

CIRCULATING PROGENITOR CELL THERAPEUTIC POTENTIAL
IMPAIRED BY ENDOTHELIAL DYSFUNCTION AND RESCUED BY A
COLLAGEN MATRIX

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Abstract:

Angiogenic cell therapy is currently being developed as a treatment for coronary artery disease (CAD); however, endothelial dysfunction (ED), commonly found in patients with CAD, impairs the ability for revascularization to occur. We hypothesized that culture on a collagen matrix will improve survival and function of circulating progenitor cells (CPCs) isolated from a mouse model of ED. Overall, ED decreased the expression of endothelial markers in CPCs and impaired their function, compared to normal mice. Culture of CPCs from ED mice on collagen was able to increase cell marker expression, and improve migration and adhesion potential, compared to CPCs on fibronectin. Nitric oxide production was reduced for CPCs on collagen for the ED group; however, CPCs on collagen had better viability under conditions of serum deprivation and hypoxia, compared to fibronectin. This study suggests that a collagen matrix may improve the function of therapeutic CPCs that have been exposed to ED.

Keywords:

Coronary artery disease; circulating progenitor cells; endothelial dysfunction; nitric oxide; collagen matrix

Statement of Contribution

Dr. Erik Suuronen and I (Jenelle Marier) designed my experiments. I synthesized all collagen matrices (hydrogels), carried out all cell-material interaction studies (adhesion, viability, and migration), cytokine arrays, and nitric oxide detection and total cholesterol experiments. I also prepared all samples for flow cytometry analysis, however, Suzanne Crowe and Branka Vulesevic (technicians) ran my samples using the FACS Aria™ flow cytometer so that I could identify cell populations. Additionally, I analyzed all data.

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List of Abbreviations

bET-1	Big Endothelin-1
bFGF	Basic Fibroblast Growth Factor
BM	Bone Marrow
BM-EPC	Bone Marrow Endothelial Progenitor Cell
BM-MNC	Bone Marrow Mononuclear Cell
CABG	Coronary Artery Bypass Grafting
CAC	Circulating Angiogenic Cell
CAD	Coronary Artery Disease
CD	Cluster of Differentiation
CPC	Circulating Progenitor Cell
CS-C	Chondroitin-Sulfate C
CVD	Cardiovascular Disease
CXCR4	Chemokine Receptor 4
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
dPBS	Distilled Phosphate Buffer Saline
EC	Endothelial Cell
ECM	Extracellular Matrix
ED	Endothelial Dysfunction
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
EDTA	Ethylenediaminetetraacetic Acid
EF	Ejection Fraction
EGM-2	Endothelial Growth Medium-2
eNOS	Endothelial Nitric Oxide Synthase
EPC	Endothelial Progenitor Cell
E-selectin	Endothelial Selectin
ET-1	Endothelin-1

FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
GCSF	Granulocyte-Colony Stimulating Factor
GH	Growth Hormone
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
HFD	High Fat Diet
HIF-1 α	Hypoxia Inducible Factor-1 Alpha
ICAM-1	Intercellular Adhesion Molecule 1
IFN- γ	Interferon-Gamma
IGF-1	Insulin-Like Growth Factor 1
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
MMP-2	Matrix Metalloproteinase-2
ND	Normal Diet
nNOS	Neuronal Nitric Oxide Synthase
NO	Nitric Oxide
NO ₂	Nitrate
NO ₃	Nitrite
PBMC	Peripheral Blood Mononuclear Cell
PDGF	Platelet-Derived Growth Factor
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SDF-1	Stromal Cell Derived Factor-1
TNF- α	Tumor Necrosis Factor-Alpha
VCAM-1	Vascular Adhesion Molecule-1
VE-Cadherin	Vascular Endothelial-Cadherin
VEGF	Vascular Endothelial Growth Factor
VEGRF2	Vascular Endothelial Growth Factor Receptor-2

1. INTRODUCTION

1.1 Coronary Artery Disease

Coronary artery disease (CAD) continues to be a leading cause of death in patients worldwide, and is often only detected following hospitalization. This form of heart disease is characterized by the narrowing of the blood vessels that supply the heart. Due to plaque progression, consisting of fat, cholesterol, calcium, and other substances, blood flow to the myocardium is restricted (Profumo et al. 2011). This leads to a process known as atherosclerosis which is a systemic inflammatory disease characterized by a loss of the endothelium's integrity resulting in endothelial dysfunction (ED) and atherosclerotic lesion formation (Petoumenos et al. 2009).

In order to prevent severe lesion formation, restoration of the endothelium is vital. In the normal redox state endothelial cells (EC) lost through apoptosis can be regenerated by neighbouring ECs as well as circulating bone marrow (BM)-derived endothelial progenitor cells (EPC) (Walter et al. 2002; Wassmann et al. 2006; Werner et al. 2006). However, severe cell damage from various cardiovascular risk factors works negatively against the regeneration potential and results in irreversible endothelial damage (Dimmeler et al. 2004; Werner et al. 2006). Furthermore, several studies have demonstrated that cardiovascular risk factors have negative effects on any form of regeneration (Tepper et al. 2002; Heeschen et al. 2004; Imanishi et al. 2004). There are many risks factors associated with CAD, including hypertension, smoking, diabetes, obesity, hypercholesterolemia, and excess alcohol consumption (John et al. 1998). In patients suffering from cardiovascular disease (CVD), endothelial progenitor cell (EPC) numbers are severely lowered and exhibit impaired function compared to EPCs from a healthy individual (Vasa et al. 2001).

Current treatments aiming to slow heart disease include drugs such as cholesterol lowering medications, beta-blockers, nitroglycerin, and calcium antagonists and percutaneous coronary interventions such as angioplasty and coronary stent-implantation. Additionally, coronary artery bypass grafting (CABG), a more invasive procedure is commonly considered. Patients who are not suitable for the above treatments have the option of receiving a heart transplant, if eligible; however, organ availability is limited (Velazquez et al. 2007). Nevertheless, the current treatments do not fully address the heart's underlying disease. Some recent research efforts focus on new angiogenic treatment modalities and various adult stem cell therapies for tissue regeneration.

1.2 Endothelial Dysfunction

ED is a condition which exposes the endothelium to oxidative stress, inflammation, erosion, and vasoconstriction. ED is often present in patients with acute or stable CAD; however, patients with angina without proof of any coronary lesions are at risk also. In a recent study, ED was most frequently present in patients presenting with acute CAD, and the presence of a large number of cardiovascular risk factors greatly increased the prevalence of ED in patients without CAD (Toggweiler et al. 2010). Evidence shows that ED is associated with cardiovascular risk factors, such as smoking, hypercholesterolemia, diabetes mellitus, and family history of premature CAD (Vita et al. 1990; Celermajer et al. 1994; John et al. 1998).

Impaired endothelial-dependent nitric oxide (NO) production is the hallmark of ED, which can be related to the inactivation of NO by reactive oxygen species (ROS). The presence of ED can be tested by a variety of methods including: iontophoresis of acetylcholine, direct administration of various vasoactive agents to segments of blood vessels, and localised heating

of the skin and temporary arterial occlusion by inflating a blood pressure cuff to high pressures. The most widely used non-invasive test for assessing ED is flow-mediated dilation. This technique measures endothelial function by inducing reactive hyperemia via temporary arterial occlusion and measuring the resultant relative increase in blood vessel diameter via ultrasound (Bonetti et al. 2003).

1.3 Nitric Oxide

NO is a key modulator in the protection against the negative effects of CAD. Its production is initiated by the Ca^{2+} dependent isoform of endothelial NO synthase (eNOS) (Dudzinski et al. 2006; Feron et al. 2006). This powerful vasodilator is not only responsible for the normal vasoconstriction of a vessel, but is also known to inhibit platelet aggregation, expression of adhesion molecules at the surface of endothelial cells (adhesion of macrophages), and the release and action of endothelin-1. When there is local presence of thrombin and other substances, NO gets released into the circulation. However, any interference with this normal process will result in an inflammatory response thereby initiating the onset of atherosclerosis (Vanhoutte 2002; Voetsch et al. 2004).

Nitric oxide synthases are a family of enzymes that catalyze the production of NO from L-arginine which include neuronal nitric oxide synthase (nNOS or NOS1), inducible nitric oxide synthase (iNOS or NOS2), and endothelial nitric oxide synthase (eNOS or NOS3). nNOS can be found in nervous tissue and skeletal muscle type II and functions in cellular communication. iNOS can be located in the immune system and cardiovascular system and is responsible for creating an immune defense against pathogens. eNOS is found within the endothelium and is responsible for the vasodilation of blood vessels.

NO, whose bioavailability is altered in ED, plays a critical role in EPC recruitment and spontaneous collateral formation. One study confirmed that eNOS deficiency causes an inhibition of stem and progenitor cell mobility which resulted in the impairment of neovascularization (Aicher et al. 2003). The endothelium of blood vessels utilizes NO to signal the surrounding smooth muscle to relax, thus resulting in vasodilation and increasing blood flow. It also contributes to vessel homeostasis by inhibiting vascular smooth muscle contraction and growth, platelet aggregation, and leukocyte adhesion to the endothelium.

When shear stress is placed upon the endothelial cell, the only way for NO to be released into the circulation is the activation of certain receptors from various factors from outside the cell, such as growth factors, hormones, purines, histamines, thrombin, and endothelin-1 (ET-1). Once activated, these receptors further undergo specific pathways, all needing eNOS to convert L-arginine into NO (O'Rourke et al., 2006; Figure 1.1)

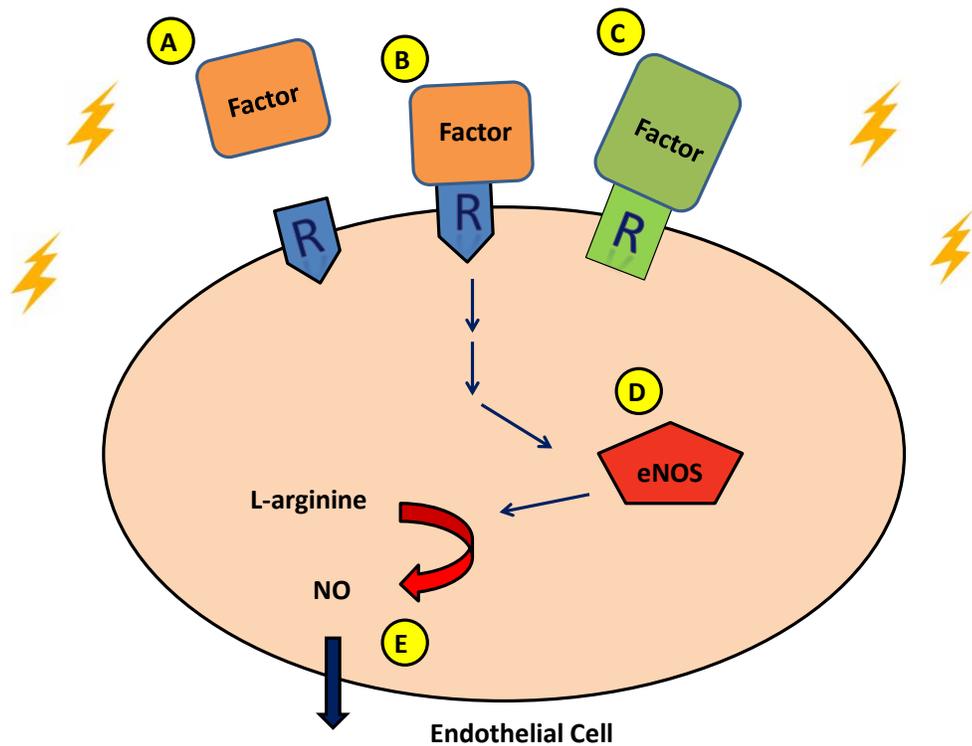


Figure 1.1. Production of nitric oxide within the endothelial cell.

Various factors from outside the cell (A) will bind to receptors on the cell (B) and the resulting complexes activate specific intracellular pathways (C). The downstream effectors stimulate eNOS to convert L-arginine into NO (D) so that NO can be released into the circulation (E).

1.4 Angiogenic Therapy

1.4.1 Protein-based therapies

There are several cytokines including members of the fibroblast growth factor (FGF) family, vascular endothelial growth factor (VEGF) family, platelet-derived growth factor (PDGF) family and angiopoietins that are involved in angiogenesis. Of these, VEGF and FGF are the most extensively studied factors for angiogenesis (Rosinberg et al. 2004). Phase I clinical trials of coronary angiogenesis have demonstrated promising results for myocardial ischemia using intramyocardial injection of FGF-1 and FGF-2. The safety and efficacy of these two treatments was further confirmed at 3 years follow-up (Pecher et al. 2000). However, Phase II trials have shown modest, if any, benefit. Although improved perfusion was observed at 32 months in test patients, the study population was rather small and a much larger populated trial would be needed to confirm these results (Ruel et al. 2002). Ultimately, it has been determined that the angiogenic response observed in these clinical trials of patients with CAD may have been inhibited due to endothelial dysfunction, administration of single agents, as well as the inefficiencies of currently available delivery methods.

1.4.2 Gene-based therapies

Gene therapy can be delivered as naked plasmid DNA with a liposomal or polymer capsule or in a viral vector, known as an adenovirus (Yla-Herttuala et al. 2000). This ensures a constant exposure to angiogenic proteins compared to the short plasma half-life when these proteins are administered directly; therefore, the use of single-dose and cell-specific therapy still remains. The safety of therapeutic angiogenesis by gene transfer has been accepted in phase I clinical trials. Some studies have demonstrated the benefit of therapeutic angiogenesis in

myocardial ischemia through gene transfer (Giordano et al. 1996; Mack et al. 1998; Lee et al. 2000). Although gene therapy has shown promise, like protein-based therapy, it also has several disadvantages. For example, adenoviral vectors promote an inflammatory response, transfection is often transient, and safety issues remain when gene transfers are made to other cells (Rosinberg et al. 2004). Of the genes studied, VEGF is the most common one used in cell therapy, in addition to FGF-1 and FGF-4. Another target for this therapy is hypoxia inducible factor (HIF)-1 α which leads to expression of angiogenesis-related genes. HIF-1 α , an upstream regulator of multiple angiogenic cytokines, is known to produce a greater angiogenic response than one growth factor alone. With this, issues ensue regarding the nonspecific action of these genes and the failed ability to completely turn them off (Khan et al. 2003).

1.4.3 Cell-based therapies

Cell-based therapies are currently being studied as a means to treat CAD, and its underlying endothelial dysfunction. Many different cell types have been exploited as potential therapeutic agents for tissue damage/injury; these include skeletal myoblasts, embryonic stem cells, adult mesenchymal stem cells, and adult hematopoietic progenitor cells. The cell type of interest here is the endothelial progenitor cell (EPC) which is derived from the bone marrow and can also be found in peripheral blood, as well as the vessel wall. They home to sites of injury in need of revascularization, differentiating into mature endothelial cells, and furthermore, contributing to angiogenesis (Asahara et al. 1999).

Cell-based approaches are attractive for treating CAD as this can be done minimally invasive, using one's own cells to restore tissue function. Initially, it was hoped that transplanting cells into ischemic tissues would replace the lost ones; however, transplanted cells

alone have low viability and several groups have reported over 90% cell death within the first hour after injection (Zhang et al. 2001). Extensive research is now being conducted on new strategies to improve the survival of transplanted cells. In addition, in order to bring cell therapy to clinical use, a number of issues need to be addressed. It is still very unclear as to which cell type to use, the optimal number of cells to transplant, the ideal delivery method and timing of transplantation, the possibility of administering proteins, and the use of biomaterials to enhance cell retention and survival. Although cell therapy remains a challenge for complete tissue restoration, the delivery combination of cells and biomaterials is very promising.

1.5 Endothelial progenitor cells

1.5.1 EPC origin and definition

EPCs were first isolated from adult peripheral blood in 1997 (Asahara et al. 1997), and were further shown to originate from bone marrow (BM) and other tissues (Asahara et al. 1999). It was believed that EPCs could reside in their primitive state and once stimulated could migrate, proliferate or differentiate into a more selective endothelial lineage; they could contribute to or even support the regeneration of injured tissues. A true definition has not yet been identified for these cells. Their phenotype and function can vary depending on their source and/or method of selection, and have been given various names including circulating progenitor cells (CPCs), circulating angiogenic cells (CACs) or EPCs; however, the function that they all share is their ability to restore vascularization to damaged tissues after cell transplantation (van Os et al. 2004). Common markers used to identify these cells include CD34, VEGFR-2, VE-cadherin, c-kit, CD31, and CD133 (Asahara et al. 1997; Ziegler et al. 1999; Burger et al. 2002).

The cell markers used in this study for characterizing cell phenotype within blood samples and cultured supernatants by flow cytometry are as follows: VEGFR2, CD31, CD34, CD45, CXCR4, and VE-cadherin. CD45 is a known marker for detecting presence of leukocytes; CD34 is a marker for identifying progenitor cell lineage, while markers VEGFR2, VE-cadherin, CD31, and CXCR4 are of angiogenic/endothelial lineage (Table 1.1).

Cell Marker	Function
VEGFR2	- Angiogenic/Endothelial marker - Receptor for VEGF
CD45	- Hematopoietic lineage - Marker for leukocytes
CD31	-Endothelial cell marker -Cell adhesion molecule
CXCR4	- Angiogenic/Endothelial marker - Receptor for SDF-1
VE-Cadherin	-Endothelial cell marker - Cell adhesion molecule
CD34	- Progenitor cell marker - Possible adhesion molecule

Table 1.1. Surface markers for cell characterization. This table identifies the individual cell markers used to assess the cell phenotype (for this project) within blood samples and cultured supernatants, and indicates their known function.

1.5.2 Role of EPCs in neovascularization

Neovascularization was originally believed to be based on angiogenesis which is the formation of new blood vessels from pre-existing vessels (Folkman et al. 1992). However, the concept that BM-derived EPCs can also contribute to neovascularization has now been demonstrated in several animal models. A model has been established for enabling the detection of BM-derived EPCs and utilizing the transplantation of BM cells from transgenic mice wild-type control mice. This model has shown that BM-derived Flk-1 and/or Tie-2-expressing endothelial cells can be localized to vascular tissues during wound healing (Bauer et al. 2006), and in skeletal muscle (Asahara et al. 1999) and cardiac ischemia (Hur et al. 2007). Also, progenitor cells with the ability to differentiate into myocytes or endothelial cells were isolated from skeletal muscle tissue in murine hindlimbs and results showed that the origin of these cells was inconclusive (Tamaki et al. 2002). This suggests that the origin of EPCs may not only be within bone marrow. Regardless of origin, it is generally accepted that EPCs play a role in contributing to neovascularisation through vasculogenesis in ischemic tissues.

1.5.3 EPC transplantation in animal models

Therapeutic approaches using EPCs have been successful in promoting revascularization and the restored function of ischemic tissues when administered on their own. The use of EPCs alone without the help of growth factors was first reported using human blood derived cultured EPCs for the transplantation into immunodeficient mice with hindlimb ischemia (Kalka et al. 2000). One study demonstrated that transplanted EPCs homed to ischemic myocardium and differentiated into endothelial cells in sites of neovascularization in a rat model of myocardial ischemia (Kawamoto et al. 2001). The same methods were repeated in another study, where

EPCs were transplanted in a nude rat hindlimb ischemia model and results further confirmed enhanced neovascularization in ischemic tissues (Murohara et al. 2000).

1.5.4 EPC transplantation in clinical trials

There are many clinical trials attempting to elucidate the benefits of cell therapy seen in animal models of ischemic diseases. The BOOST study was the first large-scale study to have shown a benefit at 6 months post BM-MNC infusion (Wollert et al. 2004); however, this was not sustained at 18 months (Meyer et al. 2006) or 5 years after cell therapy (Meyer et al. 2009). Another study (REPAIR-AMI) demonstrated significant improvements in ejection fraction (EF) at 4 months (Schachinger et al. 2006), whereas another study, where BM-MNCs were infused within 24 hours after reperfusion, showed no improvement in EF at 4 months, although a significant decrease in scar size in the cell-treated group was observed (Janssens et al. 2006). Furthermore, well-designed randomized, placebo-controlled, double-blind, and large-scale clinical trials with not only short-term but long-term follow ups are very much needed to determine whether the results seen thus far are in fact due to increased survival and reduced morbidity.

1.5.5 Proposed mechanisms for cell therapy

Although several mechanisms associated with cell-based therapies have been identified, they are still not fully understood. Some proposed mechanisms for the transplantation of EPCs: (i) EPCs can aid in the new vasculature by incorporating into new vessels (Ziegelhoeffer et al. 2004), or (ii) these cells act in a paracrine manner by secreting various cytokines, chemokines, and growth factors to initiate new vessel formation, inhibit cell death, and recruit progenitor cells to sites of injury (Kamihata et al. 2001; Kinnaird et al. 2004). Some experiments reveal that the

percentage of cells that remain within the target area is quite low. The number of cells that actually home to infarcted area greatly depends on the route of administration and unfortunately, rapidly decreases over time (Aicher et al. 2003; Brenner et al. 2004). It is well understood that response to cell therapy is dose-dependent due to poor retention and viability within affected areas and therefore limit their regenerative potential. The enhancement of EPC delivery is currently being developed by numerous groups and is of great interest (Bahlmann et al. 2005; Kuraitis et al. 2011).

The paracrine mechanism is thought to be the most important mechanism involved in the benefits that can be derived from EPC therapy. Cytokines and growth factors play a crucial role to prevent apoptosis of cardiomyocytes or in regenerating ischemic/damaged tissues. These powerful agents are thought to benefit damaged tissues by indirectly stimulating stem/progenitor cells. Of these known agents, fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), granulocyte colony-stimulating factor (GCSF), growth hormone (GH), and insulin-like growth factor (IGF)-1 have all been trialed for cytokine therapy in cardiovascular disease patients (Beohar et al. 2010). FGF promotes angiogenesis and arteriogenesis with the help of endothelial and smooth muscle cells. When administered to ischemic hearts, cell proliferation and secretion of metalloproteinases was induced, as well as VEGF which resulted in the process of angiogenesis (Grines et al. 2002). VEGF has a protective role in cardiac tissue repair. During hypoxia, stimulation of VEGF increases endothelial cell proliferation, migration, and survival, which ultimately leads to neovascularization (Vandervelde et al. 2007; Voo et al. 2008).

1.5.6 Limitations of cell therapy

There are several factors such as aging, diabetes, hypercholesterolemia, hypertension, and smoking that reduce the number or circulating BM-EPCs in patients and which lead to possible limitations for successful EPC transplantations (Alev et al. 2011). Despite the promise of cell therapy, several limitations have been identified which include: host environment, required number and function of cells needed for efficient cardiac repair, low cell dosages, route of administration, and low rates of cell engraftment (Templin et al. 2011). Regarding autologous EPC therapy, improvements including local delivery of EPCs, endogenous mobilization of EPCs with regards to administration of cytokines/growth factors to promote BM-derived EPC mobilization (Asahara et al. 1999), enhancing EPC function with gene transduction, and culture of EPCs from primitive cells isolated from other sources are needed for improving cell homing, survival, engraftment and repair capacity of transplanted cells.

1.5.7. Effects of endothelial dysfunction on regeneration

There are many proposed mechanisms for the inhibition of tissue regeneration in the presence of ED, although much extensive research needs to be conducted to validate these statements. It was recently confirmed in an animal model that ED negatively influenced the in vivo response to cell therapy (Suuronen et al. 2010). In addition, other studies demonstrated that progenitor cells taken from CVD patients may also have defects in their regenerative capacities (Vasa et al. 2001; Walter et al. 2005). The mechanism remains unclear as to why endothelial dysfunction inhibits the benefits of cell therapy, whether the host environment is responsible and/or whether paracrine/humoral factors that result from progenitor cell recruitment are lost (Vasa et al. 2001; Cho et al. 2007). Transplanted cells can rapidly disappear from the target

tissue, but they induce humoral effects, which are sustained by the host tissues - this mechanism was deemed vital in tissue recovery after cell therapy (Cho et al. 2007). If the sustained humoral effects are indeed the mechanism responsible for the therapeutic benefit of cell transplantation, there is reasonable expectation that ED will minimize or abrogate the effects of cell therapy. Suuronen et al. 2010 confirmed that the level of human cell engraftment at 3 weeks was not affected by ED suggesting that possible reasons for the observed inhibitory effects may be due to host-sustained humoral effects rather than engraftment and differentiation. Treatment with L-arginine improves the angiogenic response and offers the possibility that host and transplanted cells interact with one another as well as humoral effects which potential play a role in this improvement (Nakai et al. 2005). As stated previously, the mechanism responsible remains unknown and continues to be an area for future research.

1.6 Biomaterials in cell therapy

When stem cells are injected into the myocardium, the majority of them fail to engraft (Su et al. 2010). Therefore, strategies to improve the retention and engraftment of cells upon delivery are needed. Biomaterials can be effective for transplanting stem cells and delivery methods can be minimally invasive, particularly if injectable materials are used. Several types of materials have been tested in animal models of disease for the delivery of cells for therapy.

1.6.1 Collagen

Most of the tissues within the human body contain a protein known as collagen. To date, 28 different types of collagen have been identified (Plumb et al. 2007). Each collagen type is made up of three α -chains which form the polypeptide. Due to its integration with other

components of the extracellular matrix (ECM), collagen offers structural support and has been shown to aid in cell migration, adhesion and differentiation (Kucharz 1992).

The most commonly explored collagen is type I, as it is the most abundant type of collagen within the ECM. Water-soluble procollagen type I is synthesized by fibroblasts and secreted into the extracellular space *in vivo* (Zhang 2008). Procollagen I is converted to insoluble collagen once proteolytic removal of C- and N- propeptides has taken place. Since collagen is biocompatible with cells and tissues, it is the most sought-after material for tissue engineering.

Cross-linking is currently being investigated as a means to facilitate tissue engineering *in vitro*. This process occurs when intra-molecular or inter-molecular bonds are formed where loss of water solubility is attained. Of the various cross-linking strategies being extensively researched, the use of glutaraldehyde is most common (Charulatha et al. 2003). Other chemical reagents including 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) are also being tested to cross-link collagen type I. Since these chemical cross-linkers are associated with potential cytotoxic effects (Olde Damink et al. 1996; Liu et al. 2006), many physical methods are being investigated such as dehydrothermal treatment, ultraviolet and photo-chemical cross-linking (Pieper et al. 1999; Billiar et al. 2001; Ibusuki et al. 2007). However, physical cross-linking has been shown to be weak and further research using these methods is needed (Billiar et al. 2001; Ibusuki et al. 2007).

Interactions between collagens and cells are crucial for the properties and maintenance of a tissue. As migration of endothelial cells is important for angiogenesis, this solely depends on the interaction between collagens and cell surface receptors. Integrins are important modulators in cell-collagen interactions, some of which include $\alpha 1\beta 1$ and $\alpha 2\beta 1$ (Senger et al. 2002). These cell surface receptors initiate the migration of cells by enabling attachment to the ECM.

Cell homing and retention of transplanted cells within the target tissue relies on the adhesion of donor and host cells, and its survival rate which has been shown to be less than 15% (Muller-Ehmsen et al. 2002). Previous studies have shown that collagen-based matrices can support the differentiation of endothelial progenitor cells into mature endothelial cells, can improve the survival and function of EPCs in a serum deprivation environment, and can also produce a population of progenitor cells with enhanced functional capacities, such as cell migration, adhesion, and angiogenesis (Kuraitis et al. 2011). There are several advantages in using collagen-based matrices as they offer a large surface area for cell seeding, are very stable for mechanical support, its' porosity for vessel ingrowth, biodegradable, have minimal immunogenicity, and overall have had many successful years of use (Kofidis et al. 2003; Gonen-Wadmany et al. 2004; Boccafoschi et al. 2005).

1.7 Hypotheses and objectives

Although cell therapy remains a promising therapy for treating CAD, several limitations have been identified that limit its effectiveness in the clinic, as described above. Our lab and others have demonstrated success for the use of collagen-based matrices in improving EPC delivery, retention, and function for the treatment of ischemic tissue. Therefore, the hypothesis of this study is that culturing circulating progenitor cells (CPCs) from animals with endothelial dysfunction on collagen matrix will improve the function of these cells, including: viability, adhesion, migration, nitric oxide production and angiogenic cytokine profile.

Our objectives are to elucidate the effects by which a matrix may improve the function of CPCs for therapy in the presence of endothelial dysfunction. Specifically, the aim is to use *in*

vitro functional assays to compare collagen matrix versus fibronectin for the culture of CPCs from mice fed a high fat diet versus normal diet.

2. Materials and Methods

MATERIALS

All reagents were obtained from Sigma-Aldrich (Oakville, Canada), unless indicated otherwise.

2.1 ANIMAL MODEL

2.1.1 Inducing Endothelial Dysfunction

All procedures were performed with the approval of the University of Ottawa Animal Care Committee, in accordance with the National Institute of Health's Guide for the Care and Use of laboratory Animals. C57BL/6J wild-type 6-week old mice were ordered from Jackson Labs (Bar Harbor, USA) and fed for 4 weeks with an assigned diet that consisted of either regular mouse chow (19% protein) or a 1.25% cholesterol and 16% butter fat diet (Harlan; Madison, USA).

2.1.2 Confirmation of Endothelial Dysfunction

After 4 weeks of diet, blood from the sacrificed animals was collected by cardiac puncture. Blood was layered onto Histopaque and spun for 30 min. The plasma layer obtained from separation was prepared for anti-mouse custom cytokine arrays (Raybiotech, Norcross, USA). Protocols were carried out according to manufacturer's recommendations. Levels for each cytokine in the high fat diet group were normalized to mean level values calculated for the normal diet group. An endothelial dysfunction index was calculated from the ratio of the average changes of circulating adhesion molecules: ICAM-1, VCAM-1, and E-selectin. ED Index = (Δ I-

CAM + Δ V-CAM + Δ E-selectin/ Δ control), where Δ represents the average change in concentration.

The serum collected from sacrificed mice was also analyzed to determine total cholesterol levels using a Cholesterol E enzymatic colorimetric assay kit (Wako Diagnostics, USA). The cholesterol produced and the free cholesterol already present in the serum is oxidized in reaction catalyzed by cholesterol oxidase that generates hydrogen peroxide. Briefly, 200 μ l of color reagent is added to all standards and samples, and the 96-well plate is then incubated at 37 °C for 15min. The absorbance of the blue color was read at 600nm using a Beckman Coulter AD340 plate reader.

2.2 CPC ISOLATION and CULTURE

A cardiac puncture was performed and approximately 1 ml of peripheral blood was procured from mice, and density-gradient centrifuged on Histopaque 1077. Peripheral blood mononuclear cells (PBMCs) were removed with the buffy coat, washed twice (0.11% EDTA, 1% FBS, in distilled phosphate buffered saline (dPBS)) and plated on fibronectin-coated tissue culture polystyrene (TCPS) plates in Endothelial Growth Medium-2 (EGM-2; Clonetics, Guelph, Canada; FBS, VEGF, R3-IGF-1, hEGF, and GA-1000 supplements added). After 4 days in culture, the supernatant and non-adherent cells were discarded. Adherent cells were trypsinized (0.25%), lifted with dPBS, and considered to be circulating progenitor cells (CPCs) (Ruel et al., 2005).

2.3 BIOMATRIX PREPARATION (see Figure 2.1)

Matrix was prepared from rat tail collagen I (BD Bioscience, Mississauga, Canada) and chondroitin sulfate-C (CS-C; 9:1 CS-C: CS-A). Collagen (0.375%) was mixed in a cold glass tube with 500 μ l of buffer (29 ml buffer made from: 9 ml FBS, 9 ml 10 \times DMEM, 10 ml 200 mMHEPES, 3–5 drops gentamycin) on ice. The final concentration of collagen was 2.35 mg/ml. CS-C was added to a total concentration of 11.49 mg/ml. Glutaraldehyde (1.5%) in 1 \times DMEM was used to cross-link the CS-C and collagen and left for 45 min on ice; 20% glycine was then added to react with excess glutaraldehyde and left for an additional 45 min on ice. Matrix solution (pH of 7.2-7.4) was added to 12-well plates (100 μ l/well was evenly spread), and gels were formed at 37°C for 30 min. After gelation, EGM-2 media was added to each gel to prevent dehydration and pH was maintained between 7.2 and 7.4.

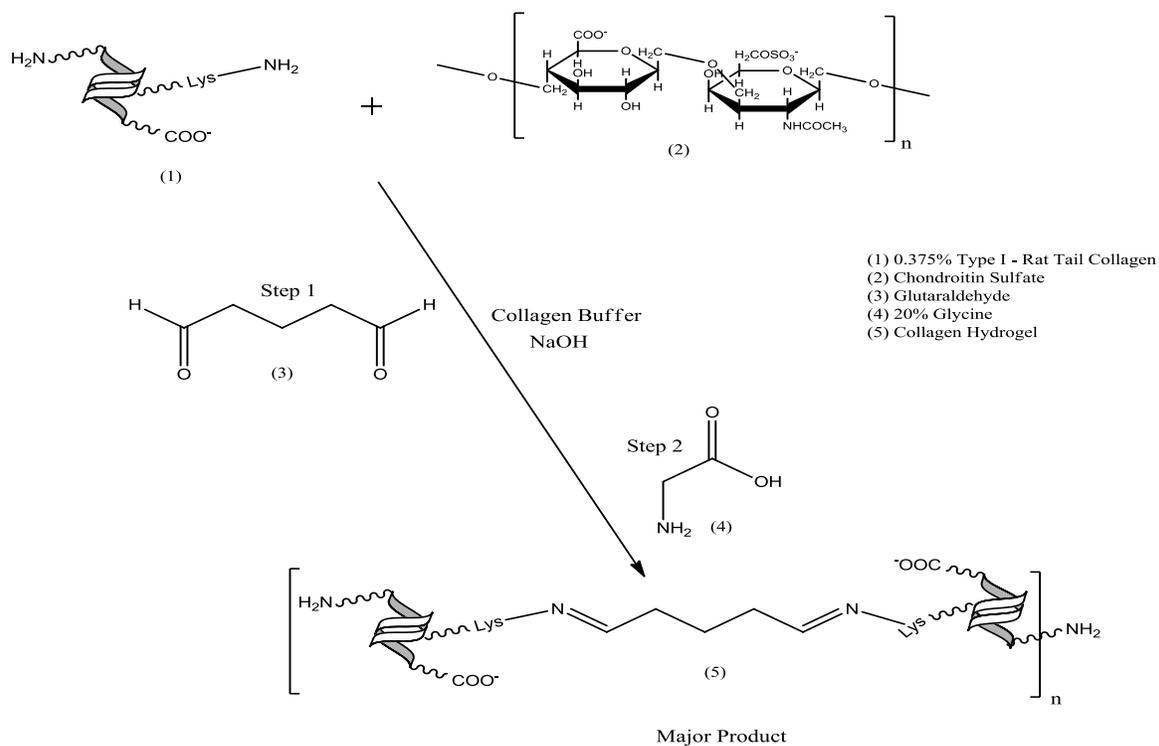


Figure 2.1. Collagen Glutaraldehyde Pathway. The collagen matrix containing two HC=N bonds is the major product and is formed from the two aldehyde groups on the bifunctional cross-linking agent (glutaraldehyde) reacting with the two $-\text{NH}_2$ groups from Type 1 Collagen.

2.4 NORMOXIC EXPERIMENTS (see Figure 2.2)

2.4.1 Flow Cytometry

The 4-day CPCs were plated for an additional 4 days on fibronectin or collagen in normoxia, lifted, and incubated with assigned antibodies at 4°C for 30 minutes. The following antibodies were used in two different combinations; **COMB 1**: VEGFR2-PB (eBioscience, San Diego, USA), CD34-PE (eBioscience, San Diego, USA), and CD45-FITC (R&D Systems, Minneapolis, USA); **COMB 2**: CD31-FITC (BD Biosciences, Mississauga, Canada), CXCR4-APC (BD Biosciences, Mississauga, Canada), and VE-cadherin-PE (Santa Cruz Biotechnology, California, USA). In controls, isotype-matched FITC-, PE-, FITC-, PB-, APC-, and PE-conjugated antibodies were used. After 30 min incubation, 1mL of dPBS was added and spun for 10 min. Cells were then resuspended in 300ul dPBS for analysis. All samples flow cytometry were performed on a FACS Aria™ (BD Biosciences, Mississauga, Canada) and data was analyzed using FACSDiva software. All marker expression values obtained from high fat diet mice (HFD) were normalized to normal diet (ND) control.

2.4.2 Adhesion Assay

The 4-day CPCs were cultured on fibronectin and collagen for an additional 4 days in normoxia. After 4 days of culture, cells were lifted as previously described. CPCs (2×10^4) were resuspended in 300 µl of EGM-2 and seeded in 12-well plates containing fibronectin-coated coverslips. After 1 h at 37 °C, the media was aspirated and adherent cells were fixed with 4% paraformaldehyde. Coverslips were washed twice with dPBS, and placed on slides with 4',6-diamidino-2-phenylindol- (DAPI) containing mounting medium (Vector Laboratories). Six

random fields-of-view were imaged using an Olympus BX60 fluorescent microscope, and DAPI+ cells were counted (Suuronen et al. 2006).

2.4.3 Migration Assay

The 4-day CPCs were further plated on fibronectin and collagen for an additional 4 days in normoxia. CPCs were subsequently lifted and (2×10^4) cells were added to the upper chamber insert (3.0um) of a 12-well plate (Fisher Scientific, Ottawa, ON) in 200ul serum- and growth factor-free media. The lower chamber contained EGM-2 with serum and VEGF (50ng/ml). After 24 h, cells that had penetrated the basement membrane and adhered to the lower insert membrane were fixed with 4% paraformaldehyde, and stained with 4',6-diamidino-2-phenylindol- (DAPI) containing mounting medium (Vector Laboratories). Six random fields-of-view were imaged using an Olympus BX60 fluorescent microscope, and DAPI+ cells were counted (Kuraitis et al., 2011).

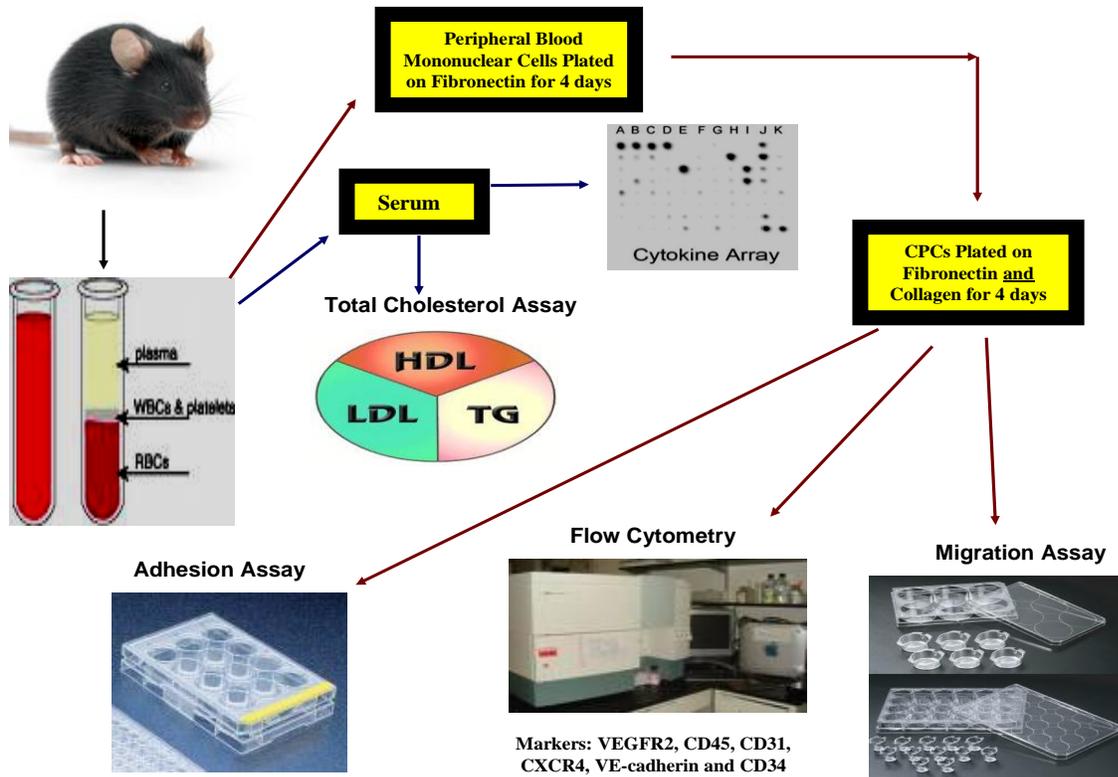


Figure 2.2. Experiments conducted in normoxic conditions. Cardiac puncture was performed on all mice; collected blood was layered onto Histopaque and spun for 30 min. The plasma was collected for total cholesterol assay and cytokine assay. The buffy coat containing peripheral blood mononuclear cells (PBMCs) was plated on fibronectin for 4 days to generate circulating progenitor cells (CPCs). These cells were then lifted and further plated for an additional 4 days on fibronectin or collagen in normoxia. These cultured cells were analyzed by flow cytometry, and used in cell adhesion migration assays.

2.5 HYPOXIC EXPERIMENTS (see Figure 2.3)

2.5.1 Flow Cytometry

The 4-day CPCs were plated (250,000 cells) for an additional 48 hrs on fibronectin or collagen in hypoxia, lifted, and incubated with assigned antibodies at 4°C for 30 minutes. The following antibodies were used in two different combinations; **COMB 1:** VEGFR2-PB (eBioscience, San Diego, USA), CD34-PE (eBioscience, San Diego, USA), and CD45-FITC (R&D Systems, Minneapolis, USA); **COMB 2:** CD31-FITC (BD Biosciences, Mississauga, Canada), CXCR4-APC (BD Biosciences, Mississauga, Canada), and VE-cadherin-PE (Santa Cruz Biotechnology, California, USA). In controls, isotype-matched FITC-, PE-, FITC-, PB-, APC-, and PE-conjugated antibodies were used. After 30 min incubation, 1mL of dPBS was added and spun for 10 min. Cells were then resuspended in 300ul dPBS for analysis. All flow cytometry was performed on a FACS Aria™ (BD Biosciences, Mississauga, Canada) and data was analyzed using FACSDiva software. All marker expression values obtained from high fat diet mice (HFD) were normalized to normal diet (ND) control.

2.5.2 Live/Dead Assay

The 4-day CPCs were plated for an additional 48 h on fibronectin or collagen in hypoxia, where 250,000 cells were subjected to serum starvation to induce apoptosis by culture in 1 ml EGM-2 without serum and VEGF. After 48 h, cells were lifted and stained using a Live/Dead Viability/Cytotoxicity Kit (Invitrogen, Burlington, ON) which quickly discriminates live from dead cells by simultaneously staining with green-fluorescent calcein-AM to indicate intracellular esterase activity (live cells) and red-fluorescent ethidium homodimer-1 to indicate loss of plasma

membrane integrity (dead cells). Six random fields-of-view were imaged using an Olympus BX60 fluorescent microscope, and both red and green stained cells were counted.

2.5.3 Nitric Oxide Assay

The 4-day CPCs were plated for an additional 48 h on fibronectin or collagen matrix in hypoxia, where 250,000 cells were subjected to serum starvation to induce apoptosis by culture in 1ml EGM-2 without serum and VEGF. After 48 h, the supernatant was collected and nitric oxide levels were detected using a colorimetric non-enzymatic nitric oxide assay (Oxford Biomedical Research, Oxford, USA) which allows you to measure total nitric oxide (NO) produced for in vitro experimental systems following conversion of nitrate (NO₃) to nitrite (NO₂) by metallic cadmium. Briefly, 1 g of cadmium beads was measured out and washed twice with H₂O, HCL, and NH₄OH. 20ul of 30% ZnSO₄ was added to 400ul of supernatant and mixed vigorously for 15min. Samples were then centrifuged for 5 min at 4°C. This supernatant was added to cadmium beads overnight and assayed the next day. The complete reaction was read at an absorbance of 540nm using a Beckman Coulter AD340 plate reader.

2.5.4 Cytokine Array

The 4-day CPCs were plated for an additional 48 h on fibronectin or collagen matrix in hypoxia, where 250,000 cells were subjected to serum starvation to induce apoptosis by culture in 1ml EGM-2 without serum and VEGF. After 48 h, the supernatant was collected and prepared for anti-mouse custom cytokine arrays (Raybiotech, Norcross, USA). Protocols were carried out as per the manufacturer's recommendations. The high fat diet group had the plasma level for each cytokine normalized to the mean level calculated for the normal diet group. An angiogenic index

was calculated from the ratio of the average changes of SDF-1, VEGF, bFGF and GM-CSF. Angiogenic index = $(\Delta\text{SDF-1} + \Delta\text{VEGF} + \Delta\text{bFGF} + \Delta\text{GM-CSF}/\Delta\text{control})$, where Δ represents the average change in concentration.

2.6 Statistical Analysis

All comparisons between groups were calculated using a 2-tailed unpaired Student's t-test. All values of $P < 0.05$ were taken as significant, and error bars represent standard error of the mean.

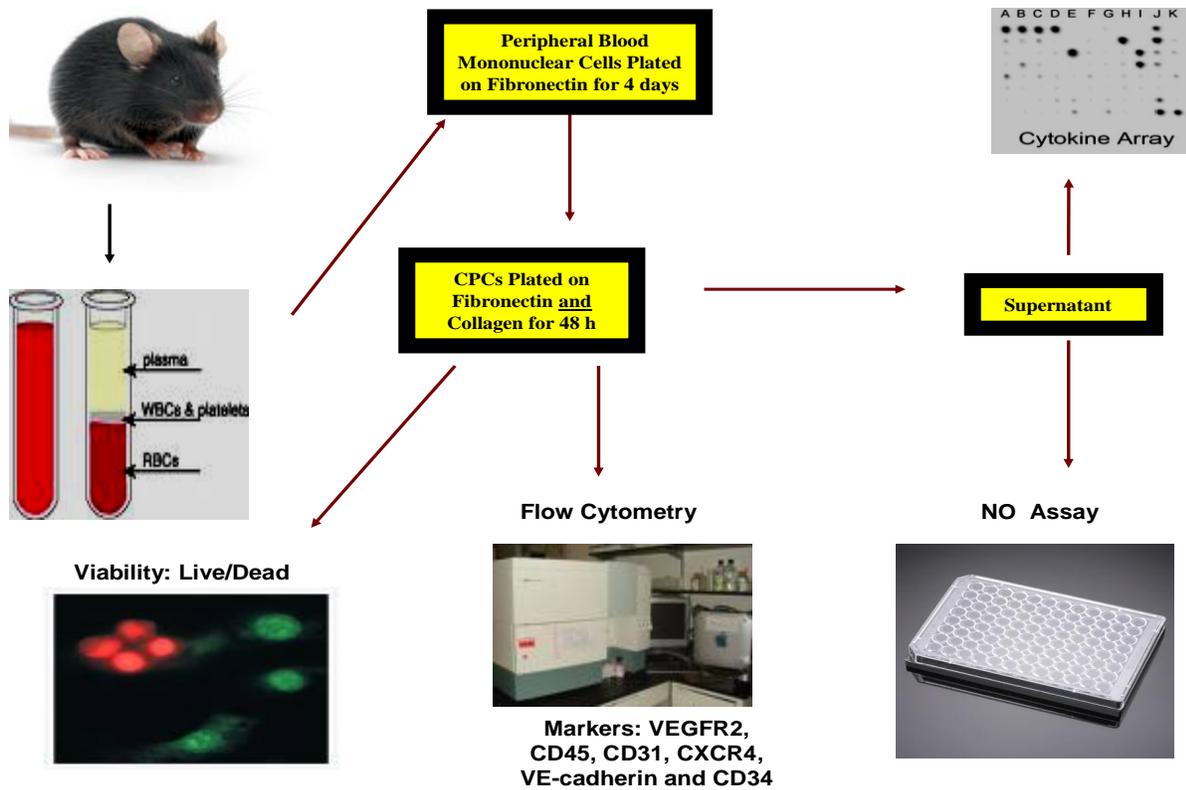


Figure 2.3. Experiments conducted in hypoxic conditions. Cardiac puncture was performed on all mice; collected blood was layered onto Histopaque and spun for 30 min. The buffy coat containing peripheral blood mononuclear cells (PBMCs) was plated on fibronectin for 4 days to generate circulating progenitor cells (CPCs). These cells were then lifted and further plated for an additional 48 h on fibronectin and collagen in hypoxia. These cultured cells were analyzed by flow cytometry, or used in live/dead assays, and the supernatant was assayed for nitric oxide (NO) and cytokine levels.

3. Results

3.1 Animal model

3.1.1. Confirmation of endothelial dysfunction in high fat diet group

To confirm that the mice fed a high cholesterol diet developed endothelial dysfunction, a custom cytokine array was used to analyze the serum collected from mice sacrificed after 4 weeks of diet. ICAM-1, VCAM-1 and E-selectin, which were included in the array, are cytokines used to indicate the presence of endothelial dysfunction (ED) (Burger et al. 2012). ICAM-1 was increased 170%, VCAM-1 was increased 120%, and E-selectin was increased 170% in the high fat diet mice compared to the circulating levels found in normal diet mice (Figure 3.1; $P=0.05$, 0.04 , and 0.01 for ICAM-1, VCAM-1 and E-selectin, respectively). The ED index was calculated from ratio of the sum average changes of ICAM-1, VCAM-1 and E-selectin and was found to be significantly greater in mice fed a high fat diet (1.5 ± 0.2) compared to mice fed a normal diet (1.0 ± 0.05) (Figure 3.2; $P=0.04$) The ED index was solely calculated to assess the overall degree of endothelial dysfunction in the animals after 4 weeks of diet. Next, we sought to establish that this high fat diet was producing high levels of cholesterol within the plasma of the mice.

3.1.2 Total Cholesterol Assay

Total cholesterol was measured using serum obtained from C57BL/6J mice fed a normal diet or a high fat diet for 4 weeks. The high fat diet mice displayed a 350% increase in total cholesterol (mg/mL) levels compared to the normal diet group (Figure 3.3; $P=0.00009$). Overall, the high fat diet induced not only endothelial dysfunction, but produced high levels of total cholesterol in the animals. Next, we looked at whether this high fat diet was inducing an inflammatory response in the animals by examining plasma levels of selected cytokines.

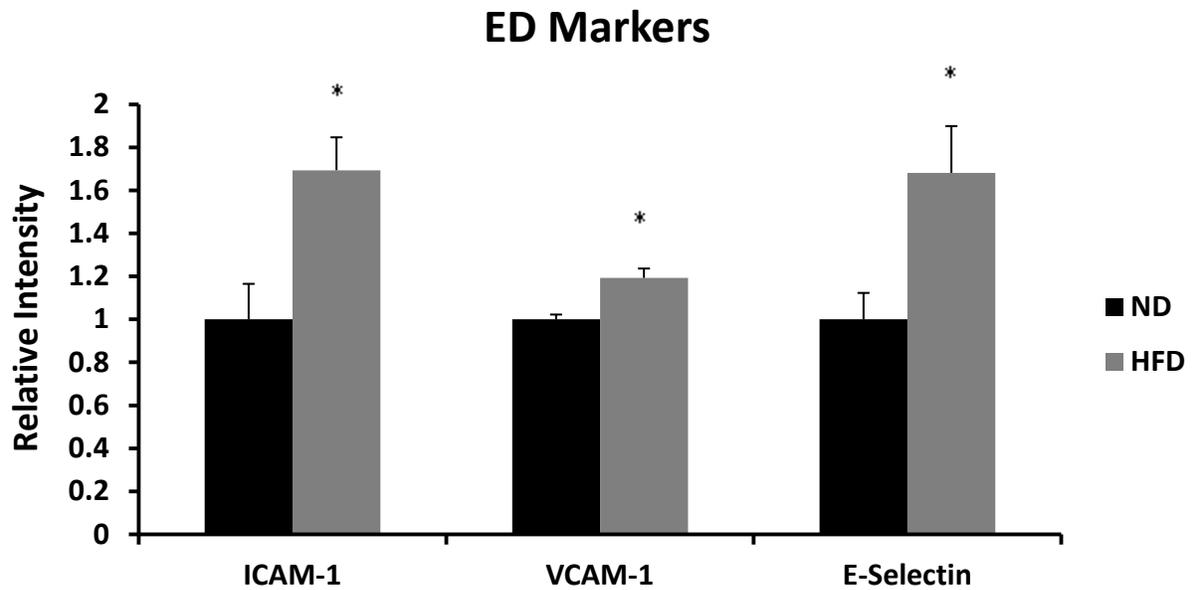


Figure 3.1. Biochemical analysis of endothelial function (n=7). After 4 weeks of diet, serum was collected from normal diet (ND) mice and high fat diet (HFD) mice to assess circulating levels of known adhesion markers to detect presence of endothelial dysfunction. Serum levels of cell adhesion markers ICAM-1, VACM-1 and E-Selectin displayed significant increases in HFD mice: I-CAM (* $P=0.05$); V-CAM (* $P=0.04$); and E-selectin (* $P=0.01$) compared to ND mice.

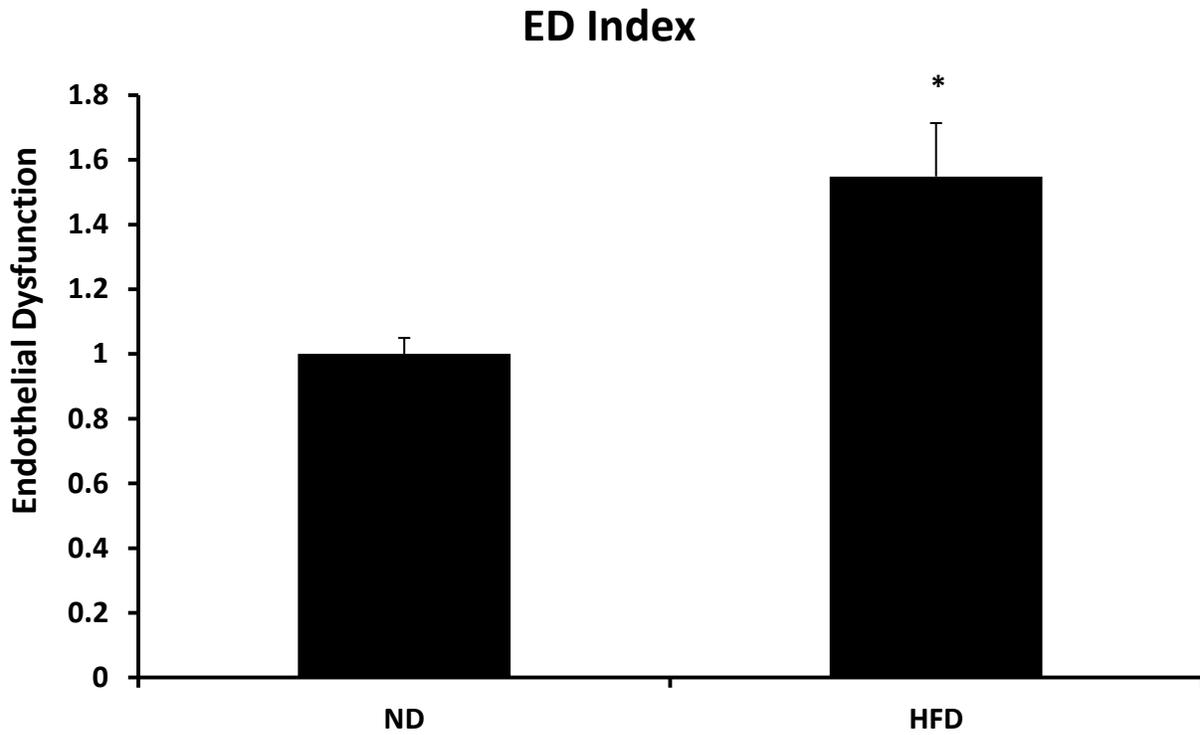


Figure 3.2. Endothelial dysfunction index. After 4 weeks of diet, serum was collected to confirm presence of endothelial dysfunction in the animals. An index was calculated from the sum average changes of ED markers ICAM-1, VCAM-1 and E-Selectin. High fat diet (HFD) mice had an increased endothelial dysfunction index after 4 weeks of diet compared to the normal diet (ND) mice (* $P=0.04$).

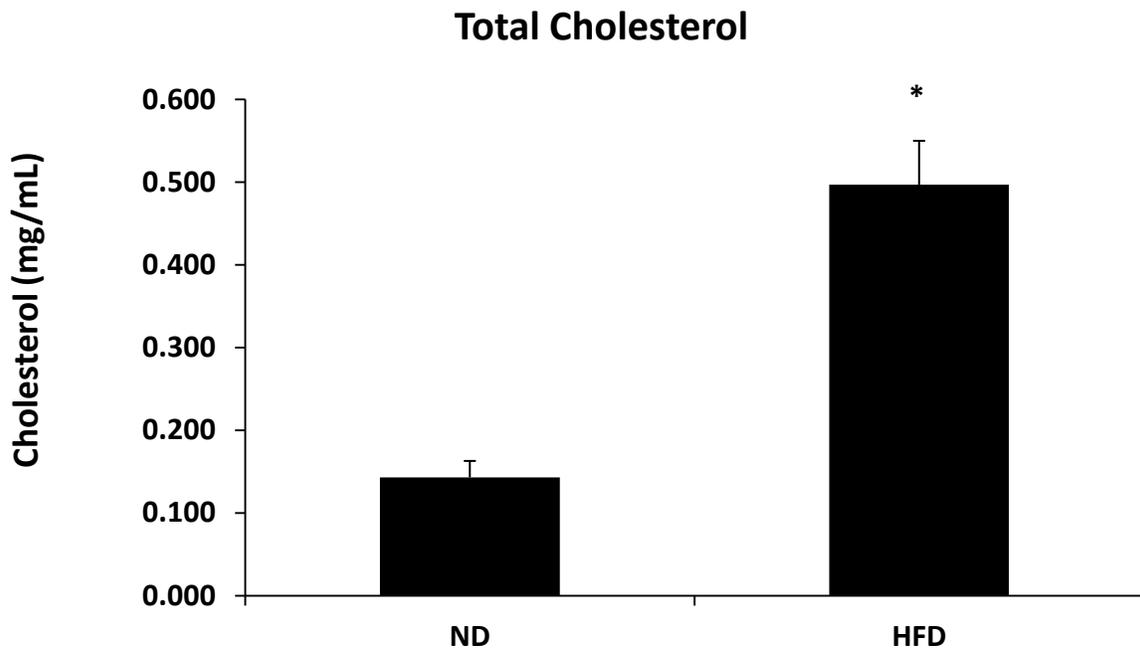


Figure 3.3. Total cholesterol levels in serum (n=6). Total cholesterol levels were measured using a Cholesterol E enzymatic colorimetric assay kit where the cholesterol produced and the free cholesterol already present in the serum is oxidized in a reaction catalyzed by cholesterol oxidase that generates hydrogen peroxide. After 4 weeks of diet, serum was collected to assess levels of total cholesterol in the animals. As shown, mice fed a high fat diet (HFD) had increased total cholesterol in serum compared to mice fed a normal diet (ND) for 4 weeks (* $P=0.00009$).

3.1.3 Inflammatory Cytokines

A custom cytokine array was used to detect cytokine levels in serum samples. In high fat diet-induced endothelial dysfunction, an inflammatory response is expected in the animals. Serum was collected from mice after 4 weeks of being fed the normal (ND) or high fat diet (HFD). Overall, a proinflammatory response was observed in the high fat diet mice, with increased serum levels of the following individual cytokines: IL-4 by 210% ($P=0.003$), IL-6 by 170% ($P=0.05$), and TNF- α by 150% ($P=0.02$), compared to normal diet animals (Table 3.1). There was also a trend for increased levels of IFN- γ by 160% ($P=0.09$), IL-1 α by 200% ($P=0.07$), IL-2 by 2010% ($P=0.1$) and anti-inflammatory cytokine IL-10 by 160% ($P=0.09$) for mice on the high fat diet (Table 3.1). These results suggest an inflammatory response in the high fat diet group compared to normal diet. Next, we sought to characterize circulating cell phenotype of both diet groups in normoxic conditions.

Cytokines in Serum			
Cytokine	ND	HFD	P-value
bFGF	0.04	0.01	0.17
E-selectin	0.86	1.26	0.17
GM-CSF	0.01	0.02	0.15
ICAM-1	0.03	0.04	0.30
IFN-γ	8.11E-03	0.01	0.09
IGFBP-3	0.73	0.82	0.37
IL-1α	0.02	0.04	0.07
IL-1β	1.43E-03	1.97E-03	0.64
IL-2	5.42E-03	0.09	0.13
IL-4	0.01	0.03	2.51E-03
IL-6	0.02	0.03	0.05
IL-10	0.01	0.02	0.10
MMP-2	0.05	0.05	0.92
SDF-1	1.64E-04	7.29E-04	0.21
TNF-α	0.02	0.03	0.02
VCAM-1	3.24	3.43	0.55
VEGF	9.41E-03	9.49E-03	0.96

Table 3.1. Cytokine profile analysis of serum (n=7). After 4 weeks of diet, serum was collected from all animals and was analyzed for the presence of pro- and anti-inflammatory cytokines. Custom cytokine array analysis of serum from normal diet (ND) mice and high fat diet (HFD) mice revealed increased levels of cytokines IL-4 ($P=0.003$), IL-6 ($P=0.05$), and TNF- α ($P=0.02$) for mice on the high fat diet. There was also a trend for increased levels of cytokines IFN- γ ($P=0.09$), IL-1 α ($P=0.07$), IL-2 ($P=0.1$) and anti-inflammatory cytokine IL-10 ($P=0.1$), for mice on the high fat diet.

3.2 CPCs in normoxia

3.2.1 Cell Phenotype

CPCs from mice of both diet groups were plated on fibronectin for 4 days. Flow cytometry was performed to examine the phenotype of CPCs using markers CD45 (marker for leukocytes), VEGFR2, VE-cadherin and CD31 (markers of endothelial lineage), CXCR4 (angiogenic cell marker), and CD34 (marker of progenitor lineage). The following antibodies were used in two different combinations; **COMB 1**: VEGFR2, CD34, and CD45; **COMB 2**: CD31, CXCR4, and VE-cadherin. CPCs from the high fat diet mice had significant decreases in the expression of VEGFR2 (by 99%), CD45 (by 80%), CD34 (by 99%), VE-cadherin (by 70%) and CD31 (by 70%), compared to CPCs from the normal diet animals (Figure 3.4A; Appendix Table 1) ($P=0.04$, 0.007 , 0.00000003 , 0.02 and 0.004 for VEGFR2, CD45, CD34, VE-cadherin and CD31, respectively). This reduced marker expression in CPCs from the high fat diet group was not observed when the cells were cultured on collagen matrix (Figure 3.4B; Appendix: Table 7.1). Rather, there was no significant difference in the expression of CD45, VEGFR2, VE-cadherin, CD31, CXCR4 and CD34 in collagen matrix-cultured CPCs from the high fat diet versus the normal diet mice. Next, we wanted to test the adhesion potential of the dysfunctional cells.

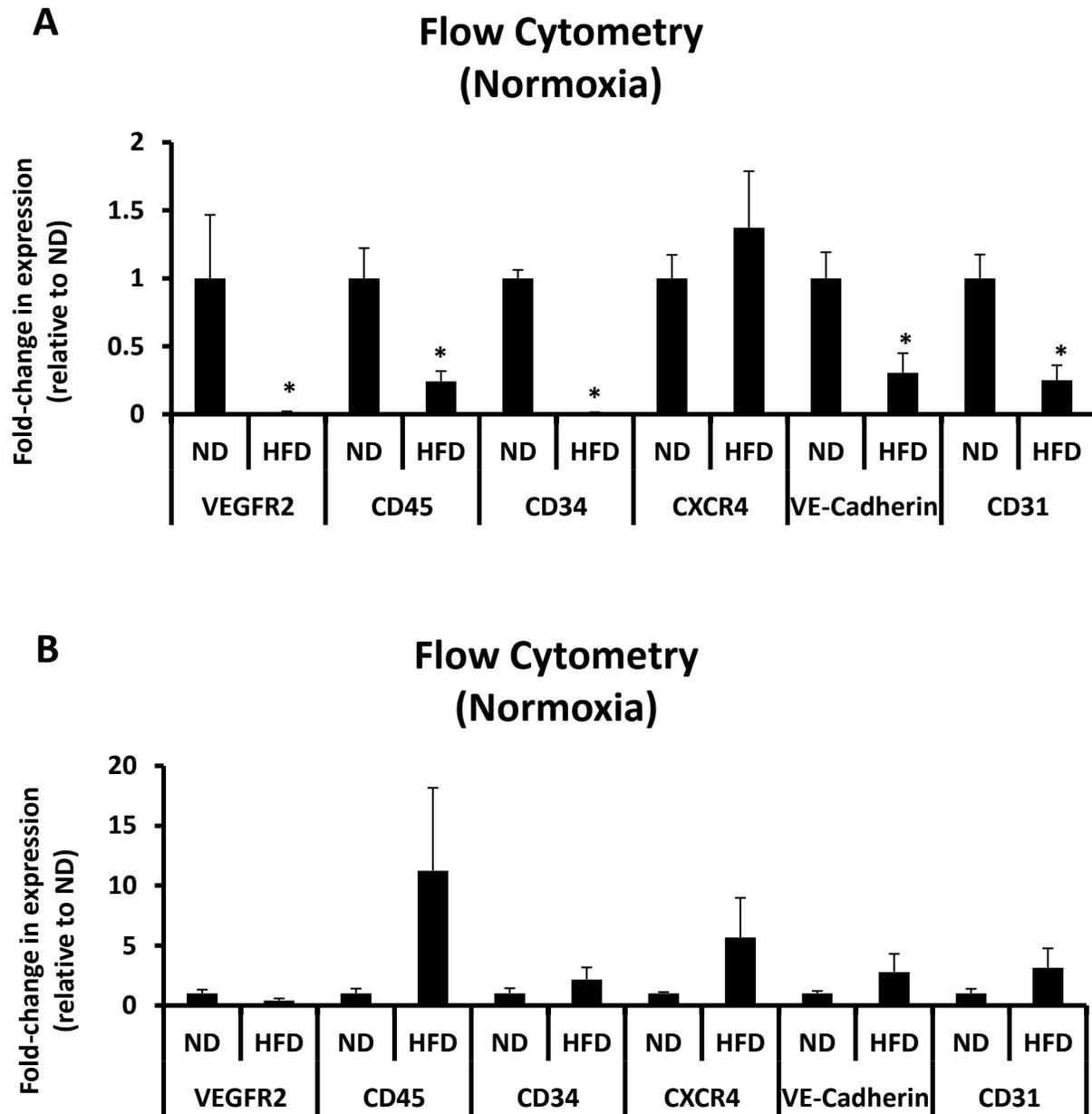


Figure 3.4. Normoxic flow cytometry analysis (n=6). After 4-day culture on fibronectin, CPCs were cultured for an additional 4 days on fibronectin (A) or collagen matrix (B) in normoxic conditions and stained with selected antibodies to assess cell phenotype. The graphs represent the expression of antigens VEGFR2, CD45, CXCR4, CD31, VE-Cadherin, and CD34 on CPCs in normoxia obtained from mice fed a normal diet (ND) or high fat diet (HFD) for 4 weeks. (A) The high fat diet group displayed decreased expression of VEGFR2, CD45, CD34, VE-cadherin and CD31 when CPCs were cultured on fibronectin. (B) No differences in marker expression were observed for CPCs cultured on collagen matrix. * $P < 0.05$ versus ND group for that marker.

3.2.2 Cell Adhesion

An adhesion assay was used to detect the cells' ability to adhere to either fibronectin or collagen matrix. After 1 hr (under normoxia), increased adhesion was observed for collagen-cultured cells by 430% compared to fibronectin-cultured cells for CPCs from the normal diet mice ($P=0.00008$). However, collagen-culture was not similarly able to improve adhesion for CPCs from the high fat diet group. There was no significant difference in adhesion to the 2 substrates for CPCs from the high fat diet mice (Figure 3.5). However, CPCs from normal diet mice display greater adhesion to collagen matrix by 40% ($P=0.0002$), compared to CPCs from the high fat diet mice. Next, we sought to determine the potential for these dysfunctional cells to migrate towards a chemotactic signal (VEGF).

3.2.3 Migratory Capacity

CPCs from normal diet and high fat diet mice that were cultured on fibronectin or on collagen matrix for a total of 4 days were then tested in a migration assay using VEGF as the chemotactic stimulus. In the normal diet group, there was a trend for increased migratory capacity of collagen-cultured CPCs (by 240%), compared to fibronectin culture (Figure 3.8; $P=0.08$). For CPCs from the high fat diet mice, a 220% increase in cell migration was seen for collagen-cultured cells compared to fibronectin, suggesting that collagen may have the potential to restore migratory capacity to the dysfunction cells (Figure 3.6; $P=0.03$). Although collagen improved the migration of CPCs from the high fat diet group compared to fibronectin, there was a trend for reduced migratory capacity compared to the CPCs from the normal diet group (Figure 3.6; $P=0.15$). Next, we sought to characterize cell phenotype of both diet groups in hypoxic conditions.

Adhesion

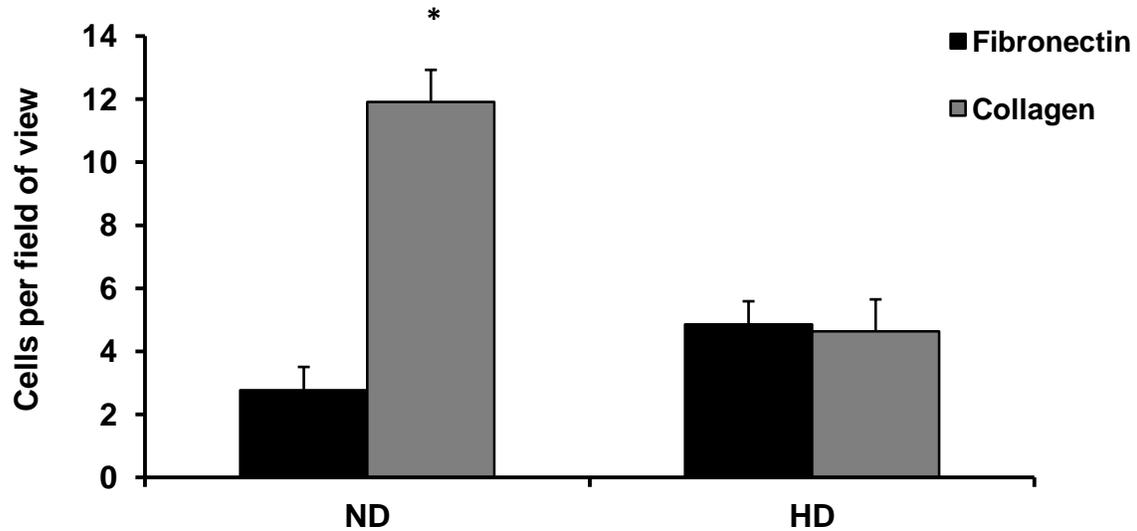


Figure 3.5. Cell adhesion analysis (n=8). Adhesion of fibronectin-cultured CPCs and collagen matrix-cultured CPCs (in normoxia) from mice fed a normal diet (ND) or high fat diet (HFD) after 4 weeks of diet. After 1 hr in normoxia, increased adhesion was observed for collagen matrix-cultured CPCs compared to fibronectin-cultured CPCs for the normal diet group (* $P=0.00008$), but not for CPCs from the high fat diet group.

Migration

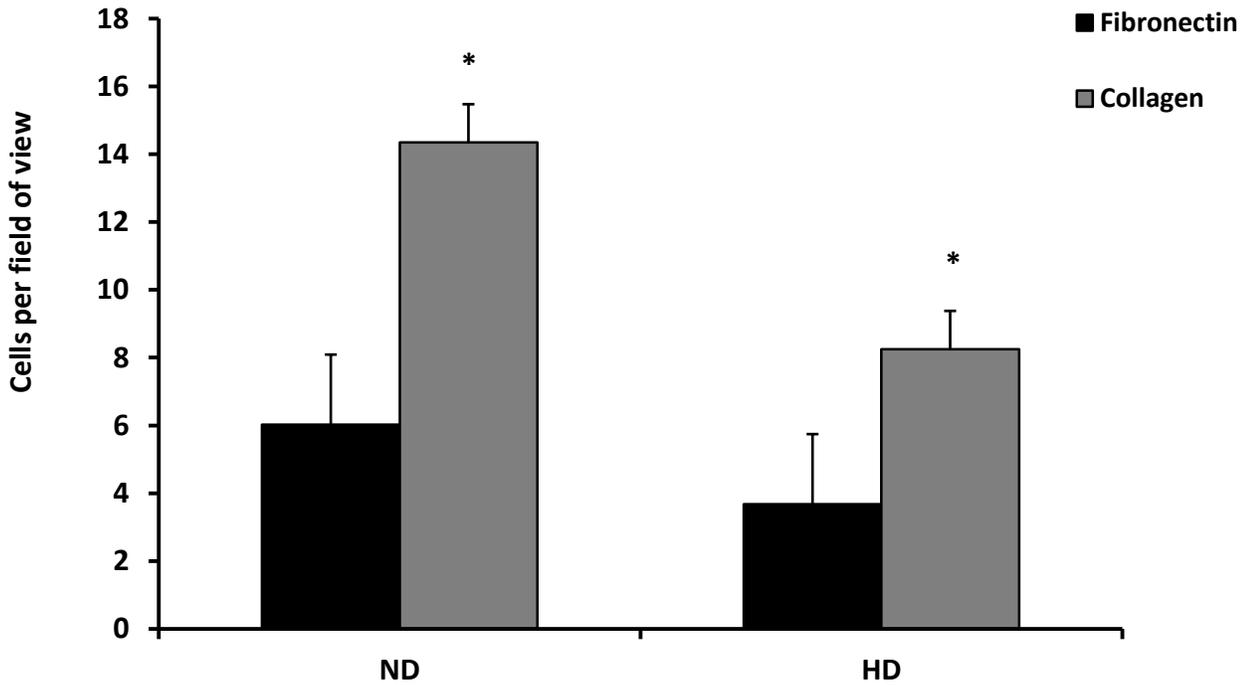


Figure 3.6. Cell migration analysis (n=7). Migration (in normoxia) of fibronectin- or collagen matrix-cultured CPCs obtained from mice fed a normal diet (ND) or high fat diet (HFD) after 4 weeks of diet. After 24 hr in normoxia, increased migration towards VEGF was observed for collagen-cultured CPCs compared to fibronectin-cultured CPCs for high fat diet mice (* $P=0.03$). There was also a trend for increased cell migration for collagen-cultured CPCs in the normal diet mice in comparison to fibronectin (* $P=0.08$).

3.3 CPCs in Hypoxia

3.3.1 Cell Phenotype

CPCs from normal diet mice and high fat diet mice were cultured on fibronectin or collagen matrix and subjected to 48 h of hypoxia, and then collected for flow cytometry analysis. Data revealed no significant differences in marker expression (CD45, VEGFR2, VE-cadherin, CD31, CXCR4 and CD34) between normal diet and high fat diet mice when CPCs were cultured on fibronectin (Figure 3.7A), or when they were cultured on collagen matrix (Figure 3.7B; Appendix: Table 7.2). Next, we sought to establish whether the cells could survive when placed in a harsh environment with the help of collagen.

3.3.2 Cell Viability

A live/dead stain was performed on CPCs cultured on fibronectin or collagen matrix subjected to serum and growth factor starvation for 48 h in hypoxia to induce apoptosis. It was observed that cell viability was increased for collagen-cultured CPCs from normal diet mice (by 320%; $P=0.0001$) and from high fat diet mice (by 170%; $P=0.0002$) compared to their respective fibronectin controls, suggesting that the properties of the collagen matrix may support the survival of CPCs in an environment of endothelial dysfunction (Figure 3.8). Next, we sought to examine nitric oxide production in the dysfunctional CPCs cultured on either fibronectin or collagen matrix when placed in the same apoptosis-inducing conditions as stated above.

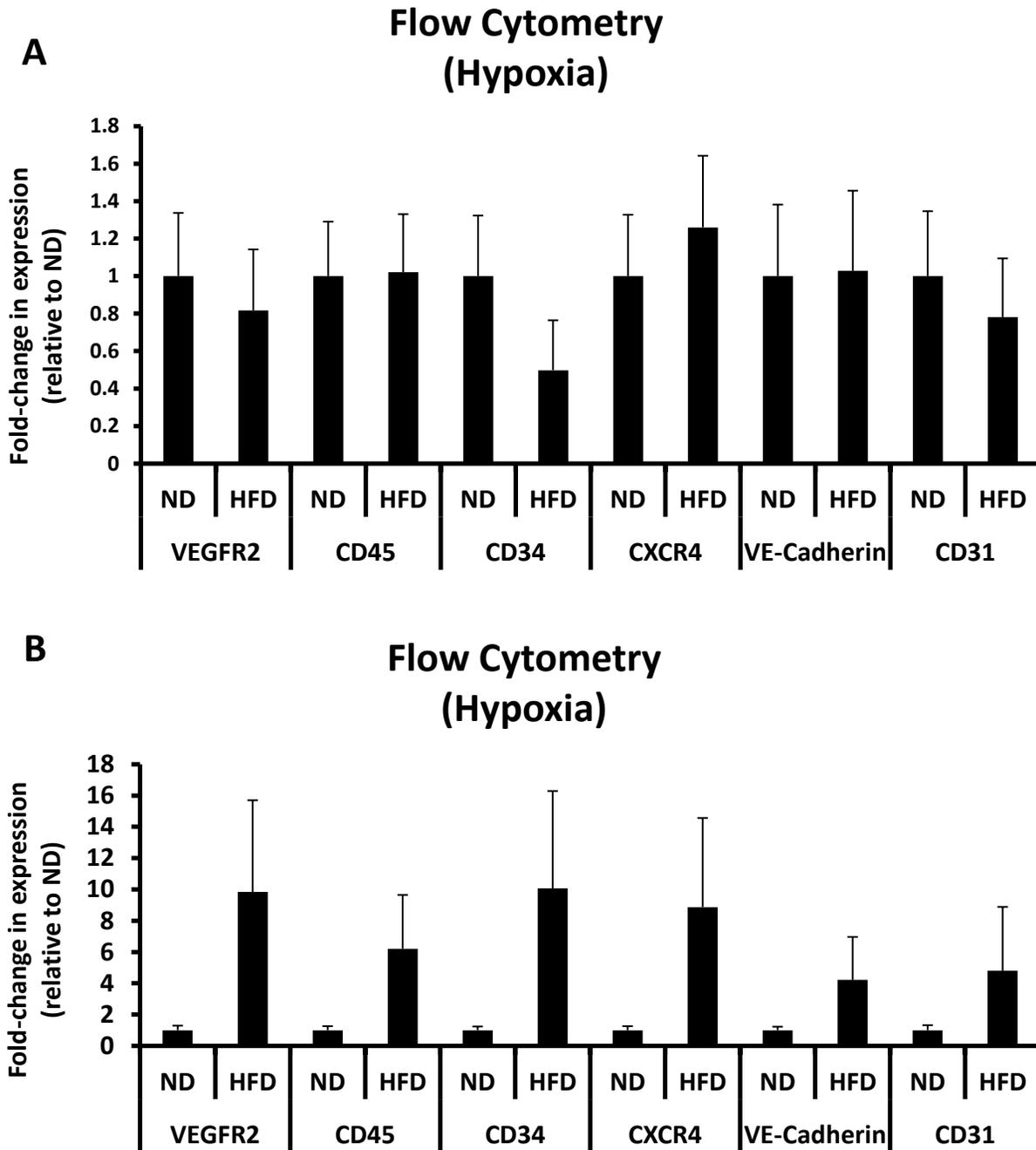


Figure 3.7. Hypoxic flow cytometry analysis (n=5). CPCs were cultured for 48 h in hypoxic conditions and stained with selected antibodies to assess cell phenotype. The graphs represent the expression of antigens VEGFR2, CD45, CXCR4, CD31, VE-Cadherin, and CD34 on CPCs in hypoxia obtained from C57BL/6J mice fed a normal diet and high fat diet for 4 weeks. No significant differences were observed between normal diet and high fat diet mice for fibronectin or collagen; A and B.

Live/Dead

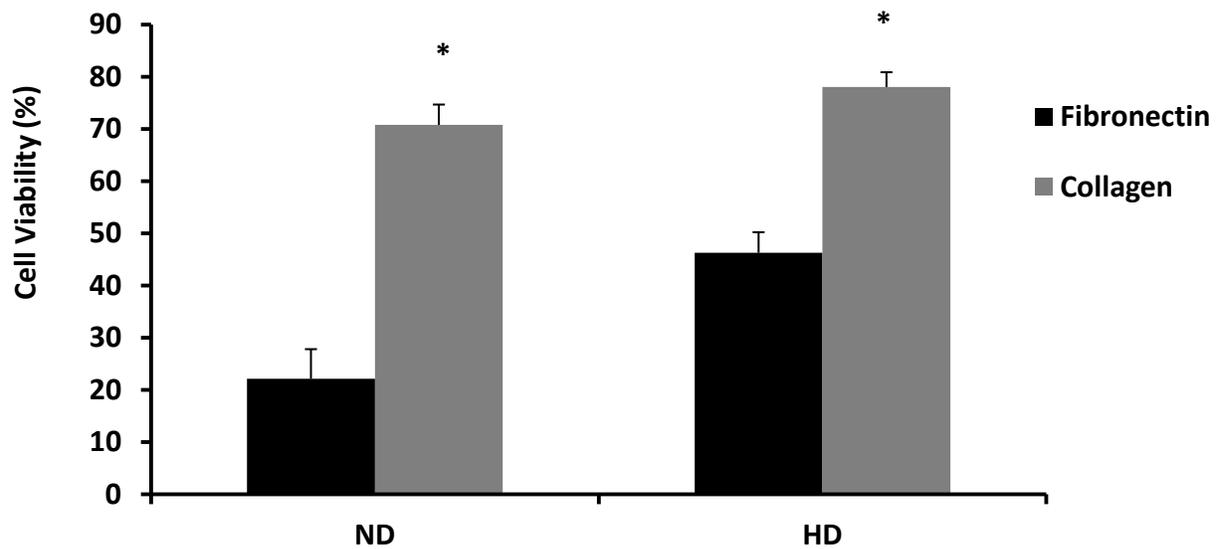


Figure 3.8. Cell viability analysis (n=5). Live/dead staining was performed after 48 h in hypoxia for fibronectin- and collagen matrix-cultured CPCs from normal diet (ND) and high fat diet (HFD) mice. This assay quickly discriminates live from dead cells by staining green-fluorescent calcein-AM to indicate intracellular esterase activity (live cells) and red-fluorescent ethidium homodimer-1 to indicate loss of plasma membrane integrity (dead cells). Both diet groups displayed increased cell viability when CPCs were cultured on collagen compared to fibronectin (ND; * $P=0.0001$ and HFD; * $P=0.0002$).

3.3.3 Nitric Oxide Production

Nitric oxide (NO) levels were measured using supernatant collected from 250,000 CPCs cultured on fibronectin or collagen matrix for 48 h in hypoxia. CPCs from normal diet mice displayed a 240% increase in nitric oxide production when cells were cultured on collagen compared to fibronectin ($P=0.04$; Figure 3.9; Appendix: Figure 7.1). There was a trend for decreased NO production in CPCs from the high fat diet group by 50% when cells were cultured on collagen in comparison to fibronectin, suggesting that properties of collagen do not enhance the production of NO in a harsh environment ($P=0.09$). CPCs from the normal diet mice also displayed a 730% increase in NO production compared to CPCs from high fat diet mice, when cells were cultured on collagen ($P=0.002$). Next, we looked at cytokine levels in the supernatant of CPC cultures to examine potential signaling mechanisms.

3.3.4 Cytokine Profile and Angiogenic Index

Supernatant was collected from CPCs for the 2 diet groups cultured on fibronectin or collagen matrix (250,000 cells) after 48 h in hypoxia and was subsequently analyzed using a custom cytokine array. Overall, there were no differences observed in the CPC culture supernatant cytokine levels between the diet groups or between the fibronectin and collagen matrix substrates; however, an increase was observed for TNF- α when CPCs from high fat diet mice were cultured on collagen ($P=0.05$; Table 3.2).

Since the secretion of angiogenic cytokines is an important aspect of the CPC's ability to repair tissue after an ischemic injury, an angiogenic index was calculated from the sum average changes of SDF-1, VEGF, bFGF and GM-CSF. No significant difference in the angiogenic index

was observed between CPCs from mice fed a normal diet versus a high fat diet when comparing culture on fibronectin versus collagen substrates (Figure 3.10).

Nitric Oxide

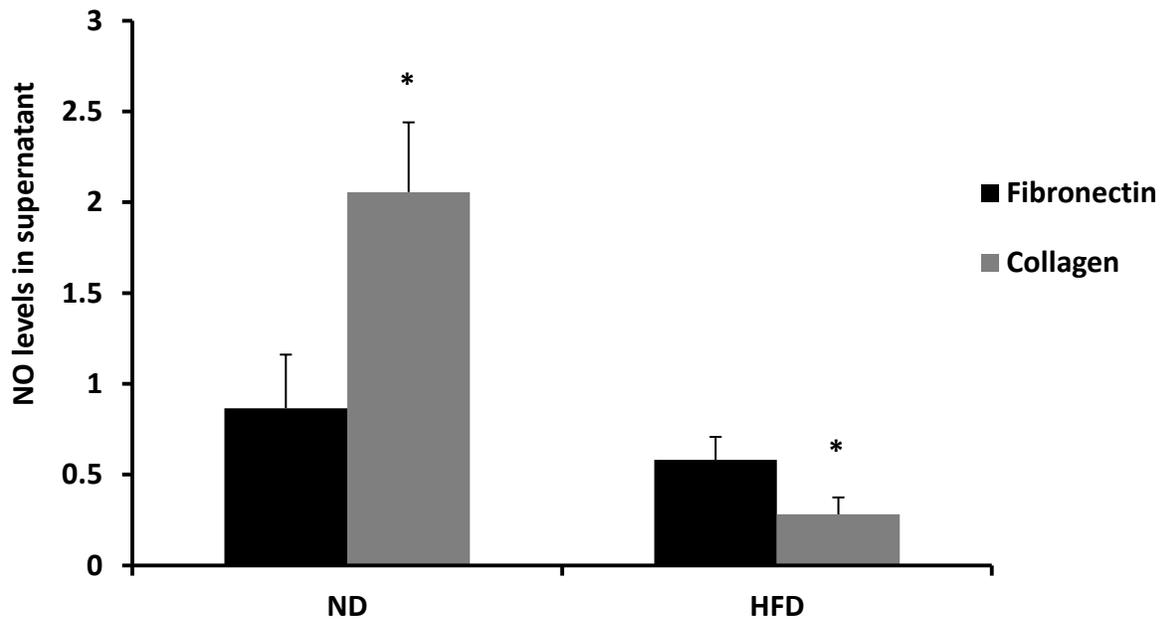


Figure 3.9. Nitric oxide production analysis (n=5). Nitric oxide (NO) levels were measured in the supernatant of CPCs, from normal diet (ND) and high fat diet (HFD) mice, cultured on fibronectin or collagen matrix for 48 h in hypoxia. This assay allows you to measure total NO by the conversion of nitrate (NO_3) to nitrite (NO_2). CPCs from normal diet mice displayed an increase in nitric oxide production when CPCs were cultured on collagen matrix versus fibronectin (* $P=0.04$). There was a trend for decreased NO production observed in the cell supernatant from the high fat diet group when CPCs were cultured on collagen compared to fibronectin (* $P=0.09$).

Cytokines in Supernatant						
Cytokine	ND		P-value	HFD		P-value
	Fibronectin	Collagen		Fibronectin	Collagen	
bFGF	2.63E-03	4.22E-03	0.25	5.70E-03	4.15E-03	0.44
E-selectin	8.55E-03	0.01	0.29	0.01	0.01	0.18
GM-CSF	7.91E-03	0.01	0.19	8.44E-03	9.20E-03	0.27
ICAM-1	0.01	0.01	0.54	0.01	0.02	0.20
IFN-γ	8.25E-03	0.01	0.15	9.52E-03	0.01	0.48
IGFBP-3	3.16E-03	3.95E-03	0.55	5.40E-03	5.58E-03	0.89
IL-1α	5.41E-03	8.67E-03	0.21	7.48E-03	9.40E-03	0.30
IL-1β	2.67E-03	9.51E-03	0.22	0.00	4.58E-03	0.64
IL-2	8.93E-03	5.83E-03	0.55	7.05E-03	7.46E-03	0.83
IL-4	8.27E-03	0.02	0.28	0.01	0.01	0.27
IL-6	9.07E-03	0.01	0.21	0.01	0.01	0.53
IL-10	8.92E-03	4.89E-03	0.18	0.01	9.79E-03	0.39
MMP-2	1.98E-03	2.36E-03	0.70	5.42E-03	3.68E-03	0.29
SDF-1	3.76E-03	4.94E-03	0.36	4.83E-03	6.40E-03	0.44
TNF-α	0.01	0.01	0.65	0.01	0.02	0.05
VCAM-1	2.99E-03	6.43E-03	0.48	0.02	4.83E-03	0.41
VEGF	3.26E-03	6.36E-03	0.47	4.54E-03	4.48E-03	0.95

Table 3.2. Cytokine profile analysis of CPC supernatant (n=4). Custom cytokine array analysis using supernatant collected after CPC culture on fibronectin and collagen for 48 h in hypoxia for normal diet (ND) and high fat diet (HFD) mice. Overall, there were no significant differences observed between normal diet and high fat diet groups when comparing supernatants obtained from both fibronectin and collagen substrates; however, an increase in cytokine level TNF- α was observed for the supernatant of collagen-cultured CPCs from the high fat diet mice compared to the supernatant from fibronectin-cultured CPCs (P=0.05).

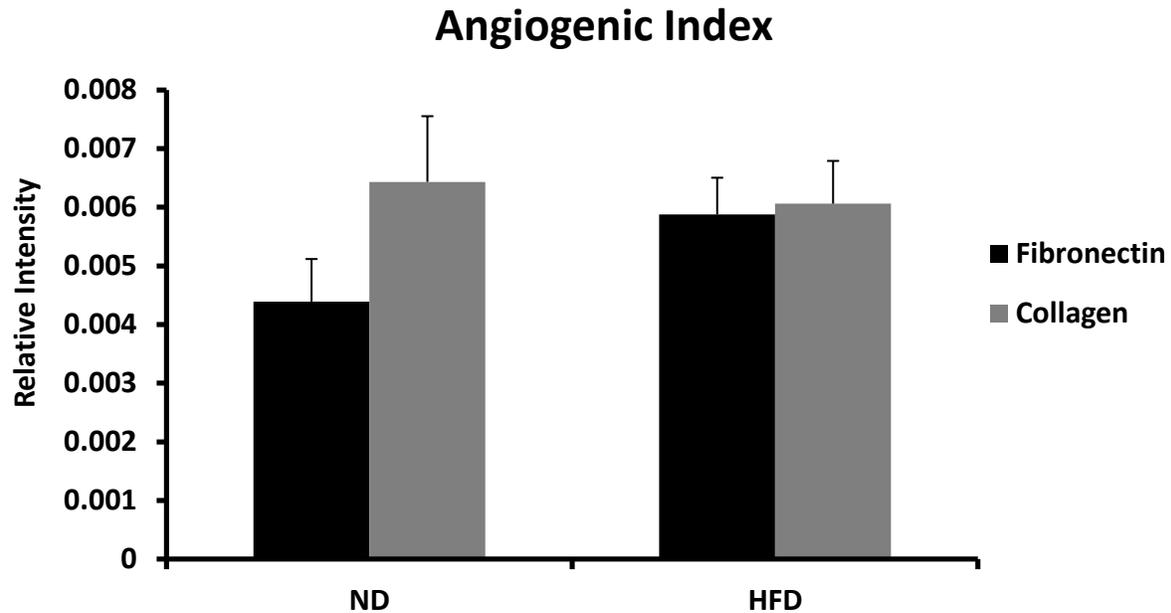


Figure 3.10. Angiogenic index. To assess the angiogenic potential of CPCs, a custom cytokine array analysis was performed using the supernatant collected after CPC culture on fibronectin and collagen for 48 h in hypoxia (for CPCs from normal diet (ND) and high fat diet (HFD) mice). The angiogenic index was calculated from the sum average levels of SDF-1, VEGF, bFGF and GM-CSF in supernatant for CPCs from high fat diet (HFD) and normal diet (ND) groups displayed no significant differences between fibronectin and collagen substrates after 48 h of culture in hypoxia.

4. Discussion

In this study, we examined the survival and function of CPCs cultured on standard fibronectin or on collagen matrix. CPCs were obtained from mice that were assigned a normal mouse chow diet or a high fat diet for 4 weeks to induce endothelial dysfunction. There were four main findings:

- (1) The expression of several cell surface markers was reduced for CPCs from high fat diet mice when plated onto fibronectin; but with culture on a collagen matrix, these decreases disappeared;
- (2) The migration and adhesion function of CPCs can be enhanced with culture on collagen matrix for cells from both normal diet and high fat diet mice;
- (3) Culture of CPCs on collagen matrix increased nitric oxide production in cells from normal diet mice, but not in cells from high fat diet mice; and
- (4) Collagen has positive effects on cell viability in both diet groups.

Overall, this study elucidates that culturing CPCs on collagen may be superior to fibronectin with respect to cell function capacities, such as migration and adhesion, and can improve the survival of CPCs in a serum-deprived environment. This suggests that CPC function and survival may be restored, at least in part, in a model of ED with the provision of a collagen matrix.

As a first step in this work, I sought to characterize and confirm the establishment of ED in my animal model. A high cholesterol diet has been shown previously to induce not only ischemia, but ED in a mouse model after as little of 4 weeks of diet (Baumhake et al. 2010). In the present study, the high fat diet, which was administered for 4 weeks, was successful in increasing the total cholesterol levels within the serum in comparison to the normal diet animals.

Therefore, the diet was able to induce the high cholesterol blood content that is a prerequisite for the development of endothelial dysfunction in this model.

Adhesion molecules play a vital role in inflammation and are responsible for recruiting leukocytes and regulating cell migration within the vessel wall. It is believed that soluble adhesion molecules such as vascular adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1) and endothelial cell-selectin (E-selectin); all of which are found within the plasma are the product of an induced inflammatory response which correlates with ED (Guarneri 2010). These biomarkers have been used to identify the presence of ED (Goligorsky 2006; Burger et al. 2012). To establish that the high fat diet animal group in the present study had developed ED, the circulating levels of ICAM-1, VCAM-1, and E-selectin were assessed.

ICAM-1 is present in low concentrations in the membranes of leukocytes and endothelial cells. In the present study, ICAM-1 serum levels were elevated in the mice fed the high fat diet, compared to normal diet mice. VCAM-1 mediates the adhesion of lymphocytes, monocytes, eosinophils, and basophils to vascular endothelium. A high fat diet resulted in increased circulating levels of VCAM-1 in the animals of this study. E-selectin is a cell adhesion molecule expressed only on endothelial cells activated by cytokines, and plays a crucial role in inflammation. During inflammation, it is responsible for recruiting leukocytes to the sites of injury. As was observed with ICAM-1 and VCAM-1, the high fat diet also increased blood serum levels of E-selectin, compared to mice fed the normal diet.

In addition to evaluating the levels of the individual ED markers, an index was also calculated by combining serum levels of ICAM-1, VCAM-1 and E-selectin. ED was defined as a significant increase in the levels of these three markers compared to normal diet control levels, as

was previously reported (Baumhakel et al. 2010). This calculation further confirmed that ED was induced after 4 weeks of being fed a high fat diet.

Some inflammatory cytokines have been shown to contribute to ED, while anti-inflammatory treatment methods have sought to improve endothelial function (Bhagat et al. 1997; Raza et al. 2000). Endothelial damage is often accompanied by inflammation and therefore, markers of inflammation have been used to detect the presence of ED and the severity of the induced inflammatory response. The serum from the high fat diet group after 4 weeks of diet contained higher levels of cytokines IL-1 α , IL-2, IL-4, IL-6, IFN- γ , and TNF- α suggesting the presence of a proinflammatory response in the high fat diet group. The majority of these cytokines stimulate the proliferation and differentiation of immune cells located at sites of injury. While the inflammatory responses provide a defense mechanism to injury, healthy cells surrounding the site of injury could also be damaged (Sprague et al. 2009), and prolonged negative effects of the inflammatory response can lead to extracellular matrix (ECM) deposition, and progression to vascular disease (Kliche et al. 2011).

Overall, these analyses support that increased cholesterol levels observed in the high fat diet mice, led to increased systemic inflammation and the development of ED, as was reported for other high fat diet models (Ross 1993; Luscher et al. 1997; Davignon et al. 2004).

There are additional methods for establishing ED that were not implemented in my study. For example, Suuronen et al. 2010 measured: 1) the ratio of serum endothelin-1 (ET-1) to big endothelin-1 (bET-1); 2) the nitrotyrosine level; and 3) the expression of endothelial nitric oxide synthase (eNOS), to establish the effects of ED in animals fed a high-cholesterol diet. Notably, the gold standard for establishing the presence of ED is the *in vitro* aortic ring vasodilatory assay and previous invasive methods involved the examination of vascular responses to infused

acetylcholine via catheterization or venous plethysmography (Ludmer et al. 1986; Fish et al. 1988; Benjamin et al. 1995). This analysis was not a possibility for the current project as the required organ chamber instrument and technical expertise for such experiments were not available. Nevertheless, the combined results of the methods that were employed to establish ED strongly support the presence of ED in the high fat diet mouse model used.

Fibronectin is the historically preferred adhesive substrate for obtaining CPCs through culture (Asahara et al. 1997; Kalka et al. 2000). CPCs have been shown to adhere to fibronectin, however, the proliferation and survival rate is very limited (Kawamoto et al. 2001). Thus, biomaterials (such as collagen) are being developed to enhance CPC adhesion, migration, proliferation, retention and survival in various models of cardiovascular disease (Simmons et al. 1993; Tolan et al. 1995; Rosinberg et al. 2004). In this study, it was hoped that a collagen-based matrix could improve the function and survival of CPCs (adhesion, migration, viability, and NO production) obtained from animals with ED.

The percentage of cells expressing leukocyte marker CD45, progenitor cell marker CD34, and angiogenic/endothelial cell markers VEGFR2, VE-cadherin and CD31 was decreased for the high fat diet mouse group when CPCs were cultured on fibronectin compared to CPCs from the normal diet mice. Surprisingly, the results demonstrated reduced expression of several markers for CPCs cultured on collagen matrix compared to fibronectin cultures (for normal diet group; data not shown). This was unexpected, since collagen-cultured CPCs have been shown previously to exhibit increases in progenitor and endothelial cell markers (Suuronen et al. 2006; Kuraitis et al. 2011). This previous work used trypsin digestion methods to free the cells from the matrix for analysis, which was not performed in the present study. Therefore, it is possible that the most adherent cells on collagen matrix are the ones expressing the endothelial/angiogenic

markers; and perhaps the cell collection method did not efficiently remove these cells from the matrix. If they were not successfully freed from the matrix, then this would result in a low expression result by flow cytometry analysis. For this reason, the collagen-cultured CPCs were compared between the normal diet and high fat diet groups, but collagen matrix versus fibronectin comparisons were not considered. The results of CPC culture on collagen matrix showed no differences in cell marker expression between the 2 diet groups. This suggests that, unlike the reduced marker expression observed for fibronectin-cultured CPCs, the collagen matrix may support the maintenance of CPC phenotypes for cells from the high fat diet mice.

Regarding function, CPC adhesion was increased for the normal diet group when cells were cultured on collagen matrix, while CPCs from the high fat diet group displayed no significant differences between substrates. The result obtained for the CPCs of normal diet animals is similar to reports in previous studies. The successful use of collagen-culture to increase adhesion of human CPCs isolated from healthy donors has been seen by various groups (Kutschka et al. 2006; Suuronen et al. 2006; Kutschka et al. 2007; Kuraitis et al. 2011). However, such improvement in adhesion was not seen for the CPCs from the high fat diet group. Vasa et al. 2001 demonstrated that patients with CAD have lower numbers of EPCs, which are functionally impaired, including reduced migration and adhesion, compared to otherwise healthy individuals. Previous work in our lab has demonstrated that the ERK pathway is involved in regulating the adhesive properties of collagen-cultured human CPCs (Kuraitis et al., 2011). Although not investigated in the present study, it is possible that the ERK pathway may be affected and plays a role in the defective adhesion response of CPCs that have been exposed to ED. Given the results stated above, whatever is the pathway responsible for collagen matrix-

mediated improvement in adhesion for normal cells, it cannot be activated in CPCs from the high fat diet mice.

The migratory capacity of CPCs from both diet groups was enhanced when cells were cultured on collagen as a substrate versus fibronectin. The benefits of collagen-cultured CPCs for enhanced cell migration have also been noted by various groups (Kutschka et al. 2006; Kuraitis et al. 2011), but not previously in the context of ED. The fact that the CPCs obtained from mice on the high fat diet were better able to migrate towards VEGF after culture on collagen suggests that collagen may have the potential to restore migratory properties to these dysfunctional cells. Since VEGF is a main homing and angiogenic cytokine produced by tissues in need of reperfusion (Thanigaimani et al. 2011), the use of a collagen matrix may be promising to help improve the migration of dysfunctional CPCs towards VEGF-producing tissue, leading to improved neovascularization and repair. The collagen matrix offers the possibility for better recruitment of these cells to sites of injury even in patients with underlying endothelial dysfunction, giving hope for improved autologous cell therapy in these patients.

Flow cytometry analysis of CPCs after 48 h culture in hypoxia displayed some interesting results, yet not expected once again. No significant differences were observed between normal diet mice and high fat diet mice when CPCs were cultured on fibronectin or collagen substrates. Other studies have shown the positive effects of collagen on CPCs in serum deprived environment, but this benefit was seen in cells isolated from healthy individuals/models (Dai et al. 2008; Kuraitis et al. 2011). Again, it is possible that not all the cells were effectively lifted off the collagen matrix resulting in a large cell fraction, possibly the progenitor/endothelial cells, adhering to collagen, resulting in a less than ideal phenotype analysis.

An important property of cells to consider in transplantation therapy is the ability to survive upon delivery to the target tissue. In the present study, upon exposure to serum deprivation and hypoxia, viability was increased by 320% and by 170% for CPCs of normal and high fat diet groups, respectively, when cultured on collagen matrix compared to fibronectin. These results suggest that provision of a collagen matrix substrate may improve the resistance of CPCs to apoptosis in an environment of endothelial dysfunction. Collagen has been shown previously to increase cell viability in apoptosis-inducing conditions (Kuraitis et al. 2011); however, to my knowledge, this is the first time it has been demonstrated to be effective at promoting survival of CPCs in the context of ED. The survival rate of transplanted cells into a target tissue varies from 1 to 10%; therefore, improvements in cell delivery, survival and engraftment are very much sought-after (Aicher et al. 2003; Retuerto et al. 2004; Kim et al. 2008). As cell survival is essential for cell therapy to be successful; the longer the transplanted cells survive, the better the therapeutic outcome (Reinlib et al. 2000; Templin et al. 2011). This said, our collagen matrix has promise to improve the effects of cell therapy by increasing their survival upon transplantation, which may be effective even for rescuing viability of dysfunctional CPCs from a donor with ED.

ED has negative effects on regeneration, and nitric oxide (NO), whose bioavailability is reduced in ED, plays an important role in endogenous progenitor cell recruitment and spontaneous collateral formation (Kapila et al. 2005; Suuronen et al. 2010). Although the supernatant from collagen-cultured CPCs after 48 h in hypoxia displayed an increase in NO content for the normal diet animals, there was no similar increase seen for NO production in collagen-cultured CPCs for the high fat diet group. This suggests that the culture of CPCs after removal from the harsh environment cannot reverse the defect in the generation of NO. Also,

collagen is ineffective at enhancing NO production in dysfunctional CPCs from animals with ED, at least for the duration of culture tested. Methods of increasing NO production within the endothelial and/or therapeutic cell population would be desirable. For example, Kuboki et al. demonstrated that insulin could increase the expression of eNOS in endothelial cells resulting in vasodilation of blood vessels (Kuboki et al., 2000). Another study demonstrated that L-arginine (a nitric oxide donor) supplementation rescued animals from the hypercholesterolemia-induced endothelial dysfunction (Suuronen et al. 2010). Due to the NO results obtained in this project, the idea of looking at eNOS and iNOS levels (RNA and protein) within the cell could elucidate whether the decreased NO levels observed are due to an issue with the enzyme itself.

Supernatants of CPC cultures were also collected for cytokine profiling after 48 h in hypoxia. Overall, data revealed no significant differences in the secretion of 16 of 17 selected cytokines for this study between CPCs of normal diet and high fat diet groups when cultured on either fibronectin or collagen substrate; the exception was an increase in TNF- α , which was observed in the high fat diet group when CPCs were cultured on collagen. Furthermore, there were no differences between the diet groups or substrates in the levels of the angiogenic cytokines SFD-1, VEGF, bFGF and GM-CSF. This is promising, since the paracrine mechanism is a main contributor to the benefit that can be achieved with angiogenic cell therapy, as previously discussed. The fact that cells from animals with ED do not appear to lose their ability to secrete important angiogenic cytokines, yet are able to survive and migrate better when cultured on collagen matrix, suggests that the collagen matrix is a promising approach for improving the efficacy of these dysfunctional cells in therapy.

The results obtained from this project were derived solely from *in vitro* culture assays. By using conditions hypoxic culture conditions, I tried to mimic the hostile environment present in

ischemic tissues, and observed that culture on collagen could restore some level of function to CPCs from ED mice. However, it would be necessary to confirm the *in vitro* results with appropriate application in *in vivo* models of disease. To this end, there are two areas for future research of particular interest. First, it would be interesting to see how effective transplantation of CPCs in combination with collagen is for the treatment of ischemic tissue in an *in vivo* mouse model of endothelial dysfunction. Secondly, a future study might investigate the use of collagen matrix culture for “restoring” autologous CPC function prior to transplantation to ischemic tissue in need of re-vascularization in a host with ED, similar to a previous study that used collagen matrix to enhance the therapeutic potential of CPCs from healthy donors (Kuraitis et al. 2011).

5. Conclusions

Patients with coronary artery disease can be surgically treated if eligible, however; the benefits remain relatively short-term as they do not fully address the underlying disease. Cell therapy is currently being developed as a means of treatment for revascularization of ischemic tissues, most commonly observed in patients suffering from CAD. In these patients, endothelial dysfunction is often present, which in turn impairs the ability for revascularization to occur. It has been shown that collagen scaffolds can improve the survival and function of transplanted cells (Kuraitis et al. 2011). Here, I provide evidence that culturing CPCs on a collagen matrix is a good candidate for increasing cell survival and function in a model of endothelial dysfunction. Our hope is that use of this matrix will improve transplanted cell retention and survival *in vivo* in the presence of endothelial dysfunction for future use in patients with cardiovascular disease.

6. References

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7. Appendix

Normoxic Flow Data Analysis						
Cell Marker	ND			HFD		
	Fibronectin	Collagen	P-value	Fibronectin	Collagen	P-value
VEGFR2	2.48	1.55	0.52	0.03	0.62	0.04
CD45	31.16	0.93	6.24E-03	7.57	10.40	0.66
CD34	33.72	0.18	2.04E-06	0.38	0.38	0.99
CXCR4	5.20	1.80	0.01	7.13	10.22	0.61
VE-cadherin	22.94	2.18	2.04E-06	7.03	6.08	0.84
CD31	31.64	0.83	1.73E-03	7.95	2.60	0.21

Table 7.1. Flow cytometry analysis of CPCs in normoxia. Data in the table represents the percentage of cells that express each individual marker. Normal diet mice displayed an increase in expression for cell markers CD45 (P=6.24E-03), CD34 (P=2.04E-06), CXCR4 (P=0.01), VE-cadherin (P=2.04E-06), and CD31 (P=1.73E-03), when CPCs were cultured on fibronectin compared to collagen matrix. High fat diet mice had increased VEGFR2 (P=0.04) expression when CPCs were cultured on collagen compared to fibronectin, while the other markers displayed no significant differences between substrates.

Hypoxic Flow Data Analysis						
Cell Marker	ND			HFD		
	Fibronectin	Collagen	P-value	Fibronectin	Collagen	P-value
VEGFR2	8.50	1.14	0.03	6.94	11.22	0.57
CD45	38.88	0.48	9.37E-03	39.68	2.98	0.02
CD34	13.14	0.28	0.02	6.54	2.82	0.37
CXCR4	36.46	1.26	0.02	45.94	11.18	0.06
VE-cadherin	35.98	4.84	0.05	36.98	20.38	0.44
CD31	28.76	2.38	0.03	22.48	11.44	0.43

Table 7.2. Flow cytometry analysis of CPCs in hypoxia. Data in the table represent the percentage of cells expressing each individual marker. Normal diet mice displayed an increase in expression for cell markers VEGFR2 (P=0.03), CD45 (P=9.37E-03), CD34 (P=0.02), CXCR4 (P=0.02), VE-cadherin (P=0.05), and CD31 (P=0.03), when CPCs were cultured on fibronectin compared to collagen. High fat diet mice had increased CD45 (P=0.02) expression when CPCs were cultured on fibronectin compared to collagen, while the other markers displayed no significant differences between substrates.

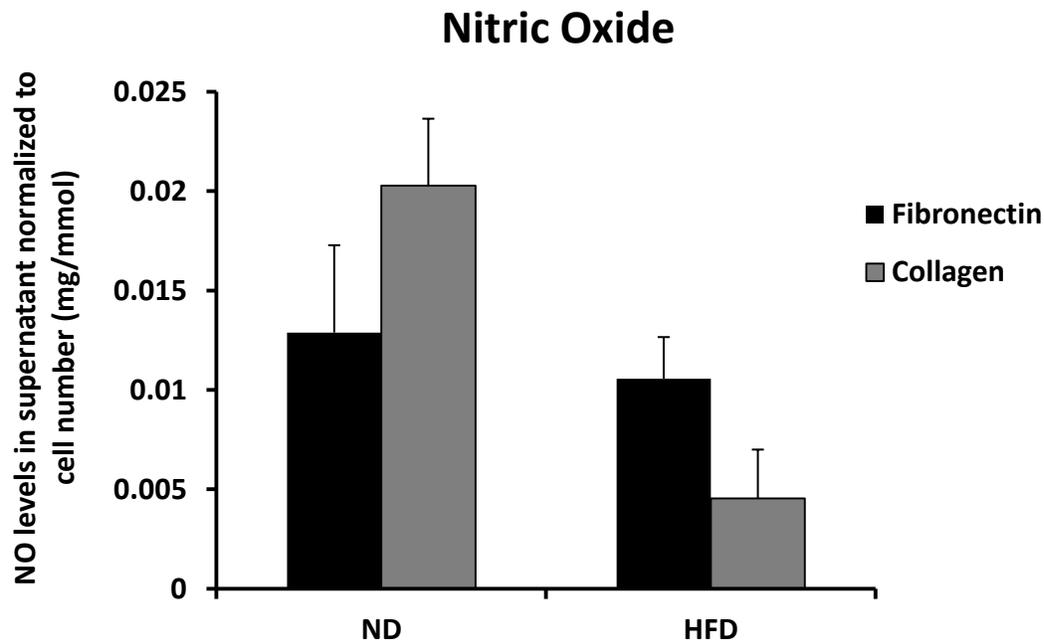


Figure 7.1. Nitric oxide levels in supernatant normalized to total cell number (n=5). Supernatant was collected from 250,000 CPCs cultured on fibronectin or collagen for 48 h in hypoxia from normal diet animals (ND) and high fat diet (HFD) animals. The graph represents nitric oxide levels in supernatant (mg/mmol) normalized to the total number of viable cells that remain after 48 h in hypoxia. No significant differences were observed between fibronectin- and collagen-cultured CPCs from ND and HFD mice.