ISCHEMIA IMPAIRS VASODILATION IN SKELETAL MUSCLE RESISTANCE
ARTERY

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ABSTRACT

Ischemia Impairs Vasodilation in Skeletal Muscle Resistance Artery

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Functional vasodilation in arterioles is impaired with chronic ischemia. We sought to examine the impact of chronic ischemia and age on skeletal muscle resistance artery function. To examine the impact of chronic ischemia, the femoral artery was resected from young (2-3mo) and adult (6-7mo) mice and the profunda femoris artery diameter was measured at rest and following gracilis muscle contraction 14 days later using intravital microscopy. Functional vasodilation was significantly impaired in ischemic mice ($14.4 \pm 4.6\%$ vs. $137.8 \pm 14.3\%$, $p < 0.0001$ $n=8$) and non-ischemic adult mice ($103.0 \pm 9.4\%$ vs. $137.8 \pm 14.3\%$, $p=0.05$ $n=10$). In order to analyze the cellular mechanisms of the impairment, a protocol was developed to apply pharmacological agents to the experimental preparation while maintaining tissue homeostasis. Endothelial and smooth muscle dependent vasodilation were impaired with ischemia, $39.6 \pm 13.6\%$ vs. $80.5 \pm 11.4\%$ and $43.0 \pm 11.7\%$ vs. $85.1 \pm 10.5\%$, respectively. From this data, it can be supported that smooth muscle dysfunction is the reason for the observed impairment in arterial vasodilation.

Keywords: Vascular Function, Age, Impaired Vasodilation, Endothelial Dysfunction, Ischemia, Arteriogenesis, Mouse, SDF Imaging, Gracilis, Peripheral Artery Disease

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“Not laughing for seven days makes one weak”

Mort Walker

TABLE OF CONTENTS

	Page
LIST OF TABLES	x
LIST OF FIGURES	xi
INTRODUCTION	1
CIRCULATORY FUNCTION	1
Circulatory Dysfunction	5
PERIPHERAL ARTERY DISEASE	6
Treatment of PAD	8
MECHANISMS OF VASCULAR GROWTH	10
Angiogenesis	11
Arteriogenesis	12
MODELS OF ISCHEMIA	17
Mouse and Rat Models	18
Rabbit and Large Animal Models	19
MECHANISMS OF VASCULAR FUNCTION	20
Vasoconstriction	21
Vasodilation	21
VASCULAR DYSFUNCTION	22
Effects of Age	23

THERAPEUTIC METHODS OF INCREASING VASCULAR FUNCTION	24
OBJECTIVES AND HYPOTHESIS	25
METHODS	27
HUSBANDRY	27
FEMORAL ARTERY RESECTION PROTOCOL	27
INTRAVITAL MICROSCOPY WITH FUNCTIONAL VASODILATION PROTOCOL	31
INTRAVITAL MICROSCOPY WITH SUPERFUSION PROTOCOL	33
DRUG MECHANISMS OF ACTION	35
Norepinephrine	35
Acetylcholine	37
Sodium Nitroprusside	39
SUPERFUSION PLUS FUNCTIONAL VASODILATION PROTOCOL	40
IMAGING AND STATISTICAL ANALYSIS	40
RESULTS	42
EFFECTS OF AGE & ISCHEMIA ON FUNCTIONAL VASODILATION	42
Impact of Ischemia	44
Impact of Age	46
Impact of Age and Ischemia	48
SUPERFUSED FUNCTIONAL VASODILATION PROTOCOL	52

SUPERFUSED VASOACTIVE AGENTS PROTOCOL	55
EFFECTS OF ISCHEMIA ON ENDOTHELIAL AND SMOOTH MUSCLE DEPENDENT REACTIVITY	58
DISCUSSION	61
RESTATEMENT OF EXPERIMENTAL OBJECTIVES	61
AIM 1: IMPACT OF AGE ON FUNCTIONAL VASODILATION	61
Aim 1: Impact of Age on Functional Vasodilation	62
Aim 1: Impact of Ischemia and Age on Functional Vasodilation	62
Aim 1: Future Recommendations	65
AIM 2: DEVELOPMENT OF FUNCTIONAL VASODILATION PROTOCOL FOR PHARMACOLOGICAL DELIVERY	67
Aim 2: Completed Modifications	68
Aim 2: Future Recommended Modifications	70
AIMS 3 AND 4: IMPACT OF ISCHEMIA ON CELL DEPENDENT DILATION AND CONSTRICTION	71
Aim 4: Possible Smooth Muscle Dysfunction	72
Differences between Superfusion and Functional Vasodilation	73
FINAL CONCLUSIONS	74
APPENDIX A	85
RESECTION PROTOCOL	85

APPENDIX B	86
APPENDIX C	88
APPENDIX D	90
SUPERFUSED FUNCTIONAL VASODILATION PROTOCOL	90
APPENDIX E	92
SDF IMAGING ANALYSIS USING AVA SOFTWARE	92

LIST OF TABLES

	Page
Table 1: Ischemia Data Table (Young Mice)	45
Table 2: Age Data Table (Sham Limb).....	47
Table 3: Age and Ischemia Data Table (Adult Mice).....	49
Table 4: Age and Ischemia Data Table (Young and Adult Mice)	50
Table 5: Superfused Functional Vasodilation Data Table.	53
Table 6: Superfused Functional Vasodilation Data Analysis Table	54
Table 7: NE Dose Response Curve.....	56

LIST OF FIGURES

	Page
Figure 1: Circulatory System Anatomy	1
Figure 2: Vessel Architecture and Function.	2
Figure 3: Blood Vessel Anatomy.....	3
Figure 4: Vasoconstriction and Vasodilation.....	5
Figure 5: Peripheral Artery Disease (PAD)	6
Figure 6: Angioplasty.	9
Figure 7: Angiogenesis	12
Figure 8: Effects of Shear Stress on Arteriogenesis	13
Figure 9: Relation between Collaterogenesis and Atherogenesis.....	15
Figure 10: Surgical Setup.....	28
Figure 11: Resected Region	29
Figure 12: Suture Timeline	30
Figure 13: Electrode Placement.	32
Figure 14: Superfusion Circuit.....	34
Figure 15: Neuroepinephrine Pathway	36
Figure 16: Acetylcholine Pathway	38
Figure 17: Sodium Nitroprusside Pathway	39
Figure 18: Example of Profunda Femoris Measurements	44
Figure 19: Young Ischemic vs. Young Sham	44
Figure 20: Young Ischemic vs. Young Sham % Change.....	45
Figure 21: Adult Sham vs. Young Sham	46

Figure 22: Adult Sham vs. Young Sham % Change.....	47
Figure 23: Adult Ischemic vs. Adult Sham.....	49
Figure 24: Adult Ischemic vs. Adult Sham % Change	50
Figure 25: Adult Ischemic vs. Young Ischemic.....	51
Figure 26: Adult Ischemic vs. Young Ischemic % Change	51
Figure 27: Superfused Functional Vasodilation: Right vs. Left Comparison.....	53
Figure 28: Superfused Functional Vasodilation: Right vs. Left % Change Comparison .	54
Figure 29: Dose Response Curve to Neuroepinephrine.....	55
Figure 30: Superfusion: Right vs. Left	57
Figure 31: Superfusion: Right vs. Left % Change.....	57
Figure 32: Superfusion Dynamic Range.....	58
Figure 33: Ischemic vs. Sham Superfusion Response	59
Figure 34: Ischemic vs. Sham % Change	60
Figure 35: Dynamic Range	60
Figure 36: Poor Vessel Images	74
Figure 37: Resection Protocol.....	85
Figure 38: Functional Vasodilation Protocol (Page 1)	86
Figure 39: Functional Vasodilation Protocol (Page 2)	87
Figure 40: Superfusion Protocol (Page 1).....	88
Figure 41: Superfusion Protocol (Page 2).....	89
Figure 42: Superfused Functional Vasodilation Protocol (Page 1)	90
Figure 43: Superfused Functional Vasodilation Protocol (Page 2)	91
Figure 44: AVA	93

Figure 45: Image Analysis	94
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INTRODUCTION

CIRCULATORY FUNCTION

The circulatory system is composed of the heart, which pressurizes the blood, and a complex network of blood vessels which deliver nutrients and remove wastes from tissues through the continuous circulation of blood (**Figure 1**) [1, 2]. Blood flow begins in the heart and is pumped through the body by muscular constriction of the left ventricle. From the heart, blood enters elastic conduits called arteries (**Figure 2**). From there, arteries stem smaller conduits called arterioles and further down the line these feed into capillaries. The venous circulation, venuoles and veins, return blood back to the heart [1].

Figure 1: Circulatory System Anatomy. Diagram showing the heart, arteries, and veins of the circulatory system.

Figure 2: Vessel Architecture and Function. Diagram showing the direction of flow from the heart through the various blood vessels, back to the heart. Adapted from [3].

Vessel function is determined by tissue structure (**Figure 3**). Arteries have a large amount of elastic tissue which allows them to absorb the large pressure differentials that the beating heart creates [1]. The arterioles are responsible for flow control to the distal tissues, which is enabled by the large amount of smooth muscle in its medial layer [1]. Nutrient exchange is facilitated in capillaries due to their single layer of intimal endothelial cells [1].

Figure 3: Blood Vessel Anatomy. Diagram shows the various tissue layers in an artery, vein and capillary. Adapted from [1].

The volume of blood in the circulatory system is relatively constant, however the distribution of this volume in different regions of the body is highly variable and can be altered by factors such as the output of the left ventricle and the contractile state of the resistance vessels of these regions [2]. With increased sympathetic activity, blood flow to the body increases due to an increased heart rate [1]. It also causes rapid firing of sympathetic fibers which innervate the vascular system, causing blood vessels to constrict and increase peripheral resistance [1].

In the regions with increased metabolic demand, which causes an increase in $p\text{CO}_2$, a higher exchange rate is needed due to nutrient consumption and waste formation rate [1]. For example, during exercise, muscles require more blood flow to match the increase in energy expenditure. This flow control is accomplished by the arterioles, located immediately upstream of the capillaries, which increase the lumen diameter by relaxation of the surrounding smooth muscle [1]. In metabolically high areas of the body, the density of these vessels is greater to allow for more nutrient and waste exchange [2].

The spontaneous, rhythmic contraction of blood vessels, specifically arterioles and feed arteries, is involved with regulating blood flow throughout the body (**Figure 4**) [2]. Vasoactivity serves either of two physiological purposes: regulation of systemic blood pressure or redirecting blood flow to different regions of the body [4]. This happens through vasoconstriction and vasodilation; which decreases and increases blood perfusion to distal tissues respectively [4]. One example of the profound effect vasoactivity can have on blood flow is the observation that aerobic exercise can increase perfusion nearly 100 times when compared to rest [5]. When working properly, different tissues can control the perfusion of their tissue through autoregulation. Autoregulation is the ability of tissues to adjust blood flow with regards to the levels of tissue metabolism, and vessel pressure [2, 4]. One of the main interests in this area of research is the future ability to control vascular function, in regions of dysfunction, through the use of drugs.

Figure 4: Vasoconstriction and Vasodilation. Vasodilation is an increase in luminal diameter and vasoconstriction is a decrease in luminal diameter. Adapted from [6].

Circulatory Dysfunction

Blood flow supply to tissues must match metabolic demand to maintain tissue function, otherwise a medical pathology occurs. For example, if resistance vessel tone is not maintained, increased capillary hydrostatic pressure will cause blood filtration and edema¹ can occur [2]. If the blood flow is reduced below the proper range, it causes a decrease in blood supply to a distal tissue and it becomes ischemic (**Figure 5**) [1]. Ischemia is insufficient tissue blood flow and results in hypoxia and poor nutrient delivery to cells [7]. In healthy tissue, vasodilation appears immediately after the beginning of hypoxia and can be maintained for several hours [7]. Unfortunately, the vasodilation process is impaired in patients with Peripheral Artery Disease (PAD), and the treatment of this dysfunction is the motivation for this research [8].

¹ Edema is a condition in which tissue becomes swollen and puffy due to increased fluid concentrations [1]

Figure 5: Peripheral Artery Disease (PAD). Peripheral artery disease is a progression of atherosclerosis in the distal limbs. It is characterized by a narrowing of the lumen of an artery. Adapted from [9].

PERIPHERAL ARTERY DISEASE

Ischemic vascular disease is the leading cause of morbidity and mortality in the developed world [10]. In the United States alone, PAD affects 8 million adults, a number that will more than likely get larger as the population ages [11]. Many factors may lead to

this disease, including hypertension, smoking, diabetes mellitus and hypercholesteremia [12]. At the cellular level, inflammation and endothelial dysfunction are contributing factors to this disease [12-14], and there is general agreement that atherosclerosis begins with a damaged or dysfunctional endothelium [2, 13, 15]. Endothelial dysfunction during atherogenesis is characterized by increased expression of adhesion molecules and an increased synthesis of inflammatory and pro-thrombotic factors [13]. Adhesion molecules enhance the attachment of monocytes, T lymphocytes, and platelets to the endothelium [2]. Once macrophages extravasate into the artery wall, they begin modifying the structure of the surrounding smooth muscle cells [2]. Later in the process, cholesterol begins to build-up in the sub-endothelial space [14].

As this process progresses, the diameter of the lumen of the vessels becomes smaller and smaller, known as stenosis. One of the symptoms of this disease is intermittent claudication, or ischemic leg pain when the patient walks or climbs stairs [2]. With minimal walking, in patients with PAD, the resistance vessels lose the ability to become maximally dilated. When the oxygen demand increases with more rapid walking or climbing, blood flow cannot increase sufficiently to meet the muscle needs for oxygen, and pain caused by muscle ischemia results [2]. It is also thought that the ischemia–reperfusion injury associated with intermittent claudication could be among the causes for increased inflammatory activity and endothelial dysfunction in PAD patients [16]. One percent to two percent of PAD patients over the age of 50 develop critical limb ischemia (CLI) which begins with pain of the limb at rest and leads to tissue ulcers, necrosis and eventually amputation [17]. The one year mortality rate of patients with CLI is 20%, and at five years this rate increases to 50% [15]. Clearly, there is a need for

treatment of this disease and the goal of this research is to address the problem of impaired vasodilation in these patients.

Treatment of PAD

The goal of contemporary treatments of PAD is to reverse conduit artery stenosis and restore tissue blood flow through percutaneous angioplasty or by-pass surgery (**Figure 6**) [18]. Endovascular angioplasty, the process of using a balloon to open up occluded arteries in the peripheral vascular system, has been shown to increase flow-mediated dilation [16]. Another avenue besides surgical treatments is drug therapy. In addition to these surgical approaches, endogenous compensation of arterial stenosis during PAD can occur through the development of collateral flow paths [18]. Therapeutic stimulation of this process has received substantial attention in patients for which surgical approaches are ineffective or contraindicated [18]. In order to properly stimulate this process, the cellular mechanisms must be fully understood.

Figure 6: Angioplasty. Vascular angioplasty involves the insertion of a balloon catheter into the stenosed area. The balloon is then inflated to increase the luminal diameter, increasing perfusion of downstream tissue. Adapted From [20].

There have been many advances in the understanding of the molecular and cellular mechanisms responsible for collateral development [19]. Some of these molecules include VEGF, FGF-2, FGF-1 and MCP-1, which have been shown to enhance collateralogenesis [19]. Despite our improved understanding of arteriogenesis and significant success of preclinical trials, therapeutic vascular growth has not been successful in a clinical setting [18, 19]. It is unknown why arteriogenesis therapies have not worked, but it could be due to the competing mechanisms of atherosclerosis and arteriogenesis [19]. Regardless, it is necessary to study the process more. However, collateral growth is a complex remodeling process, and it may be more straight-forward to improve vasodilation in these patients.

MECHANISMS OF VASCULAR GROWTH

Following an occlusive event, the peripheral circulation remodels in response to the altered flow and pressure through vasculogenesis, angiogenesis, and arteriogenesis [21]. Vasculogenesis occurs primarily during embryonic development when angioblasts differentiate to form primitive capillary plexuses [21], but has been observed in response to hindlimb ischemia [22]. The peripheral circulation remodels in response to altered flow and pressure through arteriogenesis in response to cellular hypoxia through angiogenesis, these processes form collaterals and an expanded exchange network, respectively [1, 2].

Angiogenesis

Angiogenesis is the growth of new capillaries from existing capillaries. Although angiogenesis can be induced by increased shear stress [23], in the context of ischemia, angiogenesis is primarily induced by hypoxia (**Figure 7**) [18]. Hypoxia increases the activity of hypoxia inducible factor-1 (HIF-1), a transcription factor that increases the expression of hypoxia-sensitive genes, such as VEGF, which is the most well-studied angiogenic factor in the context of both tumor growth and ischemic revascularization [18]. Various growth factors, such as VEGF, promote the division of endothelial cells, which organize into a hollow tube that sprouts out of the existing capillary. The resulting sprouting capillary reconnects to its original capillary branch or a different one, completing the angiogenic process [2]. Increasing capillary number will improve diffusive transport in the ischemic tissue. However, arteriogenesis affects larger vessels, which are more relevant to this work.

Figure 7: Angiogenesis. Diagram showing the growth of capillaries through angiogenesis. Note the growth of capillaries from preexisting capillaries.

Arteriogenesis

Arteriogenesis refers to the transformation of pre-existing collateral artery pathways into conduit arteries (**Figure 8**) [24]. The process begins when a sudden arterial occlusion or a slowly progressing stenosis causes an increase in fluid shear forces on the endothelium of the collateral flow path [21]. Normal shear values in arterial circulation ranges between 10 and 70 dyn cm⁻² [18]. When the range is breached, the elevated shear activates the endothelial cell signaling pathways and results in increased transcription of nitric oxide synthase (NOS), platelet-derived growth factor (PDGF) and monocyte chemoattractant protein-1 (MCP-1) [21]. The activity of NOS is also increased and its activity accounts for the vasodilation observed in response to increased endothelial shear [7]. The elevated shear also causes adhesion molecule presentation on the luminal surface

of the endothelium. Adhesion molecules, such as ICAM are necessary to “grab” circulating monocytes, which adhere in response to MCP-1 [21]. Monocyte adherence potentiates endothelial activation, which causes tumor necrosis factor- α (TNF- α) release, further enhancing monocyte adhesion to the endothelium [21]. At the same time, platelets adhere to the endothelium and produce interleukin-4 (IL-4), which amplifies the expression of adhesion molecules [21].

Figure 8: Effects of Shear Stress on Arteriogenesis. The effects of a reperfusion study on a rabbit hindlimb. (A) Reperfusion treatment (B) ligation treatment. Image was a post mortem angiography. Adapted from [26].

Once they extravasate, monocytes produce many growth factors, such as fibroblast growth factor-2 (FGF-2), which cause mitosis of the endothelial and smooth muscle cells [21]. Mast cells, which are converted from basophils, secrete basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) and may remain in the tissue for up to 6 weeks [25]. Finally, matrix-metalloproteinases begin to digest the extracellular matrix and provide the space for new cells and enable smooth muscle cells

to migrate towards the intima [25]. This generates an inflammatory response that attracts T cells which causes smooth muscle cells to undergo apoptosis, creating space for the increasing collateral vessel [21, 25]. Those smooth muscle cells that proliferate change their phenotype and lose most of their contractile function [25] when they become “synthetic”, or proliferative [21]. Once the vessel diameter has increased enough to return shear stress back to the baseline levels, arteriogenesis will stop. This process increases flow to the downstream tissue until the endothelial cells sense normal shear stress ranges.

There are several methods of increasing growth of collateral arteries. One of the first substances found to increase arteriogenesis was MCP-1 [27]. Unfortunately this compound also was shown to be pro-atherogenic, precluding its testing beyond preclinical trials (**Figure 9**) [18, 19].

Figure 9: Relation between Collateralogenesis and Atherogenesis. This diagram shows pathways that are common to atherogenesis and collateralogenesis. Adapted from [19].

Monocytes recruitment can also be improved by granulocyte-monocyte colony stimulating factors (GM-CSF) and granulocyte colony stimulator factor (G-CSF) [18],

which along with transforming growth factor beta (TGF- β), enhance arteriogenesis [24]. Unfortunately, both of these compounds promote tumor angiogenesis and atherosclerosis [18].

VEGF expression is critical for the presence of collaterals and collateral growth, likely through stimulation of endothelial proliferation. Unfortunately, the positive effect of VEGF on vascularization in animal models [10, 28] has not been observed in large clinical trials [29]. In addition to a lack of efficacy, VEGF therapies are associated with edema, due to the ability of VEGF to increase endothelial permeability [19, 24].

To diminish these risks of side effects, many efforts have been made to deliver the arteriogenic drugs locally [24], rather than intravascularly [18, 30]. Current research predicts future methods to deliver these drugs through therapeutic devices such as stents. These stents would elute a pro-arteriogenic compound into the affected artery over a period of time, mitigating current delivery complications [18, 24]. Additionally, the risk factors that cause PAD such as age, hypercholesterolemia, genetic susceptibility, diabetes and smoking may also impair the efficacy of these therapeutic interventions [31]. Due to the complexity of the arteriogenesis pathway, multiple therapeutic agents or cell-based therapies may be needed to achieve efficacy [18, 31].

Cell based therapies refer to the process of isolating autologous cells and delivering them to the patient [32]. Their usefulness, however, has been debated because the process of growing collateral arteries is a local and self-sufficient process that is not dependent on the circulating stem cells [25]. However, with dysfunction the arteriogenesis pathway is broken, so delivering cells that can secrete many cytokines

could be an effective modality to stimulate the process. Bone marrow derived progenitor cells improve arteriogenesis, but their clinical efficacy remains inconclusive [18, 19].

MODELS OF ISCHEMIA

The main challenge of therapeutic arteriogenesis is there has been a history of therapeutic efficacy in animal models followed by disappointing results in blinded and randomized clinical trials [18, 31]. While there is no perfect model of PAD, many efforts have been made to mimic the symptoms and pathologies of PAD to provide a better idea of efficacy of treatments further down the line. [25]. Often these procedures involve surgery, which takes a matter of minutes to hours; the time it takes for a human to develop an atherosclerotic lesion is years to decades [33]. These inherent differences require a better understanding of the animal models that are currently used. Fully understanding these differences will help lead to more reasonable interpretations of the results.

Experimental models of PAD can be divided into human and animal models. Human models are limited from the perspective that invasive studies cannot be performed and long-term studies are difficult. This is due to increased regulation enforced by the Food and Drug Administration (FDA), specifically through the Office of Good Clinical Practice (OGCP). Even with these difficulties human models are utilized. In one human study, arterioles were isolated from amputated limbs for highly controlled studies of vascular reactivity [34]. Conversely, non-invasive assessments, such as laser Doppler perfusion imaging, can be performed routinely in patients with PAD [15].

Comparatively, animal models can be used for long-term invasive studies.

Unfortunately, variability can be introduced through the numerous species and strains of animals that can be used. Different types of surgical methods used to induce ischemia also increase this variability. There are several experimental assessment endpoints that must be addressed to properly analyze the efficacy of a therapy, such as vascular cell proliferation, vascular number and size, and blood flow perfusion [35]. Many studies aim to look at all of these factors; however there is no standard method to address these experimental endpoints. This creates a need to understand the model being used in order to develop experiments that properly evaluate the problem or efficacy of the solution.

Mouse and Rat Models

One of the most variable and simple of the animal models are rodents.

Advantages of these animals are their low cost, high reproductive rate, and general similarity to human physiology. Historically, rats were the most common, but with the emergence of mouse embryonic cell culture and genetic modification, mice have become a more popular choice for experiments designed to establish causal relationships [36].

Through genetic manipulation, different co-morbidities can be induced, such as hypercholesterolemia following the disruption of the genes involved in cholesterol trafficking[37].

For ischemic research, two different surgical techniques are frequently used: ligation and resection. During a ligation, the femoral artery is occluded with a suture, preventing downstream flow [38]. The result of this occlusion is the enlargement of superficial collateral arteries, which can be viewed *in vivo* one to three weeks after

femoral occlusion [38]. A resection surgery involves the removal of the femoral artery and all of its branches from the hindlimb [39, 40]. Excision of the femoral artery results in occlusion of the external iliac artery; blood flow to the ischemic limb is consequently dependent on deep collateral vessels originating from the internal iliac artery [39, 40].

Often, a larger rodent model is used, such as a rat, which allows a greater range of experimental options with regards to surgical methods [41]. There are still inherent differences between the physiology of a human and a rodent. One example is the difference between blood type proteins of a mouse and a human [42]. This can be seen on the cellular level in embryonic stem cells [42]. This may explain why mice and rats have not been predictive of a therapeutic effect on humans. To better match the diseased human pathology and anatomy, a large animal model is considered.

Rabbit and Large Animal Models

Rabbit models are used for a variety of FDA and international biocompatibility testing standards [43], chiefly in vascular research because their iliac artery diameter is comparable to that of the human coronary artery and has similar thrombogenic properties [43]. Other than the previously stated advantages, rabbits do not provide many more advantages when compared to rats and larger animal models. However, they are used frequently in ischemia research.

The most common ischemia models involve ligations and arteriovenous shunts, which can be used to assess revascularizations in the rabbit hindlimb [28]. Arteriovenous shunts involve the ligation of both femoral arteries and for the side-to-side anastomosis operation of the distal stump of the left femoral artery with the accompanying vein [26].

This surgery re-routes vasculature and causes arterial vessels to drain into veins, which have a lower hydrostatic pressure than downstream arterioles and hence causes an increased pressure gradient and increased flow, resulting in an increased shear stress [26]. Similar to rodent studies, rabbits have predicted safety and positive responses to biologic therapeutics [44].

When even larger models are needed, the most commonly used in ischemia research are the porcine and canine model. Canines are often used because they are easy to train and their circulation contains many collaterals for arteriogenesis research [35, 45]. The porcine model is advantageous because they can be given a high calorie diet and gain similar pathologies to humans with PAD [43]. Animal species, surgical technique, treatment and size must all be analyzed in order to improve their predictive capacity in humans. Additionally, the differences in animal models can lead to differences in responses when comparing across animal models. Thus inferring animal response to humans needs to be done with caution and with careful consideration of the potential differences. With a history of efficacy of therapies in animal models and inadequate results seen in randomized patient trials, a different approach is needed if we want to improve distal perfusion in those patients that are suffering from impairment [18, 31]. Therefore a better understanding of vascular function is needed.

MECHANISMS OF VASCULAR FUNCTION

Collateral growth is a complex process and more effective results could be observed and complement or potentially replace therapeutic arteriogenesis by determining how to improve vasodilation. As stated previously in the introduction, blood

flow is controlled by the increase and decrease of lumen diameter in the resistance vasculature, arterioles, and feed arteries [1].

Vasoconstriction

Vasoconstriction involves the constriction of smooth muscle in a blood vessel to decrease the luminal diameter and decrease blood velocity and distal perfusion [4]. Normally, vessel tone is maintained through constant activity from the sympathetic nervous system [2]. Control of the vessel diameter can be divided into three separate categories: local, neural, and hormonal mechanisms [4]. One of the most recognizable ways of local vasoconstriction is in response to a cold stimulus which is a method of preserving core body temperature [2]. Stimulation of this control pathway causes various systemic effects, one of which is most relevant to this research, the increasing in peripheral resistance through the use of neuroepinephrine [2]. Vessel pressure also has an effect on vessel tone due to the myogenic response; vessels constrict in response to high pressure [2]. There are several hormones that cause vasoconstriction, such as Angiotensin II, which is a potent vasoconstrictor and a systemic way that blood pressure is regulated [4]. Just as blood pressure can be increased through vasoconstriction, it can be lowered through vasodilation.

Vasodilation

Vasodilation involves the relaxation of smooth muscle in a blood vessel to increase the luminal diameter and increase blood velocity and distal perfusion [4]. Local

factors of vasodilation include substances that are released from the vessel endothelium such as prostacyclin (PGI₂) and nitric oxide (NO) [2]. Hormonal mechanisms of vasodilation include atrial natriuretic peptide which causes vasodilation in the cardiac vasculature [4]. Vasodilation in skeletal muscle is known to be regulated by muscle metabolism, but to effectively increase flow, there must be coordination with vessels outside of the metabolic field, such as feed arteries. This occurs through a mechanism known as ascending vasodilation [46].

Ascending vasodilation is the process of the “spread” of a vasomotor response along a blood vessel [5]. Research has demonstrated that this is due to electrical signals that are capable of traveling for millimeters along the arteriolar endothelium [5, 47]. Ischemia may impair this process due to the resulting vascular dysfunction [48].

VASCULAR DYSFUNCTION

A healthy resistance vessel should dilate in response to ischemia. When this mechanism is dysfunctional, the downstream tissue becomes transiently ischemic [2], producing intermittent claudication. It is also thought that the injury associated with intermittent claudication could be among the causes for increased inflammatory activity and endothelial dysfunction in PAD patients [16]. One of the most common causes of endothelial dysfunction and inflammation is diabetes mellitus [37, 49, 50].

Endothelial dysfunction and inflammation in diabetes is due to hyperglycemia and it causes a decreased endothelial dependent relaxation due to increased thromboxane receptors, enhancing vasoconstriction [49]. Similarly, endothelial dysfunction observed in patients with PAD could be due to impaired endothelial vasodilator responses [11].

In patients with chronic limb ischemia, feed arteries from ischemic skeletal muscle showed exaggerated vasoconstrictor responses [51]. This was thought to be due to an increase in both α -1 and α -2 adrenergic receptors in the ischemic tissue [51]. The goal of this research is to better understand this mechanism to create efficacious treatments for impaired α -adrenergic receptor responsiveness

Effects of Age

Age is one of the greatest risk factors for developing chronic ischemia [52]. Also, there are greater numbers of older people in hospitals when compared to younger people [53]. The effects of age on the human body are complex and include such as increased blood pressure (hypertension) and impaired wound healing [4, 17]. Advanced age also reduces muscle mass and increases vascular resistance [54]. Peripheral resistance is directly tied to the material properties and the structural presence of blood vessels, which changes with hypertension of blood vessels. As people get older, arteries lose their elasticity and become stiffer with atherosclerosis and other pathologies [4]. Sympathetic nerve activity also increases with age, causing vessels to be more constricted [54]. These changes may explain the negative effect of age on arteriogenesis [31].

These vascular impairments are recapitulated in animal models, in which VEGF expression was reduced in the ischemic limbs of aged mice and rabbits compared to young animals [55]. Endothelial dysfunction could represent a putative basis for age-dependent impairments in angiogenesis [55]. Further, collateral numbers and endothelial-dependent vasodilation were reduced in adult mice (6mo of age) [37]. Senescent mice, aged 20 months exhibited reduced functional vasodilation, likely due to an increase in

concentration of α -adenoreceptors in the microvasculature of an aged mouse [54]. From this research, it is hypothesized that endothelial dysfunction is responsible for this decrease in vasodilation in an aged, ischemic mouse.

THERAPEUTIC METHODS OF INCREASING VASCULAR FUNCTION

One of the only therapies currently known to increase vascular function is exercise. Exercise therapy for patients with intermittent claudication increases pain-free walking time, maximal walking time and overall physical capacity [41]. PAD patients that are more physically active have increased flow-mediated dilation [11]. Unfortunately, many patients with PAD are restricted or prevented from participating in these supervised exercise programs due to limited ability or lack of medical insurance coverage [11]. Additionally, the molecular mechanism of this benefit is not understood, consequently its therapeutic efficacy cannot be maximized. Additionally, to fully understand how exercise benefits vasodilation, we must understand why vasodilation is impaired in the first place. Therefore there is an obligation to discover alternative methods of treatment such as drug and surgical intervention.

Some drug therapies to increase revascularization have been ineffective and have many counterproductive side effects [19]. It is imperative for these new vessels to have proper vascular function, unfortunately research has shown otherwise [5]. One possible solution to this problem is the mechanism of eNOS. It is thought that eNOS causes an increase in endothelial NO production which is a known vasodilator [2], and it has been shown to increase skeletal muscle perfusion in a rat model [56].

Despite our improved understanding of arteriogenesis and significant success of preclinical trials, therapeutic vascular growth has not been successful in a clinical setting [18, 19]. It is unknown why arteriogenesis therapies have not worked, but it could be due to the competing mechanisms of atherosclerosis and arteriogenesis [19]. Regardless, it is imperative that the mechanism is fully understood in order to properly address the problem. More effective results could be observed and complement therapeutic arteriogenesis by determining how to improve vasodilation in these patients. Vascular function must improve in an ischemic environment, and resolving this problem is the basis of this research.

OBJECTIVES AND HYPOTHESIS

The goal of this research is to better understand how ischemia affects vascular function. This knowledge will help develop more effective therapies in the patient population. To achieve this goal, the following Specific Aims were completed:

1. *Specific Aim 1 –Test the hypothesis that age will decrease the functional vasodilation during ischemia.* The objective was to validate previous data on 3mo old mice, and advancing the data on 6mo old mice. It was also the intention to validate results with a different group's research performed on mice at 3 and 6 months [37].
2. *Specific Aim 2 - Establish a consistent superfused functional vasodilation protocol using healthy mice.* The goal of this aim was to create a reproducible

protocol that allows studies to eventually use pharmacological agents during functional vasodilation to assess specific aspects of dysfunction.

3. *Establish an experimental protocol for assessing vascular reactivity using pharmacological agents.* The purpose of this aim was to explore the vascular reactivity to endothelial dependent and smooth muscle dependent vasodilators, as well as obtain a vascular range of motion with vasoconstrictors.
4. *Test the hypothesis that the ischemic profunda femoris artery will exhibit impaired endothelial-dependent vasodilation.* The purpose of this aim was to explore the vascular reactivity to endothelial dependent and smooth muscle dependent vasodilators in response to ischemia, as well as obtain a vascular range of motion with vasoconstrictors.

Completing these aims will help further the experimental options of the lab while discovering the potential mechanisms of dysfunction when ischemia is present.

METHODS

HUSBANDRY

All procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the California Polytechnic State University in accordance with the Guide for the Care and Use of Laboratory Animals. Male C57 mice aged 7-9 weeks were housed in the Cal Poly vivarium, with four mice to a micro-isolator cage. The room was temperature controlled with a 12 hour to 12 hour light-dark cycle. Harlan 18% protein rodent chow and water were provided *ad libitum*. During cage changes, isolation attire was worn to maintain aseptic conditions. Mice were monitored on a daily basis and any notes were taken if irregularities in behavior were noticed.

FEMORAL ARTERY RESECTION PROTOCOL

Surgeries were performed using a stereomicroscope on male C57 mice under sterile conditions using Isoflurane anesthesia (APPENDIX A). The hindlimb area was first shaved and removed of hair. Nolvasan was used to disinfect the surgical field and reduce the likelihood of infection. The mice were kept at a temperature of ~35 °C using a rectal-thermistor controlled heating pad. A sterile drape was placed over the mouse with an opening over the surgical area (**Figure 10**). **Figure 11** shows the approximate area of femoral artery and vein resected from the hindlimb. The first incision was made with a straight cut over the medial thigh of the mouse. The cut was extended proximally until the abdominal wall was visible. It was also extended distally to midway between the knee and ankle. Sterile saline solution was used to irrigate the incision and prevent tissue

desiccation. Connective tissue was blunt dissected over the femoral neurovascular bundle. Then, the epigastric fat pad and epigastric artery-vein pair were dissected using blunt dissection and heat cauterization.

Figure 10: Surgical Setup. Picture showing the surgical suite. The mouse is shown underneath the sterile drape. All surgical instruments and objects that touched the mouse during surgery were kept sterile to prevent infection.

Figure 11: Resected Region. (A) Hindlimb before resection. (B) Hindlimb with resected area denoted by a black line. The distal hindlimb area is not fully visible.

Once the fat pad was removed, the femoral artery and vein pair was separated from the nerve using gentle blunt dissection. The femoral artery and vein pair was then ligated proximal to the profunda femoris with a 6-0 silk suture. The profunda femoris was then cauterized as close to the femoral artery and vein pair as possible to minimize hemorrhage following femoral artery-vein resection. The femoral artery-vein pair was then ligated halfway between the knee and ankle. Veins branching off of the femoral

artery-vein pair were cauterized to leave some native circulation intact. Great care was taken by the surgeon to leave as many branches as possible. A cotton swab was used to apply counter-pressure on the tissue while the femoral artery vein pair was carefully pulled and separated from the underlying tissue and cut just distal to the proximal ligation. Finally, the skin incision was closed using 7.0 polypropylene suture, **Figure 12**.

Figure 12: Suture Timeline. (A) Hindlimb before incision is made (B) Hindlimb after resection surgery and suturing protocol (C) Hindlimb 14 days after surgery.

An incision, equivalent in length and placement, was made on the contralateral limb. Sterile saline was used to irrigate the incision and prevent tissue desiccation. Connective tissue over the femoral neurovascular bundle was blunt dissected similar to the previous limb. This was done to create a sham limb, designed to control for any effects of general surgical trauma. Finally, the skin incision was closed using 7.0 polypropylene suture. The mouse was then given a subcutaneous injection of buprenorphine (0.07 mg/kg) and placed in a warm recovery bin until ambulatory, then was returned to the vivarium.

INTRAVITAL MICROSCOPY WITH FUNCTIONAL VASODILATION PROTOCOL

SDF Imaging was used to evaluate profunda femoris artery diameter (APPENDIX B). Experimental preparation/dissections were performed using a stereomicroscope. The hindlimb area was first shaved and removed of hair and mice were maintained at ~35 °C using a rectal-thermistor controlled heating pad. The initial incision was made with a straight cut over the medial hindlimb of the mouse. The cut was extended proximally until the abdominal wall was visible. Phosphate Buffered Saline (PBS) solution was used to keep the incision irrigated and prevent tissue desiccation. Connective tissue was carefully dissected away from the leg, as to not harm the underlying muscle. The epigastric fat pad was then blunt dissected away from the abdominal wall, and cautery was used to bisect the fat pad into two parts. This was done to allow placement of the MicroScan over the profunda femoris artery.

Once the dissection was complete, the MicroScan was then placed over the profunda femoris artery and positioned such that the anterior gracilis muscle was visible at the bottom of the field of view. Two electrodes were then placed on the mouse hindlimb: the ground electrode on the skin of the mouse and stimulating electrode above the gracilis muscles (**Figure 13**). It was important to not puncture the muscle because this caused unwanted hemorrhage. To test proper placement, the gracilis muscle was stimulated with 1mA square waves 100 μ s in duration at 1 Hz until proper electrode placement was confirmed. If the hindlimb twitched and was visible on the video image, proper electrode placement was confirmed. At this point a KimWipe was used to remove the remaining PBS solution from the hindlimb. Mineral oil was then placed on the hindlimb using a pipette and covered with plastic wrap.

Figure 13: Electrode Placement. Areas of interest are labeled. Note the placement of the electrodes: on the gracilis muscle and on the skin. A retractor was used to fully show the entire hindlimb area.

The mineral oil prevents the tissue from desiccating and creates an effective barrier to prevent oxygen diffusion [57]. High pO_2 may cause vasoconstriction and alter basal tone as well as demand for oxygen. Therefore, to keep a controlled system, an environment free from oxygen diffusion is very important.

The experimental preparation was allowed to recover for 30 minutes. Following this recovery period, a 10 second video baseline measurement was taken. The gracilis muscle was then stimulated with 1mA square waves 200 μ s in duration at 8 Hz for 90 second. 10 second videos were taken before stimulation, after stimulation, and every two minutes afterwards until the resting diameter was reached, $\pm 5\mu$ m. The gracilis muscle was then stimulated with 1mA square waves 500 μ s in duration at 8 Hz for 90 seconds. Again, 10 second videos were taken before stimulation, after stimulation, and every two minutes afterwards until the baseline diameter was measured. After both stimulation

protocols were complete, the hindlimb was covered with plastic wrap and the process was repeated on the contralateral limb; the order of measurement was randomized between the two limbs. This was done to prevent experimental error that was found when limb order was not randomized [58]. After the protocol was completed on both limbs, the mouse was euthanized by cervical dislocation. Sacrificed mice were stored in a -20°C freezer and disposed in a biohazard waste container.

INTRAVITAL MICROSCOPY WITH SUPERFUSION PROTOCOL

In order to test the effects of pharmacological agents on the current model, a method to apply them to the preparation without the use of mineral oil was needed. Therefore a superfusion protocol was proposed (APPENDIX C). The superfusion solution was a physiological salt solution (PSS) composed of the following compounds (in mM): NaCl 137, KCl 4.7, MgSO₄ 1.2, CaCl₂ 2, and NaHCO₃ 18. Prior to each experiment, the PSS was warmed in a water bath at 45°C and deoxygenated/pH-balanced by bubbling with a 95%:5% N₂:CO₂ mixture for approximately 15 minutes. After the solution was prepared, a portion of the liquid was poured into the superfusion circuit (**Figure 14**).

Figure 14: Superfusion Circuit. Diagrams of the circuit used to apply superfusion solution on the hindlimb of the mouse. (A) Basic block diagram of the circuit (B) Image of the actual circuit used in experiments.

SDF Imaging was again used to evaluate the diameter of the profunda femoris artery, and the animal was prepared as described above. Once the dissection was complete, the MicroScan was then placed over the profunda femoris artery and positioned with the gracilis muscle at the bottom of the field-of-view. Superfusion solution was adjusted to flow at $\sim 2\text{mL}\cdot\text{min}^{-1}$ and a thermistor was used to ensure that a temperature of 35°C was maintained.

The limb was then allowed to recover for 30 minutes from the dissection. Following this recovery period, a 10 second video baseline measurement was taken

before the addition of pharmacological agents in the following order: neuroepinephrine (10^{-4}M), acetylcholine (10^{-4}M), and sodium nitroprusside (SNP) (10^{-4}M). Agents were allowed to flow over the experimental preparation for 5 minutes; 10 second videos were taken within the last minute. After the protocol was complete, the hindlimb was covered with plastic wrap and the process was repeated on the contralateral limb. The order of experimentation was alternated between the right and left hindlimbs for each mouse [58]. After the protocol was completed on both limbs, the mouse was euthanized by cervical dislocation. Sacrificed mice were stored in a -20°C freezer and disposed of in a biohazard waste container.

DRUG MECHANISMS OF ACTION

Norepinephrine

Norepinephrine is one of the most important regulators of vasoconstriction in skeletal muscle resistance vessels [1]. Norepinephrine is a catecholamine, which is a class of amines that act as chemical transmitters [1], other examples include epinephrine and dopamine [1]. In a stressful situation, neuroepinephrine is synthesized from tyrosine and is secreted from sympathetic neurons that synapse on the smooth muscle [1]. Smooth muscle cells then have different receptors that receive the neurotransmitter. Specifically α_1 -adenoreceptors and α_2 -adenoreceptor and β -adenoreceptors [2], arterial vasoconstriction is predominantly mediated by α_1 -adenoreceptors with a small contribution from α_2 -adenoreceptors [34]. Previous studies have shown an increase in

certain receptors due to ischemia, thus the motivation for using this drug in the research [34]. **Figure 15** shows the cellular mechanism of this endogenous neurotransmitter.

Figure 15: Neuroepinephrine Pathway. Concept map showing the cellular mechanism of neuroepinephrine. It binds to specific receptors and results in smooth muscle contraction, or in the case of blood vessels, vasoconstriction.

Acetylcholine

Acetylcholine is a neurotransmitter that is released by nerve endings, specifically the cholinergic fibers (**Figure 16**) [1]. Acetylcholine receptors are found on the endothelial cell and when activated result in vasodilation [59, 60]. This compound is typically used in order to assess endothelial dysfunction in vasodilation due to its ability to produce a robust dilation that is endothelial dependent, which was the main motivation in using this drug in the protocol [55]. However, there has been some debate regarding this due to a group that showed that vasodilatory chemicals may act differently than the natural pathway [61].

Figure 16: Acetylcholine Pathway. Concept map showing the cellular mechanism of acetylcholine. The process begins with acetylcholine binding to a cholinergic receptor and results in vessel vasodilation. This is an endothelial dependent vasodilator.

Sodium Nitroprusside

This mechanism is very similar to acetylcholine; only it completely skips the endothelial process and goes directly to giving rise to stimulating sGC (**Figure 17**). NO donors are typically used to assess smooth muscle functionality due to its mechanism, which was the main motivation for using this drug in the study [55]. Nitrates are also used as treatments for angina and cardiac ischemia in order to achieve greater blood flow in the heart [62].

Figure 17: Sodium Nitroprusside Pathway. Concept map showing the cellular mechanism of sodium nitroprusside. It begins with the donation of nitric oxide to the smooth muscle cell, which results in vasodilation of the vessel. This is a smooth muscle dependent vasodilator.

SUPERFUSION PLUS FUNCTIONAL VASODILATION PROTOCOL

Superfusion intravital microscopy was performed as described above, with the following modification (APPENDIX D). Once the MicroScan was placed over the profunda femoris artery, a ground electrode and placed on the skin and a stimulating electrode was placed above the gracilis muscles. Proper placement was confirmed with test contractions and the superfusion solution was adjusted to $\sim 2\text{mL}\cdot\text{min}^{-1}$.

After the 30-minute recovery period, the gracilis muscle was stimulated with 1mA square waves for 200 μs or 500 μs in duration at 8 Hz for 90 seconds. Ten-second videos were taken before stimulation, after stimulation, and every two minutes afterwards until baseline diameter was measured. After both stimulation protocols were complete, the hindlimb was covered with plastic wrap and the process was repeated on the contralateral limb. The order of experimentation was alternated between the right and left hindlimbs for each mouse [58]. After the protocol was completed on both limbs, the mouse was euthanized by cervical dislocation. Sacrificed mice were stored in a -20°C freezer and disposed of in a biohazard waste container.

IMAGING AND STATISTICAL ANALYSIS

Automated Vascular Analysis (AVA) was used to measure diameters from stabilized videos (APPENDIX E). Data was analyzed using the statistics package in Microsoft Excel. Paired t-tests were used for data sets that showed a linear association which was indicative of dependence between the two sets of data. This was true for all data sets in the first Aim except for when comparing between adult and young mice, in

which a one-way ANOVA was used. In Aims three and four, groups were compared by using a one-way ANOVA. Summary data are represented as mean values $\pm$ standard error.

RESULTS

EFFECTS OF AGE & ISCHEMIA ON FUNCTIONAL VASODILATION

For Specific Aim 1 (Page 26), the hypothesis that age and ischemia reduce functional vasodilation in the profunda femoris feed artery was tested. It was hypothesized that the effects of these treatments would be additive, which was tested by comparing age and ischemia individually against a control. Arteries were measured as stated in the methods section (**Figure 18**).

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Impact of Ischemia

In young mice (2-3mo), the resting ischemic artery was significantly larger than the control artery, $103.1 \pm 10.3 \mu\text{m}$ vs. $54.1 \pm 3.6 \mu\text{m}$, $p = 0.0006$ ($n=8$), (**Figure 19**). However, arterial diameter was not different between ischemic and control arteries following functional vasodilation (**Table 1**). Therefore, percent change in diameter compared to resting state was examined as the more accurate indicator of vascular reactivity (**Figure 20**). When the gracilis muscles were stimulated with 200 μs duration square waves, control arteries dilated significantly more than ischemic arteries, $104.1 \pm 13.0\%$ vs. $15.2 \pm 5.0\%$, $p < 0.001$, ($n=8$) (**Figure 20**). When the gracilis muscles were stimulated with 500 μs duration square waves, control arteries dilated significantly more than ischemic arteries, $137.8 \pm 14.3\%$ vs. $14.4 \pm 4.6\%$, $p < 0.0001$ ($n=8$).

Figure 19: Young Ischemic vs. Young Sham. A bar graph of the profunda femoris artery diameter during rest, after a 200 μs stimulation and 500 μs stimulation * $p=0.004$

Table 1: Ischemia Data Table (Young Mice). Average values taken from functional vasodilation protocol with young mice (2-3mo), p value < 0.5 is significant

	Young Sham	Young Ischemic	p value
Rest	54.1 ± 3.6 µm	103.1 ± 10.3 µm	0.0006
Moderate Contraction (200µs duration)	108.3 ± 6.0 µm	116.8 ± 10.0 µm	0.48
Intense Contraction (500µs duration)	125.8 ± 4.6 µm	117.0 ± 11.7 µm	0.50
Rest vs. Post 200µs Stimulation % Change	104.1 ± 13.0 %	15.2 ± 5.0 %	0.0002
Rest vs. Post 500µs Stimulation % Change	137.8 ± 14.3 %	14.4 ± 4.6 %	0.00003

Figure 20: Young Ischemic vs. Young Sham % Change. A bar graph of the profunda femoris artery diameter percent change comparing post 200 µs stimulation to rest and post 500 µs stimulation to rest *p<0.001 x p<0.0001

Impact of Age

To test a corollary of the main hypothesis for this aim, the impact of age alone on function vasodilation was examined. There was a trend towards a larger resting diameter in the adult mice, $62.4 \pm 4.9 \mu\text{m}$ vs. $54.1 \pm 3.6 \mu\text{m}$, this difference did not achieve statistical significance, $p=0.21$ ($n=10/8$) (**Figure 21**). However, arterial diameter was not different between young and adult arteries following functional vasodilation (**Table 2**). Therefore, percent change in diameter compared to the resting state was examined as the more accurate indicator of vascular reactivity (**Figure 22**). When the gracilis muscles were stimulated with 200 μs duration square waves, there was a trend towards larger young artery dilation, $104.1 \pm 13.0\%$ vs. $79.8 \pm 11.9\%$, this difference did not achieve statistical significance, $p=0.19$ ($n=8/10$) (**Figure 22**). When the gracilis muscles were stimulated with 500 μs duration square waves, young arteries dilated significantly more than adult arteries, $137.8 \pm 14.3\%$ vs. $103.0 \pm 9.4\%$, $p=0.05$ ($n=8/10$).

Figure 21: Adult Sham vs. Young Sham. A bar graph of the profunda femoris artery diameter. Note the increase in adult resting diameter compared to the young resting diameter.

Table 2: Age Data Table (Sham Limb). Average values taken from functional vasodilation protocol with adult mice (6-7mo) and young mice (2-3mo) from the sham limb, p value < 0.5 is significant

	Young Sham	Adult Sham	p value
Rest	54.1 ± 3.6 µm	62.4 ± 4.9 µm	0.21
Moderate Contraction (200µs duration)	108.3 ± 6.0 µm	108.0 ± 5.5 µm	0.98
Intense Contraction (500µs duration)	125.8 ± 4.6 µm	123.8 ± 7.8 µm	0.84
Rest vs. Post 200µs Stimulation % Change	104.1 ± 13.0 %	79.8 ± 11.9 %	0.19
Rest vs. Post 500µs Stimulation % Change	137.8 ± 14.3 %	103.0 ± 9.4 %	0.05

Figure 22: Adult Sham vs. Young Sham % Change. A bar graph of the profunda femoris artery diameter percent change comparing post 200 µs stimulation to rest and post 500 µs stimulation to rest. Note the increase in adult resting diameter compared to the young resting diameter. *p=0.05

Impact of Age and Ischemia

A similar pattern of profunda femoris diameters was observed in adult mice (6-7mo). The resting ischemic artery was significantly larger than the control artery, $102.9 \pm 10.3 \mu\text{m}$ vs. $62.4 \pm 4.9 \mu\text{m}$, $p=0.004$ ($n=10$), (**Figure 23**). However, arterial diameter was not different between ischemic and control arteries following functional vasodilation (**Table 3**). Therefore, percent change in diameter compared to the resting state was examined as the more accurate indicator of vascular reactivity (**Figure 24**). When the gracilis muscles were stimulated with 200 μs duration square waves, control arteries dilated significantly more than ischemic arteries, $79.8 \pm 11.9\%$ vs. $7.6 \pm 3.4\%$, $p < 0.00001$ ($n=10$) (**Figure 24**). When the gracilis muscles were stimulated with 500 μs duration square waves, control arteries dilated significantly more than ischemic arteries, $103.0 \pm 9.4\%$ vs. $16.8 \pm 5.9\%$, $p=0.000002$ ($n=10$). Age did not affect the resting diameter or functional vasodilation of the ischemic artery (**Table 4**, **Figure 25**, and **Figure 26**).

Figure 23: Adult Ischemic vs. Adult Sham. A bar graph of the profunda femoris artery diameter during rest, after a 200 μ s stimulation and 500 μ s stimulation *p=0.0006

Table 3: Age and Ischemia Data Table (Adult Mice). Average values taken from functional vasodilation protocol with adult mice (6-7mo). Chart shows average vessel diameters and percent change from rest at rest, moderate contraction and intense contraction. p value < 0.5 is significant.

	Adult Sham	Adult Ischemic	p value
Rest	62.4 $\pm$ 4.9 μ m	102.9 $\pm$ 10.3 μ m	0.004
Moderate Contraction (200μs duration)	108.0 $\pm$ 5.5 μ m	111.8 $\pm$ 12.2 μ m	0.78
Intense Contraction (500μs duration)	123.8 $\pm$ 7.8 μ m	119.2 $\pm$ 12.2 μ m	0.75
Rest vs. Post 200μs Stimulation % Change	79.8 $\pm$ 11.9 %	7.6 $\pm$ 3.4 %	0.00003
Rest vs. Post 500μs Stimulation % Change	103.0 $\pm$ 9.4 %	16.8 $\pm$ 5.9 %	0.000002

Figure 24: Adult Ischemic vs. Adult Sham % Change. A bar graph of the profunda femoris artery diameter percent change in adult mice comparing post 200 μ s stimulation to rest and post 500 μ s stimulation to rest *p<0.0001 x p<0.00001

Table 4: Age and Ischemia Data Table (Young and Adult Mice). Average values taken from functional vasodilation protocol with young mice (2-3mo) and adult mice (6-7mo). Chart shows average vessel diameters and percent change from rest at rest, moderate contraction and intense contraction. p value < 0.5 is significant

	Young Ischemic	Adult Ischemic	p value
Rest	103.1 $\pm$ 10.3 μ m	102.9 $\pm$ 10.3 μ m	0.99
Moderate Contraction (200μs duration)	116.8 $\pm$ 10.0 μ m	111.8 $\pm$ 12.2 μ m	0.77
Intense Contraction (500μs duration)	117.0 $\pm$ 11.7 μ m	119.2 $\pm$ 12.2 μ m	0.90
Rest vs. Post 200μs Stimulation % Change	15.2 $\pm$ 5.0 %	7.6 $\pm$ 3.4 %	0.21
Rest vs. Post 500μs Stimulation % Change	14.4 $\pm$ 4.6 %	16.8 $\pm$ 5.9 %	0.76

Figure 25: Adult Ischemic vs. Young Ischemic. A bar graph of the profunda femoris artery diameter averages in adult and young ischemic mice. No clear difference was seen.

Figure 26: Adult Ischemic vs. Young Ischemic % Change. A bar graph of the profunda femoris artery diameter percent change in young and adult ischemic mice comparing post 200 µs stimulation to rest and post 500 µs stimulation to rest.

SUPERFUSED FUNCTIONAL VASODILATION PROTOCOL

For Specific Aim 2 (Page 26), a protocol was developed for a superfused functional vasodilation. This was completed to eventually test the effects of pharmacological agents on the current model during a functional vasodilation protocol. This protocol solves the problem of not being able to add drugs to the preparation through mineral oil. By adding the drugs to a superfusion solution, pharmacological agents can be easily added to the preparation. The goal was to create a consistent protocol with minimal dilation caused by the superfusion solution.

No significant difference in diameter was noticed between the right and left limb (**Figure 27, Table 5**). However, arterial diameter increased following the addition of the superfusion solution $89.7 \pm 11.3 \mu\text{m}$ vs. $50.4 \pm 6.8 \mu\text{m}$, $p=0.0007$ ($n=10$) (**Table 6**). Looking at percent change in diameter, vascular reactivity to the superfusion solution was more accurately examined (**Figure 28**). Following the addition of the superfusion solution, arterial diameter increased $84.3 \pm 13.0\%$ for the left limb and $65.6 \pm 17.2\%$ for the right limb.

Figure 27: Superfused Functional Vasodilation: Right vs. Left Comparison. Profunda femoris artery diameter measurements at rest, 30 minutes after superfusion solution was applied, and after a 500 μ s stimulation. No significant differences were observed between right and left limbs.

Table 5: Superfused Functional Vasodilation Data Table. Average values taken from superfused functional vasodilation protocol. Chart shows average vessel diameters and percent change from rest at rest, moderate contraction and intense contraction.

	Left Limb	Right Limb
Rest	50.4 $\pm$ 6.8 μ m	56.6 $\pm$ 4.7 μ m
Post Superfusion	89.7 $\pm$ 11.3 μ m	89.9 $\pm$ 9.0 μ m
Post Stimulation	101.5 $\pm$ 9.6 μ m	112.6 $\pm$ 5.7 μ m
Rest vs. Post Superfusion % Change	84.3 $\pm$ 13.0 %	65.6 $\pm$ 17.2 %
Rest vs. Post Stimulation % Change	116.0 $\pm$ 16.6 %	108.0 $\pm$ 14.0 %

Table 6: Superfused Functional Vasodilation Data Analysis Table. Statistical analysis of data in Table 5 n=10

	Left Limb p values	Right Limb p values
Rest vs. Post Superfusion	0.0007	0.003
Post Superfusion vs. Post Stimulation	0.04	0.004
Rest vs. Post Stimulation	0.00001	0.00000009
% Change	0.07	0.004

Figure 28: Superfused Functional Vasodilation: Right vs. Left % Change Comparison. Profunda femoris artery diameter percent change measurements. No significant differences were observed between right and left limbs.

SUPERFUSED VASOACTIVE AGENTS PROTOCOL

For Specific Aim 3 (Page 26), a protocol was developed for superfusing vasoactive agents. This was completed to test the effects of pharmacological agents on the current model by creating a method to apply them to the preparation without the use of mineral oil. Previous work completed by this lab (Thomas Kessler) resulted in a protocol for the use of two different drugs, Ach and SNP. The goal of this protocol was to assess the potency of NE and examine the feasibility of using three different drugs in one superfusion experiment. First, a dose response curve to NE was generated (**Figure 29**). No significant difference between limbs was observed (**Table 7**).

Figure 29: Dose Response Curve to Neuroepinephrine. A plot of the diameters of the profunda femoris artery with increasing doses of NE (in M). Note that the final dose is KCl, a potent vasoconstrictor. No significant difference was found between limbs.

Table 7: NE Dose Response Curve. Average values taken from superfusion protocol, p value < 0.5 is significant

	Left Limb	Right Limb	p value
Rest	110 ± 6 µm	105 ± 20 µm	0.78
10⁻⁸ M NE	107 ± 4 µm	106 ± 21 µm	0.98
10⁻⁷ M NE	105 ± 2 µm	106 ± 23 µm	0.96
10⁻⁶ M NE	108 ± 3 µm	105 ± 18 µm	0.87
10⁻⁵ M NE	106 ± 2 µm	102 ± 16 µm	0.82
10⁻⁴ M NE	41 ± 15 µm	66 ± 29 µm	0.32
10⁻⁴ M KCl	42 ± 9 µm	47 ± 12 µm	0.34

No significant was noticed between limbs for the protocol setup (**Figure 30**, **Figure 31**). Due to the large difference in diameter between ACh/SNP and NE, dynamic range was calculated. No significant difference between limbs was noticed (**Figure 32**). Because no significant differences were found between all groups, the protocol was completed with sham and experimental groups.

Figure 30: Superfusion: Right vs. Left. A bar graph of the profunda femoris artery diameter and its response to three vasoactive agents: NE, Ach, and SNP. No significant difference was found between limbs.

Figure 31: Superfusion: Right vs. Left % Change. A bar graph of the profunda femoris artery diameter percent change compared to resting diameter and its response to three vasoactive agents: NE, Ach, and SNP. No significant difference was found between limbs.

Figure 32: Superfusion Dynamic Range. Dynamic ranges of the right and left limb. The maximum was determined from the value of the SNP diameter and the minimum from the NE diameter. No significant difference was found between limbs.

EFFECTS OF ISCHEMIA ON ENDOTHELIAL AND SMOOTH MUSCLE DEPENDENT REACTIVITY

For Specific Aim 4 (Page 26), the hypothesis that ischemia reduces vascular reactivity in the profunda femoris feed artery was tested. It was hypothesized that the cause of this difference would be endothelial dysfunction.

There was no trend or significant difference noticed between the ischemic and sham limbs for both the resting and NE diameters (**Figure 33**). There was a trend towards a larger diameter in the sham artery when superfused with ACh, $133.8 \pm 7.5 \mu\text{m}$ vs. $109.6 \pm 12.3 \mu\text{m}$, this difference did not achieve statistical significance, $p=0.12$ ($n=8$). A similar trend was seen with SNP, $137.5 \pm 7.6 \mu\text{m}$ vs. $113.3 \pm 12.7 \mu\text{m}$, this difference did not achieve statistical significance, $p=0.12$ ($n=8$). Due to the lack of significance, percent

change in diameter compared to the resting state was examined as the more accurate indicator of vascular reactivity (**Figure 34**). Again, when superfused with NE, no significance was noticed between groups. When the hindlimb was superfused with ACh, control arteries dilated significantly more than ischemic arteries, $80.5 \pm 11.4\%$ vs. $39.6 \pm 13.6\%$, $p=0.04$, ($n=8$). When the hindlimb was superfused with SNP, control arteries dilated significantly more than ischemic arteries, $85.1 \pm 10.5\%$ vs. $43.0 \pm 11.7\%$, $p=0.02$, ($n=8$). When dynamic range was calculated, the control arteries had a significantly larger range, $112.4 \pm 6.9 \mu\text{m}$ vs. $87.5 \pm 9.4 \mu\text{m}$, $p=0.05$, ($n=8$) (**Figure 19**).

Figure 33: Ischemic vs. Sham Superfusion Response. A bar graph of the profunda femoris artery diameter and its response to three vasoactive agents: NE, Ach, and SNP. No significant difference was found between limbs; however a trend was noticed for ACh and SNP between groups.

Figure 34: Ischemic vs. Sham % Change. A bar graph of the profunda femoris artery diameter percent change compared to resting diameter and its response to three vasoactive agents: NE, ACh, and SNP. A significant difference was observed between ischemic and sham groups for the ACh percent change and SNP percent change * $p < 0.05$

Figure 35: Dynamic Range. Dynamic ranges of the ischemic and sham limb. The maximum was determined from the value of the SNP diameter and the minimum from the NE diameter. A significant difference was observed between ischemic and sham groups * $p = 0.05$.

DISCUSSION

RESTATEMENT OF EXPERIMENTAL OBJECTIVES

To help guide the discussion of experimental results, the primary objectives of the thesis are listed below:

1. *Test the hypothesis that age will decrease the functional vasodilation during ischemia.*
2. *Establish a consistent superfused functional vasodilation protocol using healthy mice.*
3. *Establish an experimental protocol for assessing vascular reactivity using pharmacological agents.*
4. *Test the hypothesis that the ischemic profunda femoris artery will exhibit impaired endothelial-dependent vasodilation.*

AIM 1: IMPACT OF AGE ON FUNCTIONAL VASODILATION

Age is one of the greatest risk factors for developing chronic ischemia [52]. Systemic pathologies such as hypertension and impaired wound healing are also present with advanced age [4, 17, 54]. One of the most pertinent effects of age, with regards to this research, is its negative effect on revascularization, which have been recapitulated in animal models [37, 54]. Acetylcholine-dependent dilation *in vitro* can be impaired as early as 6 months of age [37], while *in vivo* functional vasodilation is known to be decreased in mice of advanced age, 20 months [54]. Given the impact of age on

vasodilation we hypothesized that age plus ischemia would have an additive effect on reducing functional vasodilation.

Aim 1: Impact of Age on Functional Vasodilation

Age reduced the functional vasodilation response observed in the sham limb. In young mice, the profunda femoris dilated 140% in response to 500 μ s pulses vs. 100% in adult mice. Furthermore, the young profunda femoris artery showed a non-significant trend of a lower resting diameter 54 μ m, compared to 62 μ m in the adult animal. This was similar to values from previous research, in which adult C57 mice exhibited less resting tone than young C57 mice [37]. It was observed that the average weight of the young mice was ~20g and that of the older mice was ~30g. There was also a considerably higher amount of visible adipose tissue around the artery in the adult mice compared to the young mice. Adipose tissue has been shown to increase vessel relaxation due to adipocyte-derived relaxing factor (ADRF) [63], opens K^+ channels in vascular smooth muscle cells [64]. Therefore, an increase in adiposity may explain the lower resting tone observed in the adult mice.

Aim 1: Impact of Ischemia and Age on Functional Vasodilation

Ischemia impaired functional vasodilation in both young and adult mice with only 10-15% increase in diameter of the ischemic leg, compared to 100-140% for the sham limb. Ischemia causes cells to release vasoactive products such as NO [65]. The response

to this hypoxia such as molecule concentration and duration, depends upon degree and duration of the hypoxic injury [7]. The ischemia surgery induces a significant amount of inflammation, which was visually assessed through the observation of connective tissue over the surgical site after 14 days. Even though it is such a long time after an inflammatory response, the mouse could have aggravated the inflammatory response through scratching or biting of the surgical area during the 14 days of recovery. Several mice bit off their tails after 14 days; this could be due to the pain at the surgical site, however great efforts were taken to mitigate this through an injection of buprenorphine after surgery. Increased monocyte recruitment to the area due to the inflammation could have led to a larger resting diameter through the release of vasodilatory factors.

Due to the significantly different vasodilatory response between the sham and ischemic arteries, the increase in diameter could also be attributed to improper surgical technique. The only time the profunda femoris artery was touched during the resection surgery was when the artery and vein pair was cauterized as proximal to the femoral artery as possible. One of the problems with using cautery is heat propagation through the surrounding tissue [66]. When heat propagates through a tissue, it causes cellular temperature to rise, leading to death of the cell. If the heat propagates too far or gets too hot, less viable tissue remains. Thus, with increased heat propagation, a smaller section of artery can be analyzed 14 days later. Also, this heat could have propagated into the artery and caused a significant amount of endothelial dysfunction, creating a difference in diameter. Heat ablation causes intimal thickening as well as activation of the coagulation cascade, which is “dose dependent”; meaning a larger amount of heat application from the cautery would increase the negative effects [67, 68]. Due to the fact that the heat

cautery is battery operated, the actual temperature of the cautery tip, and the time spent cauterizing the artery was variable for each surgery. If the cautery tip was left near the tissue for too long, the surrounding area would become burnt and charred. This occurred only a few times, but was the result of improper cautery equipment or technique. Extra effort was made to make sure that this never happened to the tissue; however the variable cautery temperatures made this difficult. After a new cautery was acquired, resting diameters and tissue charring decreased.

Another possible explanation for the increased artery diameter, which may have reduced the dynamic range and dilation potential of the vessel, is the tissue dissection technique of the ischemic limb before the intravital microscope and microelectrodes were placed. If there was a significant inflammatory response to the surgery, there was a significant amount of connective tissue that needed to be dissected in order to clearly visualize the artery. Even if it was visible through the light microscope, a layer of connective tissue on top of the profunda femoris artery could prevent successful focusing of the intravital microscope. Also, if the tissue around the artery was agitated during dissection, this could have injured and dilated the profunda femoris artery. Extra care was taken to ensure that the connective tissue was carefully dissected away from the artery before measurements were taken.

Several other precautions were taken during the experiment in order to maintain consistency between subjects. The ischemic and sham legs measurements were alternated as recommended by a previous researcher in the lab, Matt Yocum [58]. Even with efforts to improve consistency, there was still a significant difference between the two groups. This further supports the theory that the surgery affected the artery in some way.

The result of a high resting diameter was most likely due to the cautery and surgical/dissection technique. After performing several surgeries and dissections, my technique and consistency improved. Additionally, with the acquisition of the new cautery, the issue of large resting diameters disappeared.

Aim 1: Future Recommendations

Several problems were observed with the current imaging protocol. As previously mentioned, the MicroScan intravital microscope has an immersion lens, leaving very little space between the microscope tip and underlying tissue. Contact between these two surfaces may irritate the artery and cause it to dilate. Also, due to the nature of the surgery that is used and the experimental model, finding a clear picture of the artery is sometimes difficult (**Figure 36**). An elevated inflammatory response due to surgical trauma can cause a buildup of connective tissue and/or increase vascularization. This causes a reduction in image clarity and sometimes vascular response to stimulus. Furthermore, with older or larger mice, increased adipose tissue can cause similar difficulties.

Figure 36: Poor Vessel Images. Examples of poor image quality of vessel due to (A) vascularization due to inflammatory response (B) overlying adipose tissue due to larger or older mouse

For future researchers interested in assessing the impact of age and ischemia on vasodilation, several topics must be analyzed further. Further research is needed to determine why there was a significant difference between resting diameters in the experimental and sham profunda femoris. By using a superfusion setup, pharmacological agents can be added to the artery and help further explain the cellular pathways that could be dysfunctional. First, this experiment should be repeated using the proper cautery to potentially eliminate that variable. Eventually, pharmacological inhibitors to vasodilation could be superfused before and after stimulation to block hypothesized pathways.

AIM 2: DEVELOPMENT OF FUNCTIONAL VASODILATION PROTOCOL FOR PHARMACOLOGICAL DELIVERY

In the lab, there currently is no way to electrically stimulate the gracilis muscle while delivering pharmacologic agents to the area. This protocol solves the problem of not being able to add drugs to the preparation through mineral oil. By adding the drugs to a superfusion solution, pharmacological agents can be easily added to the preparation. The goal was to create a consistent protocol with minimal dilation caused by the superfusion solution. A superfused functional vasodilation protocol would allow for further research into the impact of ischemia on the cellular pathways controlling vasodilation. Pharmacological factors could be added to the superfusion solution to enhance or inhibit factors in the cellular pathways, creating an opportunity to assess causal relationships. Irrigating the exposed tissue with an isotonic, degassed solution allows for the delivery of pharmacological agents while preventing desiccation and minimizing oxygen diffusion.

The results of the protocol showed a significant increase in diameter, approximately 75%, after the addition of the superfusion solution to the preparation. Because of the pre-dilation, it creates a higher resting diameter for the artery, reducing its maximal dilation potential. The intention of the protocol development was to minimize this increase in response to the superfusion solution and increase the consistency of the data collection. Consistency is desired due to the variable nature of the surgical model that is used. Dr. Steven Segal of the University of Missouri, a veteran in superfusion protocols, was consulted during this protocol development and made some recommendations on how to improve the superfusion experimental protocol.

Aim 2: Completed Modifications

One of the first controls put into place was monitoring the pH of the physiological salt solution, because changes in pH can alter vessel tone and oxygen exchange with tissues [1]. Another recommendation was bubbling the solution with CO₂:N₂ gas before superfusing it over the hindlimb. N₂ gas is used because helps deoxygenate the solution. This is important because oxygen transport from the atmosphere directly to skeletal muscle will cause the smooth muscle to constrict [2]. The combination of these modifications produced an interesting result. Right after the PSS was mixed, the pH was measured and averaged 7.9; after the PSS was bubbled, pH decreased to 7.1. Both of these values are above and below the desired pH values (7.30 - 7.35), respectively. The pH was then measured after it had flowed through the circuit and was within desired values, this was possibly due to the carbon dioxide diffusion out of the solution during the time it was in the circuit. Efforts were made to pH balance the solution before adding it to the irrigation circuit; however this did not produced positive results. Further, application of varying pH levels, demonstrated that a change in pH was not the cause of the resting vasodilation.

In addition to pH, vascular tone is also affected by temperature. Thus, another modification was to vary the temperature and flow rate of the solution. Previous investigators maintained the PSS at 35 °C with a flow rate of ~2mL·min⁻¹ [47]. Adjusting the syringe heater the flow rate could both change the temperature due to the gain and loss of temperature through the circuit, respectively. Great efforts were taken to minimize this temperature loss by insulating the circuit, but a temperature loss of about 10 °C from the beginning to end of the circuit was still observed. Both the temperature and flow rate

changed throughout the experiment and extra care was taken to maintain both of these values close to preferred ranges. Dr. Segal recommended that a temperature of 37 °C should be used. Unfortunately, a similar loss of vessel tone was seen at both high (40 °C) and low (30 °C) temperatures.

Given that changes in PSS properties did not affect vascular tone, it was also speculated that the shear stress between the surface of the profunda femoris artery and the intravital microscope lens caused vasodilation. To test this, the intravital microscope was removed and the vessel was observed with a stereo light microscope, which has a ~10 cm working distance and does not directly contact the tissue. Unfortunately, altering the flow conditions of the PSS over the profunda femoris did not impact the resting vasodilation.

After eliminating temperature and pH, other variables that were analyzed were the freshness of the solution and the cleanliness of the superfusion circuit equipment. This recommendation was made because the salts in the solution and superfusion circuit precipitate in the solution and adsorb in the circuit, causing changes in the concentration of the different compounds. Due to the potential buildup of salts in the circuit, the dishes cleaning protocol was changed, implementing a different rinse into the process, and changing out all of the tubing that was used with new tubing. It was also determined that a piece of metal in the circuit was corroding from the acid rinse during the cleaning protocol. The metal was replaced with a plastic piece to prevent this corrosion. Again, no difference in vessel tone was observed with these changes.

Since precipitated salts were not causing the problem, the final variable to change was the actual salts used in the solutions. The compounds used were the same; however

the product numbers were different from those of Dr. Segal's group. Even after changing to the same product numbers, similar results were seen with loss of vessel tone 30 minutes after the superfusion solution was applied.

Aim 2: Future Recommended Modifications

Recommended modifications involve gaining more control of the superfusion circuit. The following variables changed throughout the experiment: gas bubbling rate, flow rate and temperature. Often times during the experiment, the rate of bubbling in the solution was variable. To solve this problem the bubbling rate of the superfusion solution needs to be higher to prevent a decrease in bubbling rate that was seen. The level of superfusion liquid was kept at 60 mL in the syringe heater, leaving a small volume for the bubbling solution. Decreasing this volume to 50 mL in the syringe will allow more room for bubbling at a higher rate without the solution spilling out of the top of the syringe. To maintain temperature and flow rate, flow rate must be more accurately controlled. Currently, flow rate is monitored through compressing a tube with a screw clamp. It is difficult to obtain and maintain using this method.

Flow over the profunda femoris artery must also be consistent. Often, the intravital microscope prevented flow over the artery if it was depressed too much. The positive effect was lack of vasodilation of the artery, however if a drug was in the superfusion solution, it had no effect on the artery. To combat this problem, a different intravital microscopy method is needed. This could be accomplished by using a microscope with a larger working distance, such as a compounded reflected light

microscope; so more space could be put between the microscope and the profunda femoris artery.

The development of a consistent superfused functional vasodilation protocol is imperative for future understanding of endothelial dysfunction due to ischemic injury. The ability to target and inhibit or enhance specific cellular mechanisms during a functional vasodilation experiment is essential for determining the impact of ischemia on this process.

AIMS 3 AND 4: IMPACT OF ISCHEMIA ON CELL DEPENDENT DILATION AND CONSTRICTION

Previous work has demonstrated impaired vasodilation in arterioles, but the effect of ischemia on feed arteries is unknown. Understanding how ischemia affects feed arteries is important because feed arteries play a critical role in determining tissue blood flow. A healthy artery has the ability to vasodilate and vasoconstrict in order to ensure the homeostasis of oxygen and other nutrients of the downstream tissues. In response to acute ischemia/hypoxia, vessels dilate to increase the blood flow to the affected area. Unfortunately, chronic ischemia leads to vasodilatory dysfunction. Ischemic arterioles in the lower leg have impaired functional vasodilation, which could be explained by impaired endothelial-dependent dilation [69, 70]. Similar results were found in rat collateral arteries in vitro [71]. In both studies, exercise restored endothelial function. Due to this result, it was hypothesized that post-resected mice would present a similar endothelial dysfunction and dilate less to ACh than SNP.

Aim 4: Possible Smooth Muscle Dysfunction

Due to the fact that ACh and SNP produced similar results and there was no significant difference between the two treatments, smooth muscle dysfunction is the best reason for this result. Since only three pharmaceuticals were used in the experiment, further research is needed to discover what mechanism is dysfunctioning. One explanation for why smooth muscle dependent process would be impaired is due to the pressure in the profunda femoris artery. The surgical removal of the femoral artery, which feeds the profunda femoris, is assumed to decrease pressure the ischemic side. Knowing the pressure could help further differentiate the role of cellular dysfunction in smooth muscle cells versus altered vasodilation due to changes in hemodynamics. There could be a biochemical difference in the smooth muscle causing dysfunction as well.

To better understand the resulting pathology of the resection surgery, further research must be done to fully rule out endothelial dysfunction. In addition to using endothelial-dependent vasodilators, the impact of ischemia on the endothelium can be assessed by damaging or removing the endothelium. The endothelium can be damaged by perfusing an air bubble through the vessel or by using an intravascular dye that heat-injures the endothelium following excitation [47].

Vasoconstriction was also analyzed to fully understand the dynamic range of the artery. Previous groups have shown an increased vasoconstrictive response by ischemic limbs [51] while others have shown a reduction in sympathetic tone with chronic ischemia [41]. In this study, both groups responded similarly to neuroepinephrine. This discrepancy could have contributed to the difficulty in measuring the vessel when it was vasoconstricted, as the differences in such a small diameter were difficult to differentiate

due to the microscope resolution. Also, due to the fact that the previous demonstration of enhanced constriction was performed with human tissue, there are several comorbidities involved in the patient when compared to the mouse that could explain this difference, such as hypertension.

Differences between Superfusion and Functional Vasodilation

There are some observed differences between the functional vasodilation and superfusion results. The measured resting values of the ischemic limbs are different. For both the young and adult mice in the first aim, they are around 100 μm . In the fourth aim, the resting diameter is around 80 μm .

First, the differences in ischemic resting diameter will be addressed. In previous work from Matt Yocum, possible causes of an increased diameter was a decreased time between dissection and measurement, and contact between the MicroScan tip and the profunda femoris artery [58]. These recommendations were understood before both aims. When a higher resting diameter was noticed, more time was added to the timer (more than 30 minutes) and great care was taken to move the MicroScan as high as possible to prevent contact between it and the underlying tissue.

One possible hypothesis to this observed difference in resting values could be the difference in surgical equipment and technique used between the two aims. The cautery used in the first aim was old and inconsistent and was replaced with brand new cautery after Aim 1 was completed. This created a more consistent heat profile that burned through the artery faster, allowing for more visible artery length 14 days later. There was

also a difference in technique that was used during dissection before the superfusion experiment in aim 4. Microdissection scissors were used to carefully cut away the connective tissue above the profunda femoris artery, as opposed to careful blunt dissection that was previously used. This could have caused less stretching of the artery, allowing for less distress to the artery and surrounding tissue.

In summary, ischemia impairs vascular reactivity however it remains unclear if it is endothelial dysfunction or smooth muscle dysfunction. The proposed data implies smooth muscle dysfunction; however more research needs to be completed to fully rule out endothelial dysfunction.

FINAL CONCLUSIONS

This study has shown that ischemia and age significantly affect vascular function. Determining the cause of this dysfunction requires additional studies and protocol improvements. This leads to further possibilities of research with this model. First a few key problems need to be addressed.

The resection model proves to be difficult due to the current experimental setup. This model could become a more viable option if the surgical protocol did not involve cautery, a method that as stated previously is hard to control with current equipment. Also, using a microscope that allows for a larger focal distance that restricts tissue contact will help control the model to allow for more consistent results. Digital calipers will also provide a more controlled and consistent method to measure arterial diameters. Once these problems are addressed future experimental methods can be attempted.

As stated in the introduction, mice have a large number of transgenic options to allow experiments that test causal relationships. Surgical methods also provide opportunities to look at the possibility of endothelial dysfunction. Superfusion protocols with different drugs such as inhibitors can help address specific cellular pathway dysfunctions. Also, with improvements made in superfusion, the effects of age using cell-specific vasoactive agents can be addressed. Exercise is also a possible treatment to be attempted, as previous research has showed improvement in endothelial function [41, 70, 71] following exercise. It is still unclear what specifically impairs vascular function in ischemic conditions; it is the goal of this research to solve those questions. There has been a history of efficacy of therapies in animal models and disappointing results seen in randomized patient trials, perhaps the current way this model is used is the answer to this problem.

To better understand the current model and its dysfunction, age must be addressed with the superfusion protocol to determine if endothelial dysfunction exists in adult mice. Also, old mice, that could present more dysfunction than young mice, should be examined. Finally, different pharmaceuticals such as inhibitors need to be used in the superfusion setup to better differentiate between endothelial dysfunction and smooth muscle dysfunction.

With the knowledge gained from these results, it is shown that ischemia and age significantly impairs vasodilation in a feed artery. By improving vasodilation in patients with ischemia, blood flow could be partially restored. More specifically, therapies targeting smooth muscle could prove more effective.

REFERENCES

1. Marieb, E.N. and K. Hoehn, *Human Anatomy & Physiology*. 7th ed. 2007, San Francisco, CA: Pearson Benjamin Cummings.
2. Koeppen, B.M. and B.A. Stanton, *Berne and Levy Physiology*. 6th ed. 2008, Philadelphia, PA: Mosby Elsevier.
3. Klabunde, R.E. *Cardiovascular Physiology Concepts*. Systemic Circulation 2008 May 17, 2011]; Available from:
<http://www.cvphysiology.com/Blood%20Pressure/BP019.htm>.
4. Saladin, K.S., *Anatomy and Physiology: The Unity of Form and Function*. Fifth ed. 2010, New York, NY: McGraw-Hill.
5. Segal, S.S., *Regulation of blood flow in the microcirculation*. Microcirculation, 2005. **12**(1): p. 33-45.
6. Shufelt, C.L. and C.N. Bairey Merz, *Contraceptive Hormone Use and Cardiovascular Disease*. J Am Coll Cardiol, 2009. **53**(3): p. 221-231.
7. Paternotte, E., et al., *Review: behaviour of endothelial cells faced with hypoxia*. Biomed Mater Eng, 2008. **18**(4-5): p. 295-9.
8. Mohler, E.R., et al., *Impaired Exercise-Induced Blood Volume in Type 2 Diabetes With or Without Peripheral Arterial Disease Measured by Continuous-Wave Near-Infrared Spectroscopy*. Diabetes Care, 2006. **29**(8): p. 1856-1859.

9. Institute, N.H.L.a.B. *Peripheral Artery Disease*. September 2008 [cited 2011 March 7]; Available from:
http://www.nhlbi.nih.gov/health/dci/Diseases/pad/pad_what.html.
10. Clayton, J.A., D. Chalothorn, and J.E. Faber, *Vascular Endothelial Growth Factor-A Specifies Formation of Native Collaterals and Regulates Collateral Growth in Ischemia*. *Circ Res*, 2008. **103**(9): p. 1027-1036.
11. Hamburg, N.M. and G.J. Balady, *Exercise Rehabilitation in Peripheral Artery Disease: Functional Impact and Mechanisms of Benefits*. *Circulation*, 2011. **123**(1): p. 87-97.
12. Brevetti, G., et al., *Inflammation in Peripheral Artery Disease*. *Circulation*, 2010. **122**(18): p. 1862-1875.
13. Sitia, S., et al., *From endothelial dysfunction to atherosclerosis*. *Autoimmunity Reviews*, 2010. **In Press, Corrected Proof**.
14. Zardi, E.M. and A. Afeltra, *Endothelial dysfunction and vascular stiffness in systemic lupus erythematosus: Are they early markers of subclinical atherosclerosis?* *Autoimmunity Reviews*, 2010. **9**(10): p. 684-686.
15. Newton, D.J., et al., *Improvement in systemic endothelial condition following amputation in patients with critical limb ischemia*. *Int Angiol*, 2008. **27**(5): p. 408-12.
16. Husmann, M., et al., *Successful lower extremity angioplasty improves brachial artery flow-mediated dilation in patients with peripheral arterial disease*. *Journal of Vascular Surgery*, 2008. **48**(5): p. 1211-1216.

17. Semenza, G.L., *Vascular Responses to Hypoxia and Ischemia*. Arterioscler Thromb Vasc Biol, 2010. **30**(4): p. 648-652.
18. Grundmann, S., et al., *Arteriogenesis: basic mechanisms and therapeutic stimulation*. European Journal of Clinical Investigation, 2007. **37**(10): p. 755-766.
19. Epstein, S.E., et al., *Janus Phenomenon: The Interrelated Tradeoffs Inherent in Therapies Designed to Enhance Collateral Formation and Those Designed to Inhibit Atherogenesis*. Circulation, 2004. **109**(23): p. 2826-2831.
20. Institute, N.H.L.a.B. *Angioplasty*. September 2008 [cited 2011 March 7]; Available from:
http://www.nhlbi.nih.gov/health/dci/Diseases/Angioplasty/Angioplasty_howdone.html.
21. Buschmann, I. and W. Schaper, *Arteriogenesis Versus Angiogenesis: Two Mechanisms of Vessel Growth*. News Physiol Sci, 1999. **14**: p. 121-125.
22. Isner, J.M. and T. Asahara, *Angiogenesis and vasculogenesis as therapeutic strategies for postnatal neovascularization*. The Journal of Clinical Investigation, 1999. **103**(9): p. 1231-1236.
23. Rivilis, I., et al., *Differential involvement of MMP-2 and VEGF during muscle stretch- versus shear stress-induced angiogenesis*. American Journal of Physiology - Heart and Circulatory Physiology, 2002. **283**(4): p. H1430-H1438.
24. van Royen, N., et al., *Arteriogenesis: mechanisms and modulation of collateral artery development*. J Nucl Cardiol, 2001. **8**(6): p. 687-93.

25. Schaper, W. and D. Scholz, *Factors Regulating Arteriogenesis*. Arterioscler Thromb Vasc Biol, 2003. **23**(7): p. 1143-1151.
26. Eitenmuller, I., et al., *The Range of Adaptation by Collateral Vessels After Femoral Artery Occlusion*. Circ Res, 2006. **99**(6): p. 656-662.
27. Ito, W.D., et al., *Monocyte Chemotactic Protein-1 Increases Collateral and Peripheral Conductance After Femoral Artery Occlusion*. Circ Res, 1997. **80**(6): p. 829-837.
28. Schierling, W., et al., *The role of angiogenic growth factors in arteriogenesis*. J Vasc Res, 2009. **46**(4): p. 365-74.
29. Shyu, K.-G., et al., *Intramuscular vascular endothelial growth factor gene therapy in patients with chronic critical leg ischemia*. The American Journal of Medicine, 2003. **114**(2): p. 85-92.
30. Bobek, V., et al., *Gene therapy of the ischemic lower limb -- Therapeutic angiogenesis*. Vascular Pharmacology, 2006. **44**(6): p. 395-405.
31. Kinnaird, T., et al., *Cardiovascular risk factors impair native collateral development and may impair efficacy of therapeutic interventions*. Cardiovascular Research, 2008. **78**(2): p. 257-264.
32. Dimmeler, S., A.M. Zeiher, and M.D. Schneider, *Unchain my heart: the scientific foundations of cardiac repair*. The Journal of Clinical Investigation, 2005. **115**(3): p. 572-583.

33. Shamoun, F., N. Sural, and G. Abela, *Peripheral artery disease: therapeutic advances.(Report)*. Expert Review of Cardiovascular Therapy, 2008. **6**(4): p. 539(15).
34. Jarajapu, Y.P., et al., *Functional characterization of alpha(1)-adrenoceptor subtypes in human skeletal muscle resistance arteries*. Br J Pharmacol, 2001. **133**(5): p. 679-86.
35. Unger, E.F., *Experimental evaluation of coronary collateral development*. Cardiovascular Research, 2001. **49**(3): p. 497-506.
36. Troidl, K., et al., *Effects of endogenous nitric oxide and of DETA NONOate in arteriogenesis*. J Cardiovasc Pharmacol, 2010. **55**(2): p. 153-60.
37. Belin de Chantemele, E.J., et al., *Obesity-induced insulin resistance causes endothelial dysfunction without reducing the vascular response to hindlimb ischemia*. Basic Res Cardiol, 2009.
38. Scholz, D., et al., *Contribution of Arteriogenesis and Angiogenesis to Postocclusive Hindlimb Perfusion in Mice*. Journal of Molecular and Cellular Cardiology, 2002. **34**(7): p. 775-787.
39. Van Belle, E., et al., *Hypercholesterolemia Attenuates Angiogenesis but Does Not Preclude Augmentation by Angiogenic Cytokines*. Circulation, 1997. **96**(8): p. 2667-2674.
40. Takeshita, S., et al., *Therapeutic angiogenesis. A single intraarterial bolus of vascular endothelial growth factor augments revascularization in a rabbit ischemic hind limb model*. The Journal of Clinical Investigation, 1994. **93**(2): p. 662-670.

41. Patrick N. Colleran, et al., *Vasoresponsiveness of collateral vessels in the rat hindlimb: influence of training*. The Journal of Physiology, 2010. **588**(8): p. 1293-1307.
42. Ginis, I., et al., *Differences between human and mouse embryonic stem cells*. Dev Biol, 2004. **269**(2): p. 360-80.
43. Byrom, M.J., et al., *Animal models for the assessment of novel vascular conduits*. Journal of Vascular Surgery, 2010. **52**(1): p. 176-195.
44. Li, J., et al., *Synergistic effects of FGF-2 and PDGF-BB on angiogenesis and muscle regeneration in rabbit hindlimb ischemia model*. Microvascular Research, 2010. **80**(1): p. 10-17.
45. Banai, S., et al., *Angiogenic-induced enhancement of collateral blood flow to ischemic myocardium by vascular endothelial growth factor in dogs*. Circulation, 1994. **89**(5): p. 2183-2189.
46. Welsh, D.G. and S.S. Segal, *Coactivation of resistance vessels and muscle fibers with acetylcholine release from motor nerves*. American Journal of Physiology - Heart and Circulatory Physiology, 1997. **273**(1): p. H156-H163.
47. Emerson, G.G. and S.S. Segal, *Endothelial cell pathway for conduction of hyperpolarization and vasodilation along hamster feed artery*. Circ Res, 2000. **86**(1): p. 94-100.
48. De With, M.C.J., et al., *Ischemia-Reperfusion Impairs Ascending Vasodilation in Feed Arteries of Hamster Skeletal Muscle*. Microcirculation, 2005. **12**(7): p. 551-561.

49. Xiang, L., et al., *Chronic hyperglycemia impairs functional vasodilation via increasing thromboxane-receptor-mediated vasoconstriction*. Am J Physiol Heart Circ Physiol, 2007. **292**(1): p. H231-236.
50. van Golde, J.M., et al., *Impaired collateral recruitment and outward remodeling in experimental diabetes*. Diabetes, 2008. **57**(10): p. 2818-23.
51. Jarajapu, Y.P.R., et al., *Increased α_1 - and α_2 -adrenoceptor-mediated contractile responses of human skeletal muscle resistance arteries in chronic limb ischemia*. Cardiovascular Research, 2001. **49**(1): p. 218-225.
52. Parfrey, P.S., et al., *Outcome and risk factors of ischemic heart disease in chronic uremia*. Kidney Int, 1996. **49**(5): p. 1428-1434.
53. Gray, L. and F.C. Martin, *Classifying older patients in hospital*. Age Ageing, 2005. **34**(5): p. 422-4.
54. Dwayne, N.J., W.M. Alex, and S.S. Steven, *Blunting of rapid onset vasodilatation and blood flow restriction in arterioles of exercising skeletal muscle with ageing in male mice*. The Journal of Physiology, 2010. **588**(12): p. 2269-2282.
55. Rivard, A., et al., *Age-Dependent Impairment of Angiogenesis*. Circulation, 1999. **99**(1): p. 111-120.
56. Brevetti, L.S., et al., *Overexpression of endothelial nitric oxide synthase increases skeletal muscle blood flow and oxygenation in severe rat hind limb ischemia*. Journal of Vascular Surgery, 2003. **38**(4): p. 820-826.

57. Young, D.A., M.M. Chi, and O.H. Lowry, *Energy metabolism of skeletal muscle biopsies stimulated anaerobically without load in vitro*. Am J Physiol Cell Physiol, 1986. **250**(6): p. C813-820.
58. Yocum, M.D., *Arteriogenic Revascularization Does Not Induce Vascular Function Impairment*, in *Biomedical Engineering*. 2009, California Polytechnic State University, San Luis Obispo: San Luis Obispo. p. 102.
59. Goud, C., et al., *Cyclic GMP-Independent Mechanisms of Nitric Oxide-Induced Vasodilation*. General Pharmacology, 1999. **32**(1): p. 51-55.
60. Thomsen, K., I. Rubin, and M. Lauritzen, *In vivo mechanisms of acetylcholine-induced vasodilation in rat sciatic nerve*. American Journal of Physiology - Heart and Circulatory Physiology, 2000. **279**(3): p. H1044-H1054.
61. Rodenwaldt, B., U. Pohl, and C. de Wit, *Endogenous and exogenous NO attenuates conduction of vasoconstrictions along arterioles in the microcirculation*. American Journal of Physiology - Heart and Circulatory Physiology, 2007. **292**(5): p. H2341-H2348.
62. Samim, A., et al., *Treatment of Angina and Microvascular Coronary Dysfunction*. Current Treatment Options in Cardiovascular Medicine, 2010. **12**(4): p. 355-364.
63. Bunker, A.K. and M.H. Laughlin, *Influence of exercise and perivascular adipose tissue on coronary artery vasomotor function in a familial hypercholesterolemic porcine atherosclerosis model*. J Appl Physiol, 2010. **108**(3): p. 490-497.

64. Dubrovská, G., et al., *Mechanisms of ADRF release from rat aortic adventitial adipose tissue*. American Journal of Physiology - Heart and Circulatory Physiology, 2004. **286**(3): p. H1107-H1113.
65. Arnet, U.A., et al., *Regulation of Endothelial Nitric-oxide Synthase during Hypoxia*. Journal of Biological Chemistry, 1996. **271**(25): p. 15069-15073.
66. Dodde, R.E., et al., *Thermal-Electric Finite Element Analysis and Experimental Validation of Bipolar Electrosurgical Cautery*. Journal of Manufacturing Science and Engineering, 2008. **130**.
67. Sato, K., et al., *The influence of radiofrequency ablation on hepatic vessels in porcine liver*. Hepatogastroenterology, 2005. **52**(62): p. 571-4.
68. Bulava, A., et al., *[Endothelial injury and activation of the coagulation cascade during radiofrequency catheter ablation]*. Vnitr Lek, 2004. **50**(4): p. 305-11.
69. Tsang, G.M., et al., *Chronic muscle stimulation improves ischaemic muscle performance in patients with peripheral vascular disease*. Eur J Vasc Surg, 1994. **8**(4): p. 419-22.
70. Kelsall, C.J., et al., *Arteriolar endothelial dysfunction is restored in ischaemic muscles by chronic electrical stimulation*. J Vasc Res, 2004. **41**(3): p. 241-51.
71. Colleran, P.N., et al., *Vasoresponsiveness of collateral vessels in the rat hindlimb: influence of training*. The Journal of Physiology, 2009. **588**(8): p. 1293-1307.

APPENDIX A

RESECTION PROTOCOL

Date _____	Hindlimb Ischemia Surgery - Resection	Initials _____
Mouse Information		
DOB: _____		
Sex: _____		
Tag: _____		
Genotype/strain: _____		
Cage: _____		
Materials		
Sterilize- autoclave or flash autoclave		
____ 1.	forceps (2)	
____ 2.	fine forceps (2)	
____ 3.	ultrafine forceps (1)	
____ 4.	fine scissors (1)	
____ 5.	microscissors spring loaded (1)	
Pre-sterilize in autoclave		
____ 6.	cotton gauze (2)	
____ 7.	cotton swabs (12)	
____ 8.	6.0 silk suture (2 x 1-inch)	
____ 9.	needle holder (1)	
Obtained in surgery suite		
____ 10.	sterile Petri dish w/ sterile saline	
____ 11.	sterile gloves	
____ 12.	sterile 7.0 prolene suture	
____ 13.	heat-cautery	
____ 14.	FST heat pad w/ rectal probe	
____ 15.	heat pad	
____ 16.	recovery bin & weigh boat	
____ 17.	depilatory cream	
____ 18.	non-sterile cotton swabs	
____ 19.	non-sterile cotton gauze	
____ 20.	isolation mask & cap	
____ 21.	analgesic (Buprenorphine)	
Surgery preparation		
____ 22.	Spray surgery area with Nolvasan	
____ 23.	Weigh animal in weigh boat	
____ 24.	Place animal in anesthesia box	
____ 25.	Open the oxygen cylinder and set anesthesia-machine flow meter to $\sim 3 \text{ l} \cdot \text{min}^{-1}$	
____ 26.	Anesthetize animal w/ 5% isoflurane	
____ 27.	Affix non-rebreathing circuit to bench-top with tape	
____ 28.	Reduce flow rate to $0.5\text{-}1.0 \text{ l} \cdot \text{min}^{-1}$ 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	
____ 35.	Apply 4x4 gauze sponge to heat pad to protect animal from excessive heat	
____ 36.	Affix non-rebreathing circuit to surgery table w/ chemistry clamp	
____ 37.	Lay animal supine on circulating heat pad w/ nose in nose-cone	
____ 38.	Insert rectal probe and set thermo-controller to 37°C	
____ 39.	Apply veterinary ointment to eyes to avoid drying during procedure	
____ 40.	Apply veterinary ointment to anus and place rectal probe $\sim 1 \text{ cm}$ into anus to monitor core-body temperature	
Surgery		
____ 41.	Make a small incision on the middle, medial aspect of the left thigh	
____ 42.	Extend the incision up to the abdominal wall	
____ 43.	Blunt dissect the subcutaneous connective tissue to maximize surgical exposure	
____ 44.	Use cautery to remove fat pad overlying femoral a-v pair & cauterize epigastric a-v pair	
____ 45.	Blunt dissect the femoral artery-vein pair from the nerve starting just upstream to the muscular branch & extending to half-way between the knee & ankle	
____ 46.	Tie off the saphenous a-v pair with 6.0 silk suture, halfway between the knee & ankle	
____ 47.	Tie off the femoral a-v pair with 6.0 silk suture, halfway just upstream to the muscular branch	
____ 48.	Grasping the distal ligature, use the cautery & microscissors to remove the a-v pair up to the upstream ligature	
____ 49.	Gently rest cotton swabs on hemorrhage sites	
____ 50.	Use 6.0 polypropylene suture to close the skin	
____ 51.	Make a small incision on the middle, medial aspect of the right thigh	
____ 52.	Extend the incision up to the abdominal wall	
____ 53.	Blunt dissect the subcutaneous connective tissue to maximize surgical exposure	
Post-Surgical		
____ 54.	Give the animal an subcutaneous injection of buprenorphine (0.075 mg/kg)	
____ 55.	Place the animal in the recovery bin, on a blue bench cover, above a heat pad and allow to recover	
____ 56.	Turn flow meter down to 0, turn off isoflurane, and close the oxygen cylinder	
____ 57.	Indicate surgery on cage card	
Notes		

Figure 37: Resection Protocol

APPENDIX B

DUAL FUNCTIONAL VASODILATION PROTOCOL

Date _____	Functional Vasodilation	Initials _____
Mouse Information		
DOB: _____		
Sex: _____		
Tag: _____		
Genotype/strain: _____		
Cage: _____		
Materials		
Instruments		
____ 1. forceps (2)		
____ 2. fine forceps (2)		
____ 3. ultrafine forceps (1)		
____ 4. fine scissors (1)		
Obtained in surgery suite		
____ 5. Petri dish w/ sterile saline		
____ 6. gloves		
____ 7. FST heat pad w/ rectal probe		
____ 8. heat pad		
____ 9. non-sterile cotton swabs		
____ 10. non-sterile cotton gauze		
Surgery preparation		
____ 11. Weigh animal in weight boat		
____ 12. Place animal in anesthesia box		
____ 13. Open the oxygen cylinder and set anesthesia-machine flow meter to $\sim 3 \text{ l}\cdot\text{min}^{-1}$		
____ 14. Anesthetize animal w/ 5% isoflurane		
____ 15. Reduce flow rate to $0.5\text{-}1.0 \text{ l}\cdot\text{min}^{-1}$ and the isoflurane to 1-3%		
____ 16. Lay animal supine with nose in nose-cone		
____ 17. Lay animal supine on circulating heat pad w/ nose in nose-cone		
____ 18. Insert rectal probe and set thermo-controller to 35°C		
____ 19. Apply veterinary ointment to eyes to avoid drying during procedure		
Measurement preparation		
____ 20. Make a small incision on the middle, medial aspect of the left thigh		
____ 21. Extend the incision up to the abdominal wall		
____ 22. Blunt dissect the subcutaneous connective tissue to maximize surgical exposure		
____ 23. Maximize visibility of muscular branch		
____ 24. Place wooden end of cotton swab on knee and secure to allow for isometric contraction and insure that the hind limb is level		
____ 25. Place black electrode on the center of the gracilis anterior and secure electrode		
____ 26. Place red ground electrode at the lateral aspect of the knee underneath the skin. Secure electrode.		
____ 27. Place mineral oil over exposed areas		
Microscan		
____ 28. Place microscan in stand and insure proper connectivity to computer.		
____ 29. Open AVA instruments and create new folder containing the date of procedure and make this the directory save location.		
____ 30. Select capture on AVA main menu for microscan imaging		
____ 31. Label patient I.D. with the number of patient first followed by exp for experimental hind limb or sham for control hind limb and frequency EX: 25exp200		
____ 32. Locate muscular branch and adjust microscan for best resolution.		
____ 33. Allow 30 minute time period to pass before taking measurement.		
____ 34. Capture video file of muscular branch to capture resting diameter		
____ 35. Open Lab Chart 6 open file in blood pressure measurement folder then open microscan default settings.		
____ 36. Set frequency to 8Hz, duration to $200\mu\text{s}$, and 1mA		
____ 37. Turn on stimulator panel		
____ 38. Stimulate muscle for 90 sec		
____ 39. Immediately capture video and continue to capture every two minutes for 10 minutes		
____ 40. Set frequency to 8Hz, duration to $500\mu\text{s}$, and 1mA		
____ 41. Turn on stimulator panel		
____ 42. Stimulate muscle for 90 sec		
____ 43. Immediately capture video and continue to capture every two minutes until vessel has returned to resting diameter		
____ 44. Perform same procedure on control limb		
Analysis		
____ 45. Open analysis section in AVA and open file of interest		
____ 46. Set Frames from 0 to 100 and stabilize file		
____ 47. Analyze vessel diameter by manually drawing diameter		
____ 48. Record results in provided table		
Post-Surgical		
____ 49. Cervical dislocation to euthanize animal		
Notes		

Figure 38: Functional Vasodilation Protocol (Page 1)

Date _____

Functional Vasodilation

Initials _____

Experimental Limb

File ID #:	Diameter
Resting: exp201	
PostStim: exp202	
exp203	
exp204	
exp205	
exp206	
File ID #:	Diameter
Resting: exp501	
exp502	
exp503	
exp504	
exp505	
exp506	
exp507	
exp508	
exp509	
exp510	

Control Limb

File ID #:	Diameter
Resting: sham201	
PostStim: sham202	
sham203	
sham204	
sham205	
sham206	
File ID #:	Diameter
Resting: sham501	
sham502	
sham503	
sham504	
sham505	
sham506	
sham507	
sham508	
sham509	
sham510	

Figure 39: Functional Vasodilation Protocol (Page 2)

APPENDIX C

SUPERFUSION PROTOCOL

Date _____	Intravital Microscopy with Superfusion	Initials _____
Mouse Information		
DOB: _____	_____ 34. Insert rectal probe and set thermo-controller to 37°C	
Sex: _____	_____ 35. Apply veterinary ointment to eyes to avoid drying during procedure	
Tag: _____	_____ 36. Open stopcock on delivery tubing to check flow rate of superfusion solution and use thermister to measure temperature	
Genotype/strain: _____	_____ 37. Adjust flow rate or temperature to achieve ~2ml·min ⁻¹ and ~35°C at tip	
Cage: _____		
Materials	Measurement preparation	
_____ 1. forceps (2)	_____ 38. Make a small incision on the middle, medial aspect of the left thigh	
_____ 2. fine forceps (2)	_____ 39. Extend the incision up to the abdominal wall	
_____ 3. ultrafine forceps (1)	_____ 40. Blunt dissect the subcutaneous connective tissue to maximize surgical exposure	
_____ 4. fine scissors (1)	_____ 41. Maximize visibility of muscular branch- minimize contact/trauma of the artery	
_____ 5. 60ml syringe		
_____ 6. Volumetric flask	Intravital Microscopy	
_____ 7. Side arm flask	_____ 42. Recheck flow rate and temperature of superfusion solution	
_____ 8. Kim wipes	_____ 43. Place microscan in stand and insure proper connectivity to computer.	
_____ 9. Vacuum pump evacuation flask	_____ 44. Open AVA instruments and create new folder containing the date of procedure and make this the directory save location.	
_____ 10. Vasodilator/vasoconstrictor	_____ 45. Select capture on AVA main menu for microscan imaging	
_____ 11. 20X PSS	_____ 46. Label patient I.D. with the number of patient first followed by left or right hind limb or sham for control hind limb.	
_____ 12. 20X NaHCO ₃	EX: 25LeftAch8	
_____ 13. non-sterile cotton swabs	_____ 47. Locate muscular branch and adjust microscan for best resolution.	
_____ 14. non-sterile cotton gauze	_____ 48. Position superfusion delivery tubing with ball-bearing manipulator at microscan lens to ensure flow over the artery	
_____ 15. 10ml Graduated cylinder	_____ 49. Place kim-wipe wick on thigh of animal (avoid contact with muscle)	
_____ 16. Stopwatch	_____ 50. Attach tubing from evacuation container/vacuum line to kim wipe	
Instrument Preparation	_____ 51. Use AVA to measure muscular branch diameter every 5 minutes until diameter stabilizes (~30 minutes)	
_____ 17. Turn on ultrasonic bath to 45°C		
_____ 18. Transfer 50ml of 20x PSS into 1L volumetric flask		
_____ 19. Transfer 50ml of 20x NaHCO ₃ into 1L volumetric flask		
_____ 20. Dilute PSS & NaHCO ₃ to 1L with 18MΩ H ₂ O		
_____ 21. Transfer 500 ml of 1X PSS to side-bar flask & place on stir plate		
_____ 22. Fill syringe in syringe heater with 50ml of 1x PSS & turn on syringe heater		
_____ 23. Place thermister in syringe heater		
_____ 24. Weigh out or thaw vasoactive agents		
Surgery preparation		
_____ 25. Weigh animal in weight boat		
_____ 26. Place animal in anesthesia box		
_____ 27. Open the oxygen cylinder and set anesthesia-machine flow meter to ~3 l·min ⁻¹		
_____ 28. Anesthetize animal w/ 5% isoflurane		
_____ 29. Reduce flow rate to 0.5-1.0 l·min ⁻¹ and the isoflurane to 1-3%		
_____ 30. Lay animal supine with nose in nose-cone		
_____ 31. Lay animal supine on preparation bench in nose-cone		
_____ 32. Use trimming clippers & depilatory cream to remove hair on the medial aspect of the hindlimb		
_____ 33. Transfer mouse to stage on FST heat pad		

Figure 40: Superfusion Protocol (Page 1)

Date _____	Intravital Microscopy with Superfusion	Initials _____
____ 52.	Ensure that 60ml syringe contains 60ml of PSS & close stopcock from side-arm flask	Limb 1
____ 53.	Add first dose of vasoactive agent	Left or Right _____
____ 54.	Allow superfusion to flow for 5 minutes and record video in the final minute	Resting Diameter _____
____ 55.	Clear the syringe	Vasoactive agent 1 _____
____ 56.	Open stopcock from side-arm flask and refill 60ml syringe to 60ml before re-closing	Dose 1 volume & concentration _____
____ 57.	Add second vasoactive agent	Dose 1 diameter _____
____ 58.	Allow superfusion to flow for 5 minutes and record video in the final minute	Vasoactive agent 2 _____
____ 59.	Clear the syringe	Dose 2 volume & concentration _____
____ 60.	Open stopcock from side-arm flask and refill 60ml syringe to 60ml before re-closing	Dose 2 diameter _____
____ 61.	Add third vasoactive agent	Vasoactive agent 3 _____
____ 62.	Allow superfusion to flow for 5 minutes and record video in the final minute	Dose 3 volume & concentration _____
____ 63.	Repeat procedure on the contralateral limb	Dose 3 diameter _____
Analysis		Limb 2
____ 64.	Open analysis section in AVA and open file of interest	Left or Right _____
____ 65.	Set Frames from 0 to 160 and stabilize file	Resting Diameter _____
____ 66.	Analyze vessel diameter by manually drawing diameter and chaining sections together	Vasoactive agent 1 _____
____ 67.	Record results in provided table	Dose 1 volume & concentration _____
Post-Experiment		Dose 1 diameter _____
____ 68.	Cervical dislocation to euthanize animal	Vasoactive agent 2 _____
____ 69.	Rinse superfusion line (bubbler, superfusion tubing, syringe, & side-arm flask) with water (flush tubing w/ syringe)	Dose 2 volume & concentration _____
____ 70.	Flush superfusion line with 1M HCl & rinse with water	Dose 2 diameter _____
____ 71.	Flush superfusion line with 18MΩ H ₂ O	Vasoactive agent 3 _____
		Dose 3 volume & concentration _____
		Dose 3 diameter _____
		Notes

Figure 41: Superfusion Protocol (Page 2)

APPENDIX D

SUPERFUSED FUNCTIONAL VASODILATION PROTOCOL

Date _____	Superfused Functional Vasodilation	Initials _____
Mouse Information		
DOB: _____		
Sex: _____		
Tag: _____		
Genotype/strain: _____		
Cage: _____		
Materials		
_____ 1.	forceps (2)	
_____ 2.	fine forceps (2)	
_____ 3.	ultrafine forceps (1)	
_____ 4.	fine scissors (1)	
_____ 5.	60ml syringe	
_____ 6.	Volumetric flask	
_____ 7.	Side arm flask	
_____ 8.	Kim wipes	
_____ 9.	Vacuum pump evacuation flask	
_____ 10.	Vasodilator/vasoconstrictor	
_____ 11.	20X PSS	
_____ 12.	20X NaHCO ₃	
_____ 13.	non-sterile cotton swabs	
_____ 14.	non-sterile cotton gauze	
_____ 15.	10ml Graduated cylinder	
_____ 16.	Stopwatch	
Instrument Preparation		
_____ 17.	Turn on ultrasonic bath to 45°C	
_____ 18.	Transfer 50ml of 20x PSS into 1L volumetric flask	
_____ 19.	Transfer 50ml of 20x NaHCO ₃ into 1L volumetric flask	
_____ 20.	Dilute PSS & NaHCO ₃ to 1L with 18MΩ H ₂ O	
_____ 21.	Check pH of diluted PSS solution	
_____ 22.	Bubble N ₂ gas into diluted PSS solution	
_____ 23.	Weigh out or thaw vasodilator/chemical	
Surgery preparation		
_____ 24.	Weigh animal in weight boat	
_____ 25.	Place animal in anesthesia box	
_____ 26.	Open the oxygen cylinder and set anesthesia-machine flow meter to ~3 l·min ⁻¹	
_____ 27.	Anesthetize animal w/ 5% isoflurane	
_____ 28.	Reduce flow rate to 0.5-1.0 l·min ⁻¹ and the isoflurane to 1-3%	
_____ 29.	Lay animal supine with nose in nose-cone	
_____ 30.	Lay animal supine on preparation bench in nose-cone	
_____ 31.	Use trimming clippers & depilatory cream to remove hair on the medial aspect of the hindlimb	
_____ 32.	Transfer mouse to stage on FST heat pad	
_____ 33.	Insert rectal probe and set thermo-controller to 35°C	
_____ 34.	Check pH of diluted pSS solution	
_____ 35.	Open stopcock on delivery tubing to check flow rate of superfusion solution and use thermister to measure temperature	
_____ 36.	Adjust flow rate or temperature to achieve ~2ml·min ⁻¹ and ~35°C at tip	
Measurement preparation		
_____ 37.	Make a small incision on the middle, medial aspect of the left thigh	
_____ 38.	Extend the incision up to the abdominal wall	
_____ 39.	Blunt dissect the subcutaneous connective tissue to maximize surgical exposure	
_____ 40.	Maximize visibility of muscular branch-minimize contact/trauma of the artery	
_____ 41.	Place black electrode on the center of the gracilis anterior and secure electrode	
_____ 42.	Place red ground electrode at the lateral aspect of the knee underneath the skin. Secure electrode.	
Intravital Microscopy		
_____ 43.	Recheck flow rate and temperature of superfusion solution	
_____ 44.	Place microscan in stand and insure proper connectivity to computer.	
_____ 45.	Open AVA instruments and create new folder containing the date of procedure and make this the directory save location.	
_____ 46.	Select capture on AVA main menu for microscan imaging	
_____ 47.	Label patient I.D. with the number of patient first followed by mus for experimental hind limb or sham for control hind limb. EX: 25mus	
_____ 48.	Locate muscular branch and adjust microscan for best resolution.	
_____ 49.	Position superfusion delivery tubing with ball-bearing manipulator at microscan lens to ensure flow over the artery	
_____ 50.	Place kim-wipe wick on thigh of animal (avoid contact with muscle)	
_____ 51.	Attach tubing from evacuation container/vacuum line to kim wipe	
_____ 52.	Use AVA to measure muscular branch diameter every 5 minutes until diameter stabilizes (~30 minutes)	
_____ 53.	Open Lab Chart 6 open file in blood pressure measurement folder then open microscan default settings.	
_____ 54.	Set frequency to 8Hz, duration to 500μs, and 1mA	
_____ 55.	Turn on stimulator panel	
_____ 56.	Stimulate muscle for 90 sec	
_____ 57.	Immediately capture video and continue to capture every two minutes until vessel has returned to resting diameter	
_____ 58.	Perform same procedure on control limb	

Figure 42: Superfused Functional Vasodilation Protocol (Page 1)

Date _____

Superfused Functional Vasodilation

Initials _____

Analysis

- ____ 59. Open analysis section in AVA and open file of interest
- ____ 60. Set Frames from 0 to 160 and stabilize file
- ____ 61. Analyze vessel diameter by manually drawing diameter and chaining sections together
- ____ 62. Record results in provided table

Post-Experiment

- ____ 63. Cervical dislocation to euthanize animal
- ____ 64. Rinse superfusion line (bubbler, superfusion tubing, syringe, & side-arm flask) with water (flush tubing w/ syringe)
- ____ 65. Flush superfusion line with 1M HCl & rinse with water
- ____ 66. Flush superfusion line with 18MΩ H₂O

Limb 1

- Vasoactive agent _____
- Pre-stimulation diameter _____
- Post-stimulation diameter _____
- 2 min post diameter _____
- 4 min post diameter _____
- 6 min post diameter _____
- 8 min post diameter _____
- 10 min post diameter _____
- 12 min post diameter _____

Limb 2

- Vasoactive agent _____
- Pre-stimulation diameter _____
- Post-stimulation diameter _____
- 2 min post diameter _____
- 4 min post diameter _____
- 6 min post diameter _____
- 8 min post diameter _____
- 10 min post diameter _____
- 12 min post diameter _____

Notes

Figure 43: Superfused Functional Vasodilation Protocol (Page 2)

APPENDIX E

SDF IMAGING ANALYSIS USING AVA SOFTWARE

In order to produce accurate measurements, the MicroScan was first calibrated according to the manufacturers specifications. For the data collection portion, the MicroScan was placed on a horizontal plane. This was usually accomplished through secure placement in the boom stand. It was also important for the MicroScan to not be in contact with the profunda femoris, but to be as far above it as possible while keeping the vessel in focus.

In order to record a video, AVA 3.0 was first opened which led to a main menu screen (**Figure 44**). After selecting the “data” button, the specific folder where the videos were saved was chosen. The “capture” button was then selected and specific information regarding the experiment was entered (mouse tag, sham or exp, etc.). Videos were taken for 10 seconds by pressing the “start capture” button. These videos were automatically saved in the folder that was determined earlier in the process.

For data analysis, selecting the “analysis” button opened a video file (**Figure 45**). Once the specific video file was chosen, sliding the white bars at the bottom of the screen chose the number of frames. Then, the “stabilize video” button was chosen and the result was a video file that stabilized and accounted for the breathing of the mouse. Then, the outline of the profunda femoris artery was carefully traced and the average diameter was recorded. This process of analysis was repeated for each video file and was performed the same way each time.

Figure 44: AVA

(A) Front screen where options to create folders [Settings], create videos for analysis [Capture] and analyze videos to create data points [Analysis]. (B) Once selecting “Capture” the menu allows for creation of videos with [Start capture]

Figure 45: Image Analysis

(1) Stabilize image before analysis (2) Draw outline of artery (3) Example of artery outline (4) Delete outline of drawing if not accurate on vessel wall (5) Average diameter measurement