

The Effects of Ischemia and High-Fat Diet on Pericyte Fate

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Abstract:

The purpose of my thesis was to assess the effects of high fat (HF) diet and ischemia on skeletal muscle pericytes. Pericytes are mural cells on the abluminal surface of capillaries that contribute to capillary stabilization and the regulation of angiogenesis, but their roles in the events that occur during muscle recovery from ischemia in patients with or without metabolic disorders remain to be established. I hypothesized that the number of capillary-associated pericytes and their phenotype will be altered by HF diet and ischemia. I used mice with fluorescently labelled pericytes (Ng2/DsRed) that were fed normal or HF diet and then underwent unilateral femoral artery ligation surgery to induce ischemia. My data showed that ischemia increased pericyte number through proliferation. Pericyte phenotype and morphology changed with ischemia, together with increased transcript levels of destabilizing factor Angiopoietin 2 mRNA. Overall, this study can be the first step in understanding the participation of pericytes in ischemic muscle recovery.

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Statement of contributions

All chapters of this thesis were written by George Nader and further revised with recommendations from Dr. Tara Haas.

Most aspects of the study were conducted by me although some of the ligation surgeries were conducted by Emmanuel Nwadozi and some of the cryosectioning, RNA data and image quantification were done by Amin Hatamnejad.

Table of content

Abstract.....	ii
Acknowledgments.....	iii
Statement of contributions	iv
Table of content	v
Contents Abbreviations.....	ix
List of Figures	xii
Chapter 1: Literature Review.....	1
1.1 The microvasculature.....	1
1.2 Pericytes	2
1.3 Pericyte-Endothelial interactions	3
1.3.1 Neural cadherin (N-cadherin).....	3
1.3.2 Notch.....	3
1.3.3 Platelet-derived growth factor-BB (PDGF-BB)	4
1.3.4 Angiopoietin 1 (Angpt1)	4
1.3.5 Angiopoietin 2 (Angpt2)	5
1.3.6 Neural/glial antigen 2 (NG2)	5
1.4 Angiogenesis	6
1.5 Regulation of angiogenesis	6
1.5.1 Vascular endothelial growth factor A (VEGFA)	7
1.5.2 Matrix metalloproteinases (MMPs).....	7

1.5.3 Thrombospondin 1 (TSP1).....	8
1.6 Pericyte role in angiogenesis	8
1.6.1 Pericytes in early stage of angiogenesis.....	8
1.6.2 Pericyte in late stage of angiogenesis.....	9
1.7 Pericyte plasticity	10
1.8 Skeletal muscle ischemia	11
1.9 Muscle tissue recovery from ischemic damage	12
1.10 Pericytes in the recovery of ischemic skeletal muscle	13
1.11 Ischemia and Insulin resistance effects on skeletal muscle	15
1.12 Insulin resistance effects on skeletal muscle fiber functionality.....	15
1.13 Insulin resistance effects on skeletal muscle blood flow	16
1.14 Insulin resistance effects on skeletal muscle capillarization.....	16
1.15 The effects of HF diet on pericytes	17
1.16 The combined effects of ischemia and HF diet on pericyte.....	18
Chapter 2: Rationale and Objectives.....	19
2.1 Objective 1	19
2.2 Objective 2	20
Chapter3: Methods	21
3.1 Ethical Approval	21
3.2 Mouse Model of Femoral Artery Ligation.....	21
3.3 Histological assessment.....	22

3.3.1 Muscle preparation	22
3.3.2 Cryotome	23
3.3.3 Pericyte and vasculature assessment.....	23
3.3.4 Proliferating pericyte assessment	23
3.3.5 Pericyte fat deposition assay	24
3.4 RNA extraction from muscle tissue	24
3.5 In-vitro Cell assay.....	24
3.5.2 MSCs treatment.....	24
3.5.3 RNA extraction from cultured cells.....	25
3.6 qPCR	25
3.7 Statistics	26
Chapter 4: Results	27
4.1 Pericyte number relative to muscle and capillary area increased significantly 14 days post ischemia.....	27
4.2 Pericyte number increased 8 days post-ligation	29
4.3 Pericyte proliferation was evident in the ischemic muscle 8-days post-ligation.....	31
4.4 Distinct pericyte morphologies were observed post-ischemia.....	33
4.5 The relative expression of <i>Angpt2</i> but not <i>Angpt1</i> and <i>Cspg4</i> genes altered in response to ischemia.....	35
4.6 Collagen expression is altered by glucose and hypoxic-mimetic Cobalt chloride.....	36
Chapter 5: Discussion.....	38
5.1 Overview of findings.....	38

5.2 The impact of critical limb ischemia on skeletal muscle	38
5.3 Pericyte contribution to CLI recovery.....	39
5.4 Pericyte number in ischemic muscle	40
5.5 High fat diet and muscle ischemia outcomes.....	42
5.6 The additive effect of HF diet and ischemia on skeletal muscle pericyte	43
5.7 Limitations of the study	45
5.7.1 Experimental design	45
5.7.2 Technical issues.....	45
5.8 Future directions	46
5.9 Conclusion	47
References	49

Contents Abbreviations:

AKT - Protein Kinase B

Angpt 1 - Angiopoietin 1

Angpt 2 - Angiopoietin 2

ATP - Adenosine Triphosphate

B1-Integrin - Integrin Beta-1

CD31 - Cluster of Differentiation 31

CLI - Critical limb Ischemia

CoCl₂ – Cobalt Chloride

Cspg4 - Chondroitin Sulfate Proteoglycan 4

EDL - Extensor Digitorum Longus

EdU - 5-ethynyl-2' -deoxyuridine

eNOS - Endothelial Nitric Oxide Synthase

HIF 1 α - Hypoxia Inducible Factor 1 α

HF - High Fat

IFN- γ - Interferon Gamma

Il-12 - Interleukin 12

IRS - Insulin Receptor Substrate Proteins

MMPs - Matrix metalloproteinases

NADP - Nicotinamide Adenine Dinucleotide Phosphate

NO - Nitric Oxide

N-cadherin - Neural Cadherin

NC – Normal Chow

NG2 - Neural/Glial Antigen 2

NF- κ B - Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells

PAD - Peripheral Artery Disease

PDGF-BB - Platelet-Derived Growth Factor-BB

PDGFR α - Platelet-Derived Growth Factor Receptor Alpha

PDGFR β - Platelet-Derived Growth Factor Receptor Beta

PECAM-1 - Platelet endothelial cell adhesion molecule 1

PI 3-Kinase - Phosphoinositide 3-Kinase

PPAR γ - Peroxisome Proliferator Activated Receptor Gamma

ROS - Reactive Oxygen Species

SMC - Smooth Muscle Cells

T2D – Type 2 Diabetes

TA - Tibialis Anterior

TBP - TATA-Box Binding Protein

TGF β 1 - Transforming Growth Factor Beta 1

Tie 2 - Tyrosine Kinase Receptor 2

TSP1 - Thrombospondin 1

VEGFA - Vascular Endothelial Growth Factor A

VEGFR2 - Vascular Endothelial Growth Factor Receptor 2

Zfp 423 - Zinc Finger Protein 423

List of Figures:

Figure 2.1: The potential effects of ischemia and HF diet on pericyte fate.....21

Figure 3.1: Schematic of study timeline (14-day time point mice).....23

Figure 3.2: Schematic of study timeline (1,4- and 8-day time points).....23

Figure 4.1: Pericytes in ischemic and non-ischemic muscle from NC and HF diet fed mice.....28

Figure 4.2: Pericyte density increased in response to ischemia at 14 days post-ligation regardless of diet.....29

Figure 4.3: Representative images of pericytes and capillaries in non-ischemic and ischemic muscle at 8 days post-ligation.....30

Figure 4.4: Pericyte number relative to muscle area increased in response to ischemia at 8 days post-ligation.....31

Figure 4.5: Representative images of pericyte proliferation in non-ischemic and ischemic muscles from mice at 8 days post-ligation.....32

Figure 4.6: Pericyte number and proliferation are significantly increased in response to ischemia at 8 days post-ligation.....33

Figure 4.7: Distinct pericyte morphologies and phenotypes in ischemic muscle.....34

Figure 4.8: The expression of genes involved in pericyte-endothelial interactions in response to ischemia.....36

Figure 4.9: Glucose concentration and CoCl₂ alter collagen type 1 expression in MSCs.....37

Chapter 1: Literature Review

1.1 The microvasculature

Blood vessels supply the body's organs with the oxygen and nutrients that they need and help to remove the waste that the organs produce (Watson and Adams 2018). Major arteries transport blood to each organ, where they branch into the arterioles, capillaries and venules that comprise the microvasculature. Despite some organ-specific differences, microvascular networks share the following general structural and functional features (Pugsley and Tabrizchi 2000):

Arterioles regulate blood flow into the tissue by relaxing or contracting their smooth muscle cells to change the lumen diameter (Muller-Delp et al. 2002). The inner wall of arterioles is composed of a single layer of endothelial cells, whereas single or multiple layers of smooth muscle cells wrap the arterioles peripherally (Parise et al. 2020). Each arteriole gives smaller branches called capillaries, which are the smallest units for blood flow regulation in the tissue. Capillaries provide an extensive surface area that allows for oxygen, waste and signaling molecules exchange between the tissue and the blood (Poole et al. 2013). Capillaries empty into venules that collect the blood to return to the heart via the venous side of the circulation (Pugsley and Tabrizchi 2000). Capillaries are well studied because of their importance in maintaining health tissue function. Capillaries are largely composed of a single layer of endothelial cells that are in direct contact with plasma on the inner side and attach to the basement membrane on their outer (abluminal) surface. The basement membrane is a three-dimensional protein network. It is dominantly composed of collagen type 4, the glycoprotein laminin, nidogens, and the heparan sulfate proteoglycan perlecan (Hallmann et al. 2005). The basement membrane plays important functional roles. It contributes to the structural stability of capillaries and to the barrier function of the endothelium by selectively hindering the movement of soluble molecules and migration of

leukocytes (Hallmann et al. 2005). In most vascular beds, each capillary is partially covered by cells called pericytes (Parise et al. 2020; Korthuis 2011; Gerhardt and Betsholtz 2003).

1.2 Pericytes

Pericytes are a heterogeneous population of mural cells that are found on the outer surface of capillaries and also some pre-capillary arterioles and post-capillary venules (Díaz-Flores et al. 2009). Pericytes typically have multiple cytoplasmic processes that emanate from the cell body (Ribatti et al. 2011; Berthiaume et al. 2018). In a cross-section of human skeletal muscle, 95% of capillaries were found to have an associated pericyte and 20% of the surface of an individual capillary was covered by pericyte protrusions (Tilton et al. 1979). Pericytes have been reported to play important roles in the different pathological and physiological conditions. For example, pericytes support capillary maturation and stabilization either by the synthesis of basement membrane components or by covering the capillary wall to support its structure and to induce endothelial cell quiescence (Stratman et al. 2009; Díaz-Flores et al. 2009). On the other hand, pericytes are thought to support angiogenesis by basement membrane degradation and the induction of endothelial cells activation and migration (Díaz-Flores et al. 2009; Girolamo et al. 2004). Lastly, pericytes behave as perivascular cell precursors given that isolated pericytes in vitro showed a potential to differentiate into different cells types including adipocytes, fibroblast, smooth muscle cells (Birbrair et al. 2013; Volz et al. 2015; Birbrair et al. 2014). Pericytes and endothelial cells share the capillary basement membrane (Baum et al. 2020). The basement membrane forms a barrier that generally minimizes contact between the two cell types. However, holes in the membrane allow the two types of cells to be in direct contact at specific locations. Multiple cytoplasmic processes arise from the pericyte cell body and insert into endothelial cell invaginations, referred to as peg and socket contacts (Baum et al. 2020). These

cell-cell connections are responsible for supporting the quiescent status in the two cell types through signaling molecules, providing structural support to the blood vessels and regulating capillary permeability either by inducing or debilitating pericyte recruitment (Geevarghese and Herman 2014). These interactions are discussed below in greater detail.

1.3 Pericyte-Endothelial interactions

Pericyte-endothelial cell connections are maintained by multiple structural proteins that hold the cells in close contact and paracrine signaling molecules that support either the stabilization or destabilization of capillaries:

1.3.1 Neural cadherin (N-cadherin)

N-cadherin is an adhesion molecule that is reported to be expressed in the peg and socket connection spots between pericyte and endothelial cells. N-cadherin form homophilic N-cadherin to N-cadherin connections (Gerhardt et al. 2000, Lee et al. 2001). Moreover, N-cadherin is thought to suppress pericyte and endothelial proliferation by sequestering β -catenin to the plasma membrane, which prevents β -catenin translocation to the nucleus where it acts as a transcriptional co-activator of cell cycle genes (Nadanaka et al. 2018; Bonewald and Johnson 2008). N-cadherin is also implicated in reducing glycolytic activity in the pericytes, which further enforces their quiescence (Cantelmo et al. 2016).

1.3.2 Notch

Transcriptional regulation through Notch is essential for many processes related to vascular development (Polacheck et al. 2017). For example, Notch signaling contributes to the quiescence of pericytes through the interaction that occurs between Jagged-1 on the endothelial

cells and Notch3 on pericytes. The latter interaction not only enhances the adhesion between pericytes and endothelial cells but also inhibits the migration and division of pericytes (Liu et al. 2009). In some types of cells, Notch signaling is also involved in the regulation of cellular metabolism. The downregulation of glycolytic enzymes, for example The glycolytic activator PFKFB3, is one of the mechanisms by which the latter regulation is achieved (Liu et al. 2010; De Bock et al. 2013). Therefore, the Notch signaling pathway that leads to pericyte quiescence may include suppression of glycolytic activity.

1.3.3 Platelet-derived growth factor-BB (PDGF-BB)

PDGF-BB is one of the isoforms of PDGF family (Fredriksson et al. 2004). PDGF-BB is made by endothelial cells. PDGF-BB binds to PDGFR- β on the membrane of pericytes. PDGF-BB/PDGFR β interaction induce pericyte recruitment to the blood vessels. Any disruption in this interaction will result in capillaries without pericyte coverage. The loss of pericyte coverage will lead to vessel instability (Lindahl et al. 1997).

1.3.4 Angiopoietin 1 (Angpt1)

Angiopoietin 1 (Angpt1) is made constitutively and predominantly by pericytes and binds to tyrosine kinase receptor Tie-2 on endothelial cells (Ribatti et al. 2011). The binding of Angpt1 to Tie-2 leads to the activation of PI 3-Kinase, which in turn leads to AKT phosphorylation and activation. The survival of the endothelial cells is induced, and their quiescence is maintained, by AKT signaling (Fiedler and Augustin 2006; Otrrock et al. 2007). Moreover, NF- κ B signaling is suppressed by the interaction between A20 binding inhibitor of NF- κ B activation 2 (ABIN-2) and phosphorylated Tie-2. The prevention of NF- κ B signaling inhibits the expression of inflammatory associated adhesion molecules such as P and E Selectin, ICAM-1 and VCAM-1 (Fiedler and Augustin 2006; Otrrock et al. 2007). As a result, Angpt1/Tie 2 signaling increases pericyte-

endothelial cell interaction and plays an essential role in lowering vascular permeability and tightening blood vessels, resulting in stable, mature capillaries (Ribatti et al. 2011; Minoshima et al. 2018).

1.3.5 Angiopoietin 2 (Angpt2)

Angpt2 is expressed by endothelial cells at the early stage of angiogenesis and interacts with B1-integrin. This Angpt2-B1-integrin interaction will cause the movement of the activated B1-integrin away from the peripheral side of the cell. This movement leads to the disruption of cell-cell adhesion and vessel stability. Moreover, Angpt2 competes with pericyte-derived Angpt1 for binding to Tie2 receptor, which causes disruption of the stabilizing effects of Angpt1. Thus, Angpt2 appears to be an autocrine signal that induces vessel instability and pericyte detachment from the vessels and decreases the pericytes coverage in response to stimuli that promote angiogenesis (Hakanpaa et al. 2015; Carmeliet and Jain 2011).

1.3.6 Neural/glial antigen 2 (NG2)

Neural/glial antigen 2 (NG2) is a surface glycoprotein that is expressed by pericytes. NG2 is used as a pericyte marker that can be used to detect pericytes in peripheral tissues in pathological and health conditions. Utilizing NG2 enable the distinguish of pericyte from other peri-vascular cells such as smooth muscle cells (Ozerdem et al. 2001; Ozerdem et al. 2002). NG2 has a functional role in pericyte where it is a supplementary receptor that improves the signaling through integrins and tyrosine kinase growth factor receptors by attaching to the growth factor and directing it to bind to its appropriate receptors (Couchman 2003). In this way, NG2 helps to support the motility and recruitment of endothelial cells to form a vascular network and pericyte survival and motility (Stallcup 2018; Fukushi et al. 2004).

1.4 Angiogenesis

In most adult organs, endothelial cells are non-proliferative and quiescent (Potente et al. 2011). Exceptions to this include: in females during the menstrual cycle and pregnancy; in expanding adipose tissue; during wound healing; or, in skeletal muscle with exercise training and in response to ischemic damage (Gerhardt and Betsholtz 2003; Eelen et al. 2015; Chinsomboon et al. 2009). Angiogenesis is the process by which new capillaries are formed from already existing ones, which results in an increase in endothelial cell surface area to allow for more efficient exchange of substances between blood and the cells comprising that tissue. Angiogenesis is an essential process for tissue development, reproduction and wound repair (Otrock et al. 2007). Angiogenesis is stimulated when there is increased metabolic demand such as during development and tissue expansion (Potente et al. 2011). Angiogenesis consists of an organized sequence of events that includes the activation and proliferation of endothelial cells, spatially regulated proteolytic degradation of the basement membrane, the formation of tip cells and their migration through the interstitial matrix to form a new sprout (Uchida et al. 2015). Finally, when the endothelial cells of the elongated vessels contact another capillary, they will fuse and form a lumenized vessel (Eelen et al. 2015). The maturation and stability of this newly sprouted capillary will be guaranteed by the formation of new basement membrane and recruitment of pericytes. Pericytes are suggested to play an essential role in angiogenesis. This role will be discussed in detail in Section 1.6.

1.5 Regulation of angiogenesis

Angiogenesis is well regulated by the balance between the pro and anti-angiogenic factors that respectively favour either the growth or the stasis of the blood vessel (Hoier et al. 2012). In skeletal muscle, multiple cell types such as myocytes, fibroblasts and immune cells are

implicated in the communication with endothelial cells by releasing growth factors or cytokines in response to exercise or damage (Newman et al. 2011; Olfert et al. 2010).

1.5.1 Vascular endothelial growth factor A (VEGFA)

The pro-angiogenic VEGFA (34-46 kDa glycoprotein) is the most studied member of the family (Otrock et al. 2007). By binding to VEGFR2 on endothelial cells, VEGFA promotes endothelial cell proliferation, sprouting and the formation of new capillaries (Otrock et al. 2007). In humans, eight different isoforms of VEGFA formed by alternative splicing were found (Bouïis et al. 2006). Within skeletal muscle, VEGFA is produced in greatest quantity by skeletal myocytes, which release it to the extracellular fluid in response to hypoxia and exercise (Olfert et al. 2009 ; Hoier et al. 2013). However, pericytes also are reported to produce VEGFA (Hoier et al. 2013). In ischemia, the hypoxic conditions induce the production of VEGFA through the stabilization of hypoxia inducible factor 1α (HIF 1α) (Wagner 2011).

1.5.2 Matrix metalloproteinases (MMPs):

Degrading the basement membrane is an essential step to initiate angiogenesis (Otrock et al. 2007). This degradation allows the endothelial cells to migrate and form new sprouts. Matrix metalloproteinases (MMPs) are a family of enzymes that are responsible for extracellular matrix degradation; therefore, they may be considered as pro-angiogenic factors (Otrock et al. 2007). In skeletal muscle, it was reported that muscle contraction leads to an increase in MMP-2 and MMP-9 production (Koskinen et al. 2002). Also, our lab reported that chronic stimulation of rat skeletal muscle promotes MMP production and improve angiogenesis (Haas et al. 2000). This may be explained by the enhanced degradation of the basement membrane, which allows for endothelial cells migration (Haas et al. 2000).

1.5.3 Thrombospondin 1 (TSP1):

Thrombospondin 1 is the first discovered member of the Thrombospondins (TSPs), a family of extracellular proteins (Lawler 2000). TSP1 is a major component of blood platelets (Lawler 2000). TSP1 is an antagonist for VEGFA that suppresses angiogenesis by inhibiting endothelial cell proliferation, migration and survival (Lawler et al. 2012). In skeletal muscle, the levels of TSP1 increase significantly after 1 bout of exercise. However, multiple repeated bouts of exercise were able to reduce TSP1 expression (Olfert et al. 2006). This information suggests that TSP1 plays a regulatory role in exercise-induced angiogenesis. Moreover, TSP1 was reported to suppress blood flow recovery in the ischemic muscle by inhibiting the NO-mediated vasodilation (Isenberg et al. 2007).

1.6 Pericyte role in angiogenesis

Most of the data about pericyte roles in angiogenesis comes from studies of the retina. These steps likely also occur in skeletal muscle angiogenesis. However, the role of pericytes in skeletal muscle angiogenesis is not well established.

1.6.1 Pericytes in early stage of angiogenesis

During the early stages of angiogenesis, pericytes retract their processes, increase their central body volume and initiate proliferation (Diaz-Flores et al. 1992). This may occur because activated endothelial cells secrete Angpt2, which competes with Angpt1 for binding to Tie2. Angpt2/Tie2 attachment leads to disruption of the contact between endothelial cells and pericytes, supporting the pro-migratory phenotype of both endothelial cells and pericytes (Augustin et al. 2009; Saharinen et al. 2008). The intact basement membrane is an impediment

to the migration of the endothelial cells and pericytes. Both pericytes and endothelial cells contribute to the degradation of the basement membrane by secretion of matrix metalloproteinases (MMPs), which eases their detachment process (Carmeliet and Jain 2011; Glaser et al. 1983). Nehls et. al reported that pericytes were seen on top and in front of the sprouting endothelial tip cells (Nehls et al. 1992). This finding supports the idea that the detached pericyte migrates to the tip of the growing endothelial cell sprout and releases VEGFA to induce the sprout and to guide the newly formed endothelial tubes (Minoshima et al. 2018; Nehls et al. 1992). The released VEGFA binds to VEGFR2 on endothelial cells of the pre-existing vessels and leads to increase endothelial cell proliferation to rapidly elongate the newly forming capillaries (Eelen et al. 2015).

1.6.2 Pericyte in late stage of angiogenesis

The activated endothelial cells release greater levels of PDGF-BB, which plays a role in increasing pericyte proliferation and recruitment (Otrock et al. 2007). Hellstrom et. al reported that endothelial cells in immature vessels express PDGF-BB, which induces the proliferation and recruitment of the PDGFR β positive pericyte to the newly formed vessels (Hellström et al. 1999). This pericyte recruitment to the new sprouts will guarantee their maturation and stability. Pericytes contribute to the production of basement membrane proteins such as laminin and matrix bridging proteins such as fibronectin and nidogen-1 that are important to attach the basement membrane protein components together (Stratman et al. 2009). Moreover, re-association of pericyte-endothelial interaction supports vessel stability through ligand-receptor signaling pathways such as Angpt1/Tie2. These signaling pathways induce the quiescence mode in both pericyte and endothelial cells (Armulik et al. 2006). However, more studies are needed to fully understand the behavior of pericytes during the process of angiogenesis in skeletal muscle.

1.7 Pericyte plasticity

Pericytes also may participate in many cellular processes implicated in tissue homeostasis and regeneration since a growing body of evidence shows that pericytes are multipotent cells that have the capacity to differentiate into different types of cells (Ferland-McCollough et al. 2017). It was reported that pericytes may differentiate into smooth muscle cells (SMC) (Volz et al. 2015). Pericytes of bigger diameter vessels (arterioles) have higher amounts of SM contractile proteins such as α smooth muscle actin and myosin heavy chain11 in comparison with capillary-associated pericytes (Nehls et al. 1992; Nehls and Drenckhahn 1991; Hess et al. 2019). Moreover, reports suggested that pericytes differentiate into smooth muscle cells and participate in arteriolarization, which is the process by which capillaries transform into bigger arterioles (Volz et al. 2015; Nehls and Drenckhahn 1991). However, pericyte participation in the arteriolarization process in skeletal muscle pathological conditions such as ischemia needs further investigation. Pericytes also may contribute to fibrogenesis. Barron et. al reported that pericytes can increase the expression of PDGFR α (Fibroblast precursor marker) in response to lung injury (Barron et al. 2016). Cultured pericytes have the ability to differentiate into adipocytes, osteoblasts and chondrocytes (Geevarghese and Herman 2014; Crisan et al. 2008). Nevertheless, not all pericytes within a microvascular bed appear to have the same differentiation potential. Birbrair et. al identified two distinct subtypes of pericytes in murine skeletal muscle that have a distinct differentiation potential in vitro, type-1 (PDGFR α positive and Nestin negative) that have adipogenic potential and type-2 (PDGFR α negative and Nestin positive) that have myogenic differentiation capacity (Birbrair et al. 2013). However, skeletal muscle resident pericyte differentiation processes in pathological and physiological conditions is poorly understood to date.

1.8 Skeletal muscle ischemia

Femoral artery occlusion causes a reduction in blood flow to the lower limb, which leads to insufficient oxygen and nutrient delivery and metabolic waste removal (Chouchani et al. 2014).

This usually happens in humans following the accumulation of atherosclerotic plaque or thrombus formation in the major arteries leading to the extremities (Lovell et al. 2009).

Peripheral artery disease (PAD) can be asymptomatic or symptomatic depending on the severity of the disease. Early and mild stages of PAD are characterized by small reductions of blood flow to the distal limb and minimal muscle damage and often no symptoms are experienced. The second stage of PAD is characterized by intermittent claudication which is identified as leg pain felt while walking that usually leads to abnormal gait. At this stage, arterial blood flow is further diminished and myofibers receive less oxygen and nutrients to maintain adequate health (Rutherford et al. 1997; McDermott 2015). This intermittent claudication can progress to the more severe stage in which individuals experience resting leg pain. (Rutherford et al. 1997 ; Norgren et al. 2007). The most severe form of PAD is known as critical limb ischemia (CLI). CLI is characterized by severe and chronic reduction in blood supply, resting pain, gangrene and may lead ultimately to limb amputation (Varu et al. 2010). Ischemic conditions will cause oxidative and nutrient-deprivation stress on muscle tissue's resident cells that will disrupt the synthesis of Adenosine triphosphate ATP. The continued consumption of ATP will lead finally to the formation of adenosine, hypoxanthine and xanthine (Defraigne and Pincemail 1998). The low availability of ATP will impair the activity of ion transporter pumps, which disrupts transmembrane ionic gradients, thus leading to increases in sodium cell content and decreases in potassium levels. Therefore, the cytosolic concentration of calcium will be elevated (Defraigne and Pincemail 1998). These high levels of calcium will activate proteases that convert the xanthine dehydrogenase into xanthine oxidase. Moreover, phospholipases will be activated, resulting in de-esterification of membrane lipids with high levels of free

polyunsaturated fatty acids. These conditions will cause myocytes damage and death if sustained (Defraigne and Pincemail 1998).

1.9 Muscle tissue recovery from ischemic damage

The tissue damage resulting from prolonged severe ischemia will induce repair processes that include inflammation, revascularization, blood flow recovery and myocyte regeneration (Tidball 2017; Arpino et al. 2017; Couffinhal et al. 1998). The recovery process occurs in three different stages. Firstly, post-ischemia damage is characterized by a strong pro-inflammatory response. The tissue resident macrophages will release cytokines that attract neutrophils from the circulation (Dimitrova et al. 2018). The recruited neutrophils will initiate the removal of damaged cell debris and release M1 pro-inflammatory macrophages phenotype biased cytokines such as interferon gamma (IFN- γ) and interleukin 12 (IL-12) (Dimitrova et al. 2018; Tateda et al. 2001). These cytokines will push the recruited monocytes and tissue resident macrophages into an M1 pro-inflammatory phenotype (Dimitrova et al. 2018). Moreover, at this stage, muscle stem cells (satellite cells) become activated and re-enter the cell cycle, as a result of growth factor production by immune cells. Thus overall, the inflammatory cells support tissue repair by removing debris from dead myofibers and promoting the proliferation of myogenic precursor cells to expand their population (Tidball 2017). Secondly, angiogenesis will be initiated, which will support tissue reperfusion. Significant increases in capillary and arteriole density and upregulation in VEGFA expression in the myocytes of the hypoxic tissue are detected (Couffinhal et al. 1998). This high level of VEGFA can induce endothelial cell proliferation. The majority of these proliferative endothelial cells are localized within small capillaries whereas a small portion are localized within the arterioles (Tidball 2017; Couffinhal et al. 1998). Also at this stage, the conversion of M1 macrophages to an M2 phenotype promotes the myogenic precursor cells to differentiate into myocytes (Tidball 2017; Couffinhal et al. 1998). TGF- β 1

secreted by M2 macrophages is implicated in this differentiation process, as its neutralization decreases the diameter of recovering myofibers (Tidball 2017; Arnold et al. 2007). The last stage is ideally characterized by complete resolution of inflammation and full blood flow recovery. Moreover, the myocytes will elongate into multinucleated myotubes. The formed myotubes will fuse with or replace the damaged myofiber and undergo maturation and growth (Tidball 2017; Couffinhal et al. 1998; Yin et al. 2013). However, this is the optimal scenario that occurs in experimental models involving young and healthy animals.

In contrast, CLI patients develop blood flow impairment and microvascular dysfunction. This microvascular dysfunction is characterized by endothelial impairments that lead to microthrombi formation at the microvessel level and the development of tissue edema due to capillary leakage (Varu et al. 2010). Furthermore, the endothelial dysfunction causes an upregulation of free radical production, abnormal platelet activity and white blood cells adhesion that all lead to capillary 'plugging', which greatly impairs oxygen and nutrient exchange at the capillary level (Varu et al. 2010). Moreover, CLI patients develop chronic myofiber impairment and atrophy, abnormal fat accumulation, fibrosis, distal limb necrosis and gangrene. CLI can lead ultimately to limb amputation (Rutherford et al. 1997; Norgren et al. 2007).

1.10 Pericytes in the recovery of ischemic skeletal muscle

The role of pericytes in ischemic skeletal muscle has been minimally investigated to date. However, a growing body of evidence suggests that pericytes may be a potential therapeutic approach to repair muscle injury. Firstly, this is due to their positive roles in guiding angiogenesis and preserving viability of capillary ECs. Angiogenesis is a crucial process to improve ischemic muscle tissue perfusion by the formation of micro vessels that rescues the functionality and integrity of the muscle tissue by compensating for the reduction in blood delivery (Couffinhal et al. 1998; Teng et al. 2021). Studies showed that there is an increased

protein expression of VEGF-A and Angpt2 in the ischemic muscle in comparison with the non-ischemic muscle (Brandão et al. 2011). These findings suggest that the pro-angiogenic environment is favoured in the ischemic muscle and that disruption of pericyte-endothelial interactions might also take place. Pericyte implantation experiments showed that type 2 but not type 1 pericytes have angiogenic potential and participate in the formation of new blood vessels in the injured muscle (Birbrair et al. 2014). Altogether, these findings suggest a role of pericytes in supporting the pro-angiogenic environment in the ischemic muscle.

Secondly, pericyte multipotency and differentiation potential into different cell types may suggest a role in the ischemic recovery. However, this role may be either positive, if it involves pericyte myogenic differentiation capacity or negative, if it promotes fibrosis or adipocyte accumulation. For example, pericytes may participate in the myofiber regeneration process. Birbrair et al. showed that type 2 pericytes transplanted into injured skeletal muscle contributed to the formation of new myofibers (Birbrair et al. 2013). Dellavalle et al. supported this finding by showing that, in the injured skeletal muscle, muscle resident pericytes differentiate into myocytes and participate in the formation of the regenerating myofibers (Dellavalle et al. 2011). On the other hand, pericytes may participate negatively in the ischemic recovery. Abnormal fat deposition is seen in the ischemic muscle in CLI patients. This ectopic adipocyte accumulation impairs muscle recovery and causes muscle weakness and atrophy (Nwadozi et al. 2020). Type 1 pericytes, which have adipogenic differentiation potential in vitro, may be the source of this fat deposition. (Birbrair et al. 2013). These cells were able to differentiate into adipocytes and participate in the fat accumulation when transplanted into injured muscle (Birbrair et al. 2013). Moreover, CLI patients may develop skeletal muscle fibrosis where collagen substrates will accumulate into the inter-myofiber space. This abnormal collagen deposition will cause muscle loss of function (Stepien et al. 2020). Type 1 pericytes express PDGFR α , commonly considered to be a marker of the fibro-adipogenic precursor cells (Birbrair et al. 2013). These cells may be

implicated in this pathological skeletal muscle fibrosis and adipogenesis. Furthermore, it was reported that pericytes may play a negative role in cardiac post-ischemia recovery by becoming more contractile, which results in the constriction of cardiac capillaries and lowered blood flow (O'Farrell et al. 2017). However, further investigation is needed to understand the behavior of pericytes in response to skeletal muscle ischemia and how their physiological or pathological functions may shape the recovery process.

1.11 Ischemia and Insulin resistance effects on skeletal muscle

Diabetes and insulin resistance promote negative peripheral artery disease (PAD) outcomes. These risk factors increase the danger of vasculature pathologies, systemic inflammation, and skeletal muscle dysfunction. Furthermore, these risk factors are implicated in an increase in the blood levels of triglyceride by disturbing metabolic processes (Nwadozi et al. 2020; Hiatt et al. 2015).

1.12 Insulin resistance effects on skeletal muscle fiber functionality

The majority (~ 2/3) of systemic glucose disposal takes place in skeletal muscle using insulin dependent mechanisms (Abdul-Ghani and DeFronzo 2010). However, high levels of plasma free fatty acids induce insulin resistance, which is a major contributor to the development of obesity and type 2 diabetes, key risk factors of PAD (Silveira et al. 2008). Sustained insulin resistance in skeletal muscle induces mitochondrial dysfunction and reduces the mitochondrial oxidative capacity that increase the lipids deposition into myocytes. Moreover, T2D is reported to cause systematic oxidative stress (Ramakrishna and Jaikhanani 2008). The dominant source of superoxide, NAD(P)H oxidase, is present in skeletal muscle and activated by hyperglycemia and hyperinsulinemia (Yokota et al. 2009). This insulin resistance combined with ROS elevation

has deleterious and damaging effects on skeletal muscle myocytes (Di Meo, Iossa, and Venditti 2017). Insulin resistance also contributes to skeletal muscle fibrosis (Pincus et al. 2015). Insulin resistance promotes skeletal muscle macrophages to release transforming growth factor β 1 (TGF β 1). TGF β 1 increases collagen 1 gene expression and leads ultimately to excessive collagen deposition surrounding the muscle fibers (Pincus et al. 2015).

1.13 Insulin resistance effects on skeletal muscle blood flow

It was reported that insulin signaling increases the production and bioavailability of nitric oxide in the vasculature, promoting vasodilation (Manrique and Sowers 2014). That happens normally by the activation of the insulin receptor substrate proteins (IRS) by the receptor tyrosine kinase. IRS phosphorylation will activate PI3K that induces serine phosphorylation of eNOS through Akt activation, thus increasing NO production (Manrique and Sowers 2014). In the pathological conditions of type 2 diabetes or obesity, insulin resistance and its metabolic abnormalities such as glucotoxicity, lipotoxicity, and inflammation cause endothelial dysfunction that impairs the production and bioavailability of nitric oxide and causes vasoconstriction (Muniyappa and Sowers 2013). This vasoconstriction would impair blood flow to the skeletal muscle and the production of NO-dependent pro-angiogenic factors such as VEGFA (Uchida et al. 2015).

1.14 Insulin resistance effects on skeletal muscle capillarization

Type 2 diabetes is implicated in angiogenesis inhibition and potentially in capillary rarefaction (Groen et al. 2014; Galiano et al. 2004). The effect of T2D on the microvasculature is well studied in retina. In diabetic retinopathy, inhibition of angiogenesis can be caused by a reduction in the expression of pro-angiogenic growth factors and/or by the induction of anti-angiogenic

pathways (Jumar et al. 2016). It is also reported that disruption of pericyte-endothelial interaction and pericyte death is implicated in diabetic retina capillary rarefaction (Geraldes et al. 2009). The diabetic-induced oxidative stress combined with the downregulation of PDGF-BB, PDGFR β dephosphorylation and endothelial cell overexpression of Ang2 may cause pericyte apoptosis or detachment that leads to destabilization of capillaries (Beltramo and Porta 2013; Hammes et al. 2004; Solomon et al. 2011; Groen et al. 2014; Geraldes et al. 2009). However, further studies are needed to understand if T2D causes angiogenesis inhibition or capillary rarefaction in skeletal muscle.

1.15 The effects of HF diet on pericytes

High fat (HF) diet frequently is used as an experimental model to cause chronic elevation of circulating fatty acids, leading to systemic insulin resistance (Costa et al. 2011). The effects of HF diet on skeletal muscle pericyte survival and trans-differentiation are not known. It has been suggested that HF diet could inhibit pericyte proliferation and induce their death. In vitro experiments have shown that high levels of fatty acid can inhibit cultured pericyte proliferation and may induce them to undergo apoptosis (Yamagishi et al. 2002). This disruption of cell viability could be explained by the elevated oxidative stress (Cacicedo et al. 2005). Data from HF diet-induced diabetic retinopathy showed that putting mice on HF diet leads to loss of retina pericytes (Rajagopal et al. 2016). Moreover, HF diet could promote a shift in pericyte phenotype. The exposure to fatty acids increases the production of TGF- β (Mishra and Simonson 2008) and it is well known that pericyte differentiation to smooth muscle cells (SMC) is promoted by TGF- β -Smad/Notch signaling (Matthew and Owens 2012; Volz et al. 2015). However, our lab also reported that feeding mice with HF diet for a prolonged period leads to the elevation of leptin and Zfp423 (pre-adipocyte commitment markers) levels in pericytes (Nwadozi et al. 2019). This observation indicates that HF diet may increase the adipogenic

potential of pericytes. However, the levels of PPAR γ (mature adipocyte marker) did not change, suggesting that the terminal adipogenic differentiation did not occur (Nwadozi et al. 2019). Therefore, further studies are needed to understand the effect of HF diet on skeletal muscle pericytes.

1.16 The combined effects of ischemia and HF diet on pericyte

The combined effects of ischemia and insulin resistance on pericyte skeletal muscle is not understood. Revascularization is important for the success of ischemic recovery (Couffinhal et al. 1998). It is possible that pericytes support the arteriole remodeling process in the ischemic muscle by differentiating into smooth muscle cells to support blood flow recovery. This hypothesis may be supported by the idea that the exposure to circulating fatty acids increases the production of TGF- β and the observation made by our lab that the HF diet increased the number of smooth muscle actin positive vessels in the ischemic muscle (Nwadozi et al. 2020). Interestingly, we also saw that adipocyte accumulation in the regenerating ischemic muscle appeared to be suppressed by HF diet (Nwadozi et al. 2020). It is not clear whether this indicates that HF diet can suppress pericyte differentiation into adipocytes in the ischemic muscle. To investigate that more studies are needed.

Chapter 2: Rationale and Objectives

PAD has a detrimental impact on public health, causing impairment in life quality and functional status. PAD risk factors such as obesity and T2D increase the severity and threat of the disease. There is a lack of effective clinical therapies for muscle damaged by CLI. Even re-vascularization of the major leg arteries fails to recover muscle function. Thus, it is important to find an alternative therapeutic approach that can restore muscle functionality.

Pericytes are suggested to have potential therapeutic approach for ischemic muscle both due to their importance in angiogenesis and arteriolarization and their multipotency. Pericytes have the potential to act as stem cells that support the regeneration processes. But they might also promote the negative accumulation of adipose or fibrous tissue in the regenerating ischemic muscle.

We have some indications of possible roles of pericytes, but insufficient knowledge of specific mechanisms by which pericytes might participate in ischemic tissue remodeling. Furthermore, there is little information about the added influence of HF diet on these functions. This lack of knowledge combined with the potential important role of pericytes in PAD treatment leads me to investigate the impact of ischemia on pericyte fate, and if this is altered in mice pre-conditioned with short term HF diet.

2.1 Objective 1: Examine the effect that ischemia has on pericyte survival, proliferation and capillary coverage, under normal or high fat diet conditions.

Hypothesis: Ischemia will invoke some pericyte death but surviving pericytes will be activated and increase proliferation. High fat diet will decrease the number of pericytes and the capillary coverage in skeletal muscle capillaries.

2.2 Objective 2: Study pericyte differentiation into adipocytes and fibroblasts in response to ischemia and short-term HF diet:

Hypothesis: The ischemic condition induces pericyte differentiation into adipocytes and fibroblasts; however, high fat diet suppresses the differentiation process.

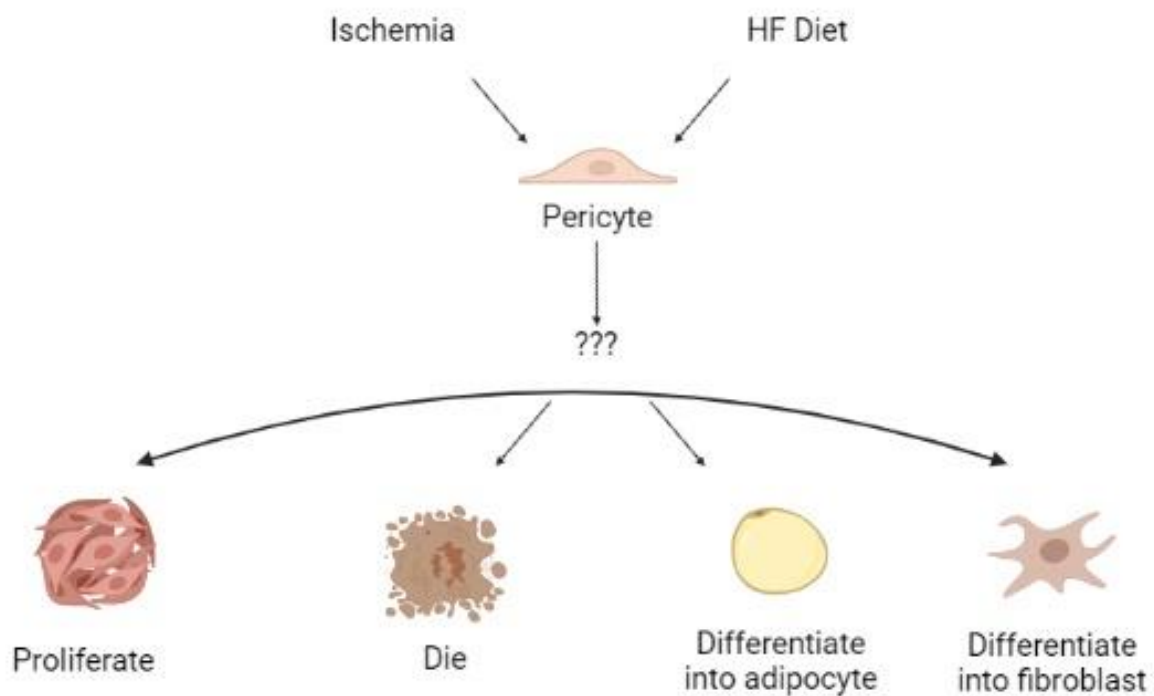


Figure 2.1 The potential effects of ischemia and HF diet on pericyte fate: HF diet and the ischemic conditions may exert pericyte proliferation or death or induce phenotype changes towards adipocytes or fibroblasts.

Chapter3: Methods

3.1 Ethical Approval

Animal studies were approved by the York University Committee on Animal Care (#2017-19R3, #2017-20R3).

3.2 Mouse Model of Femoral Artery Ligation

NG2/DsRed mice (Tg(Cspg4-DsRed.T1)1Akik/J Stock No: 008241) were fed NC or HF diet (10% or 60% kcal from fat, respectively) for 2 weeks and then underwent unilateral femoral artery ligation surgery. Hair was removed using depilatory cream that was applied in the surgery site. Inhaled isoflurane was used to anesthetize the mice. At the right leg, an incision was made to expose the common femoral artery distal to its exit from the abdominal wall. The artery was tied by double knot using a (6-0) silk suture resulting in ~90% reduction in blood flow to the distal part of the leg (confirmed using laser doppler imaging). The incision was closed using 5-0 nylon suture. Post-surgery, buprenorphine administration (0.05 mg/kg) was done for pain relief and ampicillin (20mg/kg in drinking water) to prevent infection. The left leg was used as a control to compare with the ligated leg. 5-ethynyl-2' -deoxyuridine (EdU) (Abcam #ab146186) daily i.p. injections of 100 μ l at dose (50mgEdU/gr of mouse weight) were administered on different time courses (day 2 and 3 for the 4-day time point mice and from day 4 to 8 for 8-day time points) to detect the proliferating cells. Mice were sacrificed and skeletal muscles collected at 1,4,8 or14 days post-ligation.

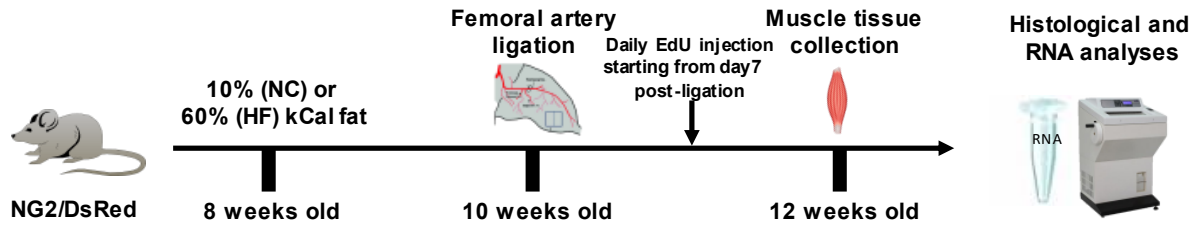


Figure 3. 1: Schematic of study timeline (14-day time point mice): 8 weeks old mice were fed NC or HF diet for 2 weeks then underwent unilateral femoral artery ligation surgery. EdU I.P injections were administered on daily basis starting from day 7 post ligation and muscles were collected for analyses 14 days post-ligation.

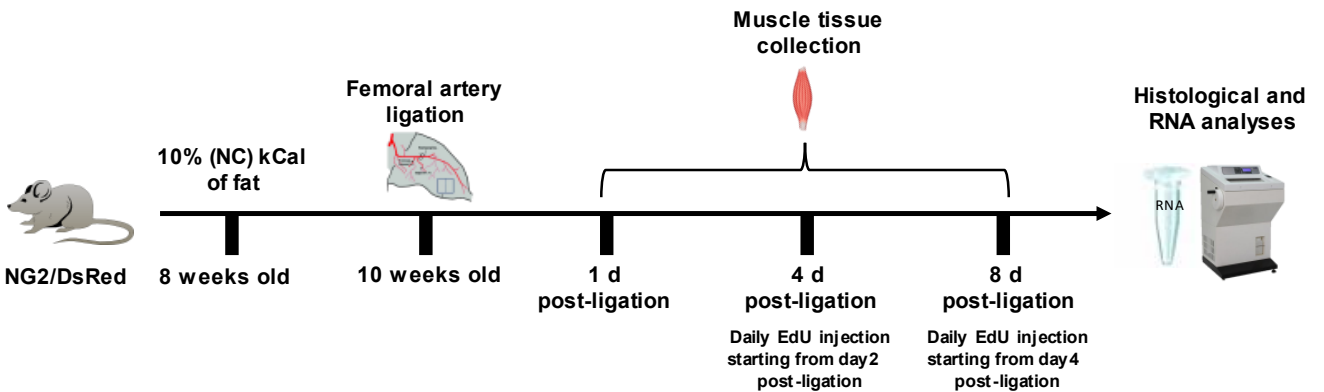


Figure 3. 2: Schematic of study timeline (1, 4- and 8-day time points): 8 weeks old mice were fed NC diet and underwent unilateral femoral artery ligation surgery. EdU I.P injections were administered on daily basis at different time courses post-ligation and muscles were collected for future analyses.

3.3 Histological assessment

3.3.1 Muscle preparation

Soleus, plantaris, extensor digitorum longus (EDL) and tibialis anterior muscles were fixed in 4% paraformaldehyde and infiltrated with 15% and 30% sucrose for 1 hour and overnight

respectively. The muscles were then embedded into optimal cutting temperature compound (OCT) and frozen in liquid nitrogen-cooled isopentane and then immersed in liquid nitrogen and stored at -80C. Gastrocnemius muscle was snap frozen in liquid nitrogen for RNA and protein analysis.

3.3.2 Cryotome

EDL and TA muscles were sectioned longitudinally (20 μ m thickness) using the cryostat. The sections were collected on slides and stored at -80C.

3.3.3 Pericyte and vasculature assessment

EDL muscle sections were stained with Griffonia simplicifolia lectin I (GSL)-FITC (1:100; Vector Laboratories, Inc) or CD31 (1:200 ;DSHB #2H8) to detect capillaries. Z stacks were taken using confocal or non-confocal microscopes, at 20x and 40x magnifications. Image J software was used for image analysis. Capillary area per field of view was measured by thresholding the lectin+ or CD31+ area, muscle longitudinal area was manually traced and measured. The number of pericytes was assessed by the manual counting of DsRed positive nuclei, and expressed as a density, relative to vascular area or to muscle area. Multiple fields of views (3-4 fields of 20x magnification images or 8-10 fields of 40x magnification images) were assessed per muscle and the averages of these assessments were used as final values.

3.3.4 Proliferating pericyte assessment

EdU incorporation was visualized in TA muscle sections using click reaction combined with azide-fluorochrome that allows detection of DNA synthesis in proliferating cells. Samples were viewed by taking images at 40x magnification using a spinning disc confocal microscope. Image

J software was used to assess the percentage of proliferating pericytes ($\frac{\text{\#DsRed+EdU+ pericytes}}{\text{total \#DsRed pericytes}} \times 100$).

3.3.5 Pericyte fat deposition assay

Bodipy (1:200 for 1 hour; Invitrogen #D3823) staining was applied on EDL muscle sections and samples were viewed by taking images at 40x magnification using a spinning disc confocal microscope.

3.4 RNA extraction from muscle tissue

Muscle tissue was lysed, and RNA was extracted using Biobasic Mini Kit (Biobasic #Bs583). RNA concentrations and purity was then quantified.

3.5 In-vitro Cell assay

3.5.1 Muscle mesenchymal stromal cells isolation:

Hindlimb muscles of mice were extracted. Isolated tissues underwent mincing and collagenase D (Millipore Sigma #11088882001) digest with agitation. The digested fractions washed once with alpha MEM. The fraction then resuspended with dispase (Sigma #D4693) solution and incubated at 37C for 5 mins then diluted with Alpha MEM, passed through cell strainer, washed, resuspended in DMEM with 10% FBS, and plated on gelatin coated flasks.

3.5.2 MSCs treatment

MSCs were plated in 12 well plates with media contains different glucose concentrations (1mM, 5.5mM or 25mM). Some of the cells were treated with 2.5mM CoCl₂ (Sigma #5862) for three

days to mimic hypoxia. Then cells were lysed for RNA extraction using Qiazol lysis reagent (Qiagen #79306).

3.5.3 RNA extraction from cultured cells

Following Qiazol lysis, chloroform was added to separate RNA from protein and DNA. RNA was collected by pipetting up the upper aqueous phase of the lysate and precipitated by addition of isopropyl alcohol. The precipitated RNA was washed with 75% ethanol and resuspended in 40µl of RNase free water. RNA concentrations and purity was then quantified.

3.6 qPCR

For each sample, 300 ng of RNA was reversed transcribed to cDNA on a Thermal cycler (2720 cycler, Applied Biosystems) using [dNTP (New England Biolabs #N0447S), Oligo DT (Invitrogen #100002344), Ribolock RNase inhibitor (Thermo Fisher Scientific #EO038), Random hexamers (Invitrogen #100026484), M-MuLV reverse transcriptase and buffer (New England Biolabs)].

The cDNA was diluted in 80 µl of RNase free water. 2µl of cDNA was combined with Taqman® Fast Advanced Master Mix (Applied Biosystems, Thermo Fisher Scientific #4444557) and TaqMan® FAM-Labelled probe sets for murine *Tbp*, *Angpt1*, *Angpt2*, *Cspg4* and *Col1a1*. q-RT PCR was performed using the PCR cycler Rotor-Gene Q system (Qiagen) in the following thermal conditions: 60 °C for 30 minutes, 95 °C for 10 minutes for 40 cycles. Samples were assessed in duplicate. To determine the relative amount of target sample mRNA, the average cycle threshold (CT) was calculated and compared to the average CT of *Tbp* (housekeeping gene) for the same samples with the formula $\Delta CT = \text{Average CT (gene of interest)} - \text{Average CT (housekeeping gene)}$. The amount of target gene amplification relative to the control was calculated using the formula $2^{-\Delta CT}$.

3.7 Statistics

Results were expressed as mean \pm SEM. Ischemic effects combined with diet data was analyzed using a two-way ANOVA repeated measures followed by Bonferroni post hoc tests. One way ANOVA was used for analysis of the effect of the ischemia time course on mRNA (Prism4; Graphpad Software Inc.) The ischemic effect data was analyzed using a two-tailed Student's *t* test. A $P < 0.05$ was considered to represent a statistical significance.

Chapter 4: Results

4.1 Pericyte number relative to muscle and capillary area increased significantly 14 days post ischemia

I first looked at pericyte number in ischemic muscle at 14 days post-ligation in mice that were fed HF or NC diet for 4 weeks. This time point was chosen because there is significant capillary perfusion and regeneration of muscle fibers in healthy mice. The diet duration was chosen based on a previous study in our lab that showed that this duration of HF diet induced a shift in pericyte gene expression and phenotype. I examined the capillary network density and pericyte density (number of pericytes relative to capillary area and muscle area) in longitudinally sectioned EDL muscle from NG2/DsRed mice, which enabled visualization of capillary networks and their associated pericytes (Figure 4.1). In these mice, DsRed fluorescent protein is produced under the control of the promoter that drives expression of *Cspg4*, the gene encoding pericyte marker NG2. Individual pericytes were identified and counted based on the presence of intense red fluorescence in their nuclei. The number of pericytes was calculated both relative to muscle area and to capillary area. Capillary area was quantified based on Griffonia simplicifolia lectin-positive fluorescence (Figure 4.1). There was a significant increase in pericyte density (relative to capillary area and relative to muscle area) in response to ischemia (Figure 4.2A and 2B). However, there was no significant effect of diet on pericyte density (Figure 4.2A and 4.2B). Furthermore, there was no change in capillary area in response to ischemia or the diet (Figure 4.2C).

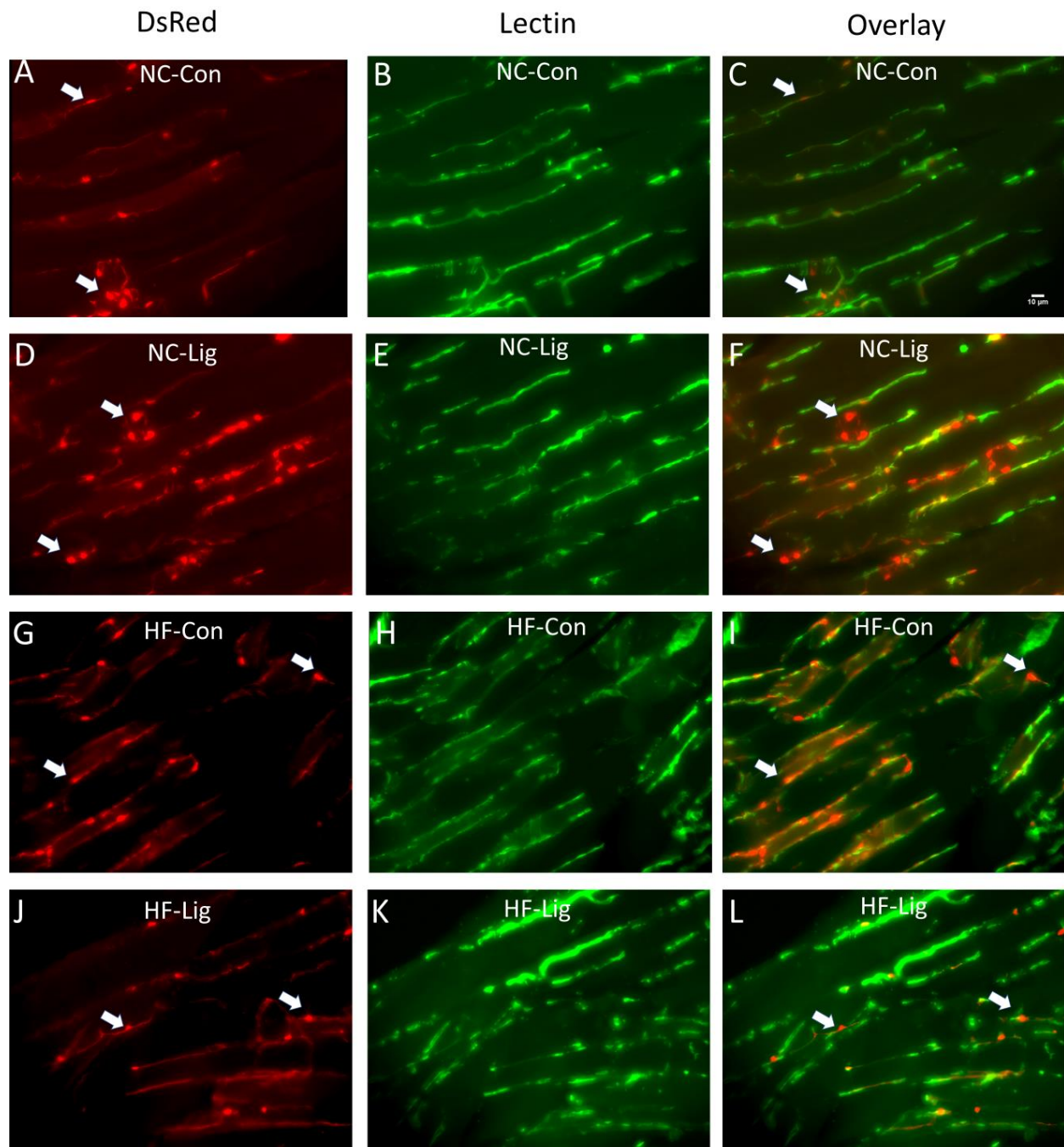


Figure 4. 1 Pericytes in ischemic and non-ischemic muscle from NC and HF diet fed mice: Hind limb ischemia was induced by femoral artery ligation after 2 weeks of either NC or HF diet. Mice recovered for 14 days before sacrifice. EDL muscle from non-ligated (Con) and ligated (Lig) limbs was examined histologically to visualize DsRed+ pericytes and lectin-stained capillaries (green). Non-confocal Z-stack images were visualized and captured using a motorized inverted fluorescent microscope with 20x objective. DsRed (A,D,G,J), Griffonia Lectin (B,E,H,K), Overlay (C,F,I,L). Arrows denote DsRed+ pericytes. Scale bar = 10 μ m.

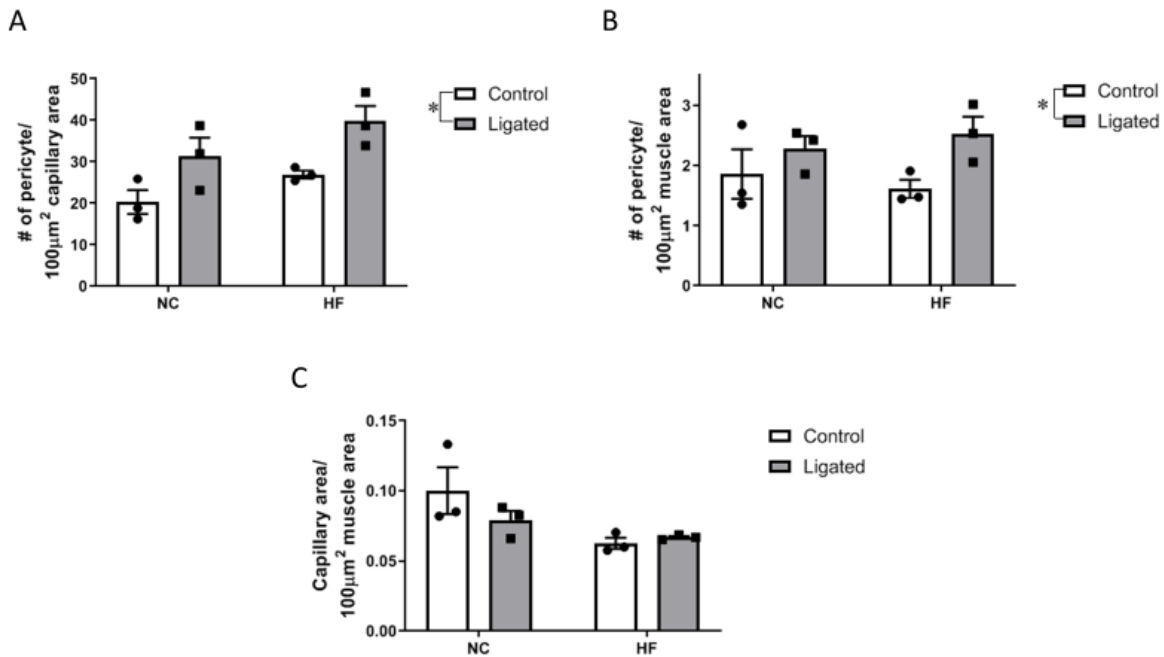


Figure 4. 2: Pericyte density increased in response to ischemia at 14 days post-ligation regardless of diet: A) Pericyte density was expressed as pericyte number relative to $100\mu\text{m}^2$ capillary area (main effect of ligation; * $P < 0.05$ two-way ANOVA. B) Pericyte density was expressed as pericyte number relative to $100\mu\text{m}^2$ muscle area (main effect of ligation). C) Capillary area relative to muscle area (No effect of ligation or diet).

4.2 Pericyte number increased 8 days post-ligation

Next, I attempted to assess a time course of pericyte number in the recovering ischemic muscle, by examining muscles at 1-, 4- and 8-days post-ligation. Due to the lack of apparent effect of diet on pericyte density at day 14 post-ligation, we focused only on NC fed mice to assess pericyte number. Unfortunately, technical problems with the 1- and 4-day post-ligation samples prevented accurate quantification of pericyte numbers. However, I was able to analyze the 8-day post-ligation muscle (for $n=3$ mice). Individual pericytes were identified and counted based on the presence of intense red fluorescence in their nuclei. Capillary area was quantified based on the positive fluorescence of platelet endothelial cell adhesion molecule (PECAM1) also known as CD31+ (Figure 4.3). There was a significant increase in pericyte number relative to

muscle area (Figure 4.4B). However, pericyte number relative to capillary area and capillary area/muscle area did not change significantly in response to ischemia although there is a trend for both to be higher post-ligation (Figure 4.4A and C).

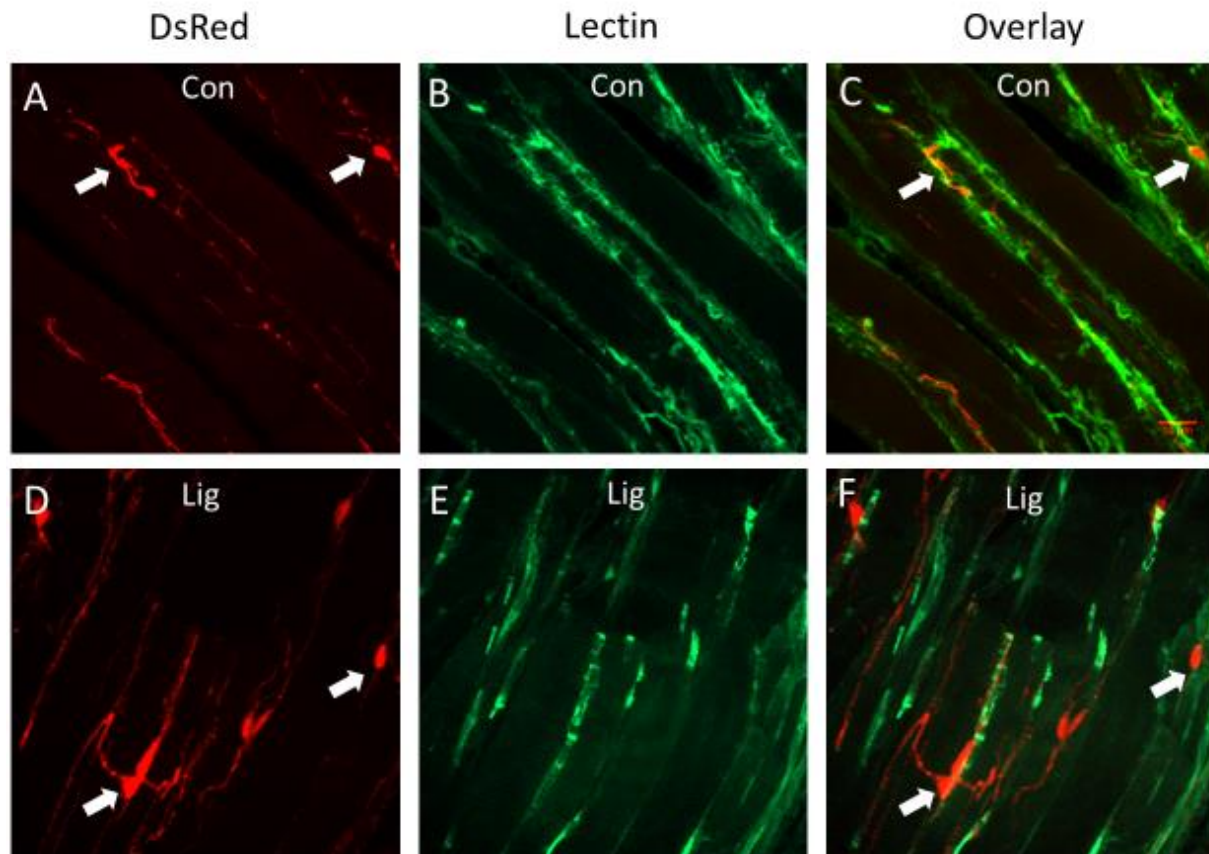


Figure 4. 3: Representative images of pericytes and capillaries in non-ischemic and ischemic muscle at 8 days post-ligation: Hind limb ischemia was induced by femoral artery ligation and mice recovered for 8 days before sacrifice. EDL muscles from non-ligated and ligated limbs were examined histologically using a Zeiss spinning disc confocal microscope and 40x objective. DsRed (A,D), CD31+ capillaries (B,E), Overlay (C,F). Arrows point to DsRed+ pericyte nuclei (A,D); Overlay (C,F). Scale bar = 10 μ m.

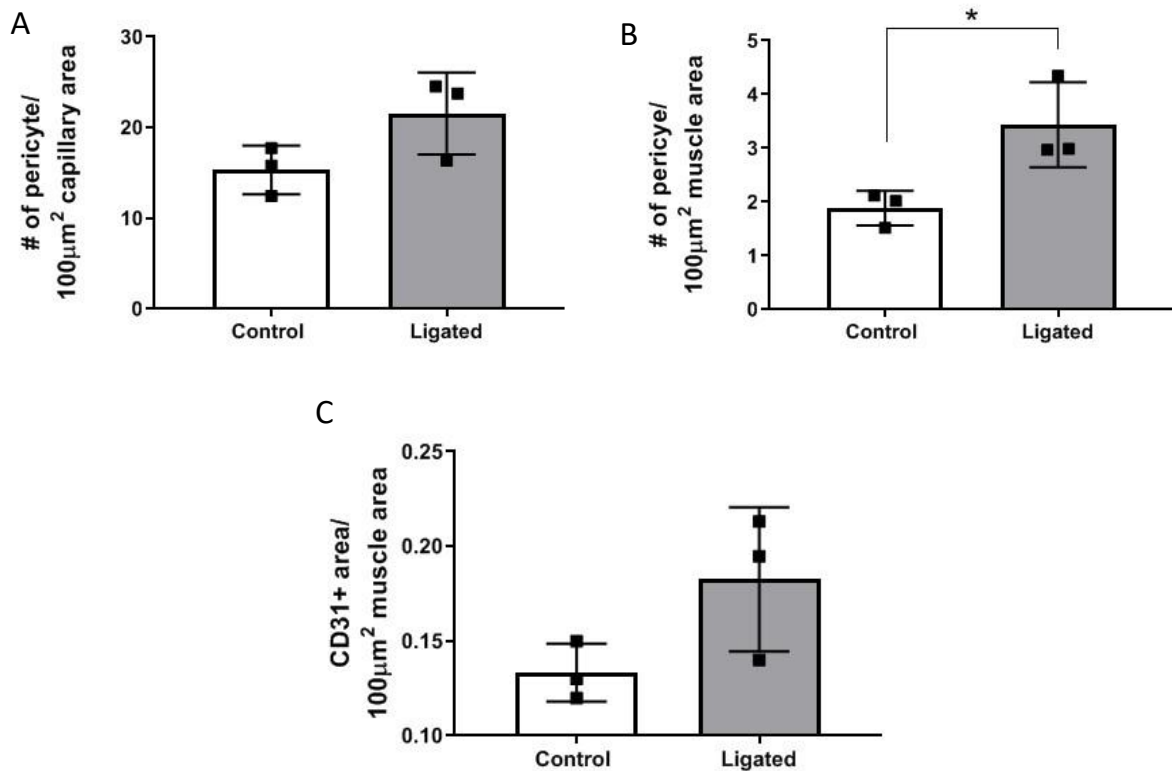


Figure 4. 4: Pericyte number relative to muscle area increased in response to ischemia at 8 days post-ligation: Pericyte density was calculated A) relative to 100µm² capillary area (P=0.1092) and B) relative to 100µm² muscle area (*P<0.05). C) Capillary area was calculated relative to muscle area (P=0.1057). Unpaired t-test.

4.3 Pericyte proliferation was evident in the ischemic muscle 8-days post-ligation

The same group of NG2/DsRed mice used to investigate pericyte density at 8 days post-ligation had been injected with EdU on days 4-7 post-ligation to label proliferating cells. Due to technical issues with tissue processing prior to freezing, I have not been able to detect EdU in the EDL muscles. Thus, tibialis anterior (TA) muscle was used to conduct these assessments. Pericyte density in the TA muscle was similar to that observed in the EDL muscle and showed a significant increase in the ischemic muscle compared to control muscle (Figure 4.6A). Minimal incorporation of EdU was detected in control muscle, as expected. Many cells were EdU+ in

the ligated muscle: these are likely to include myocytes, endothelial cells, smooth muscle cells, pericytes (Figure 4.5). Proliferating pericytes were quantified by counting the percent of DsRed+ cells that were EdU+. A significant increase in the number of EdU+ pericytes was detected in the ischemic muscle (Figure 4.6B).

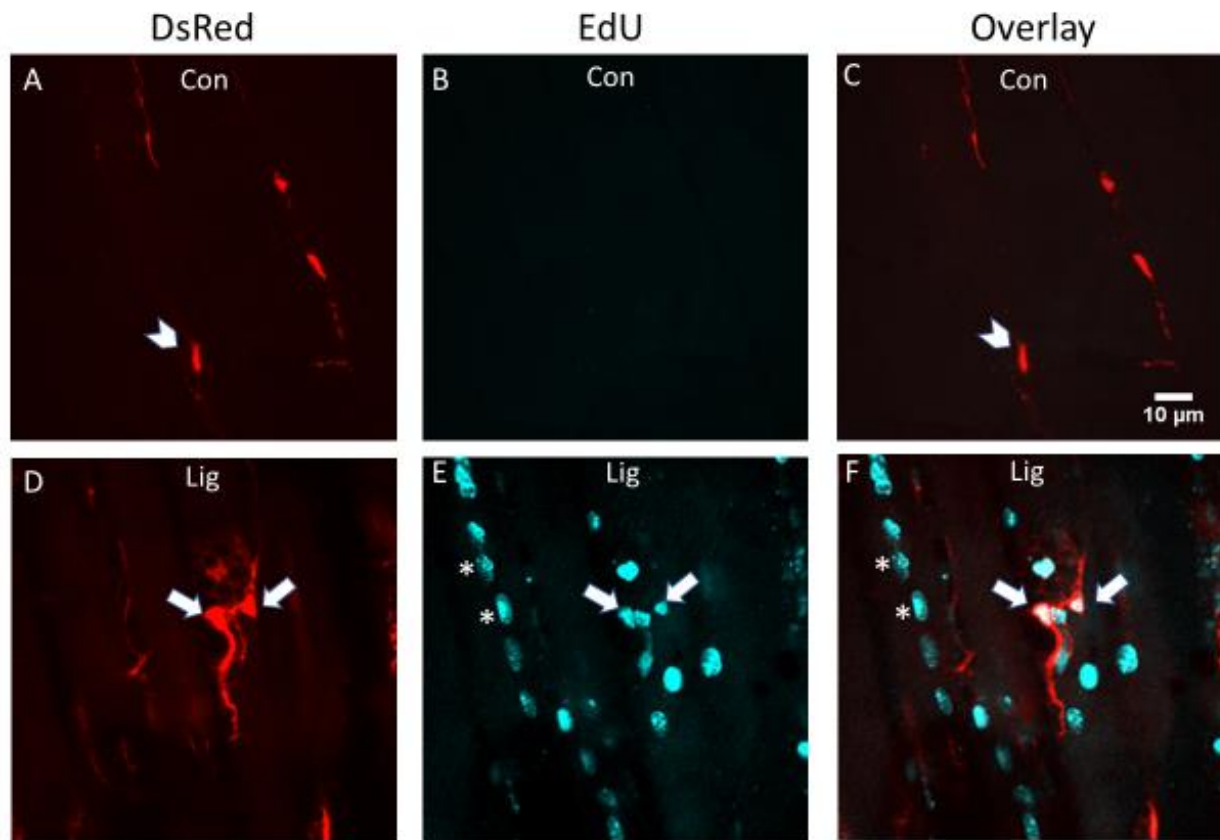


Figure 4. 5: Representative images of pericyte proliferation in non-ischemic and ischemic muscles from mice at 8 days post-ligation: Unilateral hind limb ischemia was induced by femoral artery ligation. EdU injections (i.p.) were conducted daily from days 4-7 before sacrifice at day 8 post-ligation. TA muscles from non-ligated and ligated limbs were examined histologically using a Zeiss spinning disc confocal microscope and 40x objective. DsRed+ pericytes (A,D), Cy5-EdU (B,E) ,Overlay (C,F). Proliferating pericytes (Arrows), non-proliferating pericytes (arrow heads), non-pericyte proliferating cells (asterisk). Scale bar=10μm.

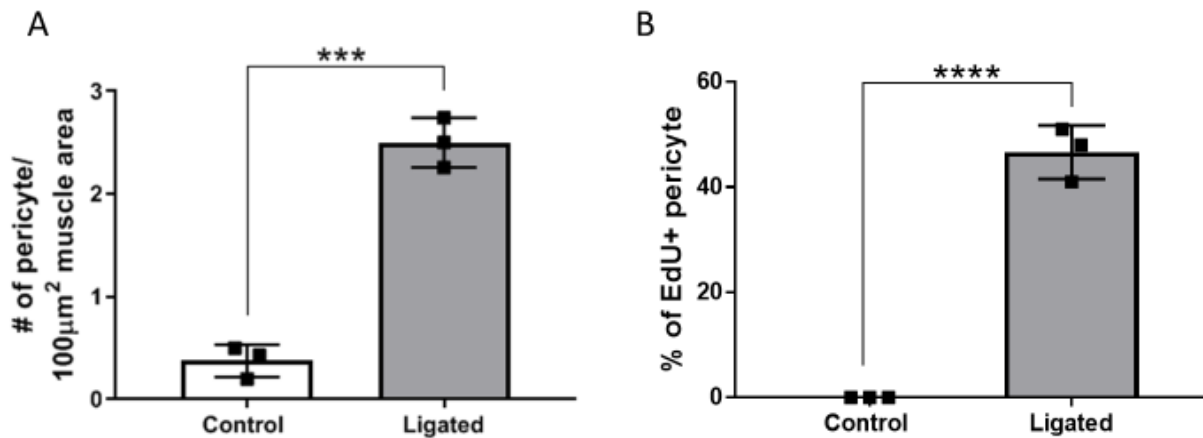


Figure 4. 6: Pericyte number and proliferation are significantly increased in response to ischemia at 8 days post-ligation: A) Pericyte density was expressed as number of pericyte relative to 100µm² of muscle area; ***P < 0.005. B) Pericyte proliferation was measured as the percentage of EdU+ DsRed+ cells. **** P < 0.0005. Unpaired t-test.

4.4 Distinct pericyte morphologies were observed post-ischemia

Our data also provided information about pericyte morphology and phenotype. High magnification images from ischemic and non-ischemic EDL muscle showed distinct pericyte morphologies (Figure 4.7). In control non-ischemic muscle, pericyte cell bodies mostly lie flat against the capillary as expected from a quiescent cell. In contrast, various pericyte morphologies are seen in ischemic muscle. For example, there appeared to be many short pericytes in an area of myofiber regeneration and many pericytes displayed a round bulky cell bodies. Moreover, some pericytes were seen to have developed extensive lipid droplet formation (based on Bodipy fluorescence) in the ischemic muscle. These findings suggest that ischemia causes pericytes to change their morphology and their interaction with capillary and appears to induce a shift in some pericytes towards an adipocyte-like cell.

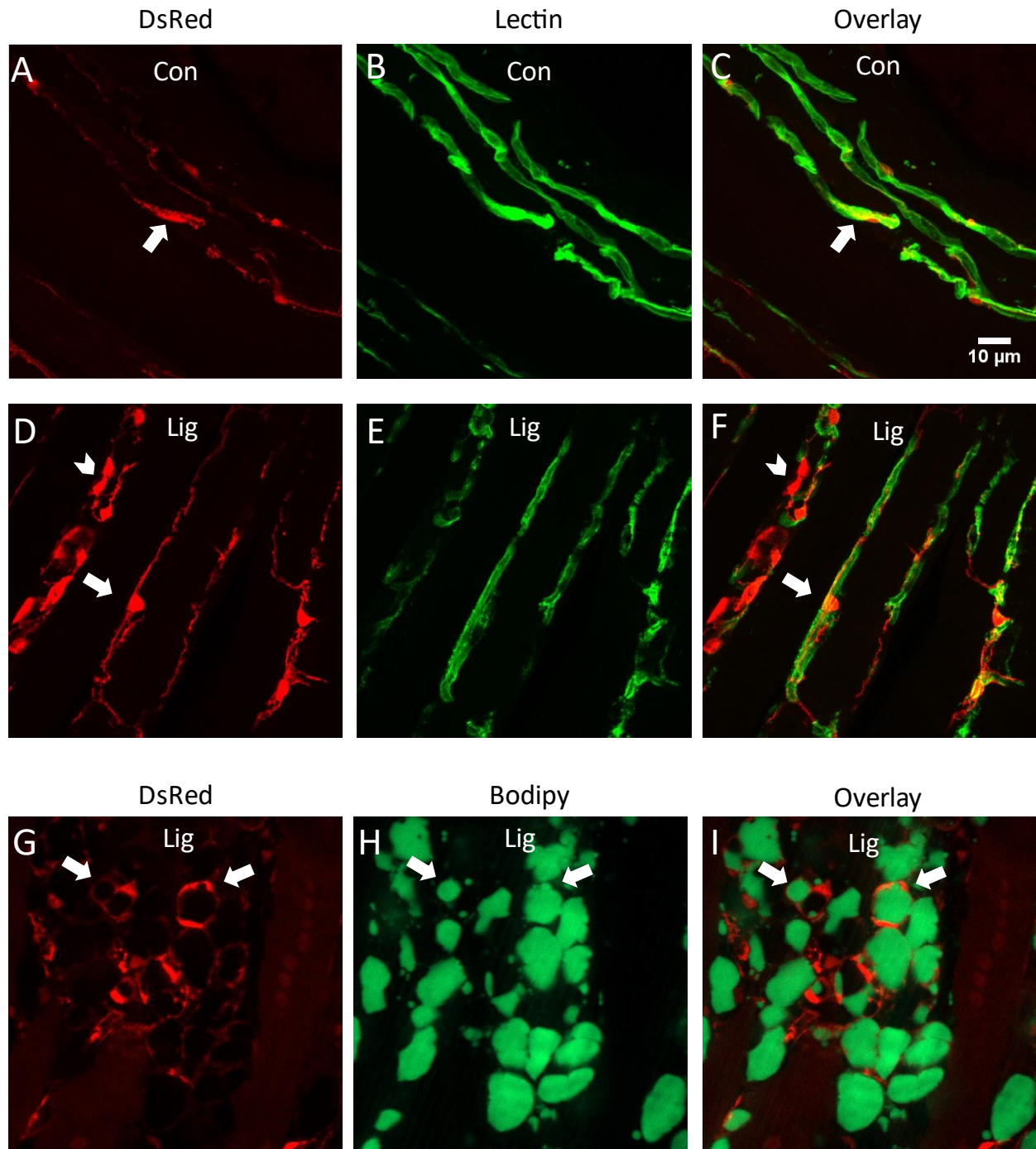


Figure 4. 7: Distinct pericyte morphologies and phenotypes in ischemic muscle: Pericyte visualization was conducted in longitudinal sections of EDL muscle using a Zeiss spinning disc confocal microscope and 40x objective. A,B,C) In control muscle, pericytes (DsRed+; Arrows) mostly lie flat against the capillary (stained with Griffonia -lectin; green). In ischemic muscle, various morphologies are seen: In 14 day-post-ligation muscles, a regeneration area contained many short pericytes (arrowheads) and round bulky pericytes (D,E,F; Arrows). In 8-day

ischemic muscle, some pericytes showed adipocyte-like appearance and contained fat depots (stained with the lipid stain Bodipy) (G,H,I; Arrows). Scale bar=10 μ m.

4.5 The relative expression of *Angpt2* but not *Angpt1* and *Cspg4* genes altered in response to ischemia

Next, I wanted to extend the pericyte histological data with gene expression measurements. I investigated the effect of ischemia on the expression of genes related to pericyte-endothelial interaction that may provide molecular insight into changes in pericyte-capillary association. I measured the gene expression of *Cspg4* (encodes the pericyte marker NG2), *Angpt1* which is a stabilizing factor for pericyte-endothelial interaction and *Angpt2* that is associated with disrupting the association between the two cell types. Those genes were measured in gastrocnemius muscle collected from mice 1-, 4-, and 8-days post-ligation relative to the housekeeping gene Tata-box binding protein (*Tbp*). There was a main effect of ligation on increasing the relative expression of *Angpt2*. However, there was no significant effect at individual time points. Moreover, the relative expression of *Cspg4* and *Angpt1* did not change with ischemia (Figure 4.8).

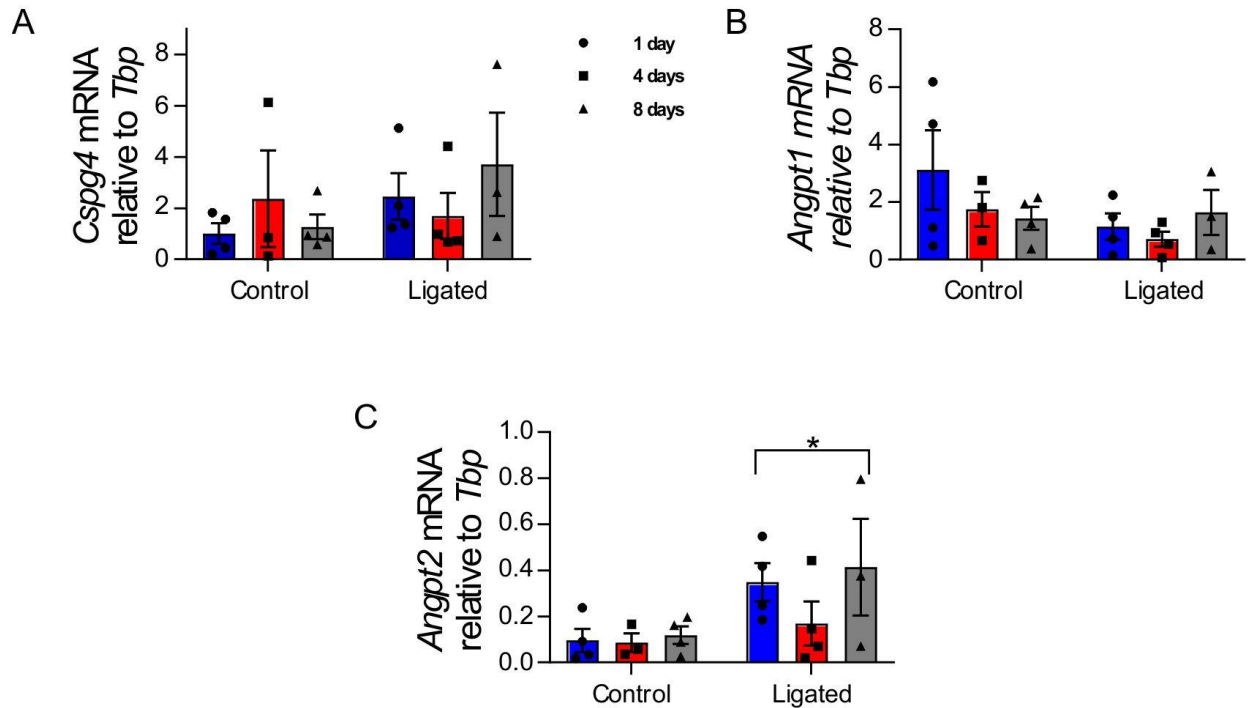


Figure 4. 8: The expression of genes involved in pericyte-endothelial interactions in response to ischemia: A) *Cspg4* gene expression relative to *Tbp*. B) *Angpt1* gene expression relative to *Tbp* C) *Angpt2* gene expression relative to *Tbp* (main effect of ligation * $P < 0.05$). One way ANOVA with Bonferroni post-hoc test.

4.6 Collagen expression is altered by glucose and hypoxic-mimetic Cobalt chloride

Ischemic muscle is characterized by nutrient and oxygen deprivation. I aimed to test if these factors alter pericyte phenotype. Due to challenges with pericyte isolation, these experiments were conducted on skeletal muscle-derived mesenchymal stromal cells (MSC), which include pericytes. Cultured MSCs were grown in media containing different glucose concentrations with or without the addition of hypoxia-mimetic cobalt chloride. The conditions were 1 mM glucose, 1mM glucose with CoCl₂ (ischemic nutrient and oxygen deprivation), 5 mM (normal glucose concentration), 5mM with CoCl₂ (hypoxic effects only), 25 mM glucose (hyperglycemia) and 25mM with CoCl₂ (hyperglycemia with hypoxia). Some cells were treated with the hypoxia-mimetic cobalt chloride (2.5mM). This experiment has been conducted only once to date. We

assessed collagen type 1 expression as a marker of a fibrosis phenotype. The glucose concentration correlated inversely with collagen type 1 relative expression. Moreover, CoCl₂ treatment substantially impaired cellular expression of collagen regardless the combined glucose concentration (Figure 4.9).

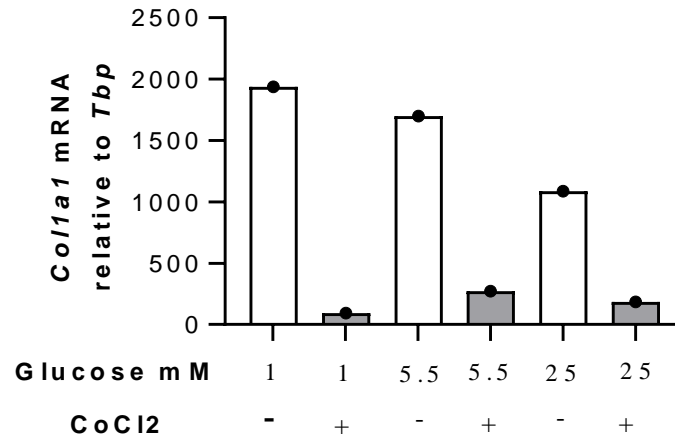


Figure 4. 9: Glucose concentration and CoCl₂ alter collagen type 1 expression in MSCs: The relative expression of *Co/1a1* in MSCs treated for three days with 1mM, 5.5mM and 25mM glucose with or without 2.5mM CoCl₂. N=1

Chapter 5: Discussion

5.1 Overview of findings

The goal of this study was to investigate the effects of ischemia and HF diet on pericyte fate. I assessed pericyte content and morphology at multiple time points following induction of ischemia. Our data showed that pericyte number in muscle increased significantly at 8- and 14-days post ischemia in both NC and HF fed mice. We demonstrated that pericyte proliferation occurred within days 4-8 post-ligation. Moreover, ischemia induced morphological changes and potential phenotype changes in skeletal muscle pericytes. Our data also demonstrated that ischemia increased the relative expression of the *Angpt2* gene that is responsible for disruption of pericyte-endothelial cell interaction. However, differences in the relative gene expression of the pericyte marker *Cspg4* and the pericyte-endothelial interaction stabilizing factor *Angpt1* were not detectable in the post-ligation in gastrocnemius muscle at 1-, 4- or 8-days. Lastly, in cultured muscle mesenchymal stromal cells, the relative expression of collagen showed a tendency to increase as glucose levels drop whereas the hypoxia-mimetic cobalt chloride greatly decreased collagen expression.

5.2 The impact of critical limb ischemia on skeletal muscle

Critical limb ischemia (CLI) is caused by the chronic blood flow reduction to the lower limbs that induces cellular damage (Varu, Hogg, and Kibbe 2010, Paoni et al. 2002). Recovery ideally includes the recruitment of immune cells that help with the removal of dead cell debris, re-establishment of the muscle vascular network to restore blood flow recovery, and the regeneration of muscle fibers (Paoni et al. 2002). These recovery events are impaired in CLI patients, who instead develop microvascular dysfunction, chronic myofiber impairment and atrophy, abnormal fat accumulation, fibrosis, distal limb necrosis and gangrene (Rutherford et

al. 1997 ; Norgren et al. 2007; Varu et al. 2010). The current conventional treatments of CLI are not adequate. Cellular therapies may be a means to improve muscle quality in the CLI patients (Powell et al. 2012). However, the specific contribution of different cell types to the pathology still needs to be established.

5.3 Pericyte contribution to CLI recovery

Pericytes may be used as a potential therapeutic approach to support the positive aspects of the ischemic recovery and suppress the bad outcomes. However, the exact roles of pericytes in the ischemic recovery and their survival capacity in response to the ischemic trauma are not understood. It is known that ischemia is a stimulus for angiogenesis. However, newly formed capillaries are structurally and functionally aberrant (Arpino et al. 2017). Since pericytes are responsible for the guidance of the vessel sprouts and the maturation and stability of this newly formed sprouts (Minoshima et al. 2018), this capillary dysfunction may be explained by a loss in pericyte coverage. For example, it was reported that cerebral ischemia leads to pericyte loss (Zehendner et al. 2015). However, it is unknown if this is the case in the skeletal muscle.

To try to pave this gap in knowledge, I investigated pericyte number in ischemic mouse skeletal muscle. This was achieved using the NG2/DsRed (NDS) mouse model, in which DsRed fluorescent protein is expressed under the control of the pericyte NG2 gene promoter. The pericytes express DsRed dominantly in their nuclei, which enables direct and accurate detection and counting of pericytes without any additional staining.

5.4 Pericyte number in ischemic muscle

The main finding from my thesis is that ischemia increased pericyte number. Pericyte density (relative to either capillary area or to muscle area) increased significantly 14-days post-ligation. This increased density can be interpreted by either an increase in pericyte number or a reduction in the capillary area. Since capillary area did not change in response to ischemia at the day 14 timepoint, this suggests that pericyte number increased. At day 8 post ligation I also detected a significant increase in pericyte number relative to muscle area in response to ischemia. Although pericyte number relative to capillary area did not change significantly, it showed a tendency to be higher in the ischemic muscles. A potential explanation could be that the few data points and their variability may prevent the statistical difference. However, the expression levels of *Cspg4* (pericyte marker) did not change in the gastrocnemius muscle at 1-, 4-, and 8-days post-ligation. The potential explanation of that result could be that *Cspg4* gene has low abundance in the muscle tissue. Therefore, the changes in the gene expression may not be detectable. Moreover, it is possible that measuring *Cspg4* in whole muscle is not a reliable estimate of pericyte content because *Cspg4* production may vary with the experimental conditions present in ischemia (Ampofo et al. 2017).

The increase in pericyte number may be the result of pericyte proliferation. My goal was to assess pericyte proliferation at multiple time points post-ischemia. Two groups of DsRed mice were injected with EdU during the muscle recovery period post-ligation surgery. The first group was injected daily from day 2 to 4 then sacrificed and the second group was injected from day 4 to 8 then sacrificed. Unfortunately, technical issues prevented from detecting Edu in the 4 days group. Nonetheless, there was substantial evidence of EdU incorporation in pericytes at the 8-day timepoint, providing clear evidence of pericyte proliferation in the ischemic muscle. Pericyte proliferation was negligible in the counterpart control muscles. Future experiments should be done to better identify the time window during which pericyte proliferate.

Pericyte proliferation may follow the same time course as angiogenesis. Starting from approximately 4 days post-ischemia, the angiogenic environment starts to be favoured in the ischemic muscle. An increase in VEGF-A production has been reported at this time point (Couffinhal et al. 1998). This VEGF-A upregulation will lead to endothelial cell activation, proliferation and new sprout formation (Couffinhal et al. 1998). In parallel with this sprouting angiogenesis, the activated endothelial cells express PDGF-BB that induces the activation and proliferation of pericytes and the subsequent recruitment of the PDGFR β positive pericytes to the newly formed vessels (Hellström et al. 1999). My data showed a change in pericyte morphology from the flat layout in the non-ischemic muscle to the round bulky cell body with short extensions in regions of regeneration in the ischemic muscle. This is accordance with the reports say that activated pericytes increase their body size and shorten their extension (Díaz-Flores et al. 2009).

Since pericytes are thought to play an essential role in angiogenesis, this increase in pericyte activation and proliferation may be implicated in supporting ischemic angiogenesis. Birbrair et al reported that pericytes have angiogenic potential in skeletal muscle by providing evidence that it supported enhanced blood flow when injected into ischemic muscle and pushed endothelial cells towards new vessel formation when co-cultured (Birbrair et al. 2014). I measured the relative expression of *Angpt1* and *Angpt2* in the gastrocnemius muscle over the recovery time course to test changes in molecules known to affect the association between pericytes and endothelial cells. My data showed that ischemia increased *Angpt2* but not *Angpt1*. This increase in the relative level of the destabilizing factor *Angpt2* supports the notion that pericyte-endothelial cell interaction is disrupted in the ischemic muscle and that the pro-angiogenic environment may be favoured.

Some reports have implicated pericytes in the trans-differentiation into different cell types in response to muscle injury. This trans-differentiation could also explain the vascular network abnormality in the ischemic muscle where the differentiated pericyte will lose its vessel stabilizing role. Moreover, pericyte phenotype change towards different cell types like myocyte, adipocyte and fibroblast like cells can participate either positively or negatively in the ischemic recovery. Birbrair et. al found two different subtypes of pericyte that have distinctive differentiation potential. Type 2 (NG2+ Nestin+) pericytes are pro-myogenic. Transplanted type 2 pericyte can differentiate into skeletal myocytes in response to muscle injury, which may improve muscle recovery. In contrast, type 1 pericytes (NG2+ Nestin-) have pro-adipogenic and pro-fibrotic potential. The type 1 pericyte can increase the expression of collagen in the injured lung and have the ability to differentiate into adipocytes when transplanted in injured muscle (Birbrair et al. 2013; Birbrair et al. 2014). My investigation found signs of pericyte phenotype changes in the ischemic muscle. I saw a potential change in pericyte phenotype towards adipocyte, based on pericyte morphologies that displayed unilocular large central fat droplets. Co-staining with bodipy showed evidence that these cells accumulated lipids while maintaining the expression of the DsRed protein. However, further study is needed to confirm this phenotype shift and their matching with the pro-adipogenic pericyte subtype (type 1).

5.5 High fat diet and muscle ischemia outcomes

HF diet is used as an the experimental model to induce insulin resistance by increasing the circulating fatty acids and mimic the effects of type 2 diabetes (T2D) (Costa et al. 2011). This is relevant for skeletal muscle ischemia since metabolic disorders such as T2D are risk factors for PAD incidence and severity (Freisinger et al. 2017). However, there is variability in the outcomes reported for HF diet effects. Some studies showed that HF-induced insulin resistance and hyperglycemia are implicated in endothelial dysfunction, inflammation, mitochondrial

dysfunction, myofiber atrophy, impaired capillary recruitment and abnormal fat accumulation and fibrosis (Mather et al. 2013; Di Meo et al. 2017 ; Varu et al. 2010). However, it was reported by our lab that HF pre-conditioning improved many aspects of the ischemic recovery. HF diet reduced the abnormal accumulation of adipocytes and fibrosis and promoted endothelial proliferation, angiogenic responsiveness and vasculature growth and increased capillary oxygen delivery during ischemic recovery (Nwadozi et al. 2020).

5.6 The additive effect of HF diet and ischemia on skeletal muscle pericyte

I wanted to determine the effect of HF diet on pericyte survival in ischemic skeletal muscle. In diabetic retinopathy, vasculature rarefaction occurs secondary to the loss of capillary pericytes (Rajagopal et al. 2016; Dalkara et al. 2011). Impaired PDGFR β signaling has been implicated in this pericyte loss. Hyperglycemia led to PDGFR β dephosphorylation, which impaired pericyte survival pathway and induced pericyte death (Geraldles et al. 2009). Based on retina studies, I hypothesized that skeletal muscle resident pericytes would be damaged/lost with HF diet. Surprisingly, I found that that HF diet did not change pericyte density (pericyte number normalized to capillary and muscle area). A possible explanation for this lack of effect is that the short-term HF diet was not capable of inducing severe hyperglycemia and insulin resistance in the mice and to cause the deleterious effects that were seen in the retina studies. The extent of metabolic adaptation to the 2-week HF diet is not known because we did not test plasma glucose levels or insulin sensitivity in the mice prior to the induction of ischemia. Moreover, my data did not show any improvement in ischemic angiogenesis in the HF versus NC diet fed mice, unlike the finding reported by our lab that prolonged HF diet feeding (8 weeks) in mice improved capillary growth and arteriolarization in the ischemic muscle (Nwadozi et al. 2020). The difference in results may be explained by the fact that long term HF diet helps to form large adipose depots prior to the ischemic damage induction. This stored fat could serve as an energy

source to support the regeneration processes and cells activation and proliferation. Moreover, this fat storage may be also implicated in the expression of cytokines and growth factors that can induce the regeneration processes including angiogenesis and blood flow recovery (Chen et al. 2018). On the other hand, short-term HF diet may be not capable to induce enough deposition of fat, thus resulting in less energy sources, less growth factor production and ultimately less regeneration capacity.

Lastly, I aimed to test what aspect of the ischemic environment is implicated in pericyte phenotype and gene expression shift. Moreover, I wanted to investigate the additive effect of high glucose levels as a side effect of HF diet. For this aim I used muscle mesenchymal stromal cells that include pericyte (Armulik et al. 2011). The cells were cultured with media containing different glucose conditions, low glucose to mimic the ischemic glucose deprivation, normal glucose concentration and high glucose level to mimic hyperglycemia. The cells then were treated with cobalt chloride that mimics hypoxia by stabilizing the hypoxia induced factor 1α and 2α under normoxic conditions. (Muñoz-Sánchez and Chánez-Cárdenas 2019). I started with measuring the relative gene expression of pro-fibrotic type 1 collagen, because it is thought that ischemia and HF diet may promote pericytes to increase their expression of collagen (Stepien et al. 2020, Pincu et al. 2015). My data showed that increasing the glucose concentration lowered collagen production. This is in accordance with the finding by our lab that HF diet preconditioning decreased the fibrosis in the ischemic muscle (Nwadozi et al. 2020). On the other hand, cobalt chloride dramatically decreased collagen expression. These results could be explained by that the cobalt chloride had a deleterious effect on cell viability and activity because we detected low transcript levels of the housekeeping gene in the cobalt chloride treated cells in comparison with un-treated counterparts. A second potential explanation could be that cobalt chloride is not an ideal way to mimic hypoxia. Therefore, in the future growing the cells under actual hypoxic conditions is needed.

5.7 Limitations of the study

5.7.1 Experimental design

Our 8- and 14-day data sets have a low sample size (underpowered experiments) that may lead to a type 2 statistical error that could result in false negative conclusion. Both female and male mice were used in the 8-day group. This could be a source of variability in the results because of potential sex differences, since sex steroids may regulate angiogenesis where sex steroids are essential modifiers of EC growth and function and vascular homeostasis (Cignarella et al. 2021). This shortage of mice was because of time problems related to York Covid lockdown and some technical issues in expanding the DsRed mice colony. To overcome this limitation, we are raising additional mice to add them to the comparison groups and increase the sample size.

To assess the effect of the ischemic injury on muscle resident pericytes, we induced the ischemic injury in the mouse right limb by conducting femoral artery ligation surgery and used the contralateral leg of the same mouse as comparison control. Conducting the comparison between the ischemic and non-ischemic (control) muscle from the same mouse helps reduce the potential variety in the ischemic response between different mice and lowers the total number of mice needed for the experiment. However, the ligation surgery procedure (incision and the separation of the connective tissue) may cause inflammation that can alter the muscle regeneration process. Therefore, we need to conduct sham surgery on one leg of a group of mice and compare it with the contralateral legs to determine the effects on the surgery procedure on muscle tissue.

5.7.2 Technical issues

In our histological assessments, I aimed to both count individual pericytes and visualize their morphology and association with capillaries. For this reason, we used longitudinal oriented

muscle sections because it can show the morphology of capillaries and associated pericytes. However, in the 14-day group, some of the muscle sections had cross-oriented fiber areas that needed to be excluded from the quantification. This problem came from poorly aligned muscles when embedded in OCT for freezing, which was fixed in the next assessed group and the muscle was straight aligned in the OCT to get a full longitudinal cryo-sections.

To assess cell proliferation, we injected the mice with EdU during the recovery period post ischemia. EdU incorporates into the DNA of the proliferating cells and can be detected in the muscle section using a chemical reaction with an azide-fluorochrome. Although the 14-day group was injected with EdU, we were not able to successfully detect the proliferating cells. Moreover, we were able to get the EdU detection to work only in the TA muscle in the 8-days group. If we can overcome this challenge, we will be able to assess pericyte proliferation in multiple muscle types and time points post ischemia. Moreover, technical issues also were faced in 1 and 4-day mice muscle tissues. After freezing and sectioning processes, the muscle sections lost the DsRed fluorescent signal. This problem may have resulted from the leakage of it out of the cells during the freezing and sectioning processes. This problem was solved in the 8-day cohort by muscle fixation prior to the freezing process.

5.8 Future directions

Our data showed that the pericyte density increased 14 days post-ischemia. It is important to determine the reason behind this increase in cell number. To satisfy this objective EdU injections must be administered to the mice at multiple time points during the ischemic recovery period to be able to identify the timing of cell proliferation. Moreover, assessment of cell death during the immediate post-ischemic period should be done. These assessments would help to more understand the behavior of pericytes in response to skeletal muscle ischemia.

I want to study the trans-differentiation potential of pericyte in response to ischemia and HF diet. For this reason, the NDS mice model is not adequate to conclusively conduct trans differentiation experiments. To test pericyte differentiation and fate in vivo we need to use lineage tracer mice. For example, mTmG mice and ribosomal tagging strategy that allows analysis of the mRNA translated in the target cells (that can be identified by the exclusive expression of HA-Tagged ribosomal protein). These mice will be crossed to mice that express Cre recombinase specifically in pericytes (tamoxifen-induced NG2/Cre-ERT2 mice). This will enable pericyte-selective expression of the downstream HA epitope-tagged exon or fluorescent marker.

Because ischemic recovery is a series of events, it is necessary to assess pericyte behavior at different stages of the ischemic recovery. This will lead to understand the stimuli that trigger pericyte proliferation and potential for trans-differentiation. This can be studied in parallel using manipulation of pericytes in cell culture.

Lastly, it is important to answer the question of whether long-term HF diet will exert a different effect on pericyte survival and capillary density in response to ischemia. To do so, we need to use the same experimental model but exposing the mice to a longer duration HF diet prior to the ischemic injury induction.

5.9 Conclusion

There is currently a lot of interest in pericytes as a potential therapeutic approach to treat patients with T2D and CLI. However, the potential that pericytes may participate negatively or positively in the ischemic recovery and the treatment of T2D requires extensive investigation.

My thesis project provided evidence that pericytes proliferate and increase in number in response to the ischemic damage. This finding can be the first step in understanding the participation and the role that pericytes play in the ischemic recovery. Moreover, this study gave a preliminary sign that pericytes start to change in morphology and phenotype in response to ischemia. This potential pericyte differentiation may help with the understanding of the adipose degeneration in the ischemic muscle and the arteriolarization and blood flow recovery process and may suggest pericyte as a potential therapeutic target to optimize the muscle health in CLI patients.

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