Impaired Resistance Artery Reactivity Following Arteriogenesis

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“Talent is a dull knife that will cut nothing unless it is wielded with great force.”

Stephen King, *Danse Macabre*
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Patients suffering from tissue hypoxia and claudication due to arterial occlusion, such as those dealing with Peripheral Arterial Occlusive Disease (PAOD), have been the target of numerous therapeutic strategies over the past few decades. These patients demonstrate inadequacies both in the volume of their collateral circulation as well as in the ability of the vessels to dilate in response to increases in metabolic demand. While much research has looked into increasing the size of the collaterals, an alternative strategy includes exogenous manipulation of the vasodilatory capacity of the vessels. It has been fairly well established that there is a change in vasoactivity in response to elevated shear stress and during the outward remodeling phase, but the extent of this impairment as well as the pathways responsible for it are poorly understood. Therefore, an effective remedial strategy would include restoration of typical vasoactivity to the collaterals while they undergo arteriogenesis. This study aimed to determine the impact of vascular occlusion on vascular reactivity through femoral artery ligation and functional vasodilation assessments. Following ligation of the artery, luminal diameter measurements were taken in the muscular branch artery at 3 and 7 days post-occlusion using intravital microscopy. At the day 3 time point, there is no difference between the resting diameters (45.9±10.92 µm control, 57.13±14.43 µm experiment) nor between the dilated diameters (101.04±29.77 µm control, 104.72±20.76 µm experiment). One week following the ligation, there is a difference between the control and experiment diameters both at resting (50.19±12.36 µm control, 67.47±13.51 µm experiment) and dilated (95.95±16.45 µm control, 119.05±18.59 µm experiment). Finally, a comparison of the percent change over resting diameter between the two time points trends toward less reactivity in the experimental limbs, but with no statistical significance, suggesting that the vasomotor tone is not impaired. Due to slight variations between this research and previous work, further investigation is requisite to establish solid evaluations of vascular reactivity in response to arterial occlusion.
Introduction

Critical Limb Ischemia (CLI) is characterized by chronic resting pain, claudication, superficial ulcers, and/or gangrene directly related to arterial occlusions. These occlusions are the result of atherosclerotic plaque deposits on the lumen of conduit arteries. While these depositions can be attributed to atherogenesis, advanced stages of this response due to acute and severe endothelial inflammation can force the progression of plaque buildup to a critical state. At this state, the lumen becomes completely occluded and blood is rerouted through the collateral circulation. The physiological consequence of such a diseased state is that the target tissue becomes hypoxic. This deficiency in metabolic exchange and the formation of multiple acute atherosclerotic lesions are indicative of Peripheral Arterial Occlusive Disease (PAOD). Major risk factors for this disease include smoking, high cholesterol, diabetes, high blood pressure and genetic predisposition. Because of the many factors that contribute to the development of this disease, it is estimated to affect between 8-10 million Americans, and the prevalence is expected to rise worldwide with the aging population.

The etiology of PAOD begins with the formation of atherosclerotic plaque (see Figure 1). The buildup of this plaque is due to the presence of turbulent shear stress at the vessel wall, leading to an activated endothelium. When major conduit arteries become chronically stenosed to the point that downstream tissue cannot receive adequate blood flow, collateral vessels begin to dilate and grow in order to restore blood flow (see Figure 2). This process is known as arteriogenesis.

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Figure 1 - Cross-sectional view of arterial lumen being occluded by plaque deposits. Atherogenesis leads to the narrowing of arteries due to the buildup of this atherosclerotic plaque.

Figure 2 - Progression of Arteriogenesis from occlusion to collateral enlargement.
a sufficient amount of nutrients, the tissue will become necrotic unless collateral vessels can support the change in flow conditions.

The current methods for treating PAOD patients suffering from CLI include surgical bypass, endovascular procedures and in extreme cases amputation. Specific treatment options can range from non-invasive techniques such as prescribing an exercise regimen to more extreme methods such as surgical intervention including aortofemoral bypass surgery and angioplasty. Treatment decisions for these patients are made on an individual basis and take into account the patient’s age, progression of the disease, surgical risk and life expectancy. Currently, there have been no efficacious demonstrations of pharmacologic or biologic therapeutic treatments for the disease. Therefore, there is a dire necessity for the development of a biological- and/or physiological-based treatment.

A physiological adaptation to arterial occlusions is the enlargement of collaterals due to increases in blood flow and shear rate through the collateral circuit (see Figure 2). These increases are due to a reduction in distal pressure, allowing for a higher pressure gradient throughout the alternate pathways. Arteriogenesis is characterized by the formation of functional collateral arteries arising from pre-existing arterio-arteriolar anastomoses. It is important to note that that morphological

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Figure 3 - Anatomy of artery, vein and capillary.

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Figure 2 - Anatomy of artery, vein and capillary.
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Changes to these collateral vessels is solely due to increased shear stress and enhanced pressure gradients. Tissue ischemia does not appear to affect arteriogenesis, partly because the vessels that are collaterally enlarging are typically not surrounded by hypoxic tissue. To fully appreciate this vascular response, it is necessary to understand the anatomy of the vessels themselves and the pathways responsible for vascular reactivation. Vasoactivity is determined by the smooth muscle cell layer, which responds to signals from the inner endothelial cell layer, neurons and hormones (see Figure 3). This layer of smooth muscle cells is much thicker in the arterial than the venous system (see Figure 4), and is a large contributor to changes in blood pressure and flow control. This endothelial cell layer is the innermost tunic, and is the only portion of the vessels in direct contact with blood and blood components. Once these cells experience an increase in shear stress and an elevated pressure gradient during arteriogenesis, they release paracrine signals to initiate the remodeling process.

The requisite pathways for vasodilation can arise through endothelial cell activation, nervous stimulation, interstitial metabolites, endocrine factors and changes in vascular pressure. For example, The release of nor epinephrine from local neurons will result in smooth muscle cell activation and ultimately, vasoconstriction. Similarly, the synthesis of nitric oxide (NO) from nitric oxide synthase (NOS) produced in the endothelium will result in smooth muscle cell relaxation and vasodilation.

It has been postulated that exogenous manipulation of vessel reactivity could have a positive effect on PAOD. Therapeutic arteriogenesis could relieve some of the symptoms caused by CLI by stimulating collateral enlargement, effectively increasing the size of available alternative flow paths. Some studies have suggested the use of growth factors such as granulocyte-macrophage colony-stimulating factor to stimulate the growth of certain collaterals. However, this therapeutic strategy has largely failed clinically due, in part to an inadequate understanding of the timeline surrounding the interplay between collateral enlargement and vascular reactivity.

In order for therapeutic arteriogenesis to be an effective remedial treatment strategy, it is foremost important to develop an understanding of the chronology.
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of vascular growth and repair. The time course for collateral growth has been established by Glass et al. and Hoefer et al. to be 10 days post occlusion. However, there is no indication of the extent to which the arterioles have enlarged. In particular, it is unclear whether this time period of 10 days is sufficient to allow these alternate flow paths to remodel to the point at which they completely mimic a mature vessel.

In addition to a chronological assessment of vascular repair, it is essential to understand the transient impairment to vascular reactivity in response to arterial occlusion. It has been observed that patients experiencing large occlusions resulting in reduced metabolic exchange at the capillary level exhibit a reduction in vasoactivity. This offers a unique therapeutic strategy—to restore or enhance the natural vasoreactivity of the blood vessel, that it may react to the changes in metabolic demand more effectively. To develop such a strategy however, requires an enhanced understanding of the mechanisms for abnormal vasoactivity in response to arterial stenosis.

The Microcirculation and Tissue Repair lab at Cal Poly has investigated a number of mechanisms to explain this reduction in vasoactivity, including surgical models assessed using brightfield microscopy, microscan equipment, and histological analysis. Some groups have considered ischemic surgical models to characterize both angiogenesis and arteriogenesis. The result of atherogenesis is a decrease in vascular activity at 7 and 14 days post occlusion. The focus of this paper is to delineate the time course of the decrease in vasoactivity due to the onset of collateral enlargement. To accomplish this, an additional time point (3 days post ligation) has been added to gain a better understanding of the remodeling process throughout a recovery period of 2 weeks.

It is hypothesized that a significant degree of collateral enlargement will have occurred one week following the ligation, and that the dilative ability of the vessel (percent increase from resting) will not differ from a control vessel. At day 3 however, we expect to see no significant change in luminal diameter, but a statistical difference between the experiment and control vessel’s ability to respond to changes in metabolic demands.

Methods

Husbandry

Two-month-old male C57Bl/6 mice were used for all time points in this study. All husbandry procedures maintained through the course of this research strictly adhered to The Guide for the Care and Use of Laboratory Animals (commonly referred to as “The Guide”) per National Institute of Health (NIH) regulations, Office of Laboratory Animal Welfare (OLAW) mandates and all Institutional Animal Care and Use
Committee (IACUC) protocols. Each mouse was housed together in groups of four inside a microisolator cage located in the Vivarium at Cal Poly. Food pellets and water were provided ad libitum along with enrichment items such as a house, tubes, and nesting material. Cages were inspected daily for mouse health and mold accumulation and were changed using aseptic technique, on average, every two weeks. The room housing the microisolator cages for all of the mice was maintained between 68-72 °F and was set to a 12 hour light/12 hour dark schedule. Following the initial survival surgery, the animals were housed in a separate cage to discourage barbering and other detrimental behavior.

**Surgical Procedures**

Two distinct procedures, including a surgery and a dissection, were performed throughout this study (the detailed protocol for each can be found in Appendices A and B, respectively). Each mouse included in the study first underwent a proximal saphenous artery ligation followed by an intravital microscopy procedure at a given time point later (either 3 or 7 days).

In preparation for the ligation surgery, a sterile pack along with the surgical instruments was placed in an autoclave. The mouse was then placed into an induction chamber and anesthetized with a 1-3% Isoflurane/Oxygen gas mixture at a flow rate of 0.5-1.0 LMin⁻¹. Once the mouse was determined to be at a surgical plane of anesthesia (respiratory rate of 1-2 breaths per second), the anesthetic gas was redirected to the surgical prep bench and the animal was placed in a supine position with its nose inside the nose cone (see Figure 5). The surgical area was prepared by first removing the hair on the medial aspect of both hindlimbs using hair clippers followed by administration of depilatory cream. Once clear of all hair, the hindlimbs were sanitized using Nolvasan and aseptic technique. The anesthetic gas was then redirected to the surgical bench and the animal was placed on a large gauze pad and laid on top of a heat pad under the brightfield microscope. A rectal thermometer was inserted to provide internal body temperature feedback to the heat pad to maintain

![Figure 5](image-url) - Surgical setup showing mouse in supine position with hindlimbs taped apart and nose directed into the nose cone to receive a continuous supply of anesthetic gas.
a temperature close to 35 °C. Additionally, PuraLube® Veterinary Ointment was administered to each eye to prevent desiccation. Both hindlimbs were outstretched and fixed to the gauze pad using medical tape. Once under the dissection scope, appropriate sterile clothing was donned (including mask, hair net, sterile gloves and isolation gown/surgical scrubs) and the sterile pack and instruments were opened.

The first incision was made on top of and parallel to the saphenous neurovascular bundle using standard pattern forceps and iris scissors. The incision was extended from near the knee up to the fat pad and blunt dissected using the S&T forceps. Note that a solution of sterile Phosphate Buffered Saline was continuously administered to the surgical site to prevent desiccation. Once adequately exposed, all connective tissue including the fat pad was blunt dissected to ensure optimal viewing of the ligation site. It was then necessary to remove the entire fat pad. To accomplish this, the adipose was dissected away from the abdominal wall and on either side of the epigastric artery/vein pair and was then cauterized. The saphenous artery was then isolated from the vein and nerve using a pair of 5-45 forceps. Once adequately dissected, a suture was tied around the vessel with a double knot. The incision was then sewn closed with 6-0 polypropylene suture using a spiral-pattern suture technique, and was anchored at both ends with surgeon’s square knots.

A sham surgery was then performed on the contralateral limb in order to provide adequate control measurements. This surgery consisted of the same incision above and parallel to the saphenous neurovascular bundle, blunt dissection of all connective tissue (excluding cauterization and resection of the fat pad) and wound closure using the spiral-pattern suture method.

Following this surgery, a subcutaneous injection of an analgesic (buprenorphine, 0.075 mg/kg) was administered. The animal was placed in a heated recovery bin until ambulatory, at which time it was placed in a new microisolator cage in the Vivarium.

The follow-up experiment for these animals included analysis of vascular diameter in the muscular branch using intravital microscopy. This imaging technique uses sidestream darkfield imaging to observe details on the order of a couple hundred micrometers.

Depending on the time point of the study, the animals were allowed to recover for a period of 3 or 7 days. At this point, each mouse was brought back to the surgery suite, anesthetized and the surgical area prepared as previously described. Each hindlimb was opened to sufficiently expose the muscular branch. While still under the brightfield microscope, two (stimulus and ground) tungsten needle electrodes were set in place. The positive lead was inserted under the skin or into the adipose tissue and acted as the ground. The negative lead was rested on the tissue surface, orthogonal to the motor
end plate of the gracilis muscle (this effectively creates a negative extracellular potential, forcing an action potential within the muscle fibers). Using LabChart software (AD Instruments), a test excitation was performed to ensure accurate placement of the electrodes. After administering enough mineral oil to coat the exposed area, the MicroVision Medical MicroScan scope was set in place on top of the profunda. Following microscope placement, a small amount of plastic wrap was placed over the exposed tissue and a rest period of 30 minutes was taken to provide ample time for the vasculature to return to resting state.

An initial 10s video of the muscular branch artery was taken using MicroVision Medical AVA software. The gracilis muscle was then stimulated for 90s at a frequency of 8Hz, duration of 500µs and amplitude of 1mA. Immediately following this stimulation, an additional video was recorded of the profunda artery along with subsequent videos every 2 minutes until the vessel returned to baseline. For each video taken, a stabilized video was constructed which filtered out the respiratory movement of the animal. Using this stable video, intraluminal diameter measurements were taken for the resting and stimulated vessel along with measurements as the vessel returned to its resting state.

The decision to perform the MicroScan measurements first on the experimental or sham limb was made at random to increase the robustness of the research. Immediately following this procedure, the animal was sacrificed via cervical dislocation.

Data analysis was performed using Microsoft Excel. The data set for each individual surgery (including resting, dilated, and return to resting diameters for sham and experimental limbs) was imported into a spreadsheet. The mean, standard deviation, standard error, and percent change from resting values were calculated for each hindlimb. Using these values, a student’s t-test was performed to examine significant differences between the data sets. A p-value ≤ 0.05 represented a statistically significant difference between groups with a confidence level of 95%. The data was graphically represented using a bar chart including error bars representing ± standard deviation.

Results

To ensure proper surgical technique throughout all aspects of this procedure (including saphenous artery ligation and microscan measurements), an initial D7 pilot study was performed. Additionally, this study allowed for verification of the collected data set against previously performed intravital measurements thought to be accurate. In this way, the data collected can be translated across many similar studies to suggest trends and show significance without the danger of using inaccurate diameter measurements.
The pilot study presented in this paper was conducted in a similar manner to the d7 experiments performed by another member of the Microcirculation lab at Cal Poly, including operation on both the sham and “experimental” limb (no ligation placed around the artery), a recovery period of 7 days and stimulation and measurement of the luminal diameter for both hindlimbs. There was no statistical difference between the control and experimental resting diameters or between the control and experimental functional vasodilation (see Figure 6). This is to be expected, as there was no experimental difference between either limb for this study. Additionally, there was a significant difference between the resting and dilated valued for both sham and experimental limbs. These results support previous diameter measurement studies, which have found a typical stimulated luminal diameter to be 200% of resting (see Figure 7). A typical stimulation and relaxation curve (see Figure 8) for the muscular branch artery shows an initial, statistically significant spike in luminal diameter followed by a gradual return (on the order of 5-8 µm min\(^{-1}\)) to resting diameter over a period of 8-12 minutes.

Upon verification of gathering reproducible data, vessel functionality following occlusion was investigated. Two time points were investigated – 3 and 7 days.
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post ligation. Using the intravital microscope, luminal diameter measurements for six induced ischemic animals was collected.

The protocol for this study consisted of ligation placement in the experimental limb and a sham surgery for the control. Following one week of recovery time, an intravital microscopy procedure was performed for both hindlimbs (see Figure 9). This study showed a significant difference between the experimental and control resting diameters with a p-value of 0.0434 (control 50.19±12.36 µm, experimental 67.47±13.51 µm). Likewise, there was a significant difference between the experimental and control dilated diameters with a p-value of 0.0458 (control 95.95±16.45 µm, experimental 119.05±18.59 µm). Both of these differences are indicative of vascular remodeling due to the ligation over the recovery period of 7 days.

As an additional check to ensure consistency among the data sets, a comparison of the percent change from resting diameter for resting and dilated diameters at both D3 and D7 was prepared. The data shows that the muscular branch in the experimental limbs at both time points was able to dilate to roughly 90% over the resting value (see Figure 12). Although not statistically different from the experimental limbs, the control limbs for both time points was able to dilate to a greater extent of nearly 120% above resting values. This trend showing
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A difference in vasoreactivity suggests that, due to the remodeling process occurring post-ligation, the vessels are less able to react to changes in metabolic demand than non-treated vessels.

Following the D7 procedure, an additional time point was necessary to assess vascular remodeling and behavior trends throughout the process of collateral enlargement. Because later time points (D14, D28) had already been characterized or were currently under investigation, a day 3 model was utilized. This model mimicked the previous protocol for arterial ligation and microscan measurements, however, following the initial ligation surgery the animal was allowed a recovery period of 3 days. The results of this study showed no statistical differences between the sham and control limbs (see Figure 10). The control resting (45.9±10.92 µm) and the experimental resting (57.13±14.43 µm) diameters were not different, with a p-value of 0.1596. However, the data does show a trend that the luminal diameter is beginning to enlarge 3 days following upstream ligation. The control dilated (101.04±29.77 µm) and the experimental dilated (104.72±20.76) diameters also exhibited no statistical significance between the groups. This lack of statistical significance shows that at 3 days following occlusion of a feed artery, collateral enlargement has not begun or progressed to the point at which there is a significant difference between the vessel diameters.
Discussion

Upon characterization of vessel phenotype and reactivity at both time points, a comparison between each group was made. This assessment of the relationship between the D7 and D3 studies provides a more robust and coherent representation of the remodeling process (see Figure 11). An initial comparison of the control limbs was made between both time points to establish a sense of congruency throughout the data. There was no significant difference between the 3-day and 7-day resting and dilated diameters for the control limbs. Likewise, there was no statistical difference between the time points for the experimental limbs. Although the data did not support any statistical significance, there are slight trends suggesting enlargement due to remodeling. Specifically, the luminal diameter for the experimental resting limb at D3 is less than the diameter for the same limb at D7 (~10µm). Likewise, the D7 experimental dilated diameter was larger than the diameter at D3. Both of these observations, while not significant, show a trend toward increasing luminal diameter at later time points. This trend is to be expected, as an increased period of remodeling will of course lead to more robust collateral enlargement.

Arterial occlusion leading to tissue hypoxia and ischemia is a complex disease that ultimately results in vascular dysfunction, both in terms of vascular growth and vasoreactivity to changes in metabolite concentration.12 While many current investigations into PAOD are

Figure 11 - Comparison of Day 3 and 7 time points. There was no statistical difference between the minimum and maximum diameters for the profunda.

Figure 12 - Mean luminal diameter percent change from baseline for both sham and experimental limbs. There was no significant difference between the dilative capacity of the vessel at both time points.
concentrating on establishing protocols for increasing vascular growth and repair, few researchers are looking into the possibilities of treatments surrounding enhanced vasoreactivity.\textsuperscript{13,14} These changes in vasomotor tone have been shown to dramatically affect the recovery from an ischemic event.\textsuperscript{3} To evaluate the extent to which vasoactivity effects an individual’s ability to handle vascular occlusions, this study investigates abnormal vasoactivity due to upstream occlusion in order to form the foundational knowledge for the eventual development of therapeutic strategies to treat PAOD.

It was initially necessary to establish consistent data collection methods to ensure that the information did not vary from researcher to researcher. This was accomplished through the use of a pilot study, which provided practice in collecting data through measurement of luminal diameter in the muscular branch using intravital microscopy. The results of the pilot study showed no statistical difference between the limbs along with a luminal diameter percent change that was consistent with previous work. Because of the reliability of this data, it can be assumed that the methods employed for data collection accurately adhere to the provided protocol, and that any differences found in the future studies are due to the physiological response to the ligature and not experimental error.

The following study included collection of luminal diameter measurements in the profunda of the left hindlimb 7 days following the ligation of the proximal saphenous artery. Upstream occlusion of feed arteries such as the saphenous artery lead to the shunting of blood through alternate or collateral pathways. This increase in mass flow rate, along with changes in the pressure gradient, lead to the enlargement of the cross-sectional area of the lumen of these collateral arteries. This study showed a statistically significant difference between the resting values of the experimental and control limbs (p-value of 0.0434). There was also a significant difference between the dilated diameters for the experimental and control limbs (p-value of 0.0458). These differences show that one week following occlusion of upstream arteries, the vessel has begun to remodel and enlarge to handle the changes in shear stress.

Although this study does correlate with previous D7 studies and confirm the incidence of collateral enlargement at this time point, there is a major difference between the confidence levels for each study. The previous work on collateral enlargement 7 days post ligation concluded that there was indeed a statistical difference between the luminal diameters for the experimental and sham limbs, but with a p-value of 4.997E-6. This suggests that we can be more than 99\% confident that collateral enlargement has taken place. However, the p-value obtained in this study (~0.045 for both resting and dilated diameters) suggests only 95\% confidence. This incongruence between the data can be attributed to
slightly inconsistent surgical methods between researchers, both for the ligation and the microscan surgeries. Until the completion of this investigation, each experimenter followed the surgical protocol under his/her own discretion. Upon observation of the variation in the data sets between the same experiments, it became necessary for each person performing a study to scrutinize others’ surgeries. By doing so, it is possible to obtain information about minute details of the surgery that each person is inadvertently performing differently. For example, it was observed that there were inconsistencies between researchers in the method and order of electrode placement on the gracilis muscle; sometimes the electrodes were placed using the brightfield microscope to ensure correct placement, or they were pressed too firmly on top of the tissue. Likewise, there were discrepancies with how proximal a location the ligature was placed and how well the incision was sutured closed. Each of these irregularities between researchers could lead to decreased collateral enlargement, possibility of infection, and decreased functional vasodilation.

In addition to dissimilarities between discrete surgical procedures across researchers, variations in the stimulation parameters led to distinct dilation responses. In terms of the resection surgery, a pulse duration of 200 µs resulted in no dilation response whereas a 500 µs duration led to a robust response when performed on separate animals. If both of these parameters were tested on the same animal, the larger pulse duration led to a negligible dilation response. Whether or not this (lack of) response was due to muscle fatigue is poorly described, but did provide us with an appropriate pulse duration of 500 µs for use in this study.

Finally, a time point of 3 days post-occlusion was included. The protocol for this study mimicked that of the D7, except that the recovery period was shortened to 3 days following the initial surgery. The results of this study only partially supported the initial hypothesis. There was no evidence of a statistical difference between the experiment and sham limbs for either the resting or dilated measurements (p-value of 0.1596 and 0.8092, respectively). This information does support the initial estimate that there would be no observable or significant change in vessel cross-sectional area at this time point. However, there was also no evidence of a difference between the vasoactivity of the experiment and control vessels, suggesting that the experimental vessel (which should be enlarging due to increases in shear stress) is still able to function similar to a typical vessel. Based on this information, it is appropriate to conclude that 3 days following occlusion of feed arteries, collateral vessels are only beginning to react to the change in flow conditions; a negligible amount of collateral enlargement has occurred in these vessels, and the vasoactivity of the collateral has been preserved.
Based upon previous work, the collateral vessels supplying the gracilis muscle have a dilative capacity close to 200% that of resting. The data collected for this study relating to the D3 and D7 time points both exhibited close to 220 percent change from resting. This 20% disparity can most likely be attributed to recruitment of extraneous muscle groups. Specifically, muscles in addition to the gracilis (such as the gastrocnemius, and/or the abductors and adductors on the posterior and anterior aspects of the hindlimb, respectively). This only reinforces the importance of accurate and precise electrode placement on the hindlimb. As previously stated, these slight discrepancies between this study and previous studies necessitate the development of standard surgical and experimental practice across researchers, ensuring consistent data.

The experimental limbs both demonstrated a slight reduction in the percent change from resting. Although not statistically significant, this reduction to near 180% of resting diameter reveals a trend that suggests a retarded ability of the ligated vessel to dilate in response to changing metabolic demands. This impaired vasodilation is consistent with our knowledge of the arteriogenic process, but the extent to which the vasoactivity is compromised is still questionable. Previous studies have demonstrated a reduction to 140% change from resting diameter for the vessels at the D7 time point. Again, this inconsistency can be correlated to imprecise stimulation of the gracilis muscle. As such, further research is necessary to establish any sound evidence of a statistically significant change to vasoactivity of the vessels.

Additional research is necessary to establish an appropriately complex and detailed model of the inhibition to vascular reactivity in response to luminal occlusion. Specifically, additional time points need to be incorporated to provide adequate representations of the progression of the diseased state, along with varying methods for initiating a vasodilative response.

Because the D3 time point showed no significant change in the luminal diameter along with no change to ability of the vessel to dilate, a D5 study is recommended to study the changes in vascular morphology and physiology between 3 and 7 days post occlusion. Additionally, the progression of arteriogenesis should be observed past one week. A two and three week time point is suggested to provide adequate representations of the progression of the vessel from occlusion to complete repair. With the addition of data from these studies, it would be possible to establish a robust description of the changes to vascular reactivity from onset to complete maturation.

At each of these time points, it is also necessary to understand how variations in the mechanism for initiating vasodilation affect the inhibition to vasoactivity. The electrical stimulation of the gracilis muscle used
in this study should be carried out at each of the other time points to create an all-inclusive study of the effect of electrode stimulation directly to the gracilis muscle on the vasoactivity of a muscular branch experiencing increases in mass flow rate. Akin to this study, direct stimulation of the nerve that innervates the gracilis muscle would provide nice contrast from the previous needle electrode stimulation study. To be consistent, 3, 5, 7, 14 and 21 days post-occlusion studies should be conducted using nerve stimulation. If the discrepancies between previous work were indeed due to issues with electrode placement, then stimulation of the nerve should eliminate these errors and provide a more accurate picture of the arteriogenic process in the profunda.

Along with direct electrical stimulation of the gracilis muscle or the nerve innervating this muscle, the effects of chemical stimulation of vasodilation should be studied. Specific pharmacologic agents to be used include acetylcholine (Ach), which is an endothelium-specific agent that results in vasodilation by stimulating the production of nitric oxide (NO) from endothelial nitric oxide synthase (eNOS), and sodium nitroprusside (SNP), which facilitates smooth muscle relaxation by donating NO to the smooth muscle cell receptors. Both of these chemical agents should be included in day 3, 5, 7, 14 and 21 superfusion studies. These studies will mimic the surgeries and microscan experiments conducted in the mechanical stimulation studies, but will replace the electrodes with these Ach or SNP. These agents will be administered using a superfusion setup that will provide a constant flow of Ach or SNP over the exposed portion of the hindlimb while the measurements are being taken.

The combination of both electrical and chemical stimulation of vasodilation over each of the established time points will provide detailed model of arteriogenesis due to arterial occlusion and aid with the development of strategies to manipulate these pathways to promote collateral enlargement and the appropriate magnitude of vasodilation.

Conclusion

It is important to realize that the animal models used in this research were both young (around 2 months old) and healthy. This issue is critical because the majority of patients suffering from PAOD or other types of extreme atherosclerotic occlusion disorders are typically a part of the aging population. Use of young and healthy animals models is an excellent first attempt at understanding the mechanisms for vasomotor disorder during arteriogenesis, but it lends itself to further research. Specifically, observation and characterization of vascular reactivity in other non-healthy animal models would provide sufficient data to extrapolate the behavior of the vessels over a wider range of disease states. Because all of the data collected in this study
came from surgical models of arterial occlusion, future animal models should include some genetic modeling, such as apolipoprotein E $^{-/-}$ or LDL receptor $^{-/-}$.

Due to the high prevalence of chronic limb ischemia and PAOD in elderly patients, the development of novel alternative treatments is a necessity. Potential remedies include therapeutic arteriogenesis, which has largely failed clinically, or manipulation of vasomotor behavior to increase perfusion to ischemic tissues. An even more effective treatment strategy would include a combination of both of these methods, however a more complete understanding of the pathways resulting in changes to vascular reactivity is imperative. This research showed a decrease in vasomotor tone during arteriogenesis and as such, provides an excellent stepping stone for future work in manipulating vasodilation to compensate for arterial occlusion.
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Appendix A

Date__________________________

Hindlimb Ischemia Surgery - Ligation

Initials______________________

Mouse Information
DOB:__________________________
Sex:___________________________
Tag:___________________________
Genotype/strain:________________
Cage:__________________________

Materials
Sterilize- autoclave or flash autoclave
1. Standard pattern forceps (1)
2. S&T forceps (2)
3. 5-45 forceps (1)
4. Iris scissors (1)
5. Microdissection scissors (1)
6. Castroviejo (1)
7. Retractor (1)

Pre-sterilize in autoclave
8. cotton gauze (2)
9. cotton swabs (12)
10. 6.0 silk suture (2 x 1-inch)

Obtained in surgery suite
11. sterile Petri dish w/ sterile saline
12. sterile gloves
13. sterile 7.0 prolene suture
14. heat-cautery
15. FST heat pad w/ rectal probe
16. heat pad
17. recovery bin & weigh boat
18. depilatory cream
19. non-sterile cotton swabs
20. non-sterile cotton gauze
21. isolation mask & cap
22. analgesic (Buprenorphine)

Surgery preparation
23. Spray surgery area with Nolvasan
24. Weigh animal in weight boat
25. Place animal in anesthesia box
26. Open the oxygen cylinder and set anesthesia-machine flow meter to ~3 l·min⁻¹
27. Anesthetize animal w/ 5% isoflurane
28. Affix non-rebreathing circuit to bench-top with tape
29. Reduce flow rate to 0.5-1.0 l·min⁻¹ and the isoflurane to 1-3%
30. Apply ear tag high on left ear
31. Lay animal supine with nose in nose-cone
32. Shave hair on the right hindlimb & lower abdomen with clippers
33. Remove excess hair with depilatory cream
34. Spray right hindlimb with Nolvasan
35. Return animal to anesthesia box
36. Apply 4x4 gauze sponge to heat pad to protect animal from excessive heat
37. Affix non-rebreathing circuit to surgery table w/ chemistry clamp
38. Lay animal supine on circulating heat pad w/ nose in nose-cone
39. Insert rectal probe and set thermo-controller to 37°C
40. Apply veterinary ointment to eyes to avoid drying during procedure
41. Apply veterinary ointment to anus and place rectal probe ~1cm into anus to monitor core-body temperature
42. Make a small incision on the middle, medial aspect of the left thigh
43. Extend the incision up to the abdominal wall
44. Blunt dissect the subcutaneous connective tissue to maximize surgical exposure
45. Use cautery to remove fat pad overlying femoral a-v pair & cauterize epigastric av-pair
46. Blunt dissect the femoral artery from the neurovascular bundle just downstream from the deep femoral branch
47. Tie off the femoral artery & vein with 6.0 silk suture, just downstream to the deep femoral branch
48. Use 6.0 polypropylene suture to close the skin
49. Make a small incision on the middle, medial aspect of the right thing
50. Extend the incision up to the abdominal wall
51. Blunt dissect the subcutaneous connective tissue to maximize surgical exposure
52. Use 6.0 polypropylene suture to close the skin
53. Give the animal an subcutaneous injection of buprenorphine (0.075mg/kg)
54. Place the animal in the recovery bin, on a blue bench cover, above a heat pad and allow to recover
55. Turn flow meter down to 0, turn off isoflurane, and close the oxygen cylinder
56. Indicate surgery on cage card

Notes
__________________________________________
__________________________________________
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# Appendix B

<table>
<thead>
<tr>
<th>Date</th>
<th>Microscan</th>
<th>Initials</th>
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<tbody>
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## 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 ~3 l min⁻¹
- **14.** Anesthetize animal w/ 5% isoflurane
- **15.** Reduce flow rate to 0.5-1.0 l 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 37°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 mus for experimental hind limb or sham for control hind limb. EX: 25mus
- **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 500µs, and 1mA
- **37.** Turn on stimulator panel
- **38.** Stimulate muscle for 90 sec
- **39.** Immediately capture video and continue to capture every two minutes until vessel has returned to resting diameter
- **40.** Perform same procedure on control limb

## Analysis

- **41.** Open analysis section in AVA and open file of interest
- **42.** Set Frames from 0 to 160 and stabilize file
- **43.** Analyze vessel diameter by manually drawing diameter and chaining sections together
- **44.** Record results in provided table

## Post-Surgical

- **45.** Cervical dislocation to euthanize animal

### Notes

- ____________________________________________
- ____________________________________________
- ____________________________________________
- ____________________________________________
- ____________________________________________