A MOUSE MODEL OF AORTIC ENDOTHELIAL DENUDATION AND NEOINTIMAL FORMATION

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PROJECT INFORMATION

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ABSTRACT

MOUSE MODEL OF AORTIC ARTERIAL INJURY AND DENUDATION AND POLYMERIC STENT DELIVERY IN MICE

Ashkon C. Nehzati

More than 385,000 people die from coronary heart disease (CHD) annually and treatment costs $108.9 billion each year including the cost of health care services, medications, and lost productivity. CHD decreases heart function by limiting oxygen and nutrient transport carried through the coronary arteries. A complete block to the coronary arteries causes a myocardial infarction in response to an elimination of blood supply to cardiomyocytes. Partial occlusion results in insufficient blood supply to cardiomyocytes, producing myocardial ischemia and angina, which are usually treated with intravascular stents deployed percutaneously, before myocardial infarction occurs. Stents are the most common ways to expand occluded vessels to treat CHD, with over 1 million stents placed in coronary arteries each year. Unfortunately restenosis, the narrowing of vessels due to endothelial damage and inflammation, is a common complication found after stenting with rates for bare metal stents reported to be between 16% and 44% or about 160,000 to 440,000 patients. Even with the recent advancement in stent technology and the introduction of drug eluting stents (DES), restenosis has continued to be a significant problem associated with stenting. DES restenosis is estimated to occur in 200,000 patients in the United States. It has recently been found that DES may also put patients at risk for late-stent thrombosis due to the delayed healing effects of the antiproliferative coating. With medicine growing at a rapid pace, new stent designs are researched to address new complications such as late-stent thrombosis. These newer designs need newer animal models to better understand the response of the vascular wall to newer stent technologies. The purpose of this study was to develop a mouse model of neointimal hyperplasia in response to aortic endothelial denudation. This model would allow for examination of the neointimal hyperplasia and endothelialization responses to next generation intravascular technologies, such as polymeric stents.

Keywords: Stents, Neointimal Hyperplasia, Restenosis, Late-Stent Thrombosis, Coronary Heart Disease, Ischemia, Bare Metal Stents, Drug Eluting Stents, Polymer Stents

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"A river cuts through rock, not because of its power, but because of its persistence."
-Unknown

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Chapter 1: INTRODUCTION

CORONARY HEART DISEASE

Overview

Coronary heart disease (CHD) is an ischemic disease caused by the narrowing or blockage of the coronary arteries due to atherosclerosis. The buildup of plaque on the inner walls of the arteries physically restricts blood flow to the myocardium. Without a sufficient supply of blood, the heart is unable to receive vital nutrients as well as a consistent flow of oxygen, inducing myocardial ischemia, which presents as angina to the patient. If the coronary artery becomes completely occluded, a myocardial infarct will occur, and cardiomyocytes will necrose. Over time, CHD can cause enlargement of the left ventricle, which can lead to heart failure and arrhythmias.

CHD is the most common type of heart disease and is the number one cause of death for both men and women in the United States. More than 385,000 people die from CHD annually and treatment costs $108.9 billion each year, including the cost of health care services, medications, and lost productivity. Undiagnosed, CHD in sedentary patients can lead to chest pain and myocardial infarction. Every year about 715,000 Americans have a myocardial infarction and in a 2005 survey, only 27% of the individuals were aware of all major symptoms and to call emergency response during a heart attack [1].

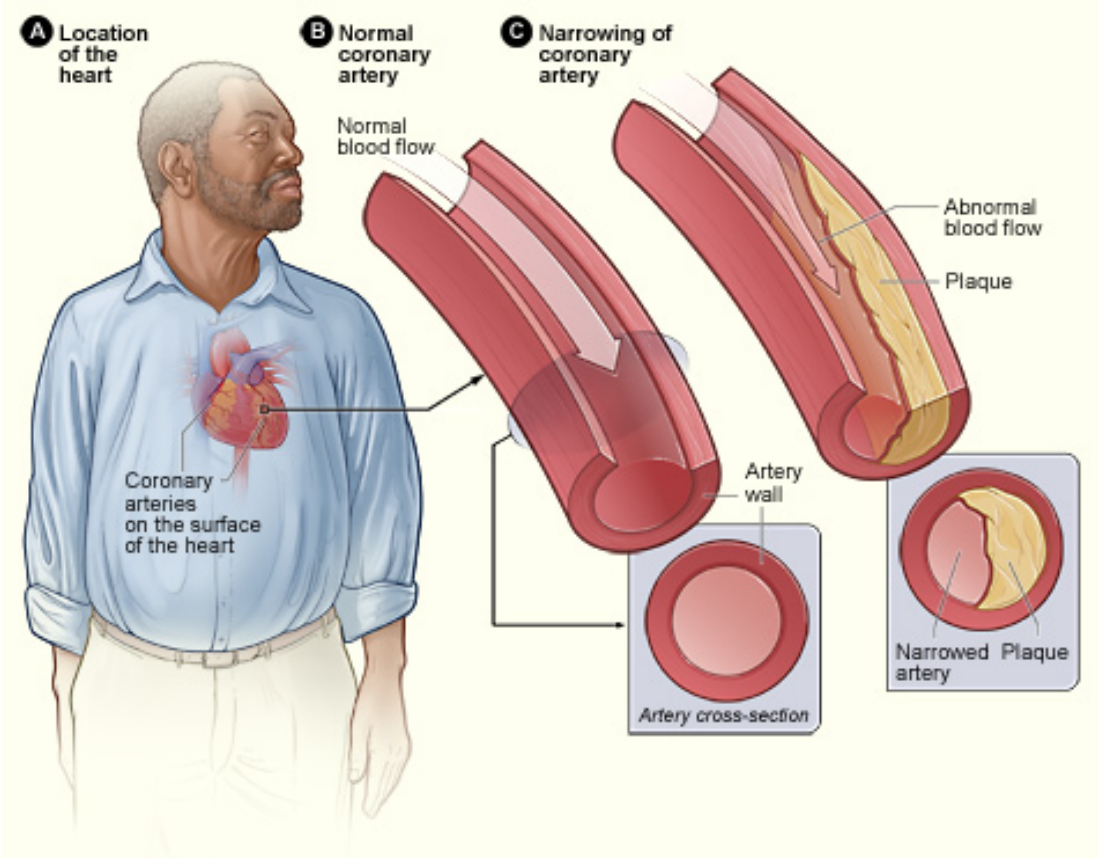

Figure 1 – Atherosclerosis in Coronary Arteries. B, healthy coronary artery with normal blood flow. C, diseased coronary artery with plaque buildup which leads to abnormal blood flow [2].

The major risk factors for CHD include family history of the disease, being of male sex, African-American lineage, and advanced age. Other risk factors include dyslipidemia, smoking, diabetes, obesity, hypertension, stress, poor diet, physical inactivity, and excessive alcohol use.

Atherosclerosis, which is characterized by plaque formation in conduit vessels, is the cause of CHD. Atherosclerosis pathology is hypothesized to be caused by endothelial injury and inflammation which can often lead to the formation of atheromas. Risk factors for atherogenesis can further aggravate the process leading to atherosclerotic plaque accumulation. This plaque accumulation can restrict blood flow to the heart and eventually result in infarcted cardiac tissue.

Atherogenesis

Atherogenesis is the process that leads to the formation of atherosclerotic plaques. Atherogenesis begins at bifurcations in response to turbulent shear stress by recruiting inflammatory cells to the sub-endothelial space. This leads to unresolved inflammation and a positive feedback mechanism which further aggravates the process. Endothelial activation caused by inflammation induces the separation of endothelial cell junctions, adhesion molecule presentation (e.g. VCAMs), and the secretion of chemokines (e.g. MCP-1) to activate adhered monocytes. Activated monocytes extravasate through endothelial cell gaps and become as macrophages under the influence of Granulocyte-macrophage colony stimulating factor (GM-CSF). In addition to leukocytes, plasma components such as LDL also extravasate into the sub-endothelial. Extravasated LDL is oxidized by macrophage-derived reactive oxygen species, and oxLDL is phagocytosed by the macrophages. Proinflammatory cytokines are released following oxLDL phagocytosis, further activating macrophages and the endothelium. This aggravation causes the release of vasoconstrictors, coagulation factors, and smooth muscle mitogens that promote cell proliferation and migration into the sub-endothelial space, eventually forming a neointima (Figure 2). While this process occurs naturally in the body, it can also occur with treatment of atherosclerotic plaques after stent deployment.

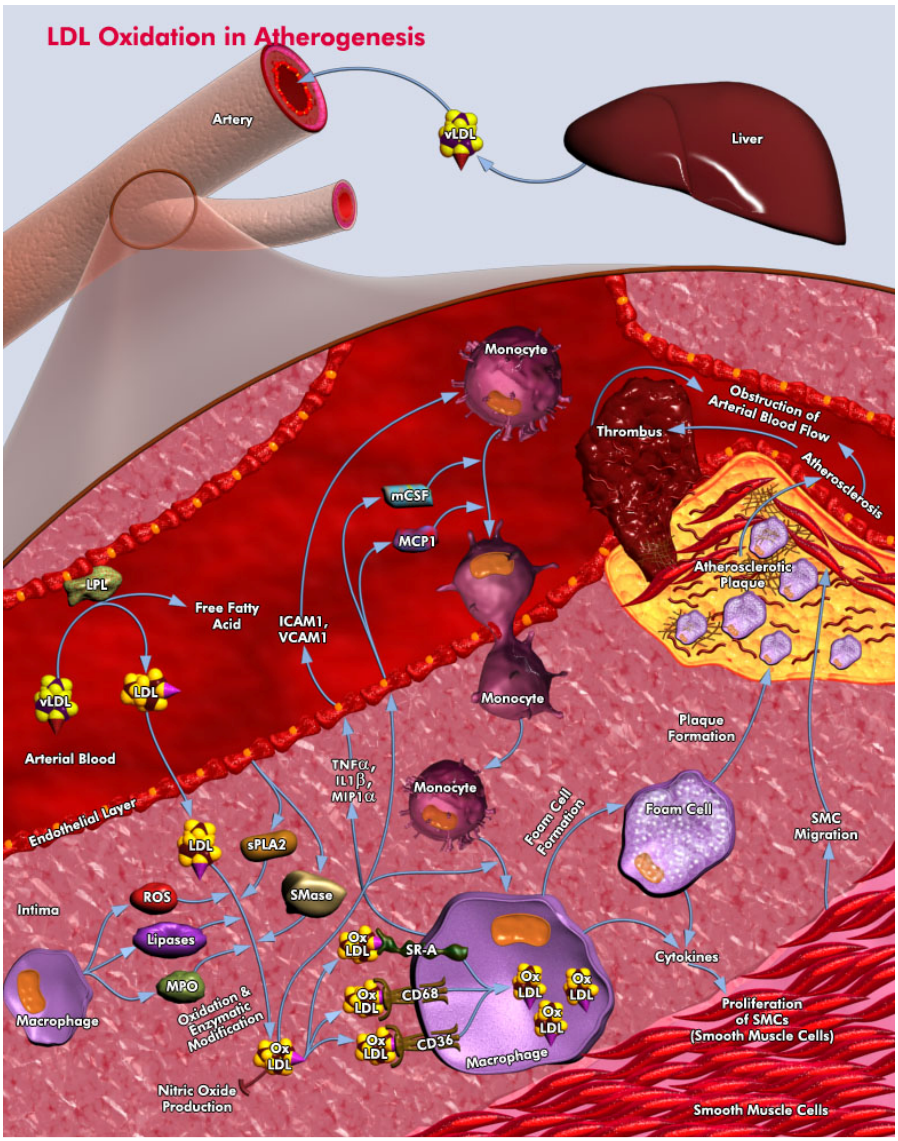

Figure 2 – Atherogenesis. The mechanistic etiology of atherogenesis from the cellular perspective [3].

Current Treatment for CHD

Catheter-based therapies are used as an alternative to bypass surgery for CHD treatment due to its minimally invasive nature. The development of catheter-based therapies began with balloon angioplasty, where plaque-occluded vessels were expanded. The balloon angioplasty procedure deploys bare metal stents and addresses the poor expansion reliability. Unfortunately, 3 years after bare metal stent implantation, 53% of patients experienced in-stent restenosis (ISR), a phenomena in which smooth muscle cell

proliferation results in re-occlusion of the artery [4] . Restenosis was addressed by coating bare metal stents with antiproliferative drugs that slowly elute over time. DES elute antiproliferative agents to delay the restenosis response, but can undergo early-stent thrombosis as soon as 30 days or late-stent thrombosis even years after implantation. The mechanism for late-stent thrombosis is due to delayed healing by the drug on the stent [5]. With further research, biomaterial design in stents will improve drug and physiological interactions to help increase safety and efficacy (Figure 3).

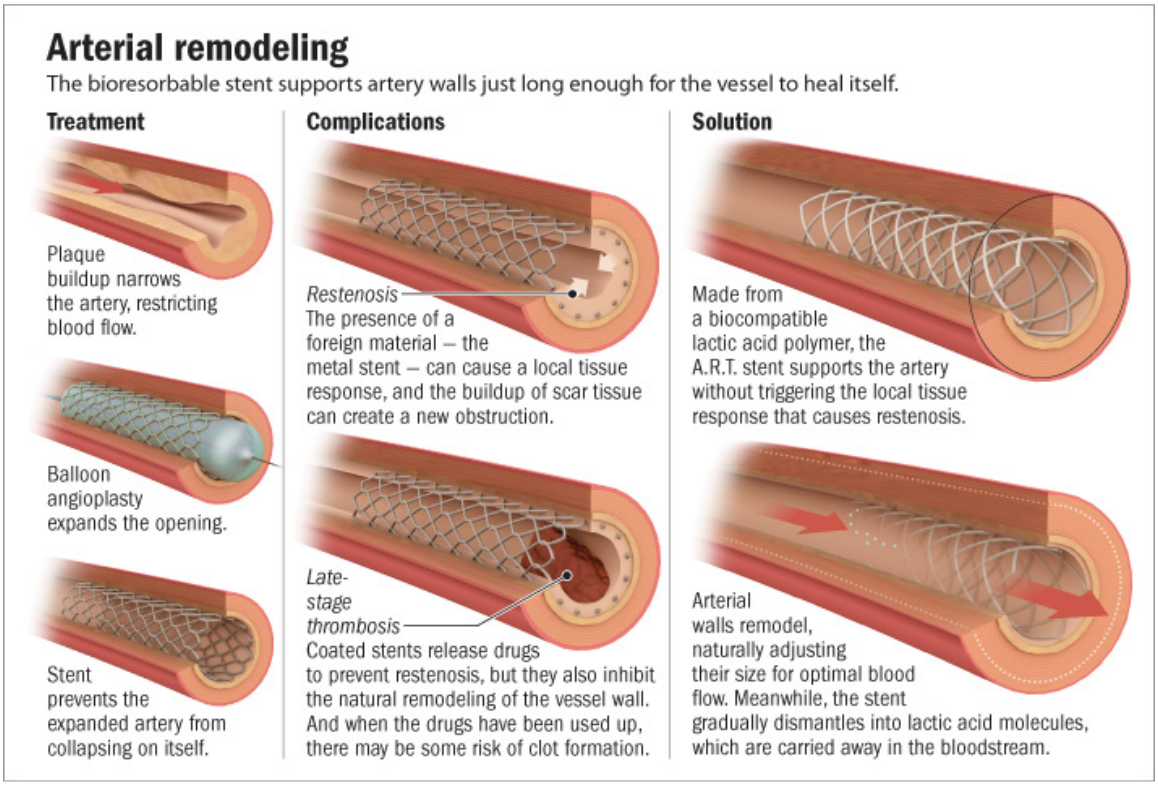

Figure 3 - Stent Material Comparison. A stent is implanted by balloon angioplasty, which expands the stent into its optimal diameter (left panel). The bare metal stent (top middle) undergoes restenosis while the drug eluting stent (bottom middle) undergoes late-stage thrombosis. Both of these are problems that stenting should prevent. The bioabsorbable polymer stent (right panel) reduces the risk of restenosis and returns normal vessel function by dissolving into the blood stream [6].

As knowledge of stents and stent effects continue to grow, stent design will evolve to address the various complications reported over time. This evolution will lead to more sophisticated mechanisms used to manipulate the biologic response to stent implantation. DES today manipulate known biological responses with an antiproliferative. A better

understanding of the underlying biology is needed to prevent proliferation of or recruit a single cell type. To achieve this, a more sophisticated animal model will allow the evaluation of the role of single proteins with knock-out mice or disease-like states with specific animal models such as Apo $^{-/-}$ or non-obese diabetic (NOD) mice.

STENT USE AND ROLE IN CHD

Testing

Animal models used for stenting are restricted due to limited number of animals with comparable human vasculature. Typical models include pigs and rabbits with the porcine model widely accepted for the final stages of pre-clinical studies, due to the geometric similarities to the human heart and coronary vasculature. The downside to these animal models is that they develop very few to no atherosclerotic lesions, even with a high-fat "western diet" [7]. As a solution, mice are widely used for early preclinical research with the ability to easily manipulate genetics while being affordable. Current genetically-modified mice for modeling CHD are ApoE $^{-/-}$ and LDL receptor $^{-/-}$ mice that are susceptible to atherosclerosis and plaque development in turbulent flow areas (aortic arch and abdominal aorta).

OBJECTIVE

The intent of this study was to better understand the neointimal hyperplasia response to intravascular stents, which are the most commonly used strategy to re-open occluded coronary arteries in human patients. In previous studies, a neointima was formed in response to endothelial damage caused by stenting in mice [8]. In a similar fashion, comparable stent injury was reproduced by catheterization of the abdominal aorta.

Objective: *Denude the abdominal aortic endothelium using an injury catheter to induce neointima formation.* The objective was to validate previous research and to optimize the protocol for denuding the abdominal aorta.

Chapter 2: METHODS

Animal Housing and Care

Seven- to 9-week-old male C57Bl/6 mice from Taconic Farms were used for all experiments according to approved protocols by the Cal Poly State University Institutional Animal Care and Use Committee (IACUC). Mice were housed at the University Vivarium in temperature-controlled rooms in microisolator cages. The following enrichment items were provided: a "mouse house", nesting material, and a plastic tube. Mice were monitored daily, given water and rodent chow *ad libitum*, and maintained on a 12 hour light/dark cycle (LD 12:12). Cages were changed weekly using aseptic technique.

Aortic Endothelial Denudation Protocol

An induction chamber with 5% isoflurane in oxygen flowing at ~3 $l \cdot min^{-1}$ was used to anesthetize the mouse. The mouse was then weighed and transferred to a preparatory bench where anesthesia was reduced to ~1-3% isoflurane in oxygen flowing at 0.5-1.0 $l \cdot min^{-1}$. Hindlimb hair was removed with clippers and depilatory cream before placing the animal supine on a heated surgical stage under a dissection scope. Body temperature was kept at ~35° C by a heat pad controlled with a rectal thermistor probe.

A small incision was made on the middle, medial aspect of the left hindlimb, directly over the femoral neurovascular bundle, and extended proximally to the abdominal wall. The area was continually irrigated with phosphate buffered saline (PBS) throughout the procedure to prevent desiccation. Connective tissue was blunt dissected while a heat cautery was used to remove the epigastric fat pad and a majority of the

epigastric neurovascular bundle. The superficial femoral artery was separated from its vein and nerve pair, and arteriotomy performed just upstream to the profunda femoris. The catheter was inserted into the femoral artery just proximal to the profunda femoris branch (Figure 4) and advanced into the abdominal aorta to denude the endothelium. Following aortic endothelial denudation, the catheter was partially retracted and 100 I.U./mL of heparinized saline was injected through the catheter followed by 50 µL of 40 mg/mL Evans blue dye.

After the dye circulated, the animal was perfuse fixed with 4% paraformaldehyde (PFA). For the perfusion fixation procedure, a thoracotomy was performed to expose the heart. The left ventricle was then catheterized and connected to a syringe pump for the infusion of PBS following by PFA. An incision was made in the right atrium for solution drainage; for a detailed description of the surgical procedure refer to the Appendix.

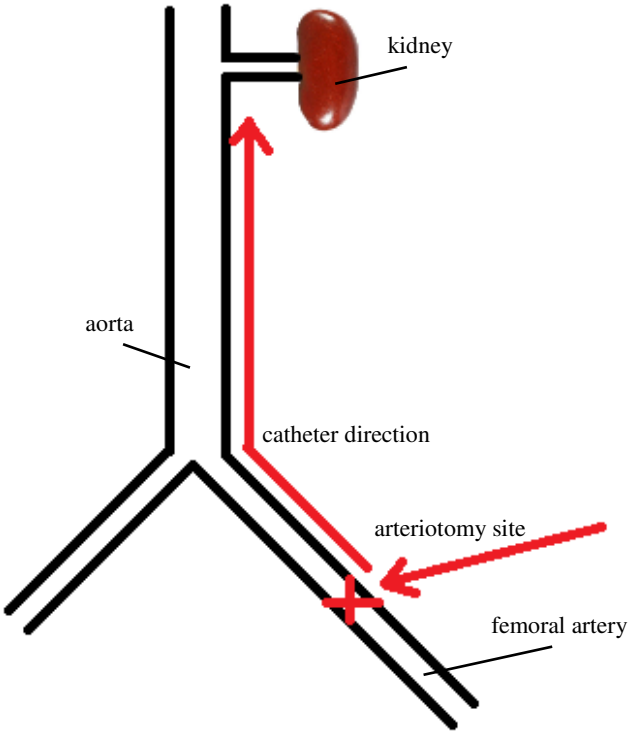

Figure 4 – Catheterization Procedure Diagram. Illustration of the aortic endothelial denudation protocol procedure.

Imaging Analysis

Infinity imaging software (Lumenera) was used to capture images and ImageJ was used to add scale bars. ImageJ was calibrated using a stage micrometer scale at the appropriate magnification.

Chapter 3: RESULTS

The results demonstrate a progression of adjustments made to the protocol while using the injury catheter. Figure 5 illustrates a poor Evans Blue and PBS perfusion in the abdominal aorta, where the dye was injected into the left ventricle of the heart after denudation. Modification to the Evans Blue injection is reflected in Figures 6-8 where the dye was introduced through the injury catheter following endothelial denudation.

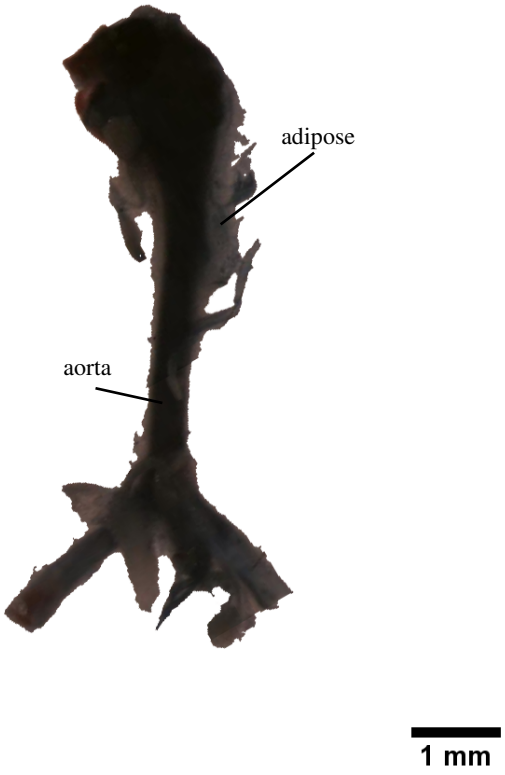

Figure 5 – Poor Evans Blue Stain and Perfusion Fixation of Abdominal Aorta. Evans Blue stain is too dark due to poor perfusion fixation.

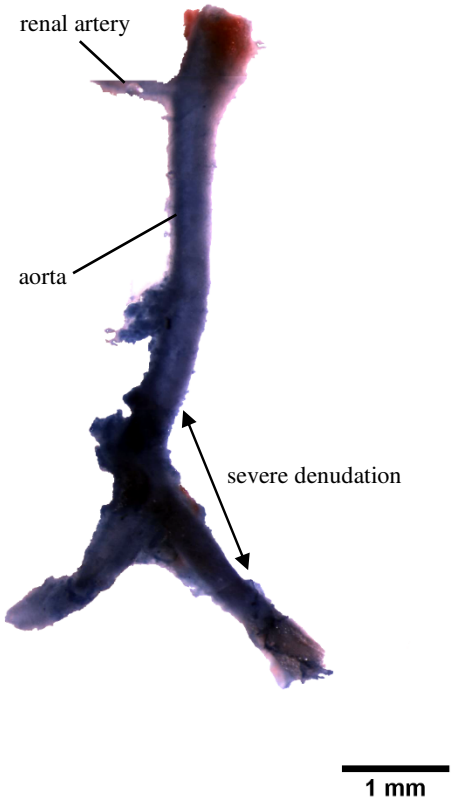

Figure 6 – Ineffective Evans Blue Stain and Denudation of Abdominal Aorta. Blue staining is inconsistent throughout aorta. More damage near iliac bifurcation (darker Evans Blue) due to difficulties advancing injury catheter.

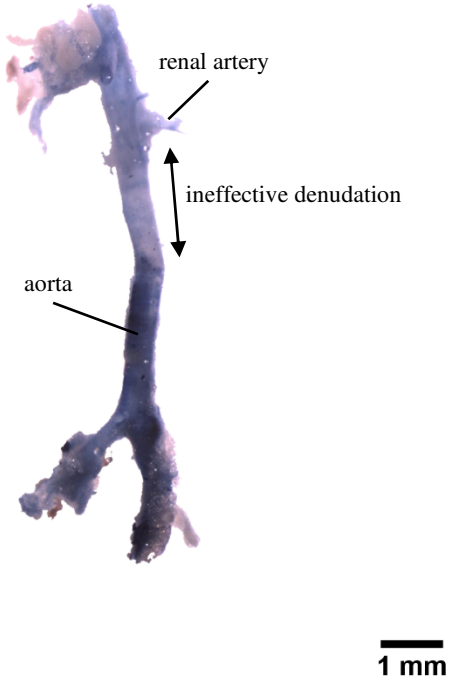

Figure 7 – Evans Blue Stain with Ineffective Denudation of Abdominal Aorta. Denudation of aorta can be seen distal the left renal artery and needed to be more proximal.

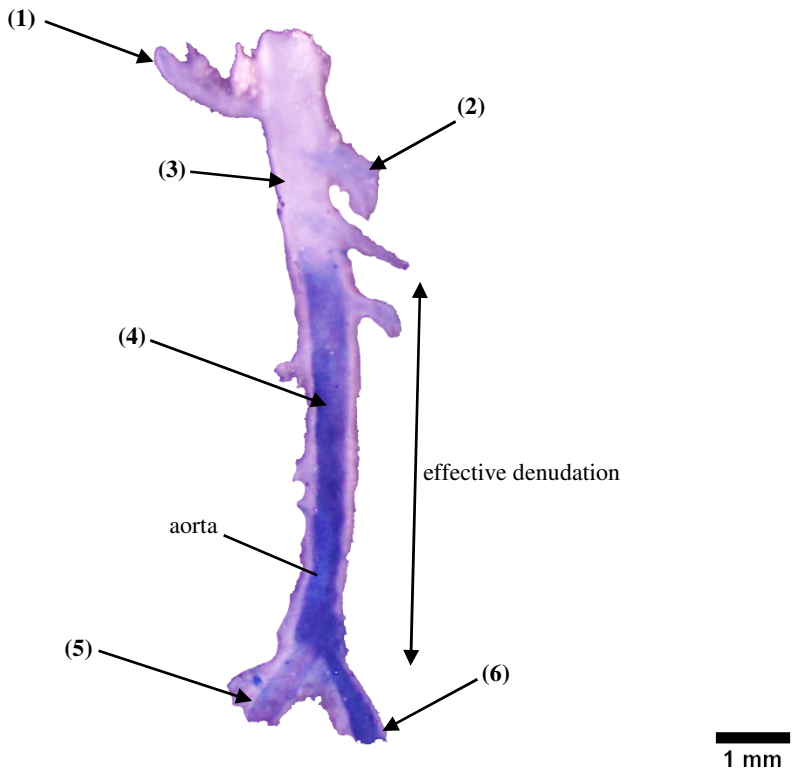

Figure 8 – Successful Evans Blue Stain and Denudation of Abdominal Aorta. The blue staining of the vessel indicates where endothelial denudation has occurred. (1) - Right renal artery (clear) (2) - Left renal artery (clear) (3) – Upper abdominal aorta (clear) (4) – Lower abdominal aorta (blue) (5) - Right iliac (clear) (6) - Left iliac (blue).

Figure 8 shows an image of a specimen's dissected aorta stained with Evans Blue dye, indicating successful endothelial denudation. The Evans Blue dye was used to confirm denudation and works by staining albumin which adheres to the basement membrane of the vessel in areas where the endothelium is damaged.

Chapter 4: DISCUSSION

CHD decreases heart function and blood flow through the coronary arteries due to atherosclerotic plaque buildup, which is commonly treated by stenting. New types of stent designs yield new challenges to solve, like the advent of DES and problems with late-stent thrombosis. To address this, we need additional research models to assist understanding the responses of the vascular wall to stent implantation. This mouse model can serve as the foundation to build upon and to help with the design of newer research models.

Success of the Protocol Development

The developed protocol was demonstrated successfully with the refinement of the surgical procedure to produce endothelial denudation (Figure 8). The injury catheter was advanced through the left iliac and into the abdominal aorta to the left renal artery. Due to the damage, the basement membrane was exposed, which allows the Evans Blue to stain the tissue by binding to the localized albumin. In the clear areas, the basement membrane is not exposed, which does not allow albumin to adhere, allowing the vessel to remain unstained.

Challenges and Improvements

Initial experiments revealed difficulty with inserting the injury catheter into the femoral artery. This was due to the device and vessel diameter being similar in size. To overcome this challenge, a smaller diameter catheter was used while learning how to properly perform the arteriotomy and gradually increased to the required catheter size to denude the vessel. The purpose of the larger catheter size was twofold. The larger size

allowed for easier endothelial denudation without tearing, as well as a vehicle for deployment and future testing of experimental polymeric stents.

Later experiments were difficult due to tactile unfamiliarity with vessel anatomy and blind catheterization with the injury catheter. If the vessel tore at the arteriotomy site during an experiment, the mouse was used to practice perfusion fixation. If the vessel perforated in the abdominal region, a practice dissection of the abdominal aorta was performed. Utilization of the mouse in failed attempted procedures proved helpful to gain more experience in mastering other areas of the protocol. After many experiments, confidence with tactile feedback grew along with technical skill. Another challenge included poor Evans Blue dye perfusion and blood coagulation during perfusion fixation. This was addressed with the addition of polyethylene (PE-100) tubing joining the injury catheter to a Luer-Lok mechanism (Figure 9 in Appendix). It is important to note that there should be sufficient slack available in the tubing so that the Luer-Lok will not catch onto the surgical stage. Also, more slack allows for more tactile feedback from the catheter due to the weight distribution over a longer length of the tubing preventing it from "pulling" the catheter as much.

Learning the Evans Blue staining protocol revealed inconsistencies with the acute denudation experiment completed before. A heterogeneous denudation pattern was observed throughout the vessel where the catheter traveled. Improvements made in the protocol involved the twisting movement of the catheter during retraction. This increased the amount of damage and homogeneity observed by the Evans Blue stain, particularly inferior to the left renal artery.

Future Work

This optimized protocol will be used in future experiments which will include a chronic 28-day study to determine if the formation of a neointima occurs in the denuded regions of the abdominal aorta. We are interested in assessing neointimal formation in response to injury and/or bioresorbable stent implantation. Neointima formation is clinically caused by the endothelial damage left by stent implantation. This damage causes smooth muscle cell proliferation into the intima of the artery. The intimal thickening causes the artery to undergo restenosis where the vessel wall grows around the stent struts, occluding a majority of the blood flow through the vessel. Therefore prevention of this neointima is key to solving the issues plaguing stent and angioplasty usage. In the future, this model can be used to characterize gene expression and assess specific molecular pathways that contribute to restenosis and thrombosis.

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APPENDIX

Aortic Endothelial Denudation Protocol

Date_____ **Hindlimb Ischemia Surgery – Abbott Project** Initials_____

Mouse Information

DOB: _____
Sex: _____
Tag: _____
Genotype/strain:_____
Cage: _____

Materials

Sterilize- autoclave or flash autoclave

____1. Standard pattern forceps (1)
____2. S&T forceps (2)
____3. 5-45 forceps (1)
____4. Iris scissors (1)
____5. Microdissection scissors (1)
____6. Castroviejo (1)
____7. Retractor (1)

Pre-sterilize in autoclave

____8. cotton gauze (2)
____9. cotton swabs (12)
____10. 6.0 silk suture (2 x 1-inch)

Obtained in surgery suite

____11. sterile Petri dish w/ sterile saline
____12. sterile gloves
____13. sterile 7.0 prolene suture
____14. heat-cautery
____15. FST heat pad w/ rectal probe
____16. heat pad
____17. recovery bin & weigh boat
____18. depilatory cream
____19. non-sterile cotton swabs
____20. non-sterile cotton gauze
____21. isolation mask & cap
____22. analgesic (Buprenorphine)

Surgery preparation

____23. Spray surgery area with Nolvasan
____24. Weigh animal in weight boat
____25. Place animal in anesthesia box
____26. Open the oxygen cylinder and set anesthesia-machine flow meter to ~3 $l \cdot min^{-1}$
____27. Anesthetize animal w/ 5% isoflurane
____28. Give the animal an subcutaneous injection of buprenorphine (0.075mg/kg)
____29. Affix non-rebreathing circuit to bench-top with tape
____30. Reduce flow rate to 0.5-1.0 $l \cdot min^{-1}$ and the isoflurane to 1-3%
____31. Apply ear tag high on left ear
____32. Lay animal supine with nose in nose-cone
____33. Shave hair on the right hindlimb & lower abdomen with clippers
____34. Remove excess hair with depilatory cream
____35. Spray right hindlimb with Nolvasan
____36. Return animal to anesthesia box
____37. Apply 4x4 gauze sponge to heat pad to protect animal from excessive heat
____38. Affix non-rebreathing circuit to surgery table w/ chemistry clamp
____39. Lay animal supine on circulating heat pad w/ nose in nose-cone
____40. Insert rectal probe and set thermo-controller to 35°C
____41. Apply veterinary ointment to eyes to avoid drying during procedure
____42. Apply veterinary ointment to anus and place rectal probe ~1cm into anus to monitor core-body temperature

Surgery

____43. Make a small incision on the middle, medial aspect of the left thigh
____44. Extend the incision up to the abdominal wall
____45. Blunt dissect the subcutaneous connective tissue to maximize surgical exposure
____46. Use cautery to remove fat pad overlying femoral a-v pair & cauterize epigastric av-pair
____47. Blunt dissect the femoral artery from the neurovascular bundle just downstream from the deep femoral branch
____48. Tie off the femoral artery & vein with 6.0 silk suture, proximal to the profunda
____49. Using the micro-vascular clamp, clamp off the femoral artery at the abdominal wall
____50. Cut the femoral artery halfway through the vessel with microdissection scissors
____51. Use 6.0 polypropylene suture to loosely tie a ligature around the artery upstream of the incision
____52. Slowly insert the 5-45 forceps into the hole in the femoral artery and slowly open the forceps to stretch the artery
____53. Grab the top flap of artery wall where the incision was made with the 5-45 forceps
____54. Gently insert the catheter until it reaches the vascular clamp
____55. Carefully tighten the ligature around the femoral artery making sure the catheter is also within this ligature
____56. Remove the vascular clamp
____57. Hold the ligature with an S&T forceps and slowly advance the catheter ~20 mm
____58. (As needed) unsheathe the catheter to expose the injury coil
____59. Re-sheathe the injury coil
____60. Pull the catheter within 5 mm of the insertion point

____61. Use 6.0 polypropylene suture to close the femoral artery upstream of the catheter tip and remove catheter

____62. Use 6.0 polypropylene suture to close the skin

Post-Surgical

____63. Place the animal in the recovery bin, on a blue bench cover, above a heat pad and allow to recover

____64. Turn flow meter down to 0, turn off isoflurane, and close the oxygen cylinder

____65. Indicate surgery on cage card

Notes

_____
_____
_____
_____

Evans Blue & Perfusion Fixation Protocol

Date _____ **Evans Blue Injection & Perfusion Fixation** Initials_____

Mouse Information
DOB: _____
Sex: _____
Tag: _____
Genotype/strain: _____
Cage: _____
Weight(g): _____

Materials
Non-Sterilize Dissection Instruments
____1. Forceps (2)
____2. Fine Forceps (2)
____3. Bone Scissors(1)
____4. Skin Scissors (1)
____5. Dissection Scissors (1)
____6. Hemostats(1)
____7. Vascular clamp (1)

Obtained in surgery suite
____8. Tape
____9. 10mL Syringes (2)
____10. 30 gauge needle
____11. Bench cover
____12. Heating pad
____13. Catheter
____14. Non-sterile saline
____15. Cotton swab

Dye and Fixative Preparation
____16. Fill PE-100 tubing attached to injury catheter with 100 I.U./mL of heparinized saline
____17. Obtain 50µL of 40mg/ml Evans Blue dye in a 10mL syringe with a 30 gauge needle or 5 units in a U-100 BD insulin syringe
____18. Obtain a 5 mL syringe of 4% PFA

Procedure Preparation
____19. Obtain saline filled beaker, cotton swab, and instruments
____20. Spray surgery area with Nolvasan
____21. Weigh animal in weight boat
____22. Place animal in anesthesia box
____23. Open the oxygen cylinder and set anesthesia-machine flow meter to ~3 $l \cdot min^{-1}$
____24. Anesthetize animal w/ 5% isoflurane
____25. Reduce flow rate to 0.5-1.0 $l \cdot min^{-1}$ and the isoflurane to 1-3%
____26. Lay animal supine with nose in nose-cone
____27. Remove excess hair with depilatory cream
____28. Spray right hindlimb with Nolvasan
____29. Return animal to anesthesia box
____30. Apply 4x4 gauze sponge to heat pad to protect animal from excessive heat
____31. Affix non-rebreathing circuit to surgery table w/ chemistry clamp
____32. Lay animal supine on circulating heat pad w/ nose in nose-cone
____33. Insert rectal probe and set thermo-controller to 35°C
____34. Apply veterinary ointment to anus and place rectal probe ~1cm into anus to monitor core-body temperature
____35. Tape animal down to heated bench cover

Evan's Blue Dye Injection
____36. Inject 50µL of 40mg/ml Evans Blue dye into PE tubing attached to catheter followed by a PBS rinse
____37. Allow the dye to circulate in the mouse for 2 minutes

Fixation
____38. Separate skin from muscle from the abdomen to the top of the thoracic cavity
____39. Cut through abdomen close to diaphragm
____40. Quickly cut through the ribs and diaphragm to open chest cavity and clamp back with hemostats
____41. Cut away excess tissue around the heart
____42. Make a small incision at the apex of the heart (Left Ventricle)
____43. Insert PE-100 tubing and clamp with vascular clamp and cut right atrium
____44. Inject 10 mL of PBS at a rate of 4ml min^{-1} with the automatic syringe depressor
____45. Repeat with another 10 mL of PBS if needed
____46. Fix with 10 mL of PFA at a rate of 4 mL min^{-1}
____47. Resect the abdominal aorta upstream of the renal arteries and downstream of the iliac bifurcation

Notes
_____
_____

Photographs of Procedure

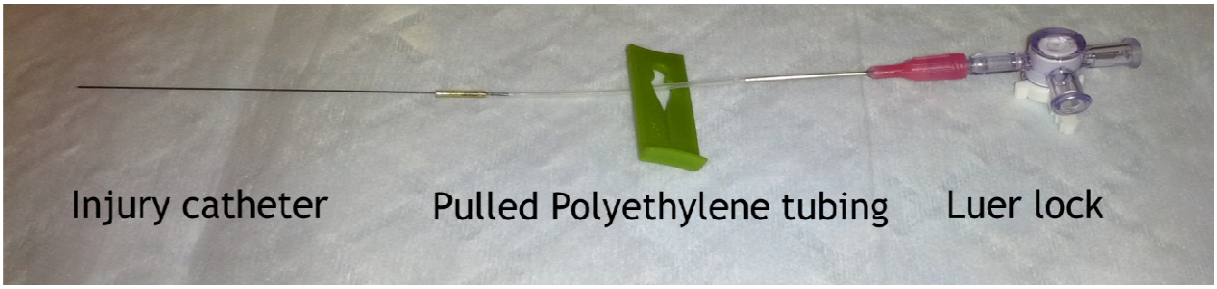

Figure 9 – Assembled Injury Catheter. Preparation of injury catheter with heparinized saline 100 I.U./mL.

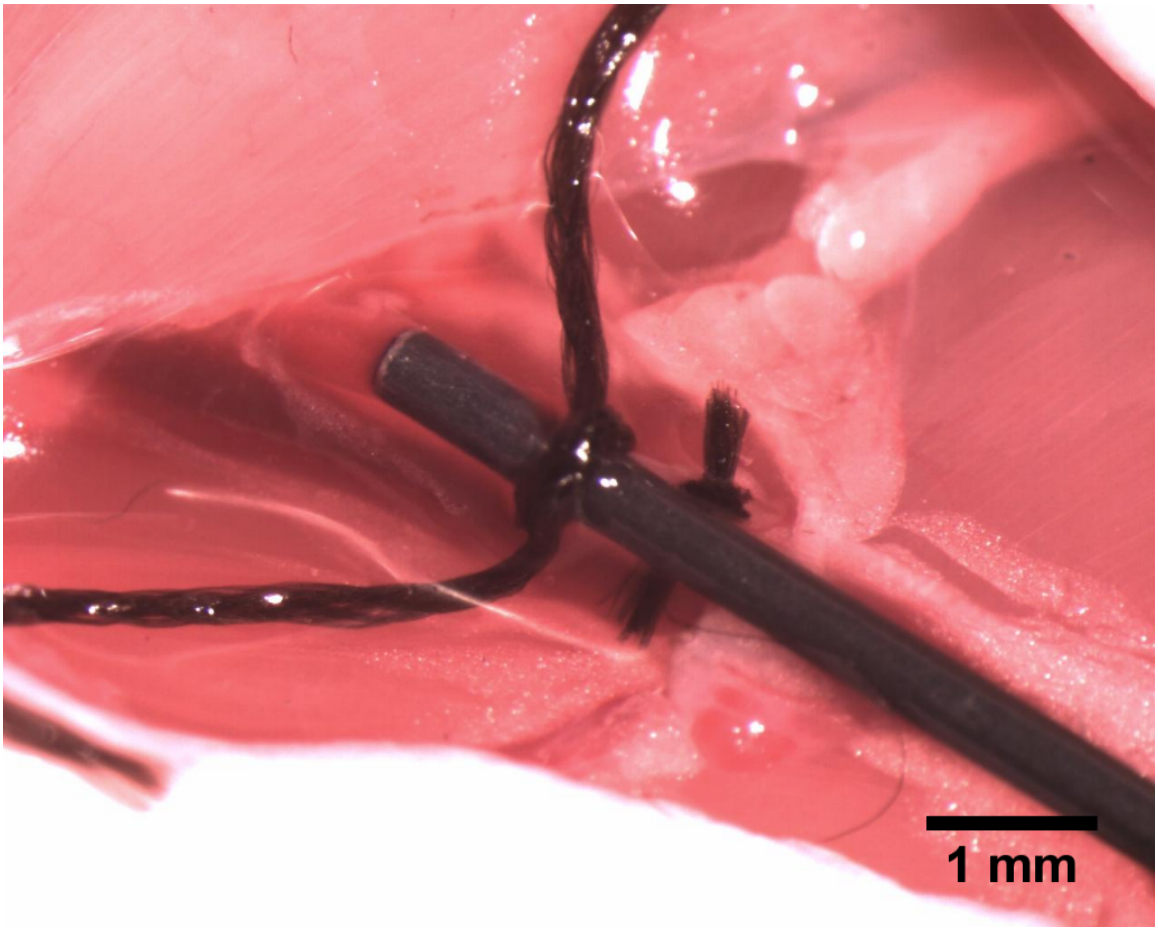

Figure 10 – Femoral Artery Catheterization. Successful catheterization of the injury catheter before advancement into the abdominal aorta.

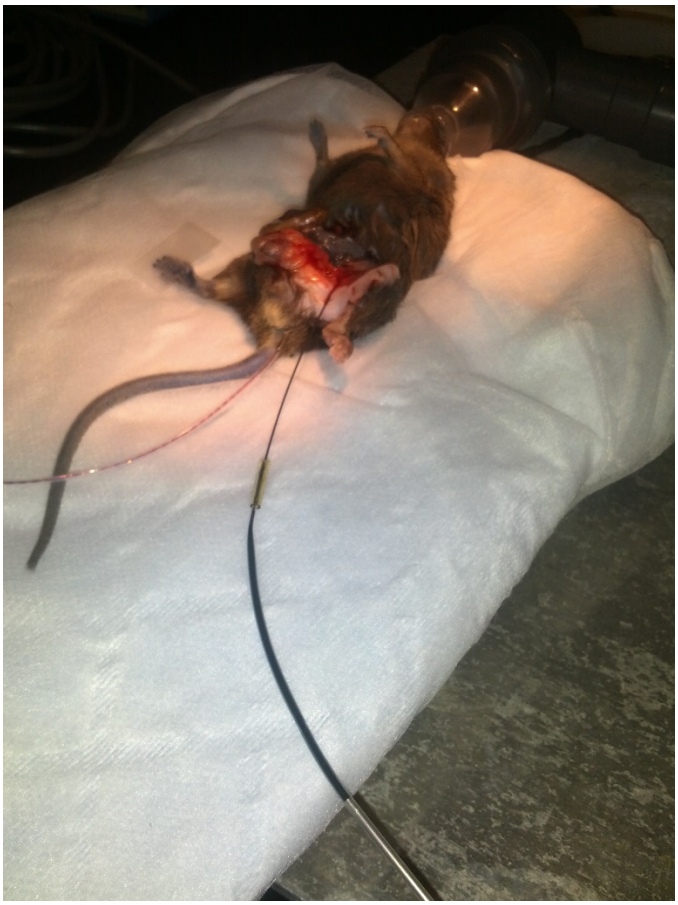

Figure 11 – Evans Blue Injection. 50 µL of 40 mg/mL of Evans Blue delivered directly into the blood with 100 I.U./mL heparinized saline through the injury catheter.

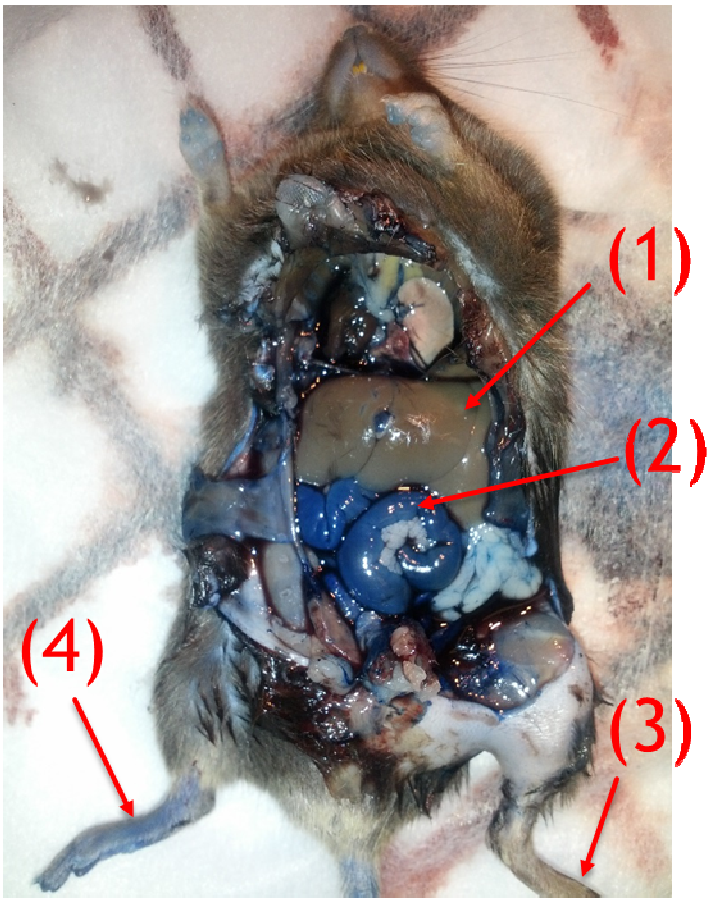

Figure 12 – After a Perfusion Fixation Protocol. (1) - Clear liver indicates successful perfusion of 4% PFA/PBS and removal of blood (2) - Blue stained intestine indicates perfusion of Evans blue dye (3) - Left hind paw not stained blue due to ligation distal to insertion site (no blood flow) (4) - Right hind paw stained indicates circulation of dye throughout extremities