# SMOOTH MUSCLE CELL ORGANIZATION IN THE STEM REGION OF THE GRACILIS COLLATERAL CIRCULATION

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## PROJECT INFORMATION

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## ABSTRACT

SMOOTH MUSCLE CELL ORGANIZATION IN THE STEM REGION OF THE GRACILIS COLLATERAL CIRCULATION

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Many patients who suffer from the ischemic Peripheral Arterial Occlusive Disease (PAOD) experience intermittent claudication, which can be attributed to impaired vasodilation. Collateral vessels are the primary site of resistance to blood flow downstream; therefore maximizing vasodilation in collaterals is crucial for efficient circulation. Collaterals function as natural bypasses around the occluded arteries and the increase in flow into these vessels causes them to outwardly remodel into conduit vessels. However, functional vasodilation in the stem region of collateral vessels is impaired at day 7 following femoral ligation, which can be attributed to smooth muscle cell malfunction. However, the increase in vessel diameter in outwardly remodeled collaterals is not due to cell proliferation. One possible explanation for the diameter increase in the stem region of the collateral is that the vascular smooth muscle cells undergo mechanoadaptation to acclimate to the increase in blood flow and fluid shear stress. To test this hypothesis, outward remodeling was induced in the gracilis collateral stem via femoral artery ligation. At day 7 following surgery, maximal vasodilation in the profunda femoris artery (collateral stem) was evaluated before perfusion fixation. The profunda was then resected and immunostained before measuring smooth muscle cell length and overlap using confocal microscopy. Average SMC overlap was significantly less in the collateral artery, 43 ± 1 μm versus 51 ± 2 μm in the control artery. Also, average SMC length was significantly longer in the collateral artery 249 ± 13 μm versus 205 ± 10 μm in the control. These results indicate that mechanoadaptation occurred in the collateral stem and its correlation to impaired vasodilation suggests that the reorientation and remodeling associated with mechanoadaptation may play a causal role in vessel impairment. Further studies need to be performed to determine if mechanoadaptation causes impaired vasodilation for patients with PAOD.

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"You're only given one little spark of madness. You mustn't lose it."

- Robin Williams

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## INTRODUCTION

*Peripheral Arterial Occlusive Disease*

Peripheral Arterial Occlusive Disease (PAOD) affects 12 million Americans, and disease prevalence increases with age [1]. PAOD is characterized by the narrowing of arteries due to atherosclerotic plaque accumulation in the lower extremities and is a strong predictor for coronary heart disease. These obstructions can lead to ischemia, or an insufficient blood supply. Subsequently, there is an inadequate amount of oxygen and glucose required for metabolic function in the peripheral tissues [2].

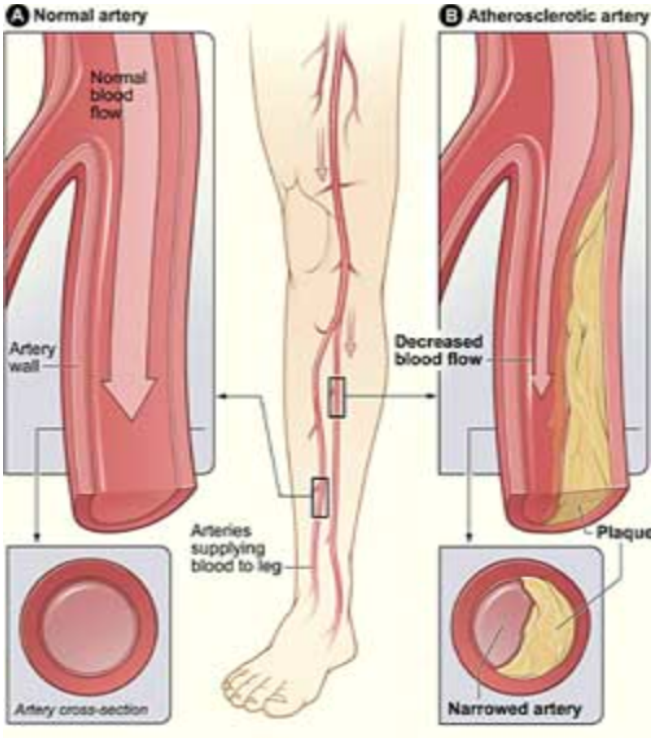


**Figure 1: Peripheral Arterial Occlusive Disease**. Narrowed arteries and reduced blood flow due to plaque buildup that causes PAOD [3].

Intermittent claudication, which is pain caused by deficient blood flow during exercise, is the result of ischemia and is the primary symptom of patients diagnosed with PAOD. For these individuals, pain occurs during increased activity and is alleviated during rest; however, if left untreated, the disease can progress to Critical Limb Ischemia (CLI), a very severe form of PAOD characterized by chronic ischemic pain at rest [4]. Risk factors for PAOD include age, obesity, cigarette smoking, diabetes mellitus, physical inactivity, high blood pressure, and high cholesterol. Patients who have PAOD experience a lower the quality of life because they must constantly endure uncomfortable physical pain during daily activity [2].

*Current Treatment Methods for Patients with PAOD*

The effectiveness of current treatment options for PAOD is limited. The first step following diagnosis is lifestyle changes, such as increasing exercise and eating a balanced, healthy diet. Other treatment options include medications that prevent blood clots, lower cholesterol, or relieve symptoms to improve claudication [5]. Often these drugs are palliative, and alleviate the primary symptoms but do not treat the underlying issue of impaired vasodilation. In more severe cases, angioplasty or bypass surgery may be performed. Angioplasty is a minimally invasive procedure that involves placing a balloon catheter at the blockage site and inflating it to widen the artery. In many cases a stent is deployed in the artery to ensure that the vessel remains patent. During this procedure, the stent is mounted onto the balloon and when the balloon expands, the stent is compressed against the arterial wall. The balloon is then deflated and removed while the stent remains in its position (**Figure 2**). Stenting generally provides immediate relief; however, the

vessel can undergo restenosis, or the reduction in the lumen diameter following stent intervention [6]. Bypass surgery is performed to circumvent an occlusion by surgically anastomosing a vein graft proximal and distal to the site of occlusion, as an alternative route for blood flow around the blocked artery. As with any surgical procedure there are risks associated with bypass surgery and eventually graft occlusion can occur, which causes a recurrence of ischemia [7].

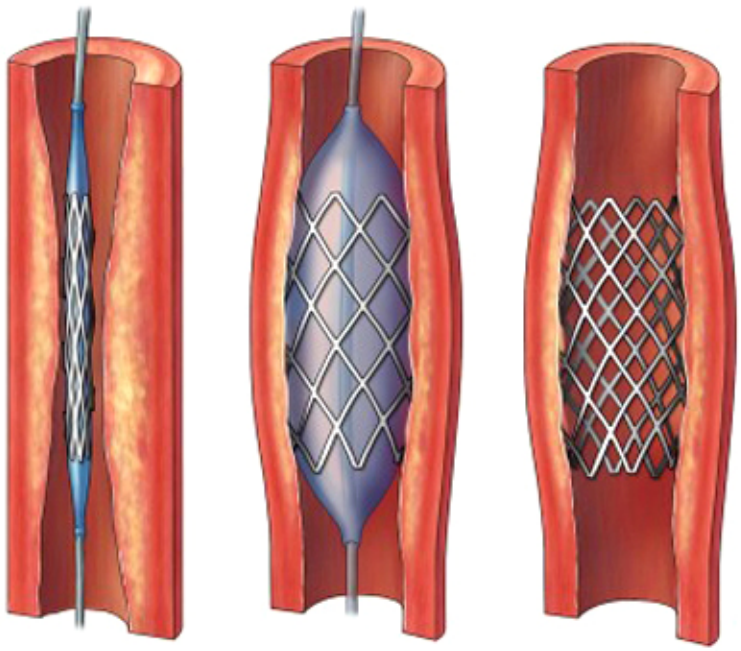

**Figure 2: Angioplasty and Stent Deployment**. A balloon catheter is guided to the blockage site and inflated to widen the artery. A stent is left behind at the blockage site to keep the affected vessel open [8].

Due to the limitations of the current intervention methods, an alternative therapy is desired. Establishing a better understanding of the body's natural physiologic response to an arterial occlusion may be beneficial in developing an

effective treatment for patients with PAOD. When an arterial occlusion restricts blood flow, blood is redirected down alternative pathways via collateral vessels, pre-existing arterial-arterial connections, which can act as natural bypass routes around occlusions. The pressure is lowered downstream of an occlusion, which increases blood flow and pressure in the collateral network. The increased blood flow increases the fluid shear stress along the wall of collateral vessels and stimulates arteriogenesis, the outward remodeling of collateral vessels [9].

*Arteriogenesis in Collaterals*

Arteriogenesis begins when the endothelial cells that line the luminal surface in the collateral vessel are activated in response to the increase in fluid shear stress. The endothelial cells display adhesion proteins and express monocyte chemoattractant protein-1 (MCP-1) on their surface to recruit monocytes to adhere to the endothelium. The monocytes extravasate and become macrophages that secrete matrix-metalloproteinases (MMPs), which degrade the matrix to allow the collateral to be remodeled [10]. Concomitantly, the endothelial cells secrete vascular endothelial growth factor (VEGF) and tumor necrosis factor-α (TNF-α) to cause endothelial cell and vascular smooth muscle cell (SMCs) proliferation, respectively [11]. This process enlarges the lumen diameter until the shear stress is normalized.

Arteriogenesis is most often described in the mid-zone of the collateral circuit, however the SMC proliferation that is characteristic of arteriogenesis is absent from the stem region of the collateral circuit, despite an increase in resting diameter that is suggestive of outward remodeling. The increased resting diameter of the stem region in the absence of SMC proliferation may be explained by SMC

mechanoadaptation, the rearrangement of SMCs through changing cell length and cell overlap without proliferation to chronically resize the lumen diameter.

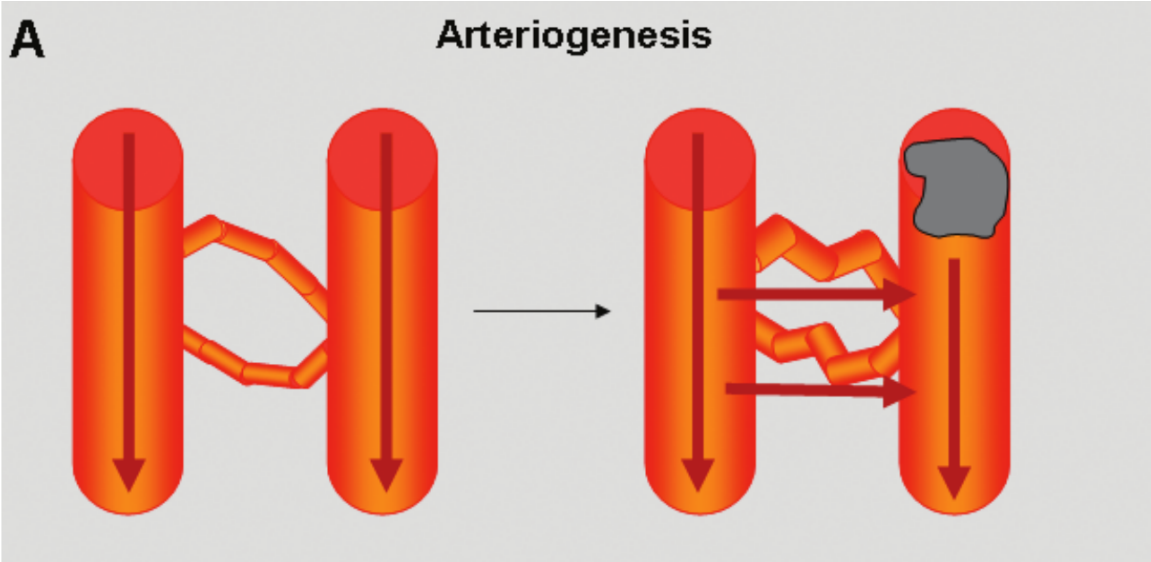


**Figure 3: Arteriogenesis**. Progression of arteriogenesis, the outward remodeling of collateral vessels, from normal blood flow (left) to altered blood flow due to occlusion (right) [12].

*Impaired Vasodilation*

Impaired vasodilation, which is observed in patients with PAOD, is a possible explanation for intermittent claudication because sufficient vasodilation is imperative for supplying adequate blood flow and nutrients to meet tissue metabolic demand, especially during periods of physical exertion [13]. It is also known that during muscle contraction arterioles vasodilate to increase blood flow to tissues [14]. If vasodilation was impaired this could result in inadequate nutrient delivery and thus pain to the extremities. Impaired vasodilation is observed in patients with PAOD, therefore it is useful to further study this phenomenon [15].

It was previously hypothesized that SMC proliferation during arteriogenesis was the cause of impaired vasodilation. During the outward remodeling process, SMCs change phenotype from contractile to synthetic since the synthetic phenotype is more proliferative. This results in an increase in number of SMC cells with non-contractile function, which would enlarge the diameter, but may impair the vessel's reactivity [16]. However, proliferation was not observed in the collateral stem and could not be a cause of impairment, therefore SMC mechanoadaptation has been considered. Changing of SMC length and overlap causes substantial structural changes to the cell components, which may have debilitating effects on cell contraction and relaxation.

*Previous Work*

Resting diameter of the profunda femoris artery (stem to the gracilis collateral circuit) is increased at day-7 following femoral artery occlusion, however the percent change from resting to maximally dilated diameter is reduced, which indicated a decreased dynamic range in the vessel. There is also an increase in blood flow in the profunda femoris artery at day-7. Also at day 7 in the occluded hindlimb, smooth muscle-dependent vasodilation is significantly impaired in the profunda femoris artery [17]. Surprisingly the proliferation of SMCs did not occur in the profunda femoris artery following femoral ligation and there was no significant difference in the number of SMCs between the ligated and non-ligated hindlimbs, indicating that the increase in vessel diameter was not due to cell proliferation. This suggests that the cause of the impairment of the collateral stem needs to be studied further [16].

Given the absence of SMC proliferation, mechanoadaptation has been further investigated. SMCs in rat arterioles undergo mechanoadaptation in response to prolonged exposure to the vasoconstrictor norepinephrine (NE). The vessels showed a decrease in vessel diameter as well as an increase in SMC overlap, indicating that the SMCs mechanically reoriented themselves to acclimate to the change in their environment and cause remodeling of the vascular wall (**Figure 4**) [18]. Mechanoadaptation can work in the opposite direction and may cause an increase in lumen diameter through an increase in SMC length and a decrease in SMC overlap.

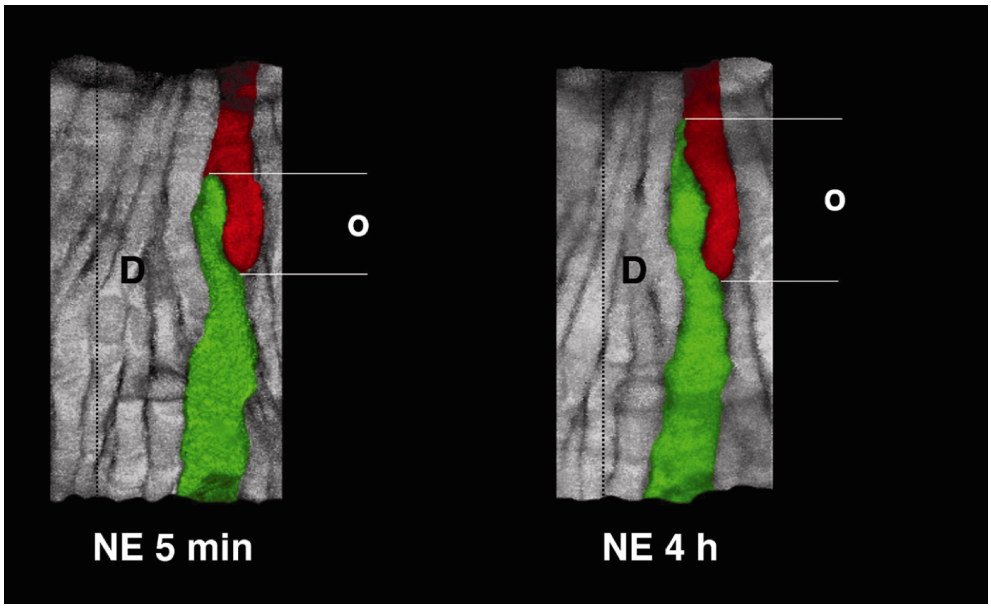


**Figure 4: Mechanoadaptation in Rat Arterioles**. Study investigated by Martinez-Lemus et al. showed an increase in SMC overlap when rat arterioles were exposed to vasoconstrictors for extended periods of time [18].

*Specific Aims*

Patients with PAOD experience intermittent claudication, which may be explained by impaired vasodilation of the collateral network. Impaired vasodilation

cannot be explained by SMC proliferation; therefore SMC mechanoadaptation and its role in vessel impairment must be further studied. The objectives of this study were to:

1. Test the hypothesis that the collateral stem undergoes outward remodeling by comparing resting diameter to papaverine and SNP induced maximal vasodilation
2. Test the hypothesis that the outward remodeling that occurs can be explained by smooth muscle cell mechanoadaptation

## MATERIALS AND METHODS

### *Animal Husbandry*

Male C57BL/6 mice were used for all procedures according to experimental protocols that were approved by the Institutional Animal Care and Use Committee (IACUC) of California Polytechnic State University. The mice were housed in the Cal Poly vivarium in micro-isolator cages within a temperature controlled room on a 12 hour light-dark cycle. Mice were monitored daily and were provided with enrichment, and feed and water ad libitum.

### *Femoral Artery Ligation Surgery*

An arterial occlusion was simulated in the mouse hindlimb via femoral artery ligation. Prior to surgery, the instruments and materials were sterilized in an autoclave and the surgery bench was disinfected with 70% isopropyl alcohol. Mice were anesthetized with 1-3% isoflourane gas in oxygen at a flow rate between 0.8-1.2 $l \cdot min^{-1}$. The hair on the anterior hindlimb was depilated and the underlying skin disinfected with Nolvasan. The animal was then placed supine on a heated surgical stage and a rectal probe thermometer was inserted to maintain internal body temperature at $35^{o}C$.

An incision was made in the medial aspect of the hindlimb and extended proximally to the abdominal wall. Phosphate buffered saline (PBS) was applied to the surgical site throughout the procedure to prevent tissue desiccation. The connective tissue was blunt dissected and the epigastric fat pad was repositioned medially using a retractor to expose the ligation site. At the ligation site, S&T forceps were used to separate the nerve from the femoral artery-vein pair and #5/45 Dumont forceps were used to separate

the vein from the artery. The femoral artery was ligated distal to the epigastric branch and proximal to the popliteal branch (**Figure 5**) using polypropylene suture. The contralateral hindlimb was un-operated for control.

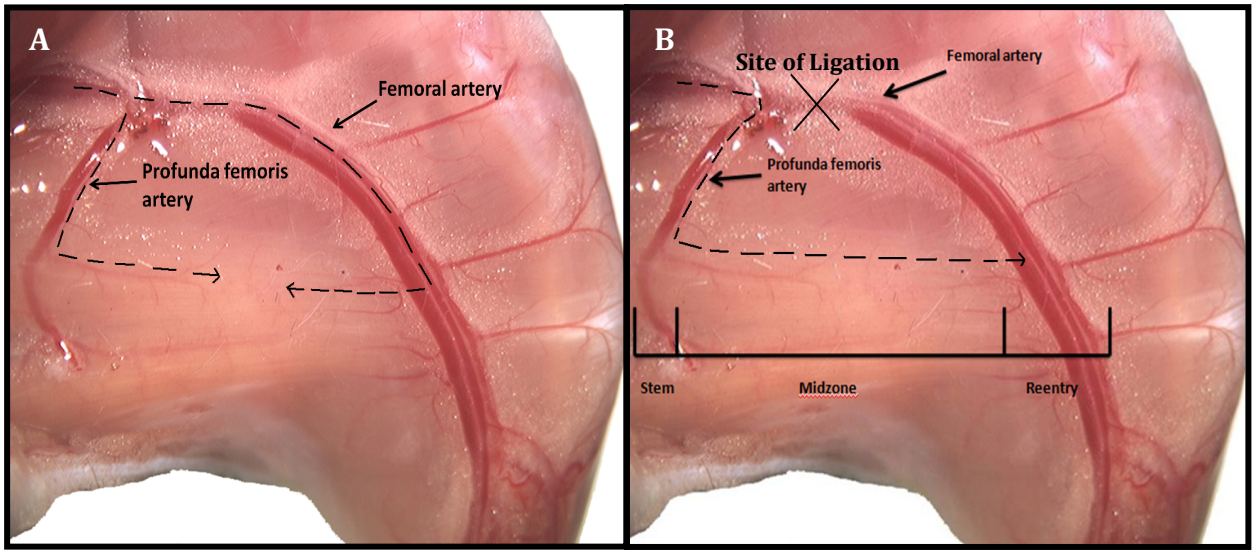


**Figure 5: Femoral Ligation Procedure and Blood Flow Path**. A) Normal blood flow in a hindlimb. B) Site of ligation (X) and consequent blood flow pathway through gracilis collateral network.

*Intravital Microscopy*

Intravital microscopy was used to assess vasodilation of the collateral stem artery 7 days following femoral artery ligation. Mice were anesthetized and prepared as discussed in the previous section. An incision was made in the hindlimb and the epigastric fat pad was resected using a heat cautery. Plastic wrap was placed over the exposed tissue to prevent desiccation and the profunda femoris artery was given 30 minutes to reach a stable resting diameter.

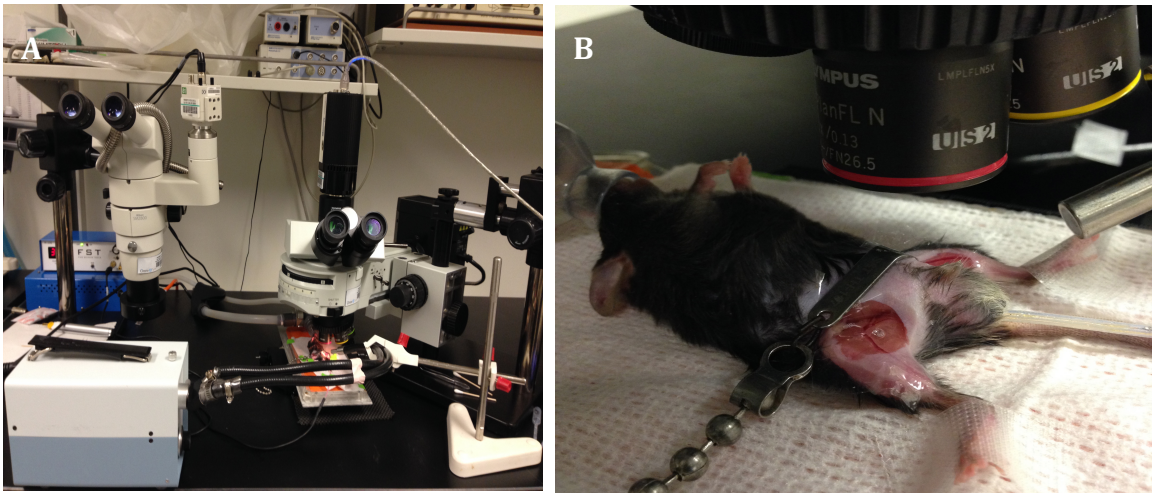


**Figure 6: Intravital Microscopy Preparation.** A) Experimental set-up during day-7 procedure. B) Exposed hindlimb on mouse, images of the profunda femoris were taken with an intravital microscope.

An intravital microscope (Olympus BXFM) with a 5x objective and QCapture Imaging software was used to capture images of the profunda femoris artery after the initial 30 min equilibration period at rest, and following maximal vasodilation (**Figure 6**). Next, vasodilators, sodium nitroprusside (SNP, $10^{-3}$) and papaverine ($10^{-2}$), were applied to the profunda femoris artery and left to maximally dilate the artery for 5 minutes before another set of images was taken on both the ligated and control hindlimbs.

*Sample Fixation and Processing*

After intravital imaging, tissues of interest were perfusion fixed. Briefly, a catheter was inserted into the left ventricle through a thoracotomy, and the cardiovascular system was perfused with 10mL PBS, 5mL 4% paraformaldehyde (PFA), and 10mL PBS using a syringe pump at 4 ml⍰min$^{-1}$ to maintain the cylindrical, in situ geometry of the profunda femoris artery. During the perfusion,

SNP and papaverine were periodically superfused to both hindlimbs to ensure the profunda femoris retained maximum diameter during perfusion fixation.

Following perfusion fixation, the gracilis muscle and profunda femoris artery were resected and post-fixed in 4% PFA overnight 4°C before rinsing with PBS and stored at 4°C prior to staining.

An antibody solution was prepared containing 1:200 1A4 clone (alpha-smooth muscle actin, Cy3 conjugate) in 0.1% saponin (in PBS) and 2% BSA, Bovine Serum Albumin (in PBS). Samples were removed from storage and placed into individual wells of a culture plate with 0.3mL of antibody solution and incubated for 7 days at 4°C. After incubation, the samples were washed in 0.1% saponin in PBS and covered with foil for 20 minutes; this process was repeated 3 times. The samples were then placed in plain PBS for 30 minutes before preparing for depression slide mounting. One to two droplets of 50/50 PBS and Glycerol were placed onto each depression slide using a transfer pipette and each sample was placed onto a slide, covered with cover glass, sealed with clear nail polish to prevent tissue desiccation, and stored at 4°C covered in foil until imaging.

*Confocal Microscopy*

Images of the profunda femoris artery were captured with a confocal microscope at 40x oil immersion objective using Fluoview Viewer (FV10-ASW 4.1) software using the Texas Red filter for light emission. The profunda artery was imaged at the collateral stem, just proximal to the collateral branch and light power was adjusted individually per sample to optimize brightness and SMC clarity as well as minimize light saturation. A z-stack with 1µm slices was captured for

approximately half the circumference of each artery setting the anterior portion of the artery as the top of the z-stack and extending towards the posterior side. See APPENDIX D.

*Image Analysis*

ImageJ was used to measure vessel diameter at rest and at maximum dilation from the intravital images. ImageJ was also used to merge the z-stacks of the profunda femoris into 3-D reconstructions to then create a 2-D image, and from these images, average smooth muscle cell overlap between the individual cells could be measured. SMC overlap was calculated by measuring all of the observable overlaps between cells to compute an average; the average length of the SMCs was also measured from these images.

Several considerations were made for measuring SMC length including attempting to rotate the 3-D reconstruction of the vessel to measure the length of each cell individually, ultimately however, a formula from previous studies was chosen to calculate the average smooth muscle cell length using a 2-D image view. To calculate average SMC length in a single viewing plane several assumptions must be made [19]:

1. The vessels are fully dilated and are cylindrical in shape.
2. There is only one layer of SMCs on the vessel.
3. The SMCs have only two end points.

4. There are two visible types of cells: “wrap” cells, which extend across the entire visible portion of the vessel, and “taper” cells, which partially extend the visible portion of the vessel (**Figure 7**).

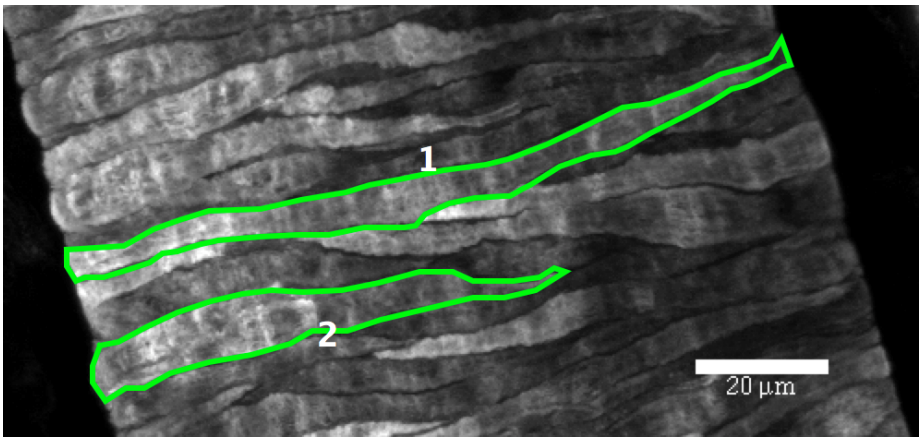


**Figure 7: Confocal microscopy view of SMCs.** 1) Wrap cell extends across entire visible portion of the artery. 2) Taper cell partially extends across visible portion of the artery (40x).

Average smooth muscle cell length was calculated based on a formula used in previous studies [18, 19], which uses statistical patterns of smooth muscle cell orientation to calculate average smooth muscle cell lengths using a single view of an image.

For each 3-D reconstruction, a 100µm length of artery was delineated for measurement. Average smooth muscle cell length for each vessel was determined using the following equation [18, 19]:

$$Lc = 4\pi RvF(Sw + St)/Tv$$

where Lc is average SMC length; Rv is the radius of the artery; F is the fraction of the vessel circumference that measurements were made; Sw is the number of wrap cells; St is the length of all tapered cells combined and divided by the length of a single wrap cell; and Tv is the number of taper cells.

*Statistical Analysis*

Differences in the profunda femoris artery at rest and maximum dilation as well as differences in SMC overlap and length between the ligated and control limbs were determined using a student t-test. A p-value of less than 0.05 determined statistical significance. Data are presented as bar graphs for visual comparison.

## RESULTS

The goal of this study was to test the hypothesis that smooth muscle cell, SMC, mechanoadaptation occurs during outward remodeling of the collateral stem following an upstream occlusion. The first objective was to measure the profunda femoris artery diameter *in vivo* to confirm that the vessel outwardly remodeled and exhibited impaired vasodilation at day-7 following occlusion.

### *Diameter*

As expected based on previous work, both resting and maximally dilated diameters were greater in the profunda femoris artery from the ligated hindlimb than the control hindlimb, 72.7 ± 4.3μm vs. 51.2 ± 3.1μm, respectively ($p<0.05$, Figure 8) for resting diameter, and 121.6 ± 2.6μm vs. 109.1 ± 3.4μm, respectively ($p<0.05$, Figure 8) for maximally dilated diameter. Also as predicted, the percent change in diameter from resting to maximum dilation was greater in the profunda femoris artery from control hindlimb than the ligated hindlimb, 117.1 ± 14.1% vs. 69.3 ± 7.6%, respectively ($p<0.05$, Figure 8).

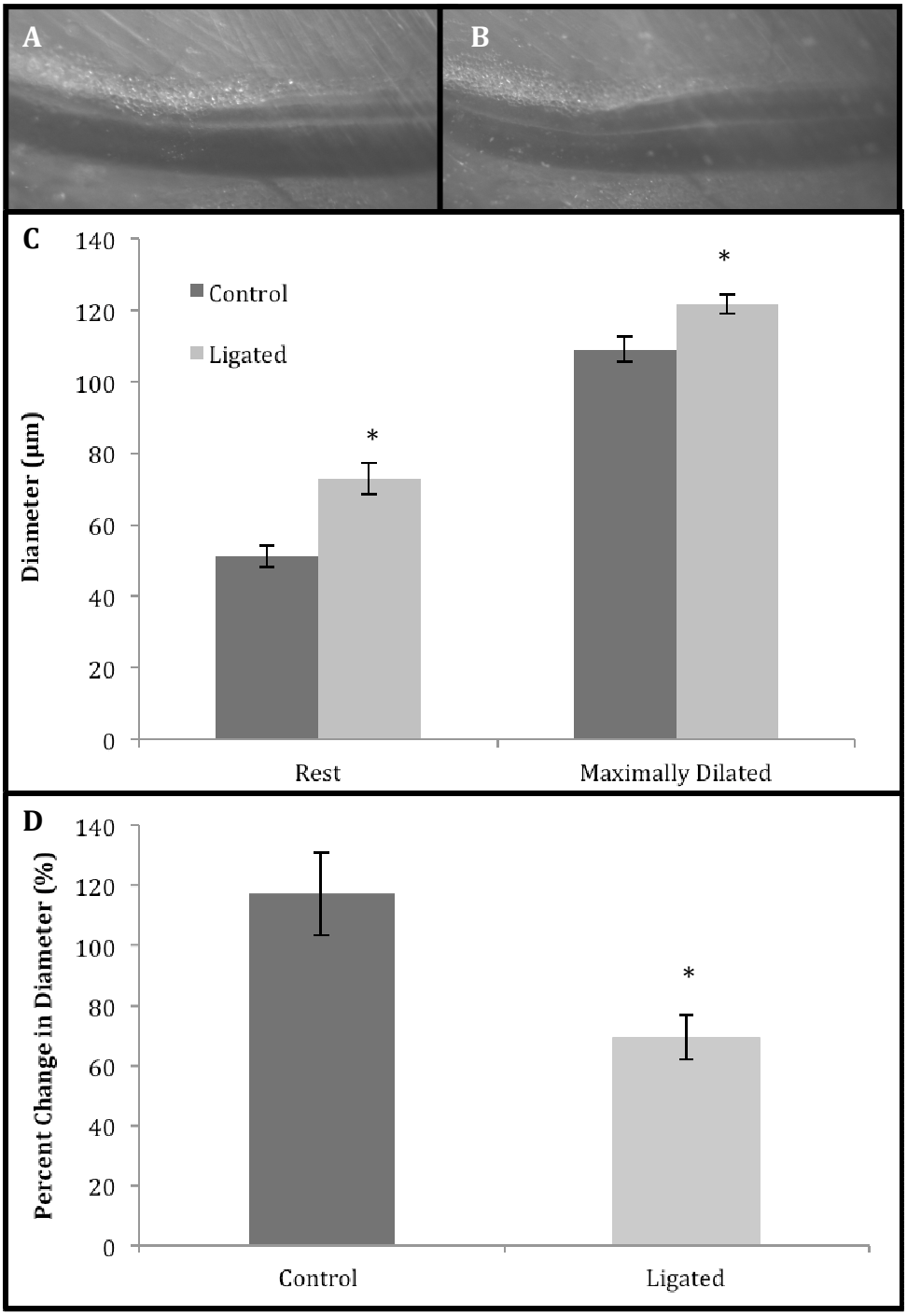


**Figure 8: Profunda Femoris Diameter**. A) Image of profunda femoris at resting diameter. B) Image of profunda femoris at maximally dilated diameter. C) Vessel diameter before and after dilation on control and ligated limbs. D) Percent change in vessel diameter from resting to maximum dilation, n=6; *p<0.05.

*SMC Overlap and Length - Mechanoadaptation*

Mechanoadaptation is expected to occur during the outward remodeling process because based on previous studies, SMC proliferation does not occur. The SMC overlap in the ligated hindlimb was shorter than the SMC overlap in the control hindlimb, 43.8 ± 1.4μm vs. 50.5 ± 1.9μm, respectively ($p<0.05$, Figure 9). Smooth muscle length was longer in the ligated hindlimb than in the control limb, 248.9 ± 13.1μm vs 205 ± 9.6μm, respectively, ($p<0.05$, Figure 10).

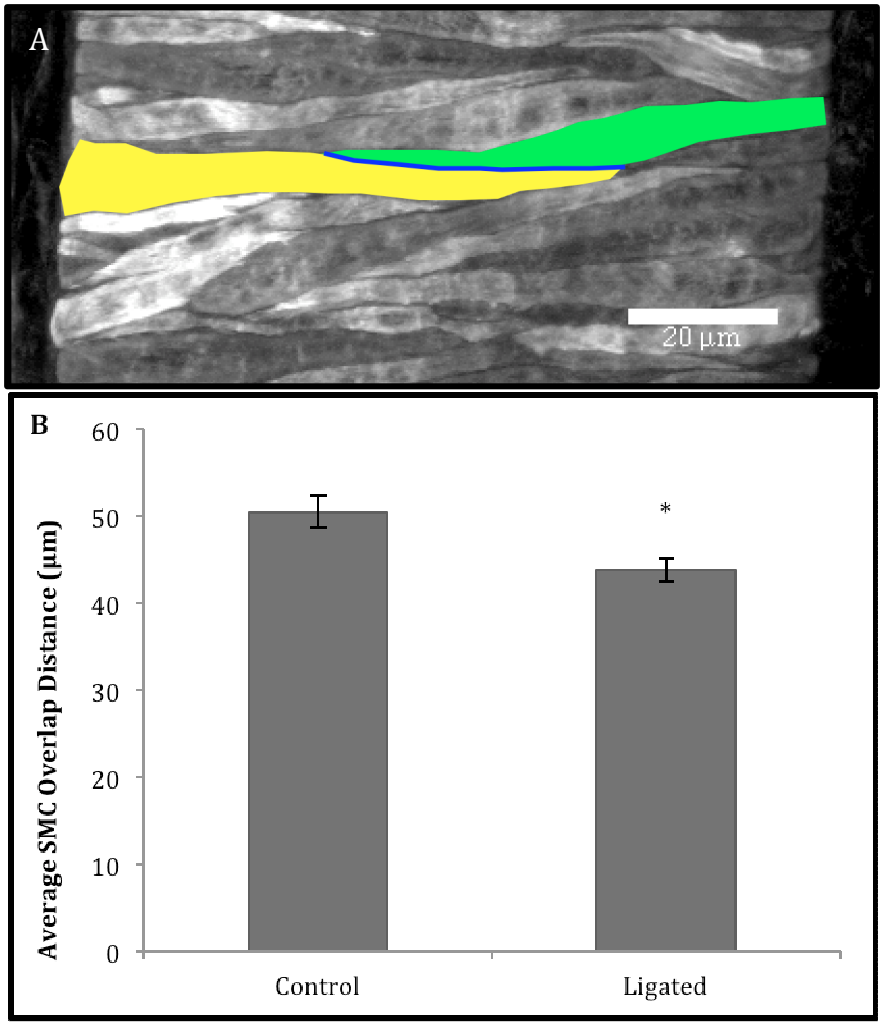


**Figure 9: Smooth Muscle Cell Overlap**. A) Overlap of two smooth muscle cells denoted in blue (40x). B) Average SMC overlap in the profunda femoris artery in the control and ligated hindlimbs, n=6; *$p<0.05$.

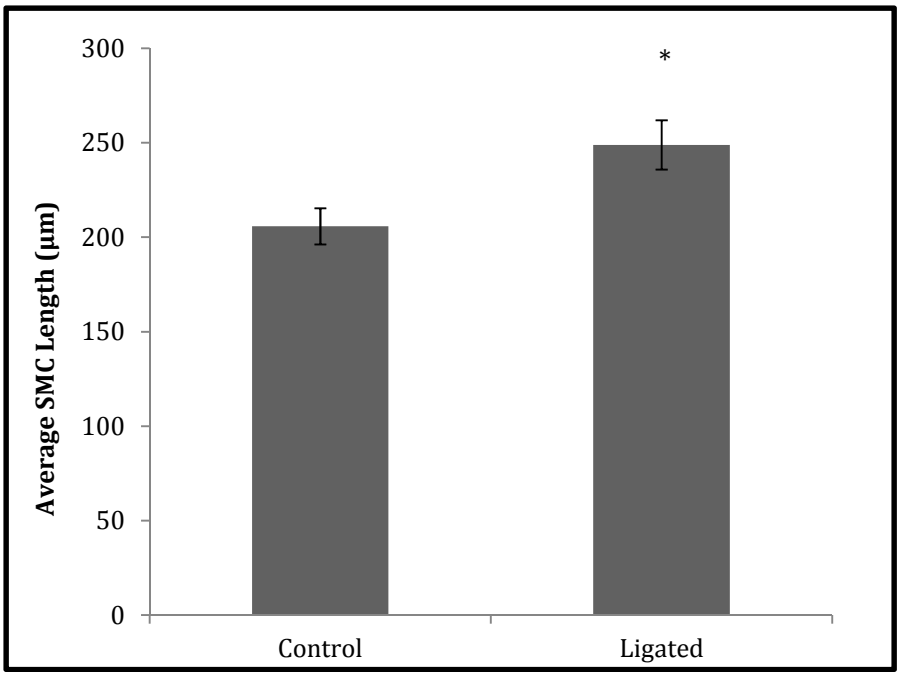


**Figure 10: Smooth Muscle Cell Length**. Average smooth muscle cell length in the profunda femoris artery in the ligated and control hindlimbs, n=6; *p<0.05.

## DISCUSSION

Patients with PAOD experience intermittent claudication, which may be explained by impaired vasodilation, therefore, restoring vasodilation is a potential method to alleviate symptoms. In animal models, impaired vasodilation in the stem of the collateral network can be attributed to smooth muscle cell dysfunction [16]. It has been previously established that smooth muscle cell, SMC, proliferation does not occur in the collateral stem and therefore is not the cause of the impaired vasodilation. To further investigate the cause of the impaired vasodilation, the goal of the study was to test the hypothesis that the vascular smooth muscle cells in the collateral stem artery underwent mechanoadaptation, or the reorientation of the cells in response to a mechanical stimulus.

The first step to test this hypothesis was to reaffirm that outward remodeling and impaired vasodilation occurred in the collateral stem following an upstream arterial occlusion by measuring the diameters of the profunda femoris artery, the collateral stem, *in vivo* at rest and at maximum dilation. We found that the ligated hindlimb exhibited a larger diameter at rest and at maximum dilation than the control, suggesting that outward remodeling had occurred. However, the percent change in the diameter from rest to maximum dilation was larger in the control hindlimb, indicating that vasodilation was impaired in the ischemic hindlimb.

Having confirmed that vasodilation was impaired; the second objective was to measure the lengths and overlaps of the individual smooth muscle cells in the profunda femoris artery to determine whether mechanoadaptation had occurred. It was found that SMCs had less overlap in the ligated hindlimb than the control. It

was also found that SMCs had a longer average cell length in the ligated hindlimb. These findings suggest that the SMCs of the collateral stem in the ischemic hindlimb have undergone mechanoadaptation.

The results from this study indicate that there is a correlation between impaired vasodilation and SMC mechanoadaptation occurrence in the collateral stem artery. We want to further investigate whether mechanoadaptation is both sufficient and necessary for vasodilation impairment. One way to determine whether it is necessary is to block mechanoadaptation from occurring after femoral artery ligation. One possible method for this is to use endothelial nitric oxide synthase (eNOS) inhibitors to prevent outward remodeling post-ligation. It is believed that eNOS plays a role in collateral remodeling because an increase in fluid shear stress activates eNOS and causes vascular smooth muscle relaxation in arteries, this is also known as flow-mediated dilation which, when chronic, leads to outward remodeling of the vessel [20]. It is of interest to inhibit eNOS and therefore block outward remodeling to determine whether vasodilation under these conditions exhibits impairment.

PAOD patients with pre-existing collaterals have a better prognosis than those who do not. In this study, C57BL/6 mice, which have pre-existing collaterals, were studied. A further experiment would be follow the same protocol using Balb/c mice, which have fewer pre-existing collaterals and less collateral remodeling [21], to see whether similar effects in smooth muscle cell mechanoadaptation are observed. This would be a useful study to complete to better understand how

different collateral anatomies react to an arterial occlusion because there is evidence that collateral density and remodeling varies among humans [21].

When making conclusions and interpretations from this data, it is important to consider that there were limitations in this study. In calculating SMC length, it was assumed that all vessels were perfectly cylindrical. Although the vessels might not be perfectly cylindrical, several actions were taken to address this including using perfusion fixation to maintain the cylindrical shape and using depression slides to mount the samples to prevent the arteries from being pressed and misshapen between the glass. It is believed that these actions aided in reducing the variability and error.

Further understanding of impaired vasodilation will lead to greater understanding of intermittent claudication and PAOD, which affects millions of Americans. Investigating smooth muscle cell orientation, which may play a role in impaired vasodilation, leads us to gain a better insight of the disease in the hopes of creating an effective treatment in the future.

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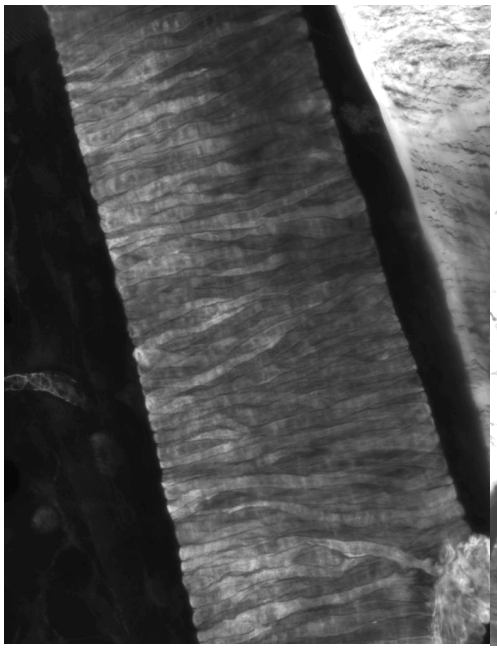
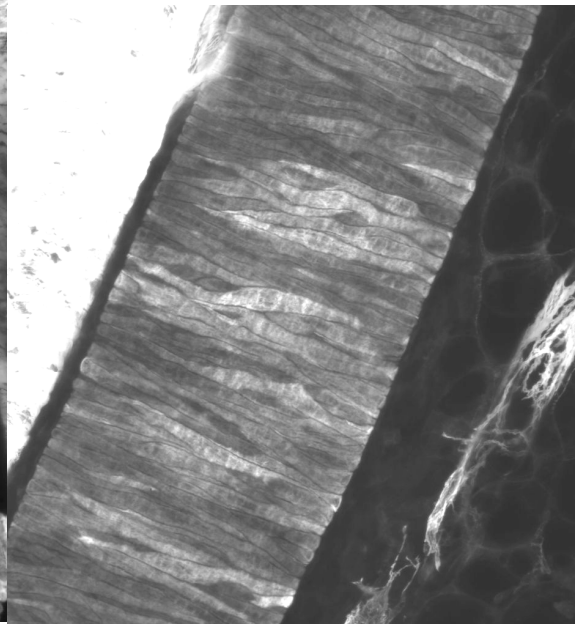
**APPENDIX A**

*Confocal Images of the Profunda Femoris Artery*



N=8 Control



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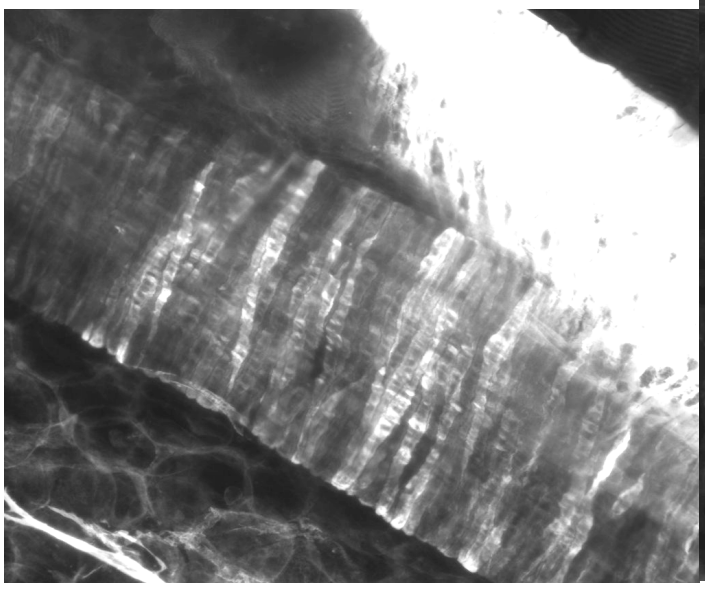

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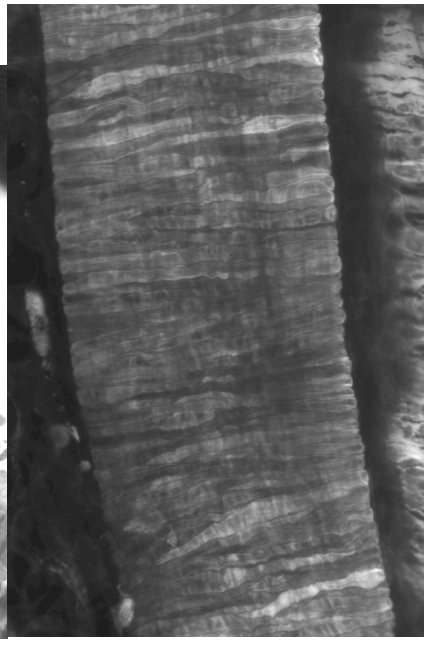

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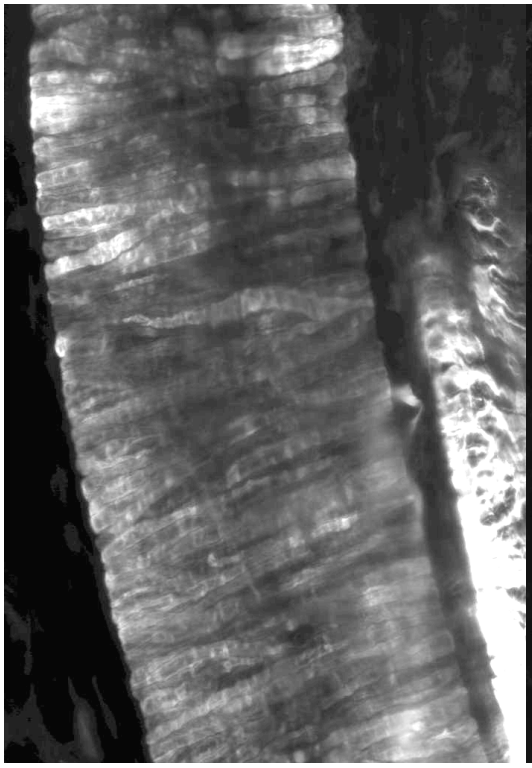

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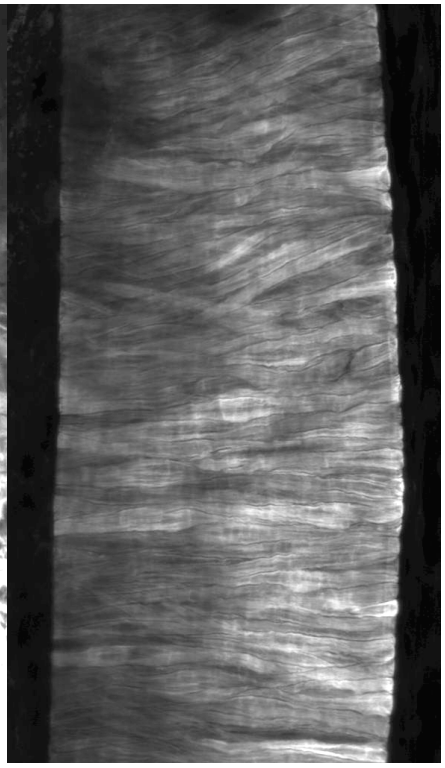

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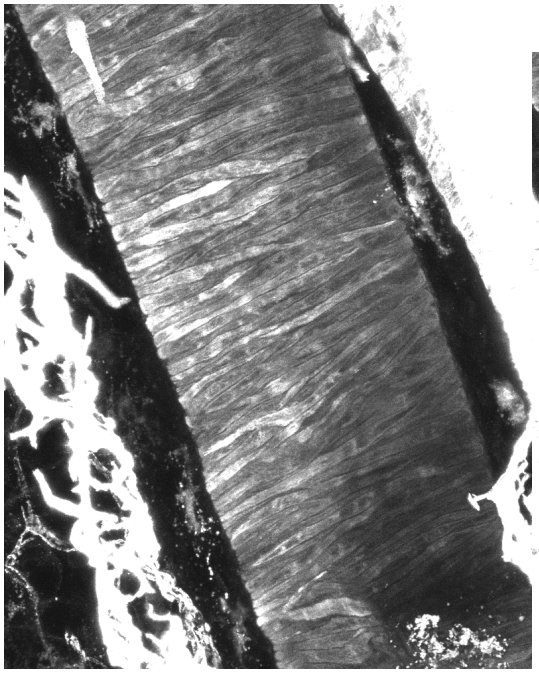

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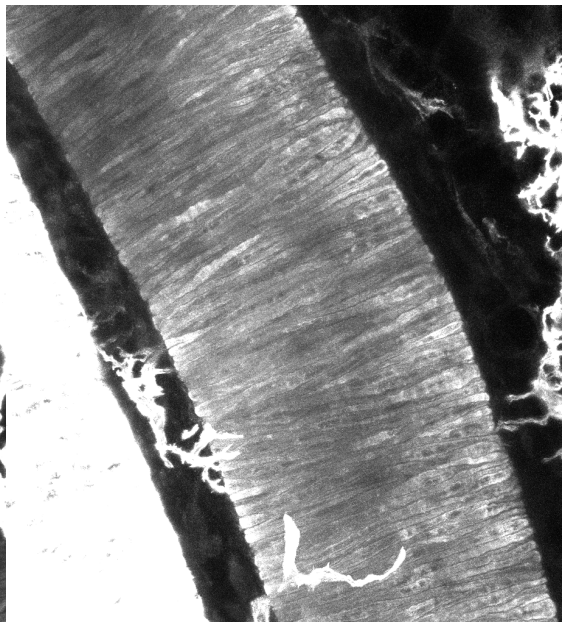
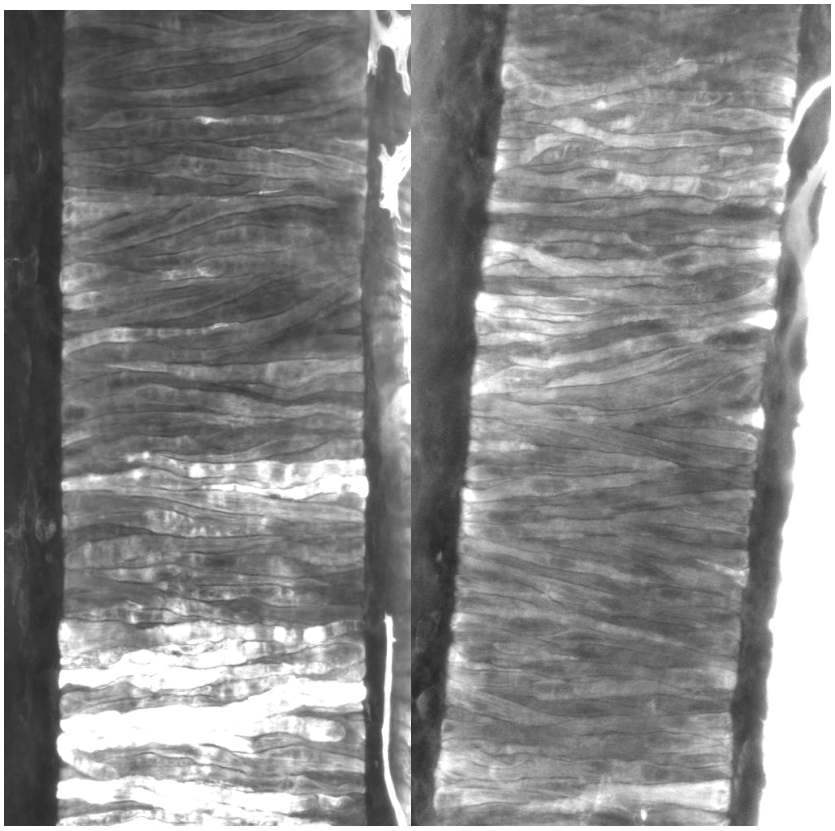


N=4 Ligated



N=9 Control N=9 Ligated

N=10 Control N=10 Ligated

## APPENDIX B

*Femoral Ligation Protocol*

Date _____ **Hindlimb Ischemia Surgery - Ligation** Initials _____

**Mouse Information**
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 a 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 desired 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. Blunt dissect the fat pad from the skin and use a retractor to pull back to expose ligation location
____47. Blunt dissect the femoral artery from the neurovascular bundle
____48. Tie off the femoral artery with 6.0 silk suture proximal to the popliteal branch and distal to the epigastric branch
____49. Use 6.0 polypropylene suture to close the skin
____50. Make a small incision on the middle, medial aspect of the right thing
____51. Extend the incision up to the abdominal wall
____52. Blunt dissect the subcutaneous connective tissue to maximize surgical exposure
____53. Use 6.0 polypropylene suture to close the skin

**Post-Surgical**
____54. Place the animal in the recovery bin, on a blue bench cover, above a heat pad and allow to recover
____55. Give the animal a second subcutaneous injection of buprenorphine (0.075mg/kg)
____56. Turn flow meter down to 0, turn off isoflurane, and close the oxygen cylinder
____57. Indicate surgery on cage card

**Notes**
_____
_____
_____
_____

## APPENDIX C

*Perfusion Fixation Protocol*

Date _____ **Perfusion Fixation** Initials _____

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

**Materials**
Non-Sterilize Dissection Instruments
____1. forceps (1)
____2. Bone Scissors(1)
____3. Iris Scissors (1)
____4. Vascular clamp (1)
____5. Castroviejo(1)

Obtained in surgery suite
____6. Surgical tape
____7. 10mL Syringe (1)
____8. Bench cover
____9. Heating pad
____10. Catheter
____11. Isoflurane Anesthetic
____12. Non-sterile saline
____13. Cotton swab
____14. 4x4 sponges (2)
____15. 20mL PBS
____16. 5mL 4% Paraformaldehyde (PFA)
____17. Syringe pump

**Procedure Preparation**
____18. Obtain beaker with 20mL PBS
____19. Fill 10mL syringe with PBS and place in syringe pump
____20. Place bench cover on top of heating pad

**Fixation**
____21. Anesthetize animal w/ 5% isoflurane
____22. Reduce flow rate to 0.5-1.0 $l \cdot min^{-1}$ and the isoflurane to 1-3%
____23. Tape animal down to bench cover
____24. Separate skin from muscle from the abdomen to the top of the thoracic cavity
____25. Locate the sternocleidomastoid (about halfway down abdomen) cut through muscular layer on top using forceps to pull back the layer and iris scissors to cut through it to expose the sternocleidomastoid
____26. Using the Castroviejo's, clamp the sternocleidomastoid
____27. Quickly cut through the ribs all the way up to the forelimbs using bone scissors
____28. Quickly cut through the diaphragm and pull back Castroviejo's cranially to open the chest cavity
____29. Cut away excess tissue around the heart by gently grabbing the excess tissue with forceps and cutting with iris scissors
____30. Gently grasp the heart to hold it steady using forceps and make a small incision in the apex of the heart in the left ventricle making sure to cut deep enough past the muscle layer to create a small hole in the ventricle using iris scissors
____31. Insert the catheter into the hole created and clamp down on it using the vascular clamp
____32. Make an incision in the right atrium to allow the blood to flow out using 4x4s for absorption
____33. Inject10mL PBS via catheter and syringe pump
____34. Inject 5mL of 4% PFA
____35. Inject 10mL PBS
____36. Resect the gracilis muscle and profunda femoris artery
____37. Leave in 4% PFA overnight at 4°C
____38. Remove PFA and store in PBS at 4°C

**Telling Signs of a Successful Perfusion**
- The liver will change color from red to gray as the blood and solutions perfuse through it
- Typically, the blood and solutions can be seen moving through the arteries and veins in the hindlimbs
- Fixation is successful upon seeing the 'formalin dance', the spontaneous movement of the limbs and tail while perfusing the PFA

**Notes**
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_____
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_____
_____
_____

## APPENDIX D

*Confocal Imaging Protocol*

Date _____ **Confocal Imaging** Initials _____

**Materials**

___1. Depression slide
___2. Coverglass (.08-.13mm thick)
___3. Clear nail polish
___4. 50/50 PBS-glycerol
___5. Slide box
___6. Forceps (1)

**Slide Preparation**

___7. Using forceps, gently place the sample into the well on the depression slide with the anterior of the sample facing up
___8. Drop 50/50 glycerol onto the sample
___9. Place coverglass on top of sample and use nail polish to seal the edges

**Confocal Microscope Setup and Imaging**

___10. Turn on the confocal microscope by switching the seven power buttons starting from top to bottom going from left to right (at the one with the keys, wait for the steady green light to move on)
___11. Log into the log book
___12. Log on to computer: password: fluoview
___13. Open Fluoview Program
___14. Choose "Load Acquisition" to pull up parameters of an old file
___15. Place a single drop of oil onto the 40x objective (this will be the viewing objective)
___16. Turn off the light before removing sample from slide box
___17. Place the sample onto the stage with the coverslip facing downward, toward the objective
___18. Carefully bring the objective closer to the sample using the coarse adjustment until the slide contacts the oil
___19. Open the shutter on the tube as well as the stage to let the fluorescent light in
___20. Turn on the fluorescent light via the icon on the program that has a diamond on top of a circle
___21. Locate the desired location of the vessel for imaging through the eye piece using the coarse and fine objective adjustments located on the bottom of the microscope near to the left and right of the stage
___22. Press XY Repeat on Fluoview program to bring image onto computer
___23. Adjust the power (do not increase the power above 20) to see the clearest image of the sample, making sure that image is not saturated
___24. Find the top of the vessel by altering the Z-plane, moving the fine adjustment clockwise to move to the surface of the sample and counterclockwise to go deeper into the sample
___25. Once the top is determined, click the "Set" button on the bottom left of the program
___26. Next find the end of the Z-stack desired by going deeper into the vessel and set this as the end point
___27. Set the step size to 1µm
___28. Click the Z-stack "XYZ" button to take the Z-stack
___29. Save the file as an .oib onto the computer and export it as ›a. tiff

**Microscope Clean Up**

___30. Turn off the microscope in the opposite way it was turned on, from right to left, bottom to top
___31. Make sure to close the shutters
___32. Log out in the log book
___33. Fold a lens sheet and drip isopropyl alcohol (IPA) onto it then make a single swipe over the objective to clean the oil from it, repeat until the lens is clear using several lens sheets, use a cotton tip applicator with IPA to clean the area around the lens on the objective
___34. Cover confocal microscope

**Notes**

_____
_____
_____