MECHANISMS OF REDUCED VASCULAR TONE FOLLOWING ARTERIOGENESIS

INDUCED BY FEMORAL ARTERY LIGATION

A Senior Thesis

Presented to

the Faculty of California Polytechnic State University, San Luis Obispo

In Partial Fulfillment

of the Requirements for the Degree in

Bachelor of Science in Biomedical Engineering

By

Christopher Justus Hatch

August 2019

TITLE: MECHANISMS OF REDUCED VASCULAR TONE

FOLLOWING ARTERIOGENESIS INDUCED BY FEMORAL

ARTERY LIGATION

AUTHOR: CHRISTOPHER JUSTUS HATCH

DATE SUMBITTED: AUGUST 2019

ADVISOR: TREVOR CARDINAL, ASSOCIATE PROFESSOR

ABSTRACT

Mechanisms of Reduced Vascular Tone Following Arteriogenesis Induced by Femoral Artery

Ligation

Chris Hatch

The presence of a developed, native collateral network can decrease the severity of ischemic injury proceeding arterial occlusion. The collateral network must under arteriogenesis to enlarge and increase blood flow to the ischemic region. Although there has been tremendous effort attempting to understand the mechanisms of arteriogenesis, no therapies have been successful in improving patient outcome. To better understand the mechanisms involved in arteriogenesis, the effect of nitric oxide production, myogenic tone, and α-adrenergic receptors were evaluated as these have been identified as playing an important role in vascular injury. Arteriogenesis was induced by ligating the femoral artery between the epigastric and popliteal branches in male C57/BL6 mice between two to four months old. Pharmacological agents were dissolved in a physiological salt solution that was superfused over the exposed gracilis anterior to generate does response curves. The collateral diameter was measured using intravital microscopy. Diameter measurements were normalized to resting diameter to create percent changes for the operated vessels and contralateral sham. Procedures were performed at both seven and twenty-eight days following femoral artery ligation to evaluate how pathways changed with the restoration of vascular tone. Nitric oxide production does not appear to play an important role as the values for the day seven ($-47 \pm 7\%$ for the operated and $-43 \pm 5\%$ for the contralateral control) were similar to day twenty-eight ($-31 \pm 5\%$ vs $-27 \pm 4\%$, control and operated respectively). Myogenic tone does not appear to play an important role as the values for day seven ($19 \pm 3\%$ for ligated and $31 \pm 7\%$ for the sham) are similar to day twenty-eight ($25 \pm 3\%$ vs $39 \pm 6\%$, ligated and sham respectively). α-adrenergic receptor stimulation appears to play an important role as there is a heightened response at day seven ($-71 \pm 7\%$ vs $-39 \pm 6\%$, ligated vs sham respectively) compared to day twenty-eight ($-44 \pm 4\%$ vs $-31 \pm 9\%$, ligated vs sham respectively). However, inhibition did not appear to be significant because there is a lack of response at both day seven ($16 \pm 9\%$ vs $73 \pm 15\%$, ligated vs sham, respectively) and day twenty-eight ($16 \pm 7\%$ vs $50 \pm 7\%$, ligated vs sham, respectively). These findings suggest that there is lack of sympathetic innervation seven days after ligation that is restored twenty-eight days later.

Keywords: arteriogenesis, α-adrenergic receptors, endothelial nitric oxide synthase, L-type voltage gated calcium channels, sympathetic innervation, vascular reactivity

ACKNOWLEDGMENTS

"Hmmmmm"

TABLE OF CONTENTS

Page

LIST OF FIGURES

Introduction

Peripheral artery disease (PAD) affects approximately 8.5 million people in the United States and manifests as intermittent claudication, or pain while walking [National, Di Minno, Criquie]. PAD is a type of ischemic disease caused by atherosclerosis of the lower extremities [Allen], and the most severe form of PAD is critical limb ischemia (CLI) which effects roughly 11% of patients (figure 1) [Nehler]. CLI manifests itself as severe blockage in the arteries of the lower extremities that results in ischemic rest pain, gangrene, and ulcer formation [Samura]. Approximately 20-30% of the individuals with CLI cannot receive the common treatments of angioplasty or bypass due to comorbidities [Davies, Norgen]. As a result, about 20% of the CLI patient population faces mortality within five years [Norgen]. Therefore, there is a need for alternative therapeutic options to treat individuals with PAD or CLI that cannot receive the common treatments.
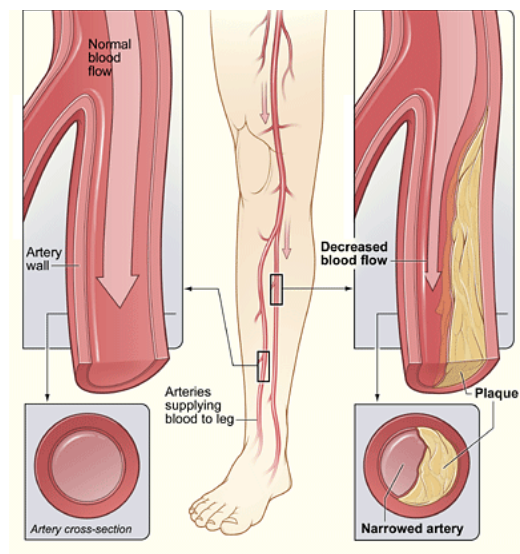

Figure 1. Peripheral Artery Disease (PAD). PAD is the accumulation of plaque in the peripheral circulation, typically in the lower extremities [Peripheral]

The presence of collaterals, or natural bypasses around the occluded region, can mitigate the severity of PAD [Annex] as increased collateral number is associated with improved functional performance [McDermott, Keeling]. Arteriogenesis, collateral growth, is a response mechanism to provide an ischemic region with sufficient blood flow by increasing the hemodynamic capability of the collaterals (figure 2) [Yu]. Many groups have tried to stimulate arteriogenesis in clinical trials by targeting the key processes involved. [Krishna, Annex, Raval, Maufus, Perin]. Arteriogenesis is caused by an increase in shear stress due to the occlusion that stimulates structural wall remodeling in an attempt to normalize shear stress [Troidl, Mack]. Additionally, the occluded vessel can create a hypoxic region downstream that leads to cells releasing pro-angiogenic cytokines and growth factors [Landázuri, Annex]. These growth factors and increased shear stress mobilize progenitor and inflammatory cells from the bone marrow to enter the surrounding tissue and start the process of arteriogenesis [Landázuri, Mack]. Numerous cells and pathways have been targeted to develop therapeutic treatments stimulating arteriogenesis [Krishna, Annex, Raval, Maufus, Perin]. The pro-angiogenic vascular endothelial growth factor (VEGF) was regarded as one of the most likely candidates for treatment. VEGF induces monocyte chemoattractant protein -1 (MCP-1) in endothelial cells (ECs) which leads to monocyte recruitment, and it stimulates EC proliferation and migration [Jazwa, Schaper]. Other approaches focused on colony stimulating factor, progenitor cells, and autologous bone marrow derived mononuclear cells [Raval, Maufus, Perin]. Although there were promising results in preclinical models, there has been no conclusive efficacy in larger human randomized clinical trials [Krishna, Annex, Raval, Maufus, Perin].
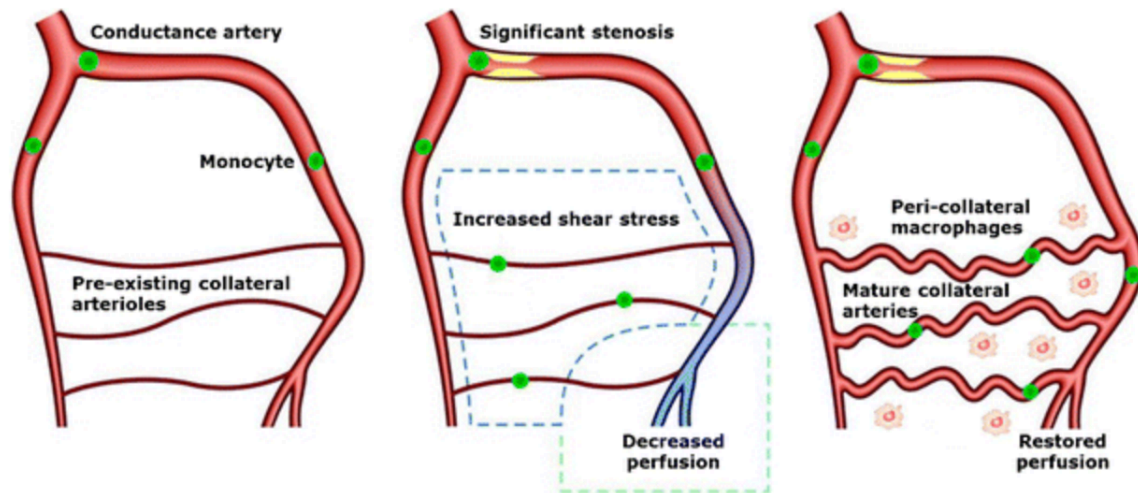
Figure 2. Mechanisms of Arteriogenesis. Increased shear stress leads to monocyte recruitment that stimulates arteriogenesis [Schirmer]

Therefore, it might not be sufficient for these collaterals to reperfuse downstream tissue by acting solely as larger conductance pathways because abnormal vascular reactivity might limit the therapeutic effects of collaterals [Bauters]. Collaterals should also be able to regulate blood flow to match oxygen delivery with tissue metabolic demand [Rey]. Normally, blood flow is locally controlled to ensure sufficient oxygen to tissues. However, following ischemia, blood flow is controlled by a high resistance collateral circuit that must have a functional range that can accommodate varying levels of tissue metabolic demand [Taylor]. Therefore, it is interesting that most investigators focus on maximum diameter measurements, which does not provide information about the resting diameter and vascular reactivity, which define the ability of a resistance vessel to regulate blood flow. [Gruionu, Heuslein, Clayton]. In order to develop new therapeutic strategies, a greater understanding of the processes of vessel adaption is needed, which can be accomplished by evaluating vascular reactivity [Jaipersad].

Although clinical observations in patients with PAD show a loss of vascular reactivity, there is no consensus on the underlying mechanisms [Silva, Langham]. One explanation is endothelial

dysfunction, as patients with PAD have greater endothelial dysfunction with impaired vasodilation [Parshakov]. However, patients with PAD have significantly higher plasma NO levels compared to non-PAD patients [Miralles], suggesting that the lack of vasodilation is caused by insensitivity of SMCs to NO [Silva]. SMCs display both contractile and synthetic phenotypes, each with diverse migration rates and protein expression [Rensen], and SMCs switch between the two phenotypes based on environmental stimuli [Gomez]. A pro-inflammatory environment causes SMCs to transition into a synthetic, largely noncontractile phenotype [Brozovich]. During arteriogenesis, there is an increase in regulator of G-protein signaling 5 (RGS5), which terminates signaling cascades that control contractility of SMCs [Arnold]. Both factors could affect the ability of SMCs to regulate vascular reactivity, which would lead to reduced functionality. In newly formed collaterals of the mouse spinotrapezious muscle, there is a loss of vascular reactivity via impaired vasodilation and lack of vasoconstriction seven days after arteriogenesis begins, which is caused by a lack of SMCs contractility. However, these are presumably new SMCs in the region, which might explain the restoration of vascular reactivity at twenty-one days [Gouin III]. The lack of agreement on the cause of a loss of vascular reactivity in PAD patients suggests the need to further examine the basic mechanisms driving collateral remolding . If important signaling pathways can be identified, better therapeutics might be developed [Heuslein, Jaipersad].

Vascular tone is regulated by vasoconstriction and vasodilation of the collaterals that include many signaling pathways (figure 3) [Korthuis, Tykocki, Durand, Dora]. Myogenic tone, the mechanism responsible for pressure-dependent, steady state contractility of SMCs, plays a prominent role in regulating vascular tone [Jackson]. Specifically, L-type voltage Ca^{2+} channels (LVCCs) are integral as they are major source of activator Ca^{2+} in myogenic tone [Tykochi]. Fluid shear stress regulates vascular tone by activating endothelial nitric oxide synthase (eNOS)

that creates NO [Iring]. NO then acts as a vasodilator of SMCs, thus regulating vascular tone [Zhao Vascular nitric oxide]. Release of norepinephrine (NE) from sympathetic nerves targets α-adrenoreceptors thus helping to regulate vasoconstriction [Calzada]. In α_1-adrenoreceptors, NE increases the inositol triphosphate (IP3) pathway which leads to an increase in intercellular Ca^{2+} and SMC vasoconstriction. On the other hand, in α_2-adrenoreceptors, NE inhibits the adenylyl cyclase (cAMP) pathway leading to vasoconstriction. Although there are other pathways, in peripheral vascular disease these have been identified as being the most important [Frisbee].
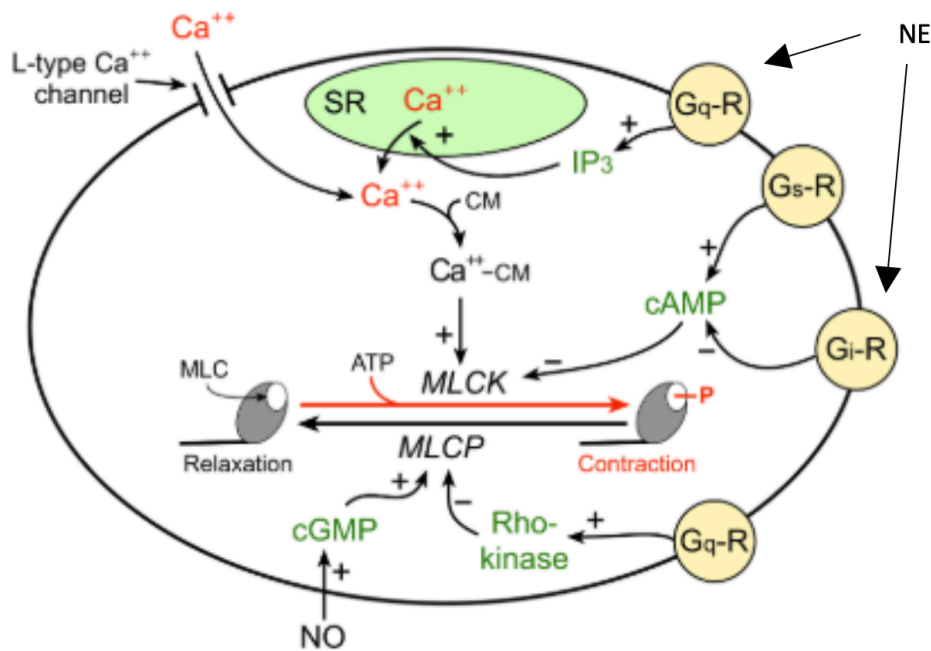
Figure 3. Regulation of Vascular Tone. L-type Ca^{2+} channels increase SMC intercellular Ca^{2+} leading to increased vasoconstriction. NE stimulates vasoconstriction. NO produced from shear stress leads to vasodilation

Previous work in the lab has demonstrated that arteriogenesis following femoral artery ligation induces a loss of vascular tone that is restored at a later point in time [Chu]. The goal of this work was to evaluate the signaling pathways that might be responsible for the loss.

Methods

 Animals: All procedures were performed according to a protocol approved by the California Polytechnic State University Institutional Animal Care and Use Committee. Male C57BL/6 mice (The Jackson Laboratory; Sacramento, CA) between two and four months were used for all experiments. The mice were maintained on a twelve-hour light/dark cycle with ad libetum access to food and water. Mice were housed in microisolator cages and provided nesting material, plastic tube, and "housing".

 Femoral Artery Ligation: Chronic hindlimb ischemia was induced by ligating the femoral artery between the epigastric and popliteal branches [Limbourg]. Animals were anesthetized in an induction chamber with 5% isoflurane in oxygen flowing at 1L/min. Hair was removed using depilated cream and skin was disinfected with chlorhexidine diacetate. Ophthalmic ointment was applied to prevent corneal desiccation. Pre- and post-surgical buprenorphine analgesic (0.075 mg·kg^{-1}) was administered and body temperature was maintained at 35°C through the use of a temperature-controlled heat pad and rectal thermistor probe (CWE, Inc.; Ardmore, PA). Following a skin incision above the neurovascular bundle in the hindlimb, the left femoral artery was separated from the neurovascular bundle distal to the epigastric branch and proximal to the deep femoral branch. The femoral artery was ligated with 6-0 silk suture. The incision site was then closed with 7-0 polypropylene suture. A sham surgery was performed on the contralateral hindlimb, in which the skin was exposed and the connective tissue overlying the femoral neurovascular bundle was blunt dissected before skin closure. Mice recovered on a heat pad until ambulatory.

 Solution Preparation: The physiological salt solution (PSS, ~35°C, pH~7.4) was composed of (in μM): NaCl 137, KCl 4.7, MgSO$_4$ 1.2, CaCl$_2$ 2, and NaHCO$_3$ 18. Nitric oxide synthase inhibitor N$_\omega$-Nitro-L-arginine methyl ester hydrochloride (L-NAME, Sigma-Aldrich,

Lot No: MKBV9596V) and norepinephrine (NE, Sigma-Aldrich, Lot No: BCBR1921V) were dissolved at 10^{-3} M and stored at -20°C until the day of the procedure. Norepinephrine receptor inhibitor (α_1) prazosin (Cayman Chemical, Lot No: 0446327-23), (α_2) rauwolscine (Cayman Chemical), and calcium channel blocker nifedipine (Cayman Chemical) were dissolved at 10^{-3} M and freshly prepared.

Superfusion: 7 or 28 days following femoral artery ligation, animals were prepared as previously described. The gracilis muscle was exposed via skin incision and connective tissue was removed. A trans-illuminating LED probe was placed under the hindlimb to allow visualization of the primary collateral. 60 ml of PSS was placed in a syringe heater (custom built) and deoxygenated with 5% CO_2 - 95%N_2. PSS was then passed over the exposed collateral at ~35°C at ~2 ml/min for an equilibration period of at least thirty minutes. Resting images were captured using an intravital microscope (Olympus BXFM) with a 10X objective (Olympus LMPlanFL N) and a digital imaging software (QCapture Pro). Responses to pharmacological agents were determined from 10^{-8} M to 10^{-4} M over a period of five minutes. The procedure was repeated on the sham side; if necessary, the entire process was then repeated for additional agents.

Perfusion Fixation: 7 or 28 days post-ligation, perfusion fixation was used to preserve gracilis tissues. Before perfusion, gracilis anterior muscles were separated from surrounding fascia to facilitate resection. After exposing the thoracic cavity, a 27G catheter was inserted into the left ventricle via the apex of the heart, and the right atrium was cut to create an open system. A 60 ml syringe loaded with a warm solution of 40 ml PBS containing 10^{-3} M Sodium Nitroprusside, 10^{-4} M Adenosine, and 100 U/ml Heparin as an anticoagulant was perfused at 4ml·min^{-1} using a 10 ml syringe. Afterwards, animals were fixed with 5 ml of cold 4%

paraformaldehyde. Once fixed, gracilis anterior muscles were resected from the profunda femoris artery to the distal saphenous artery.

Immunofluorescent: Gracilis anterior muscles were whole-mount immunostained to visualize vasculature (α-smooth muscle actin) and sympathetic innervation (tyrosine hydroxylase). Following resection, muscles were incubated in 2% Triton-X-100 in 1X PBS for 2 hours at room temperature. Muscles were then stained with 0.1% Triton-X-100, 2% BSA, 1:300 Anti-Actin α-smooth muscle – Cy3 antibody mouse monoclonal (Sigma-Aldrich C6198), 1:1000 Anti-Tyrosine Hydroxylase Antibody (Millipore Sigma AB152) in PBS at 4°C for 96 hours. They were then washed in PBS three times for 10 minutes at room temperature. Muscle were incubated with 1:100 of secondary Goat anti-Rabbit IgG Cross-Adsorbed Secondary Antibody, Alexa Flour 488 (ThermoFisher A11008) for 24 hours at room temperature and washed in 0.1% Triton-X-100 in PBS three times for 10 minutes at room temperature followed by a 30 minute wash in PBS at room temperature. Muscles were mounted on depression slides with 50/50 glycerol (Sigma G7757-1L) and PBS and stored in 4°C for one day before imaging.

Imagining: Whole muscles were imaged at 10X and 20X on an Olympus FV1000 confocal microscope using the Alexa Fluor 488 and Texas red dye (594 nm) filter settings. Images were captured using the Multi-Area Time-Lapse feature by identifying the region of interest using the mosaic outline.

Measurements and Statistics: Vessel diameters were measured with Image/J software at three different regions in the primary collateral. Differences between all concentrations and sham and ligated sides were tested using repeated measured ANOVA followed by Tukey pairwise comparisons. Percent change was calculated as the difference between a given time point and resting diameter divided by resting diameter. All measurements are displayed as mean ± standard error.

Results:

   The goal of this work was to identify signaling pathways that contribute to the restoration of vascular tone following arteriogenesis. The three pathways that were evaluated were nitric oxide production, myogenic tone, and α-adrenoreceptors because they have been identified as being the most important in peripheral vascular disease [Frisbee].

   The first pathway that was evaluated was NO production by inhibition with LNAME. Seven days post femoral artery ligation the resting diameter on the ligated side was $83 \pm 9\mu m$ with a minimum diameter of $51 \pm 5\mu m$ (Figure 4C). The contralateral sham had a resting diameter of $44 \pm 3\mu m$ with a minimum diameter of 30 ± 2 μm (Figure 4C). In order to gain greater clarity, values were converted to percent change. The percent change normalized measurement to the resting diameter, eliminating any bias from varying resting measurements. This translated to a functional range of $-47 \pm 7\%$ for the operated and $-43 \pm 5\%$ for the contralateral control (Figure 4E). Twenty eight days later both the contralateral control and operated had similar percent changes, $-31 \pm 5\%$ vs -27 ± 4 %, respectively (Figure 4F). The operated side has a resting diameter of $44 \pm 3\mu m$ and a minimum diameter of $30 \pm 2\mu m$; whereas, the contralateral control has a resting diameter of $33 \pm 2\mu m$ and a minimum diameter of $24 \pm 2\mu m$ (Figure 4D). Because there is no statistical significance between the percent change at day seven and day twenty eight, it is likely that NO production is not responsible for the loss of vascular tone and later restoration.
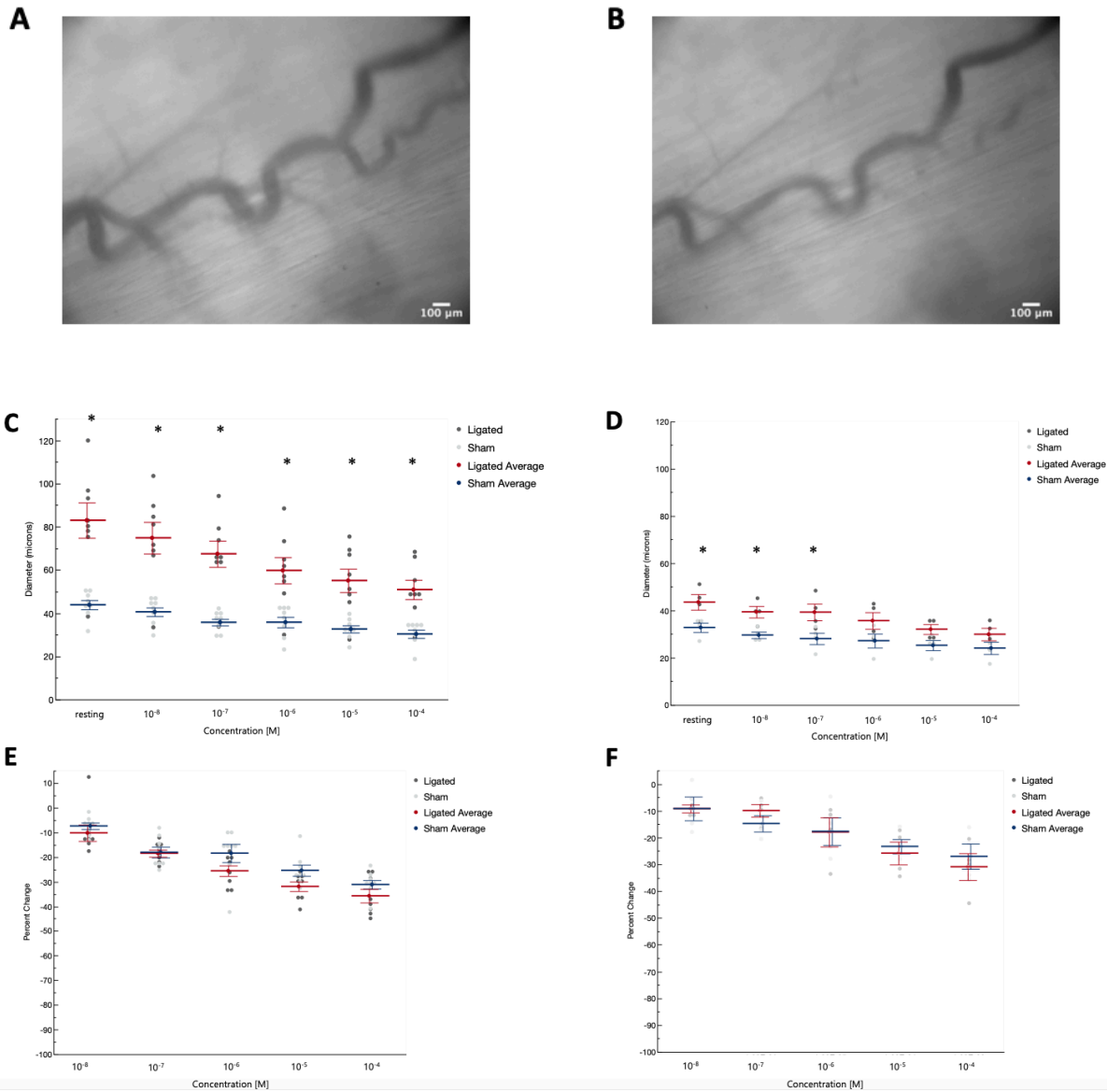
Figure 4. The effect of nitric oxide on collateral growth. NO does not appear to play a significant role in restoring vascular reactivity. The representative images of the resting (A) and 10^{-4} M (B). There appears to be no significant difference in vascular reactivity at either 7 days (C,E) or 28 days (D,F) post-femoral artery ligation. The difference observed in the micron measurements is caused by an increased resting diameter on the ligated side * = $p<0.05$ between ligated and sham. % = $p<0.05$ between ligated day seven and day twenty-eight.

Because an excess vasodilating influence does not appear to be responsible, another possibility was an insufficient vasoconstricting influence. Therefore, the second pathway that

was evaluated was myogenic tone. Specifically, L-type voltage gated calcium channels (LVCCs) were inhibited with nifedipine. tone. If myogenic tone played an important role, it would be expected that there is a reduced influence of inhibition at day seven compared to day twenty eight. Seven days following ligation the resting diameter of the ligated side was $86 \pm 3\mu m$ with a max diameter of $102 \pm 2 \mu m$. The sham side had resting diameter of $41 \pm 3\mu m$ and a max diameter of $53 \pm 4\mu m$ (Figure 5C). This corresponds to a $19 \pm 3\%$ functional range for the ligated side compared to a $31 \pm 7\%$ range for the sham (Figure 5E). Twenty eight days later, the functional range for the ligated side remained relatively unchanged with a resting diameter of $70 \pm 6\mu m$ and a max diameter of $88 \pm 7\mu m$. The sham has a resting diameter of $39 \pm 6\mu m$ and a max diameter of $53 \pm 6\mu m$ (Figure 5D). This produces a functional range of $25 \pm 3\%$ for the ligated side compared to $39 \pm 6\%$ for the sham (Figure 5F). Because there is only one concentration between both time points that is statistically different between the ligated and sham, it is likely that the loss and restoration of vascular tone is not caused by LVCCs and myogenic tone. Therefore, the hypothesis that myogenic tone plays a vital role is rejected because there was not a reduced impact at day seven.
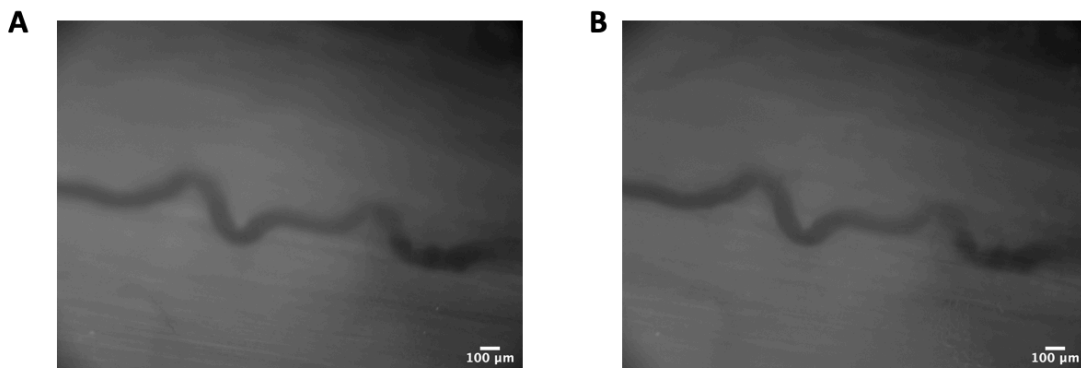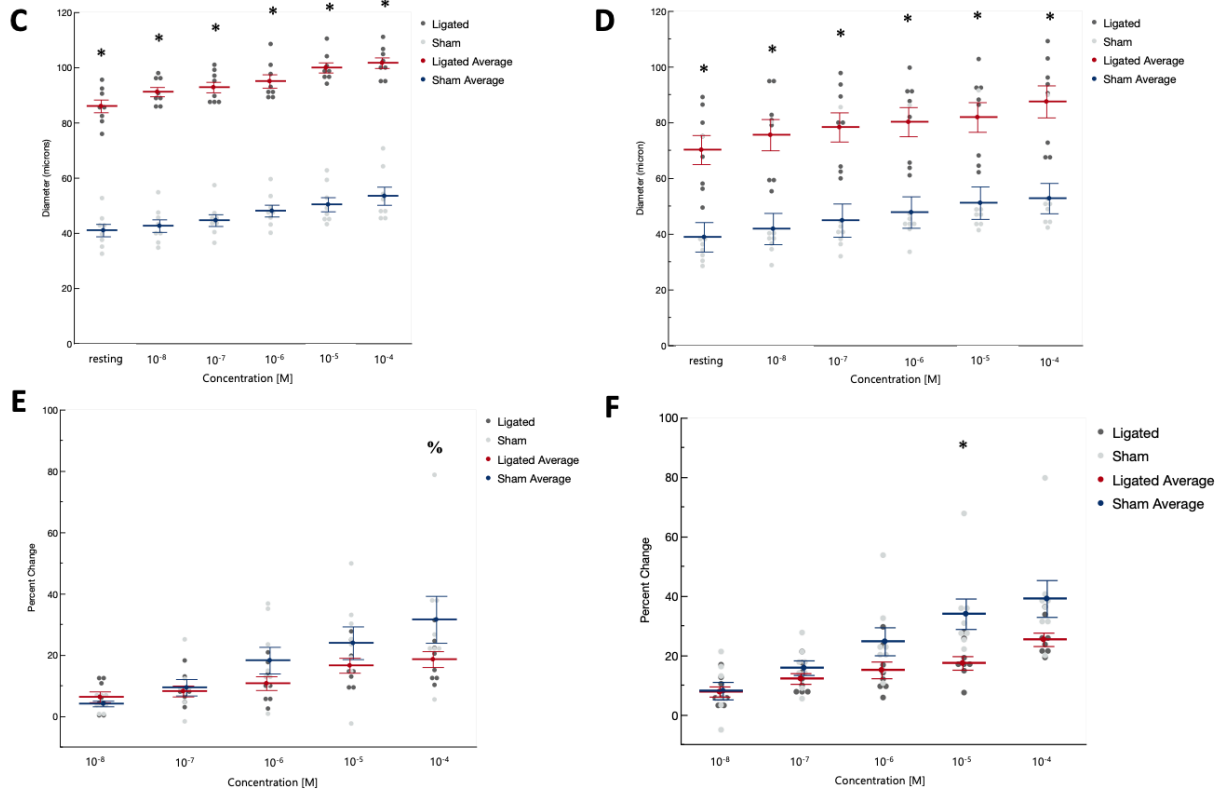
Figure 5. Effect of myogenic tone on collateral growth. Myogenic tone does not appear to play an important role in regulating vascular tone following arteriogenesis. The representative images at day seven between resting (A) and 10^{-4} M (B). There appears to be no significant difference in vascular reactivity at either 7 days (C,E) or 28 days (D, F) post-femoral artery ligation. The difference observed in the micron measurements is caused by an increased resting diameter on the ligated side. * = $p<0.05$ between ligated and sham. % = $p<0.05$ between ligated day seven and day twenty-eight.

The third pathway that was evaluated was α-adrenergic receptors. In order to support a hypothesis that VSMCs were in a state of proliferation, a minimal response to stimulation of α-adrenergic receptors at day seven and increased response at day twenty-eight would be expected. To test this, the receptors were stimulated with NE. At seven days post femoral artery ligation, the collaterals on the operated side had a resting diameter of $78 \pm 8\mu$m and a minimum diameter of $28 \pm 5\mu$m, while the contralateral control had a resting diameter of $48 \pm 3\mu$m with a minimum

diameter of $30 \pm 3\mu m$ (Figure 6C). The functional range of the operated side was $-71 \pm 7\%$ compared to $-39 \pm 6\%$ for the sham, suggesting that VSMCs were hypersensitive to stimulation (Figure 6E). However, at day twenty eight, vasoconstriction caused by NE was not statistically different between the operated side and contralateral control, $-44 \pm 4\%$ vs $-31 \pm 9\%$, respectively (Figure 6F). The resting diameter for the operated side at day twenty-eight was $46 \pm 3\mu m$ and the minimum diameter was $26 \pm 2\mu m$. The resting diameter for the contralateral sham was $33 \pm 3\mu m$ with a minimum diameter of $23 \pm 3\mu m$ (Figure 6D). This would suggest that VSMCs phenotype is not responsible for the loss and restoration or vascular tone. Interestingly, it appears that α-adrenergic receptors are hypersensitive to stimulation suggesting that there might be insufficient NE signaling.

To evaluate the presence of α-adrenergic stimulation, prazosin, an α_1-adrenergic antagonist was applied to induce vasodilation. At day seven, prazosin reduced vascular tone in the contralateral control significantly more than in the enlarged collateral, increasing diameter $73 \pm 15\%$ vs $16 \pm 9\%$, respectively (Figure 6I). The resting diameters for the resting of contralateral sham and operated were $25 \pm 2\mu m$ and $47 \pm 10\mu m$, respectively. The maximum diameters were $44 \pm 8\mu m$ and $53 \pm 7\mu m$, control vs operated, respectively (Figure 6G). This trend was consistent at day 28 where the vasodilation between the operated and contralateral was significantly different, $16 \pm 7\%$ vs $50 \pm 7\%$, respectively (Figure 6J). The resting diameter and max diameter for the contralateral control were $28 \pm 3\mu m$ and $42 \pm 4\mu m$, respectively. For the operated, the resting diameter was $40 \pm 3\mu m$ and the max diameter was $47 \pm 6\mu m$ (Figure 6H).
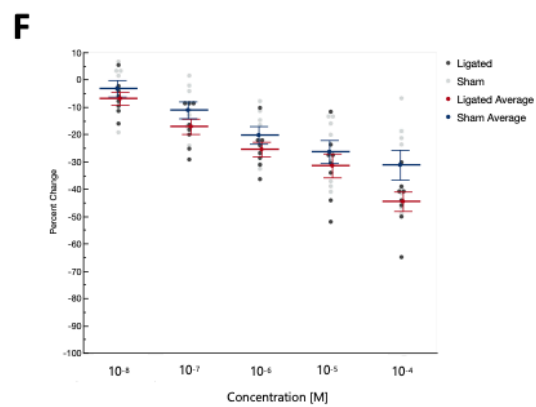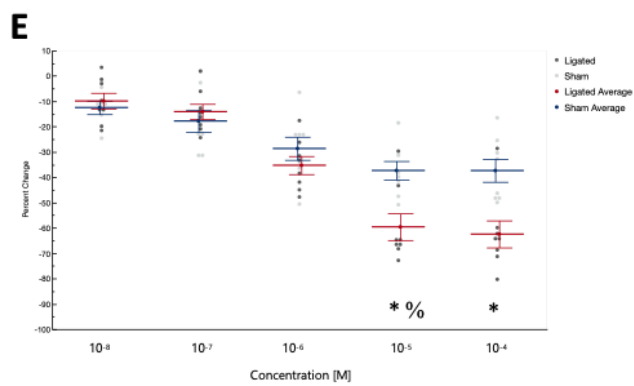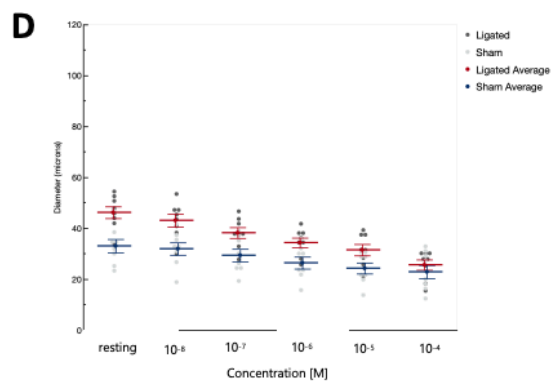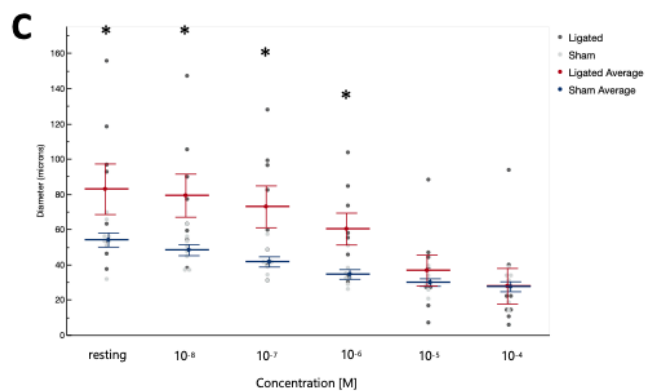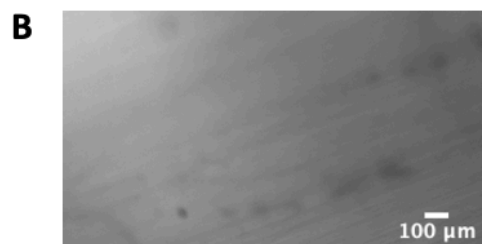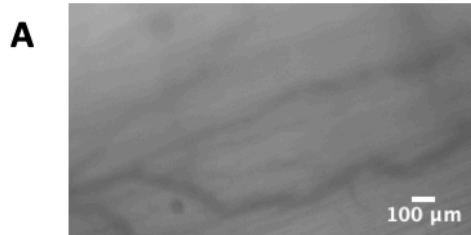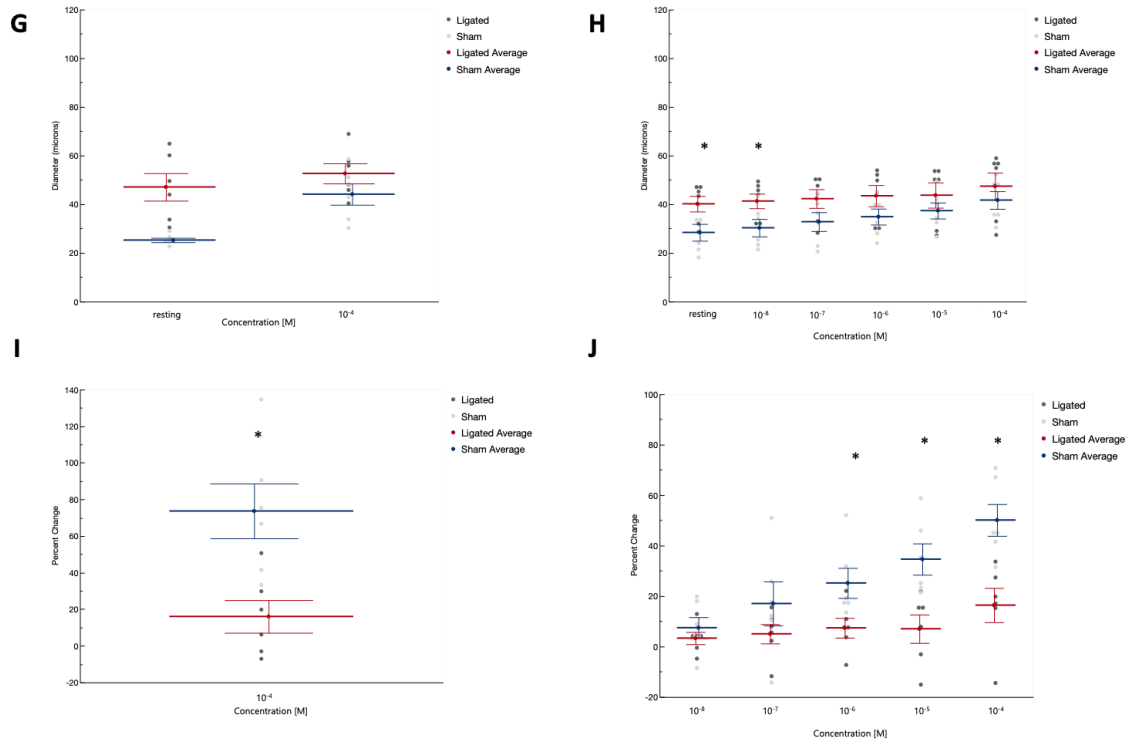
Figure 6. The role of α-adrenergic receptors in collateral growth. Stimulation, but not inhibition, of α-adrenergic receptors appear to play a role in the restoration of vascular tone at day twenty-eight. Collaterals demonstrate regional reactivity to α-adrenergic agonists at 7 days post femoral artery ligation as shown by the difference between resting (A) and stimulation with 10^{-4} M NE (B). Collaterals appear to be hypersensitive to α-adrenergic agonists at 7 days (C,E), but not 28 post femoral artery ligation (D,F). α-adrenergic antagonists appear to be similar to contralateral sham at both time points (G-J). The difference observed in the micron measurements is caused by an increased resting diameter on the ligated side. * = $p<0.05$ between ligated and sham. % = $p<0.05$ between ligated day seven and day twenty-eight.

15
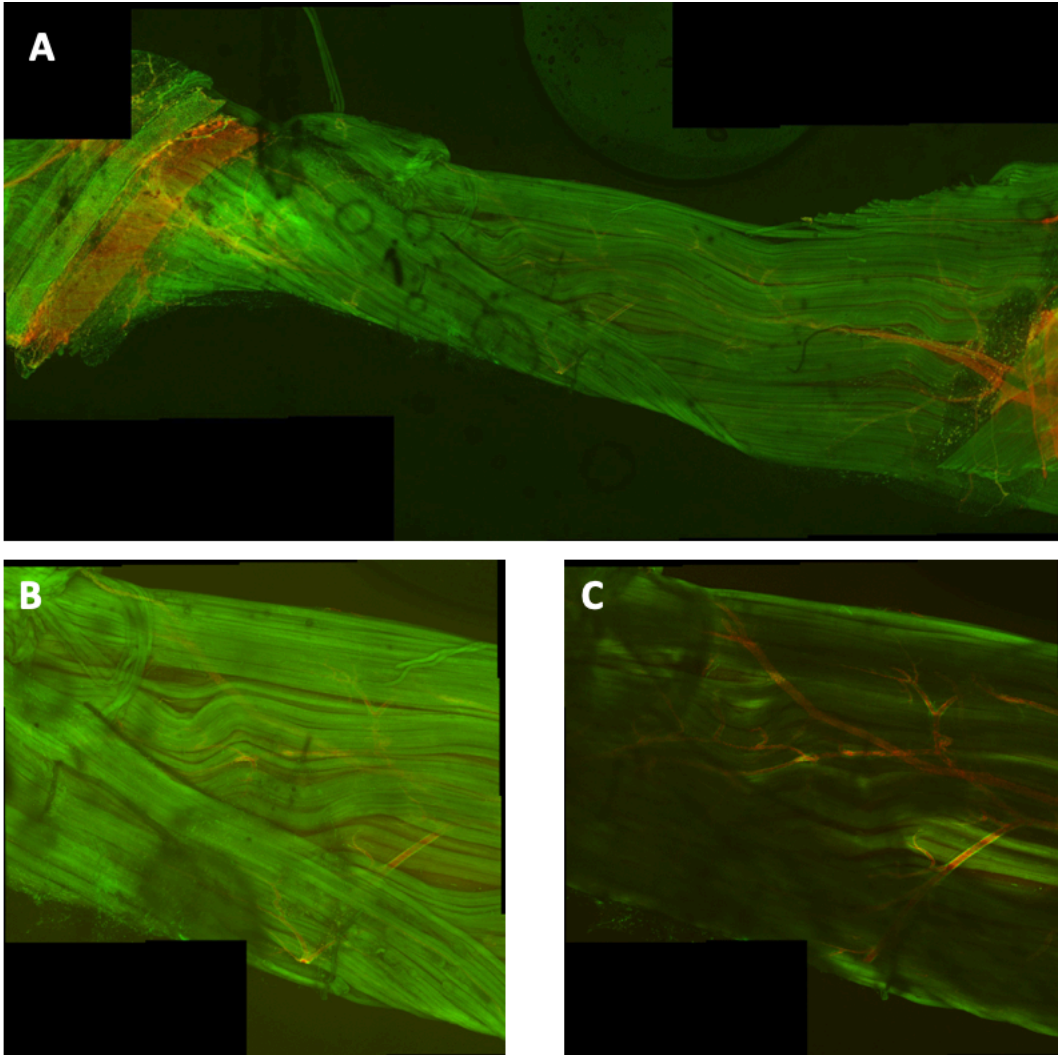
Figure 7. Role of Sympathetic Innervation in Arteriogenesis. Sympathetic innervation should play an important role in arteriogenesis. α-smooth muscle actin (red) and tyrosine hydroxylase (red). There is significant background noise/autofluorescence from the tissue. The entire gracilis anterior of unligated Balb/C at 10X magnification (A). Midzone of gracilis anterior at 20X (B). Midzone at 20X with fewer z stacks of tyrosine hydroxylase to enable viewing of innervation (C). Innervation is solid green surrounding collateral

Discussion

Femoral artery ligation is a viable model to study the loss and restoration of vascular tone [Chu]. Seven days post femoral artery ligation there is an increased resting diameter and a

decreased functional range that is restored at twenty-eight days [Chu]. It was hypothesized that either nitric oxide (NO) production, myogenic tone, or α-adrenoreceptors were responsible for causing this because these mechanisms were identified as being the most important in peripheral vascular disease [Frisbee]. However, an increase in NO production is not a factor (Figure 4) as inhibition of NO via LNAME had similar effects on both the sham and operated at both day seven and day twenty-eight. Also, myogenic tone is not responsible for the restoration of vascular tone (Figure 5) as there were no statistically significant trends in the percent change for sham and operated at both time points. α-adrenoreceptors appear to play an important role in regulating vascular tone following femoral artery ligation (Figure 6). At day seven, but not day twenty-eight, there is increased sensitivity to stimulation of α-adrenoreceptors with NE (Figure 6).

It is unlikely that excess NO production from increased shear stress plays an important role in the loss of vascular reactivity following collateral growth. There has been conflicting results on the role of endothelial nitric oxide synthase and arteriogenesis. Systemic inhibition of NO by treatment with LNAME from three to twenty-eight days after femoral artery resection led to a decreased recruitment of vascular progenitor cells, specifically hemangiocytes [Park/Hoffman]. Furthermore, this led to a decreased collateral diameter compared to mice without inhibition, leading to the conclusion that NO is important in regulating arteriogenesis during the first month following an ischemic event. [Park/Hoffman]. However, eNOS is necessary for vasodilation immediately after femoral artery ligation, but not for collateral growth [Mees]. In eNOS knockout mice blood flow is only decreased compared to wild type until day 7, where afterwards they become similar [Mees]. Furthermore, a possible explanation for the disagreement in literature is that more invasive and damaging methods, such as resection, might induce both arteriogenesis and angiogenesis and thus might provide information that is not

unique to arteriogenesis [Mees]. All of this would suggest that another pathway should play a more prominent role.

Myogenic tone plays an important role in determining resting vascular tone in healthy individuals [Howitt], and it has been hypothesized that SMCs phenotype switching relies on suppression of L-type voltage gated calcium channels (LVCCs) [Wamhoff]. In a rat aortic balloon injury, at day two and seven SMC proliferation increased and LVCCs were reduced [Quignard]. However, LVCCs were restored by day thirty [Quignard]. This is not consistent with the femoral artery ligation, as there appears to be no downregulation in LVCCs at either time points (Figure 2). Interestingly, seven days post femoral artery ligation SMC proliferation peaks (Bynum, unpublished). Therefore, it would be expected to see a loss of LVCCs influence at day seven. A possible explanation is that an aortic balloon injury causes more damage than a femoral artery ligation. This increased damage might correspond to greater SMC proliferation and thus greater downregulation of LVCCs. However, a more likely explanation is that another pathway plays a more prominent role. In other models, NO deficiency leads to T-type calcium channels become more important in regulating vascular tone as shown by the ability to increase vessel diameter greater than inhibition with LVCCs [Howitt]. Although it is unlikely there is NO deficiency in arteriogenesis, this raises the possibility that other pathways that regulate myogenic tone might become more important in regulating vascular reactivity. Transient receptor potential cation channel subfamily V member 4 (TRPv4) activation enhances arteriogenesis, suggesting that it might play a more prominent role in regulating vascular tone [Troidl, Schierling]. Inositol-1,4,5-triphosphate receptors (IP3R) and ryanodine receptors (RyR) regulate intracellular Ca^{2+} concentration. During SMC proliferation there is a reduction in RyR, but not IP3R [Vallot]. After SMC differentiation, the number of RyR matches those found before proliferation [Vallot]. Evaluation of RyR receptors following arteriogenesis might provide insight into the role of

myogenic tone in regulating vascular tone; however, it is challenging to find pharmacological agents that selectively target these pathways *in vivo* [Tykocki].

Inhibition of α-adrenergic receptors is probably not responsible for the loss of vascular tone. When only α-1-adrenegic receptors are inhibited following femoral artery occlusion there is no change in the conductance of the collateral circuit [Taylor]. However, catecholamine synthesis appears to play an important role in collateral vessel growth. When catecholamine synthesis is knocked out via gene deletion of dopamine β-hydroxylase, there is reduced collateral vessel growth [Chalothorn]. Furthermore, denervation upstream of the gracilis anterior before ligation leads to impaired arteriogenesis as demonstrated by a decreased collateral diameter [Cen]. On the other hand, stimulation of α-adrenergic receptors leads to increased SMC proliferation following aorta balloon injury [Erami]. Although there was no change between inhibition of α-adrenergic receptors at day seven and day twenty eight, there was an increased response to α-adrenergic stimulation at day seven, but not at day twenty-eight that would imply stimulation of α-adrenergic might be responsible for the restoration of vascular tone. Interestingly, this trend is evident in patients with CLI, as there is increased vasoconstriction in ischemic skeletal muscle compared to non-ischemic [Jarajapu]. Furthermore, a three hour acute ischemic condition in canine femoral arteries showed not only increased contractility towards norepinephrine, but also a decrease in prazosin and Rauwolscine induced vasodilation [Sapienza]. The increase in vasoreactivity was caused by increased density and functional activity of α-adrenergic receptors [Sapienza]. Therefore, it is possible that there is an increase in functional activity and density of α-adrenergic receptors at day seven leading to increased hypersensitivity that is restored at day twenty eight.

On another note, the hypersensitivity of NE and reduced α-adrenergic receptor activity at day seven could be symptomatic of a lack of sympathetic innervation. In rat soleus muscles there is an increase in chemosensitivity following resection of the sciatic nerve in the thigh [Jones]. This suggests that the hypersensitivity of NE at day seven could be caused by lack of innervation. During arteriogenesis, MMPs degrade the tissue structure [Cai 2000]. We hypothesize that during this remodeling the sympathetic innervation is either damaged or distances itself from the collaterals. Following nerve crush, blood vessel diameter increases until nerve regeneration leads to a greater regulation of vascular tone [Podhajsky]. Further support of this concept comes from angiogenesis where vascularization precedes innervation As time proceeds, MMPs are downregulated [Cai 2000], which could allow the sympathetic nerves to fully innervate collaterals leading to greater regulation of vascular tone. Therefore, the restoration of vascular tone that is seen at day twenty-eight likely correlates with an increase in vascular tone. A possible way to evaluate this is to stain for sympathetic innervation and α-smooth muscle actin at both day seven and day twenty-eight. In Balb/C mice, anti-tyrosine hydroxylase was used to stain for sympathetic innervation (Figure 7). Some optimization is necessary before this stain can be implemented. Furthermore, changing the secondary to a different wavelength, such as 647 nm, might remove the background noise from the tissue.

Interestingly, treatment with nerve growth factor (NGF) improves arteriogenesis by increasing arteriole density [Emanueli]. In spite of this, there appears to be a lack of interest in sympathetic innervation in clinical trials, possibly because there is no significant difference between biomarker expression of NGF in healthy individuals compared to individuals with PAD [Gardner]. Future studies could evaluate the role of Netrin-1 and arteriogenesis. Netrin-1 is an axon guidance cue produced by SMCs that regulates arterial innervation, and a lack of Netrin-1 leads to defective vasoconstriction [Brunet]. Future studies could determine how Netrin-1

expression changes during SMC phenotype switching to see if it might play a role in re-innervation of sympathetic neurons.

Ultimately, stimulation of arteriogenesis has the potential to be a therapeutic treatment for PAD. Although there has been success in preclinical models, no treatment has had clinical success, which points to the need for a greater understanding of the underlying mechanisms. Evaluation of vascular reactivity has the potential to provide insight into novel therapeutic options. There is a larger resting diameter and increased sensitivity to NE seven days post femoral artery ligation, but by twenty-eight days post femoral artery ligation the resting diameter has decreased and NE sensitivity is on par with the contralateral sham. This suggests that there is a loss of sympathetic innervation at day seven that is restored at day twenty-eight. Therefore, future therapeutics should target sympathetic innervation to improve patient outcome.

References

1. National Center for Chronic Disease Prevention and Health Promotion, Division for Heart Disease and Stroke Prevention. Peripheral Arterial Disease (PAD) Fact Sheet. Hyattsville, Maryland. 2016.

2. Di Minno, Giovanni, et al. "Systematic reviews and meta-analyses for more profitable strategies in peripheral artery disease." Annals of medicine 46.7 (2014): 475-489.

3. Criqui, Michael H., and Victor Aboyans. "Epidemiology of peripheral artery disease." Circulation research 116.9 (2015): 1509-1526.

4. Allen, Jason D., Tony Giordano, and Christopher G. Kevil. "Nitrite and nitric oxide metabolism in peripheral artery disease." Nitric Oxide 26.4 (2012): 217-222.

5. Nehler, Mark R., et al. "Epidemiology of peripheral arterial disease and critical limb ischemia in an insured national population." Journal of vascular surgery 60.3 (2014): 686-695.

6. Samura, Makoto, et al. "Therapeutic strategies for cell-based neovascularization in critical limb ischemia." Journal of translational medicine 15.1 (2017): 49.

7. Davies, Mark G. "Critical limb ischemia: cell and molecular therapies for limb salvage." Methodist DeBakey cardiovascular journal 8.4 (2012): 20.

8. Norgren, Lars, et al. "Inter-society consensus for the management of peripheral arterial disease (TASC II)." Journal of vascular surgery 45.1 (2007): S5-S67.

9. Annex, Brian H. "Therapeutic angiogenesis for critical limb ischaemia." Nature Reviews Cardiology 10.7 (2013): 387.

10. McDermott, Mary M., et al. "Proximal superficial femoral artery occlusion, collateral vessels, and walking performance in peripheral artery disease." JACC: Cardiovascular Imaging 6.6 (2013): 687-694.

11. Keeling, Aoife N., et al. "Clinical correlates of size and number of collateral vessels in peripheral artery disease." Vascular Medicine 17.4 (2012): 223-230.

12. Yu, Jun, et al. "Endothelial nitric oxide synthase is critical for ischemic remodeling, mural cell recruitment, and blood flow reserve." Proceedings of the National Academy of Sciences 102.31 (2005): 10999-11004.

13. Krishna, Smriti Murali, Safraz Mohamed Omer, and Jonathan Golledge. "Evaluation of the clinical relevance and limitations of current pre-clinical models of peripheral artery disease." Clinical Science 130.3 (2016): 127-150.

14. Raval, Zankhana, and Douglas W. Losordo. "Cell therapy of peripheral arterial disease: from experimental findings to clinical trials." Circulation research 112.9 (2013): 1288-1302.

15. Maufus, Mario, et al. "Critical limb ischaemia and the response to bone marrow-derived cell therapy according to tcPO2 measurement." Vasa 46.1 (2016): 23-28.

16. Perin, Emerson C., et al. "Evaluation of cell therapy on exercise performance and limb perfusion in peripheral artery disease: the CCTRN PACE trial (patients with intermittent claudication injected with ALDH bright cells)." Circulation135.15 (2017): 1417-1428.

17. Troidl, C., et al. "Calcium-dependent signalling is essential during collateral growth in the pig hind limb-ischemia model." Journal of molecular and cellular cardiology 49.1 (2010): 142-151.

18. Mack, Peter J., et al. "Biomechanical regulation of endothelium-dependent events critical for adaptive remodeling." Journal of Biological Chemistry284.13 (2009): 8412-8420.

19. Landázuri, Natalia, et al. "Growth and regression of vasculature in healthy and diabetic mice after hindlimb ischemia." American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 303.1 (2012): R48-R56.

20. Jazwa, Agnieszka, et al. "Limb ischemia and vessel regeneration: is there a role for VEGF?." Vascular pharmacology 86 (2016): 18-30.

21. Schaper, Wolfgang. "Collateral circulation." Basic research in cardiology 104.1 (2009): 5-21.

22. Bauters, Christophe, et al. "Recovery of disturbed endothelium-dependent flow in the collateral-perfused rabbit ischemic hindlimb after administration of vascular endothelial growth factor." Circulation 91.11 (1995): 2802-2809.

23. Rey, Sergio, and Gregg L. Semenza. "Hypoxia-inducible factor-1-dependent mechanisms of vascularization and vascular remodelling." Cardiovascular research 86.2 (2010): 236-242.

24. Taylor, Jessica C., et al. "α-Adrenergic and neuropeptide Y Y1 receptor control of collateral circuit conductance: influence of exercise training." The Journal of physiology 586.24 (2008): 5983-5998.

25. Gruionu, Gabriel, et al. "Structural remodeling of the mouse gracilis artery: coordinated changes in diameter and medial area maintain circumferential stress." Microcirculation 19.7 (2012): 610-618.

26. Heuslein, Joshua L., et al. "Mechanisms of amplified arteriogenesis in collateral artery segments exposed to reversed flow direction." Arteriosclerosis, thrombosis, and vascular biology35.11 (2015): 2354-2365.

27. Clayton, Jason A., Dan Chalothorn, and James E. Faber. "Vascular endothelial growth factor-A specifies formation of native collaterals and regulates collateral growth in ischemia." Circulation research 103.9 (2008): 1027-1036.

28. Jaipersad, Anthony S., Gregory YH Lip, Stanley Silverman, and Eduard Shantsila. "The role of monocytes in angiogenesis and atherosclerosis." Journal of the American College of Cardiology 63, no. 1 (2014): 1-11.

29. Silva, Rita de Cassia Gengo E., et al. "Vascular reactivity is impaired and associated with walking ability in patients with intermittent claudication." Angiology 66.7 (2015): 680-686.

30. Langham, Michael C., et al. "Quantitative CMR markers of impaired vascular reactivity associated with age and peripheral artery disease." Journal of Cardiovascular Magnetic Resonance 15.1 (2013): 17.

31. Parshakov, Aleksey, et al. "Detection of endothelial dysfunction using skin temperature oscillations analysis during local heating in patients with peripheral arterial disease." Microcirculation 23.6 (2016): 406-415.

32. Miralles JH, Martinez-Aguillar E, Florez A, Vareta C, Bleda S, Acin F. Nitric oxide: link between endothelial dysfunction and inflammation in patients with peripheral arterial disease of the lower limbs. Interact Cardiovasc Thorac Surg. 2009;9(1):107-112.

33. Rensen, S. S. M., P. A. F. M. Doevendans, and G. J. J. M. Van Eys. "Regulation and characteristics of vascular smooth muscle cell phenotypic diversity." Netherlands Heart Journal 15.3 (2007): 100-108.

34. Gomez, Delphine, and Gary K. Owens. "Smooth muscle cell phenotypic switching in atherosclerosis." Cardiovascular research 95, no. 2 (2012): 156-164.

35. Brozovich, F. V., et al. "Mechanisms of vascular smooth muscle contraction and the basis for pharmacologic treatment of smooth muscle disorders." Pharmacological reviews 68.2 (2016): 476-532.

36. Arnold, Caroline, et al. "RGS5 promotes arterial growth during arteriogenesis." EMBO molecular medicine 6.8 (2014): 1075-1089.

37. Gouin III, Kenneth H., et al. "Arterialized collateral capillaries progress from nonreactive to capable of increasing perfusion in an ischemic arteriolar tree." Microcirculation 25.3 (2018): e12438.

38. Korthuis, R. J. "Regulation of vascular tone in skeletal muscle." San Rafael, CA, Morgan & Claypool Life Sciences (2011).

39. Tykocki, Nathan R., Erika M. Boerman, and William F. Jackson. "Smooth muscle ion channels and regulation of vascular tone in resistance arteries and arterioles." Comprehensive Physiology 7.2 (2011): 485-581.

40. Durand, Matthew J., and David D. Gutterman. "Diversity in mechanisms of endothelium-dependent vasodilation in health and disease." Microcirculation 20.3 (2013): 239-247.

41. Dora, Kim A. "Endothelial-smooth muscle cell interactions in the regulation of vascular tone in skeletal muscle." Microcirculation 23.8 (2016): 626-630.

42. Iring, Andras, et al. "Shear stress–induced endothelial adrenomedullin signaling regulates vascular tone and blood pressure." The Journal of clinical investigation 129.7 (2019).

43. Zhao, Yingzi, Paul M. Vanhoutte, and Susan WS Leung. "Vascular nitric oxide: Beyond eNOS." Journal of pharmacological sciences 129.2 (2015): 83-94.

44. Calzada, Beatriz Civantos, and Amaya Aleixandre de Artiñano. "Alpha-adrenoceptor subtypes." Pharmacological Research 44.3 (2001): 195-208.

45. Frisbee, Jefferson C., et al. "Integration of skeletal muscle resistance arteriolar reactivity for perfusion responses in the metabolic syndrome." American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 296.6 (2009): R1771-R1782.

46. Chu, Megan T. "Assessing Functional Vasodilation in the Gracilis Collateral Arteriole." (2015).

47. Park, B., Hoffman, A., Yang, Y., Yan, J., Tie, G., Bagshahi, H., ... & Messina, L. M. (2010). Endothelial nitric oxide synthase affects both early and late collateral arterial adaptation and blood flow recovery after induction of hind limb ischemia in mice. Journal of vascular surgery, 51(1), 165-173.

48. Mees, Barend, et al. "Endothelial nitric oxide synthase activity is essential for vasodilation during blood flow recovery but not for arteriogenesis." Arteriosclerosis, thrombosis, and vascular biology 27.9 (2007): 1926-1933.

49. Howitt, Lauren, et al. "Chronic deficit in nitric oxide elicits oxidative stress and augments T-type calcium-channel contribution to vascular tone of rodent arteries and arterioles." Cardiovascular research 98.3 (2013): 449-457.

50. Wamhoff, Brian R., Douglas K. Bowles, and Gary K. Owens. "Excitation–transcription coupling in arterial smooth muscle." Circulation research 98.7 (2006): 868-878.

51. Quignard, Jean-François, et al. "Transient down-regulation of L-type Ca2+ channel and dystrophin expression after balloon injury in rat aortic cells." Cardiovascular research 49.1 (2001): 177-188.

52. Schierling, W., et al. "Cerebral arteriogenesis is enhanced by pharmacological as well as fluid-shear-stress activation of the Trpv4 calcium channel." European Journal of Vascular and Endovascular Surgery 41.5 (2011): 589-596.

53. Vallot, Olivier, et al. "Intracellular Ca2+ handling in vascular smooth muscle cells is affected by proliferation." Arteriosclerosis, thrombosis, and vascular biology 20.5 (2000): 1225-1235.

54. Taylor, Jessica C., et al. "α-Adrenergic inhibition increases collateral circuit conductance in rats following acute occlusion of the femoral artery." The Journal of physiology 586.6 (2008): 1649-1667.

55. Chalothorn, Dan, et al. "Catecholamines augment collateral vessel growth and angiogenesis in hindlimb ischemia." American Journal of Physiology-Heart and Circulatory Physiology 289.2 (2005): H947-H959.

56. Cen, Y., Liu, J., Qin, Y., Liu, R., Wang, H., Zhou, Y., ... & Hu, Z. (2016). Denervation in Femoral Artery-Ligated Hindlimbs Diminishes Ischemic Recovery Primarily via Impaired Arteriogenesis. PloS one, 11(5), e0154941.

57. Erami, Cauveh, et al. "α1-Adrenoceptor stimulation directly induces growth of vascular wall in vivo." American Journal of Physiology-Heart and Circulatory Physiology 283.4 (2002): H1577-H1587.

58. Jarajapu, Yagna PR, et al. "Increased α1-and α2-adrenoceptor-mediated contractile responses of human skeletal muscle resistance arteries in chronic limb ischemia." Cardiovascular research49.1 (2001): 218-225.

59. Sapienza, Paolo, et al. "Ischemia-induced peripheral arterial vasospasm role of α1-and α2-adrenoceptors." Journal of surgical research 62.2 (1996): 192-196.

60. Jones, Rosemary, and Gerta Vrbová. "Two factors responsible for the development of denervation hypersensitivity." The Journal of physiology 236.3 (1974): 517-538.

61. Cai, Wei-jun, et al. "Altered balance between extracellular proteolysis and antiproteolysis is associated with adaptive coronary arteriogenesis." Journal of molecular and cellular cardiology 32.6 (2000): 997-1011.

62. Podhajsky, Ronald J., and Robert R. Myers. "The vascular response to nerve crush: relationship to Wallerian degeneration and regeneration." Brain research 623.1 (1993): 117-123.

63. Emanueli, C., Salis, M. B., Pinna, A., Graiani, G., Manni, L., & Madeddu, P. (2002). Nerve growth factor promotes angiogenesis and arteriogenesis in ischemic hindlimbs. Circulation, 106(17), 2257-2262.

64. Gardner, Andrew W., et al. "Impaired vascular endothelial growth factor A and inflammation in patients with peripheral artery disease." Angiology65.8 (2014): 683-690.

65. Brunet, Isabelle, et al. "Netrin-1 controls sympathetic arterial innervation." The Journal of clinical investigation 124.7 (2014): 3230-3240.

Appendices: Protocols

Date_____ **Hindlimb Ischemia Surgery – Femoral Artery Ligation** Initials _____

Mouse Information
Age: _____
Sex: _____
Tag: _____
Genotype/strain:_____
Cage: _____

Purpose: _____
_____

Materials

Pre-sterilize in autoclave

____1.   Standard pattern forceps (1)
____2.   Fine forceps- S&T (2)
____3.   Ultrafine forceps- 545 (1)
____4.   Curved iris scissors (1)
____5.   Microdissection scissors (1)
____6.   Gauze sponges- 2x2 and 4x4
____7.   Cotton swabs
____8.   6.0 silk suture (2 x 1-inch pieces)
____9.   7.0 prolene suture
____10.   Needle holder (1)

Obtain in surgery suite

____11.   Depilatory cream- Veet
____12.   Non-sterile cotton swabs
____13.   Non-sterile gauze sponges (2x2 and 4x4)
____14.   Chlorhexidene diacetate (Nolvasan)
____15.   1-mL insulin syringes (2)
____16.   Buprenorphine analgesic (0.03 mg·ml^{-1})
____17.   Ear punch
____18.   Veterinary ointment
____19.   Surgical tape
____20.   FST heat pad w/ rectal probe
____21.   Surgical scrubs
____22.   Sterile petri dish (1)
____23.   Sterile 5-mL syringe (1)
____24.   Sterile saline
____25.   Isolation mask and cap
____26.   Sterile gloves
____27.   Recovery bin and heat pad
____28.   70% isopropyl alcohol (IPA)

Animal preparation

____29.   Spray surgery area with Nolvasan.
____30.   Place animal in induction chamber.
____31.   Open oxygen cylinder. Set flow high and isofluorane to 5%.
____32.   Once anesthetized, weigh animal and move to preparatory bench in a supine position.
____33.   Reduce isoflurane to 1-3% and flow to 0.5-1.5 l·min^{-1}.

____34.   Gently apply veterinary ointment to eyes using a cotton swab.

____35.   Apply depilatory cream to hindlimb with a cotton swab and let sit for 1-3 minutes.
____36.   Spray a 2x2 gauze sponge with Nolvasan and wipe hindlimb clean of cream and hair.
____37.   Flip animal over and apply ear punch.
____38.   Administer pre-op buprenorphine dose (0.075 mg·kg^{-1}) by subcutaneous injection.
____39.   Cover heat pad with a 4x4 gauze sponge and transfer animal to surgery stage.
____40.   Apply lubricant to rectal probe and insert. Set thermo-controller to 35°C.
____41.   Change into surgical scrubs and wash hands/forearms.
____42.   Open sterile instrument tray and sterile pack.
____43.   Obtain sterile petri dish in sterile field and fill with sterile saline, using a 5-mL syringe.
____44.   Put on mask, cap, and sterile gloves.

Surgery

____45.   Make a small incision on the middle, medial aspect of the left hindlimb, directly over the neurovascular bundle.
____46.   Extend incision to the abdominal wall
____47.   Blunt dissect subcutaneous connective tissue to maximize surgical exposure.
____48.   Blunt dissect and retract epigastric fat pad to expose ligation site, proximal to the popliteal artery and distal to the epigastric.
____49.   Blunt dissect connective tissue over bundle and separate nerve from the artery-vein pair.
____50.   Use ultrafine forceps to separate the artery from the vein.
____51.   Tie off the femoral artery with silk suture.
____52.   Use 7-0 prolene suture to close the incision with spiral sutures.
____53.   Make a small incision in the middle medial aspect of the left hindlimb.
____54.   Extend incision to the abdominal wall
____55.   Blunt dissect subcutaneous connective tissue to maximize surgical exposure.
____56.   Use 7-0 prolene suture to close the incision with spiral sutures.

Post-Surgical

____57.   Administer post-op buprenorphine dose (0.075 mg·kg^{-1}) by subcutaneous injection.
____58.   Microwave recovery heat pad for ~1-2 min.
____59.   Transfer animal to recovery bin.
____60.   Turn off isoflurane, flow, and close oxygen.
____61.   Wipe down surgical area with IPA and wash all instruments.

Superfusion -Vascular Reactivity

Purpose: To specifically modulate different pathways affecting vasoreactivity at increasing doses on the sham and ligated side to evaluate how arteriogenesis affects the underlining pathway

Safety Concerns:
- Check SDS and SOP for all pharmacological agents.
- Be careful of residual heat from syringe heater.
- Be careful of sharp edges instruments
- Dispose of mouse in carcass container
- Dispose of all waste in appropriate labeled waste container

Materials:
1. Standard pattern forceps (1)
2. Fine forceps- S&T (2)
3. Curved iris scissors (1)
4. Depilatory cream – Veet
5. Cotton swabs
6. Gauze sponges (2X2 and 4X4)
7. Chlorhexidene diacetate (Nolvasan)
8. 70% isopropyl alcohol (IPA)
9. Jelly
10. Surgical tape
11. FST heat pad w/rectal probe
12. Petri dish w/ PBS
13. Syringe Heater
14. 10ml Graduated cylinder
15. Hot plate
16. 5% CO_2 – 95% N_2 with tubing
17. Supefusion tubing
18. Vacuum pump evacuation flask
19. Physiological salt solution (PSS) (stock solution 20X in 4°C
20. Sodium bicarbonate
21. Vasodilator/vasoconstrictor
22. Trans-illuminator
23. Intravial microscope – Qcapture Pro
24. Kim wipes
25. Plastic wrap
26. Petri dish
27. 500ml beaker
28. 60ml syringe (x2)
29. 18 ohmic water
30. 1 L volumetric flask
31. Instrument stand
32. Sodium nitroprusside (SNP)
33. 1M Hydrochloric acid (HCl)

Instrument Preparation

34. Dilute sodium bicarbonate (20x) and PSS (20x) in 18 MΩ water using 1L volumetric flask
35. Pour ~300 ml in 500ml beaker and place on hot plate. Turn hot plate on to ~85C
36. Place syringe heater on instrument stand and insert 2 60ml syringes in outside slots
37. Put stopcocks on 60ml syringes and connect tubing
38. Pour ~50ml in each 60ml syringe and turn syringe heater on
39. Put aliquoted reagent in middle slot of the syringe heater

Animal Preparation
40. Prepare animal for surgical exposure

Surgical Exposure
41. Bubble 5% CO_2 – 95% N_2 in 60 ml syringes and cover with plastic wrap
42. Make a small incision on the middle, medial aspect of the thigh
43. Extend the incision up the abdominal wall
44. Cut away the skin above the gracilis muscles and the collateral circuit to expose the region from the profundal to the saphenous
45. Very gently, blunt dissect and remove the connective tissue overlying the gracilis muscle
46. Move surgical stage underneath the BXFM
47. Position the trans-illuminator LED probe underneath the limb, below the gracilis
48. Open QCapture Pro and position the intravital microscope above the collateral arteriole in the gracilis anterior

Superfusion
49. Support superfusion tubing with instrument stand and place over exposed gracilis muscles
50. Place a kim wipe that has been torn in half on top of plastic wrap and roll it to make a wick. Place on top of thigh (make sure not to touch muscle) and the other end in a petri dish
51. Open stopcocks to syringe heater and check pH (7.2-7.4), flow rate (~2 ml min^{-1}), and temperature (~35°C) of PSS and keep running. **Continuously check throughout remainder of procedure**
52. Allow muscle to stabilize (~30 minutes)
53. Use the vacuum pump to remove PSS from petri dish as needed.
54. Add PSS to 60ml syringe as needed (roughly every 10 minutes) during the 30 minute rest period
55. Acquire resting diameter of collateral
56. Top of 60ml syringe with PSS
57. Place first dosage of reagent in appropriate 60ml syringe (left or right depending on sham or ligated side)

58. Capture images at 5 minutes (or longer if need be (look in literature or do pilot study))
59. Refill 60ml syringe
60. Place next dose of reagent in appropriate slot for 60ml syringe
61. Repeat steps 66-68 until all concentrations have been used
62. Quickly flush 60ml syringe with PSS to remove residual pharmacological agents
63. Repeat on sham side
64. Repeat with additional pharmacological agents
65. Close stopclock and stop PSS flow
66. Put .6 ml 10^{-3} M SNP into 60ml syringe and superfuse over exposed gracilis collateral. Image

Post Experiment
67. If perfuse fixing, fix mouse and remove gracilis anterior
68. Perform Cervical dislocation to euthanize animal and dispose of in bag and place in carcass box in -20C freezer
69. Turn off isofluorane, flow, and close oxygen
70. Turn off hot plate
71. Flush superfusion line with 1M HCl and rinse with distilled water

Date_____   **Perfusion Fixation**   Initials _____

Mouse Information

DOB:_____

Sex: _____

Tag:_____

Genotype/strain: _____

Cage:_____

Weight(g): _____

Materials

Non-Sterilize Dissection Instruments

____1.   Forceps (1)

____2.   Fine forceps (2)

____3.   Bone scissors (1)

____4.   Curved Iris scissors (1)

____5.   Microdissection scissors (1)

____6.   Vascular clamp (1)

____7.   Castroviejos

Obtained in surgery suite

____8.   Tape

____9.   20 mL syringes (2)

____10.   5 mL syringe (1)

____11.   Syringe pump

____12.   Petri-dish

____13.   Bench cover

____14.   Depilatory cream

____15.   Clippers

____16.   Veterinary ointment

____17.   Heating pad

____18.   Catheter

____19.   Non-sterile saline

____20.   Cotton swabs

____21.   Gauze sponges

____22.   Saran wrap

Vasodilator Cocktail Preparation

____23.   Turn on water bath to 37ºC

____24.   400 µL heparin

____25.   1mL SNP(orange)

____26.   600µL Adenosine(clear)

____27.   38mL PBS solution

____28.   5 mL 4% Paraformaldehyde (PFA)

____29.   Thaw SNP, Adenosine and PFA

____30.   Add heparin, SNP, Adenosine, and PBS solution together in a 50mL conical

____31.   Place vasodilator cocktail in water bath

Procedure Preparation

____32.   Obtain saline filled petri-dish, cotton swab, and instruments

Fixation

____33.   Remove hair on back by shaving & depilation

____34.   Tape animal in supine position to 4X4 gauze sponge over heating pad

____35.   Expose muscles of interest and blunt dissect to aid in removal post-fixation, then cover with saran wrap

____36.   Fill 20mL syringe with 20 mL warm Vaso D, load into syringe pump and attach catheter

____37.   Flow liquid through the catheter to the tip to prevent air from being injected into circulatory system

____38.   Lift skin from muscle in abdominal region and cut a window over the sternum

____39.   Lift sternum and cut connective tissue under

____40.   Use bone scissors in hole to quickly cut through the ribs to the armpit on both sides

____41.   Clamp sternum with castroviejos and reflect towards mouse's head

____42.   Cut diaphragm with curved iris scissors to open chest cavity

____43.   Cut away excess tissue around the heart

____44.   Make a small incision in the apex of the heart

____45.   Insert catheter and clamp with vascular clamp and cut right atrium

____46.   Inject Vaso D solution into animal approximately 2x 20mL (5mL/min), soaking up excess blood and fluids with gauze sponges

____47.   Inject 5 mL PFA (4 mL/min)

____48.   Dissect out muscles of interest using fine forceps and microdissection scissors

____49.   Turn off water bath, cover scope, turn off oxygen, turn off isofluorane, and clean instruments

Notes

_____

_____

_____

_____

_____

_____

Purpose: To stain gracilis anterior muscles with α-smooth muscle actin and tyrosine hydroxylase. This will provide insight into how innervation is impacted by arteriogenesis.

Materials:
- 24 well plate
- Phosphate Buffer Saline (PBS)
- TritonX-100 10% (Triton)
- Bovine Serum Albumin (BSA)
- Anti-Actin α-smooth muscle – Cy-3 antibody 1:300 (α-SMA) (C6198)
- Standard pattern forceps
- Glycerol
- Anti-tyrosine hydroxylase antibody 1:1000 (THir) (AB152)
- Alexa 488 goat-anti rabbit 1:100 (secondary) (A11008)
- Glass cover slips
- Depression slides
- Aluminum foil
- Cover slips
- Nail polish

Methods:
- Place muscle in 2 ml centrifuge tubes with 500 μL of 2% Triton in 1X PBS and incubate at room temperature (RT) for 2 hours shaking incrementally
- Place muscle in 600 μL microcentrifuge tubes with 300 μL of 0.1% Triton, 2% BSA, 1:300 α-SMA, 1:1000 THir in 1X PBS
- Wrap 24 well plate in aluminum foil and incubate at 4°C for 96 hours. Cover everything in foil from here on out
- Wash in PBS 3X for 10 minutes at RT
- Incubate with 1:100 secondary for 24 hours at RT
- Wash in 0.1% Triton in PBS 3X for 10 minutes at RT
- Wash in PBS for 30 min at RT
- Place muscle on depression slide. Using transfer pipet drip 1 to 2 drops of 50/50 glycerol PBS on muscle
- Place cover slide and paint edges with nail polish
- Store in 4°C and wait at least 1 day before imaging

Notes:

_____

_____