cage under a heating lamp and monitor until rat regains sufficient consciousness to maintain sternal recumbency. Keep the rat in a separate cage until the animal is fully alert and mobile before transferring back to their original cage.

3.2. Monitor the rat daily for the next three days and then three times per week until euthanasia. Euthanize using isoflurane overdose followed by bilateral thoracotomy as described below.

3.2.1. If any animal appears to be experiencing pain or develop any neurologic compromise, sacrifice immediately.

3.2.2. For animals that do not receive carprofen, administer acetaminophen 6 mg/mL in their drinking water 24 h prior to surgery through 48 h post-surgery. Acetaminophen provides analgesia with minimal anti-inflammatory effects.

4. **Tissue harvest and imaging**

4.1. Two weeks after surgery, euthanize the rat by overdose of anesthesia (5% isoflurane). Alternatively, euthanize rats at an earlier time point to analyze the various aspects of the arterial injury response.

4.1.1. Once breathing stops perform bilateral thoracotomy as a secondary method of euthanasia.

4.2. Make a lateral incision through the abdomen, and then cut upwards, through the diaphragm and ribs, exposing the thoracic cavity.

4.3. Perfuse and fix the arteries.

4.3.1. Insert an 18 G cannula attached to a gravitational perfusion-fixation system through the left ventricle. Maintain equivalent pressure between rats by marking the height of the perfusion system relative to the benchtop (120 cm elevation, equivalent to 91 ± 3 mmHg).

4.3.2. Clamp the cannula together with the ventricle using a curved hemostat.

4.3.3. Make a cut in the right atrium, opening the vascular circuit, and begin perfusion with PBS followed by 2-4% paraformaldehyde (about 250 mL each).

4.3.4. Prepare paraformaldehyde diluted in PBS the day of sacrifice, or at most the night before sacrifice. If preparing on the day of sacrifice, ensure paraformaldehyde has cooled to room temperature before beginning the perfusion. Store paraformaldehyde at 4 °C.

4.4. After fixation, extract the left and right carotid arteries and store at 4 °C for 2 h in 2-4% paraformaldehyde.

4.5. Transfer arteries to 30% sucrose and store overnight at 4 °C.

4.6. After 16-24 h, embed the arteries in optimal cutting temperature (OCT) compound and freeze OCT-embedded artery blocks.

4.6.1. Condition arteries in OCT at room temperature for 10 min. Place the artery parallel to the plane of the cryomold filled with OCT, marking the side of the cryomold to which the arterial bifurcation is facing. Snap-freeze in liquid nitrogen.

4.6.2. Store frozen blocks long-term at -80 °C.

4.7. Section frozen blocks using a cryostat.

4.7.1. Collect six 5 µm thick arterial cross sections per slide, with slide 1 starting at the bifurcation.

4.7.2. Section frozen blocks until hyperplasia no longer visible (around 100 slides).

4.8. Hematoxylin & eosin (H&E) stain slides[18]

4.8.1. Find the area of injury by staining one in every ten slides along the entire artery starting from the bifurcation (e.g., slides 1, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100).

4.8.2. Stain additional slides around the site of injury to find the slide with peak occlusion (e.g., slides 20, 30, and 40 had visible hyperplasia, thus stain slides 15, 25, 35, and 45).

4.8.3. Stain and quantify the slide with peak occlusion and equidistant slides before and after the peak occlusion slide (e.g., peak occlusion found at slide 35, then stain and quantify slides 25, 45, etc.) for a total of 3-10 slides per rat.

4.9. For light sheet fluorescence microscopy imaging, store arteries overnight at 4 °C after fixation in step 4.4.

4.9.1. Probe artery with 1:500 dilution of rabbit anti-CD31 primary antibody in diluent (pH 7.4) for 3 days. Then counterstain artery with 1:500 dilution of anti-rabbit Alexa Fluor 647 secondary antibody for 2 days[19].

4.9.2. Clear the artery using iDISCO+[20].

4.9.3. Image the artery using a light sheet fluorescence microscope[21]. Render images using software (e.g., Imaris)[19].

4.10. Quantify neointimal hyperplasia. Perform quantification in a blinded manner if possible.

4.10.1. Use ImageJ software to trace the perimeter of the intima, internal elastic lamina (IEL), and external elastic lamina (EEL) of an artery on each of the 3-10 slides determined above (step 4.8.3).

4.10.2. Quantify the area of each traced region in ImageJ and export these values. The intima trace yield the lumen area, the IEL trace yields the IEL area, and the EEL trace yields the EEL area.

4.10.3. Average the values obtained from the 3-10 slides to get the average injury (% occlusion, intima:media (I:M) ratio, neointimal hyperplasia) per rat carotid artery.

$$Neointimal\ Hyperplasia = IEL\ area - Lumen\ area$$

$$I{:}M\ Ratio = \frac{Intima\ area}{Media\ area} = \frac{IEL\ area - Lumen\ area}{EEL\ area - IEL\ area}$$

$$\% \, Occlusion = \frac{IEL \; area - Lumen \; area}{IEL \; area} * 100$$

REPRESENTATIVE RESULTS

Figure 1 shows all of the materials and surgical tools used to perform this surgery. Hematoxylin & eosin (H&E) staining of two-week injured arterial cross sections allows for clear visualization of neointimal hyperplasia. **Figure 2** shows representative images of H&E-stained arterial cross-sections of a healthy, injured, and treated artery. **Figure 2** also outlines how to quantify the level of neointimal hyperplasia in an injured artery using ImageJ, a widely used image processing software. Using this approach, the perimeter of the neointima, as well as the internal and external elastic lamina are traced to quantify the respective areas. The pressure-controlled segmental injury method we describe results in an intima to media ratio of 0.80 with a standard deviation of 0.29 (2 different surgeons and n=11 rats). Treatment with periadventitial application of CA in Pluronic results in an inhibition of neointimal hyperplasia, as we have shown before (61% reduction in percent occlusion)[15].

Figure 3 provides an illustration for creating an optimal arteriotomy at the bifurcation of the ECA and STA. Lastly, **Figure 4** shows how light sheet fluorescence microscopy can be used to visualize the entire region of injury along the length of the artery. CD31 staining to visualize the endothelial cells lining the intimal layer can be performed on fixed arteries. Arteries can then be embedded in 1% agarose and cleared using the iDISCO+ method to homogenize the refractive index of the sample[20]. Then the arteries can be imaged in a light sheet fluorescence microscope and the images can be rendered using software for quantifying the I:M ratio. Using this approach, we obtained an I:M ratio of 0.86, which is in agreement with the H&E results.

FIGURE LEGENDS:

Table 1. Commonly used number of arterial cross-sections for hyperplasia analysis.

Figure 1. Surgical instruments and tools. In clockwise order starting in the upper left corner of the image: (**A**) Cotton swabs; (**B**) Betadine solution; (**C**) Gauze; (**D**) 70% ethyl alcohol solution; (**E**) 1cc syringes with needle; (**F**) Atropine; (**G**) Retractors; bent paper clips used here; (**H**) Rimadyl; (**I**) Micro-serrefine clamp applying forceps; (**J**) Needle holder; (**K**) 4-0 nylon suture; (**L**) 4-0 vicryl suture; (**M**) Sterile drapes; (**N**) Mayo scissors; (**O**) Standard forceps; (**P**) Fine curved forceps; (**Q**) Microdissection scissors; (**R**) Micro serrefine clamps; (**S**) Fine scissors; (**T**) T-pins; (**U**) Curved hemostats; (**V**) Three 7-0 Prolene sutures cut to approximately 1-inch; (**W**) 100 µL of 25% pluronic-127 gel; (**X**) Lubricating eye ointment; (**Y**) 2 French balloon embolectomy catheter in sterile saline solution; (**Z**) Insufflator.

Figure 2. Hematoxylin & Eosin (H&E) staining and analysis of rat carotid artery cross sections. (**A**) Cross section of healthy, uninjured right carotid artery. IEL = Internal Elastic Lamina, EEL = External Elastic Lamina. (**B**) Cross section of two-week injured left carotid artery treated with Pluronic-F127 vehicle. (**C**) Cross section of two-week injured left carotid artery treated with 100 µM cinnamic aldehyde. Scale bar = 100 µm. (**D**) Sectioning schematic of frozen arteries for

quantifying injury. Slide 1 starts at the bifurcation and six arterial sections 5 µm in width are taken per slide. Sectioning typically continues to slide 70 as the injury usually occurs before this slide. (**E**) Cross section of injured left carotid artery treated with Pluronic vehicle (**B**). The innermost black line traces the neointima and delineates the luminal area. The middle yellow line delineates the area of the internal elastic lamina, or tunica intima. The outer blue line delineates the area of the external elastic lamina, or tunica adventitia. Scale bar = 100 µm. (**F**) Calculations used for measuring percent vessel occlusion and intima:media (I:M) ratio based on measurements obtained from (**E**).

Figure 3. Arteriotomy creation. Illustration of the steps to create a proper arteriotomy, and avoiding a false tract. CCA = Common Carotid Artery, ECA = External Carotid Artery, ICA = Internal Carotid Artery, OA = Occipital Artery, STA = Superior Thyroid Artery. Isolate the bifurcation between the ECA and STA branches. Dissect this bifurcation until the area changes to a brighter color, indicating thinning of the arterial wall, and then create an arteriotomy using microdissection scissors. Lift arteriotomy using fine forceps to assist in balloon insertion.

Figure 4. Light sheet fluorescence microscopy to visualize arterial injury. Longitudinal cross sections along the length of the common carotid artery from a 14 week old Sprague Dawley rat with a representative transverse section below. Arteries are stained with CD31 and counterstained with AF647. (**A**) Cross sections of healthy, uninjured right carotid artery. White = CD31, Green = Elastic Lamina, L = Lumen, Scale bar = 200-500 µm. (**B**) Cross sections of injured, left carotid artery treated with Pluronic-F127 vehicle. Arrowheads indicate regions of neointimal hyperplasia. (**C**) Intima to media (I:M) ratio of uninjured and injured carotid artery, with exact value noted for each group (n=1).

DISCUSSION

The rat carotid artery balloon injury is one of the most extensively used and studied restenosis animal models. Both the original balloon injury model[3] and the modified pressure-controlled segmental injury variation[10] have informed many aspects of the arterial injury response that also occurs in humans, with the few limitations being that fibrin-rich thrombus rarely develops and local inflammation is minimal compared to other injury models such as in hypercholesterolemic rabbit or porcine models[9,22]. The rats can also be sacrificed at different time-points to quantify and analyze the different aspects of the arterial injury response. For instance, earlier time points can be used to study aspects of early response to injury such as cell proliferation, phenotypic switch of vascular smooth muscle cells, and the early immune response. We have previously shown that leukocyte infiltration and cell proliferation are maximal between 3 days and 1 week[16]. Intermediate time points can be used to assess the rate of re-endothelialization. The two-week time point is the earliest suggested time point for measuring neointimal hyperplasia as the artery is mostly re-endothelialized at this point[16]. A major limitation for translating this model is that the injury is performed in a healthy artery, whereas this procedure occurs in patients with atherosclerotic disease. This limitation exists in part due to the previous lack of available rat atherosclerosis models[23,24]. However, advances in gene editing technologies have allowed for the development of reliable atherosclerotic rat models[24], which may yield novel insights in studying the pathophysiology of restenosis.

Comparatively, male rats yield a more robust injury than female rats, which typically develop less neointimal hyperplasia possibly due to a protective effect of estrogen[25]. However, the described model is still appropriate to study arterial healing in females. Male rats aging 12-16 weeks, between 300-400 g yield the most robust and reproducible neointimal formation. Rats younger than 12 weeks of age may be used; however, the arteries of these younger rats may be too small for the 2F balloon to easily enter the artery depending on the rat strain. Rats weighing under 200 grams should not be operated on with this model as the balloon does not easily fit through the arteriotomy and can actually tear the artery if forced. Additionally, using rats older than 16 weeks of age may yield a variable response in neointimal formation. Various rat strains can be used for performing this injury model, with Sprague Dawley rats being the most often used throughout the literature[26]. To start the surgery, first get the proper alignment and orientation of the incision site in the neck by feeling for the jaw bones and using the rat nose to find the midline. After the initial incision, dissect the tissue until two longitudinal muscles (sternohyoid and sternomastoid) running parallel to each other are visualized. Use the neck muscle (masseter) as the lower limit of the operation window, towards the head. Separate the parallel muscles, which run towards the body, from each other until a muscle that runs perpendicular to these two is visualized. Cutting the perpendicular muscle will allow for easy retraction of the two parallel muscles, exposing the carotid artery. As the anatomy may vary slightly from each animal, along with their positioning, there may be a minor arterial branch that rests on top of the ICA. This minor branch can be clamped together with the ICA; however, when this small branch is not clamped there should be no issues with performing the procedure. Additionally, make sure to dissect away the vagus nerve from both the ICA and CCA before any clamping and suturing takes place. It is important to be gentle and to avoid nerve damage at this point. If the animal twitches after putting on a clamp that may be a response of the vagus nerve coming in contact with the metal clamp; consider readjusting the clamp.

Arguably the trickiest step of the entire procedure is making the arteriotomy. This is because it is possible to make a ‘false’ arteriotomy, and inserting a balloon through this ‘false’ arteriotomy will cause the balloon to actually run above the artery, rather than inside the artery. If this occurs, then making a new arteriotomy closer to the bifurcation at the CCA is a possible solution, but if the balloon was forced into the artery, then the surgery may not be rescuable. To prevent a ‘false’ arteriotomy (**Figure 3**), dissect the adventitial layer at the ECA and STA bifurcation using fine forceps until the appearance is significantly redder than nearby regions, and that portion of the artery appears to protrude out. Afterwards, use the micro-scissors to create the arteriotomy by quickly inserting one prong of the scissors into the cleared area at the bifurcation and then cutting. After making the arteriotomy, use the fine forceps to lift the opening of the artery and push the balloon into the lumen. The balloon should slide easily through the arteriotomy and into the CCA. Depending on the rat positioning it may be helpful to guide the balloon into the CCA by using fine forceps to gently pull upwards on the outside of the CCA while guiding the balloon catheter into the CCA. After the balloon is inserted into the CCA, tape the catheter down so the balloon does not exit the artery as it is being inflated.

Periadventitial application of the therapeutic allows for local and directed drug delivery only at the site of injury. This approach limits potential off-target effects as well as dosing limitations compared to something delivered systemically by oral, intraperitoneal, or intravenous administrations. Pluronic-F127 is thermo-reversible, meaning it is liquid at cold temperature and gels at room temperature. This allows for the therapeutic to be easily prepared in a liquid solution before the Pluronic gels, while the gel can be evenly applied to the artery immediately after injury. Whereas the top of the CCA is easily accessible to effectively cover the entire region of injury the CCA should be gently lifted to coat the bottom portion of the CCA. However, researchers need to ensure to power the study appropriately to account for potential variability between treated animals. It is important to have an estimation of the expected effect size and the standard deviation of the outcome to power the study appropriately. The limitation of the periadventitial method of delivery is that it is not a clinically relevant approach since a patient's artery is not exposed during an angioplasty, which is performed as a percutaneous procedure. Nevertheless, periadventitial application allows for preliminary testing of molecules and biologics delivered locally to the site of injury[15,27-30].

The current standard method of quantifying neointimal hyperplasia is based on morphometric analysis of H&E stained slides. The injured carotid artery is physically sectioned onto slides in 5 µm slices. These slides are then stained using H&E and images are taken using a light microscope. ImageJ software is then used to measure the areas and perimeters delimited by the intima, internal lamina, and external lamina. Even though we have reported increased precision using 10 slides to quantify hyperplasia[19], no consensus exists in the literature about how many slides to measure, with reported methodology varying from 3 to 10 evenly spaced sections (**Table 1**)[31-36]. An I:M ratio of 0.8 with a standard deviation of 0.29 (n=11) can be expected using this methodology (Range: 0.54-1.51). We and others have previously reported light sheet fluorescence microscopy (LSFM) provides a novel approach to visualizing arterial injury[19,37]. LSFM allows for imaging of the entire carotid artery in the x, y, and z plane. LSFM allows for optical slicing to generate arterial cross-sections for analysis, yielding more precise estimates of hyperplasia (coefficient of variation: 28% by LSFM vs 41% by histology) than traditional histological approaches[19,37]. As seen in **Figure 4**, the I:M ratio obtained by LSFM (0.86, n=1) is comparable to the results we obtained through classical histological analysis (0.8 ± 0.29).

In conclusion, the pressure-controlled segmental injury recapitulates the arterial injury response that occurs after clinical revascularization procedures, making it an ideal model for studying the pathophysiology of restenosis. Periadventitial drug application is a useful proof-of-concept delivery method for assaying the therapeutic efficacy of local drug delivery, and can inform development of targeted systemic drug delivery approaches.

DISCLOSURES

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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Figure 1

Click here to access/download;Figure;Figure 1.pdf

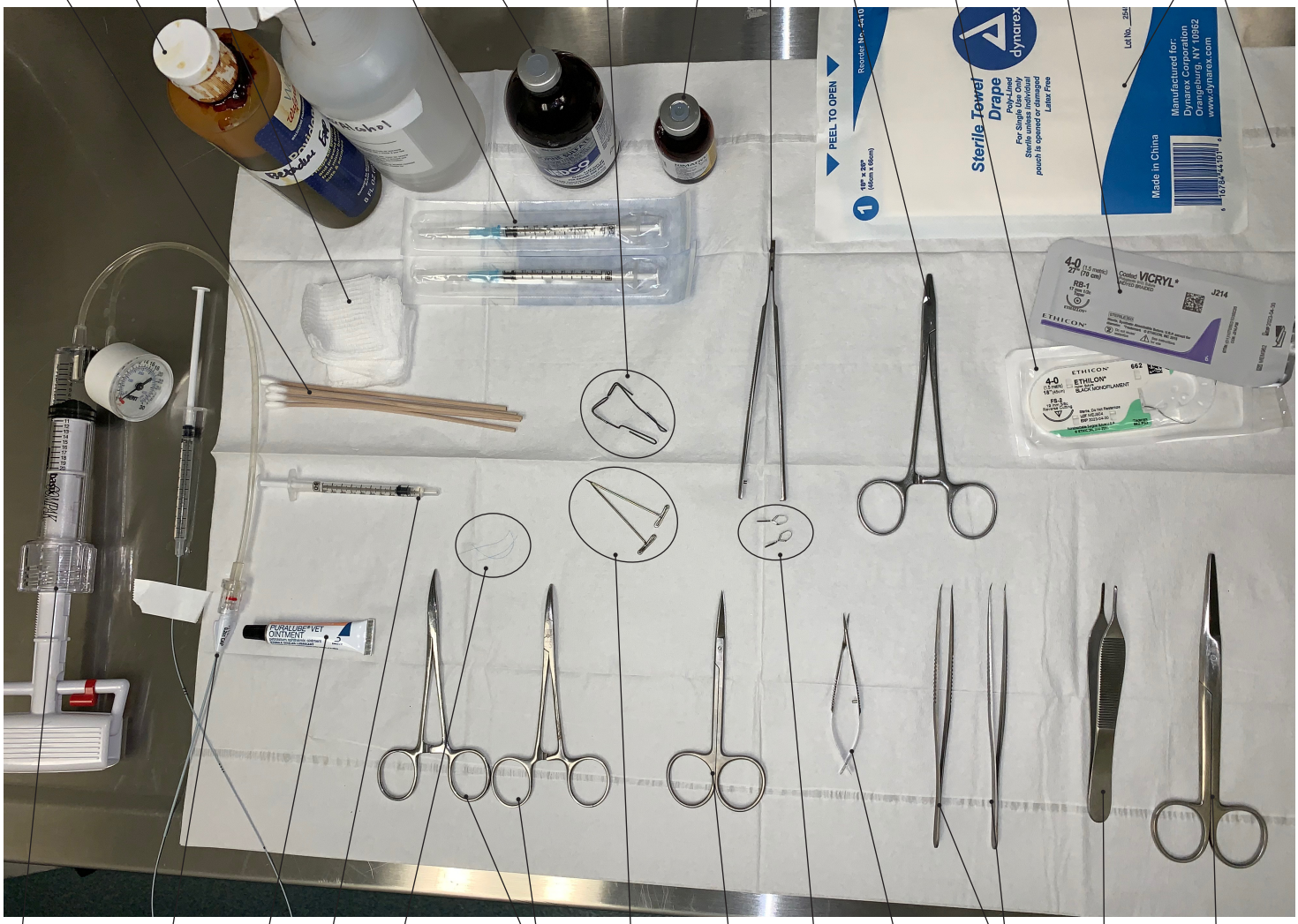
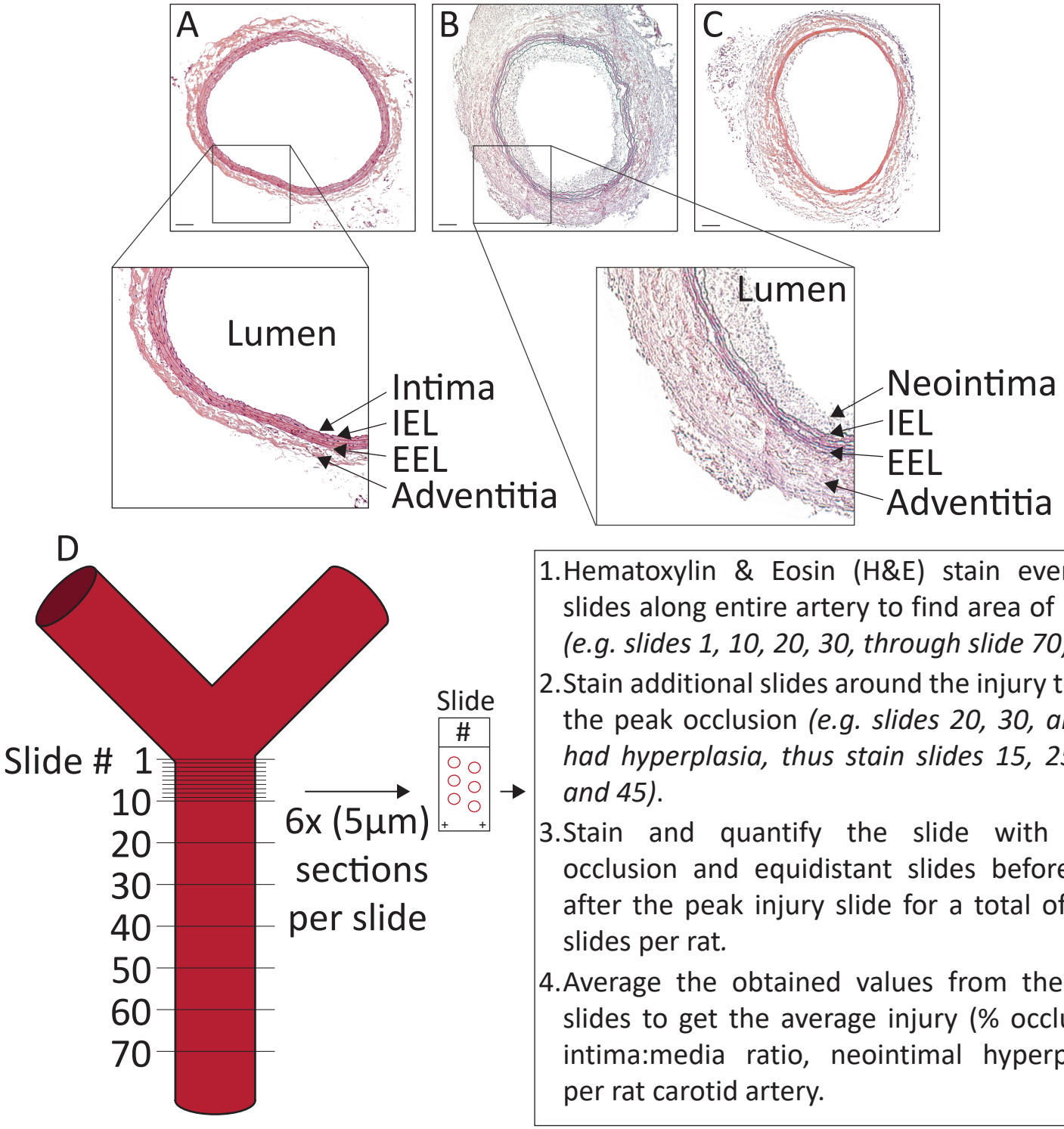
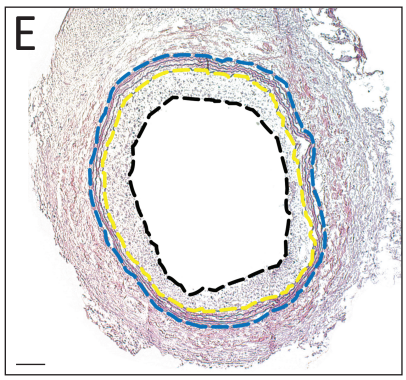


Figure 2



1. Hematoxylin & Eosin (H&E) stain every 10 slides along entire artery to find area of injury *(e.g. slides 1, 10, 20, 30, through slide 70)*.
2. Stain additional slides around the injury to find the peak occlusion *(e.g. slides 20, 30, and 40 had hyperplasia, thus stain slides 15, 25, 35, and 45)*.
3. Stain and quantify the slide with peak occlusion and equidistant slides before and after the peak injury slide for a total of 3-10 slides per rat.
4. Average the obtained values from the 3-10 slides to get the average injury (% occlusion, intima:media ratio, neointimal hyperplasia) per rat carotid artery.



F

$$\% \text{ Vessel Occlusion} = \left(\frac{\text{Area}_{\text{internal}} - \text{Area}_{\text{lumen}}}{\text{Area}_{\text{internal}}} \right) \times 100$$

$$\text{Intima:Media} = \frac{\text{Area}_{\text{intima}}}{\text{Area}_{\text{media}}} = \frac{\text{Area}_{\text{internal}} - \text{Area}_{\text{lumen}}}{\text{Area}_{\text{external}} - \text{Area}_{\text{internal}}}$$

Figure 3

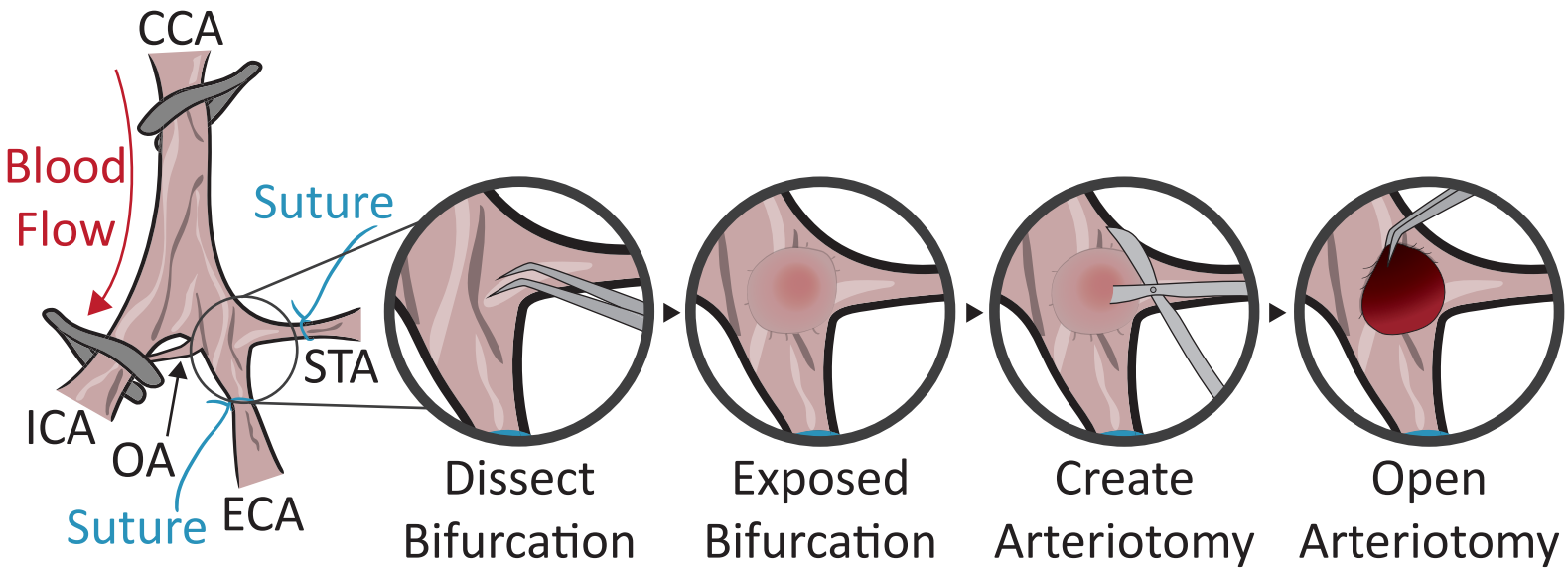
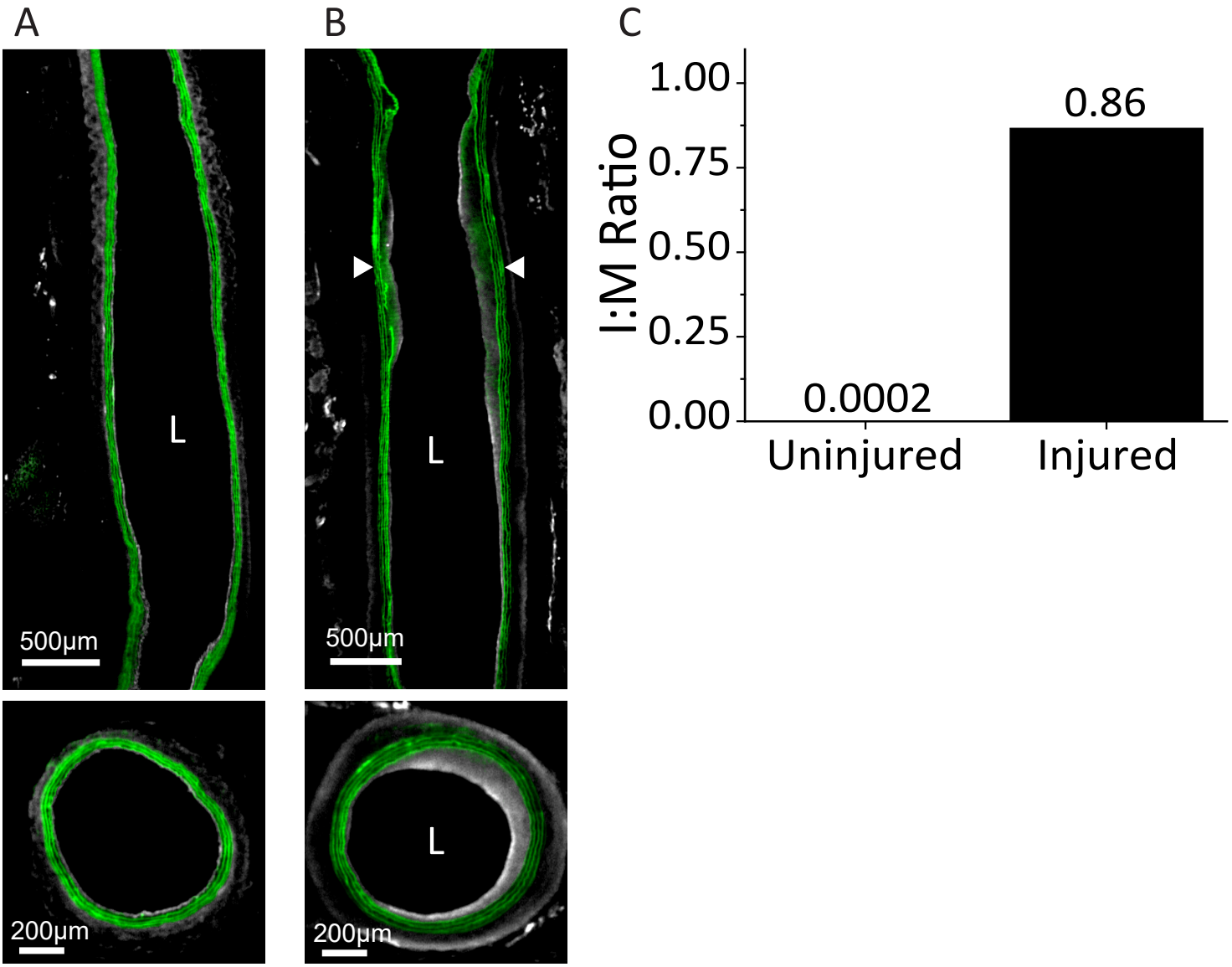


Figure 4



Section Number	Reference
10 sections	27
8 sections	28
6-10 sections	29
6 sections	30
5 sections	31
3 sections	32

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials.xlsx

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1 mL Syringe	Fisher	14955450	
1 mL Syringe with needle	BD	309626	
2 French Fogarty Balloon Embolectomy Catheter	Edwards LifeSciences	120602F	
4-0 Ethilon (Nylon) Suture	Ethicon Inc	662H	
4-0 Vicryl Suture	Ethicon Inc	J214H	
7-0 Prolene Suture	Ethicon Inc	8800H	
70% ethyl alcohol			
Anti-Rabbit Alexa Fluor 647	Thermo Fisher Scientific	A21245	
Atropine Sulfate	Vedco Inc		for veterinary use
Cotton Swabs	Puritan	806-WC	
Curved Hemostats	Fine Science Tools	13009-12	
Fine Curved Forceps	Fine Science Tools	11203-25	
Fine Scissors	Fine Science Tools	14090-11	
Gauze	Covidien	2252	
IHC-Tek Diluent (pH 7.4)	IHC World	IW-1000	
Insufflator	Merit Medical	IN4130	
Iodine solution			
Lubricating Eye Ointment	Dechra		for veterinary use
Mayo Scissors	Fine Science Tools	14010-15	
Micro Serrefines	Fine Science Tools	18055-05	
Microdissection Scissors	Fine Science Tools	15004-08	
Micro-Serrefine Clamp Applying Forceps	Fine Science Tools	18057-14	
Needle Holder	Fine Science Tools	12003-15	
Pluronic-127 (diluted in sterile water)	Sigma-Aldrich	P2443	25% prepared
Rabbit Anti-CD31	Abcam	ab28364	
Retractor			Bent paper clips work well
Rimadyl (Carprofen)	Zoetis Inc		for veterinary use
Saline solution			
Standard Forceps	Fine Science Tools	11006-12	
Sterile Drape	Dynarex	4410	
T-Pins			

We thank the editors and reviewers for their thorough evaluation of our manuscript and for the opportunity to address their critiques. We have revised the manuscript according to these critiques and provide a detail point by point answer below.

Editorial comments:

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

    We have proofread the manuscript

2. Please do not highlight any steps describing euthanasia or anesthesia.

    We have updated the manuscript to not have these steps highlighted.

3. 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 or dashes.

    All numbering has been updated

4. Please do not separate the protocol into several parts. Please number all protocol steps continuously.

    All numbering has been updated

5. Please use h, min, s for time units.

    All units have been updated

6. Line 244: Please specify the euthanasia method.

    Euthanasia method has been specified. At step 21: “Euthanize using isoflurane overdose followed by bilateral thoracotomy as described below.” The full euthanasia protocol is outlined in steps 22-23.1

7. Line 247: Please ensure that all text is written in the imperative tense.

    Updated.

8. Line 297: Please ensure that all text is written in the imperative tense.

    Updated.

Reviewers' comments:

Reviewer #1:

Major Concerns:

The presented data regarding Light Sheet Fluorescent Microscopy is insufficient to support the statement in the discussion line 469-472 "Unlike traditional..." and is misleading. The current manuscript does not provide sufficient comparative data or statistics to support such statement. This needs to be further investigated if it is to be included. If not, any information or figure regarding the light sheet fluorescent microscopy technique should be completely removed from the manuscript.

We thank the reviewer for this comment and for allowing us to clarify this point. We do not intend to overstate the utility of LSFM for analyzing this model. Thus, we updated the text accordingly. This manuscript is an instructional video on how to perform a technique and does not intend to validate a technique. We have updated the manuscript with the

references that validate LSFM for studying vascular injury and have simply mentioned that LSFM allows for visualization of the whole artery, which is indeed shown in the current manuscript [1, 2]. Again we only intend to provide a protocol on how to perform a validated technique.

Minor Concerns:
Operative procedure 3
- The authors should provide an explanation on the handling of the occipital artery. The occipital artery should also be added to the figure 3.

Handling of the occipital artery has been included in figure 3, and in the protocol. The text now reads: “Finish dissecting around the ICA, reach forceps underneath and around the ICA, and use a non-crushing vascular clamp to achieve distal control. Clamp the occipital artery together with the ICA.”

Operative procedure 4
- 4.3 Please explain the reasoning for removing the blood from the vessel lumen prior to balloon injury

This is done so there is a cleaner and clearer view of the artery to insert the balloon catheter. If blood is present at the arteriotomy site, it is more difficult to see the opening. Removing the blood facilitates insertion of the balloon catheter through the arteriotomy.

- 4.8 Please clarify whether the artery is flushed with blood before ligation of the arteriotomy, if not, any risk of air embolus or thrombus?

We thank the reviewer for pointing this out and for the opportunity to clarify this in the manuscript. The artery is indeed flushed and this has been updated in the protocol as step 17.8. “17.8. Flush the CCA by gently squeezing on the clamp at the CCA. Do not remove the clamp.”

Operative procedure
- How is "even coating" ensured?

The full volume of 100 µL of Pluronic gel effectively covers all of the CCA. Lifting the artery ensures that the underside of the CCA receives treatment. This sentence is in the discussion “Whereas the top of the CCA is easily accessible to effectively cover the entire region of injury the CCA should be gently lifted to coat the bottom portion of the CCA.” Additionally, the video portion of this manuscript will clearly demonstrate the technique.

Tissue Harvest and Imaging
- 3.1 Please provide the height from bench and equivalent pressure in mmHg or ATM. Also, have the pressure in the common carotid artery been verified, if so, how?

Thank you very much for pointing this out and providing us with an opportunity to clarify this important issue. As this is an instructional manuscript it is paramount that all methodological aspects are clear. We control the height and calculate pressure according to $P = \rho . g . h$

Where P is pressure in N/m^2
ρ is density in kg/m^3
g is the acceleration of gravity in m/s^2
and h is height in m.

We have verified that the experimentally measured pressure connecting the cannula to a manometer, agrees with the theoretical calculations. We have also checked that within the volumes we fill the bags there is <6% variation in pressure. We keep the pressure at 91 ± 3 mmHg. This information has been added to step 24.1

Reviewer #4:
Manuscript Summary:
The authors provide a methodological approach to induce stenosis in rat carotid using a pressure balloon injury.

Major Concerns:
none

Minor Concerns:
the rat strain should be mentioned

Thank you for noting this omission. We have now included this sentence in the discussion "Various rat strains can be used for performing this injury model, with Sprague Dawley rats being the most often used throughout the literature[3]."

Line 162: the authors should indicate that the salivary glands are present and should be displaced

We appreciate the opportunity to clarify this methodological detail. We have included this sentence in step 15.2 of the protocol "Displace the salivary glands underneath the skin to access the muscle tissue."

Line 233: could the authors comment on the use of a 4-0 suture that seems quick thick for the connective tissue, why aren't they use a 6-0 suture?

There is no particular reason other than 4-0 suture was available in the lab. Importantly, we have not had any issues closing the wound using this suture size. However, we updated step 19.2. to read "Close wound using interrupted 4-0 or 6-0 vicryl layer along the connective tissue."

Line 254: the name of the anesthetic and the dose should be provided.

Thank you for pointing out this important detail and giving us the opportunity to correct this omission. The anesthetic and dose have been included to step 22.

Reviewer #5:
Manuscript Summary:
The authors describe a pressure controlled balloon injury technique that is applied in carotid arteries of rats. The technique seems well suitable to study the pathophysiology of restenosis preclinically.

Major Concerns:
None.

Minor Concerns:
LSFM and CD31 staining:
1. I cannot see the endothelial monolayer on the inner surface of the vessel wall (should be imaged in white), neither in the uninjured vessel nor in the AS plaque area. Please provide better picutures (higher magnification, zoom etc).

> Updated with higher resolution photo.

2. N=1 per group is not sufficient to me. I would appreciate at least 3 animals per group, even though it is qualitative.

> We understand and appreciate the reviewer's concern. However, this manuscript does not try to validate a methodology, it is an instructional on how to perform a technique. Respecting the 3R principles of animal research, we think the sacrifice of extra animal lives is not justified. Especially because there is literature that validates the methodology. We have reference our work[1] and those of others[2] showing the validation of the technique. The results shown here are representative and validation is beyond the scope of the article.

Reviewer #6:
Manuscript Summary:
The manuscript described the procedure of carotid artery balloon injury that create neointimal hyperplasia in a rat model. The pressure-controlled procedure is similar to the angioplasty procedure in the clinics. A pluronic gel solution containing cinnamic aldehyde was applied to the injured common carotid artery (CCA) to inhibit neointimal hyperplasia. The neointimal hyperplasia of injured CCA was observed by cryosection stained with Hematoxylin and Eosin (H & E) and Light Sheet Fluorescent Microscopy (LSFM). Degree of neointimal hyperplasia was quantified by image analysis software. The manuscript provides detailed descriptions regarding preoperative procedure, operative procedure, postoperative procedure, tissue harvest and cryosection, and method of neointimal hyperplasia quantification, which allows readers to be able to repeat the experiments.

Major Concerns:
1. Page 6, operative procedure #5: Please describe the procedure and instrument used to apply the pluronic gel solution intraluminally. It is difficult to understand how to one-mL syringe (Figure 1. W) to apply the gel solution.

> We appreciate the reviewer's comment and the opportunity to clarify. The gel solution is applied periadventitially, not intraluminally. The mouth of the 1mL syringe without a needle attached is adequate to deliver the gel solution. The technique will be clearly demonstrated in the video portion of this article.

2. Page 8, tissue harvest and imaging #8: The materials and method of fluorescent staining and LSFM observation (Figure 4) should be described in the Protocol.

> We thank the reviewer for his suggestion. As a methodological paper, it is important to have clear methods. We have referenced the methodology appropriately and described it in the protocol steps 30-30.3.Including the references to the clearing and imaging steps[4, 5]

Minor Concerns:
1. Page 3, preoperative procedure #4 and 4.1: Please revise the grammar.

Thank you for catching this, we have revised the grammar.

2. Page 8, tissue harvest and imaging #9.3: Please check the misspelling in the "% occlusion" equation.

Thank you for catching this, the misspelling has been corrected.

Reviewer #7:
The paper is written clearly and nicely.

The periadventitial delivery has very limited, if at all, clinical implication. This should be highlighted and discussed. A drug delivered periadventitially is fundamentally different than the clinical application of coated balloon/stent. This is not discussed at all.

We agree with the reviewer's opinion and acknowledge this limitation. However, this is a pre-clinical model and local delivery allows to test therapeutic effects for effectiveness, and even mechanism. The appropriate drug delivery is a challenge in itself and different delivery methods could be appropriate for different drugs: from targeted delivery via systemic injection to local intraluminal balloon-mediated delivery, or even coated stents. We now address this limitation in the discussion "The limitation of the periadventitial method of delivery is that it is not a clinically relevant approach since a patient's artery is not exposed during an angioplasty, which is performed as a percutaneous procedure. Nevertheless, periadventitial application allows for preliminary testing of molecules and biologics delivered locally to the site of injury[15, 22-25]." Additionally, we mention in the end of the discussion that "Periadventitial drug application is a useful proof-of-concept delivery method for assaying the therapeutic efficacy and mechanism of action of local drug delivery, and can inform development of targeted systemic drug delivery approaches."

Several key references on periadventitial delivery in the rat model are missing (see also below) including of polymeric cuffs. Local delivery in the isolated lumen of the injured segment is more relevant in view of the clinically approved double balloon catheter. I would suggest to omit the periadventitial delivery since it is a very simple methodology with pluronic. And refer to references of periadventitial delivery (several types of delivery systems explored in the literature), and the relevancy of the results obtained.

We appreciate the comment and we agree that this delivery and other delivery approaches are well established in the literature. However, the goal of this article is to provide an instructional video of how to perform the technique. We do not claim novelty, we have not found an instructional video of the segmental pressure-controlled model with periadventitial delivery.

The rat model of restenosis, of injuring the carotid artery with a balloon catheter, is a well-known model of restenosis being used for over 20 years, and see papers by the Levy, Topol, Waltenberger, Fishbein groups). Why is it important to describe it now?

This manuscript details an injury model that varies from the classical balloon injury model originally described. Whereas other instructional videos we have found describe the original method of passing the balloon several times throughout the entire common carotid, we describe a technique where the balloon stays stationary at 5 atm for 5 min. This method has been characterized in the literature and shows some differences with the original. Shear et al. use this "modified" method in 1998 and their results describe: "Balloon injury of the common carotid artery in rats creates a reproducible injury

response with the development of a thick neointimal lesion by 14 days post-injury that measures approximately 1.5 times the depth of the medial layer"[6]. However, there is no instructional resource for researchers to perform this variation of the method. We aim to provide such resource. One important difference is that in the original model described by Clowes, the endothelial layer never returns to full coverage of the injured vessel[7]. By contrast, the pressure-controlled segmental injury we perform results in complete restoration of the endothelial layer by 2 weeks[8]. Additionally, this variation of the balloon injury more closely resembles the way angioplasty is performed in the clinic, where an inflated balloon is never passed through a vessel but inflated in the place of stenosis.

Also, there is no real discussion on the limitation of this 'proliferative' model (no atherosclerotic plaque).

> We agree that this is one of the main problems with this pre-clinical model. We have included mention of this in the discussion: "A major limitation for translating this model is that the injury is performed in a healthy artery, whereas this procedure occurs in patients with atherosclerotic disease. This limitation exists in part due to the previous lack of available rat atherosclerosis models[23,24]. However, advances in gene editing technologies have allowed for the development of reliable atherosclerotic rat models[24], which may yield novel insights in studying the pathophysiology of restenosis."

No discussion on the time points for evaluating stenosis- 30 days after injury is the optimal endpoint for assessing restenosis inhibition (for e.g., Indolfi, Esposito et al. 2000 and more).

> Thank you for bringing up this point. We now include the following in the discussion section: "The rats can also be sacrificed at different time-points to quantify and analyze the different aspects of the arterial injury response. We have previously shown that leukocyte infiltration and cell proliferation are maximal between 3 days and 1 week[16]. Therefore, earlier time points can be used to study aspects of early response to injury such as cell proliferation, phenotypic switch of vascular smooth muscle cells, and the early immune response. Intermediate time points can be used to assess the rate of re-endothelialization. The two-week time point is the earliest suggested time point for measuring neointimal hyperplasia as the artery is mostly re-endothelialized at this point[16,8]. Additionally we have previously shown that there is no difference in the I:M ratio between 2 and 8 weeks[8].

Injury can be done by denuding the endothelium of the left common carotid artery by the intraluminal passage of a 2F balloon catheter (introduced through the external carotid artery). The catheter should be passed 3 times with the balloon distended sufficiently with saline to generate a slight resistance, with no need for controlled pressure (see literature).

> We acknowledge that the way we describe the balloon injury is not technique that was originally described. However, there is extensive literature describing both methods, the "original" and the pressure-controlled "modified" injury[9-11]. Hence this method's performance has been described[6]. Shear et al. use this "modified" method in 1998 and their results describe: "Balloon injury of the common carotid artery in rats creates a reproducible injury response with the development of a thick neointimal lesion by 14 days post-injury that measures approximately 1.5 times the depth of the medial layer[6]. However, there is no instructional resource for researchers to perform this variation of the method. We aim to provide such resource. One important difference is that in the original model described by Clowes, the endothelial layer never returns to full coverage of the injured vessel[7]. By contrast, the pressure-

controlled segmental injury we perform results in complete restoration of the endothelial layer by 2 weeks[8]. Additionally, this variation of the balloon injury more closely resembles the way angioplasty is performed in the clinic, where an inflated balloon is never passed through a vessel but inflated in the place of stenosis. In the introduction we claim: “Herein we describe a pressure-controlled segmental arterial injury, a well-established, modified version of the rat carotid artery balloon injury. This pressure-controlled approach closely mimics the clinical angioplasty procedure, and allows for reproducible neointimal hyperplasia formation two weeks after injury[14,15]. Additionally, this pressure-controlled arterial injury results in complete endothelial layer restoration by 2 weeks after surgery[16]. This directly contrasts the original balloon injury model, described by Clowes, where the endothelial layer never returns to full coverage[3].”

Male rats, weighing 350-400 g, are optimal. There is no discussion on the difference between rat species.

Thank you for pointing this out and allowing us an opportunity to address it. The weight has been included in the relevant sentence in the discussion “Male rats aging 12-16 weeks, between 300-400 g yield the most robust and reproducible neointimal formation.” We agree that different strains and the different sexes respond differently to the carotid balloon injury. However, the goal of this manuscript is to describe the pressure-controlled segmental balloon injury. Evaluation of the response between different rat strains is beyond this scope of this manuscript.

Rats' arteries should be cut at 8-10 sites (600 µm apart), and sections of 5-6 µm mounted and stained with Verhoeff's elastin stain should be analyzed. The latter staining reveals important features in comparison to H&E. Eight to 10 sections of each rat artery that underwent computerized morphometric analysis should be performed by at least two researchers blinded to the treatment type. Should discuss if averaged data is used or most narrow section? The residual lumen, the area bounded by the internal elastic lamina (original lumen), and the area circumscribed by the external elastic lamina (total arterial area) are being measured.

We agree with the reviewer that 10 equally distributed sections results in more precise estimate of stenosis. We recommend this in the discussion and methods. However, we think we cannot ignore that there is no consensus in the literature as to the way to analyze hyperplasia. Careful review of the literature shows reports varying from 3 to 10 slides. We discuss this fact, and recommend the use of 10 slides. In a different manuscript, we have actually studied how precision increases and how 10 slides approaches the precision of 3D volumetric assessment[1]. In this manuscript we state in the protocol that we assess slides centered around the site of maximal injury. We average the slides per rat as technical replicates obtaining one I/M estimate per rat that is used in subsequent analysis. We agree that the analysis should be performed in a blinded manner. Even though that is not always feasible for every lab (lab with one or two people), we now explicitly recommend blinded analysis in the methods. We also note in the protocol (step 31-31.3.) to quantify up to 10 slides around the narrowest section, and average those results for the final measurement of neointimal hyperplasia.

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