SEX DIFFERENCES IN COLLATERAL REMODELING FOLLOWING HINDLIMB
ARTERIAL OCCLUSION

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by
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ABSTRACT

Sex Differences in Collateral Remodeling Following Hindlimb Arterial Occlusion

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Clinical evidence indicates a higher incidence of peripheral arterial occlusive disease and associated likelihood of critical limb ischemia in women, as well as worse prognosis and decreased survival post myocardial infarction. Therefore, understanding the possible differences in underlying vascular compensation mechanisms is crucial. With arterial occlusions, necrosis and tissue injury can be naturally mitigated by the collateral circulation, improving patient prognosis. Previous sex-comparison studies describing differences in vascular remodeling are inconsistent. Therefore, the aim of this study was to describe the effect of arterial occlusion on collateral remodeling in healthy male and healthy reproductive-stage female mice. At 7 days following femoral artery ligation in C57Bl/6 and BALB/c mice, there were no sex-related differences in functional ambulatory recovery. There were no sex-related differences in mechanoadaptation indicators in the collateral stem- vascular smooth muscle cell (VSMC) length and overlap, with the exception of longer smooth muscle cells in male C57Bl/6 mice, VSMC lengths 329 ± 19 versus $288 \pm 13 \mu\text{m}$, male and female. Collateral midzone luminal and abluminal diameters, as well as wall thicknesses were not different between sexes. As comprehensive sex-specific differences were not captured in our specific investigation of arteriogenesis, an evaluation of microvascular remodeling in the ischemic zone and collateral vasodilation would be of interest, as would evaluating arteriogenesis following oophorectomy with estrogen depletion. The determination of any underlying mechanistic sex-specific differences could be the foundation for which targeted therapeutics are developed, which will be crucial for closing the prognosis gap between men and women in the global treatment of peripheral arterial occlusive disease.

Keywords: arteriogenesis, mechanoadaptation, collateral circulation, hindlimb, mouse

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“If I have seen further, it is by standing on the shoulders of giants.”

-Sir Isaac Newton

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INTRODUCTION

Peripheral arterial occlusive disease (PAOD), an atherosclerotic narrowing of the arteries distal to the aortic bifurcation, affects an estimated 10 percent of the global population, including 27 million patients in the United States and Europe [4-7]. Critical limb ischemia (CLI), the most severe manifestation of PAOD, has 500 to 1000 new cases diagnosed yearly per million of the population, with an estimated annual cost of 40-66 billion dollars [8]. CLI can lead to amputation or death without proper treatment, with four-year survival rates as low as 40 percent [9]. Recent clinical evidence suggests a higher incidence of PAOD in females, and an associated higher likelihood of critical limb ischemia [10-15]. Women are also less likely to survive, and exhibit worse prognosis following a myocardial infarction (MI) [16, 17]. Within women, ovariectomy before age 35 increases the risk of a nonfatal MI by 7.2 times that of premenopausal women [18]. In patients with severe coronary artery lesions, the male sex was associated with increased collateralization [19], therefore, these differences in outcomes could be due to underlying molecular and cellular sex-related differences in vascular compensation methods [20, 21].

With arterial occlusions, necrosis and tissue injury can be naturally mitigated by the collateral circulation, improving patient prognosis [22-24]. When a hemodynamically-relevant stenosis occurs in a main feeding artery, blood is redirected to downstream tissues through the collateral vessel(s), which connects the high-pressure region upstream of the stenosis to the low-pressure region downstream. The increased blood velocity and shear stress through the collaterals initiates outward remodeling through arteriogenesis,

allowing the collateral to improve patient prognosis by maintaining tissue perfusion [3, 25].

During this outward remodeling, vessel caliber increases in a process involving monocyte extravasation and transformation into cytokine-, growth factor-, and enzyme-secreting macrophages. Specific secreted agents, such as vascular endothelial growth factor A (VEGF-A) and fibroblast growth factor 2 (FGF-2), promote proliferation of endothelial

and vascular smooth muscle cells (VSMCs) in classical arteriogenesis, **Figure 1** [26-32]. Although outward remodeling usually involves VSMC proliferation, inward remodeling can involve both VSMC apoptosis [33-37] and changes in VSMC length, increasing cellular overlap through a process described as mechanoadaptation [38]. As inward remodeling involves a continuum from changes in apoptosis to VSMC

morphology, outward remodeling may also involve a continuum from proliferation to changes in VSMC morphology.

In addition to VSMC proliferation, outward remodeling also requires a transient inflammatory response, moderate oxidative stress, and activation of the nitric oxide (NO) pathway, **Figure 2**, with all three processes partially modulated by estrogens [39-42]. Estradiol levels in men and postmenopausal women are lower than levels in non-

pregnant women, possibly explaining the differences in outward remodeling [43-45]. 17- β -estradiol increases the endothelial production of nitric oxide and flow-mediated outward remodeling following oophorectomy is rescued by 17- β -estradiol [42, 46]. Oophorectomy also increases blood pressure and decreases vessel densities in rabbits, indicators of angiogenesis and arteriogenesis [47].

However, sex comparison of arteriogenesis without female estrogen depletion is of value due to female hormonal cycle variation in the general population, as well as the presence of PAOD in females prior to the average age of natural menopause [11, 43, 48, 49].

Following femoral excision, similar blood pressure and vessel densities were observed in

male and non-oophorectomized female rabbits, although collateral number was not examined [47]. Furthermore, female mice without oophorectomy exhibit higher hindlimb vascular resistance, less responsiveness to vasodilators, and more sensitivity to vasoconstrictors following arterial occlusion [50].

The inconsistency in female flow-recovery impairment following femoral artery occlusion or excision within a small body of literature necessitates further study [21, 47, 50]. Increased vascular resistance in female mice is suggestive of impaired collateral enlargement, therefore we evaluated the extent and mechanisms of collateral enlargement throughout the gracilis collateral circuit in male and female mice. The aim of this study was test the hypothesis that healthy male mice exhibit superior collateral remodeling to healthy reproductive-stage female mice following femoral artery ligation. This was performed by evaluating daily hindlimb use, mechanoadaptation in the gracilis collateral stem, and collateral enlargement in the gracilis collateral midzone.

MATERIALS AND METHODS

Animals

All experimental procedures were performed according to the protocols approved by the Institutional Animal Care and Use Committee (IACUC) of California Polytechnic State University. Female and male eight-week old C57Bl/6 and BALB/c mice were obtained from Taconic Farms, Jackson Laboratories, or Charles River Laboratories. Eight female and eight male C57Bl/6, and six female and six male BALB/c mice were used for the mechanoadaptation and collateral remodeling studies. All mice were maintained on a 12:12 hour light:dark cycle in a temperature controlled room in the University Vivarium. Mice were housed by sex with littermates in micro-isolator cages, supplied with feed, water, and enrichment ('houses', nesting material, and tubes), and inspected daily.

Collateral Enlargement Model

Collateral enlargement was induced in the superficial gracilis collaterals by ligating the femoral artery between the profunda femoris and popliteal branches of the left hindlimb, as described previously [51]. Briefly, mice were anesthetized using 1-3% isoflourane gas at an oxygen flow rate of 0.8-1.2 l·min⁻¹, administered through a 'nose-cone', and given pre-operative analgesic (buprenorphine, 0.075 mg·kg⁻¹ subcutaneous). Hair on the medial hindlimb was depilated and the surgical area was disinfected with chlorohexidine diacetate. Mice were placed supine on a heated surgical stage, adjusted to maintain a core body temperature of 35°C. Ophthalmic ointment was applied to the eyes to avoid corneal desiccation.

A small skin incision (1-2 cm) was made on the middle, medial aspect of the left thigh, with subcutaneous connective tissue blunt dissected to maximize surgical exposure. Throughout the procedure, the incision site was irrigated with sterile phosphate buffered saline (PBS) to prevent tissue desiccation. To expose the ligation site, the epigastric fat pad was reflected medially, and the femoral artery was blunt dissected from the neurovascular bundle before ligation with 6.0 silk suture distal to the epigastric branch and proximal to the popliteal branch [51]. The skin was closed using 7.0 polypropylene suture. For control, the surgical procedure outlined above was performed on the contralateral hindlimb, with the exception of ligating the femoral artery. Animals were given post-operative buprenorphine ($0.075 \text{ mg} \cdot \text{kg}^{-1}$), and supplied with buprenorphine water ($0.01 \text{ mg} \cdot \text{ml}^{-1}$) for approximately 2-3 days.

Clinical Ambulation Scoring

Following surgery, animals were observed daily for ambulation, and scored from 0-3 based on hindpaw range of motion, as described previously [52]. A dragging hindpaw was scored as a 3, no plantar flexion and constant dorsiflexion a 2, some plantar flexion a 1, and normal use a 0. Animals were monitored until normal ambulation was observed on two consecutive days, or until day-7 post ligation.

Perfusion Fixation and Microfil Infusion

In preparation for vascular casting and immunofluorescence, mice were perfusion fixed with paraformaldehyde. Through a thoracotomy, an incision was made in the apex of the heart into the left ventricle, and a catheter was inserted and secured with a vascular

clamp, **Figure 3A**. A cycle of 40 ml vasodilator cocktail heated to 37 °C (5 U· μ L⁻¹ heparin, 10⁻³ M sodium nitroprusside, 10⁻⁴ M adenosine, in PBS), followed by 5 ml 4% paraformaldehyde (PFA), was injected through the catheter with a syringe pump (5 ml·min⁻¹), **Figure 3B**. Following fixation, microfil agents were combined, and 1 ml was injected (0.5 ml·min⁻¹). Once the Microfil perfused distal to the knee, both ankles were ligated with silk suture to prevent entry into venous circulation through ankle A-V shunts, **Figure 3C**. Microfil infusion continued until the casting agent reached the anterior gracilis collateral (approximately 3 ml of additional solution). Post infusion, the exposed tissue was covered with plastic wrap and Microfil polymer cured overnight at room temperature. The anterior gracilis muscle and profunda femoris artery were resected and post fixed in 4% PFA for approximately 24 hours before rinsing and storing in PBS at 4 °C.

Immunofluorescence

Immunofluorescence was used to determine collateral vessel diameters and smooth muscle cell mechanoadaptation. Whole gracilis anterior muscles were stained with α -smooth muscle actin (Sigma Aldrich Anti-Actin α -Smooth Muscle Cy3™ Antibody, C6198) in a solution with 0.1% saponin and 2% BSA in PBS for 3 days at 4°C. After staining, muscles were washed in 0.1% saponin in PBS 3x for 20 minutes at room temperature, and PBS for 30 minutes. The samples were mounted onto depression slides with 50:50 PBS:glycerol to clear.

The midzone of the anterior gracilis collateral was captured with widefield fluorescence microscopy (α -smooth muscle actin) and widefield microscopy (Microfil cast). All anterior gracilis collateral inner and outer diameters were measured at the collateral midzone in three locations using ImageJ.

Confocal microscopy was used to visualize smooth muscle cells around each artery to assess smooth muscle cell overlap and length (mechanoadaptation indicators). Utilizing a 40x oil immersion objective, images of the collateral stem region of the profunda femoris artery were captured with Fluoview Viewer software (Olympus, FV10-ASW 4.1). Image stacks in the z-direction were captured with 1-micron slices for approximately half the diameter for each artery. To assess mechanoadaptation in the collateral stem, the z-stacks of the profunda femoris were merged into single, 3-D reconstructions, and smooth muscle cell overlap between the individual cells and smooth muscle cell length were measured using ImageJ software, as described previously [38, 53]. Briefly, a 100 μ m length of

artery was delineated from each 3D reconstruction, and average VSMC length for each vessel, L_v , was determined using Equation 1 below [38, 53]:'

$$L_v = 4\pi R_v F(S_w + S_t)/T_v \quad (\text{Eq. 1})$$

In equation 1, L_v is the average VSMC length, R_v the radius of the artery, F the fraction of vessel circumference in which measurements were made, S_w the number of wrap cells, S_t the length of all tapered cells divided by the length of one wrap cell, and T_v the number of taper cells.

Statistical Analysis

Differences in daily ambulation scores were determined using a repeated measures analysis of variance (ANOVA). Differences in intersex or interstrain ambulation scores, collateral stem VSMC lengths and overlaps, collateral midzone inner and outer diameters, and collateral midzone wall thicknesses were determined using independent t-tests. All values are expressed as mean $\pm$ standard error of the mean.

RESULTS

Functional Impairment Following Femoral Artery Ligation

Previous investigations of male-female differences in response to arterial occlusion suggested that deficits observed in female mice were likely attributable to reduced collateral remodeling or fewer collateral number [50]. However, because recovery from femoral artery resection involves both collateral enlargement in the thigh along with angiogenesis and capillary arterialization in the leg [54, 55], we sought to evaluate the impact of female sex in an arterial occlusion model that involves primarily collateral enlargement [56]. Surprisingly, there was no difference in hindlimb use between male and female C57Bl/6, which was normalized by 3.9 ± 0.4 days for both, **Figure 4**. If anything, C57Bl/6 female mice may have recovered normal hindlimb use before the males, but this was only statistically different at the day-5 time point. Female C57Bl/6 mice were statistically different at days 1, 2, and 5 from the rest of the days scored for that group, and male C57Bl/6 were different at days 1 and 6. Female BALB/c mice were different at days 4 and 6 from the rest of the days scored for that group, and male BALB/c mice were different at days 4, 5, and 6. As expected, BALB/c mice regained normal hindlimb function more slowly, but there was no difference between males and females, 5.8 ± 0.2 and 6.0 ± 0 days, respectively. As ambulation was only scored up to 6 days, these data suggest that BALB/c mice hindlimb function did not return to normal use during the experiment.

Although no difference in hindlimb use suggests a similar degree of collateral enlargement between male and female mice, hindlimb use is a sufficiently coarse

assessment that depends upon more than collateral flow. Therefore, we specifically evaluated collateral remodeling between male and female mice following femoral artery ligation.

Mechanoadaptation in the Collateral Stem

Although the collateral stem enlarges following femoral artery ligation, the absence of smooth muscle cell proliferation suggests the enlargement is not due to typical arteriogenesis [57, 58]. To test the hypothesis that enlargement in the collateral stem occurs via mechanoadaptation, change in vascular diameter by the modification of VSMC length and overlap, and to determine if female mice are deficient in this process, we assessed smooth muscle cell length and overlap utilizing confocal microscopy at day 7 in both sham-operated and ligated profunda femoris arteries in male and female C57Bl/6 mice. Although the native anatomy of smooth muscle cells in the collateral stem of C57Bl/6 male and female mice was not different, VSMCs were longer in males following remodeling, and VSMC lengths in both sexes were greater than the contralateral controls, **Figure 5C**. Consistent with mechanoadaptation, smooth muscle cell overlap decreased at day-7 post ligation in both sexes, but there was no difference between male and female mice **Figure 5D**.

As BALB/c mice have lower collateral density and decreased collateral remodeling when compared to C57Bl/6 mice at day-21 following ligation, we examined enlargement in this model [52, 56]. There was no difference in VSMC lengths of male and female collateral stems in BALB/c mice, and remodeling was consistent, with all VSMC lengths greater in both male and female mice following ligation than the contralateral controls.

Smooth muscle cell overlap decreased at day-7 post ligation in both male and female BALB/c mice, consistent with mechanoadaptation, **Figure 5D**. Despite the native anatomy of the BALB/c mice differing with VSMC overlap shorter in male than female mice in the non-ligated group, there was no difference in remodeled collateral stem

VSMC overlaps between male and female BALB/c mice.

Anterior Gracilis Collateral Remodeling Post-Femoral Artery Ligation

Having compared the mechanoadaptation events between males and females, we next evaluated the arteriogenesis response in the collateral midzone within the anterior gracilis muscle [56]. To determine if female mice exhibit impaired arteriogenesis, we measured outward remodeling (based on luminal diameter) and measured abluminal diameter so that we could calculate wall thicknesses and identify any differences in the type of remodeling that occurred (i.e. hypertrophic, eutrophic, or hypotrophic), **Figure 6**, in male and female C57Bl/6 and BALB/c mice. As expected, robust outward remodeling occurred, as the luminal diameter was larger for all ligated gracilis collaterals compared to the respective contralateral control, for both male and female C57Bl/6 mice, **Figure 6E**. The abluminal diameter was also larger for both male and female C57Bl/6 mice, and even larger than expected based on the increase in luminal diameter, suggesting a hypertrophic outward remodeling. **Figure 6F**. Indeed, measurements of wall thickness confirmed a hypertrophic outward remodeling, which was not different between male and female C57Bl/6 mice. **Figure 6G**. There was no difference between male and female C57Bl/6 mice for luminal diameter, abluminal diameter, or wall thickness in the remodeled collateral.

With no evidence of intersex differences in collateral midzone outward remodeling in C57Bl/6 mice, we examined if there was an impairment in collateral remodeling for BALB/c female mice. Like the C57Bl/6 mice, the lumen was larger for all ligated gracilis collaterals compared to the respective contralateral control, within both males and females, **Figure 6E**. The abluminal diameter was also greater for both male and female

mice, comparing the ligated gracilis collaterals to the contralateral control, **Figure 6F**.

Both males and females exhibited larger wall thicknesses for the day-7 remodeled collaterals compared to the contralateral control, **Figure 6G**. There was no difference between male and female BALB/c mice for luminal diameter, abluminal diameter, or wall thickness in the remodeled or control collaterals.

DISCUSSION

Contemporary clinical evidence indicates a higher incidence of PAOD and associated likelihood of critical limb ischemia in women [10-15, 59]. Women are also less likely to survive, and exhibit worse prognosis following myocardial infarction [16, 17]. With these differences possibly due to discrepancies in vascular compensation, developing effective therapeutics necessitates understanding the mechanisms underlying these potential differences [20, 21]. Previous work is inconsistent on the impact of female sex on flow recovery following arterial occlusion, but suggested an impairment in collateral growth [21, 47, 50]. Therefore, we described the effect of arterial occlusion on collateral remodeling in healthy male and healthy reproductive-stage female mice.

In functional ambulatory recovery evaluation, both male and female C57Bl/6 mice regained normal hindlimb function more quickly than BALB/c mice post-ligation, **Figure 4**. The inter-strain findings are consistent with previous observations that BALB/c mice have greater hindlimb use impairment at days 3 and 7 when compared to C57Bl/6 models [52]. Furthermore, BALB/c mice had greater myofiber atrophy, greater myocyte apoptosis, and attenuated myogenic regulatory gene expression and stress-response signaling following hindlimb ischemia, with evidence in functional impairment [60]. No difference existed in days to normal ambulation between male and female mice with normal estrous cycles in either strain.

In exploring the outward vascular remodeling mechanisms underlying the ambulatory recovery, it is known that the collateral stem outwardly remodels following an upstream

occlusion in male mice [57]. Although the collateral stem enlarges, the lack of VSMC proliferation in this area suggests the enlargement was due to other processes [58, 61-63]. Repositioning of VSMCs due to an altered mechanical environment is established, as physical alignment and reorientation of isolated cultured VSMCs in a position perpendicular to the stretch vector occurs due to cyclic stretch, and arteriolar inward remodeling in response to norepinephrine and angiotensin [64-66]. As such, we tested the hypothesis that enlargement in the collateral stem occurs via VSMC mechanoadaptation, and that female mice are deficient in this process. Although all smooth muscle cell lengths increased relative to their contralateral control, surprisingly, the only sex difference in remodeling was longer VSMCs in C57Bl/6 males compared to females. As the native VSMC lengths between C57Bl/6 mice were not different, these males may exhibit the ability to elongate VSMCs greater than females of the same strain, although this did not affect the diameter of the vessel in a measurable way. VSMC shape and migration are altered by many different stimuli, including humoral factors (ANGII, TGF β ₁, ET-1, etc.), neuronal contributors, intercellular VSMC paracrine factors, endothelial cell influences (NO, etc.), and mechanical forces such as active stress and strain [67]. Estrogen levels in men and postmenopausal women are lower than levels in non-pregnant women [41-43], and estrogen has been shown to cause an increase in norepinephrine levels [68]. Norepinephrine exposure has resulted in shortening of VSMCs [38]. Therefore, it is possible that if the females had higher baseline levels of norepinephrine, their VSMCs lengths may not lengthen as robustly, resulting in the sexual dimorphism seen. In examining an additional aspect of mechanoadaptation, all

VSMC overlaps decreased relative to their contralateral control, but there were no sex-related remodeling differences among BALB/c or C57Bl/6 strains, **Figure 5**.

As the anterior gracilis collateral exhibits smooth muscle cell proliferation, evidence of typical arteriogenesis [56, 58], we tested the hypothesis that female mice exhibit impaired arteriogenesis in this vessel. No differences were found between C57Bl/6 and BALB/c mice, consistent with prior day-7 collateral arteriole remodeling results, in which male BALB/c collateral outward remodeling was impaired at day-3 post arterial occlusion, but showed no difference at day-7 [56]. Vessel diameters and wall thickness were larger in the remodeled vessel than the control vessel in all sexes and strains. The only exception was the wall thickness of C57Bl/6 female mice. This measurement had a large amount of variability, which may be due to variations in the mice estrous cycles. 17- β -estradiol levels are variable during the cycle [69], and 17- β -estradiol increases the endothelial production of nitric oxide [46], which is an endothelial-derived vasodilator [70]. Nitric oxide diffuses into VSMCs, initiating a cascade that activates protein kinase G. This then regulates calcium concentrations and sensitization, and causes actin and myosin dynamic alterations that result in cellular relaxation, possibly altering wall thickness [71].

However, no difference existed between male and female C57Bl/6 mice for inner diameter, outer diameter, or wall thickness in the remodeled or control collaterals, **Figure 6**. Therefore, this lack of difference in diameters may suggest the lack of increase in wall thickness to be artifact. As this finding suggests no obvious anatomical remodeling differences in the anterior gracilis collateral midzone of male and female mice, there may

be other factors accounting for any flow recovery differences previously observed, such as increased vascular resistance in the ischemic zone in females [50].

Fewer preexisting collaterals in females with unimpaired remodeling, or dysregulated capillary arterialization and angiogenesis may explain increased vascular resistance in females, with collaterals remodeling to the same extent. However, no difference between male and female C57Bl/6 mice in number of α -smooth muscle actin-positive vessels was found at baseline in the midpoint of the semimembranosus muscle [50]. Despite this lack of difference, the small sample size of no more than 5 mice in each group leaves this possibility to be considered [50]. Furthermore, the collateral distribution between male and females may be different, so although there was no difference at the midpoint of the semimembranosus muscle, there may be fewer preexisting collaterals in other areas of the hindlimb. Despite no difference at day-7 post ligation in α -smooth muscle actin-positive vessels compared to baseline for females, there was an increase in males. This increase is suggestive of preexisting capillaries being transformed into collaterals, possibly due to pericyte recruitment, known as capillary arterialization [72, 73]. In a basic examination of angiogenesis, female C57Bl/6 mice had less fewer red blood cell-containing vessels in a Matrigel Plug Assay compared to males after 5 days, indicating dysregulation [50]. An increased ability in either of these mechanisms would explain lower vascular resistance in males.

Alternatively, higher vascular resistance in female mice [46] may be due to impaired vasodilation in the ischemic zone, even without vessel growth impairment or difference

in baseline collateral density [50, 74]. Because reports concerning the impact of arterial occlusion on smooth muscle-dependent and endothelium-dependent vasodilation are inconsistent, it is unclear what component of the remodeling process may be affected. Vasodilation is impaired in response to endothelium-dependent agents in immature collaterals in animal models [75-79], but upon maturation is either restored [78] or continually impaired [79]. This may be explained by endothelial dysfunction induced by inflammatory conditions during remodeling [80], or an increase in collateral flow [81]. Hypercholesterolemia and ovariectomy in female mice each lead to impaired endothelial-dependent vasodilation in resistance vessels [102], so it is possible that additional comorbidities or estrogen deficiency may precipitate sex-dependent causal effects on vasodilation impairment, possibly leading to higher female vascular resistance depending on female mice position in the estrous cycle [82].

Impaired vasodilation may be partially contributed to smooth muscle cell dysfunction [83]. This dysfunction is suggested by diminished endothelium-dependent and independent dilation responses in immature feed arteries that are restored after maturation [83]. Although feed arteries and collaterals remodel differently, endothelium-independent vasodilation is also impaired in collateral vessels [79, 84, 85]. This impairment could be due to smooth muscle dysfunction caused by the smooth muscle cell phenotype change during proliferation in immature collaterals [86, 87]. Although there is conflicting evidence that vasodilation responses to sodium nitroprusside are intact at day-14 following the induction of chronic ischemia [79], nitric oxide-induced, smooth muscle-dependent dilation is impaired in intramuscular arterioles at 5-weeks post chronic

ischemia induction [79, 85]. Although the mechanism of the impairment is unknown in these reports, there is a possible relation to oxidative stress. This stress increases with chronic ischemia [88], impairing dilation response in the obese Zucker rat to sodium nitroprusside [89]. Impaired dilation may have increased vascular resistance in female C57Bl/6 mice, as they were less responsive to vasodilators at day-7 following femoral artery excision [50].

This research had several limitations regarding its interpretation. One of the potential limitations of this study is the non-standardization of the estrus cycle. This cycle in mice is approximately 4-5 days in length, consisting of the four phases: proestrus, estrus, metestrus and diestrus [90]. Through the duration of the study, the female mice were randomly cycling through these phases, undergoing several cycles. Although this non-standardization is not optimal in determining correlative mechanisms, it more accurately reflects the healthy female general population hormonal cycle variation. Another limitation is the variation in ambulation scorers throughout the week. A different lab member is in charge of determining an ambulation score for the mice every day, which may have led to variation in the data due to the subjective nature of the measure. To mitigate this potential variation, it may be best to have one individual assign scores throughout the duration of the experiment, possibly limiting the data collection to days 1, 3, and 6 post ligation to ease workload and still capture a comprehensive view of recovery. In addition, training videos or sessions could be created to minimize variation between scorers within the lab. Another possible limitation is the variation in time of day

in which the ambulation data was collected, so moving forward it would be recommended to score the mice at a similar time each day for consistency.

In summary, the aim of these studies was to describe the effect of arterial occlusion on collateral remodeling in male and healthy reproductive stage female mice. The collateral stem enlarged seemingly due to mechanoadaptation in both BALB/c and C57Bl/6 mice. There were no major sex-related differences in the gracilis collateral stem or midzone remodeling or resultant functional ambulation, with the exception of longer VSMCs in male C57Bl/6 mice, at day-7 post femoral artery ligation in the collateral stem. As clinical evidence indicates a higher incidence of PAOD and associated likelihood of critical limb ischemia in women, as well as worse prognosis and decreased survival post myocardial infarction, understanding the possible differences in underlying vascular compensation mechanisms is crucial [10-17, 59]. As these differences were not captured in our specific investigation of arteriogenesis, an evaluation of microvascular remodeling in the ischemic zone and collateral vasodilation would be of interest. Furthermore, as oophorectomy reduces flow-mediated outward remodeling and decreased vessel densities in mice [46], arteriogenesis should be evaluated in the hindlimb following oophorectomy with estrogen depletion. 17- β -estradiol increases the endothelial production of nitric oxide, and flow-mediated outward remodeling following oophorectomy is rescued by 17- β -estradiol, with increased age a risk factor for PAOD development [11, 42, 46]. The determination of any underlying mechanistic sex-specific differences could be the foundation in which targeted therapeutics are developed, crucial for closing the prognosis gap between men and women in the global treatment of PAOD.

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APPENDICES

Appendix A: Raw Images

Wideview Microscopy (10x)

C57Bl/6 Females:

N=1 Anterior Gracilis Collateral Day-7 Post-Femoral Artery Ligation A) Alpha-Smooth Muscle Actin B) Microfil

N=1 Anterior Gracilis Collateral Control A) Alpha-Smooth Muscle Actin B) Microfil

N=2 Anterior Gracilis Collateral Day-7 Post-Femoral Artery Ligation A) Alpha-Smooth Muscle Actin B) Microfil (Did not use)

N=2 Anterior Gracilis Collateral Control A) Alpha-Smooth Muscle Actin B) Microfil

N=3 Anterior Gracilis Collateral Day-7 Post-Femoral Artery Ligation A) Alpha-Smooth Muscle Actin B) Microfil

N=3 Anterior Gracilis Collateral Control A) Alpha-Smooth Muscle Actin B) Microfil

N=4 Anterior Gracilis Collateral Day-7 Post-Femoral Artery Ligation A) Alpha-Smooth Muscle Actin B) Microfil

N=4 Anterior Gracilis Collateral Control A) Alpha-Smooth Muscle Actin B) Microfil

N=5 Anterior Gracilis Collateral Day-7 Post-Femoral Artery Ligation A) Alpha-Smooth Muscle Actin B) Microfil

N=5 Anterior Gracilis Collateral Control A) Alpha-Smooth Muscle Actin B) Microfil
C57Bl/6 Males:

N=1 Anterior Gracilis Collateral Day-7 Post-Femoral Artery Ligation A) Alpha-Smooth
 Muscle Actin B) Microfil

N=1 Anterior Gracilis Collateral Control A) Alpha-Smooth Muscle Actin B) Microfil

N=2 Anterior Gracilis Collateral Day-7 Post-Femoral Artery Ligation A) Alpha-Smooth Muscle Actin B) Microfil

N=2 Anterior Gracilis Collateral Control A) Alpha-Smooth Muscle Actin B) Microfil

N=3 Anterior Gracilis Collateral Day-7 Post-Femoral Artery Ligation A) Alpha-Smooth Muscle Actin B) Microfil

N=3 Anterior Gracilis Collateral Control A) Alpha-Smooth Muscle Actin B) Microfil

N=4 Anterior Gracilis Collateral Day-7 Post-Femoral Artery Ligation A) Alpha-Smooth Muscle Actin B) Microfil

N=4 Anterior Gracilis Collateral Control A) Alpha-Smooth Muscle Actin B) Microfil

N=5 Anterior Gracilis Collateral Day-7 Post-Femoral Artery Ligation A) Alpha-Smooth Muscle Actin B) Microfil (Did not use)

N=5 Anterior Gracilis Collateral Control A) Alpha-Smooth Muscle Actin B) Microfil

N=6 Anterior Gracilis Collateral Day-7 Post-Femoral Artery Ligation A) Alpha-Smooth Muscle Actin B) Microfil

N=6 Anterior Gracilis Collateral Control A) Alpha-Smooth Muscle Actin B) Microfil

BALB/C Females:

N=1 Anterior Gracilis Collateral Day-7 Post-Femoral Artery Ligation A) Alpha-Smooth Muscle Actin B) Microfil

N=1 Anterior Gracilis Collateral Control A) Alpha-Smooth Muscle Actin B) Microfil

N=2 Anterior Gracilis Collateral Day-7 Post-Femoral Artery Ligation A) Alpha-Smooth Muscle Actin B) Microfil

N=2 Anterior Gracilis Collateral Control A) Alpha-Smooth Muscle Actin B) Microfil

N=3 Anterior Gracilis Collateral Day-7 Post-Femoral Artery Ligation A) Alpha-Smooth Muscle Actin B) Microfil

N=4 Anterior Gracilis Collateral Day-7 Post-Femoral Artery Ligation A) Alpha-Smooth Muscle Actin B) Microfil

N=4 Anterior Gracilis Collateral Control A) Alpha-Smooth Muscle Actin B) Microfil

N=5 Anterior Gracilis Collateral Day-7 Post-Femoral Artery Ligation A) Alpha-Smooth Muscle Actin B) Microfil

N=5 Anterior Gracilis Collateral Control A) Alpha-Smooth Muscle Actin B) Microfil

N=6 Anterior Gracilis Collateral Day-7 Post-Femoral Artery Ligation A) Alpha-Smooth Muscle Actin B) Microfil

N=6 Anterior Gracilis Collateral Control A) Alpha-Smooth Muscle Actin B) Microfil

BALB/C Males:

N=1 Anterior Gracilis Collateral Day-7 Post-Femoral Artery Ligation A) Alpha-Smooth Muscle Actin B) Microfil

N=1 Anterior Gracilis Collateral Control A) Alpha-Smooth Muscle Actin B) Microfil

N=2 Anterior Gracilis Collateral Day-7 Post-Femoral Artery Ligation A) Alpha-Smooth Muscle Actin B) Microfil

N=2 Anterior Gracilis Collateral Control A) Alpha-Smooth Muscle Actin B) Microfil

N=3 Anterior Gracilis Collateral Day-7 Post-Femoral Artery Ligation A) Alpha-Smooth Muscle Actin B) Microfil

N=3 Anterior Gracilis Collateral Control A) Alpha-Smooth Muscle Actin B) Microfil

N=4 Anterior Gracilis Collateral Day-7 Post-Femoral Artery Ligation A) Alpha-Smooth Muscle Actin B) Microfil

N=4 Anterior Gracilis Collateral Control A) Alpha-Smooth Muscle Actin B) Microfil

N=5 Anterior Gracilis Collateral Day-7 Post-Femoral Artery Ligation A) Alpha-Smooth Muscle Actin B) Microfil

N=5 Anterior Gracilis Collateral Control A) Alpha-Smooth Muscle Actin B) Microfil

N=6 Anterior Gracilis Collateral Day-7 Post-Femoral Artery Ligation A) Alpha-Smooth Muscle Actin B) Microfil

N=6 Anterior Gracilis Collateral Control A) Alpha-Smooth Muscle Actin B) Microfil

Confocal Images (40x)

C57Bl/6 Females:

N=1 A) Profunda Femoris Artery Day-7 Post-Ligation B) Profunda Femoris Artery Control

N=2 A) Profunda Femoris Artery Day-7 Post-Ligation B) Profunda Femoris Artery Control

N=3 A) Profunda Femoris Artery Day-7 Post-Ligation B) Profunda Femoris Artery Control

N=4 A) Profunda Femoris Artery Day-7 Post-Ligation B) Profunda Femoris Artery Control

N=5 A) Profunda Femoris Artery Day-7 Post-Ligation B) Profunda Femoris Artery Control

N=6 A) Profunda Femoris Artery Day-7 Post-Ligation B) Profunda Femoris Artery Control

N=7 A) Profunda Femoris Artery Day-7 Post-Ligation B) Profunda Femoris Artery Control

N=8 Profunda Femoris Artery Day-7 Post-Ligation

C57Bl/6 Males:

N=1 A) Profunda Femoris Artery Day-7 Post-Ligation B) Profunda Femoris Artery Control

N=2 A) Profunda Femoris Artery Day-7 Post-Ligation B) Profunda Femoris Artery Control

N=3 A) Profunda Femoris Artery Day-7 Post-Ligation B) Profunda Femoris Artery Control

N=4 A) Profunda Femoris Artery Day-7 Post-Ligation B) Profunda Femoris Artery Control

N=5 A) Profunda Femoris Artery Day-7 Post-Ligation B) Profunda Femoris Artery Control

N=6 A) Profunda Femoris Artery Day-7 Post-Ligation B) Profunda Femoris Artery Control

N=7 A) Profunda Femoris Artery Day-7 Post-Ligation B) Profunda Femoris Artery Control

BALB/C Females:

N=1 A) Profunda Femoris Artery Day-7 Post-Ligation B) Profunda Femoris Artery Control

N=2 A) Profunda Femoris Artery Day-7 Post-Ligation B) Profunda Femoris Artery Control

N=3 A) Profunda Femoris Artery Day-7 Post-Ligation (*Did not use*) B) Profunda Femoris Artery Control

N=4 A) Profunda Femoris Artery Day-7 Post-Ligation B) Profunda Femoris Artery Control

N=5 A) Profunda Femoris Artery Day-7 Post-Ligation B) Profunda Femoris Artery Control

N=6 A) Profunda Femoris Artery Day-7 Post-Ligation B) Profunda Femoris Artery Control (*Did not use*)

BALB/C Males:

N=1 Profunda Femoris Artery Day-7 Post-Ligation

N=2 A) Profunda Femoris Artery Day-7 Post-Ligation B) Profunda Femoris Artery Control

N=3 Profunda Femoris Artery Control

N=4 A) Profunda Femoris Artery Day-7 Post-Ligation B) Profunda Femoris Artery Control

N=5 Profunda Femoris Artery Control

N=6 A) Profunda Femoris Artery Day-7 Post-Ligation B) Profunda Femoris Artery Control

Appendix B: Tables

Table I. Days to Normal Ambulation Post-Femoral Artery Ligation in C57Bl/6 and BALB/c Mice

	Sex	n	Days to Normal Ambulation $\pm$ SE
C57Bl/6	Males	13	3.9 ± 0.4
	Females	13	3.9 ± 0.4
BALB/c	Males	6	5.8 ± 0.2
	Females	6	6.0 ± 0.0

Table II. Daily Ambulation Scoring in C57Bl/6 and BALB/c Mice

	Sex	n	Day 1 Post-Ligation $\pm$ SE	Day 2 Post-Ligation $\pm$ SE	Day 3 Post-Ligation $\pm$ SE	Day 4 Post-Ligation $\pm$ SE	Day 5 Post-Ligation $\pm$ SE	Day 6 Post-Ligation $\pm$ SE
C57Bl/6	Males	13	1.6 ± 0.2	0.8 ± 0.1	0.5 ± 0.2	0.4 ± 0.2	0.3 ± 0.1	0.0 ± 0.0
	Females	13	1.4 ± 0.2	1.0 ± 0	0.1 ± 0.1	0.1 ± 0.1	0.0 ± 0.0	0.3 ± 0.2
BALB/c	Males	6	3.0 ± 0	2.0 ± 0.0	2.4 ± 0.2	1.8 ± 0.3	1.7 ± 0.3	1.0 ± 0.3
	Females	6	2.8 ± 0.2	1.8 ± 0.4	2.3 ± 0.2	1.7 ± 0.2	1.3 ± 0.2	1.7 ± 0.2

Table III. Indicators of Mechanoadaptation in C57Bl/6 and BALB/c Mice

	Sex	Limb	n	VSMC Length (μ m) $\pm$ SE	Overlap (μ m) $\pm$ SE
C57Bl/6	Males	Day-7 Post-Ligation	6	329 ± 19	19 ± 2
		Control	7	273 ± 15	27 ± 2
	Females	Day-7 Post-Ligation	8	288 ± 13	16 ± 1
		Control	7	235 ± 19	26 ± 2
BALB/c	Males	Day-7 Post-Ligation	4	325 ± 9	24 ± 2
		Control	5	280 ± 10	29 ± 1
	Females	Day-7 Post-Ligation	4	327 ± 10	22 ± 2
		Control	5	297 ± 11	35 ± 2

Table IV. Anterior Gracilis Collateral Remodeling in C57Bl/6 and BALB/c Mice.

				Outer Diameter (μm) $\pm$ SE	Inner Diameter (μm) $\pm$ SE	Wall Thickness (μm) $\pm$ SE
	Sex	Limb	n			
<i>C57Bl/6</i>	Males	Day-7 Post-Ligation	4	93 $\pm$ 9	70 $\pm$ 7	12 $\pm$ 1
		Control	5	50 $\pm$ 5	38 $\pm$ 4	6 $\pm$ 1
	Females	Day-7 Post-Ligation	4	95 $\pm$ 12	62 $\pm$ 7	17 $\pm$ 6
		Control	5	46 $\pm$ 5	29 $\pm$ 3	8 $\pm$ 1
<i>BALB/c</i>	Males	Day-7 Post-Ligation	6	103 $\pm$ 9	67 $\pm$ 7	18 $\pm$ 2
		Control	6	50 $\pm$ 7	37 $\pm$ 4	7 $\pm$ 3
	Females	Day-7 Post-Ligation	6	100 $\pm$ 7	70 $\pm$ 6	17 $\pm$ 1
		Control	5	46 $\pm$ 3	35 $\pm$ 3	6 $\pm$ 1

Appendix C: Femoral Artery Ligation Protocol

Date _____	Hindlimb Ischemia Surgery - Ligation	Initials _____
Mouse Information		
DOB: _____		
Sex: _____		
Tag: _____		
Genotype/strain: _____		
Cage: _____		
Materials		
Sterilize- autoclave or flash autoclave		
____ 1. forceps (2)	____ 35. Apply 4x4 gauze sponge to heat pad to protect animal from excessive heat	
____ 2. fine forceps (2)	____ 36. Affix non-rebreathing circuit to surgery table w/ chemistry clamp	
____ 3. ultrafine forceps (1)	____ 37. Lay animal supine on circulating heat pad w/ nose in nose-cone	
____ 4. fine scissors (1)	____ 38. Insert rectal probe and set thermo-controller to 37°C	
____ 5. microscissors spring loaded (1)	____ 39. Apply veterinary ointment to eyes to avoid drying during procedure	
	____ 40. Apply veterinary ointment to anus and place rectal probe ~1cm into anus to monitor core-body temperature	
Pre-sterilize in autoclave		
____ 6. cotton gauze (2)	Surgery	
____ 7. cotton swabs (12)	____ 41. Make a small incision on the middle, medial aspect of the left thigh	
____ 8. 6.0 silk suture (2 x 1-inch)	____ 42. Extend the incision up to the abdominal wall	
____ 9. needle holder (1)	____ 43. Blunt dissect the subcutaneous connective tissue to maximize surgical exposure	
Obtained in surgery suite		
____ 10. sterile Petri dish w/ sterile saline	____ 44. Use cautery to remove fat pad overlying femoral a-v pair & cauterize epigastric av-pair	
____ 11. sterile gloves	____ 45. Blunt dissect the femoral artery from the neurovascular bundle just downstream from the deep femoral branch	
____ 12. sterile 7.0 prolene suture	____ 46. Tie off the femoral artery & vein with 6.0 silk suture, just downstream to the deep femoral branch	
____ 13. heat-cautery	____ 47. Use 6.0 polypropylene suture to close the skin	
____ 14. FST heat pad w/ rectal probe	____ 48. Make a small incision on the middle, medial aspect of the right thigh	
____ 15. heat pad	____ 49. Extend the incision up to the abdominal wall	
____ 16. recovery bin & weigh boat	____ 50. Blunt dissect the subcutaneous connective tissue to maximize surgical exposure	
____ 17. depilatory cream	____ 51. Use 6.0 polypropylene suture to close the skin	
____ 18. non-sterile cotton swabs		
____ 19. non-sterile cotton gauze	Post-Surgical	
____ 20. isolation mask & cap	____ 52. Give the animal an subcutaneous injection of buprenorphine (0.075mg/kg)	
____ 21. analgesic (Buprenorphine)	____ 53. Place the animal in the recovery bin, on a blue bench cover, above a heat pad and allow to recover	
Surgery preparation		
____ 22. Spray surgery area with Nolvasan	____ 54. Turn flow meter down to 0, turn off isoflurane, and close the oxygen cylinder	
____ 23. Weigh animal in weight boat	____ 55. Indicate surgery on cage card	
____ 24. Place animal in anesthesia box		
____ 25. Open the oxygen cylinder and set anesthesia-machine flow meter to ~3 l·min ⁻¹		
____ 26. Anesthetize animal w/ 5% isoflurane		
____ 27. Affix non-rebreathing circuit to bench-top with tape		
____ 28. Reduce flow rate to 0.5-1.0 l·min ⁻¹ and the isoflurane to 1-3%		
____ 29. Apply ear tag high on left ear		
____ 30. Lay animal supine with nose in nose-cone		
____ 31. Shave hair on the right hindlimb & lower abdomen with clippers		
____ 32. Remove excess hair with depilatory cream		
____ 33. Spray right hindlimb with Nolvasan		
____ 34. Return animal to anesthesia box		
	Notes	
	_____	
	_____	
	_____	
	_____	

Appendix D: Perfusion Fixation Protocol

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 37°C
- ____ 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 both legs 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 20mL X 2 (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 isoflurane, and clean instruments

Notes

Appendix E: Microfil Protocol

Microfil casting and dissection protocol

Date _____	Microfil _____	Initials _____

Mouse Information

DOB: _____

Sex: _____

Tag: _____

Genotype/strain: _____

Cage: _____

Weight (g): _____

Materials

Non-Sterilize Dissection Instruments

____ 1. Forceps (1)

____ 2. Bone Scissors (1)

____ 3. Skin Scissors (1)

____ 4. Hemostats (1)

____ 5. Spring Scissors for Ventricle (1)

____ 6. Vascular clamp (1)

Obtained in surgery suite

____ 7. Tape

____ 8. 20mL Syringe (1)

____ 9. Bench cover

____ 10. Heating pad

____ 11. Catheter/Stopcock/Blunt needle (PE-100)

____ 12. Isoflurane Anesthetic

____ 13. Gauze squares

Vasodilator Cocktail Preparation

____ 14. Turn on water bath to 37°C

____ 15. 400 μ L heparin

____ 16. 1mL SNP (orange)

____ 17. 600 μ L Adenosine (clear)

____ 18. 38mL PBS solution

____ 19. Thaw SNP and Adenosine

____ 20. Add heparin, SNP, Adenosine, and PBS solution together in a 50mL conical

____ 21. Place vasodilator cocktail in water bath

Microfil Cocktail Preparation

____ 22. 2mL Microfil MV-122

____ 23. 1.25mL Microfil HV-Diluent

____ 24. 1.25mL Microfil MV-Diluent

____ 25. Add MV-122, HV-Diluent & MV-Diluent into a 14mL conical, vortex, place in bath

Procedure Preparation

____ 26. Weigh animal

____ 27. Obtain saline filled petri-dish, cotton swab, and instruments

Fixation

____ 28. Anesthize mouse with Isoflurane

____ 29. Heat up heat pad in microwave and wrap with bench cover when warm

____ 30. Remove hindlimb hair on both legs by shaving

____ 31. Tape animal down to heated bench cover

____ 32. Separate skin from muscle from the abdomen to the top of the thoracic cavity

____ 33. Fill 20mL syringe with warm Vaso D

____ 34. Cut through abdomen close to diaphragm

____ 35. Quickly cut through the ribs and diaphragm to open chest cavity and clamp back with hemostats

____ 36. Cut away excess tissue around the heart

____ 37. Make a small incision in the apex of the heart

____ 38. Insert catheter and clamp with vascular clamp and cut right atria

____ 39. Inject Vaso D solution into animal approximately 20mL at 5mL/min x2

____ 40. Add .2mL of curing agent to Microfil

____ 41. Inject 1mL of Microfil Cocktail (.5mL/min)

____ 42. Pause microfilm and ligate both ankles with silk suture once Microfil has passed the knee. Resume Microfil.

____ 43. Repeat step 41 until satisfied with fill.

____ 44. Remove mouse from heat pad and take out catheter (keep heart clamped)

____ 45. Place mouse in bag (cover open wounds with saran wrap) and let sit overnight at room temp

____ 46. Cover scope, turn off water bath, turn off oxygen, turn off Isoflurane, clean instruments

Dissection Date: _____

Non-Sterilized Dissection Instruments

____ 47. Fine Forceps (2)

____ 48. Forceps (1)

____ 49. Iris Scissors (1)

____ 50. Dissection scissors (1)

Obtained in surgery suite

____ 51. Non-sterile saline

____ 52. Cotton swab

____ 53. Bench cover

____ 54. Tape

____ 55. Petri Dishes (2)

____ 56. Cover Slips (2)

____ 57. Disposable pipettes (1)

____ 58. 25% ethanol

____ 59. Dissect chosen muscles and place on cover slip and then in labeled petri dish of 25% ethanol

____ 60. Cover scope and clean instruments

Notes:

_____

_____

_____

_____

_____

_____

Appendix F: ASMA Staining Protocol

Materials

24-well culture plates (Cat#: 3738, Corning Incorporated)

PBS

0.1% Saponin (Cat#: 47036, Sigma-Aldrich)

2% Bovine Serum Albumin (Cat# B6917, Sigma Aldrich)

Monoclonal Anti-Alpha Smooth Muscle Actin, Cy3 Conjugate (Cat#: C6198, Sigma-Aldrich)

Slides

Coverslips

Parafilm

Aluminum foil

Staining

1. Using forceps, remove muscle from PBS (stored in microcentrifuge tube at 4°C) and place in a single well of a 24-well culture plate.
2. Prepare antibody solution containing 1:200 1A4 clone (alpha-smooth muscle actin, Cy3 conjugate) in 0.1% saponin (reconstituted in PBS), 2% BSA (reconstituted in PBS) in PBS, using 0.3mL of solution per muscle.
3. Incubate muscle in antibody solution for 3 nights (72 hours) at 4°C. (**Note: Critical step—3 nights crucial for bright staining) by gently pipetting solution over muscle.**)
4. Wash in 0.1% saponin in PBS 3x for 20 minutes at room temperature. Cover plate with foil during each wash.
5. Wash in plain PBS for 30 minutes. Cover with foil during each wash.
6. Place 1-2 drops of 50/50 PBS and Glycerol onto slide.
7. Remove muscle from well using forceps and place on a slide.
8. Add 1-2 drops of 50/50 PBS and Glycerol to the top of the muscle and place cover slips over the muscle.
9. Paint edges of coverslip with clear nail polish to create a seal and prevent tissue desiccation.
10. Store slides at 4°C wrapped in foil or an opaque container between imaging.

Imaging

11. Image using a standard fluorescent microscope. (Cy3 excitation: 550 nm, emission: 570 nm)

Appendix G: Confocal 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

- ___ 22. piece using the coarse and fine objective adjustments located on the bottom of the microscope near to the left and right of the stage
- ___ 23. Press XY Repeat on Fluoview program to bring image onto computer
- ___ 24. 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
- ___ 25. 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
- ___ 26. Once the top is determined, click the "Set" button on the bottom left of the program
- ___ 27. Next find the end of the Z-stack desired by going deeper into the vessel and set this as the end point
- ___ 28. Set the step size to 1µm
- ___ 29. Click the Z-stack "XYZ" button to take the Z-stack
- ___ 30. Save the file as an .oib onto the computer and export it as a .tiff

Microscope Clean Up

- ___ 31. Turn off the microscope in the opposite way it was turned on, from right to left, bottom to top
- ___ 32. Make sure to close the shutters
- ___ 33. Log out in the log book
- ___ 34. 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
- ___ 35. Cover confocal microscope

Notes

Appendix H: Inadequate Methods

There were several methods that did provide an adequate assessment of the data. These methods are outlined below, along with suggestions for possible mitigation of challenges.

Transilluminator

To attempt to visualize the red blood cell column to measure inner diameter in the gracilis collateral midzone, a transilluminator was used. A transilluminator is a water-resistant probe with a light at the distal end designed to fit behind the dissected anterior gracilis *in vivo*. The light was placed directly behind the collateral midzone, and images were captured utilizing an Olympus BXFM intravital microscope. However, the method did not provide images with clearly discernable red blood cell columns, so inner diameter measurements were not successfully recorded. Possible reasons for failure are depth of vessels resulting in challenging focusing for microscope, or incorrect microscope settings. Suggestions for possible mitigation include adjustment of light settings or location of probe to result in clearer images, adjustment of imaging variables to result in clearer images, removal of tissue overlying gracilis muscle, and adjustment of post-processing of images. A sample image is seen below.

In vivo gracilis collateral midzone visualization utilizing transilluminator.

Endothelial Lectin Stain

To attempt to visualize endothelial cells to measure inner diameter in the gracilis collateral midzone, a lectin stain was used. Lectin specifically binds to glycoproteins located in the basal membrane of endothelial cells [91], and can be visualized using widefield fluorescent microscopy. This protocol can be seen in Appendix I. In addition to the recommended incubation time and concentration in the protocol, I also performed 1:40 4 hour, 1:20 2 hour, 1:20 4 hour, and 1:20 overnight incubation. However, upon imaging there was only nonspecific activation of cells, not allowing clear visualization of the endothelium. As lectin depends on diffusion through tissue, it is possible that the gracilis collateral is too deep in the anterior gracilis muscle for the molecule to bind to the endothelium. Suggestions for possible mitigation include adjustment of antibody

concentrations, incubation times, and permeabilizing agents to allow for binding to the collateral endothelium, adjustment of widefield fluorescent filters, and adjustment of post-processing of images. A sample image of a lectin stained image overlaid to an alpha-smooth muscle actin image is seen below, with no clear visualization of the endothelium.

Lectin stain. (A) Lectin positive image of 1:20 dilution incubated for 4 hours. (B) Lectin positive image (green) overlaid with alpha-smooth muscle actin positive (red).

Evans Blue Dye

To visualize inner diameter of the gracilis collateral midzone, Evans Blue Dye was utilized. Evan's blue dye binds to serum albumin, resulting in luminal staining of vessels, allowing visualization upon microscopy [92]. It is injected into the ventral artery in the tail. However, upon intravital microscopy using a transilluminator, clear visualization of the inner diameter was not obtained. This may be due to diffusion of the dye to other tissues, as the myofibers also turned blue, as well as challenges previously discussed with transillumination. Potential mitigation includes adjustment of transillumination technique, concentration of dye or volume injected, a different method of injection such as intracardiac following perfusion fixation, adjustment of widefield microscopy bright-light settings, or adjustment of post-processing of images.

Estrous Cycle Determination

To assign where a female mouse was in the murine estrous cycle on day-7 post ligation, vaginal visualization was utilized, as outlined in a prior study [93]. Images were captured using a hand-held digital camera of the vaginal area, and then compared to the reference images in the publication. However, as there were no personnel in the lab trained in this subjective method, there was no way to confirm the correct assignment of the estrous cycle. To mitigate this moving forward, collaboration with the group who created this method would be ideal to ensure proper training and success in assignment.

Example image for vaginal visualization.

Length Density and Volume Fraction

To determine vessel density in the anterior gracilis muscle along the stem, midzone and reentry portions, length density and volume fraction calculations were utilized following alpha-smooth muscle actin staining as described in **Appendix I**, and imaged using widefield microscopy. Images were taken at the stem, midzone, and reentry regions of the vessel and imported into Rapid Analysis of Vessel Elements (RAVE) software. Length density is a measure of total arteriole length per unit area of tissue, and volume fraction represents the total length of all vessels divided by total pixel area, with the method outlined in a prior publication [94]. However, because images were not in focus all the way through the depth of collateral location in the muscle, it was difficult to use the software to assign a correct binary to distinguish between vascular and muscular tissue. As the images were taken on the widefield microscope, utilization of the confocal microscope and creation of a z-stack would be ideal for the uniform focus of the vessels necessary for RAVE software utilization.

Vessel Density Measurement. (A) Rapid Analysis of Vessel Elements (RAVE) Software GUI. (B,C) Representative alpha-smooth muscle actin positive images that were uploaded for assessment.

Appendix I: Lectin Stain Protocol

Staining Protocol for White Gastroc Specimens (Laughlin Work)

Tissue Harvesting:

1. Pin-out the desired Gastroc half onto 60 x 15 mm petri dish filled with SLYGARD (silicone elastomer).
2. Immerse tissue in 4% P-form in PBS for 3 hours in the fridge. Drop petri dish into 150 ml beaker. Add enough P-form solution to ensure immersion of specimen.
3. Un pin specimen and wash with PBS for 30 minutes on rotating plate. Replace the PBS solution every 10-15 minutes. For all washes muscle specimens were placed in a small 10 ml beaker or petri dish.
4. Cut off red section, leaving only the white section.
5. Store white section in PBS/Glycerol overnight. This step mimics the storage/shipping process of the harvested muscles.

Tissue Staining (upon arrival of specimens):

1. Place each muscle specimen in an individual 10 ml beaker or glass petri dish and wash with PBS for 30 minutes on rotating plate. Replace the PBS solution every 10-15 minutes.
2. Incubate white portion in 3 mg/ml type I collagenase in PBS for 30 minutes. Place muscle specimen in 10 ml beaker and add approximately 2 ml of collagenase solution.
3. Wash with 0.1% saponin in PBS for 30 minutes on rotating plate. Again, place each muscle specimen in an individual 10 ml beaker or glass petri dish. Replace the 0.1% saponin in PBS solution every 10-15 minutes.
4. Incubate in 1:200 1A4-CY3 in 0.1% saponin, 2% BSA in PBS for 3 nights at 4 deg. C. Place muscle specimen in a 10 ml falcon tube. Fill tube with 1.5-2 ml of antibody solution. Ensure that the tissue is immersed in antibody solution.
5. Wash with 0.1% saponin in PBS for 1 hour on rotating plate. Again, place each muscle specimen in an individual 10 ml beaker or glass petri dish. Replace the 0.1% saponin in PBS solution every 10-15 minutes.
6. Incubate in 1:20 BSI Lectin FITC in 0.1% saponin in PBS for 2 hours at 4 deg. C. Place muscle specimen in a 10 ml falcon tube. Fill tube with 1.5-2 ml of antibody solution. Ensure that the tissue is immersed in antibody solution.
7. Wash with 0.1% saponin in PBS for 30 minutes on rotating plate. Again, place each muscle specimen in an individual 10 ml beaker or glass petri dish. Replace the 0.1% saponin in PBS solution every 10-15 minutes.
8. Store in 50/50 PBS/Glycerol in the freezer. Place muscle specimen in a 10 ml falcon tube. Fill tube with 1.5-2 ml of glycerol solution. Ensure that the tissue is immersed in solution.