HHS Public Access

Author manuscript

J Vis Exp. Author manuscript; available in PMC 2021 January 09.

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

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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,

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Disclosures

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

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 instent 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. 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.
2. Prepare therapeutic in 25% Pluronic-127 gel (diluted in sterile distilled water).
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.
4. Induce anesthesia by placing the rat in a chamber with 5% isoflurane.
    1. Remove the rat from the chamber and record the rat's weight. Use hair clippers to shave fur on the ventral neck region.
    2. Place the rat back into the chamber with 5% isoflurane to ensure induction of anesthesia.
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. Reduce inhalational anesthesia to 1.5% isoflurane. Verify the depth of anesthesia by a toe-pinch reflex on all four feet.
    2. Tape all four legs down to the surgical platform.
6. Turn on the heat lamp.
7. Inject Atropine (0.01 mg/kg) subcutaneously to reduce airway secretions.
8. Inject Carprofen (5 mg/kg) subcutaneously for pain management.
9. Apply lubricant eye ointment to both eyes using a sterile cotton swab to prevent corneas from drying during surgery.
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.
11. Put on sterile surgical gloves before handling sterile surgical instruments and supplies.
12. Lay out all autoclaved surgical instruments on a sterile surgical sheet.
13. Cut three independent 1 inch strands of sterile 7–0 Prolene suture.
14. Place cotton swabs and gauze on surgical sheet.
15. Drape the rat with a sterile surgical sheet that only exposes the sterilized neck region.

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

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. Expose the common carotid artery (CCA).
    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. 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.
    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.
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.
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.
5. Reach forceps underneath the perpendicular muscle and cut to separate the two longitudinal muscles and expose the CCA.
6. Dissect the CCA.
    1. Dissect the CCA near the bifurcation until the internal carotid artery (ICA) and external carotid artery (ECA) are exposed.
    2. Dissect the superior thyroid artery (STA), which branches from the ECA.
    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.
    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.
    5. Dissect the CCA proximal to the bifurcation, ensuring to separate the vagus nerve from the CCA.

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.

7. Perform balloon injury.
    1. Maneuver the curved hemostats holding each ligated artery branch to expose the bifurcation between the ECA and superior branch.
    2. Gently dissect tissue at the bifurcation and then make an arteriotomy incision between the ECA and superior branch using microdissection scissors.
    3. Use a cotton swab to push all blood out of the CCA and clean up the arteriotomy site.
    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.
    5. Tape catheter to the nose cone so the balloon does not slip out of the artery during inflation.
    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.
    7. After 5 min, deflate balloon and gently remove from the CCA through the arteriotomy.
    8. Flush the CCA by gently squeezing on the clamp at the CCA. Do not remove the clamp.
    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.

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.

9. Close the wound site.
    1. Cut excess Prolene sutures.
    2. Close the wound using interrupted 4–0 or 6–0 vicryl layers along the connective tissue.
    3. Finish closing the wound using running 4–0 nylon suture along the skin.

3. Postoperative procedures

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

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.
    1. Once breathing stops perform bilateral thoracotomy as a secondary method of euthanasia.
2. Make a lateral incision through the abdomen, and then cut upwards, through the diaphragm and ribs, exposing the thoracic cavity.
3. Perfuse and fix the arteries.
    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).
    2. Clamp the cannula together with the ventricle using a curved hemostat.
    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. 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. After fixation, extract the left and right carotid arteries and store at 4 °C for 2 h in 2–4% paraformaldehyde.
5. Transfer arteries to 30% sucrose and store overnight at 4 °C.

6. After 16–24 h, embed the arteries in optimal cutting temperature (OCT) compound and freeze OCT-embedded artery blocks.
    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.
    2. Store frozen blocks long-term at −80 °C.
7. Section frozen blocks using a cryostat.
    1. Collect six 5 μm thick arterial cross sections per slide, with slide 1 starting at the bifurcation.
    2. Section frozen blocks until hyperplasia no longer visible (around 100 slides).
8. Hematoxylin & eosin (H&E) stain slides[18]
    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).
    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).
    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.
9. For light sheet fluorescence microscopy imaging, store arteries overnight at 4 °C after fixation in step 4.4.
    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].
    2. Clear the artery using iDISCO+[20].
    3. Image the artery using a light sheet fluorescence microscope[21]. Render images using software (e.g., Imaris)[19].
10. Quantify neointimal hyperplasia. Perform quantification in a blinded manner if possible.
    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).

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

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.

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 , 28 , 29 , 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 , 32 , 33 , 34 , 35 , 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

N.E.B. was supported by a training grant from the National Institute of Environmental Health Sciences (5T32ES007126-35, 2018), and an American Heart Association pre-doctoral fellowship (20PRE35120321). E.S.M.B. was a KL2 scholar partially supported by the UNC Clinical and Translational Science Award-K12 Scholars Program (KL2TR002490, 2018), and the National Heart, Lung, and Blood Institute (K01HL145354). The authors thank Dr. Pablo Ariel of the UNC Microscopy Services Laboratory for assisting with LSFM. Light Sheet Fluorescence Microscopy was performed at the Microscopy Services Laboratory. The Microscopy Services Laboratory, Department of Pathology and Laboratory Medicine, is supported in part by P30 CA016086 Cancer Center Core Support Grant to the UNC Lineberger Comprehensive Cancer Center.

References

1. Association AH Cardiovascular Disease: A Costly Burden for America, Projections Through 2035. American Heart Association CVD Burden Report. (2017).
2. Singh RB, Mengi SA, Xu YJ, Arneja AS, Dhalla NS Pathogenesis of atherosclerosis: A multifactorial process. Experimental and Clinical Cardiology. 7 (1), 40–53 (2002). [PubMed: 19644578]
3. Clowes AW, Reidy MA, Clowes MM Mechanisms of stenosis after arterial injury. Laboratory Investigation. 49 (2), 208–215 (1983). [PubMed: 6876748]
4. Clowes AW, Reidy MA, Clowes MM Kinetics of cellular proliferation after arterial injury. I. Smooth muscle growth in the absence of endothelium. Laboratory Investigation. 49 (3), 327–333 (1983). [PubMed: 6887785]
5. Sartore S et al. Contribution of adventitial fibroblasts to neointima formation and vascular remodeling: from innocent bystander to active participant. Circulation Research. 89 (12), 1111–1121 (2001). [PubMed: 11739275]
6. Tanaka K et al. Circulating progenitor cells contribute to neointimal formation in nonirradiated chimeric mice. The FASEB Journal. 22 (2), 428–436 (2008). [PubMed: 17848623]
7. Henry M et al. Carotid angioplasty and stenting under protection. Techniques, results and limitations. The Journal of Cardiovascular Surgery (Torino). 47 (5), 519–546 (2006).
8. Kounis NG et al. Thrombotic responses to coronary stents, bioresorbable scaffolds and the Kounis hypersensitivity-associated acute thrombotic syndrome. Journal of Thoracic Disease. 9 (4), 1155–1164 (2017). [PubMed: 28523173]
9. Jackson CL Animal models of restenosis. Trends in Cardiovascular Medicine. 4 (3), 122–130 (1994). [PubMed: 21244891]
10. Shears LL 2nd et al. Efficient inhibition of intimal hyperplasia by adenovirus-mediated inducible nitric oxide synthase gene transfer to rats and pigs in vivo. Journal of the American College of Surgeons. 187 (3), 295–306 (1998). [PubMed: 9740187]

11. Takayama T et al. A murine model of arterial restenosis: technical aspects of femoral wire injury. Journal of Visualized Experiments. (97) (2015).
12. Zhang LN, Parkinson JF, Haskell C, Wang YX Mechanisms of intimal hyperplasia learned from a murine carotid artery ligation model. Current Vascular Pharmacology. 6 (1), 37–43 (2008). [PubMed: 18220938]
13. Jahnke T et al. Characterization of a new double-injury restenosis model in the rat aorta. Journal of Endovascular Therapy. 12 (3), 318–331 (2005). [PubMed: 15943507]
14. Gregory EK et al. Periadventitial atRA citrate-based polyester membranes reduce neointimal hyperplasia and restenosis after carotid injury in rats. American Journal of Physiology-Heart and Circulatory Physiology. 307 (10), H1419–1429 (2014). [PubMed: 25239800]
15. Buglak NE, Jiang W, Bahnson ESM Cinnamic aldehyde inhibits vascular smooth muscle cell proliferation and neointimal hyperplasia in Zucker Diabetic Fatty rats. Redox Biology. 19, 166–178 (2018). [PubMed: 30172101]
16. Bahnson ES et al. Long-term effect of PROLI/NO on cellular proliferation and phenotype after arterial injury. Free Radical Biology and Medicine. 90, 272–286 (2016). [PubMed: 26627935]
17. Gilbert JCW,C; Davies MC; Hadgraft J The behaviour of Pluronic F127 in aqueous solution studied using fluorescent probes. International Journal of Pharmaceutics. 40 (1–2), 93–99 (1987).
18. Tulis DA Histological and morphometric analyses for rat carotid balloon injury model. Methods in Molecular Medicine. 139 31–66 (2007). [PubMed: 18287663]
19. Buglak NE et al. Light Sheet Fluorescence Microscopy as a New Method for Unbiased Three-Dimensional Analysis of Vascular Injury. Cardiovascular Research. (2020).
20. Renier N et al. iDISCO: a simple, rapid method to immunolabel large tissue samples for volume imaging. Cell. 159 (4), 896–910 (2014). [PubMed: 25417164]
21. Ariel P UltraMicroscope II - A User Guide. (2018).
22. Touchard AG, Schwartz RS Preclinical restenosis models: challenges and successes. Toxicologic Pathology. 34 (1), 11–18 (2006). [PubMed: 16507539]
23. Xiangdong L et al. Animal models for the atherosclerosis research: a review. Protein Cell. 2 (3), 189–201 (2011). [PubMed: 21468891]
24. Chen H, Li D, Liu M Novel Rat Models for Atherosclerosis. Journal of Cardiology and Cardiovascular Sceinces. 2 (2), 29–33 (2018).
25. Xing D, Nozell S, Chen YF, Hage F, Oparil S Estrogen and mechanisms of vascular protection. Arteriosclerosis, Thrombosis, and Vascular Biology. 29 (3), 289–295 (2009).
26. Tulis DA Rat carotid artery balloon injury model. Methods in Molecular Medicine. 139, 1–30 (2007). [PubMed: 18287662]
27. Pellet-Many C et al. Neuropilins 1 and 2 mediate neointimal hyperplasia and re-endothelialization following arterial injury. Cardiovascular Research. 108 (2), 288–298 (2015). [PubMed: 26410366]
28. Wu B et al. Perivascular delivery of resolvin D1 inhibits neointimal hyperplasia in a rat model of arterial injury. Journal of Vascular Surgery. 65 (1), 207–217 e203 (2017). [PubMed: 27034112]
29. Tan J, Yang L, Liu C, Yan Z MicroRNA-26a targets MAPK6 to inhibit smooth muscle cell proliferation and vein graft neointimal hyperplasia. Scientific Reports. 7, 46602 (2017). [PubMed: 28429763]
30. Pearce CG et al. Beneficial effect of a short-acting NO donor for the prevention of neointimal hyperplasia. Free Radical Biology and Medicine. 44 (1), 73–81 (2008). [PubMed: 18045549]
31. Cao T et al. S100B promotes injury-induced vascular remodeling through modulating smooth muscle phenotype. Biochimica et Biophysica Acta - Molecular Basis of Disease. 1863 (11), 2772–2782 (2017). [PubMed: 28693920]
32. Madigan M, Entabi F, Zuckerbraun B, Loughran P, Tzeng E Delayed inhaled carbon monoxide mediates the regression of established neointimal lesions. Journal of Vascular Surgery. 61 (4), 1026–1033 (2015). [PubMed: 24418641]
33. Khurana R et al. Angiogenesis-dependent and independent phases of intimal hyperplasia. Circulation. 110 (16), 2436–2443 (2004). [PubMed: 15477408]

34. Tsihlis ND, Vavra AK, Martinez J, Lee VR, Kibbe MR Nitric oxide is less effective at inhibiting neointimal hyperplasia in spontaneously hypertensive rats. Nitric Oxide. 35, 165–174 (2013). [PubMed: 24149190]

35. Chen J et al. Inhibition of neointimal hyperplasia in the rat carotid artery injury model by a HMGB1 inhibitor. Atherosclerosis. 224 (2), 332–339 (2012). [PubMed: 22857898]

36. Mano T, Luo Z, Malendowicz SL, Evans T, Walsh K Reversal of GATA-6 downregulation promotes smooth muscle differentiation and inhibits intimal hyperplasia in balloon-injured rat carotid artery. Circulation Research. 84 (6), 647–654 (1999). [PubMed: 10189352]

37. Becher T et al. Three-Dimensional Imaging Provides Detailed Atherosclerotic Plaque Morphology and Reveals Angiogenesis after Carotid Artery Ligation. Circulation Research. 126, (5), 619–632 (2020). [PubMed: 31914850]

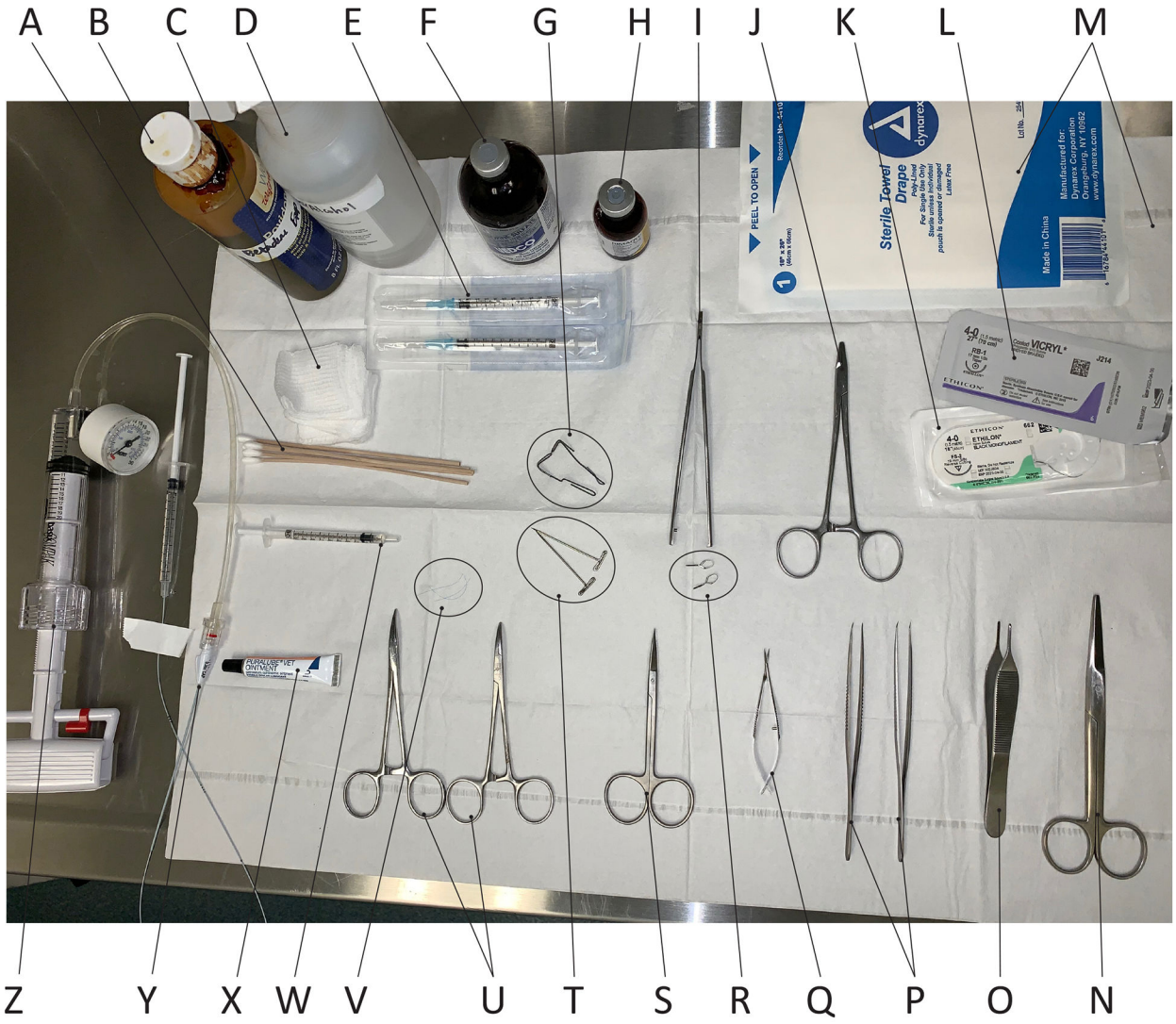

Figure 1. Surgical instruments and tools.

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.

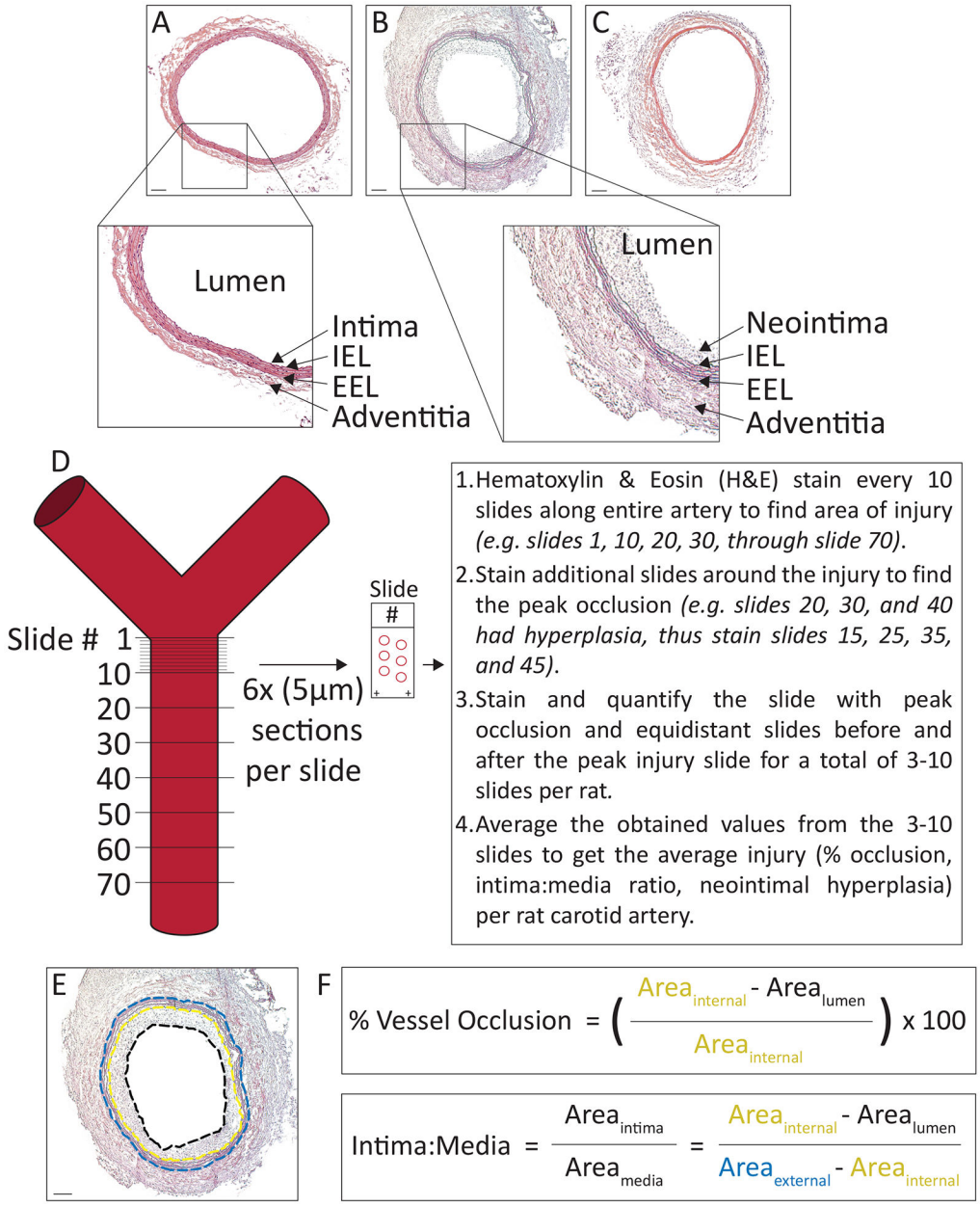

Figure 2. Hematoxylin & Eosin (H&E) staining and analysis of rat carotid artery cross sections.

(**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**).

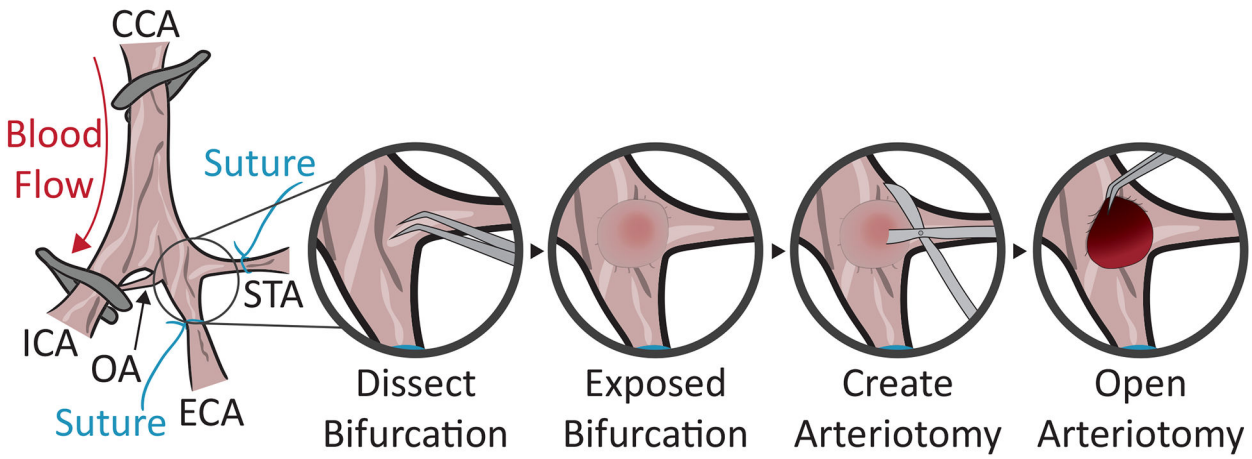

Figure 3. Arteriotomy creation.

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.

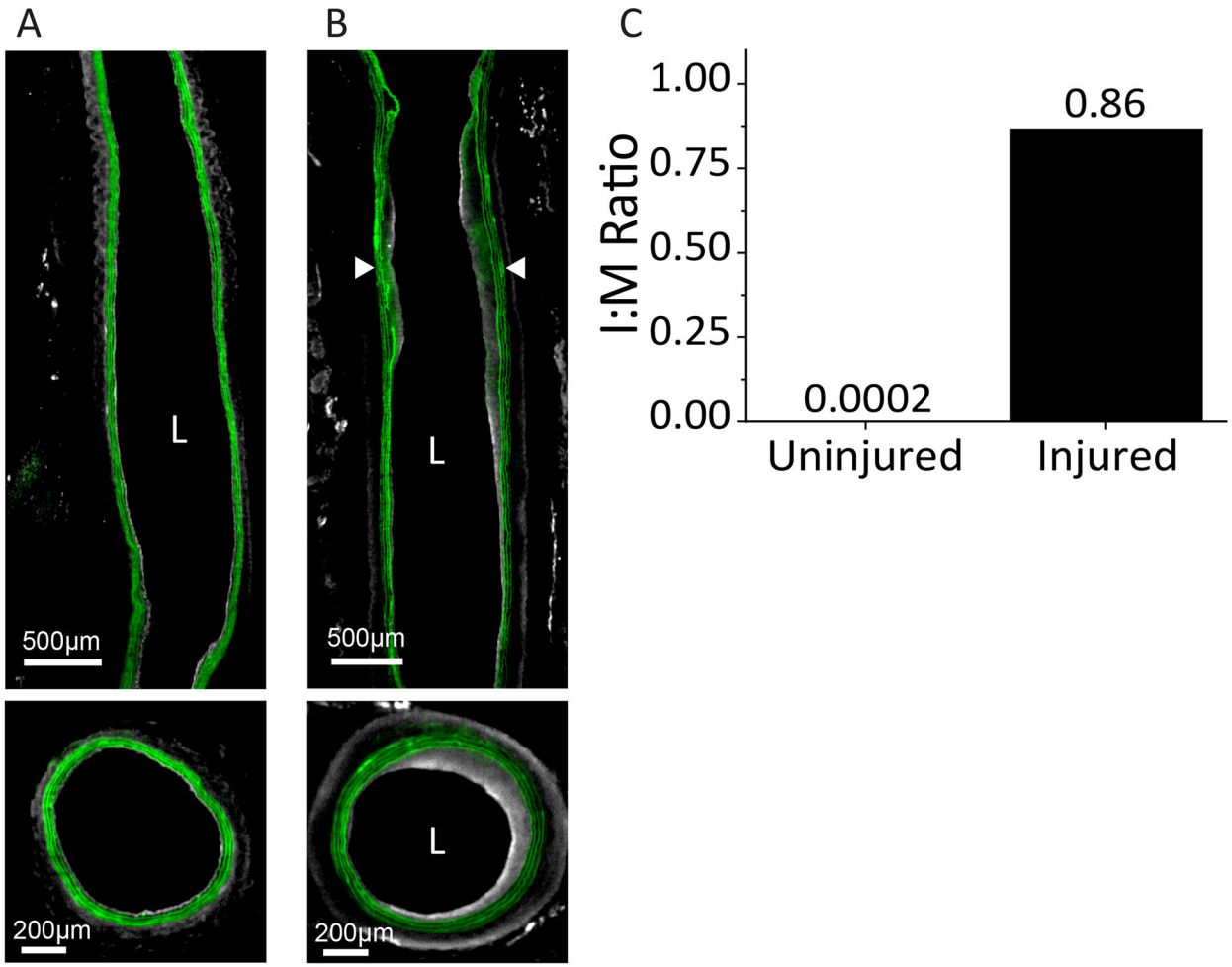

Figure 4. Light sheet fluorescence microscopy to visualize arterial injury.

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).

Table 1.

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

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