~~FUNCTIONAL~~ TISSUE ENGINEERED VASCULAR CONSTRUCT AS AN *IN VITRO* MODEL TO STUDY THE EFFECT OF HYPERGLYCEMIA AND HYPERLIPIDEMIA ON ENDOTHELIAL CELL DYSFUNCTION

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Ph.D.Thesis

2020

SREE CHITRA TIRUNAL INSTITUTE FOR
MEDICAL SCIENCES AND TECHNOLOGY
THIRUVANANTHAPURAM
INDIA

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A THESIS PRESENTED BY

RENU RAMESH

TO

SREE CHITRA TIRUNAL INSTITUTE
FOR
MEDICAL SCIENCES AND TECHNOLOGY
THIRUVANANTHAPURAM
INDIA

IN PARTIAL FULFILMENT OF THE REQUIREMENTS

FOR THE AWARD OF

DOCTOR OF PHILOSOPHY

2020

CERTIFICATE

I, **Renu Ramesh**, hereby certify that I have personally carried out the work depicted in the thesis entitled, ***"Functional tissue engineered vascular construct as an in vitro model to study the effect of hyperglycemia and hyperlipidemia on endothelial cell dysfunction"***, except where due acknowledgement has been made in the text. No part of the thesis has been submitted for the award of any other degree or diploma prior to this date.

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*Clearance was obtained from the Institutional Ethics Committee for carrying out this study.

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The thesis entitled

"Functional tissue engineered vascular construct as an in vitro model to study the effect of hyperglycemia and hyperlipidemia on endothelial cell dysfunction"

Submitted by

Renu Ramesh

for the degree of

Doctor of Philosophy

of

SREE CHITRA TIRUNAL INSTITUTE
FOR
MEDICAL SCIENCES AND TECHNOLOGY
THIRUVANANTHAPURAM

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Thesis Examiner

DEDICATED TO

ANOOP, AADI AND TO ALL THOSE WHO

BELIEVED IN ME……

ACKNOWLEDGEMENT

I take this opportunity with great pleasure to thank all those who contributed in one way or the other for the success of this study.

I consider myself privileged to have had the opportunity to carry out my doctoral studies in the Division of Thrombosis Research, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, India. I gratefully acknowledge the SRF fellowship awarded by ICMR to undertake the Ph.D. programme.

Firstly, I express my deepest gratitude to my Guide Dr. Lissy K. Krishnan for the constant encouragement, guidance and support provided to me throughout the course of the study. I thank her for all the patience, timely advice and great effort that she put in during my doctoral research. She has always been a pillar of strength and support and been the guiding light throughout the journey.

I thank members of the doctoral advisory committee, Dr. V.Kalliyanakrishnan, Scientist G (senior grade) and Head (Retd.), Department of Biomaterial Science and Technology, Dr. M. Unnikrishnan, Professor Senior grade (Retd.), Department of Cardiovascular and Thoracic Surgery and Dr. Annie John, Scientist F (Retd.), SCTIMST for their timely suggestions, ideas and comments which helped in the improvement of the quality of this work. I also take this opportunity to thank all the teaching faculty of my PhD course work.

I am extremely grateful to the Director of our institute and the Head, BMT Wing for all the support and excellent facilities provided during the course of my work.

I take this opportunity to express my deep sense of gratitude to the former and present Dean, Associate Dean, the Registrar, the Deputy Registrar and all members of Division of Academic Affairs, Director's office and Head BMT Wing office for their valuable assistance.

I express my sincere gratitude to all staff of Department of Cardiovascular and Thoracic Surgery, SCTIMST for providing adipose tissue samples during my research work. I also express my sincere gratitude to Dr. P V Sulochana (Retd.), Dr. S Sathyabhama and all staff of blood bank for providing platelet rich plasma bags during my research work.

I sincerely thank Mr. Ranjith S, Ms. Priyanka, Mr. Anilkumar and Ms. Deepa from the Division of Thrombosis Research for providing the fibrin sealant components. I am grateful to Dr.Anugyya Bhatt and Mr.Ranjith S, Division of Thrombosis Research, for providing the flow cytometry facility and the technical assistance in performing flow cytometry.

I thank Mr.Ramesh Babu and staff of Precision Fabrication Facility for fabricating the flow chamber to be used for bioreactor. My deep sense of gratitude to Dr.Lizymol and Dr.Deepu, Dental Products Lab for providing the UTM facility and the technical assistance in using the facility. All members of DPL are also acknowledged. I should also acknowledge the guidance and training given by Dr.Soumya Columbus in fabricating the scaffolds and the constant encouragement given by her during my work. I express my gratitude to Dr.Rethikala Nishanth for her support, encouragement and friendship.

I express my sincere gratitude to Dr. Anoop Kumar T, Scientist F, Division of Molecular Medicine for his guidance in performing molecular biology techniques and all staff and students of Division of Molecular Medicine and Division of Tissue Culture. My sincere thanks to Dr.Nishad for SEM analysis. I am very much thankful to Mr.Sajithlal, Mr.Binu and all staff of Network Engineering Services. The valuable support and encouragement from Dr.Rekha.M.R. and Dr.P.R.Anilkumar are sincerely acknowledged.

I am deeply indebted to all staff and colleagues at Division of Thrombosis Research. I express my sincere thanks to Dr. Anugya Bhatt for her advice and support during the course of my work. I place my sincere gratitude to my seniors Dr. Ragaseema, Dr. Unnikrishnan Sivan, Dr. Renjith P Nair and Dr. Tara S for guiding and training me on various stem cell isolation and molecular biology techniques. I sincerely thank Ms. Mary Vasantha, Ms. Juliet Baby Saroja, Dr.Sabeela Beevi, Dr.Lakshmi, Dr.Aravind, Ms. Priyanka Manoj, Mr. Ranjith S, Mr. Anilkumar V, Ms. Safeena, Ms. Deepa, Dr.Subha, Dr. Krishnapriya Chandrababu, Dr.Amita Ajit, Dr. Rashmi R, Ms. Rakhi and Ms. Athira for their support, encouragement and friendship. I also thank Ms. Usha, Ms. Bindu, Mr.Hari and Mr. Shibu for helping me in maintaining the aseptic conditions in cell culture facility.

My friends and teachers from within and outside the campus have helped in many different ways and must be acknowledged. I express my sincere gratitude to all fellow students from other departments of our campus for their support and friendship. I thank the staff of administrative department, accounts department, stores and purchase division and library for their assistance.

I cannot end without acknowledging my whole family for their endless support, patience and prayers without which this would not have been possible. Finally, I am indebted to the Divine Power who has been a guiding force throughout my life.

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ABBREVIATIONS

2D	: 2 Dimensional
3D	: 3 Dimensional
ABAM	: Antibiotic Antimycotic
ACD	: Acid Citrate Dextrose
ADMA	: Asymmetric Dimethyl Arginine
ADMSC	: Adipose Derived Mesenchymal Stem Cell
AGE	: Advanced Glycosylation Endproduct
ASC	: Adult Stem Cells
AT1R	: Angiotensin II Type 1 Receptor
bFGF	: basic Fibroblast Growth Factor
BMMNC	: Bone Marrow Mononuclear Cell
BMMSC	: Bone marrow derived Mesenchymal Stem Cell
BMP	: Bone Morphogenetic Protein
BSA	: Bovine Serum Albumin
CAD	: Coronary Artery Disease
CCR	: Cysteine-Cysteine Motif Chemokine Receptor
CD	: Cluster of Differentiation
cDNA	: Complimentary Deoxy ribo Nucleic Acid
CHD	: Coronary Heart Disease
CRP	: C - reactive protein
CVD	: Cardio Vascular Disease
DAPI	: 4', 6-diamidino-2-phenylindole
DNA	: Deoxyribo Nucleic Acid
dNTP	: deoxy Nucleotide Tri Phosphate
EC	: Endothelial Cell
ECM	: Extracellular matrix
EDHF	: Endothelium Derived Hyperpolarizing Factor
EDTA	: Ethylene Diamine Tetra acetic Acid
EGF	: Epidermal Growth Factor
eNOS	: Endothelial Nitric Oxide Synthase
EPC	: Endothelial Progenitor Cell
EPCR	: Endothelin Protein C Receptor
ePTFE	: expanded Polytetra Fluoro Ethylene
E-SEM	: Environmental Scanning Electron Microscopy
ET	: Endothelin
FACS	: Fluorescence Activated Cell Sorter
FBS	: Fetal Bovine Serum
Fib	: Fibrinogen
FMD	: Flow Mediated Dilation
GAPDH	: Glyceraldehyde 3-Phosphate Dehydrogenase

GF : Growth Factor
HA : Hydroxy Apatite
HBSS : Hank's Balanced Salt Solution
HC : High Cholesterol
HDL : High Density Lipoprotein
HE : Hypothalamus Extract
HG : High Glucose
HGF : Hepatocyte Growth Factor
HLA : Human Leukocyte Antigen
HUASMC : Human Umbilical Artery Smooth Muscle Cell
HUVEC : Human umbilical Vein Endothelial Cell
ICAM : Intercellular Adhesion Molecule
IEC : Institutional Ethics Committee
IGF-1 : Insulin-like Growth Factor 1
IL : Interleukin
INF : Interferon
iNOS : inducible Nitric Oxide Synthase
iPSC : Induced Pluripotent Stem Cell
ISCT : International Society for Cellular Therapy
LDL : Low Density Lipoprotein
LOX : Oxidized LDL Receptor
MCP : Monocyte Chemo attractant Protein
MDA : Malonidialdehyde
MHC : Myosin Heavy Chain
MHC II : Major Histocompatibility Complex II
MI : Myocardial Infarction
MMP : Matrix Metalloproteinase
MSC : Mesenchymal Stem Cell
NCEP : National Cholesterol Education Program
NM : Normal Medium
NO : Nitric Oxide
OxLDL : Oxidised LDL
PAI : Plasminogen Activator Inhibitor
PAR : Protease Activated Receptor
PBS : Phosphate Buffered Saline
PCL : Poly- €- Capro Lactone
PDGF : Platelet derived growth factor
PECAM : Platelet Endothelial Cell Adhesion Molecule
PGA : Poly Glycolic Acid
PGF : Platelet Growth Factor
PLGA : Poly-dl-Lactic-co-Glycolic Acid
PLLA : Poly-L-Lactic Acid
PROCAM : Prospective Cardiovascular Munster
PRP : Platelet Rich Plasma

qRT-PCR	: Quantitative Real Time Polymerase Chain Reaction
RAS	: Renin Angiotensin System
RNA	: Ribo Nucleic Acid
ROS	: Reactive Oxygen Species
SDBV	: Small Diameter Blood Vessel
SMC	: Smooth Muscle Cell
SMPC	: Smooth Muscle Progenitor Cell
SS	: Shear Stress
SVF	: Stromal Vascular Fraction
TCP	: Tri Calcium Phosphate
TCPS	: Tissue Culture Polystyrene
TE	: Tissue Engineering
TEVG	: Tissue Engineered Vascular Graft
TGF	: Transforming Growth Factor
TNF	: Tumor Necrosis Factor
tPA	: Tissue Plasminogen Activator
UTM	: Universal Testing Machine
VCAM	: Vascular Cell Adhesion Molecule
VEGF	: Vascular Endothelial Growth Factor
vWF	: von Willebrand Factor
αSMA	:α Smooth Muscle Actin

ANNOTATIONS

%	: Percentage
<	: Less than
>	: Greater than
α	: Alpha
β	: Beta
€	: Epsilon
cm	: Centimeter
µg	: Microgram
µl	: Microlitre
µm	: Micromolar
d	: Days
h	: hours
IU	: International Unit
Kv	: Kilovolt
mA	: Milli Ampere
min	: Minutes
mM	: Milli Molar
mm	: Millimeter
mg	: Milligram
ml	: Millilitre
N	: Newton
ng	: Nanogram
nm	: Nanometers
pmol	: Picomol

SYNOPSIS

Cardiovascular diseases (CVD) are considered to be one of the main causes of death globally. Atherosclerosis, a multifactorial disease and the dominant cause of CVD is considered as a chronic, immunoinflammatory and fibroproliferative disease. Endothelial cell (EC) lining the blood vessel is multifunctional, highly dynamic and may respond to various untoward factors in circulation causing cellular dysfunction. Endothelial dysfunction (ED) is regarded as an initial step in the pathogenesis of atherosclerosis. ED causes reduced nitric oxide (NO) activity in patients with atherogenic risk factors such as male gender, aging, hypertension, diabetes, hyperlipidemia, smoking and family history. The variations in shear stress influenced by blood flow is another contributor in the development of ED. However, the reported human and animal studies are not sensitive to capture the small changes in EC, which occur in response to risk factors. Therefore, major goal of this study is to develop an *in vitro* model of human tissue to understand the early effects of risk factors on the variable expressions of functional markers in endothelial cells.

The thesis is divided into 6 chapters.

Chapter 1 introduces the research problem in the light of current scientific knowledge in the field and relevant gaps are identified. An approach to the problem is defined, research hypothesis is developed and objectives of the study are stated. Briefly, the currently available clinical data have not yet proven definitively the impact of risk factors on ED. The problem associated with the use of animal models for studying atherosclerosis is that the lesions in other species do not resemble those seen in human atherosclerosis. The hemodynamic forces which is a critical regulator of CVD is much different in animal models, making it difficult to extrapolate the results. In *in vitro* studies, 2D cell culture methods are not adequate to consider effect of flow known as shear stress. To address these problems a fully functional tissue-engineered vascular graft (TEVG) comprising human cells and tubular conduit to circulate risk factors in the lumen seems promising.

Established methods and materials such as biodegradable bilayered conduit fabricated from poly Ɛ-caprolactone (PCL) and coated with fibrin could be used to construct TEVG. The application of adipose derived mesenchymal stem cells (ADMSCs) in constructing a TEVG is nascent; but, several studies have shown that the plasticity of these cells enable them to differentiate to the vascular cells such as endothelial cells (ECs) and smooth muscle cells (SMCs). Also the dynamic culture of the TEVG gives an opportunity to study the effect of high glucose, lipids, proinflammatory TNF-1α and shear stress simultaneously.

With this background a research hypothesis has been developed: Biomimetic functional blood vessel may be constructed by growing human ADMSC derived EPCs (endothelial progenitor cells) and SMPCs (smooth muscle progenitor cells) on suitable scaffold under shear stress, for use as an *in vitro* model system to study the subtle changes to EC phenotype upon dynamic exposure to risk factors.

To prove the hypothesis, three major objectives have been defined:

1. To establish conditions for developing human vascular cells from adipose derived mesenchymal stem cells (hADMSCs) for *in vitro* construction of small diameter blood vessels
2. To construct tissue engineered vascular graft (TEVG) on an established fibrin coated PCL scaffold. This is to study the influence of SMPC and shear stress on vessel stability and EC marker expressions, respectively
3. To establish the suitability of artificially constructed human cell grown blood vessel. This is to study the effect of risk factors on the expressions of molecular markers in the endothelial cell lining the lumen by circulating high glucose, high cholesterol in the presence and absence of TNF-1α containing medium.

Chapter 2 describes the published Literature in the selected field of research, which has been reviewed extensively to understand the physiology of endothelial function and mechanisms of dysfunction. Topics like inflammatory changes in EC and the markers that can be studied to understand the dysfunction are reviewed. Construction of tissue engineered small diameter blood vessel, the polymers used, strategies adopted for *in vitro* differentiation of hADMSCs to endothelial cells and smooth

muscle cells, cell seeding and mechanical stimulation involved in the fabrication of blood vessel are also highlighted in review of Literature.

In **chapter 3**, the experimental design adopted to achieve the proposed objectives of the study are explained in detail. The detailed description in this chapter includes materials used, fabrication of polymeric hybrid scaffold, isolation and characterization of hADMSCs, induction of hADMSCs to EPCs and SMPCs, characterization of induced cells by morphological examination, immunocytochemistry, flowcytometry and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)/qRT-PCR. For induced EPCs, marker expression at the translational level at different periods of culture are explained. Assessment of NO release to analyze the functionality of induced EPCs, assessment of cell proliferation on scaffolds and analysis of deposited extracellular matrix (ECM) proteins on scaffold by qualitative and quantitative methods are described. Furthermore, measurement of mechanical strength of cell grown scaffold and the marker expression at the translational level upon treating the cell seeded scaffolds with high glucose, high cholesterol and inflammatory cytokine are also elaborated.

In **chapter 4,** the results of the study are illustrated using figures, tables and graphs. hADMSCs are isolated, proliferated, established purity, stemness and multipotency by standard criteria. A novel method for differentiating hADMSCs to EPC is established by demonstrating expressions of multiple markers at mRNA level and protein level. The differentiated EPC released NO under shear stress demonstrated functional phenotype in the lumen. A novel method is also developed for differentiating hADMSCs to SMPCs expressing multiple markers at transcriptional and translational level. The ECM deposition by SMPC demonstrates tendency to form medial layer of blood vessel, in the electrospun fibrous compartment of scaffold, under static culture which seemed to influence tensile properties of scaffold. Constructed functional TEVG by growing SMPC in the ablumen under static condition and EPC in the lumen under dynamic culture followed by analysis of morphology of EPCs in the lumen of the scaffold and cell stability after shear stress are demonstrated. Basal level expressions of target genes in EC are determined at molecular level advocating suitability of the model for studying functional variations in response to risk factors. On exposure of EPCs to high glucose(HG) and high

cholesterol(HC), changes in gene expressions are observed as compared to that in circulated normal medium(NM). Antithrombotic factor eNOS shows an upregulated expression in HG when compared with NM and HC, while the prothrombotic factor vWF shows comparable gene expressions in all the three conditions. Pro fibrinolytic and anti-fibrinolytic markers, tPA and PAI, show an inverse correlation in expression in all three conditions. Adhesion molecules CD31, vCAM1 are down regulated upon exposure to HG and HC. MCP1, the proinflammatory marker, shows high expression in the absence of shear stress. Upon exposure to shear stress MCP1 expression is down regulated irrespective of the medium compositions. Similarly, on exposure of the cell seeded scaffolds to TNF-1α along with NM, HG and HC, change in gene expressions are quantifiable and different. eNOS, vWF, tPA and CD31 are down regulated just by adding TNF-1α in normal medium whereas PAI and vCAM1 show a slight up regulation in expression.MCP-1 shows minimal expression in the presence and absence of TNF-1α.CD31 shows a greater expression on exposure to TNF-1α in the presence of HG while vCAM1 shows greater expression in all three conditions with TNF-1α.All genes show a slightly less expression on exposure to HC with TNF1α, except vCAM1. All these quantitative findings were seen in at least 4 replicate experiments and the results are presented as average±S.D.

Chapter 5 discusses results of the study in the light of and in comparison to relevant publications in literature. This study focuses standardization of separate protocols for hADMSC differentiation to obtain EC and SMC simultaneously, from same donor cells for seeding on a bi-layered fibrin coated PCL scaffold. Several studies have used stem cell based tissue engineering to regenerate or replace blood vessels *in vivo.* This study also establishes feasibility of autologous TEVG construction by collecting adipose tissue from CVD patients undergoing bypass surgery and are differentiated to EPCs and SMPCs. Effective protocols developed for differentiation of SMPCs and EPCs seems novel in terms of achieving functional vascular cells upon seeding on the scaffold and undergoing dynamic culture. Furthermore, the study also explored the suitability of using artificially constructed blood vessel for studying the effect of high glucose/cholesterol, in the absence and presence of inflammatory cytokine-TNF-1α circulated in medium, on the phenotype of endothelial cell lining the lumen. This is a novel concept, not published elsewhere.

In **chapter 6,** results of the study are summarized and conclusions are drawn. To summarize, stem cells can be easily harvested from human adipose tissue and tissue engineered vascular graft constructed using hADMSC derived EPCs and SMPCs. Influence of static SMPC culture and dynamic EPC culture on TEVG properties are identified. The NO release established adequate function of constructed TEVG and the role of shear stress as a major contributing factor for regulating EC marker expressions. The study established that hADMSC derived EPCs are sensitive to medium composition and subtle changes in ECs are quantifiable. The TEVG comprising human EC grown under dynamic condition could be a suitable model to study long term effects of circulating risk factors at different concentrations and variable shear stress on EC phenotype. The limitations of the study have been identified and future studies are proposed to address more questions.

Lastly, **Citations** are listed in the bibliography section.

CHAPTER 1

1. Introduction

The cardiovascular system consists of the heart and the network of arteries, veins and, capillaries that transport blood throughout the body. It is powered by a hollow muscular pumping organ known as heart. The circulatory system is responsible for the delivery of oxygen and nutrients to all cells, as well as the removal of carbon dioxide, waste products, maintenance of optimum pH, and the mobility of the elements, proteins, and cells, of the immune system. Blood vessels are the body's highways that allow blood to flow quickly and efficiently from the heart to every region of the body and back again. There are three major types of blood vessels: arteries, capillaries, and veins. Arteries carry blood away from the body. Arteries have three distinct layers: intima, media, and adventitia. Tunica intima (tunica interna) is the innermost layer consisting of simple squamous endothelium, surrounded by a connective tissue basement membrane with elastic fibers. Tunica media is the middle layer primarily consists of smooth muscle and is usually the thickest layer. It not only provides support for the vessel but also changes vessel diameter to regulate blood flow and blood pressure. Tunica externa or tunica adventitia is the outermost layer that attaches the vessel to the surrounding tissue. It has varying amounts of elastic and collagenous fibers. It also contains nerves that supply the vessel as well as nutrient capillaries (vasa vasorum) in the larger blood vessels. Veins carry blood to the heart and have three layers like that of arteries, but veins have less smooth muscle and connective tissue. This makes the walls of veins thinner than those of arteries which is related to the fact that blood in the veins has less pressure than in the arteries. Due to this, veins can hold more blood. Another characteristic feature of veins is the presence of valves. Valves prevent regurgitation or reverse blood flow. Capillaries are the smallest and most numerous of the blood vessels. They form the connection between the arteries and the veins. The primary function of capillaries is the exchange of materials between the blood and tissue cells. Extracellular matrix (ECM) is a major constituent of blood vessels and

provides a framework in which various cell types are attached and embedded. The composition and organization of ECM are primarily controlled by the mesenchymal cells. ECM is responsible for the mechanical properties of the vessel wall, forming complex networks of structural proteins that are highly regulated. ECM also plays a central role in cellular adhesion, differentiation, and proliferation. In arteries and veins, the ECM constitutes more than half of the mass of the wall. Changes in the ECM have been implicated in the pathogenesis of several cardiovascular conditions including atherosclerosis and heart failure. Matrix proteins play a key role in the development of atherosclerotic complications, such as plaque rupture, aneurysm formation, and calcification.

Cardiovascular diseases (CVD) including coronary heart disease and cerebrovascular disease are considered to be the main cause of death globally and there is an increasing prevalence of cardiovascular diseases in developing countries. Atherosclerosis is the dominant cause of cardiovascular diseases and it leads to myocardial infarction, heart failure, stroke, and claudication. It is a multifactorial disease involving the interplay of genetic and environmental factors and is a subdivision of arteriosclerosis, literally means hardening of the arteries. If the walls of the arteries are elastic, they can withstand the turbulence of blood flowing under high pressure. As the arteries grow harder and thicker, they become more and more resistant to the blood flow and hence, the blood pressure keeps rising as age advances or disease worsens. There are three general patterns, with differing clinical and pathologic consequences: 1) Arteriolosclerosis that affects small arteries and arterioles, and may cause downstream ischemic injury.

2) Mönckeberg medial sclerosis is characterized by calcific deposits in muscular arteries in persons typically older than age 50.

3) Atherosclerosis

Atherosclerosis derives its name from two Greek words in which ‘athero’ means gruel (accumulation of lipid) and ‘sklerose’ means hard. The history of atherosclerosis dates back to more than 3500 years; reports suggesting its presence in

Egyptian mummies.Several risk factors may intensify or provoke atherosclerosis through their effects on low-density lipoprotein (LDL) particles and inflammation. It is characterized by the narrowing and hardening of both the medium and large-sized arteries due to excessive plaque formation within the arterial walls. Atherosclerosis can be mainly seen as lesions in the intima of medium and large-sized arteries, which progressively affect the other arterial walls such as media and adventitia. The focal intimal lesions are called atheromas or atherosclerotic plaques. These lesions protrude into the vessel lumen leading to various complications. Coronary arteries are mainly affected by these lesions. Though the occurrence of developing atherosclerosis is higher in men than in women, there is a rise in the incidence of atherosclerosis in women due to dietary habits, smoking, and mental stress.

1.1. Pathogenesis of atherosclerosis

Atherosclerosis can be considered as a chronic, immunoinflammatory and fibro proliferative disease. Atherosclerosis starts early in life. Some studies suggest that maternal hypercholesterolemia during pregnancy causes the formation of fatty streaks in the human fetus (Singh et al., 2002a). The disease appears in the aorta during fetal life, while it appears in the coronary arteries in the second decade and in the cerebral arteries in the third decade. There are three stages in the life history of an atherosclerotic plaque: initiation, progression, and complication (Singh et al., 2012).

1.1.1. Initiation

Recruitment of mononuclear leucocytes to the intima marks the initiation of the atherosclerotic lesion. Specific adhesion molecules such as selectins and members of the immunoglobulin superfamily namely Vascular Cell Adhesion Molecule-1 (VCAM-1) and Intercellular Adhesion Molecule-1(ICAM-1) are expressed on the surface of the vascular endothelial cell. When an atherogenic stimulus occurs, these adhesion molecules mediate leucocyte adhesion of mainly monocytes and to a lesser extent, T-lymphocytes to the intima. Once adherent, the leucocytes enter the intima directed by chemoattractant chemokines such as monocyte chemoattractant protein-1 (MCP-1) and Tumor Necrosis Factor-1α (TNF-1α).The monocytes get transformed

into macrophages in the intima. When chronic hyperlipidemia which is one of the risk factors for atheroma formation occurs, the lipoproteins that are accumulated inside the intima gets oxidized by the action of oxygen free radicals generated by intimal macrophages. Oxidized LDL further stimulates the release of cytokines and chemokines and flares up the ongoing inflammation. Macrophages internalize oxidized LDL through scavenger receptors and are then called foam cells. The accumulation of foam cells is considered as the hallmark of early and asymptomatic atheromatous precursor, the fatty streak.

1.1.2. Progression

As the disease progresses, the inflammatory response changes to a fibro proliferative response mediated by intimal smooth muscle cells. The atheroma progresses by the accumulation of smooth muscle cells. The smooth muscle cells produce extracellular matrix macromolecules. The collagen-rich matrix gives stability to plaques, protecting them from the consequences of plaque rupture and thrombosis. The smooth muscle cell is the principal connective tissue producing cell in the normal and atherosclerotic intima. Smooth muscle cells can be contractile or synthetic. The synthetic activity of smooth muscle cells is considered to be beneficial whereas their loss is detrimental to plaque stability. Plaque rupture occurs due to a lack of smooth muscle cells and this can be due to apoptotic cell death. It's seen that progressing atheroma accumulates calcium and this calcification has a stabilizing effect on the plaque. An atheromatous plaque can be described as a cellular component that contains macrophages, smooth muscle cells, T cells, extracellular matrix including collagen, elastic fibers, proteoglycans, and intracellular as well as extracellular lipids. Collagen and smooth muscle cells can be seen in the outer fibrous cap of the plaque. Deep inside the cap, there is a cellular area that contains macrophages, T cells, and smooth muscle cells. Beneath is a necrotic core containing lipid, the debris of dead cells, foam cells, fibrin, and smooth muscle cells.

1.1.3. Complication

On the basis of pathologic characteristics, plaques can be of two types- vulnerable plaques or non-stenotic plaques and stable plaques or stenotic plaques. Vulnerable plaque is prone to rupture, causing thrombosis and other complications of plaque. It is characterized by a large lipid core, thin fibrous cap, clusters of inflammatory cells mainly foamy macrophages and fewer smooth muscle cells. Stable plaque or stenotic plaque has a thick fibrous cap, small lipid core, more of collagen synthesizing smooth muscle cells and less inflammation. This type of plaque gradually occludes the vessel compromising blood flow and causes ischemic injury to myocardium depending on the blood supply by the affected vessel. In the coronary circulation, when there is a loss of 70% of the area through which blood can flow, it is known as critical stenosis. The clinical condition is known as stable angina. On the other hand, vulnerable plaques are more prone to acute plaque changes like rupture/fissuring, erosion/ulceration and hemorrhage into the atheroma or intraplaque rupture. Intraplaque hematoma further increases the intraplaque pressure making it prone to physical disruption. Vasospasm, low flow, decreased fibrinolytic activity, procoagulant states, etc. can also contribute towards plaque disruption. A ruptured plaque can be clinically manifested as unstable angina, myocardial infarction or sudden death. However, if the plaque disruption is minor, local flow is high, and the fibrinolytic system is active, thrombus formation may be minimal and plaque may remain silent for years and may pose life-threatening sudden complications if left undetected.

1.2. Cellular components of atherosclerosis

Endothelial cells, leukocytes, and intimal smooth muscle cells are the major cell types in the development of atherosclerosis.

1.2.1. Endothelial cells: Endothelial cell structure and functional integrity are important in maintaining the integrity of the vessel wall and circulatory function. As a barrier, the endothelium is semipermeable and regulates the transfer of small and large molecules. Endothelial cells (ECs) are dynamic and have both metabolic and

synthetic functions. Endothelial cells play a major role in maintaining a nonthrombogenic blood-tissue interface and regulate thrombosis, thrombolysis, platelet adherence, vascular tone, and blood flow. But the endothelium becomes activated due to atherogenic or proinflammatory stimuli or due to various risk factors such as hypertension, sepsis and inflammatory syndromes. This results in endothelial injury and dysfunction. As a result, the expression of the adhesion molecules, primarily vascular cell adhesion molecule-1(VCAM-1), is up-regulated, and monocytes and T cells are recruited. In addition to VCAM-1, other adhesion molecules, such as ICAM-1, E selectin, and P selectin, also contribute to the recruitment of blood-borne cells to the atherosclerotic lesion. Atherosclerotic lesions begin to develop under an intact but leaky, activated, and dysfunctional endothelium.

***1.2.2. Leucocytes*:** Leucocytes are the cells of the immune system that are involved in protecting the body against both infectious disease and foreign invaders. One of the earliest cellular responses in atherogenesis is the focal recruitment of circulating monocytes and T lymphocytes. Leukocyte recruitment involves rolling, adhesion and transmigration of cells. There are three major classes of leukocyte cell adhesion molecules: selectins, integrins and the immunoglobulin superfamily of cell adhesion molecules. The selectins are responsible for the initial leucocyte/endothelial interactions also known as tethering. Platelet endothelial cell adhesion molecule (PECAM-1/CD31), a surface receptor of the immunoglobulin superfamily, is a 130kD glycoprotein expressed on the cell surface of leucocytes, platelets, subsets of T cells and endothelial cells (Stevens et al., 2008). Since PECAM-1/CD31 expression is localized at the intercellular junction of ECs, the protein is also known as EC junction molecule. CD31 plays a role in the progression of atherogenesis, though its exact role in the biologic events of atherosclerosis remains controversial. CD31 signaling regulates leucocyte detachment, T-cell activation, platelet activation, and angiogenesis, all of which are critical to the pathogenesis of atherosclerosis. Studies suggest that PECAM-1 expressing on the surface of endothelial cells plays a major role in detecting mechanoreception (mechanical shear force) and mechanotransduction (conversion into chemical signals). Leucocytes transmigrate into the sub endothelial space via endothelial cell-cell junctions or directly through

endothelial cells and there is evidence that lack of PECAM-1 impairs leucocyte trans endothelial migration. Monocytes/macrophages are the primary white blood cells involved in the vascular response to injury and the development of neointimal hyperplasia.

***1.2.3. Smooth muscle cells*:** For the disease progression, a fibro proliferative response accompanies the immunoinflammatory response and this is mediated by intimal smooth muscle cells (SMCs). These cells are responsible for healing after arterial injury. SMCs undergo molecular changes from a contractile phenotype exhibiting specific contractile and cytoskeletal proteins such as SMC α-actin, vinculin and desmin to an activated secretory state. This phenotypic switch results in the synthesis of more cytokines and growth factors. This, in turn, activates other SMCs, facilitates leucocyte chemotaxis and infiltration into the vessel wall, upregulation of adhesion molecules expressed on endothelial cells, and stimulate the production of ECM components such as collagen, elastin, and proteoglycans. There will be an upregulated expression of VCAM-1 and MCP-1 by the SMCs and this is regulated by the NF-κ B mechanism. Activated SMCs also proliferate and migrate to the intima, ultimately contributing to the developing neointima. The size of the neointima is determined in large part by the SMC and ECM content. SMC activation and phenotypic switch is not a terminal, irreversible change.

1.3. Endothelial function

Once considered as a simple barrier between the blood and vessel wall, the endothelium is now regarded as a dynamic organelle that lines the entire vascular system. It is a multifunctional endocrine organ located strategically between the blood and the blood vessel. A healthy endothelium inhibits platelet and leukocyte adhesion to the vascular surface and maintains a balance of profibrinolytic and prothrombotic activity. The endothelial function has been assessed primarily in terms of endothelium-dependent vasomotion. A healthy endothelium produces endothelium-derived nitric oxide (NO), synthesized by the endothelial NO synthase (eNOS) from the precursor L-arginine. Endothelium-derived nitric oxide is a major

mediator of endothelium-dependent vasodilation and is also critically involved in the regulation of other protective properties of the healthy endothelium. Endothelium-derived nitric oxide has an important anti-inflammatory role too. Pharmacologic inhibition of endothelium-derived NO production leads to an increased monocyte adhesion to the endothelium and this effect is attenuated by dietary L-arginine, the substrate of eNOS (Čejková et al., 2016). Increased leucocyte endothelial cell interactions have been observed in eNOS-deficient mice (Sanz et al., 2001).At a molecular level, eNOS inhibition results in increased expression of leucocyte-adhesion molecules and chemokines, such as MCP-1. These molecules are responsible for the migration of monocytes into the intima at sites of atherosclerotic lesion formation. In addition to limiting the monocyte adhesion to the endothelium, endothelium-derived NO also limits platelet activation, adhesion, and aggregation. Antithrombotic effects of endothelium-derived NO include inhibition of plasminogen activator inhibitor-1(PAI-1) expression, a prothrombotic protein. Endothelial function can be assessed by measuring the flow-dependent dilation of the brachial artery. This is a noninvasive endothelial function test. Impaired endothelium-dependent vasomotion reflects alterations of other functions of the endothelium too. Many studies demonstrate that endothelial dysfunction in terms of reduced nitric oxide activity is one of the earliest markers in patients with atherogenic risk factors such as male gender, aging, hypertension, diabetes, smoking and family history.

1.4. Endothelial dysfunction: the determinant of progression of atherosclerosis

Endothelial dysfunction can be defined as, “the partial or complete loss of balance between vasoconstrictors and vasodilators, growth-promoting and growth-inhibiting factors, proatherogenic and anti-atherogenic factors” (Quyyumi, 1998). Endothelial dysfunction is characterized by a reduction of the bioavailability of vasodilators, particularly nitric oxide (NO), and/or an increase in endothelium-derived contracting factors (Lerman and Burnett, 1992). The functional characteristic of endothelial dysfunction is the impairment of endothelium-dependent vasodilation. In addition to this, endothelial dysfunction is also characterized by a proinflammatory,

proliferative, and procoagulatory states that favor all stages of atherogenesis. Both traditional and novel cardiovascular risk factors including smoking, aging, hypercholesterolemia, hypertension, hyperglycemia, and a family history of premature atherosclerotic disease are all associated with endothelial dysfunction. Endothelial dysfunction has also been associated with obesity, elevated C-reactive protein, and chronic systemic infection. When endothelial dysfunction occurs there is an increased oxidative stress. This is an important promoter of inflammatory processes. When oxidative stress increases, there is an increased vascular endothelial permeability and this promotes leucocyte adhesion. This is coupled with alterations in endothelial signal transduction and redox-regulated transcription factors. Out of the many risk factors, hyperlipidemia, diabetes and disturbed blood flow are considered to be major risk factors.

1.5. Risk factors for atherosclerosis

According to the Framingham risk point scores adopted by the U.S. National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III [ATP III]) the traditional (old) risk factors are age, sex, total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, systolic blood pressure, and smoking. Contrary to this, in the more recent Prospective Cardiovascular Mu¨nster (PROCAM) simple scoring scheme, 8 risk variables are identified: age, LDL cholesterol, smoking, HDL cholesterol, systolic blood pressure, family history of premature myocardial infarction (MI), diabetes mellitus, and triglycerides (Fruchart, 2004). The traditional risk factors for atherosclerosis are hyperlipidemic states, diabetes mellitus, smoking, and hypertension, but any one of these is insufficient to produce an atherosclerotic lesion. These risk factors are also known as modifiable risk factors. They can be prevented by lifestyle modification measures. The non-modifiable risk factors are increased age, male gender, and positive family history. In addition to these factors, few other factors such as increased levels of C Reactive Protein (CRP), hyperhomocysteinemia, metabolic

syndrome, increased lipoprotein A levels and elevated plasminogen activator inhibitor 1 levels are also linked to coronary artery disease, cerebrovascular disease, and peripheral vascular disease.

1.6. Influence of risk factors on endothelial dysfunction

Diabetes is a chronic metabolic disorder characterized by inappropriate hyperglycemia due to lack of or resistance to insulin, which contributes to endothelial cell dysfunction. In the diabetic state, there is an increased tendency for oxidative stress and high levels of oxidized lipoproteins, particularly low-density lipoprotein. In humans, diabetes induces a prothrombotic tendency as well as increased platelet aggregation, with tumor necrosis factor-1α (TNF-1α) implicated as a link between insulin resistance, diabetes, and endothelial dysfunction; a hypothesis has been advanced that insulin and/or insulin precursors may be atherogenic (Calles-Escandon and Cipolla, 2001a). Cholesterol is one of the well-established risk factors for coronary artery disease. Hypercholesterolemia and high levels of total cholesterol and low-density lipoprotein (LDL) cholesterol result in impaired endothelial function in both peripheral and coronary circulation. Studies have shown that cholesterol levels even in the normal range are inversely related to endothelium-dependent vasodilation, and this finding has important clinical implications. This suggests that lowering cholesterol levels even when it is within the normal range may improve the production and release of endothelium-dependent NO and hence improve endothelial function (Masumoto et al., 2002). Several *in vitro* studies have been conducted to examine the hypothesis that hyperglycemia and hypercholesterolemia modulate receptor expression and hence cause lipid accumulation. Similarly, *in vivo* studies have also been carried out to develop a useful animal model for mechanistic studies as well as to develop and test novel therapeutics for cardiovascular complications associated with diabetes and cholesterol. Systemic vascular factors such as smoking, hyperlipidemia, hyperglycemia and hypertension influence vascular shear stress, another risk factor in the progression of atherosclerotic plaque. This in turn modulates the vascular wall behavior in regions of disturbed blood flow. This

dynamic interaction between shear stress induced changes and systemic risk factors promotes atherosclerosis.

1.7. Effect of shear stress on the pathogenesis of atherosclerotic plaque

Blood vessels are constantly subjected to various hemodynamic forces. Hemodynamic forces are generated from the pulsatile nature of normal blood pressure and blood flow which can be characterized as shear stress, cyclic stretch, and hydrostatic pressure. As ECs form a monolayer in blood vessels, they come in direct contact with the blood flow and they bear most of the wall shear stress. The magnitude of shear stress in straight vessels can be estimated as being directly proportional to the viscosity of blood and inversely proportional to the third power of the inner radius of the vessel. Experimental measurements using different methods have shown that in humans the magnitude of shear stress ranges from 1 to 6 dynes/cm^2 in the venous system and from 10 to 70 dynes/cm^2 in arteries. An increase or decrease in shear stress plays critical roles in vascular remodeling and homeostasis. A disturbed or nonlaminar blood flow plays an important role in the pathogenesis of atherosclerotic plaque. It is seen that atherogenesis is promoted by decrease in shear stress (<5 dynes/cm^2) because it is associated with a reduction in several vascular wall functions including endothelial nitric oxide synthase (eNOS) production, vasodilatation and endothelial cell repair. These reductions in vascular wall functions, in turn, cause an increase in reactive oxygen species (ROS), endothelial permeability to lipoproteins, leucocyte adhesion, apoptosis, smooth muscle cell proliferation, and collagen deposition. It's a known fact that endothelial-dependent vasomotion is an independent predictor of cardiac events.

1.8. Role of inflammation in the progression of atherosclerosis

Endothelial dysfunction has been regarded as an initial step in the pathogenesis of atherosclerosis in the general population. Chronic inflammation is seen involved in the pathogenesis of accelerated endothelial dysfunction. Since atherosclerosis is considered as a chronic inflammatory disease, both innate and adaptive immune system is responsible for the progression of atherosclerosis. Both these systems are

regulated by a variety of cytokines (Ait-Oufella et al., 2011). Endothelial dysfunction is triggered by the accumulation of low-density lipoprotein (LDL) and other (Apo) B-containing lipoproteins. The accumulated LDL is then oxidized to become oxidized LDL (OxLDL). This elicits an inflammatory response in the ECs neighboring the LDL accumulation. The activated ECs then release cytokines and chemokines into the bloodstream as well as express cell adhesion molecules on their surface in order to recruit circulating monocytes and other immune cells to the site of oxLDL (Ramji and Davies, 2015). Cytokines are a group of low molecular weight proteins that are involved in cellular signaling pathways. Cytokines act on almost all cells involved in the pathogenesis of atherosclerosis. They participate in all steps of the process, from the early endothelial dysfunction to the late formation and disruption of a vulnerable plaque (Tedgui and Mallat, 2006). Depending on their effects on the formation and progression of the atherosclerotic plaque, cytokines can be broadly classified as pro or anti-atherogenic. Pro-atherogenic cytokines include tumor necrosis factor-1α (TNF-1α), interleukin (IL)-1, and IL-6. They are secreted by macrophages, lymphocytes, natural killer cells, and vascular smooth muscle cells. Contrary to TNF-1α, IL-1, and IL-6, transforming growth factor-β (TGF-β), IL-10, and IL-35 are known as anti-inflammatory and atheroprotective cytokines. Chemokines are a sub-group of cytokines that are capable of attracting cells to the desired location. In atherosclerosis development, chemokines play an important role in the recruitment of circulating monocytes and other immune cells to the site of oxLDL retention (McLaren et al., 2011). Monocyte chemoattractant protein-1 (MCP-1) is a low molecular weight (9-15KDa) chemotactic cytokine that plays an important role in the inflammatory process associated with the development and progression of atherosclerosis. MCP-1 is expressed by vascular smooth muscle cells (SMC) and endothelial cells (EC) at the site of vessel wall injury. MCP-1, in interaction with several cytokines and cell adhesion molecules, mediates the trans endothelial migration of monocytes to the sub endothelial layer *via* cysteine-cysteine motif chemokine receptor-2 (CCR-2) receptor (Hartung et al., 2004). The recruited monocytes then express MCP-1 by themselves, causing an influx of mononuclear cells. The monocytes then differentiate to macrophages, express matrix

metalloproteinases (MMPs), transform to foam cells by digesting oxidized LDL *via* expression of scavenger receptors, and form a necrotic core by undergoing apoptosis or necrosis. Since the receptor for MCP-1 is only expressed by infiltrating monocytes, the detection of MCP-1 receptors reveals the extent of inflammation in atherosclerotic plaque. Therefore targeting cytokines and their signaling pathways represents a promising therapeutic strategy.

1.9. Etiology of CVD- Attempts to study

Several human and animal studies have been conducted to analyze the proatherogenic role of hyperglycemia in vascular cells. Though the results were not as strong as for insulin resistance, there is suggestive evidence that high glucose is atherogenic, particularly at the level of the arterial endothelium. In a study conducted in human postmortem samples (Burke et al., 2004) lesions from patients with diabetes have a higher macrophage content than lesions from subjects without diabetes, in a manner that correlates with glycated hemoglobin levels rather than lipid levels. There are studies that correlate a relationship between suboptimal glycemic control and cardiovascular events and suggest a coronary artery disease (CAD) benefit of glucose-lowering in patients with type 2 diabetes (Mazzone, 2010). Studies have been conducted in animal models too, to delineate the effects of diabetes on atherosclerosis. But the results are often complicated by the presence of hyperlipidemia that overrides the effects of diabetes on atherosclerosis. In some models, diabetes increases blood glucose levels, without an increase in plasma lipids and in such models, a proatherogenic effect of diabetes can be observed (Bornfeldt and Tabas, 2011). The commonly used models are $Ldlr^{-/-}$ or $Apoe^{-/-}$ mice and they are injected with streptozotocin, a toxin that primarily targets beta-cells. There are many *in vitro* studies that examined the direct effect of high glucose on ECs, but there is a dearth in *in vivo* studies. But the results from the *in vivo* studies done both in humans and animals suggest that the endothelium is particularly sensitive to changes in glucose concentrations.

1.10. Definition of the problem

The relationship between hyperglycemia and endothelial dysfunction is still unclear. As mentioned above, atherosclerosis is associated with several cardiovascular risk factors and it is possible that hyperglycemia acts synergistically with other CAD risk factors to promote atherogenesis. Currently available clinical data have not yet proven definitively the impact of hyperglycemia on endothelial dysfunction that precedes CAD. In studies in diabetic animals with severe dyslipidemia, it is observed that lesion initiation is primarily driven by hyperlipidemia, and this overshadows the atherogenic effect of other risk factors, such as hyperglycemia or advanced glycation end products. This is probably because glucose and lipids use similar mechanisms to induce lesion formation. In humans, cholesterol levels greater than 600 mg/dL are rarely seen even in individuals with familial hypercholesterolemia. Though this is an interesting finding, its clinical significance is still unclear. Importantly, lesion initiation does not necessarily result in clinical symptoms, and it has been suggested that macrophage-rich fatty streak lesions regress unless a fibrous cap of smooth muscle exists to encapsulate the macrophages (Cho et al., 2005; Virmani et al., 2000). Many animal studies are restricted to these early lesions. The knowledge of the major characteristics of human atherosclerosis is based largely on studies of coronary artery lesions, with significant contributions from studies of closely similar lesions in the carotid arteries and aorta. As mentioned earlier, studies of experimental atherosclerosis are usually conducted in animal models, particularly genetically manipulated mice. The problem associated with the use of animal models for studying atherosclerosis is that the lesions in animal models do not resemble those seen in human atherosclerosis. In addition to this, the pattern of blood flow in animals and humans are different to a large extent. No high-risk (vulnerable) plaque can be created and the distribution and composition of lipoproteins are very different from that in humans. All the *in vitro* studies that have been explained in the literature has been carried out using EC monolayer culture.

1.11. Gaps and Goal

Despite the large volume of work, many such studies have not sufficiently delineated the role of individual risk factors and their effect on initiating pathological changes to endothelial cells.

The major reasons are lack of using functional human primary cells in 2-D culture systems, no shear stress applied on cells during cultures, and also no 3-D culture that facilitate 2 major vascular cells (SMC &EC) that are known to be involved in development of atherosclerosis have not been used in a biomimetic environment. In *in vivo* animal studies hemodynamic forces are very low as compared to that in human artery and response to risk factors in animals and human are not comparable.

- None of the reported *in vitro* studies have simultaneously applied shear stress and risk factors to evaluate changes in endothelial cells.
- None of the reported *in vitro* studies have used a biomimetic blood vessel with functional intimal and medial cell layers
- None of the reported studies quantified molecular level changes in endothelial cells as an effect of individual risk factors.

Therefore, the major goal of the current study is to address the identified gaps by developing a culture model and validate the suitability of the model by demonstrating quantifiable molecular level changes in endothelial cells upon exposure to selected most commonly known life-style related risk factors.

1.12. Approach to the problem

1.12.1. Design of 3-D culture

When designing a 3-D culture system mimicking blood vessel, an endothelial cell layer is essential, which may be exposed to risk factors. Also underneath endothelial layer, smooth muscle layer with ECM is required to maintain EC in the functional phenotype. Another requirement is opportunity to simultaneously apply shear stress and risk factors on endothelial layer. To meet all these requirements, a functional tissue-engineered vascular graft (TEVG) may be constructed on a pliable conduit

fabricated using biodegradable polymer. The selected conduit shall be of small diameter with smooth and porous lumen for growing endothelial monolayer and fibrous outer layer for growing multilayers of SMC to construct a biomimetic blood vessel. Human EC and SMC could be employed to generate luminal and underlying medial layer, respectively and shear stress could be regulated by adjusting flow rate of circulating medium using a peristaltic pump. This model can allow exposure of TEVG lumen to high glucose and /or cholesterol in the presence or absence of inflammatory cytokine. Thus the model can be used to study the response to risk factors and small changes to endothelial cell marker molecules.

1.12.2. Tissue engineering technology

Tissue engineering has been projected as a method for artificially creating blood vessels for implantation. Tissue engineering incorporates cells into a biodegradable scaffold. Components of a tissue-engineered blood vessel include:

- Polymeric Scaffold- which gives strength to the graft
- Use of endothelial cells and smooth muscle cells-which gives functionality to the graft. Most widely accepted cells are derived from multipotent stem cells.
- The use of bioreactor to apply shear stress during TEVG construction- which permits mechano-stimulation and cellular regulation due to hemodynamic forces.

Stem cells are a viable cell source due to their inherent ability to self-renew and proliferate rapidly. Stem cells from human tissue can be predifferentiated to endothelial progenitor cells (EPCs) and smooth muscle progenitor cells (SMPCs) as human cells for fabricating tissue engineered vascular graft. Mesenchymal stem cells (MSCs) have the ability to self-renew and enable rapid expansion. MSCs can be isolated from almost every organ, but there are slight variations in phenotype, mainly due to the influence of the tissue from which they are isolated (da Silva Meirelles et al., 2006). Adipose tissue provides a readily-available and relatively easily obtainable source of mesenchymal stem cells. The use of adipose derived mesenchymal stem cells (ADMSCs) in TEVG is nascent, because these cells have shown the plasticity

to differentiate into both endothelial cells and smooth muscle cells. Various studies have reported that the retention of ADMSC differentiated ECs have increased when the cells are seeded on fibronectin and exposed to shear (McIlhenny et al., 2010a). Similarly, ADMSCs have been shown to differentiate into SMCs when treated with TGF-β1 and bone morphogenetic protein 4 (BMP4). The lineage committed, proliferating EPC & SMPC may be used for functional TEVG construction. Established methods and materials such as biodegradable bilayered conduit fabricated from poly Ɛ-caprolactone (PCL) and coated with fibrin could be used to construct TEVG (Ragaseema et al., 2014). Based on these knowledge, the major goal of the study was defined as fabrication of TEVG using human ADMSC derived EPC and SMPC seeded on inner and outer layers of bilayered fibrin coated PCL conduit, respectively, and applying shear stress using a peristaltic pump based bioreactor. Another objective of the study was to prove that the constructed TEVG has functional EC in the lumen that are sensitive to components of culture medium that can be quantified using real time polymerase chain reaction (RT-PCR) of EC specific marker proteins. Based on the goal of the study, a hypothesis was developed and specific objectives were defined to prove the hypothesis.

1.13. Hypothesis

Biomimetic functional blood vessel may be constructed by growing human ADMSC derived EPCs and SMPCs on suitable scaffold under shear stress, for use as an *in vitro* model system to study the subtle changes to EC phenotype upon dynamic exposure to risk factors.

1.14. Objectives

To prove the above hypothesis, a study was designed with three major objectives. Each major objective was subdivided into specific aims as follows:

1. To establish conditions for developing human vascular cells from adipose derived mesenchymal stem cells (hADMSCs) for *in vitro* construction of small diameter blood vessels

- To isolate human adipose tissue, culture expand and characterize
- To standardize protocols for differentiation of hADMSCs to endothelial progenitor cells (EPC)
- To standardize protocols for differentiation of hADMSCs to smooth muscle progenitor cells (SMPC)
- To characterize EPC and SMPC using specific markers at mRNA level and protein level

2. To construct tissue engineered vascular graft (TEVG) on an established fibrin coated PCL scaffold to study the influence of SMPC and shear stress on vessel stability and EC marker expressions, respectively

- To establish growth of SMPC on electrospun ablumen of PCL-fibrin hybrid scaffold
- To establish ECM deposition by SMPC on the graft
- To establish effect of SMPC growth on the tensile properties of the graft
- To seed EPC in the lumen of SMPC grown graft and culture under dynamic condition
- To establish EPC function by assessing NO release

3. To establish the suitability of artificially constructed human cell grown blood vessel to study the effect of risk factors on the expressions of molecular markers in the endothelial cell lining the lumen by circulating high glucose, high cholesterol in the presence and absence of TNF-1α containing medium

- To analyze effect of high concentration of glucose and cholesterol in circulating medium on NO release
- To demonstrate independent and combinatorial effect of high glucose/cholesterol/TNF-1α on the regulation of relative gene expressions of specific markers in EPC:
    - pro/anti thrombotic molecules (vWF/eNOS)
    - pro/anti-fibrinolytic molecules (tPA/PAI)
    - adhesion molecules (CD 31,VCAM1)
    - pro inflammatory molecule (MCP-1)

1.15. The rationale of the study

The progression of an atherosclerotic plaque is a complicated process and modern technological advances in the field of stem cell biology and tissue engineering has not been explored to develop a biomimetic test model. This study considered that functional human EC and SMC could be sensitive to risk factors if such components are circulated in the lumen of tissue-engineered vascular graft. Once it is found that ECs respond to risk factors and the effects are quantifiable, the model may be used for conducting many other studies in future to understand the steps involved in initiation of endothelial dysfunction at molecular and cellular level.

CHAPTER 2

2. Literature review

The chapter describes in detail about the topics related to the field of research. A systematic account is given, starting with atherosclerosis and the role played by endothelium in the development of an atherosclerotic lesion. Elements of endothelial function, dysfunction and the effect of risk factors on endothelial physiology are included. All the risk factors are described, among them the risk factors which are of interest for the research namely hyperglycemia and hyperlipidemia are elaborated. The ways to study the endothelial dysfunction, the disadvantages of the currently available techniques to study endothelial dysfunction and the role of shear stress in endothelial functions are explained. To develop a tissue engineered graft which can be used as a model to study the endothelial dysfunction, recent advancements in the construction of a tissue engineered vascular graft with respect to the polymers and the cells are also explained. Various strategies adopted for the differentiation of stem cells to vascular cells are critically evaluated to understand the part played by growth factors and physical stimuli.

2.1. Atherosclerosis

The morbidity and mortality from cardiovascular diseases (CVD) has decreased in the last two decades, at least in the western world. But it is expected that this will reverse in the future because of a global increase in diabetes and obesity along with an alarming rise in CVD in developing countries in part due to acquisition of a sedentary lifestyle. Atherosclerosis is the primary cause of cardiovascular related events and the associated mortality and morbidity. Atherosclerosis can be described as a chronic inflammatory disease of the large and medium arteries. It is initiated by risk factors such as high plasma cholesterol levels, hyperglycemia and hypertension. Atherosclerosis develops during the lifespan of an individual and progresses through a number of steps and is characterized by the presence of atherosclerotic lesions (atheroma). They are asymmetric focal thickenings of the innermost layer of the

artery, the intima. Atheroma consists of blood-borne inflammatory and immune cells as well as endothelial and smooth-muscle cells. A fatty streak precedes an atheroma. A fatty streak is caused by accumulation of lipid-laden cells beneath the endothelium. The accumulation of plaque within vessel wall alters both the structure and function of arteries, increasing vessel wall stiffness. The elastic nature of medium and large sized arteries are known to stiffen due to healthy aging (Gepner et al., 2014),or due to atherosclerosis, diabetes, hypertension and obesity. The accumulation of extracellular lipids form lipid pools within the intima, disrupting the cellular structure of the artery, breaking elastic fibers and causing thickening of the vessel wall. As a result of this intimal disorganization, some lesions develop new connective fibrous tissue, thereby developing the 'fibrous cap' of an atherosclerotic plaque. Fatty streaks, the earliest sign of atherosclerosis, have been observed in fetal aortas and in children above 3 years of age (Hong, 2010). Recent studies indicate that deleterious alterations of endothelial physiology, also known as endothelial dysfunction, represents a key early step in the development of atherosclerosis. Endothelial dysfunction is considered as a surrogate marker of an unfavorable cardiovascular prognosis (Reriani et al., 2010). Several risk factors are associated with atherosclerosis. Though the association is well documented, the mechanism by which these risk factors induce lesion formation and lead to atherosclerosis is not entirely defined. Given its strategic location and biological properties, the endothelial cell layer is considered as the 'missing link' between any given risk factor and its detrimental vascular effects.

2.2. The endothelium

The endothelium is a cell layer lining the blood luminal surface of vessels. The term endothelium was coined in 1865 by the anatomist Wilhelm His. The endothelium was once considered as a "cellophane wrapper" of the blood vessels, with no other specific functions other than affording selective permeability to water and electrolytes (Rajendran et al., 2013). But enormous advances since 1980s revealed the distinct and unique functions of vascular endothelial cells (EC). These functions

include fluid filtration, such as in the glomeruli of the kidneys, blood vessel tone, hemostasis, neutrophil recruitment, and hormone trafficking. ECs are absent in invertebrates, cephalochordates, and tunicates but are present in the three major groups of extant vertebrates: hagfish, lampreys, and jawed vertebrates. Endothelium can be considered as a tissue structure conserved during the evolution of vertebrate. Developmentally, endothelium arises from mesoderm via the differentiation of hemangioblasts and/or angioblasts. But other cell lineages may also transdifferentiate into ECs and ECs into other lineages (Favero et al., 2014). Endothelium is an extremely complex tissue from the metabolic point of view. The EC surface in an adult human is composed approximately of 1 to 6×10^{13} cells, weighs approximately 1 kg, and covers a surface area of about 1 to $7m^2$. Endothelium is lined with several membrane bound receptors as well as specific junctional proteins and receptors that govern cell-cell and cell-matrix interactions. The endothelium is involved in the control of thrombosis and thrombolysis, platelet and leukocyte interaction with the vessel wall, the regulation of vascular tone and growth of blood vessels.

Endothelial cells have three surfaces: cohesive, adhesive, and luminal. ECs are joined with each other at the cohesive surface and this facilitates transport processes and consists of specialized intercellular junctions: gap, tight or adherent junctions and syndesmos. The adhesive surface of ECs adheres to basal lamina. The luminal side of the vascular endothelium consists of molecules and specific binding proteins to regulate the trafficking of circulating blood cells. ECs possess various contractile proteins, such as actin, myosin and tropomyosin, which allow motor activities. When shear stress as well as blood flow increases, ECs are flattened and aligned in the direction of the flow; whereas when blood flow decreases the ECs increase their volume losing the alignment and they look like cobble stones. The ECs are sensitive to changes of the intravascular tension and may increase their stiffness with an increase in the intravascular pressure. Quiescent ECs have an active antithrombotic surface that facilitates the transit of the plasma and cellular constituents throughout the vasculature, thereby regulating the blood flow. Perturbations occurring at sites of inflammation or high hydrodynamic shear stress disrupt these activities and induce

ECs to create a prothrombotic and antifibrinolytic microenvironment. Endothelium is also involved in secretion and uptake of vasoactive substances that act in a paracrine manner to constrict and dilate specific vascular beds in response to stimuli such as endotoxin.

2.2.1. Endothelial functions in physiology

Endothelium plays an important role in many physiological functions, including the control of vascular tone, blood cell trafficking, innate and adaptive immunity, and hemostasis. Endothelium produces a host of vasoactive factors, such as vasodilators and vasoconstrictors, procoagulants and anticoagulants, inflammatory and anti-inflammatory factors, fibrinolytic and antifibrinolytic, oxidizing and antioxidizing, and other factors. Endothelial cells provide a non-thrombogenic inner layer of the vascular wall, maintaining blood fluidity and providing a proper haemostatic balance. Endothelial cells prevent thrombosis by means of different anti-coagulant and anti-platelet mechanisms and they have a key role in the regulation of coagulation. The endothelium has the capacity to maintain blood in a fluid state, limiting clot formation. When there is an injury in the vascular wall, the endothelium serves as a borderline between the coagulation factors circulating in the blood and the primary initiator of coagulation within the vascular wall (Pries and Kuebler, 2006). One mechanism by which endothelial cells control the coagulation is by regulating the expression of binding sites for anticoagulant and procoagulant factors on the cell surface. In the quiescent state, endothelial cells maintain blood fluidity by activating numerous anticoagulant pathways, including the protein C/protein S pathway. ECs also express platelet selectin (P-selectin), which is stored intracellularly in Weibel-Palade bodies. It is expressed preferentially in post capillary venules. Endothelial cells produce endothelial nitric oxide synthase (eNOS) whose expression increases after higher shear stress. eNOS is responsible for inducing the parallel sustained increase in the production of nitric oxide (NO) (Uematsu et al., 1995).NO is the key endothelium-derived relaxing factor that plays a pivotal role in the maintenance of vascular tone and reactivity. In addition to being the main

determinant of basal vascular smooth muscle tone, NO also prevents the actions of potent endothelium-derived contracting factors, such as angiotensin II and endothelin-1. Angiotensin II is not only a vasoconstrictor but is also a pro-oxidant. It stimulates the production of endothelin. Endothelin and angiotensin II promote proliferation of smooth muscle cells and thereby contribute to the formation of plaque. NO inhibits platelet and white cell activation and maintains the vascular smooth muscle cells in a non-proliferative state. Endothelial heterogeneity helps in maintaining adequate homeostasis in different organs and parts of the vascular tree. ECs express tissue factor pathway inhibitor, heparin, thrombomodulin, endothelial protein C receptor (EPCR), tissue-type plasminogen activator (tPA), ecto-ADPase, prostacyclin and NO, as anti-coagulant factors, and synthesize tissue factor, plasminogen activator inhibitor-1(PAI-1), von Willebrand factor (vWF), and protease activated receptors (PARs), as procoagulant factors (Aird, 2007).

Endothelial cells regulate the transport of liquids across the semi permeable vascular endothelial barrier. ECs also function as a protective biocompatible barrier between all tissues and the circulating blood, thereby modulating the bidirectional passage of macromolecules and blood gases to and from tissues and blood. Even in the absence of any apparent morphological damage, platelets interact with endothelium. Platelets stick to an intact endothelium inflamed by different stimuli, such as infection, mechanic alteration or ischemia and reperfusion, or to endothelium located at lesion-prone sites, such as the carotid artery bifurcation. The recruitment of platelets and leukocytes at sites of vascular injury is a very rapid response. This is mediated by Weibel-Palade bodies and their major constituents, the largest multimers of von Willebrand factor, and P-selectin, which are the most active promoters of platelet and leukocyte adhesion. Certain substances are released by endothelial cells that can evoke a hyper polarization of smooth muscle cells. As discussed above, endothelial cells release several contraction-inducing factors such as endothelin, thromboxane A2, angiotensin II, superoxide, and unidentified endothelium-derived contraction-inducing factors under certain conditions. Endothelial cells are also a source of growth inhibitors and promoters, such as heparin and heparin sulphates, platelet-

derived growth factor, and thrombospondin. The endothelium is also involved in blood vessel formation. Vascular endothelial growth factor (VEGF) is a growth factor specific for the vascular endothelium. A family of growth factors known as the angiopoietins (Ang) and particular members of the very large ephrin family have also unique effects on the endothelium. Recent insights have shown that VEGF is the most critical driver of vascular formation, as it is required to initiate the formation of immature vessels by vasculogenesis or angiogenic sprouting. Ang1 and ephrinB2 are subsequently required for further remodeling and maturation of this initially immature vasculature, as endothelial cells integrate with supporting cells such as smooth muscle cells and pericytes. Following vessel maturation, Ang1 is important for maintaining the quiescence and stability of the mature vasculature. Dysfunction of the endothelium- dependent regulatory systems may play a role in cardiovascular diseases, such as hypertension and atherosclerosis (Barton et al., 2012). Endothelial cells play a key role in microcirculatory diseases such as thrombotic microangiopathies and diffuse intravascular coagulation. Endothelial dysfunction not only precedes atherogenesis but may also predispose to arterial thrombosis.

2.2.2. Endothelial dysfunction

Basic mechanisms involved in atherogenesis indicate that a deleterious alteration in endothelial physiology is one of the early steps in atherosclerosis. This deleterious alteration in endothelial physiology is known as endothelial dysfunction. When endothelial dysfunction occurs there will be a shift in the actions of endothelium toward a reduced vasodilation, proinflammatory and prothrombotic state. Usually, endothelial cells become activated according to environmental needs and act accordingly to stop the hemorrhage and repair injured vascular tissue. Endothelial dysfunction is characterized by impaired endothelium-dependent vasorelaxation in response to endothelium-dependent agonists such as acetylcholine and bradykinin or due to an increase in shear stress in conditions such as flow mediated dilation. EC dysfunction cannot be restricted anatomically to a single organ or limited in a singular disease mechanism (Aird, 2008), because the endothelium actively

maintains approximately 60,000 miles of blood vessels in the human body. Endothelial dysfunction disrupts the mechanism of vascular homeostasis regulation. This causes the vessel wall sensitive to vasoconstriction, leukocyte adhesion, platelet activation, oxidative stress, thrombosis, coagulation, and inflammation. Though the mechanisms leading to endothelial dysfunction are multiple, a reduction in NO bioavailability is largely observed in many cardiovascular disorders. Reduced NO bioavailability can be due to decreased L-arginine availability, increased asymmetric dimethyl arginine(ADMA), altered interaction with hsp90, phosphorylation of eNOS and increased NO scavenging by excessive reactive oxygen species(ROS) generated by NADPH and xanthine oxidases (Li et al., 2014).

Endothelium is highly active and constantly sensing and responding to alterations of the local extracellular environment. It is a fact that endothelium may be activated without being dysfunctional and may also contribute to disease initiation and progression. The transition between EC function and dysfunction is not always clear. Several studies have shown a correlation between endothelial dysfunction and the presence of coronary risk factors in persons with no clinical evidence of coronary disease. A disruption in the balance of nitric oxide damages the endothelium leaving it overly permeable, allowing toxins to pass into body tissues. The presence of free radicals in the body may disrupt NO balance. The human body has an adequate supply of antioxidants obtained from various foods to neutralize these free radicals; but if the body is depleted of these antioxidants, or if there are too many coexistent factors, injury to the endothelium and a change in the balance of NO may occur. The factors that contribute to the presence of free radicals in the body include obesity, smoking, sleep deprivation, acute microbial infections, high glucose intake, and exposure to metals and air pollutants. Endothelial dysfunction is associated with most forms of cardiovascular diseases such as hypertension, coronary artery disease, chronic heart failure, peripheral vascular disease, diabetes, chronic kidney failure, and severe viral infections. A study conducted by Ludmer et al in humans, using the acetyl choline test, is the first evidence that showed the presence of impaired endothelium dependent vasodilation in atherosclerosis (Ludmer et al., 1986). The

investigators observed a paradoxical constriction in the arteries of patients with mild coronary artery disease (CAD), as well as in those with advanced CAD. This shows that endothelial dysfunction is present in the early stage of atherosclerosis. The risk factors associated with the development of atherosclerosis are also associated with endothelial dysfunction. The presence of remnant lipoproteins, another proposed coronary risk factor, is also associated with impaired endothelium dependent vasodilation. Lipoprotein (a) is considered as an independent risk factor for coronary heart disease (CHD), stroke, and peripheral atherosclerosis. But in a study by Schlaich et al lipoprotein (a) did not appear to be related to impaired endothelium-dependent vasodilation (Schlaich et al., 1998). However, in subjects with elevated lipoprotein (a) levels, the production and release of basal NO was enhanced. This suggests a compensatory response to the atherosclerotic effects of lipoprotein (a).

2.2.3. Effect of risk factors on endothelial dysfunction

The traditional risk factors associated with atherosclerosis are hyperlipidemic states, diabetes mellitus, smoking and hypertension. Besides the traditional risk factors, hyperhomocysteinemia is also found to be linked to coronary artery disease, cerebrovascular disease and peripheral vascular disease. Prolonged exposure to the sulphur-containing amino acid homocysteine promotes atherogenesis by causing endothelial damage which in turn has mitogenic effects on vascular SMCs. Hyperfibrinogenemia is also considered as a risk factor for atherosclerosis and it is evident from an association between high fibrinogen and a number of other risk factors for ischemic heart disease. Genetic alterations also promote the development of atherosclerotic disease. Familial predilection is a known risk factor besides the other predisposing diseases such as polygenic disease and combined hyperlipidemia. However, any one of these alone is insufficient to produce an atherosclerotic lesion. Among the several cardiovascular risk factors, elevated plasma cholesterol level is considered to be a unique risk factor. Elevated plasma cholesterol is sufficient to drive the development of atherosclerosis, even in the absence of other known risk factors (Glass and Witztum, 2001). If all adults had plasma cholesterol levels <150 mg/dl, symptomatic disease would be rare. The other risk factors of atherosclerosis

such as male gender and inflammatory markers (e.g., C reactive protein, cytokines, and so on), appear to accelerate a disease driven by atherogenic lipoproteins, namely low-density lipoprotein (LDL). The mechanism by which the risk factors accelerate the process of atherogenesis is unknown, but they may either increase the atherogenicity of LDL (e.g., particle size, number, and composition) or increase the susceptibility of the arterial wall (e.g., permeability, glycation, inflammation, and so on). These risk factors directly contribute to endothelial dysfunction also.

Conversely, alcohol, exercise, and high-density lipoprotein (HDL) and its major Apo lipoprotein, apoA-I are known as protective factors. They confer protection against diseases caused by atherothrombosis. Among other things, HDL/apoA-I prevents the atherogenic modifications of LDL and promotes “reverse cholesterol transport”. This slows plaque progression and may induce rapid regression, documented in experimental studies and suggested by serial intravascular ultrasound examinations of patients with acute coronary syndrome (Falk, 2006).

2.2.3.1. Endothelial cell dysfunction and hyperglycemia

Diabetes, characterized by persistent elevation of blood glucose levels (hyperglycemia), occurs due to inadequate production of insulin (type 1 diabetes) or resistance to endogenous insulin. This is usually associated with metabolic syndrome and obesity (type 2 diabetes). Patients with diabetes have impaired endothelium dependent vasodilation. This is partly due to the frequent association of the disease with other cardiovascular risk factors, including hypertension, obesity, and dyslipidemia. Diabetic as well as obese patients usually consume a high-calorie diet rich in macronutrients (Rajendran et al., 2013). This increases the production of reactive oxygen species (ROS) and promotes inflammation in systemic and cerebral blood vessels. The predominant vascular sources of ROS are the superoxide-producing enzyme NADPH oxidase, xanthine oxidase, and mitochondrial enzymes, as well as the uncoupling of nitric oxide synthase (NOS). In the uncoupled state, NOS generates superoxide instead of NO. Thus, oxidative stress and vascular inflammation are major pathways through which risk factors exert their deleterious

effects on blood vessels. In type 1 diabetes, endothelial dysfunction is predominantly triggered by the metabolic changes due to hyperglycemia and microvascular complications. This occurs prevalently at retinal and kidney levels. In type 2 diabetes, the link between endothelial dysfunction and diabetes is more complex. In this case endothelial dysfunction starts well before the onset of diabetes (Favero et al., 2014)

In a disease condition, such as in diabetes, when advanced glycosylation end products, which are proteins modified by glucose, accumulate in the vasculature at a rapid rate; changes may occur in endothelial coagulant properties. This indicates the potential relevance of these mechanisms to diabetic vascular disease. Chronic hyperglycemia leads to nonenzymatic glycation of proteins and macromolecules. Diabetic state is characterized by an increased tendency for oxidative stress and high levels of oxidized lipoproteins, especially the small, dense low-density lipoprotein. When an inflammation or infection occurs, platelets adhere to and leucocytes roll up on the endothelium. This represents the initial stage of a multistep process leading to extravasation of white blood cells to sites of inflammation or infection, to platelet-leukocyte interaction and aggregation on a thrombogenic surface, and finally to vascular occlusion. Hyperglycemia causes glycosylation of proteins and phospholipids, thus increasing intracellular oxidative stress. Nonenzymatic reactive products, known as Maillard or browning reaction, glucose-derived Schiff base, and Amadori products, form chemically reversible early glycosylation products. They subsequently rearrange to form more stable products and continue undergoing complex series of chemical rearrangements to form advanced glycosylation end products (AGEs). Once formed, AGEs are stable and virtually irreversible. AGEs generate ROS with subsequent increased vessel oxidative damage. A variety of endothelial functions is compromised in diabetic conditions. This includes regulation of vascular tone and organ perfusion, inhibition of inflammation, trans endothelial transport of blood solutes, prevention of coagulation and initiation of angiogenesis (Esper et al., 2006). Recent studies have observed that hyperglycemia causes mitochondrial fragmentation and altered mitochondrial dynamics, associated with increase in mitochondrial ROS production. This impairment could be the reason for

the endothelial dysfunction observed in diabetes; however, endothelial dysfunction is often evident prior to a significant elevation in plasma glucose levels and can be induced by factors other than hyperglycemia. Hyperglycemia may lead to intracellular changes in the redox state resulting in depletion of the cellular NADPH pool. Over expression of growth factors has also been implicated in diabetes with proliferation of endothelial cells and vascular smooth muscle, thereby promoting neovascularization. The high levels of fatty acids and hyperglycemia have also been shown to induce an increased level of oxidation of phospholipids as well as proteins. In humans it is associated with a prothrombotic tendency as well as increased platelet aggregation.

2.2.3.2. Endothelial cell dysfunction and hyperlipidemia

Cholesterol is considered to be one of the well-established risk factors for premature coronary artery disease. Cholesterol levels and coronary artery disease risk have a strong and linear relationship. Hypercholesterolemia and high levels of total cholesterol and low-density lipoprotein (LDL) cholesterol result in impaired endothelial function in both peripheral and coronary circulation (Hadi et al., 2005). Studies have shown that cholesterol levels even in the normal range may be inversely related to endothelium dependent vasodilation. This suggests that lowering cholesterol levels even when it is within the normal range may improve the production and release of endothelium-dependent NO and hence improve endothelial function. This idea is supported by recent reports that lowering cholesterol levels enhances endothelium dependent vasodilation not only in subjects with massively elevated cholesterol levels but also in those with normal cholesterol levels. Lowering of average cholesterol levels in patients with documented coronary artery disease leads to decreased rates of myocardial infarction. This protective effect may in part be due to improvement in endothelial function (Uğurel et al., 2016). In addition to lipid-lowering therapy, administration of tetrahydrobiopterin, an essential cofactor for NO production, could restore NO activity in familial hypercholesterolemia. Lowering low-density lipoproteins (LDL) therapy using statins is a completely

established strategy to reduce the risk of CAD. Studies revealed that high-density lipoproteins (HDL) or LDL cholesterol affect endothelial cells and regulate the expression of cellular adhesion molecules such as intracellular adhesion molecule (ICAM) and monocyte chemoattractant protein-1 (MCP-1). A huge amount of evidence suggests that oxidized low density lipoprotein (ox-LDL) contributes to atherogenesis. Endothelial cells exposed to ox-LDL secrete adhesion molecules, chemoattractant proteins, and colony-stimulating factors that enhance the infiltration, proliferation, and accumulation of monocytes/macrophages in the arterial wall. When circulating ox-LDL is elevated, it represents an independent risk factor for acute cardiac events. Normal-HDL includes the anti-oxidant enzyme PON1, which suppresses the formation of oxidized lipids and lipoproteins, such as malonidialdehyde (MDA). Dysfunctional-HDL has a decreased PON1 enzyme activity that potentially causes a greater production of MDA, which activates the lectin-like oxidized LDL receptor-1 (LOX-1).The LOX-1 is an oxLDL receptor expressed in vascular endothelium, and a multiligand receptor implicated in endothelial dysfunction and atherosclerosis (Kratzer et al., 2014).

2.2.4. Shear stress on endothelial cell dysfunction

Hemodynamic forces, mainly shear stress, have a powerful influence on endothelial phenotype and function. Shear stress is the mechanical force created when a blood flow acts on a surface of the endothelium (Zhang et al., 2013).But there is no clear consensus on how endothelial cells sense shear. Several theories have been proposed to show that multiple endothelial cell signal transduction pathways are activated when exposed to shear stress *in vitro*. These theories include ion channel activation, caveolae-mediated regulation of Ca^{2+} G-protein-coupled receptor activation, tyrosine kinase receptor activation, adhesive protein activation, glycocalyx elongation, and bending of primary cilia (Johnson et al., 2011). Certain types of hemodynamic forces are essential for physiological functions of the EC under normal conditions. On the other hand certain types can induce endothelial dysfunction by adversely modulating EC signaling and gene expression, thus contributing to the development of vascular

pathologies in concert with the risk factors that act on the entire arterial system (e.g., genetics, biochemical factors, living habits, etc.) (Chiu and Chien, 2011). The earliest lesions of atherosclerosis characteristically develop in a nonrandom pattern, i.e., preferentially at arterial branches and curvatures (or bends), where the local flow is disturbed. This suggests the possible role of hemodynamic forces in endothelial dysfunction. The disturbed flow pattern includes recirculation eddies and changes in direction with space (flow separation and reattachment) and time (reciprocating flow). Atherosclerosis is considered as a geometrically focal disease. Though several risk factors are associated with atherosclerosis, it's mainly localized to the outer edges of blood vessel bifurcations, where blood flow is slow resulting in a weak hemodynamic shear stress. The associated low shear stress induces a sustained activation of a number of atherogenic genes in ECs, e.g., the monocyte chemotactic protein-1 (MCP-1) that induces monocyte infiltration into the arterial wall and platelet-derived growth factors (PDGFs) that result in EC turnover and SMC migration into the sub intimal space. In contrast, the straight part of the artery, which is generally spared from atherosclerotic lesions, is exposed to sustained laminar blood flow and high shear stress. This results in the associated down regulation of atherogenic genes (e.g., MCP-1 and PDGF-BB) and upregulation of antioxidant and growth-arrest genes in ECs.

Endothelial cells are subjected to different forms and magnitudes of shear stress (SS) *in vivo* depending on the type of blood flow. Laminar blood flow which is a steady undisturbed blood flow creates a constant shear stress along the EC surface. As mentioned earlier, ECs exposed to laminar blood flow exhibit an anti-atherogenic phenotype with the expression of anti-atherogenic genes. The most important anti-atherogenic molecule produced *in vivo* is nitric oxide. Production of NO is catalyzed by eNOS in response to shear stress, insulin and acetylcholine. The anti-atherogenic environment due to NO production inhibits several aspects of atherogenesis such as smooth muscle cell proliferation and migration, platelet aggregation and leukocyte adhesion to the vascular wall, improves fibrinolysis, and regulates permeability and vasomotor tone, as well as act as an antioxidant under situations of increased

superoxide anion concentrations. NO inhibits platelet aggregation by increasing platelet cyclic GMP and through the *S*-nitrosylation of platelet proteins. In addition to this, tissue plasminogen activator, thrombomodulin, and cylcooxygenase-2 gene expression are up-regulated and plasminogen activator inhibitor type-1, VCAM-1 and endothelin-1 are down-regulated in response to steady laminar flow. But on the other hand, in regions of bifurcations in the arterial tree, laminar blood flow and shear stress is modified into low shear stress and oscillatory flow patterns. Such areas of low and oscillatory shear stress are prone to atherosclerotic lesions. Oscillatory flow induces the expression of ICAM-1, E-selectin, and endothelin-1, increase monocyte-EC attachment through NF-κB, as well as increase EC macromolecule uptake. Moreover oscillatory shear stress induces oxidative stress through the increase of intracellular superoxide radicals and NADH oxidase activity. The current state of research shows that the hemodynamic forces have a direct influence on endothelial biology and pathobiology and the role of disturbed flow pattern in endothelial function and dysfunction.

2.3. Studies on endothelial dysfunction

In the coronary arteries, impairment of endothelial function occurs early in the course of atherosclerosis, with respect to systemic risk factors and abnormal hemodynamic shear stresses (Chhabra, 2009). Extensive literature survey documents that endothelial dysfunction is associated with almost every condition predisposing to atherosclerosis and cardiovascular disease (Brunner et al., 2005). There are many studies correlating endothelial dysfunction with cardiovascular risk. Endothelial dysfunction has been observed in patients with arterial hypertension, in normotensive subjects with a family history of hypertension, in smokers and passive smokers (Heiss et al., 2008), in dyslipidemia, in ageing, in diabetes mellitus, in obesity, in hyperhomocysteinaemia, in humans with low intracellular magnesium levels (Shechter et al., 2000) and in patients with inflammatory or infectious diseases (Flammer et al., 2008). The effects of cardiovascular risk on the endothelium can be seen in children as early as 8 years of age. It can be aptly said that endothelial

dysfunction may represent the effect of these risk factors on vascular health. The dysfunctional eNOS/NO pathway is considered as an early marker or a common mechanism for various cardiovascular disorders. Several studies have been conducted to understand the underlying mechanisms of endothelial eNOS/NO dysfunction in atherosclerosis. Though various mechanisms responsible for decreased endothelial NO bioactivity under the disease condition have been suggested, no single mechanism can fully explain the endothelial dysfunction. Studies have shown that patients having cardiovascular risk factors but without any clinical signs of atherosclerosis are affected by endothelial dysfunction, as indicated by their impaired response to endothelial vasodilators such as acetylcholine and bradykinin. There is a well-documented association of atherogenic lipoproteins particularly low density lipoproteins (LDLs), post-prandial chylomicron remnants and fasting triglyceride-rich particles with endothelial function. Hypercholesterolemia (HC) and high levels of total cholesterol and LDL cholesterol are inversely related to endothelium-dependent vasodilation (Norata and Catapano, 2005). High density lipoproteins (HDLs) are anti-atherogenic and they contribute majorly to reverse cholesterol transport. This seems to modulate endothelial function in a beneficial manner. Since humans spend a considerable amount of time in the post-prandial state, there is increasing evidence that post-prandial changes in the concentrations of lipoproteins promote atherogenesis at various stages of development. Triglycerides isolated from fasting plasma samples taken from hypertriglyceridemia subjects induce an inflammatory response in endothelial cells, but the molecular mechanisms and gene expression responses underlying post-prandial endothelial dysfunction is still ambiguous. The main pathway that is impaired by HC is L-arginine/NO pathway. The renin-angiotensin system (RAS) plays a crucial role in the pathogenesis of endothelial dysfunction. Hypercholesterolemia affects renin-angiotensin system. HC increases angiotensin II-type 1 receptor (AT1 R) density and its functional responsiveness to vasoconstrictors. There is also evidence that HC increases plasma angiotensinogen and angiotensin peptide production (Daugherty et al., 2004) and AT1R antagonism improves HC-associated endothelial dysfunction (Wassmann Sven et al., 2002). In a

way it can be said that there is an extensive body of evidence available that shows the association of generalized endothelial dysfunction with the early manifestation of a variety of cardiovascular diseases.

Several studies are conducted in animals to understand the importance of endogenous eNOS-derived NO production in regulation of vascular function, with implementation of NOS inhibitors. In such studies, blood pressure was seen elevated after intravenous administration of NOS inhibitors in rabbits, guinea pigs, dogs, monkeys and rats (Kolluru et al., 2012). An experimental model known as "NG-nitro-L-arginine methyl ester (L-NAME)-induced" or "NO-deficient" hypertension was established to investigate not only the role of NO in vascular function and blood pressure regulation but also in maintenance of homeostasis in the whole cardiovascular system (Dananberg J et al., 1993). These studies suggest that hypertension is a common characteristic of lack of NO production. Endothelial dysfunction is associated with both insulin-dependent and independent diabetes mellitus. In rat models with type 2 diabetes, changes in endothelium- derived hyperpolarizing factor (EDHF) causes endothelial dysfunction in which an impaired EDHF-mediated vasorelaxation was observed before marked alteration in NO-mediated responses(Gao et al., 2011). A high fat diet comprising of pig chow supplemented with 2% cholesterol, 17.1% coconut oil, 20.3% corn oil and 0.7% sodium cholate has been reported to decrease endothelium dependent relaxation and eNOS level in pigs.

Impaired NO-dependent vasodilation is of particular importance in clinical studies because its measurement helps in diagnosing malfunction of the endothelium and predicts adverse cardiovascular events. NO-dependent vasodilation in humans is assessed by measuring changes in vessel diameter in response to chemical or physical stimuli, with various detection techniques namely angiography, plethysmography, tonometry, and Doppler ultrasonography. In clinical settings, invasive methods are of limited use. The noninvasive techniques used are flow-mediated dilation (FMD) in the brachial artery, measured by ultrasonography, a method regarded as a gold standard; and reactive hyperemia (RH-PAT) in peripheral

circulation, measured by tonometry of the finger (Steyers and Miller, 2014). But the therapeutic approaches that exist or are in development are inadequate to treat the various cardiovascular diseases. These inadequacies are mainly due to the fact that discovery and therapeutic programs rely heavily on results from animal models which poorly predict the human pathophysiology and drug responses (Creager, 2016). Even though some human pathologies can be induced in animal models, the molecular mechanisms behind their onset and progression are significantly different (Grainger, 2014). The inefficacy of some drugs on humans which are successfully tested on animals can be perceived as the inability of animal model to effectively recapitulate human physiology. To fully understand the endothelial dysfunction, a patient's native tissue architecture, crosstalk between diseased and heterotypic cell populations, and the functional response of such systems should be known. To address these problems, the availability of physiologically-relevant *in vitro* model is needed. 2D cell culture models are used in preclinical studies but they lack many of the requisite phenotypic characteristics necessary for their utility in predictive drug assays. Human cell based *in vitro* models provide a first glimpse into the initial events of plaque deposition in humans (Robert et al., 2013). But most of these studies are performed using single types of cells grown on regular static plastic dishes. Such studies do have their limitations as they do not account for the complexity of the native artery environment with all the cell-cell and/or cell matrix interactions. To account for these limitations, several co-culture models of endothelial and smooth muscle cells have been established (Takaku Mikio et al., 1999). Again, such studies are laden with limitations such as unphysiological attachment of cells to plastic dishes or trans-well membranes and short co-culture times for the development of typical vascular cell-extracellular matrix interactions. To address these limitations, a long term co- culture set up on fibrin gels was established (Dorweiler et al., 2006). In that they analyzed the *in vitro* accumulation of LDL and immunocytes in a sub endothelial matrix. The static and non-dynamic co-culture system lack the circular structure of arteries and the physiological vascular hemodynamic situation characterized by flow and shear-stress. In such a scenario, 3D tissue-engineered models are expected to become useful tools in the preliminary testing and in the

investigation of the molecular mechanisms underpinning disease onset and progression. By developing a 3D *in vitro* model that can address the above said problems, dependence on ineffective and harmful treatment strategies can be reduced and bridge the treatment gaps for non-responding individuals (Dekkers et al., 2013). Advancements on tissue engineering and regenerative medicine are highly beneficial in developing an *in vitro* model to study the endothelial dysfunction. Moreover, tissue engineering has been projected as an alternative treatment by replacing the damaged tissue or organ with constructs which are bio fabricated based on the required tissue or organ features. In the tissue engineering paradigm, engineering and life science tools are combined to develop the bio artificial substitute of an organ or tissue of interest that can be used to elucidate fundamental aspects of cell functions *in vivo* or to identify mechanisms involved in aging processes and disease onset and progression. Precisely, three-dimensional (3D) *in vitro* models can more realistically reproduce a tissue or organ than two-dimensional (2D) models. Moreover, they can overcome the limitations of animal models and reduce the need for *in vivo* tests. 3D *in vitro* models allow independent identification and modulation of cellular and molecular factors responsible for disease onset and progression, allowing the investigation of the contribution of each of them on the development of a specific disease. A 3D *in vitro* model allows the cells to grow and interact with each other and with the ECM in all spatial dimensions and it consists of a 3D porous matrix (scaffold), the cells, and the applied cues (biochemical or physical). In recent years, several laboratories have developed tissue engineered artery equivalents under pulsatile and native like flow conditions for therapeutic and regenerative repair.

2.4. Inflammatory stimulus and EC activation

When an inflammation occurs in blood vessels, there will be vasodilation, increased vascular permeability, and blood stasis. Vascular permeability occurs due to disruption of EC junctions due to cytoskeletal changes in endothelial cells. A delayed sustained response involves inflammatory factors such as C-reactive protein (CRP), plasminogen activator inhibitor-1 (PAI-1), protease-activated receptor (PAR)

signaling, CD40/CD40 ligand interactions and cytokines such as interleukins, tumor necrosis factor-α (TNF-α), and interferon-γ (INF-γ). This delayed response leads to ECM deposition, granular tissue formation and connective tissue growth. Inflammation is usually terminated when the injurious stimulus is removed and all the mediators are dissipated or inhibited. If vascular inflammation progresses is unresolved, it can lead to vascular disease. Cytokines induce the expression of iNOS, thereby inducing ECs to produce NO. TNF-α in combination with IFN-γ is a potent stimulus of iNOS in EC. But when rat mesenteric arteries is treated with TNF-α, an inhibition of NO-dependent relaxation occurs. TNF-α inhibits NO-mediated endothelium dependent relaxation in small coronary arteries via sphingomyelinase activation and consequent free radical oxygen production in ECs. High concentrations of TNF-α also directly decrease the levels of eNOS mRNA in human ECs and promotes the production of oxygen-derived free radicals by neutrophils, SMCs and ECs. Reactive oxygen species in turn reduce the bioavailability of NO, and lead to reduction in vascular relaxation. As discussed in section 2.3., the renin-angiotensin system (RAS) has important modulatory activities in the atherogenic process. The AngII-induced effects on gene expression occurs through the cytoplasmic NF-κB transcription factor. TNF-α stimulates AngII production in the female reproductive tract and IL-6 upregulates the expression of AT1R in vascular smooth muscle cells (Sprague and Khalil, 2009).

2.5. Markers of inflammatory EC

When ECs undergo inflammatory activation due to various risk factors, for example the presence of oxidized lipoproteins, reduction in intracellular concentration of NO and upregulation of angiotensin II occurs. Angiotensin II can elicit the production of reactive oxygen species (ROS), increase the expression of the proinflammatory cytokines such as interleukin (IL)-6 and monocyte chemoattractant protein-1 (MCP-1). When ECs undergo inflammatory activation, there will be an increased expression of adhesion molecules namely selectins, vascular cell adhesion molecule (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1). Continued release of

cytokines, such as MCP-1, by activated ECs and foam cells not only perpetuates inflammation and lipid accumulation within the atheroma but also influences SMC activity. Inflammation participates in all steps of atherosclerosis and as a result there will be the presence of inflammatory mediators namely IL-1, TNF-α and CD40L within the plaque. Circulating high sensitivity C-reactive protein (CRP) P represents one of the strongest independent predictors of vascular death in a number of settings. In fact, CRP appears to be a stronger predictor than LDL cholesterol. CRP has been shown to stimulate endothelin -1(ET-1) and IL-6 release, upregulate adhesion molecules, and stimulate MCP-1 while facilitating macrophage LDL uptake. So CRP can be considered both as an inflammatory marker and as a mediator of the disease. IL-18 ,a proinflammatory cytokine, triggers several proatherogenic functions and induces the expression of the interstitial collagenases MMP-1 and MMP-13 as well as the gelatinase MMP-9 in human vascular SMCs and macrophages (Szmitko et al., 2003).

2.6. Tissue engineered small diameter blood vessel (TESDBV)

To construct a tissue or an organ by tissue engineering (TE) approach is extremely complex since several aspects must be taken into account and each single tissue has different features. A classic TE construct consists of a combination of living cells with a natural, synthetic, or bio artificial support or a 3D living construct that is structurally, mechanically, and functionally equal to a tissue. The fundamental elements in a small diameter blood vessel (SDBV) include scaffolds, vascular cells which include mainly endothelial cells (ECs) and smooth muscle cells (SMCs), and physical stimuli. The scaffolds can be categorized as synthetic, biological, or composite. The cell source can either be cells isolated from blood vessels or those differentiated from adult stem cells (ASC). The ultimate aim is to artificially construct a fully functional blood vessel. Functional blood vessels require a healthy and quiescent EC layer that can respond to external stimuli and SMC layer that can grow and deposit extracellular matrix proteins to impart compliance and contractility. The function of scaffold is mainly to give mechanical strength and direct cellular growth.

2.6.1. Synthetic polymer scaffolds for TE of SDBV

The selection of the most suitable biomaterial for scaffold fabrication is a key element in the construct, since it strongly influences cellular functions. In an *in vivo* scenario, complex tissue organization allows cells to interact with each other and with the surrounding ECM. So in an *in vitro* model, the scaffold must be selected keeping in mind that it can finely replicate *in vitro* the architecture of the native tissue, i.e., its ECM framework to let cells to adhere, spread, proliferate, differentiate, mature, and produce ECM, similarly to what they do *in vivo.* Regardless of the tissue type, a number of factors should be taken into consideration while choosing a scaffold. The factors to consider are a) biocompatibility b) biodegradability c) mechanical properties d) scaffold architecture and e) manufacturing technology (O'Brien, 2011). A pathologic tissue has altered ECM properties. To model a pathologic system, the scaffold should be designed to reproduce these altered ECM features. It should have a high degree of porosity with interconnected pores. This porous architecture allows migration of cells into the inner part of the 3D construct and permits nutrient/oxygen diffusion and waste removal. Experimental studies show that cells cultured on 3D scaffolds significantly differs from that of cells cultured on 2D surfaces (Miyagawa et al., 2011) and 3D patterned scaffolds promote greater cell aggregation, proliferation, and differentiation than 2D substrates (Eniwumide et al., 2014). It was also demonstrated that cell response to the substrate morphology strongly depends on the cell type, and topography can enhance the differentiation of progenitor cells into their programmed pathway (Yim et al., 2007). Scaffolds used for constructing a tissue engineered blood vessel should be resistant to thrombosis, inflammation, neointimal proliferation and they should also resemble the native vessels (Nugent Helen M. and Edelman Elazer R., 2003).

Three individual groups of biomaterials namely ceramics, synthetic polymers and natural polymers, are used in the fabrication of scaffolds for tissue engineering. Ceramic scaffolds used are hydroxyapatite (HA) and tri-calcium phosphate (TCP), for bone regeneration applications. Ceramic scaffolds are typically characterized by high mechanical stiffness (Young's modulus), very low elasticity, and a hard brittle

surface. Among the synthetic polymers, there are synthetic non degradable and degradable polymers. Synthetic non degradable polymers are expanded polytetrafluoroethylene (ePTFE), Dacron (polyethylene terephtalate fibre) and polyurethanes. ePTFE and Dacron are the most widely used synthetic materials for scaffold fabrication for large diameter high flow vessels. Synthetic degradable polymers commonly used are polystyrene, poly-l-lactic acid (PLLA), polyglycolic acid (PGA), poly-dl-lactic-co-glycolic acid (PLGA) and poly-ε-caprolactone (PCL).
PCL, a FDA approved polymer, is a semi crystalline, aliphatic polyester synthesized by the ring-opening polymerization of *ε*-caprolactone. It is a versatile polymer that shows good mechanical properties, specifically high elongation, strength and good biocompatibility. Furthermore, PCL degrades very slowly *in vivo* by enzymatic action and by hydrolysis to caproic acid and its oligomers and the resultant fragments are eliminated by macrophages and giant cells. PCL-based scaffolds are used to engineer venous blood vessels (Couet et al., 2007). A hybrid porous scaffold ,made from PCL and fibrin composite ,seeded with human umbilical vein endothelial cells (HUVEC) showed a remodeled ECM and non-thrombogenic EC phenotype (Pankajakshan et al., 2010).Third category of scaffolds used are biological materials such as collagen, various proteoglycans, alginate-based substrates and chitosan. Natural polymers are biologically active and typically promote excellent cell adhesion, growth and they are also biodegradable. But fabricating scaffolds from biological materials with homogeneous and reproducible structures presents a challenge as these scaffolds have poor mechanical properties, which limits their use in load bearing applications.

2.6.2. Cells to fabricate SDBV

The choice of the most appropriate cell source is a challenge in the design and further development of a tissue-engineered model. To produce a more biocompatible vascular substitute, viable biological components are introduced into an artificial material-based vascular conduit. To construct an engineered blood vessel, both endothelial cells and smooth muscle cells are needed to incorporate in a tubular

scaffold. Weinberg and Bell developed the first completely biological tissue-engineered blood vessels, composed of intima, media, and an adventitia, using cultured mature smooth muscle cells and endothelial cells in bovine collagen gels (Weinberg and Bell, 1986). To bridge the gap between animal models and clinical trials, *in vitro* models should include human cells (Griffith and Swartz, 2006; Maltman and Przyborski, 2010). Most of the human cells used are adult primary cells isolated from patients. These cells are representative of the functional unit of a tissue and they can be isolated from tissue biopsies from healthy or pathological patients. But these adult primary cells have limited life span and low proliferation rate, and their isolation procedure is complex (Benam et al., 2015). To overcome these problems, stem cells are used. Stem cells are undifferentiated cells that have the capacity to both self-renew and differentiate to one or more types of specialized cells, which can be isolated from different sources, such as embryos, fetuses, or adult tissues, and their differentiation capability depends on the cell source. In addition to this, adult stem cells can be isolated from a patient's own tissue and are not limited by ethical issues that surround embryonic stem cells.

2.6.3. Multipotency of mesenchymal stem cells

The main properties of stem cells are their capability to control cell differentiation pathways toward the desired lineages and the immature phenotype of the cells derived from stem cells, which have a gene expression profile similar to fetal cells. Bone marrow mononuclear progenitor cells, mesenchymal stem cells from various sources, and endothelial precursor cells are widely used in vascular tissue engineering applications. Bone marrow mononuclear cells (BM-MNCs) are a heterogeneous population consisting of late outgrowth endothelial precursor cells (EPCs), early-outgrowth EPCs, mature endothelial cells (ECs), mesenchymal stem cells (MSCs), hematopoietic stem cells, monocytes, CD4+ T cells, CD8+T cells, B cells and NK cells (Krawiec and Vorp, 2012). Studies have shown that without differentiating BM-MNCs into vascular phenotypes, a graft can be constructed. In this case, a culture period is not required which can significantly reduce the

preparation time for a tissue engineered vascular graft. BM-MNCs were seeded into poly-ε-caprolactone and poly-L-lactic acid scaffold and the scaffold was implanted into the inferior vena cava of a canine model. The scaffold remained patent for a period of two years by generating ECs and SMCs. Implanting BM-MNCs in their undifferentiated states can result in a graft composed of SMCs and ECs, but how this happens is still ambiguous. Since BM-MNC consists of a diverse population of cells, monocytes are thought to play a key role in regenerating vascular tissue and maintaining patency. Bone marrow mesenchymal stem cells (BM-MSCs), a subpopulation within BM-MNCs, and monocytes secrete a variety of angiogenic and arteriogenic growth factors and cytokines. Next set of adult progenitor cells are mesenchymal stem cells (MSCs). MSCs have the ability to self-renew allowing them to be rapidly expanded. Due to their rapid expansion property, MSCs are preferable to terminally differentiated cells. MSCs are capable of differentiating into a variety of phenotypes such as bone, fat, cartilage, muscle, tendon, ligament, and cardiovascular phenotypes. MSCs can be used allogenically due to the lack of major histocompatibility complex (MHC) II. Though MSCs can be harvested from almost every organ, there are slight variations in phenotype depending on the type of tissue from which they are isolated. Adipose MSCs (ADMSCs) could be harvested in the largest frequency, when compared with MSCs isolated from umbilical cord and bone marrow (Krawiec and Vorp, 2012). On the other hand, umbilical cord MSCs could be cultured the longest and had the highest proliferation potential while bone marrow had the lowest. Though all MSCs could differentiate into osteogenic and chondrogenic lineages under defined media, only bone marrow and adipose MSCs could differentiate toward an adipogenic lineage. There are also differences in the regeneration ability of MSCs from different sources and the ability to regenerate may be linked to CD105+ expression, which is lowest in umbilical cord MSCs.

2.6.4. Advantages of using ADMSCs

Adipose tissue provides a readily-available and relatively easily obtainable source of mesenchymal stem cells. Adipose derived mesenchymal stem cells (ADMSCs) were

first discovered by Zuk and colleagues in 2001(Zuk et al., 2001).The process of obtaining these cells involves harvesting fat, mostly by liposuction method, followed by enzymatic digestion and filtering. The stromal vascular fraction (SVF) obtained after centrifugation contains a heterogeneous population of cells including adipose-derived mesenchymal stem cells (ADMSCs), which will grow when plated and cultured. Only about 1 in 30 cells from the SVF become the ADMSC population with an approximate yield of 100,000 to 1 million ADMSCs per gram of fat. On culturing ADMSCs for several passages, the population of cells expressing stromal markers increases, resulting in a more homogeneous population. Studies have shown that ADMSCs have the plasticity to differentiate into both endothelial (EC) and smooth muscle cells (SMC). This property has been explored *in vitro* for tissue engineered vascular graft (TEVG) construction.

2.6.5. Strategies for differentiation of ADMSCs to endothelial cells

ADMSCs differentiate more quickly into ECs and they possess a stronger proliferation ability than BM-MSCs (Ma et al., 2017). ADMSCs have a paracrine effect and they could secrete various protective factors such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), and a variety of microRNAs and thus qualifies them to be better candidates than BM-MSCs for vascular differentiation. Studies showed the differentiating capability of a subpopulation of ADMSCs (CD34+ /CD31–) into ECs when cultured in endothelial growth medium supplemented with IGF and VEGF. Under this condition, the cells showed a spindle-shaped morphology and high expression of EC markers such as CD31 and vWF. On the other hand, another subset of ADMSCs (CD34– / CD31–) when cultured on a matrigel supplemented with basic fibroblast growth factor (bFGF) and VEGF differentiated into cells similar to human umbilical vein endothelial cells. ECs differentiated from ADSCs seeded on fibronectin and exposed to shear has been shown to be expressing increased retention through upregulation of the α5β1 integrin. ADMSCs also exhibited EC differentiation

capacity when they augmented capillary density after their intravenous injection into a mouse ischemic hindlimb.

2.6.6. Strategies for differentiation of ADMSCs to smooth muscle cells

Vascular smooth muscle cells are important in maintaining the physiological functions of the blood vessel wall. When ADMSCs were cultured in smooth muscle inductive medium consisting of MCDB 131 and heparin for 6 weeks, the cells expressed VSMC markers and contracted in response to carbachol (Rodríguez et al., 2006). U46619 (a thromboxane A2 analog) could induce differentiation of ADMSCs to SMCs *in vitro* and the stimulated cells expressed VSMC-specific markers such as SM α-actin, calponin, SM-MHC, and smoothelin (Kim et al., 2009). Furthermore, studies have shown that sphingosylphosphorylcholine could induce the differentiation of ADMSCs into VSMCs via modulation of the Smad2 pathway. ADMSCs have been shown to differentiate into contractile SMCs with a combination of TGF-β1 and bone morphogenetic protein 4 (BMP 4) (Wang et al., 2010a). On applying shear stress with a pulsatile bioreactor, smooth muscle markers such as α-smooth muscle actin, calponin, and myosin heavy chain were found to be increased.

2.6.7. Cell seeding and mechanical stimulation to fabricate SDBV

The differentiation capacity of human adipose derived stem cells can be explored to fabricate a 3D construct by seeding differentiated endothelial like cells in the lumen of the construct and differentiated smooth muscle like cells in the outer surface of the construct. Polymer biodegradable scaffolds are chemically modified to exhibit selective cell adhesion properties that enhance cell attachment and subsequent tissue growth. Many cell types have been successfully cultured on these scaffolds, including smooth muscle cells and endothelial cells. The challenge is to create an ideal tissue engineered vascular construct by balancing polymer degradation rates with ECM production and cellular infiltration and this has resulted in the fabrication of polymers designed with cell-binding sequences, enzymatic cleavage sites, and

tethering of chemoattractant molecules (Ravi and Chaikof, 2010). Polymers are functionalized by different methods namely coatings, chemical and protein modifications and endothelial cell seeding in the lumen of the scaffolds to improve endothelialization and inhibit inflammation. Several ECM components such as collagen I, collagen IV, laminin and fibronectin, have been used as surface coatings for biomaterials to facilitate cell attachment and growth, which promote biocompatibility. Studies have demonstrated that fibrin coating improves the adhesion and stability of endothelial cells grown on PCL surfaces (Pankajakshan et al., 2008).Furthermore, activated endothelial cells increase growth factor production and encourages SMC proliferation seeded on the outer surface of the scaffolds.

In a 3D model, cells in the middle of the construct could behave differently from cells growing on the surface, depending on nutrients concentration gradient. To avoid local concentrations of the nutrients and to overcome the diffusion limits that affect cell behavior, chemical, and mechanical signals should be coupled. In *in vivo* condition, cells are subjected to various extracellular and intracellular mechanical forces that determine their fate. Mechanical stimuli are usually provided to tissue-engineered constructs by bioreactors specifically designed to reproduce the *in vivo* conditions. Bioreactors provide mechanical or electrical stimuli and allow a fine modulation of culture conditions to reach tissue maturation (Massai et al., 2013). While constructing a 3D model to study the endothelial dysfunction, the required physico chemical stimuli can be given by bioreactors. Bioreactors simulate the physiological environment and control the parameters such as pH, temperature, pressure, nutrient supply, and waste removal in order to allow biological or biochemical processes to occur and subsequently develop the desired tissue (Nemeno-Guanzon et al., 2012). The design and functional requirements of the tissue to be engineered determine the specific design requirements in the bioreactor. A bioreactor for cardiovascular applications mimics the parameters that exist *in vivo* such as flow rate, volume, shear, pressure, resistance and compliance. A bioreactor must possess the ability to supply media to all areas of the developing tissue and prevent necrosis. Lack of perfusion, which would guarantee nutrient support, greatly limits the thickness of engineered tissue. In contrast to static culture, dynamic culture

allows active continuous transport of nutrients and gases to the cells and tissue thereby promoting aerobic metabolism. In static culture, diffusional transfer results in anaerobic cell metabolism. Many bioreactors are designed to fit entirely or partially in an incubator. The incubator has the ability to control and maintain the physiological temperature while the bioreactor is functioning (T et al., 2003).

2.6.8. Testing the SDBV function

Ideally, the blood vessels constructed should mimic the native blood vessels in both structure and functions. They should be biocompatible, bioactive and have acceptable mechanical properties. The synthesized blood vessels should be biomimetic in such a way that they should be resistant not only to thrombosis, but also to inflammation, and neointimal proliferation. For these reasons, it is necessary to investigate the physical, chemical, and biological properties and modifications of materials to further understand the molecular mechanism of the cell material interaction. Endothelial cell seeding on the luminal surface of the graft mitigates the thrombogenicity and promotes intimal proliferation within the graft. Several *in vitro* and *in vivo* studies are conducted to study the patency of the constructed cell seeded grafts. Decellularized vessels, seeded with EPCs, implanted into the common carotid arteries of sheep remained patent for 130 days due to the presence of EPCs and not seeded decellularized vessels occluded within 15 days (Catto et al., 2014). TEVGs *in vitro* seeded and cultured with bovine aortic SMCs and ECs, demonstrate good adhesion and cell proliferation up to 14 days. Natural polymers lack mechanical strength, but they possess excellent biological behavior. Synthetic polymers show controllable physical and mechanical properties but lack in biological performances. So combining natural materials with synthetic polymers (such as fibrin with PCL (Pankajakshan et al., 2008)), will result in promising results in terms of mechanical properties and cell/material interaction.

In summary, the current understanding about the risk factors leading to the disease process is evident in the reviewed literature. The relevance of studying the endothelial dysfunction is justified. Many studies evaluated NO- pathway for

understanding endothelial function and dysfunction. Therefore, this study selected both NO release and eNOS expression to distinguish the functional status of endothelial cells lined in the constructed TEVG. Also, based on the literature, the shear stress is included as an important aspect in this study, not only for TEVG construction, but also for while studying the effect of risk factors on endothelial cell phenotype. Literature highlighted that the endothelial dysfunction can be identified based on expressions of different molecules produced in EC. Out of these VCAM -1 and MCP-1 were selected as representative markers in study.

Since the mechanical properties and morphology of the scaffold is important in mimicking the biology of the grown tissue, the bi-layered PCL scaffold was chosen for TEVG construction. Based on the reviewed literature, bilayered scaffold with smooth lumen for producing continuous endothelium and 3-dimensional fibrous outer layer that can permit porous SMC growth for gas and medium exchange is selected for this study. Many studies reported use of ADMSCs for producing EC and SMC for construction of vascular graft. It was noticed that none of the reported study showed stable differentiation of ADMSC to EC and SMC. Therefore, based on the information in the literature, a multi-step strategy was adopted in this study to obtain stable EC and SMC that expressed multiple differentiation markers. The review revealed methods of scaffold development, use of different cell types for construction, and the shear stress to promote vessel stability. Methods of constructing tissue engineered vascular graft described in various studies aimed blood vessel replacement; therefore, the novelty of study topic and hypothesis is evident.

CHAPTER 3

3. Materials and methods

The first step of the study aimed at differentiation of human adipose derived mesenchymal stem cells (hADMSCs) into endothelial progenitor cells (EPCs) and smooth muscle progenitor cells (SMPCs) by demonstrating multiple lineage markers in both cells types. The next step was to fabricate bilayered poly-ε-caprolactone (PCL) scaffold, coat with fibrin to enhance seeding and growth of EPC in the lumen and SMPCs on the outer surface. Further the properties of cells established the success in functional TEVG construction. The third step was to validate the sensitivity of EPCs to high glucose, high cholesterol and an inflammatory cytokine. The materials, equipment and methods used are described below.

3.1. Isolation and Characterization of hADMSCs

Subcutaneous adipose tissue (~ 5 gm) was collected from patients admitted for coronary artery bypass graft procedure at SCTIMST, Trivandrum and the tissue was collected as per the Institute Ethics Committee approval (SCT/IEC/1231/June 2018,SCT/IC-SCR/43/March 2017). ADMSCs were isolated as described by Zuk et al (Zuk et al., 2002). Briefly, the tissue obtained was thoroughly washed with sterile Hank's Balanced Salt Solution (HBSS) after which the tissue was minced thoroughly. The chopped tissue was digested with 4mg/ml Collagenase type I enzyme (Merck Sigma, USA) for 1 h at 37°C with continuous shaking at 120 rpm in an incubator shaker (Kuhner Shaker, Switzerland). The digested cell suspension was strained (70uM, BD Falcon, USA), mixed with serum containing medium to terminate the enzyme action, centrifuged at 400g and the cell pellet was resuspended in low glucose Dulbecco's modified Eagle's medium (DMEM- LG; Gibco, USA) supplemented with 10% Fetal Bovine Serum (FBS; Gibco, USA) and Antibiotic-Antimycotic solution (ABAM; Invitrogen, USA). The cells were seeded to a $25cm^2$ tissue- culture polystyrene flask (TCPS; Nunc, Denmark) and incubated at 37°C under 5% CO_2. Medium was changed at 3 day intervals. Upon reaching ~ 80%

confluence, cells were passaged by standard trypsinization protocol using 0.25% Trypsin- EDTA (Invitrogen, USA) for expansion of cell numbers. hADMSCs from passage 2-5 were used for characterization. The multipotency of isolated hADMSCs was confirmed by tri-lineage differentiation and specific staining. Stemness of isolated hADMSCs was done by cell surface marker analysis using flow cytometry, based on International Society for Cellular Therapy (ICST) norms. Briefly, cells at passage 2 - 5 were trypsinized using standard protocol and fixed in 3.7% formaldehyde (Merck, Germany) for 20 min at room temperature. The cells were washed by centrifugation at 400g for 5 min, the supernatant was discarded and the cell pellet was dissolved in 0.3% bovine serum albumin/phosphate buffered saline (BSA/PBS) and incubated for 30 min. Characterization of surface markers was done using human MSC phenotyping kit (MACS; Miltenyi Biotec, USA). The cells were incubated with the primary antibodies against CD 105, CD 90, CD 73, CD 14, CD 45, CD 20 and CD 34 for 10 minutes in the dark in the refrigerator. After primary antibody incubation, the cells were washed by centrifugation and were analyzed by flow cytometry (Cytoflex, BC, China). The source of antibodies used for ADMSC characterization is listed in Table 1. The unstained control was used to acquire histogram of fluorescence against side scatter. The percentage of cells expressing the specific markers was estimated using FlowJo software (Tree Star Inc., USA). Adipogenic, osteogenic and chondrogenic differentiation of hADMSCs was done to prove the multipotency, using StemPro medium (Life Technologies, USA), as per the standard procedure. For adipogenic induction 1×10^4 cells/cm^2 and for osteogenic induction 5×10^3 cells/cm^2 respectively, were seeded and cultured in respective Stem Pro induction medium for 21 days with medium change once in three days. For chondrogenic differentiation, cells were seeded at high density as microdroplets and grown for 14 days in respective medium. Differentiation to adipogenic, osteogenic and chondrogenic lineages was confirmed by staining with Oil Red O, Alizarin Red S and Toluidine blue stains respectively, as per the standard protocols.

1. **Table 1 – Source of antibodies used for hADMSC characterization**

Name	Isotype	Source	Catalog No.
CD 105 PE	Mouse IgG1	MSC Phenotyping Kit, MACS, Miltenyi Biotec	130-095-198
CD 73 APC	Mouse IgG1		
CD 90 FITC	Mouse IgG1		
CD 14 PerCP	Mouse IgG2a		
CD 45 PerCP	Mouse IgG2a		
CD 20 PerCP	Mouse IgG1		
CD 34 PerCP	Mouse IgG2a		

3.2. Induction of hADMSCs to EPCs

3.2.1. Preparation of bovine hypothalamus extract (HE)

Bovine hypothalamus extract was prepared according to the protocol of Maciag et al (Maciag et al., 1979).Briefly, bovine calf brain was collected from the slaughter – house, kept in ice, dissected, hypothalamus was isolated, minced into small pieces and homogenized. The homogenized sample was stirred for 2 h in ice-cold saline and centrifuged at 13000g at 4℃ for 15 min. The supernatant was collected and mixed properly with 0.5 w/v percentage streptomycin sulphate (Sigma, USA). The mixture was given an overnight incubation at 4℃ to remove the lipid content with subsequent centrifugation at 13000g at 4℃ for 15 min. The supernatant was collected and dialyzed against 0.1M sodium chloride (NaCl) for 24h at 4℃. The dialysed sample was then sterile filtered using 0.22µm syringe filter (Merck Millipore; USA), dispensed, lyophilised (Modulyo 4K Freeze dryer, Edwards, UK) and stored at -80°C (SANYO ultralow, Japan) until use. The concentration of the extract was determined by Lowry's assay. Based on the protein concentration of HE cocktail, the quantities used in the matrix was normalised.

3.2.2. Trilevel induction protocol - Induction by growth factor and hypoxia

hADMSCs in early passages (P2-P5) were induced with a combination of growth factors and hypoxia for differentiation to EPCs.$1*10^4$ cells/cm^2 were seeded onto tissue culture polystyrene (TCPS) dishes coated with the bio-mimetic matrix prepared by mixing fibronectin-associated fibrinogen, gelatin and in-house prepared hypothalamus extract (HE).TCPS dishes were coated with fibrin matrix as described earlier (Prasad and Krishnan, 2008). Briefly, pharmacopoeia grade fibrin sealant (Drug controller approved) was reconstituted. Diluted (5IU) thrombin treated surface was layered with thin layer of (12.5 ul per cm^2) diluted (2mg/ml) fibrinogen containing 0.2 mg fibronectin, exogenous gelatin (0.2%; Sigma, USA), in-house prepared HE, and was allowed to clot. The clot formed was stabilized by incubating the culture dishes at 37°C for 30 min, lyophilized (Modulyo 4K Freeze dryer, Edwards, UK) in sterile atmosphere, and stored at 4°C until used.Seeded cells were cultured for a period of two days in MCDB 131 medium (Gibco, USA) containing 10% FBS (Gibco, USA), Antibiotic-Antimycotic solution (Invitrogen, USA), heparin sulphate (Sigma, USA), L-glutamine (Sigma, USA), L-ascorbic acid (Sigma, USA), 50ng/ml recombinant human vascular endothelial growth factor 165 (VEGF 165, Cell Signaling Technology, USA), 20ng/ml recombinant human Insulin-like growth factor -1 (IGF-1, Cell Signaling Technology, USA) and 10ng/ml recombinant human epidermal growth factor (EGF, Cell Signaling Technology, USA). After two days in static culture, the induced cells were subjected to hypoxia in a hypoxia incubator chamber (Stem Cell Technologies, USA) for 20h. Morphological analysis using phase contrast microscopy (DMIRB, Leica Microsysytems, Wetzlar, Germany) was used as the first level of evidence for transformation of cells. The morphology was continuously monitored during the culture period and was documented before further characterization.

3.2.3. Trilevel induction protocol –Induction by shear stress

The growth factor and hypoxia induced cells were seeded in the lumen of a fibrin coated bilayered PCL scaffold at a seeding density of 10,000cells/cm^2.The scaffolds

were kept in the incubator at 37°C under 5% CO_2 for 30 minutes, in a horizontal position with occasional rolling, for the proper attachment of cells in the entire inner surface of the scaffold. Afterwards, the scaffolds were connected to a bioreactor (Masterflex L/S pump, ColeParmer, USA) with a graded flow rate from 20ml/min to 100ml/min for applying shear force which gave a shear stress from 11.6 dynes/cm^2 to 58.2 dynes/cm^2.

3.2.4. Expression of EPC markers by immunocytochemistry

The cells induced to differentiate to EPCs were characterized by immunocytochemistry for EPC markers namely acetylated LDL, CD31, VCAM-1, VE-Cadherin and eNOS. Induced cells were incubated with 10µg/ml acetylated LDL (Molecular Probes, USA) for 4h in the dark in the incubator at 37°C under 5% CO_2. For CD31 (1:50),VE- Cadherin (1:50),VCAM-1(1:100) and eNOS (1:100) staining, the cells were fixed with 3.7% paraformaldehyde (Merck, Germany) for 20 minutes at room temperature. Permeabilization using 0.2% Triton X 100 (Sigma Aldrich, USA) for 5 minutes was done for VE-Cadherin, VCAM-1 and eNOS. Blocking was done with 3% bovine serum albumin (BSA; Sigma, USA) for 30 minutes. The cells were then incubated with primary antibodies overnight at 4°C, secondary antibodies at 37°C for one hour at room temperature and the cells were further stained for nucleus using DAPI (Life technologies, USA). The source of antibodies used for immunostaining is listed in Table 2.

Table 2– Sources of antibodies used for Immunostaining studies

Name	**Isotype**	**Source**	**Catalog No.**
CD31	Mouse IgG	Biolegend	303110
eNOS	Mouse IgG	Abcam	ab76198
VCAM-1	Rabbit IgG	Abcam	ab215380
VE-cadherin	Mouse IgG	SantaCruz Biotechnology	Sc52751
Anti-mouse AF 488	Goat IgG	Abcam	ab150113
Anti-rabbit AF 488	Goat IgG	Abcam	ab150077

3.2.5. Assessment of NO release

Functionality of EPCs grown inside the lumen of the scaffolds was evaluated by measuring nitrite. Nitrite is a stable metabolite of nitric oxide, which is an important cellular signaling molecule. Modified Griess reagent (Sigma, USA) method was used for measuring nitrite. For this assay, EPCs were grown on the scaffold for 4 days, 7 days and 14 days. The scaffolds were perfused with phenol red-free M199 medium (Sigma, USA) for 1 h, the medium was collected and the assay was done. To the medium an equal volume of Griess reagent was added and incubated for 15 min at room temperature. The color developed was read at 540 nm, using a diode array spectrophotometer (Hewlett-Packard 8053, USA). The amount of nitrite formed was calculated from a calibration curve obtained using serially diluted sodium nitrite as standard.

3.2.6. Assessment of cell stability in the scaffold lumen

The morphology and stability of EPCs seeded in the lumen of the hybrid scaffold was established by ESEM analysis and actin phalloidin staining. For ESEM analysis, the cell seeded scaffolds were fixed in 2% glutaraldehyde for 20 minutes after which the scaffolds were washed four times in PBS. The scaffolds were then processed by passing through ascending series of alcohol for dehydration: 30% -30 minutes, 50%-30 minutes, 70%-30 minutes, 90%-30 minutes and 100%-30 minutes. The processed scaffolds were dried and used for ESEM analysis. The ESEM analysis was carried out using 30Kv Environmentally Scanning Electron Microscope (ESEM-Quanta 200, Germany).

For confirming cell attachment and growth on scaffold, cell seeded scaffolds were stained for actin with texas red phalloidin after 4d of dynamic culture. Briefly, cell cultured scaffolds were fixed with 2% glutaraldehyde for 20 min, washed with PBS and permeated by 0.2 % triton X 100, washed and stained with Texas red conjugated phalloidin (1 : 250 dilution) for 45 min and washed. The scaffolds were then counter stained with DAPI (Life technologies, USA) and observed and imaged using fluorescent microscope (Leica, DMIRB, Germany).

3.2.7. Expression of EPC markers by Quantitative Real- Time polymerase chain reaction (qPCR)

EPC marker expression was confirmed at the transcriptional level by quantitative real time PCR. Gene expression of EPC markers CD31 (PECAM1), eNOS, vWF, VCAM-1, PAI-1, tPA and MCP-1 at different time periods were analyzed. The time periods analyzed were a) 48h after growth factor induction b) 20h after growth factor and hypoxia induction c) 4 days in static culture and d) 4 days in dynamic culture. RNA was isolated from the cells at respective time periods using TRIZOL reagent (Invitrogen, USA) based on the manufacturer's protocol. RNA quantification was done spectrophotometrically using Nanodrop 1000 Spectrophotometer (ND 2000; Thermo Scientific, USA). 200ng of total RNA was converted to cDNA using OrionX cDNA kit (Origin, India) in a thermal cycler (Master cycler; Eppendorf). Amplification of genes using specific intron-spanning primers for EPC markers was analysed by qRT-PCR. Real time PCR was carried out in a total volume of 15µl containing 20ng cDNA, 100 pmol each of respective forward and reverse primers and 7.5 µl of OrionX 2X Real time PCR master mix (Origin, India). Forty cycles of reaction were performed using the Bio-Rad iQ5 Real time PCR detection system (Bio-Rad, USA) under the following conditions: Enzyme activation, 95°C for 15 minutes; denaturation, 95°C for 30 seconds; annealing, 50°C for 20 seconds; and extension, 72°C for 20 seconds. GAPDH was used as house-keeping gene. Melt curve analysis was performed for each gene to confirm the specificity of each reaction. Products were analyzed by agarose gel electrophoresis for correct amplicon size. Fold change in expression was calculated after normalization with GAPDH expression on each day of analysis using the formula $2^{-\Delta\Delta Ct}$. List of primers used for the study are given in Table 3.

Table 3 – Primer sequences used for qRT PCR analysis

Genes	Amplicon Size	Primer sequence (5'-3')
GAPDH	120bp	FP - GAA ATC CCA TCA CCA TCT TCC AGG RP - GAG CCC CAG CCT TCT CCA TG

CD 31	118bp	FP - CAG TCA TTA CGG TCA CAA T RP - CTG AGG ACA CTT GAA CTT C
eNOS	116bp	FP- GGC ATC ACC AGG AAG AAG A RP- TCG GAG CCA TAC AGG ATTG
vWF	310bp	FP- CAC CAT TCA GCT AAG AGG AGG RP- GCC CTG GCA GTA GTG GAT A
VCAM-1	90bp	FP- CCT CCT TAA TAA TAC CTG CCA TTG RP- TCT GTG CTT CTA CAA GAC TAT ATG A
PAI-1	485bp	FP- TTG GTG AAG GGT CTG CTG TG RP- GGC TCC TTT CCC AAG CAA GT
tPA	319bp	FP- ATG GGA AGA CAT GAA TGC AC RP- GAA AGG GGA AGG AGA CTT GA
MCP -1	120bp	FP – CCG AGA GGC TGA GAC TAA C RP – ATG AAG GTG GCT GCT ATG A

3.3. Induction of hADMSCs to SMPCs

3.3.1. Preparation of Platelet growth factor (PGF)

Platelet growth factor was prepared from platelet rich plasma (PRP) based on a previously published protocol (Resmi and Krishnan, 2002). Fresh PRP bags were collected from blood bank and was washed with 10% excess acid citrate dextrose (ACD) solution and centrifuged at 750g. Following centrifugation, cells were washed thrice with ACD-Tyrode's buffer. Calcium chloride ($CaCl_2$) was added to get 2mM final concentration, and challenged with 1IU/ml thrombin for 5 min. EDTA at a concentration of 5mM was used to stop further activation of platelets. Activated platelets were then centrifuged at 36000g for 1 h at 4°C and the released proteins were collected in supernatant and dialysed against Ca^{2+} - Mg^{2+} - free Hank's Balanced Salt Solution (HBSS) overnight. The dialysed sample was then sterile filtered using 0.22µm syringe filter (Merck Millipore; USA), dispensed and stored at

-20°C until use. The concentration of PGF cocktail was determined by Lowry's assay and the concentration in the medium was adjusted as per the requirement.

3.3.2. Growth factor induction of hADMSCs to SMPCs

ADMSCs in early passages (P2-P5) were induced with a combination of growth factors for differentiation to SMPCs.$1*10^4$ cells/cm^2 were seeded onto tissue culture polystyrene (TCPS) dishes coated with the bio-mimetic matrix prepared by mixing fibronectin-associated fibrinogen, gelatin and in-house prepared platelet growth factor (PGF). TCPS dishes were coated with fibrin matrix as described earlier (Prasad and Krishnan, 2008). Briefly, pharmacopoeia grade fibrin sealant (Drug controller approved) was reconstituted. Diluted (5IU) thrombin treated surface was layered with thin layer of (12.5 ul per cm^2) diluted (2mg/ml) fibrinogen containing 0.2 mg fibronectin, exogenous gelatin (0.2%; Sigma, USA), in-house prepared PGF, and was allowed to clot. The clot formed was stabilized by incubating the culture dishes at 37°C for 30 min, lyophilized (Modulyo 4K Freeze dryer, Edwards, UK) in sterile atmosphere, and stored at 4°C until used. Seeded cells were cultured for a period of three days in MCDB 131 medium (Gibco, USA) containing 1% FBS (Gibco, USA), Antibiotic- Antimycotic solution (Invitrogen, USA), L-glutamine (Sigma, USA), L-ascorbic acid (Sigma, USA), 2.5ng/ml recombinant human Bone Morphogenetic Protein (BMP4; R&D systems, USA) and 0.6µg/ml in-house processed PGF. The growth factors were withdrawn after 72 h and the induced cells were sub cultured till passage 2 and medium change was given once in three days. Morphological analysis using phase contrast microscopy (DMIRB, Leica Microsysytems, Wetzlar, Germany) was used as the first level of evidence for transformation of cells. The morphology was continuously monitored during the culture period and was documented before further characterization.

3.3.3. Expression of SMPC markers by immunocytochemistry

The cells induced to differentiate to SMPCs were characterized by immunocytochemistry of SMPC markers α SMA, calponin, MHC 11 and PDGF R α. For α SMA (1:20), calponin (1:20), MHC 11 (1:50) and PDGF R α (1:250) staining,

the cells were fixed with 3.7% paraformaldehyde (Merck, Germany) for 20 minutes at room temperature. Permeabilization using 0.2% Triton X 100 (Sigma Aldrich, USA) for 5 minutes was done for α SMA, calponin and MHC 11. Blocking was done with 3% bovine serum albumin (BSA; Sigma, USA) for 30 minutes. The cells were then incubated with primary antibodies overnight at 4°C, secondary antibodies at 37°C for one hour at room temperature and the cells were further stained for nucleus using DAPI (Life technologies, USA). The source of antibodies used for the immunostaining is listed in Table 4.

Table 4 – Sources of antibodies used for Immunostaining studies

Name	**Isotype**	**Source**	**Catalog No.**
α SMA	Mouse IgG	ThermoScientific	MA5-11547
Calponin	Mouse IgG	ThermoScientific	MA5-11620
MHC 11	Rabbit IgG	Abcam	ab53219
PDGFRα	Mouse IgG	Abcam	ab96569
Anti-mouse AF 488	Goat IgG	Abcam	ab150113
Anti-rabbit AF 488	Goat IgG	Abcam	ab150077

3.3.4. Expression of SMPC marker by flow cytometry

The expression of SMPC specific marker α SMA was quantified using flow cytometry (Cytoflex, BC, China). Briefly, cells cultured for SMPC differentiation were harvested using the standard trypsin digestion method. The cells were then fixed in 3.7% formaldehyde, permeated using 0.1% triton X-100 for 5 min, blocked with 0.3% BSA for 30 min and incubated with primary antibody against α SMA (1:20) and corresponding secondary antibody, as listed in Table 5. Fluorescence intensity from 10,000 events was recorded using cells from at least three donors. Unstained control was used to acquire histogram of fluorescence against side scatter. The percentage of cells expressing the specific marker was estimated using FlowJo software (Tree Star Inc., USA).

3.3.5. Expression of SMPC markers by Quantitative Real- Time polymerase chain reaction (qPCR)

Gene expression of SMPC specific markers alpha smooth muscle actin (α SMA), calponin and myosin heavy chain 11 (MHC 11) were analyzed by real time PCR. RNA was isolated from the cells using TRIZOL reagent (Invitrogen, USA) based on the manufacturer's protocol. RNA quantification was done by using Nanodrop 1000 Spectrophotometer (ND 2000; Thermo Scientific, USA). 200ng of total RNA was converted to cDNA using OrionX cDNA kit (Origin, India) in a thermal cycler (Master cycler; Eppendorf). Amplification of genes using specific intron-spanning primers for specific markers was analyzed by qRT-PCR. Real time PCR was carried out in a total volume of 15µl containing 20ng cDNA, 100 pmol each of respective forward and reverse primers and 7.5 µl of OrionX 2X Real time PCR master mix (Origin, India). Forty cycles of reaction were performed using the Bio-Rad iQ5 Real time PCR detection system (Bio-Rad, USA) under the following conditions: Enzyme activation, 95°C for 15 minutes; denaturation, 95°C for 30 seconds; annealing, 50°C for 20 seconds; and extension, 72°C for 20 seconds. GAPDH was used as house-keeping gene. Melt curve analysis was performed for each gene to confirm the specificity of each reaction. Products were analyzed by agarose gel electrophoresis for correct amplicon size. Fold change in expression was calculated after normalization with GAPDH expression on each day of analysis using the formula $2^{-\Delta\Delta Ct}$. List of primers used for the study are given in Table 5.

Table 5 – Primer sequences used for qRT PCR analysis

Genes	Amplicon Size	Primer sequence (5'-3')
GAPDH	120bp	FP - GAA ATC CCA TCA CCA TCT TCC AGG RP - GAG CCC CAG CCT TCT CCA TG
α SMA	100bp	FP- GAG TTA CGA GTT GCC TGA T RP- AGA CTC CAT CCC GAT GAA
Calponin	104bp	FP- TTG AGA ACA CCA ACC ATA CA RP- CTC TGC GTA CTT CAC TCC
MHC 11	143bp	FP- ACT ACA CCT TCC TCT CCA A RP- GAC CGA TGA TAC CAC CTT C

3.3.6. Assessment of cell proliferation on scaffolds

Proliferation of hADMSC induced SMPCs, seeded on the outer surface of coated scaffolds, was assessed by DNA quantification. Briefly, 15 scaffolds of 2cm length were coated with matrix specific for differentiation of hADMSCs to SMPCs, as described in 3.3.2. hADMSCs were seeded on the ablumen of the coated scaffolds at a seeding density of 10,000cells/cm^2 and induction was initiated for 72h. After 72h, the induction was withdrawn and DNA was isolated from 5 scaffolds using TRIZOL reagent (Invitrogen, USA) based on the manufacturer's protocol. Remaining 10 scaffolds were continued in culture with medium change at every alternate day. The culture of 5 scaffolds were terminated on 7^{th} day of initial seeding and DNA was isolated. Remaining 5 scaffolds were continued in culture with medium change at every alternate day. The culture was terminated on 15^{th} day of initial seeding and DNA was isolated. DNA quantification was done spectrophotometrically using Nanodrop 1000 Spectrophotometer (ND 2000; Thermo Scientific, USA). Based on the DNA concentration of cells from three different time periods, a graph was plotted and cell proliferation was calculated.

3.3.7. Analysis of scaffolds for collagen deposition

ECM deposited on the cell seeded scaffolds were analysed by culturing induced SMPCs on the ablumen of the scaffold for 3 days (72h), 7 days and 15 days. Briefly, 15 scaffolds of 2cm length were coated with matrix specific for differentiation of hADMSCs to SMPCs, as described in 3.3.2. hADMSCs were seeded on the ablumen of the coated scaffolds at a seeding density of 10,000cells/cm^2 and induction was initiated for 72h.After 72h, the induction was withdrawn and decellularization was done for 5 scaffolds. Remaining 10 scaffolds were continued in culture with medium change at every alternate day. Culture was terminated for 5 scaffolds on 7^{th} day of initial seeding and decellularization was done. Remaining 5 scaffolds were continued in culture for 15 days with medium change at every alternate day and culture was terminated on 15^{th} day of initial seeding and decellularization was done.

Decellularization of scaffolds was done by a modified hypotonic–alkaline–detergent treatment cycle (Meyer et al., 2006).The scaffolds with cells were incubated in deionized water for 3 h at 4°C. After 3 h, the water was aspirated and a hypotonic solution containing 0.09% sodium chloride (Sigma, USA) and 0.02% sodium azide (Merck, Germany) was added and incubated for 1 h at 37°C in an environ shaker (Kuhner Shaker, Switzerland) at a shaking speed of 75 rpm. After 1 h of incubation, the solution was aspirated and the scaffolds were incubated for 1 h with a solution containing 1% Triton-X 100 (Sigma Aldrich, USA), 0.1% ammonium hydroxide (Sigma, USA) and 0.9% sodium chloride (Sigma, USA). The scaffolds were then washed thoroughly with PBS and deposition of ECM protein, collagen, was quantified by specific assay. For collagen estimation, the decellularized scaffolds were incubated overnight in 0.5M acetic acid containing 1% w/v pepsin (Sigma, USA) at 37°C. 100µl was taken from the test material and 1ml of Sircol dye reagent was added. This was given a gentle mechanical shaking for 30 minutes at a shaking speed of 75rpm at 37°C. Collagen dye complex was precipitated by centrifugation at 10000g for 10 minutes. The supernatant was removed and the pellet was dissolved in 0.5M NaOH (Merck, Germany). The absorbance was read at 555nm in a microplate

reader (iMark Microplate reader, Bio-rad, USA). Quantity of collagen was calculated from a calibration curve obtained using serially diluted collagen as standard.

3.3.8. Quantification of ECM marker expressions

Collagen and elastin deposited on the cell seeded scaffolds were estimated by quantitative real time PCR by culturing induced SMPCs on the ablumen of the scaffold for 3d, 7d and 15d. RNA was isolated from the cells at respective time periods using TRIZOL reagent (Invitrogen, USA) based on the manufacturer's protocol. RNA quantification was done spectrophotometrically using Nanodrop 1000 Spectrophotometer (ND 2000; Thermo Scientific, USA). 200ng of total RNA was converted to cDNA using OrionX cDNA kit (Origin, India) in a thermal cycler (Master cycler; Eppendorf). Amplification of genes using specific intron-spanning primers for ECM markers were analysed by qRT-PCR. Real time PCR was carried out in a total volume of 15µl containing 20ng cDNA, 100 pmol each of respective forward and reverse primers and 7.5 µl of OrionX 2X Real time PCR master mix (Origin, India). Forty cycles of reaction were performed using the Bio-Rad iQ5 Real time PCR detection system (Bio-Rad, USA) under the following conditions: Enzyme activation, 95°C for 15 minutes; denaturation, 95°C for 30 seconds; annealing, 50°C for 20 seconds; and extension, 72°C for 20 seconds. GAPDH was used as house-keeping gene. Melt curve analysis was performed for each gene to confirm the specificity of each reaction. Products were analyzed by agarose gel electrophoresis for correct amplicon size. Fold change in expression was calculated after normalization with GAPDH expression on each day of analysis using the formula $2^{-\Delta\Delta Ct}$. List of primers used for the study are given in Table 6.

Table 6 – Primer sequences used for qRT PCR analysis

Genes	Amplicon Size	Primer sequence (5'-3')
GAPDH	120bp	FP - GAA ATC CCA TCA CCA TCT TCC AGG RP - GAG CCC CAG CCT TCT CCA TG
Collagen 1	120bp	FP- CCA AGG GTA ACA GCG GTG A RP- GCT TTC CTT CCT CTC CAG CA
Elastin	79bp	FP- CCT CCA CCC CTC TCG GCC TG RP- CAG CGC TGG ATA AAA GAC TCC TCC A

3.3.9. Characterization of deposited ECM

ECM deposited on the coated scaffolds were characterised by immunostaining the ECM proteins, elastin and collagen on the ablumen of the scaffold. Briefly, scaffolds of 2cm length were coated with matrix specific for differentiation of hADMSCs to SMPCs, as described in 3.3.2. hADMSCs were seeded on the ablumen of the coated scaffolds at a seeding density of 10,000cells/cm^2 and induction was initiated for 72h. After 72h, induction was withdrawn and the cell seeded scaffolds were cultured for 15 days with medium change at every alternate day. Decellularization of the scaffolds was done by a modified hypotonic–alkaline–detergent treatment cycle (Meyer et al, 2006). After decellularization, the scaffolds were washed thoroughly with phosphate buffered saline (PBS), fixed with 3.7% paraformaldehyde (Merck, Germany) for 20 minutes at room temperature and washed. The scaffolds were incubated with primary antibody against Collagen I (ab6308, Abcam, U.K.) and elastin (ab9519, Abcam, U.K.) overnight at 4℃. Following primary antibody incubation, the scaffolds were stained with secondary antibody Alexa Fluor 488 anti - mouse IgG (ab150113, Abcam, U.K.) for 2h. Fluorescent images of immunostained Collagen I and elastin positive areas in the scaffolds were imaged using fluorescent microscope (DMIRB, Leica Microsysytems, Wetzlar, Germany).

3.3.10. Mechanical strength of SMPC seeded and bare scaffolds

Mechanical strength of cell seeded and bare scaffolds were determined using a universal testing machine (UTM, Instron3365, UK). Outer diameter and wall thickness of each scaffold was measured. Test was conducted uniaxial at cross head speed of 1 mm/min using a 10 N load cell. Maximum load (*N*) is obtained from the load-extension curve. Briefly, 12 scaffolds of 6cm length were coated with matrix specific for differentiation of hADMSCs to SMPCs, as described in 3.3.2. 6 coated scaffolds, without cells seeded on them, were immersed in MCDB 131 medium (Gibco, USA) containing 1% FBS (Gibco, USA), L-glutamine (Sigma, USA) and L-ascorbic acid (Sigma, USA) for 15 days and they were termed as bare scaffolds. hADMSCs were seeded on the ablumen of the other 6 coated scaffolds (termed as cell seeded scaffolds) at a seeding density of 10,000cells/cm^2 and induction was initiated for 72h. After 72h, the induction was withdrawn and the culture was continued for 15 days with medium change at every 48h.On day 15, all the scaffolds were taken out, washed thoroughly and mechanical strength was assessed.

3.4. Fabrication of TEVG

3.4.1. Fabrication of poly-ε-caprolactone (PCL) scaffolds

The scaffold fabrication was done as per the method of Soumya et al (Soumya et al., 2014). The polymer, PCL granules (Mn 45,000; Aldrich Chemicals, USA) were dissolved in 1:1 wt% polyethylene glycol (Mw 3400; Merck, Germany) in dichloromethane (AR grade; SD Fine Chemicals, India). Stainless steel mandrels of 3.5±0.5mm diameter was dipped in the polymer solution, dried the scaffold by air drying the solvent and was immersed in water to carefully remove tubular scaffold from the mandrel. The porogen was leached out by dipping the scaffold in distilled water for 72h to achieve a porous but smooth lumen. The scaffold was then subjected to lyophilization in a freeze dryer (Modulyo 4K Freeze dryer, Edwards, UK). After lyophilisation, another layer was laid over the solvent cast scaffold by electro spinning using high molecular weight PCL (Mn 80,000; Sigma-Aldrich Chemicals,

USA),dissolved in dichloromethane. The spinning parameters were as follows: mandrel speed-1000rpm, flow rate- 3ml/h, duration of spinning for each scaffold-12 minutes and distance from the tip to the mandrel-20cm.

3.4.2. Coating of scaffolds with Fibrin

The fabricated scaffolds were dipped in 70% alcohol for 30 minutes for sterilization, washed with sterile deionized water and lyophilized in a freeze dryer (Modulyo 4K Freeze dryer, Edwards, UK). The lyophilized scaffolds were then dipped in diluted filtered thrombin (2IU/ml) for 30 minutes at 37°C, thrombin was completely removed and the scaffolds were again lyophilized in a freeze dryer. In the next step, the lyophilized scaffolds were coated with fibrinogen (2mg/ml) containing 0.2 mg fibronectin and exogenous gelatin (0.2%; Sigma, USA), and was allowed to clot. The clot formed was stabilized by incubating the scaffolds at 37°C for 30 min, lyophilized in sterile atmosphere, and stored at 4°C until used. For cell culture experiments, scaffolds were plasma sterilized onto which hADMSCs were seeded for the differentiation experiments. The scaffolds were coated with specific matrix for each differentiation study.

3.4.3. SMPC seeding on the ablumen of the scaffold

The lyophilised scaffolds were coated on the outer surface with matrix specific for differentiation of hADMSC to SMPC as described in 3.3.2. hADMSCs were seeded on the ablumen of the coated scaffolds at a seeding density of 10,000cells/cm^2. The cell seeded scaffolds were kept in the incubator at 37°C for 30 minutes for complete attachment of cells, after which the scaffolds were kept in induction medium, as described in 3.3.2, and induction was initiated for 72h. After 72h, the induction was withdrawn and the cell seeded scaffolds were immersed in MCDB 131 medium (Gibco, USA) containing 1% FBS (Gibco, USA), L-glutamine (Sigma, USA) and L-ascorbic acid (Sigma, USA) for further analysis.

3.4.4. EPC seeding in the lumen of the scaffold

The lumen of the lyophilised scaffolds were coated with matrix as described in 3.2.2. On the 4th day of seeding SMPC on the outer electrospun compartment of scaffolds, hADMSCs induced for differentiation to EPCs, as described in 3.2.2, were seeded in the lumen of SMPC seeded scaffolds at a seeding density of 10,000cells/cm^2.The cell seeded scaffolds were kept in a horizontal position in the incubator at 37°C for 30 minutes with occasional rolling for the proper attachment of cells in the entire inner surface of the scaffold. The scaffolds were then connected to bioreactor to culture the cells under shear stress.

3.4.5. Culture of TEVG under shear stress

EPC and SMPC seeded scaffolds were connected to a bioreactor (Masterflex L/S pump, ColeParmer, USA) and the scaffolds were exposed to continuous medium flow with a flow rate of 20ml/min which gave a shear stress of 11.6 dynes/cm^2 .The cell seeded scaffolds were cultured under shear stress for 4d after which the scaffolds were disconnected and further analysis was done.

3.5. Exposure of TEVG to Risk Factors

hADMSCs induced to EPCs and SMPCs were seeded on fibrin coated bilayered PCL scaffolds for studying endothelial dysfunction by exposing the cell seeded scaffolds to high glucose, high cholesterol and the inflammatory cytokine, TNF-1α. Briefly, scaffolds of 6cm length were coated with matrix specific for differentiation of hADMSCs to SMPCs, as described in 3.3.2. hADMSCs were seeded on the ablumen of the coated scaffolds at a seeding density of 10,000cells/cm^2 and induction was initiated for 72h. After 72h, the induction was withdrawn. hADMSCs induced for differentiation to EPCs, as described in 3.2.2 were seeded in the lumen of the scaffolds grown with SMPC. The cell seeded scaffolds were connected to the bioreactor with the scaffolds exposed to a continuous medium at a high shear rate for 4 days. At a time, six scaffolds were connected to the bioreactor with 2 scaffolds exposed to normal MCDB 131 medium (Gibco, USA), 2 scaffolds exposed to

MCDB 131 medium with high glucose (Sigma, USA) and 2 scaffolds exposed to MCDB 131 medium with high cholesterol (Sigma, USA). The experiment was repeated three times to get 6 replicate samples for each analysis.

3.5.1. Exposure to normal medium (NM)

The cell seeded scaffolds connected to the bioreactor were exposed continuously to MCDB 131 (Gibco, USA) medium containing 10% FBS (Gibco, USA), Antibiotic-Antimycotic solution (Invitrogen, USA), heparin sulphate (Sigma, USA), L-glutamine (Sigma, USA) and L-ascorbic acid (Sigma, USA).

3.5.2. Exposure to high glucose (HG)

The cell seeded scaffolds connected to the bioreactor were exposed to a continuous medium of MCDB 131 (Gibco, USA) containing 10% FBS (Gibco, USA), Antibiotic-Antimycotic solution (Invitrogen, USA), heparin sulphate (Sigma, USA), L-glutamine (Sigma, USA), L-ascorbic acid (Sigma, USA) and glucose (Sigma, USA) at a concentration of 25.5mmol/l (460mg/dl).

3.5.3. Exposure to high cholesterol (HC)

The cell seeded scaffolds connected to the bioreactor were exposed to a continuous medium of MCDB 131 (Gibco, USA) containing 10% FBS (Gibco, USA), Antibiotic-Antimycotic solution (Invitrogen, USA), heparin sulphate (Sigma, USA), L-glutamine (Sigma, USA), L-ascorbic acid (Sigma, USA) and cholesterol (Sigma, USA) at a concentration of 6.5mmol/l (500mg/dl).

3.5.4. Exposure to TNF-1a

The cell seeded scaffolds connected to the bioreactor were exposed to a continuous medium of MCDB 131 (Gibco, USA) containing 10% FBS (Gibco, USA), Antibiotic-Antimycotic solution (Invitrogen, USA), heparin sulphate (Sigma, USA),

L-glutamine (Sigma, USA), L-ascorbic acid (Sigma, USA) and 4ng/ml TNF-1α (Sigma, USA).

3.5.5. Exposure to TNF-1α and high glucose

The cell seeded scaffolds connected to the bioreactor were exposed to a continuous medium of MCDB 131 (Gibco, USA) containing 10% FBS (Gibco, USA), Antibiotic-Antimycotic solution (Invitrogen, USA), heparin sulphate (Sigma, USA), L-glutamine (Sigma, USA), L-ascorbic acid (Sigma, USA) and 25.5mmol/l glucose(Sigma, USA).To the high glucose medium,4ng/ml TNF-1α (Sigma, USA) was added to prepare medium with high glucose and TNF-1α.

3.5.6. Exposure to TNF-1α and high cholesterol

The cell seeded scaffolds connected to the bioreactor were exposed to a continuous medium of MCDB 131 (Gibco, USA) containing 10% FBS (Gibco, USA), Antibiotic-Antimycotic solution (Invitrogen, USA), heparin sulphate (Sigma, USA), L-glutamine (Sigma, USA),L-ascorbic acid (Sigma, USA) and 6.5mmol/l cholesterol (Sigma, USA).To the high cholesterol medium, 4ng/ml TNF-1α (Sigma, USA) was added to prepare medium with high glucose and TNF-1α.

3.6. Analysis of effects of risk factor exposure

3.6.1. Assessment of NO release

Functionality of EPCs grown inside the lumen of the scaffolds after exposure to normal medium, medium with high glucose and medium with high cholesterol was evaluated by measuring nitrite as per the procedure described in 3.2.5. For this assay, EPCs grown in the lumen of the scaffold for 4 days were perfused with phenol red-free M199 medium and the amount of nitrite formed was calculated.

3.6.2. Quantification of EPC marker expressions

After 4 days of dynamic culture, the scaffolds were disconnected, RNA was isolated as per the procedure described in 3.2.7 and the quantitative gene expression by real time PCR, on exposure to normal medium and medium with high glucose and high cholesterol, of pro/anti-thrombotic molecules (vWF/eNOS), pro/anti-fibrinolytic molecules (tPA / PAI), pro inflammatory molecule (MCP-1) and adhesion molecules (VCAM-1, CD 31) was done. Quantitative expression of EPC genes upon activation with the inflammatory marker, TNF-1α along with glucose and cholesterol was also analyzed.

3.7. Statistical analysis

Statistical significance was calculated by Student T Test for all quantitative data. Mean values and standard deviation (SD) were calculated for all parameters and are represented in graphical form. Significance is labelled in the graphs with '***' ($P<0.001$), '**' ($P<0.01$); and '*' ($P<0.05$).The number of replicate experiments carried out is indicated in the legends of each figure.

CHAPTER 4

4. RESULTS

The results of the study are illustrated in this chapter. The results are divided into three main sections. The first section demonstrates the outcome of protocols standardized for differentiation of isolated hADMSCs to EPCs and SMPCs, in the end proving the lineage commitment. The second section demonstrates the fabrication of bilayered small diameter PCL conduits, seeding and culture of EPC and SMPC on different layers of the scaffold and establishing the cellular functions upon dynamic culture. The third section demonstrates the use of functional TEVG to quantify the subtle changes in EC phenotype on exposing the graft lumen to specific risk factors such as high glucose, cholesterol and inflammatory cytokine (TNF-1α).

4.1. Properties of isolated hADMSCs

The tissue collected from operating room was found aseptic and suitable for cell isolation and culture **(Figure 1A)**. The trimmed and washed adipose tissue showed a pink colour due to traces of contaminating blood cells **(Figure 1B)**. Enzyme digestion and repeated washing by centrifugation in culture medium eliminated most of the blood cells. The blood cells were completely eliminated by frequent medium change after plating. The plastic adherent hADMSCs **(Figure 1C)** showed spindle shape in 24h and proliferated in 4-5 days **(Figure 1D)**.

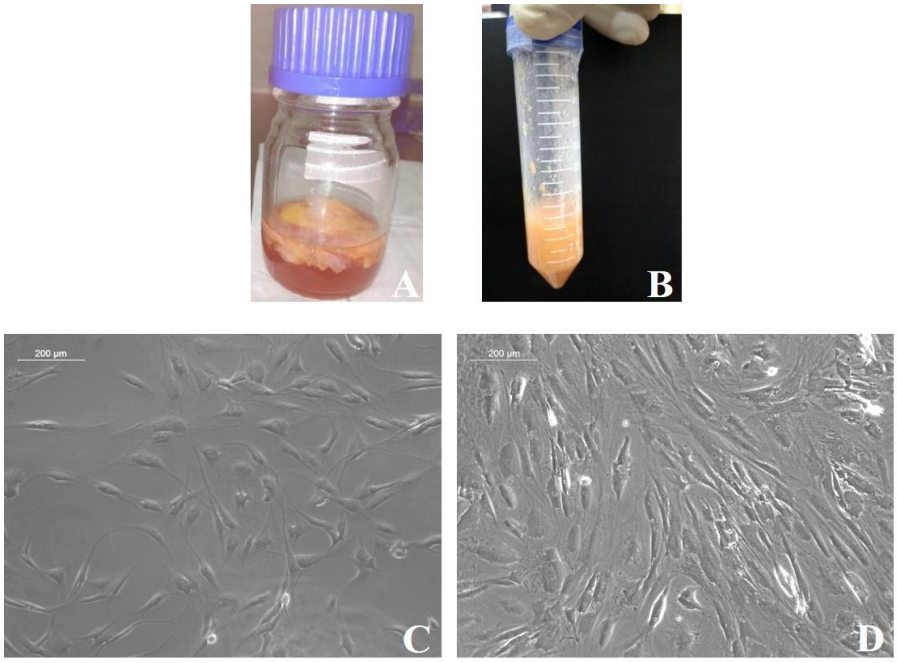

Figure 1. Different stages of hADMSCs isolation and culture. (A) Human adipose tissue; (B) Digested adipose tissue by collagenase treatment: (C) & (D) Phase contrast image of hADMSC morphology at passage 0 and passage 1, respectively. Scale bar: 200µm

Specifically stained cells in culture confirms the tri-lineage differentiation potential of isolated hADMSCs. Alizarin Red S stained **(Figure 2A)**, Oil Red O stained **(Figure 2B)** and Toluidine Blue stained **(Figure 2C)** cells in cultures of hAMDSCs grown in each specific medium confirmed the osteogenic, adipogenic and chondrogenic lineage commitment, respectively. Flow cytometry analysis of P3 hADMSCs showed positivity in acceptable range for cell surface markers CD90, CD73 and CD105 and <2% positivity for haematopoietic markers CD14, CD34 and CD45 **(Figure 2D),** confirming that the proliferating cells are relatively pure MSCs.

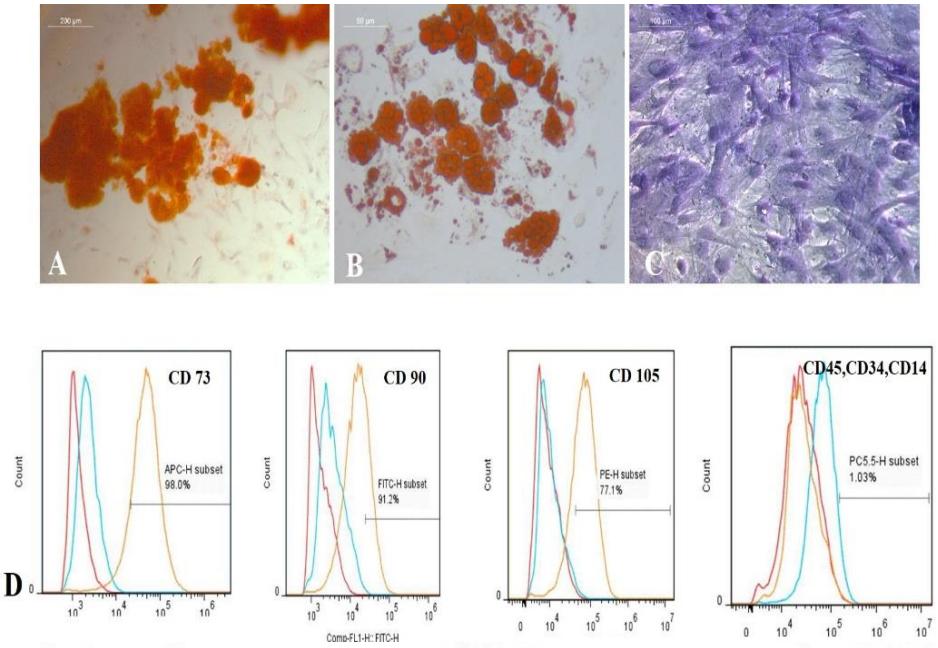

Figure 2. Purity and property of hADMSCs in culture. (A) Osteogenic differentiation (Alizarin Red S staining); (B) Adipogenic differentiation (Oil Red O staining); (C) Chondrogenic differentiation (Toluidine Blue staining). Magnification of each image is marked using scale bar; (D) Flow cytometry histogram shift of P3 hADMSC characterized using cell surface markers

4.2. Induction of hADMSCs to EPCs

Sub cultured hADMSCs in P3 showed spindle morphology before growth factor induction **(Figure 3A)**, but upon induction with growth factors for 48h, there was a visible difference in morphology. Elongated slender cells exhibited a tubular pattern, typical of angiogenic cells **(Figure 3B)**. Exposure to hypoxia **(Figure 3C)** for 20h transformed the elongated morphology to a more uniform cobblestone morphology **(Figure 3D)**, characteristic of endothelial cells.

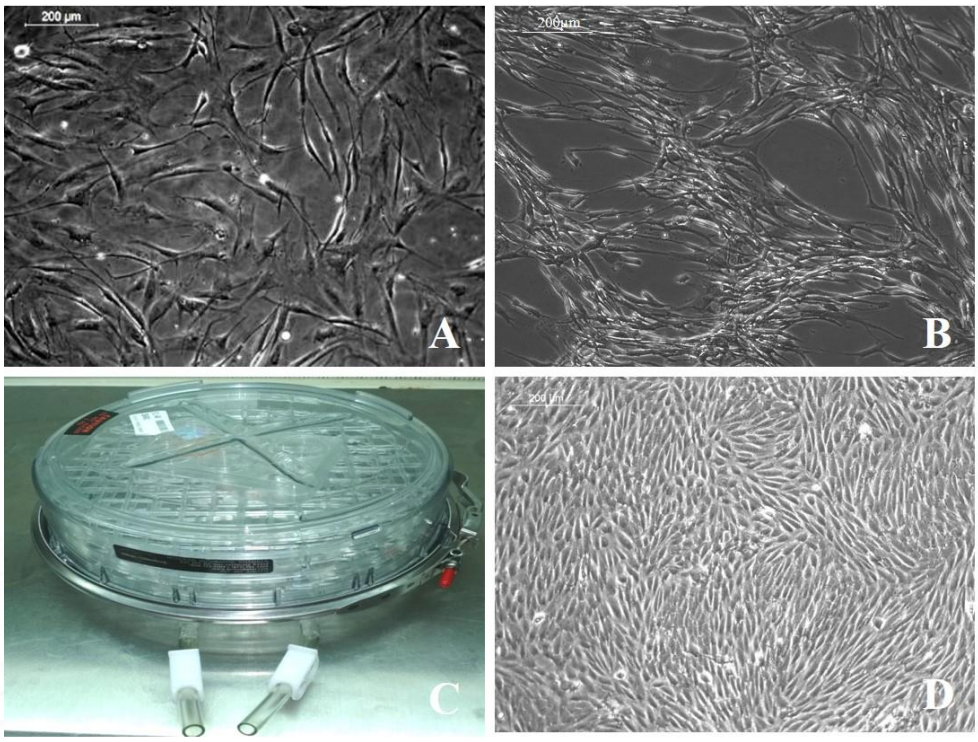

Figure 3. Characteristics of hADMSCs induced to EPCs. (A)Phase contrast image of P3 hADMSCs before GF induction; (B)Phase contrast image of P3 hADMSCs 48h after GF induction; (C) GF treated cells in hypoxia chamber; (D) Phase contrast image of P3 hADMSCs after hypoxia induction. Scale bar: 200µm

4.2.1. Identification of EPC markers by immunocytochemistry

The GF and hypoxic induction established the differentiation of hADMSCs to EPCs as evidenced by expressions of EPC specific markers at protein level. The transformation of hADMSCs into endothelial like cells (ELC) was confirmed by AcLDL uptake and expression of endothelial marker CD31. In both cases, the expression is comparable to the positive control HUVEC; AcLDL uptake **(Figure 4A, B)** and CD31 expression **(Figure 4C, D).** The hADMSC-derived EPC showed more elongated morphology as compared to HUVEC.

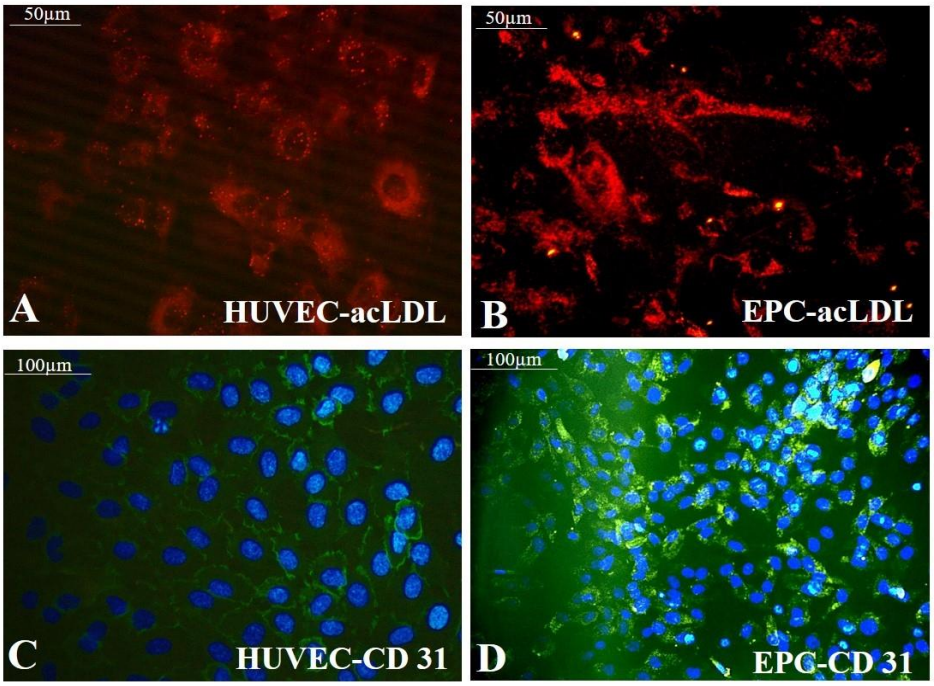

Figure 4. Identification of EPC markers against HUVEC. Fluorescent micrographs of (A) AcLDL (red) in HUVEC; (B) AcLDL (red) in EPC; (C) CD 31(green) in HUVEC; (D) CD 31(green) in EPC. Cells are counterstained with DAPI (blue). Magnification of each image is marked using scale bar.

The other marker up-regulations were compared with hADMSCs. All three antigens, eNOS **(Figure 5A)**, VCAM-1 **(Figure 5C)** and VE-Cadherin **(Figure 5E)** are seen significantly higher than the respective control-stained hADMSCs **(Figures 5B, 5D and 5F)**. The eNOS stained nucleus demonstrates cyan colour **(Figure 5A)** due to dual colours of DAPI (blue) and eNOS (green).

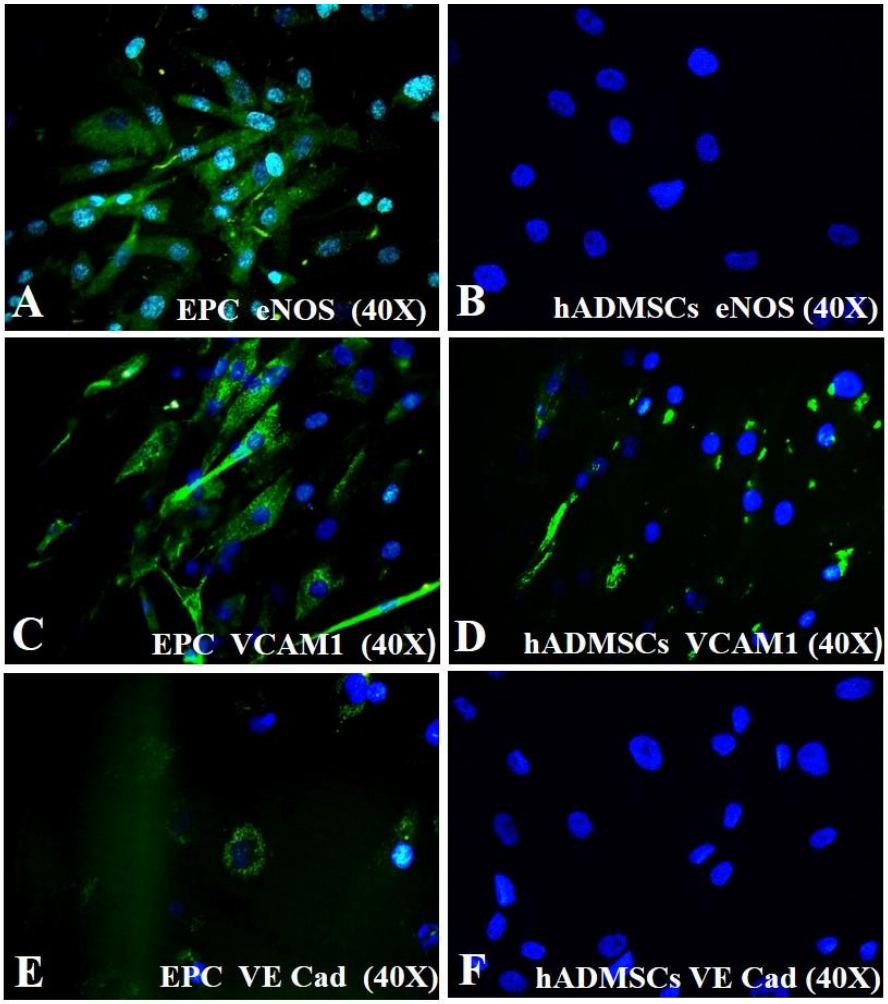

Figure 5. Identification of EPC markers against normal hADMSCs. Fluorescent micrographs of (A) eNOS (green) in EPC; (B) eNOS (green) in undifferentiated hADMSCs; (C) VCAM-1(green) in EPC; (D) VCAM-1 (green) in undifferentiated hADMSCs; (E) VE-Cadherin (green) in EPC; (F) VE-Cadherin (green) in undifferentiated hADMSCs. Cells are counterstained with DAPI (blue).Magnification of each image is represented in the figure.

4.2.2. Effect of induction step on expression of EPC markers

Gene expressions of EPC markers analyzed at different time periods is represented graphically. Significant up-regulation of endothelial specific markers CD 31 and VCAM-1 after dynamic culture are evident **(Figure 6)** indicating the importance of shear stress in differentiating the cells to endothelial lineage. Initial induction with growth factors up-regulated CD31, but after hypoxic induction, there was a down regulation. On exposure to shear stress for 4 days, the expression of CD 31 was significantly up regulated to ~9 fold in comparison with that in static culture of hADMSCs induced with GF, hypoxia and grown in static condition for 4 days. The observation highlights the importance of shear stress for CD 31 expression. Similarly, VCAM-1 also showed different expressions at different steps with statistically significant lesser expression after hypoxic induction and a higher expression after dynamic culture indicating the tendency of cells to differentiate to endothelial lineage.

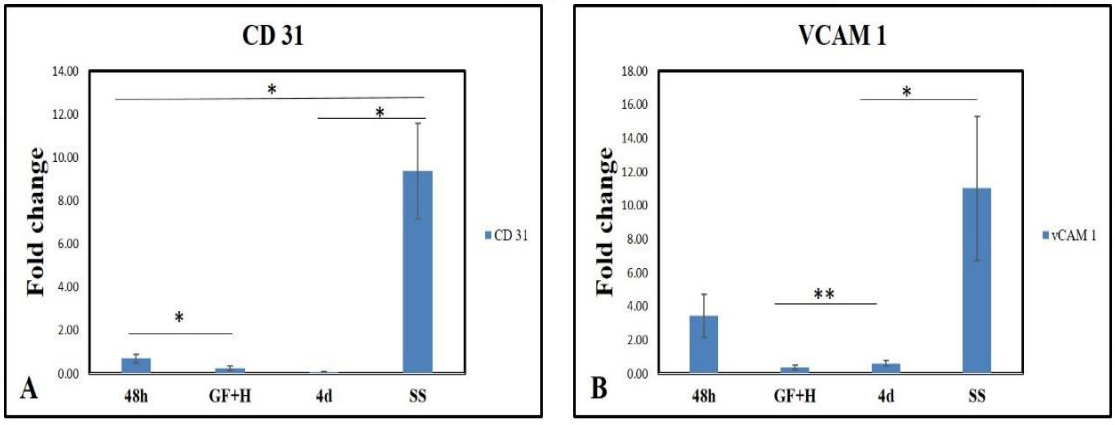

Figure 6. Graphical representation of relative gene expressions of membrane protein markers in EPCs. The relative gene expression of (A) CD 31; and (B) VCAM-1 at 48h of growth factor induction (48h), 20h of hypoxic induction (GF+H), 4d of static culture (4d), 4d of culture under shear stress (SS). All gene expressions in EPC was normalized to the control ADMSCs and represented graphically. Fold change is quantified relative to GAPDH expression on each day of analysis using the $2^{-\Delta\Delta Ct}$ method. Error bars represent standard error. (n=4). *P<0.05, **P<0.01

The pro-thrombotic molecule vWF and anti-thrombotic marker eNOS respectively, showed a similar pattern of expression at different periods of culture **(Figure 7)**. In comparison with 4 days of static culture, eNOS expression showed a significant increase of ~9 fold up regulation under the influence of shear stress. Similarly, when induction with growth factors alone was compared with the effect of shear stress, eNOS expression was upregulated under shear stress. This shows the continuous expression of eNOS, which is a hall mark of a healthy continuous monolayer of endothelium. Expression of vWF was also upregulated under shear stress as compared with other periods of culture namely 48h of GF induction, hypoxic induction and 4 days of static culture. This confirms that shear stress plays a major role in modulating the expression of functional protein marker vWF.

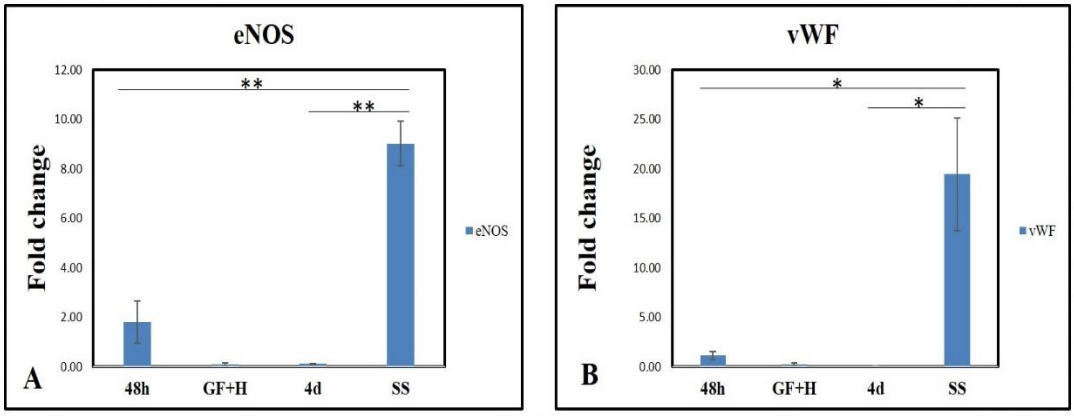

Figure 7. Graphical representation of relative gene expressions of eNOS & vWF in EPCs. The relative gene expression of (A) eNOS ; and (B) vWF at 48h of growth factor induction (48h), 20h of hypoxic induction (GF+H), 4d of static culture (4d),4d of culture under shear stress (SS), normalized to the control hADMSCs is represented graphically. Fold change is quantified relative to GAPDH expression on each day of analysis using the $2^{-\Delta\Delta Ct}$ method. Error bars represent standard error. (n=4). *P<0.05, **P<0.01

The expression of pro fibrinolytic molecule tPA was nominally higher than that in hADMSCs after all inductions **(Figure 8)**. The expression of tPA after growth factor induction and 4 days of dynamic culture was comparable. No synergistic effect of

multiple induction protocol was evident. Therefore, the basal tPA expression seems steady throughout various induction stages. On the other hand the anti-fibrinolytic molecule PAI, showed a significant up regulation after the combined induction of growth factors and hypoxia when compared to growth factor induction alone. Then the expression of PAI was similar after 4 days of static culture and 4 days of dynamic culture indicating that PAI expression is unaffected by shear stress.

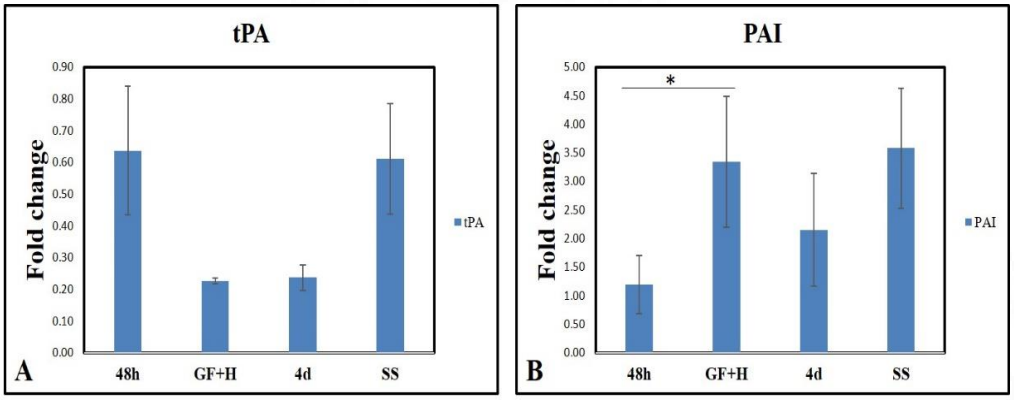

Figure 8. Graphical representation of relative gene expressions of tPA & PAI in EPCs. The relative gene expression of (A) tPA; and (B) PAI at 48h of growth factor induction (48h), 20h of hypoxic induction (GF+H), 4d of static culture (4d), 4d of culture under shear stress (SS), normalized to the control ADMSCs is represented graphically. Fold change is quantified relative to GAPDH expression on each day of analysis using the $2^{-\Delta\Delta Ct}$ method. Error bars represent standard error. (n=4). *P<0.05

The chemokine, MCP1 which is a pro inflammatory molecule was up regulated >5 folds upon GF induction alone **(Figure 9)**. The hypoxia reduced the expression level, and was further reduced by static culture. After applying shear stress, the MCP1 expression was similar to that in hADMSCs. The observation indicates that shear stress is an important parameter that regulates EPC into a non-inflammatory phenotype.

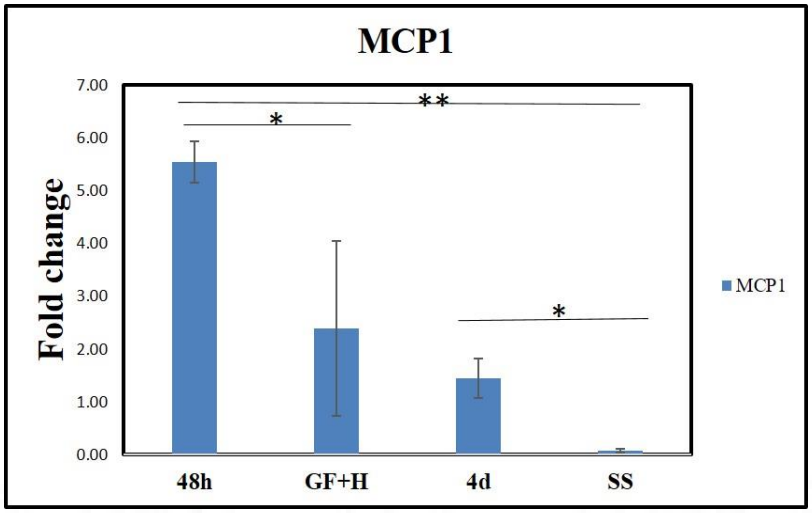

Figure 9. Graphical representation of relative gene expression of MCP1 in EPCs. The relative gene expression of MCP1 at 48h of growth factor induction (48h), 20h of hypoxic induction (GF+H), 4d of static culture (4d),4d of culture under shear stress (SS), normalized to the control ADMSCs is represented graphically. Fold change is quantified relative to GAPDH expression on each day of analysis using the $2^{-\Delta\Delta Ct}$ method. Error bars represent standard error. (n=4). *P<0.05, **P<0.01

Overall, up regulation of all 7 endothelial specific markers, in the hADMSCs induced sequentially with GF, hypoxia and shear stress, as compared to normal hADMSC cultured for similar period of time confirms the differentiation of stem cells into EPCs. Also analysis of cells after each step indicated that all 3 steps are contributing to the differentiation and for maintenance of quiescent prototype.

4.3. Induction of hADMSCs to SMPCs

P3 hADMSCs treated with the growth factors, PGF and BMP4, in combination and independently, elicited different morphological changes in the induced cells. Finally, the combinatorial use of PGF and BMP4 at 0.6µg/ml and 2.5ng/ml respectively induced hADMSCs to smooth muscle cell lineage and this concentration of growth factors was fixed for further induction studies. hADMSCs on induction with PGF at 0.4µg/ml **(Figure 10 A)** and at 0.8 µg/ml **(Figure 10 B)** did not demonstrate smooth

muscle cell morphology. Similarly, the induction of hADMSCs with 0.8 µg/ml PGF in combination with 2.5ng/ml BMP4 **(Figure 10 C)** and 5ng/ml BMP4 **(Figure 10 D)** did not induce the cells to smooth muscle lineage. Instead, broad flattened cells with myocyte-like appearance were seen. hADMSCs induced with 0.6 µg/ml PGF alone **(Fig 10 E)** also resulted in cells with broad flattened appearance. But when PGF was used at a concentration of 0.6 µg/ml along with 2.5 ng/ml of BMP4 **(Fig 10 F)** for the induction, slender elongated cells were seen.

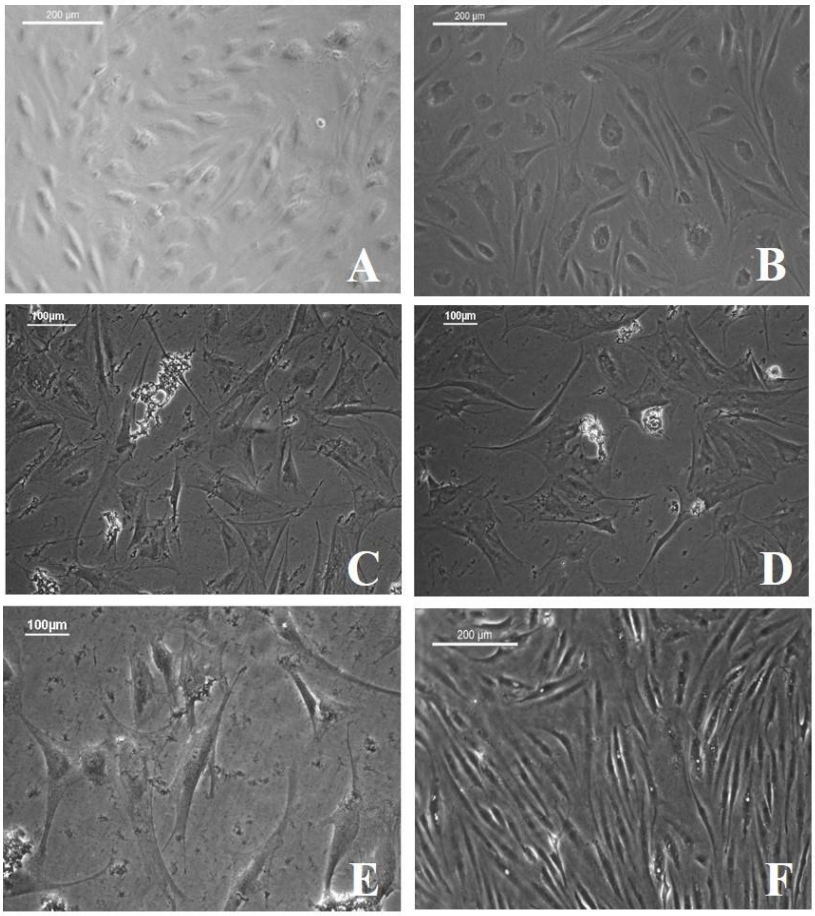

Figure 10. Morphological characteristics of hADMSCs induced to SMPCs. Phase contrast images of cells induced with (A) 0.4µg/ml of PGF; (B) 0.8µg/ml of PGF; (C) 0.8µg/ml PGF + 2.5ng/ml BMP4; (D) 0.8µg/ml PGF + 5ng/ml BMP4; (E) 0.6µg/ml of PGF; and (F) 0.6µg/ml of PGF + 2.5ng/ml of BMP4. Magnification of each image is marked using scale bar.

On further repeating the experiments, with the same combination of growth factors at different time periods such as 72h **(Figure 11 A)**, 120h **(Figure 11 B)** and 144h **(Figure 11 C)**, cells with 'hill and valley morphology', characteristic of smooth muscle cells, were observed.

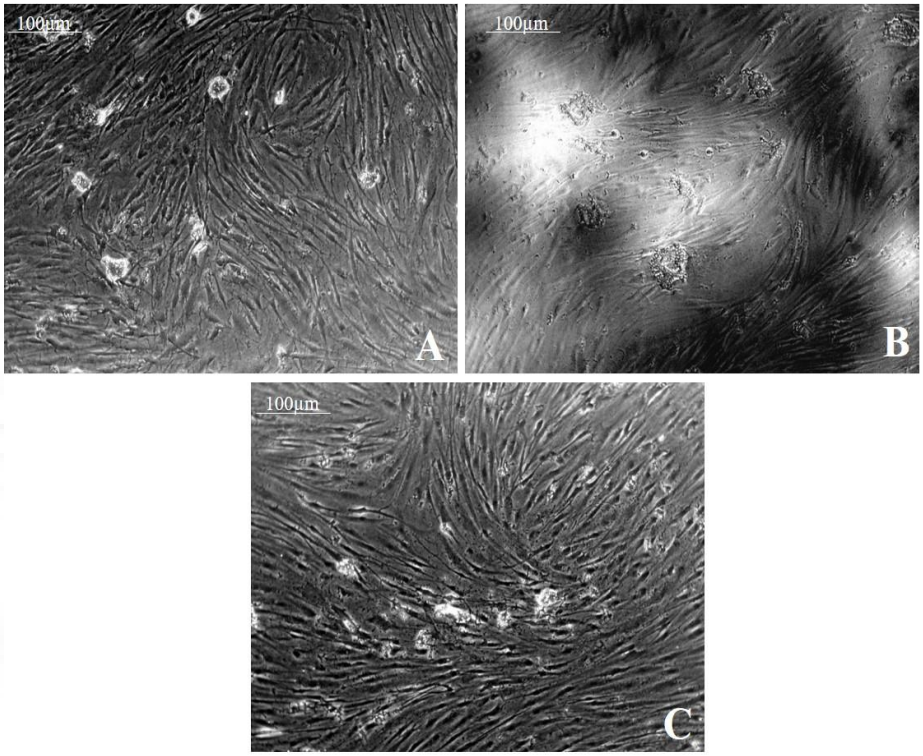

Figure 11. Effect of culture period on morphology of hADMSCs induced to SMPCs. Phase contrast images of cells induced with 0.6µg/ml of PGF+2.5ng/ml of BMP4 at (A) 72h of culture; B) 120h of culture; and (C) 144h of culture. Magnification of each image is marked using scale bar.

4.3.1. Identification of SMPC markers

The combined use of the growth factors PGF and BMP-4 resulted in the differentiation of hADMSCs to SMPCs. The differentiation was confirmed by the expression of SMPC specific markers at translational level. The smooth muscle cell lineage commitment of hADMSCs was confirmed by the presence αSMA **(Figure 12**

A, B), and calponin **(Figure 12 C, D)**, and the stained cells were compared with human umbilical artery SMCs as positive control.

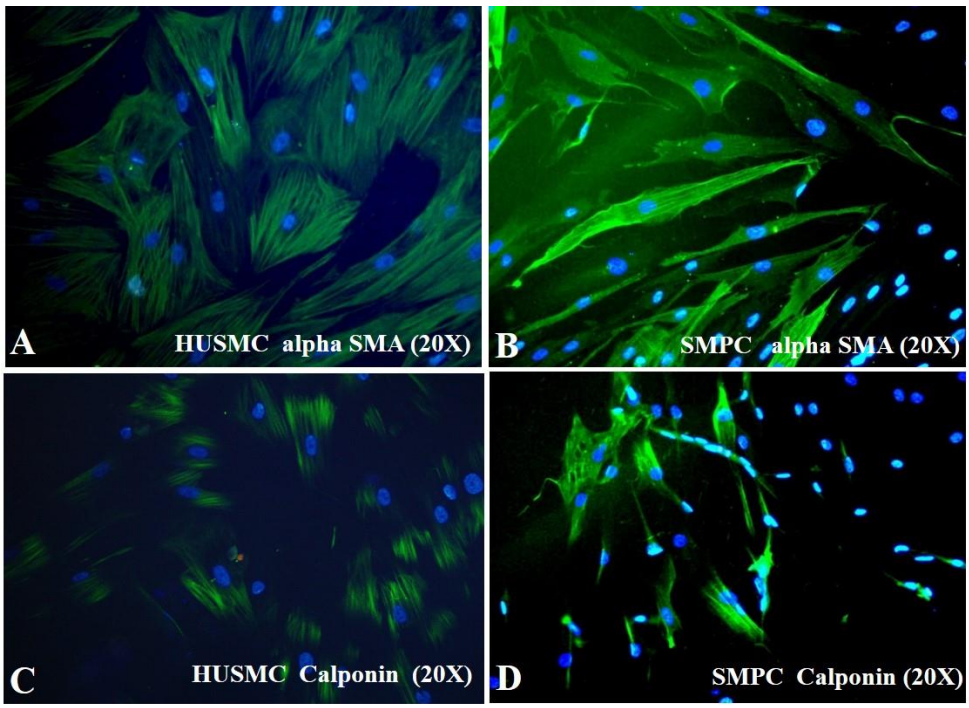

Figure 12. Identification of specific markers in SMPC vs HUASMC. Fluorescent micrographs of (A) αSMA (green) in HUSMC; (B) αSMA (green) in SMPC; (C) Calponin (green) in HUSMC ; and (D) Calponin (green) in SMPC. Cells are counterstained with DAPI (blue). Magnification of each image is represented in the figure.

Other markers detected were MHC11 **(Figure 13 A, B)** and PDGF Rα (**Figure 13 C, D)** by immunocytochemistry; undifferentiated hADMSCs were used as the negative control. Expression of αSMA, calponin and MHC11, early, mid and late SMC markers respectively, confirms the smooth muscle lineage of the induced cells. The presence of PDGF Rα shows the proliferative nature of the induced cells.

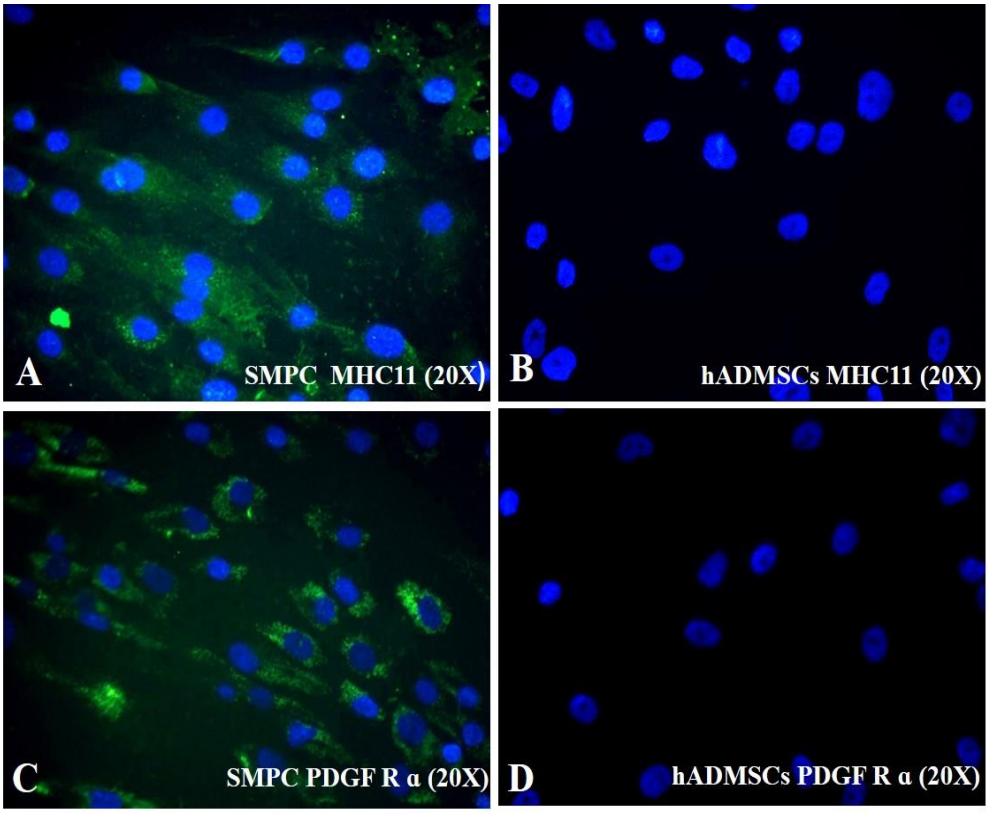

Figure 13. Identification of specific markers in SMPC vs hADMSCs. Fluorescent micrographs of (A) MHC11 (green) in SMPC; (B) MHC11 (green) in undifferentiated hADMSCs; (C) PDGF R α (green) in SMPC; and (D) PDGF R α (green) in undifferentiated hADMSCs. Cells are counterstained with DAPI (blue). Magnification of each image is represented in the figure.

Quantitative analysis of αSMA in induced cells by flowcytometry showed more than 35% positive cells expressing the marker when compared with unstained control (**Table 1).**

Quantification of SMPC marker -alpha SMA	
Donor 1	36.74%
Donor 2	42.95%
Donor 3	39.45%

Table 1. Flow cytometry analysis of αSMA in differentiated cells The experiments were carried out using cells from three donors

4.3.2. Expression of SMPC markers by qPCR

Gene expression data of SMPC markers established the lineage commitment of induced cells to smooth muscle cell phenotype. The graphical representation demonstrated that the early marker αSMA and mid marker calponin were negligibly expressed, while the expression of the late marker, MHC 11 was statistically significant **(Figure 14)**. This shows the contractile nature of the lineage committed cells.

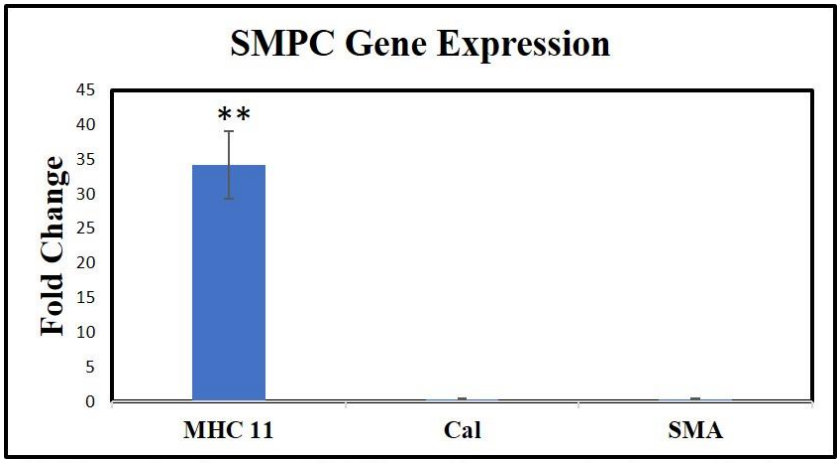

Figure 14. Relative gene expressions of SMPC markers MHC11, Calponin and α-SMA. The relative gene expressions of αSMA, Calponin (Cal) and MHC11, normalized to the control ADMSCs is represented graphically. Fold change is quantified relative to GAPDH expression on each day of analysis using the $2^{-\Delta\Delta Ct}$ method. Error bars represent standard error. (n=4). **P<0.01

4.4. Tissue engineering of vascular graft

4.4.1. Properties of bilayered scaffold

More than 50 bilayered scaffolds of 7±0.5cm length and 3.5±0.5mm diameter were fabricated for construction of tissue engineered vascular graft **(Figure 15 A, B)**. The scaffolds were subjected to leak test and about 90% of the fabricated scaffolds were leak proof. SEM analysis showed the smooth porous lumen in the solvent cast tube and the outer fibrous layer of the electrospun scaffolds **(Figure 15 C, D)**.

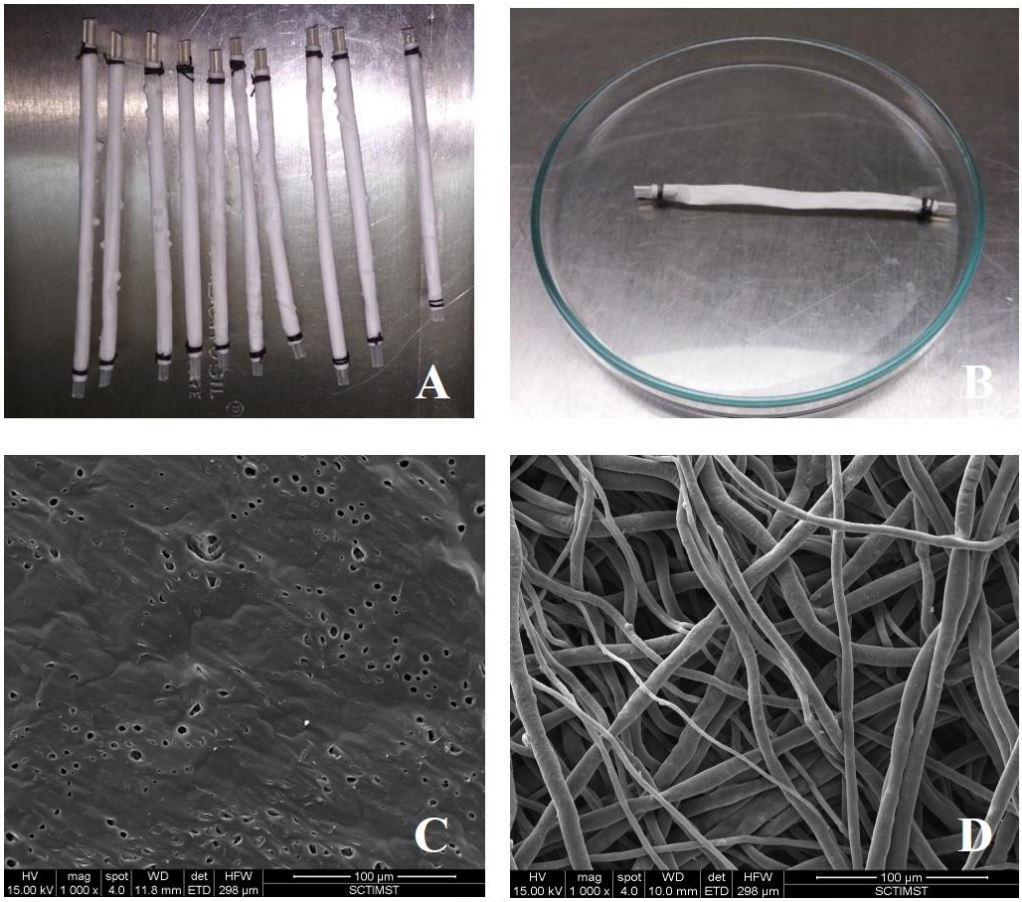

Figure 15. Properties of bilayered scaffold. (A) Gross image of the fabricated bilayered PCL scaffolds with lyophilized fibrin coating; (B) A single scaffold of 7cm length and 4mm diameter after coating with fibrin; (C) SEM image of the porous lumen of the scaffold; and (D) SEM image of the outer fibrous surface of the scaffold. Magnification 1000X

4.4.2. Seeding of cells and dynamic culture

The differentiated EPCs seeded in the lumen of fibrin coated bilayered PCL scaffold were subjected to shear stress for different days **(Figure 16 A, B, C)**.

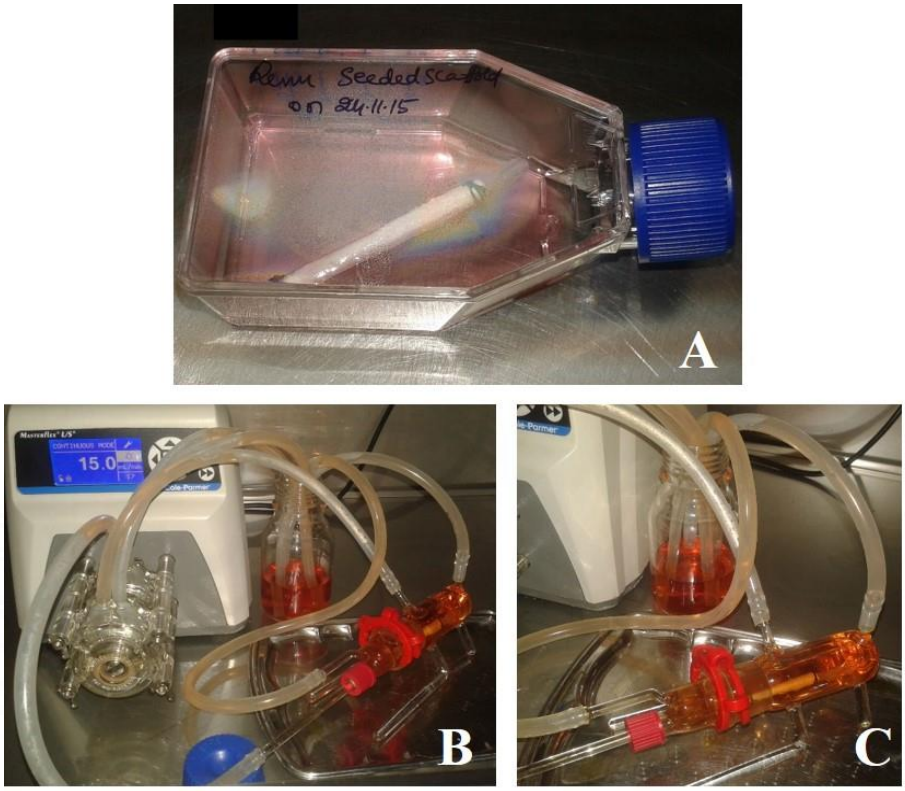

Figure 16. Various stages of construction of cell seeded graft. (A) Cell seeded graft in medium kept for attachment of cells (B) & (C) Cell seeded graft connected to bioreactor for dynamic culture

4.5. Properties of Cells in TEVG

4.5.1. EPC growth and function

SEM images established the morphology and alignment of differentiated EPCs in the lumen of the graft, after dynamic culture **(Figure 17)**. Elongated cells with cell to cell contact were seen. Stability and growth of cells in the lumen of the scaffold and aligned to direction of flow was evident from actin staining with cells clearly expressing actin filaments **(Figure 18)**.

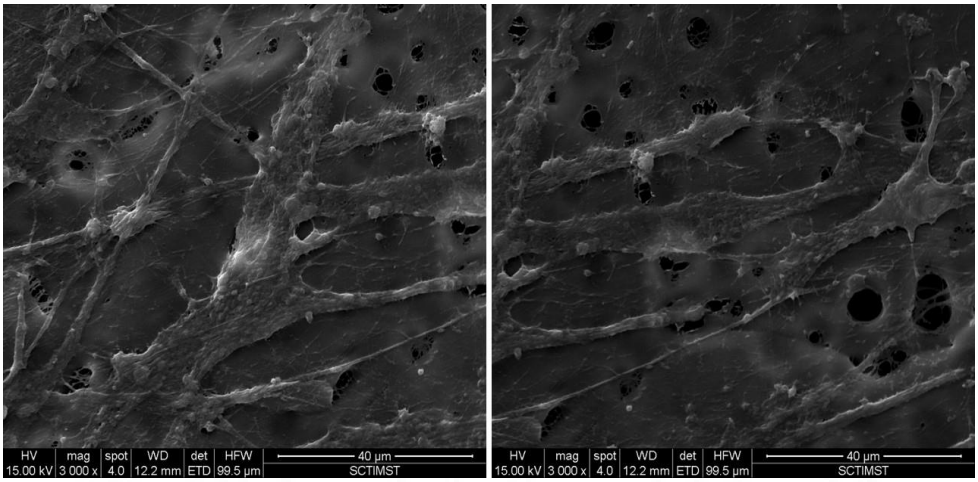

Figure 17. Morphology of cells in the lumen of the scaffold. Scanning Electron micrographs of cell seeded graft. Magnification 3000X

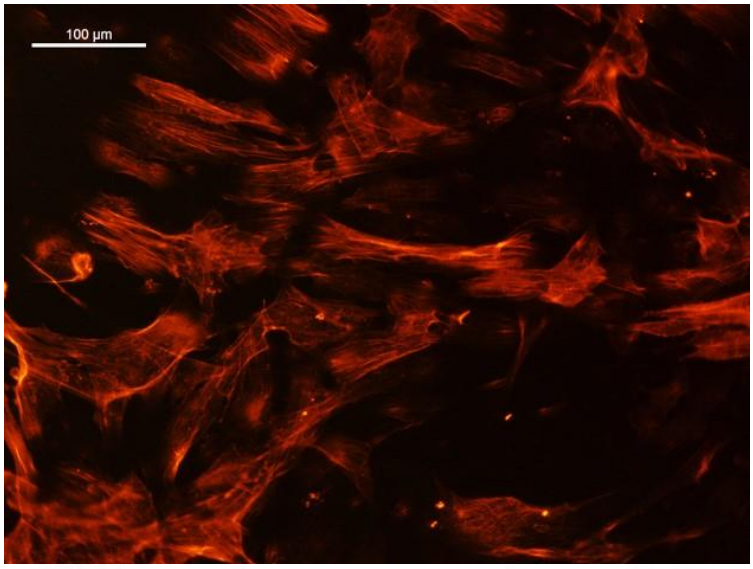

Figure 18. Cytoskeleton stained EPCs grown under shear stress. Fluorescent micrographs of Texas Red phalloidin staining of actin filaments of differentiated EPCs seeded in the lumen of PCL graft. Scale bar: 100µm

Nitric Oxide (NO) released into the medium after each study period was assessed **(Figure 19)**. The release of NO on 4th day was comparable with the release on 7th day and 14th day. Therefore, cell numbers in the monolayer were same on 4th day and

14th day and NO release was not increased by increasing the culture period. This demonstrates the formation of an endothelial monolayer in the lumen of the graft by day 4 and the regulated release of NO. Based on this observation, further dynamic cultures were limited to 4 days.

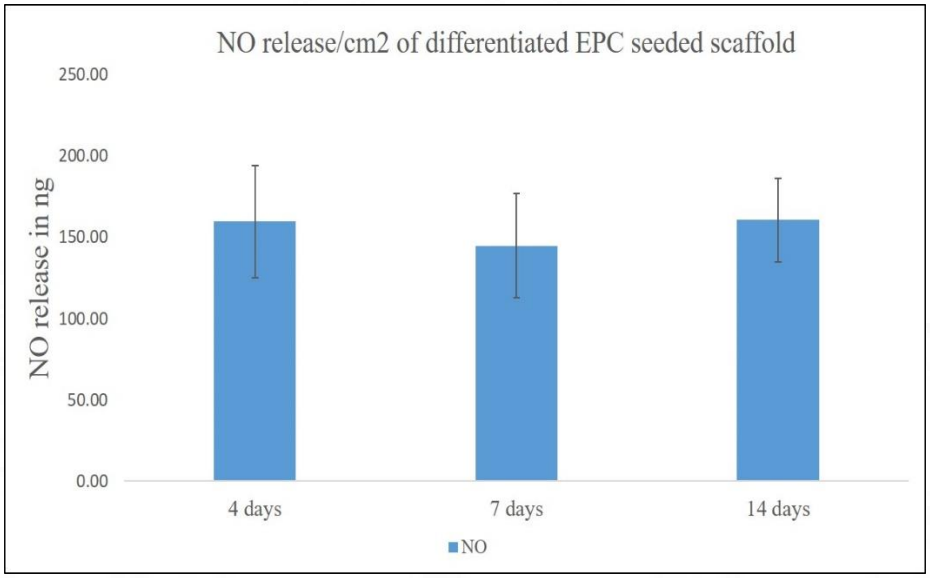

Figure 19. Graphical representation of NO release by EPCs. The pattern of NO release upon dynamic culture on different days. Error bars represent standard error. (n=4)

4.5.2. SMPC proliferation on scaffold ablumen

The extracted DNA of SMPCs was considered as a measure of proliferation. As compared to the DNA extracted within 2h of seeding, which is the baseline control, significant cell proliferation was observed by 3rd day. Since cells were grown on the fibrous side (ablumen), the extracted DNA was found to be insignificantly reduced on 7th day and 15th day with respect to 3rd day but was significantly higher with respect to the cells adhered in 2h **(Figure 20)**.

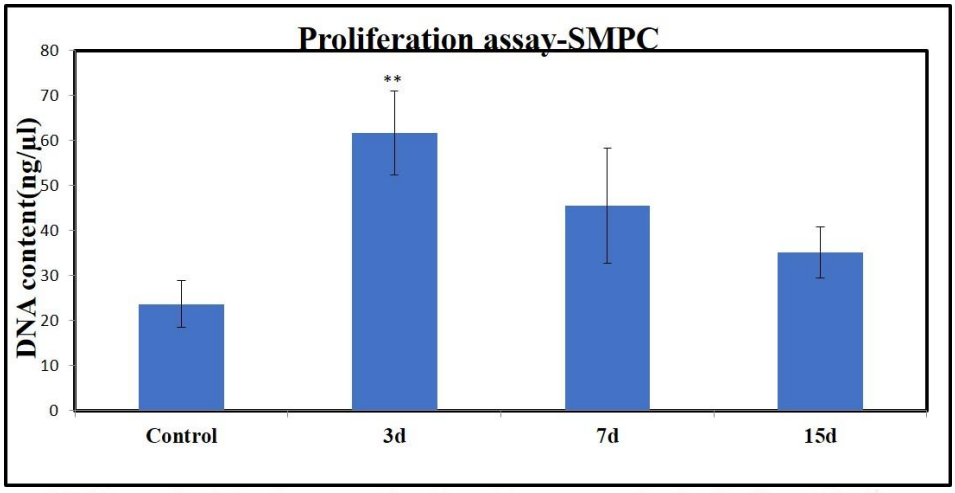

Figure 20. Proliferation of cell seeded scaffold. Graphical representation of proliferation pattern of cell seeded scaffold after 2h, on 3rd, 7th and 15th day. Error bars represent standard error. (n=6). **P<0.01

4.5.3. Quantification of extractable collagen on TEVG

Collagen deposited by SMPC seeded scaffolds at different days of culture was analysed and is graphically represented. The quantity of collagen extracted on 3rd day was found to be less with respect to 7th day. On day 7 there was a significant extractable collagen and was less extractable on 15th day. It is possible that the collagen is more organised by 15th day and was not extractable from the fibrous 3-D culture surface **(Figure 21)**.

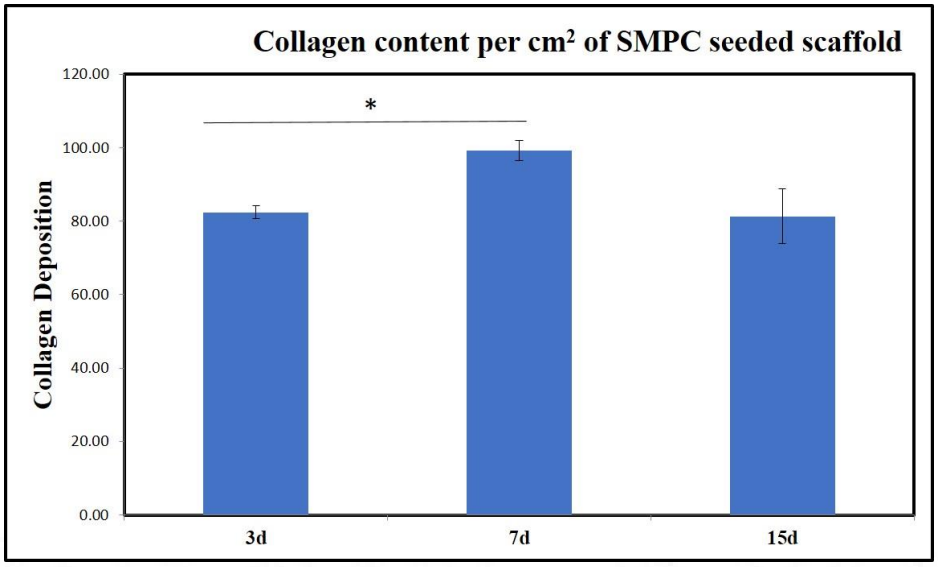

Figure 21. Collagen extracted from SMPC seeded scaffold. Graphical representation of collagen content /cm^2 of cell seeded scaffold on 3d, 7d, 15d. Error bars represent standard error. (n=5). *P<0.05

4.5.4. Gene and protein expression of ECM proteins

The expression of ECM proteins, collagen and elastin, on real time PCR analysis was found barely higher than hADMSC at all three different periods of culture. This shows the regulated expression of the ECM proteins and also the contractile phenotype of the differentiated SMPCs **(Figure 22)**.

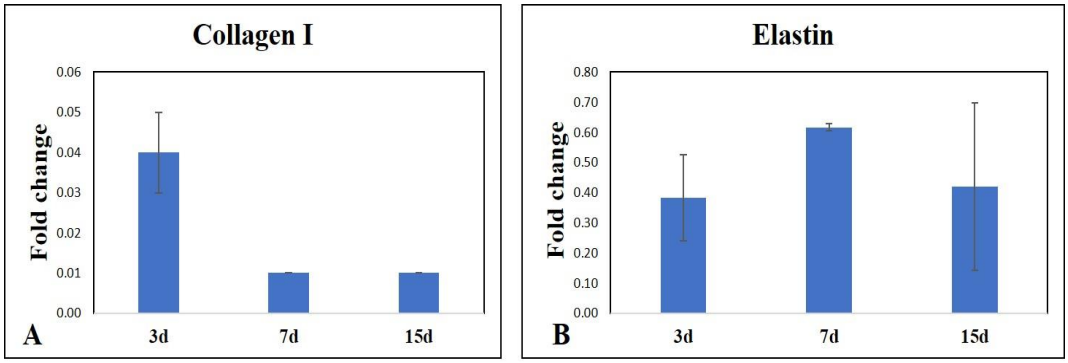

Figure 22. Relative gene expressions of SMPC markers collagen and elastin. The relative gene expression of (A) collagen I; and (B) elastin, normalized to the control ADMSCs is represented graphically. Fold change is quantified relative to GAPDH expression on each day of analysis using the $2^{-\Delta\Delta Ct}$ method. Error bars represent standard error. (n=5).

Immunocytochemical data showed the presence of the ECM proteins on the ablumen of the cell seeded scaffold. The ECM deposition by SMPC demonstrates tendency to form medial layer of blood vessel, in the electrospun fibrous compartment of scaffold **(Figure 23)**.

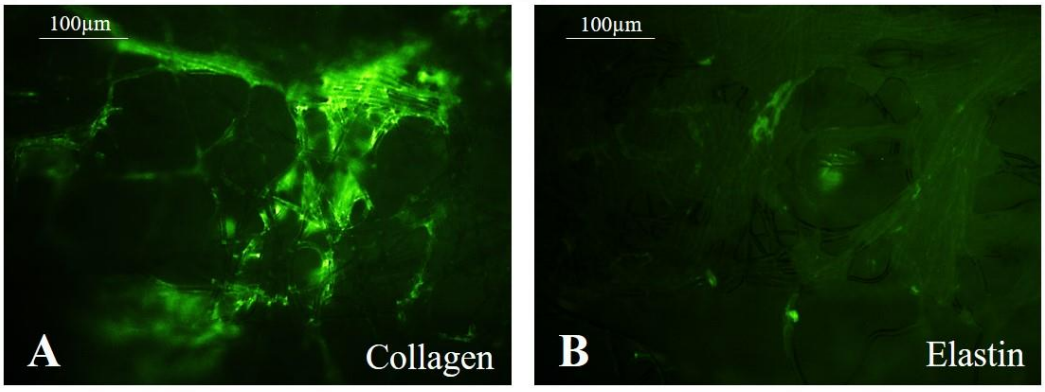

Figure 23. Immunocytochemistry of ECM proteins. Fluorescent micrographs of decellularised scaffolds immunostained for (A) Collagen (green) (B) Elastin (green).Scale bar : 100µm

4.5.5. Physical properties of SMPC grown scaffold

The cell grown scaffolds maintained the tensile properties of scaffold. In comparison with bare scaffolds, hydrated for similar period of time by incubating with culture medium, the tensile stress and modulus of the cell grown scaffolds were significantly higher. The tensile strain was comparable in both cell seeded and bare scaffolds. The ECM proteins deposited by the cells on the ablumen of the scaffold may have influenced the stability of the scaffold **(Figure 24)**.

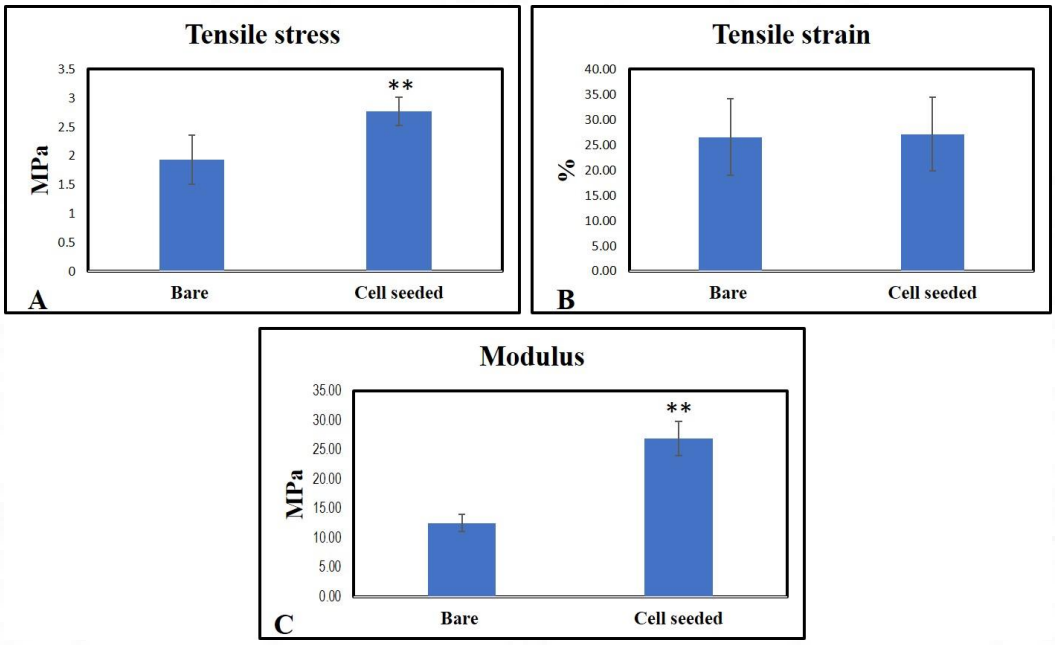

Figure 24. Tensile properties of scaffolds. Graphical representation of (A) tensile stress, (B) tensile strain and (C) modulus of cell seeded and bare scaffolds. Error bars represent standard error. (n=6). **P<0.01

4.6. Effect of risk factors on EC phenotype

4.6.1. Assessment of NO release

Effect of added glucose, cholesterol, TNF-1α and combinations of TNF-1α with glucose/cholesterol on EC growing in the TEVG lumen were compared to the cells grown in basal medium (normal medium). The release of the functional marker NO was higher when cells were exposed to high glucose and high cholesterol in medium.

The effect was insignificant and more variable with high standard deviation in replicate measurements when cholesterol was high in the medium **(Figure 25)**.

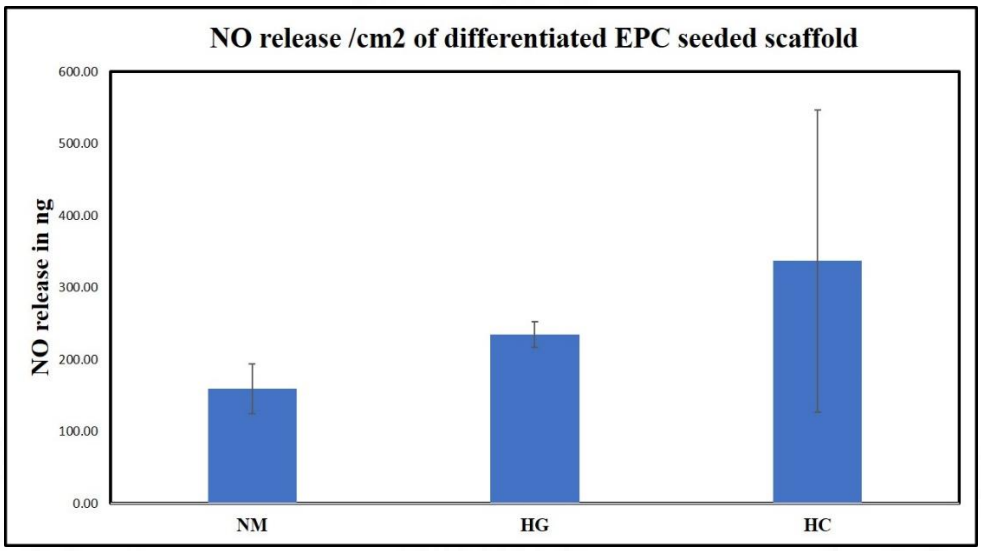

Figure 25. Quantity of NO released from medium with HG & HC. The pattern of NO release upon dynamic culture of EC seeded scaffolds under Normal medium (NM); High glucose medium (HG); and High cholesterol medium (HC). Error bars represent standard error. (n=4).

4.6.2. Influence of high glucose and high cholesterol on EC gene expression

The pro-thrombotic marker vWF and anti-thrombotic marker eNOS showed different pattern of expressions at different culture conditions **(Figure 26)**. In comparison with normal medium, eNOS expression was high in medium with high glucose, while a significantly lowered expression in medium with high cholesterol was seen. Compared to normal medium, eNOS expression was less in medium containing TNF-1α. Average expression of vWF was similar in the medium with HG and HC. In TNF-1α containing culture, vWF expression was much less compared to NM culture **(Figure 26)**.

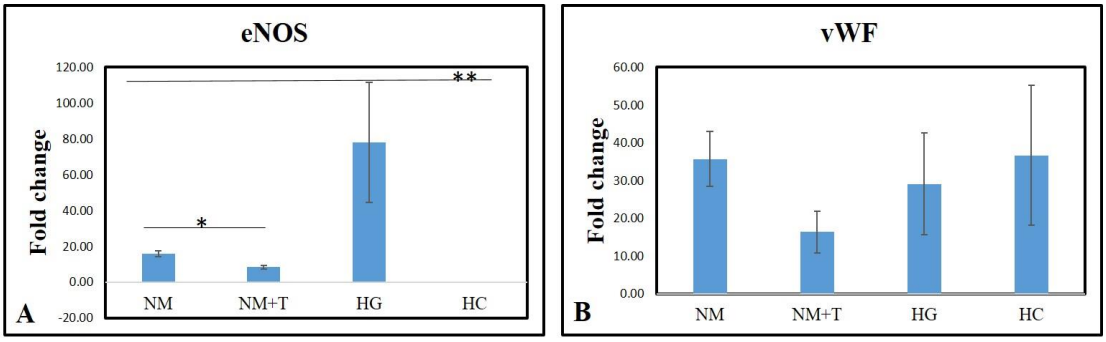

Figure 26. Independent effect of HG, HC and TNF-1α on relative gene expressions of eNOS & vWF in EC. The relative gene expression of (A) eNOS; and (B) vWF in normal medium (NM), medium with TNF-1α (NM+T), medium with high glucose (HG) and medium with high cholesterol (HC), normalized to the control ADMSCs is represented graphically. Fold change is quantified relative to GAPDH expression on each day of analysis using the $2^{-\Delta\Delta Ct}$ method. Error bars represent standard error. (n=6). *P<0.05,**P<0.01

Pro-fibrinolytic marker tPA showed reduced expressions in all the three conditions from the basal level expression **(Figure 27)**. On comparing with the expression in normal medium, tPA expression was low in medium with TNF-1α and significantly low in medium with high glucose and medium with high cholesterol. Expressions of the anti-fibrinolytic marker PAI was low in normal medium while it was slightly upregulated in medium with high glucose and was highly upregulated in medium with high cholesterol. PAI's expression in normal medium with TNF-1α was comparable with that in medium with high cholesterol **(Figure 27)**.

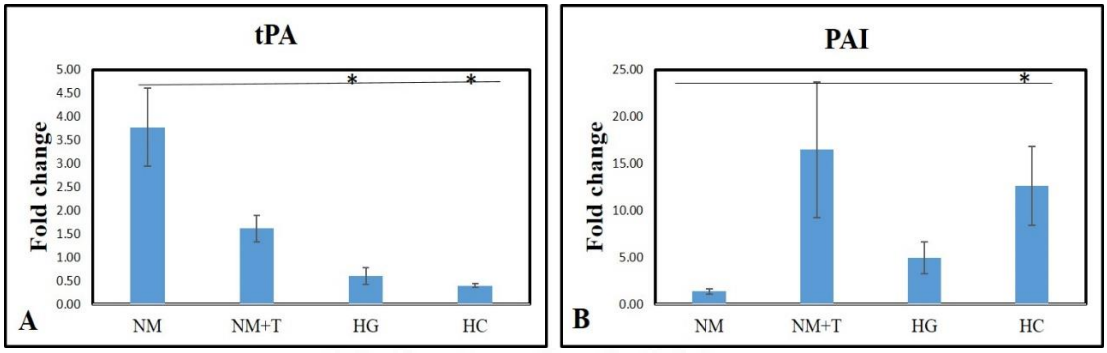

Figure 27. Independent effect of HG, HC and TNF-1α on relative gene expressions of tPA & PAI in EC. The relative gene expression of (A) tPA; and (B) PAI in normal medium (NM), medium with TNF-1α (NM+T), medium with high glucose (HG) and medium with high cholesterol (HC), normalized to the control ADMSCs is represented graphically. Fold change is quantified relative to GAPDH expression on each day of analysis using the $2^{-\Delta\Delta Ct}$ method. Error bars represent standard error. (n=6). *P<0.05

The adhesion molecule CD 31 was significantly downregulated upon exposure to medium with TNF-1α and medium with high cholesterol,when compared to normal medium **(Figure 28)**. Expression of CD31 in medium with high cholesterol was much down regulated as compared with that in normal medium. VCAM-1 showed an upregulated expression in normal medium with and without TNF-1α and both the expressions were comparable. In high glucose and high cholesterol medium, VCAM-1 expression was downregulated in comparison with normal medium **(Figure 28)**.

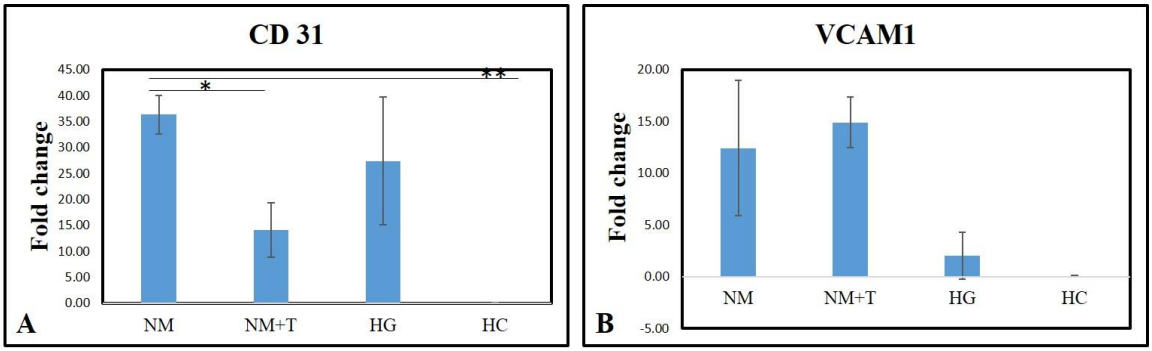

Figure 28. Independent effect of HG, HC and TNF-1α on relative gene expression of CD31 & VCAM-1 in EC. The relative gene expression of (A) CD 31; and (B) VCAM-1 in normal medium (NM), medium with TNF-1α (NM+T), medium with high glucose (HG) and medium with high cholesterol (HC), normalized to the control ADMSCs is represented graphically. Fold change is quantified relative to GAPDH expression on each day of analysis using the $2^{-\Delta\Delta Ct}$ method. Error bars represent standard error. (n=6). *P<0.05, **P<0.01

Pro-inflammatory molecule MCP1 showed minimal expression in all three medium compositions **(Figure 29)**. Within the minimal expression, MCP1 showed a significant change on exposure to medium containing TNF-1α. High glucose and high cholesterol do not elicit any change in MCP1 expression.

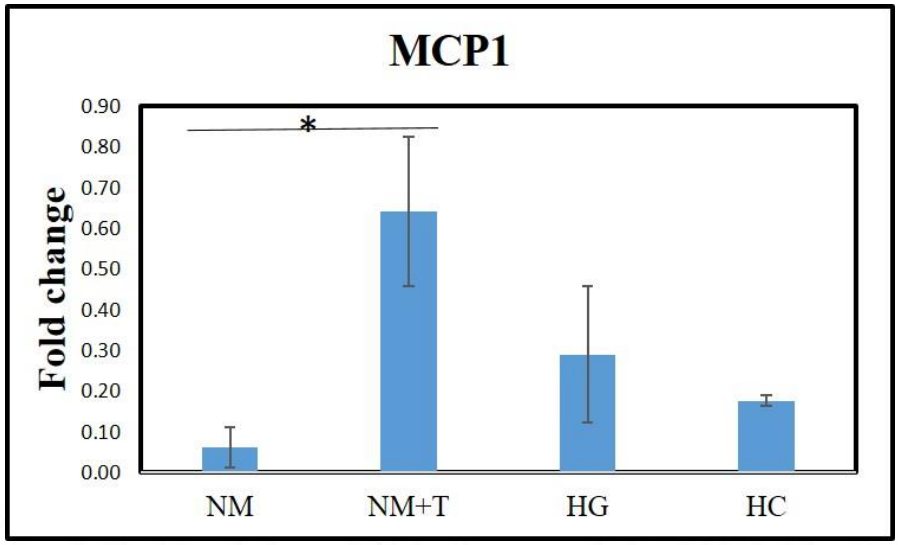

Figure 29. Independent effect of HG, HC and TNF-1α on relative gene expression of MCP1 in EC. The relative gene expression of MCP1in normal medium (NM), medium with TNF-1α (NM+T), medium with high glucose (HG) and medium with high cholesterol (HC), normalized to the control ADMSCs is represented graphically. Fold change is quantified relative to GAPDH expression on each day of analysis using the $2^{-\Delta\Delta Ct}$ method. Error bars represent standard error. (n=6). *P<0.05

4.6.3. Influence of high glucose on TNF-1α activated ECs

eNOS was seen significantly downregulated in scaffolds exposed to normal medium with TNF-1α when compared with scaffolds exposed to normal medium without TNF-1α **(Figure 30)**. eNOS was upregulated in scaffolds exposed to high glucose but on addition of TNF-1α along with glucose, expression was reduced. vWF showed a comparable expression when exposed to normal medium, medium with high glucose with and without TNF-1α, but vWF expression was less when treated with normal medium containing TNF-1α **(Figure 30)**.

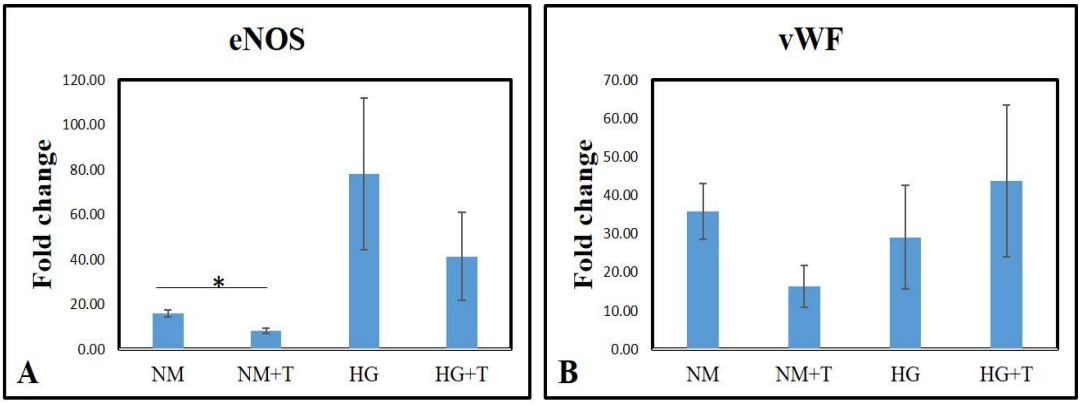

Figure 30. Combination effect of HG and TNF-1α on relative gene expressions of eNOS & vWF in EC. The relative gene expression of (A) eNOS; and (B) vWF in normal medium (NM), medium with TNF-1α (NM+T), medium with high glucose (HG) & medium with high glucose & TNF-1α (HG+T), normalized to the control ADMSCs is represented graphically. Fold change is quantified relative to GAPDH expression on each day of analysis using the $2^{-\Delta\Delta Ct}$ method. Error bars represent standard error. (n=6). *P<0.05

tPA was seen downregulated in scaffolds exposed to normal medium with TNF-1α and was significantly downregulated in scaffolds exposed to medium with high glucose in comparison with normal medium **(Figure 31)**. Expression of tPA in medium with high glucose along with TNF-1α was slightly downregulated with respect to normal medium. PAI was upregulated in normal medium with TNF-1α and downregulated in normal medium, medium with high glucose and medium with high glucose and TNF-1α **(Figure 31)**.

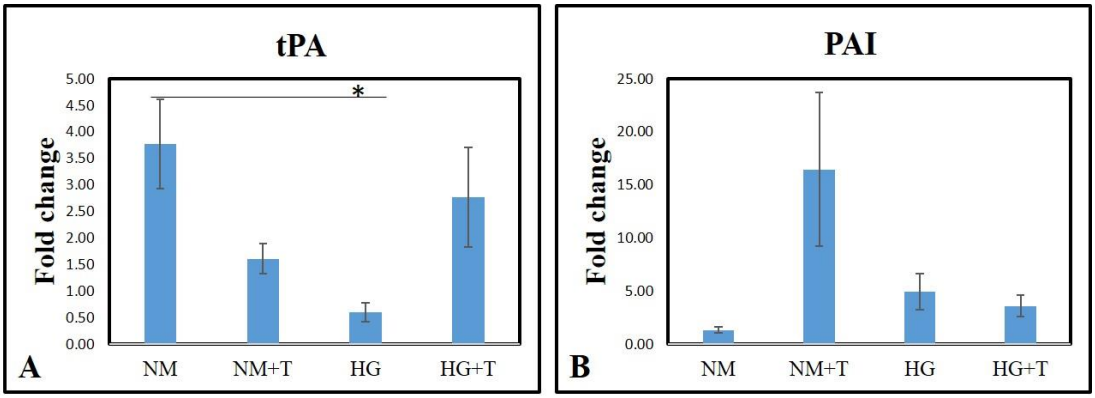

Figure 31. Combination effect of HG and TNF-1α on relative gene expressions of tPA & PAI in EC. The relative gene expression of (A) tPA; and (B) PAI in normal medium (NM), medium with TNF-1α (NM+T), medium with high glucose (HG) & medium with high glucose & TNF-1α (HG+T), normalized to the control ADMSCs is represented graphically. Fold change is quantified relative to GAPDH expression on each day of analysis using the $2^{-\Delta\Delta Ct}$ method. Error bars represent standard error. (n=6). *P<0.05

CD 31 showed comparable expressions in medium with high glucose and glucose with TNF-1α and a significantly lower expression in normal medium with TNF-1α with respect to normal medium **(Figure 32)**. On other hand, VCAM-1 showed low expressions in normal medium with TNF-1α and medium with high glucose when compared with normal medium. VCAM-1 showed a significant upregulation in medium with glucose and TNF-1α with respect to normal medium **(Figure 32)**.

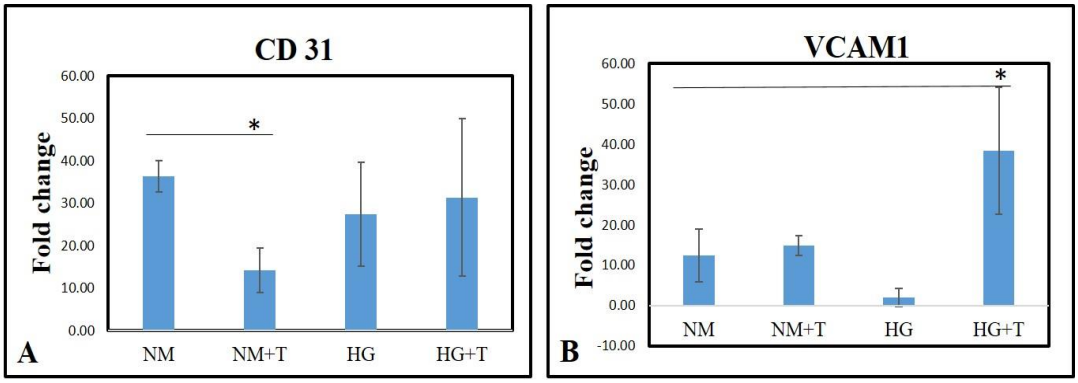

Figure 32. Combination effect of HG and TNF-1α on relative gene expressions of CD31 & VCAM-1 in EC. The relative gene expression of (A) CD 31; and (B) VCAM1 in normal medium (NM), medium with TNF-1α (NM+T), medium with high glucose (HG) & medium with high glucose & TNF-1α (HG+T), normalized to the control ADMSCs is represented graphically. Fold change is quantified relative to GAPDH expression on each day of analysis using the $2^{-\Delta\Delta Ct}$ method. Error bars represent standard error. (n=6). *P<0.05

MCP1 expression was negligible even after adding TNF-1α to normal medium, and high glucose **(Figure 33)**. But even with minimal expression, there was a significant change when the scaffolds were exposed to normal medium with TNF-1α.

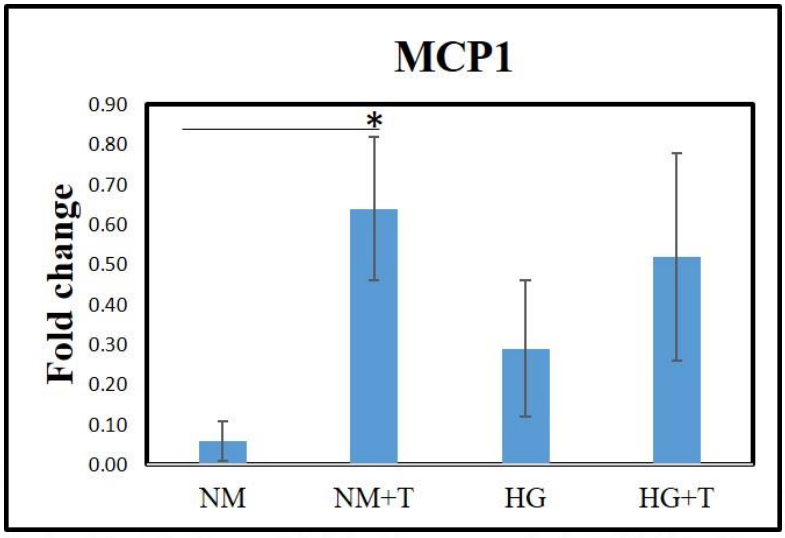

Figure 33. Combination effect of HG and TNF-1α on relative gene expression of MCP1 in EC. The relative gene expression of MCP1 in normal medium (NM), medium with TNF-1α (NM+T), medium with high glucose (HG) & medium with high glucose & TNF-1α (HG+T), normalized to the control ADMSCs is represented graphically. Fold change is quantified relative to GAPDH expression on each day of analysis using the $2^{-\Delta\Delta Ct}$ method. Error bars represent standard error. (n=6). *P<0.05

4.6.4. Influence of high cholesterol on TNF-1α activated ECs

Addition of TNF-1α to normal medium and high cholesterol medium downregulated the expression of eNOS **(Figure 34)**. But eNOS expression was significantly downregulated in scaffolds exposed to high cholesterol alone, when compared with normal medium. vWF expression in high cholesterol medium was comparable with normal medium. On adding TNF-1α to normal medium and high cholesterol medium, expression of vWF was seen downregulated **(Figure 34)**.

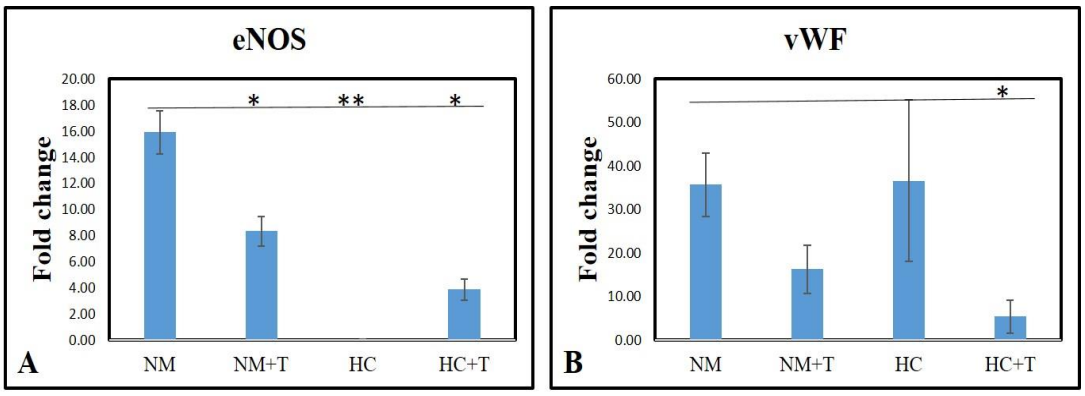

Figure 34. Combination effect of HC and TNF-1α on relative gene expressions of eNOS & vWF in EC. The relative gene expression of (A) eNOS; and (B) vWF in normal medium (NM), medium with TNF-1α (NM+T), medium with high cholesterol (HC) & medium with high cholesterol & TNF-1α (HC+T), normalized to the control ADMSCs is represented graphically. Fold change is quantified relative to GAPDH expression on each day of analysis using the $2^{-\Delta\Delta Ct}$ method. Error bars represent standard error. (n=6). *P<0.05, **P<0.01.

tPA showed reduced expressions with the addition of TNF-1α in normal medium and medium with high cholesterol **(Figure 35)**. tPA expression was significantly reduced in medium containing high cholesterol alone when compared with normal medium. PAI on the other hand showed an inverse pattern of expression with less expression in normal medium and high expression in medium with TNF-1α and medium with cholesterol. Conversely, PAI expression was significantly reduced when TNF-1α was added to medium containing high cholesterol **(Figure 35)**.

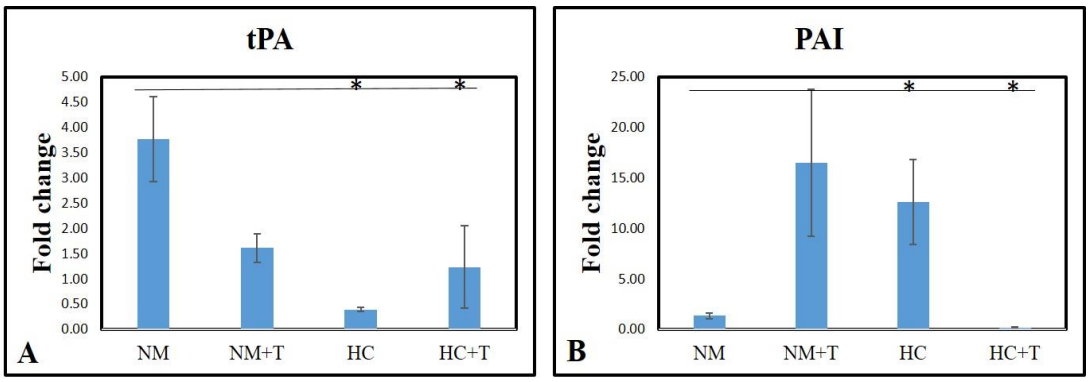

Figure 35. Combination effect of HC and TNF-1α on relative gene expressions of tPA & PAI in EC. The relative gene expression of (A) tPA and (B) PAI in normal medium (NM), medium with TNF-1α (NM+T), medium with high cholesterol (HC) & medium with high cholesterol & TNF-1α (HC+T), normalized to the control ADMSCs is represented graphically. Fold change is quantified relative to GAPDH expression on each day of analysis using the $2^{-\Delta\Delta Ct}$ method. Error bars represent standard error. (n=6). *P<0.05

With the addition of TNF-1α to the medium, CD 31 was downregulated in normal medium and minimal in medium with high cholesterol. Similar to eNOS expression, CD31 was significantly reduced in medium with cholesterol alone **(Figure 36)**.VCAM-1 expression was less in normal medium, medium with TNF-1α and was minimal in medium with cholesterol alone. But VCAM-1 expression was significantly upregulated in EPCs exposed to medium containing TNF-1α and cholesterol **(Figure 36)**, when compared to normal medium.

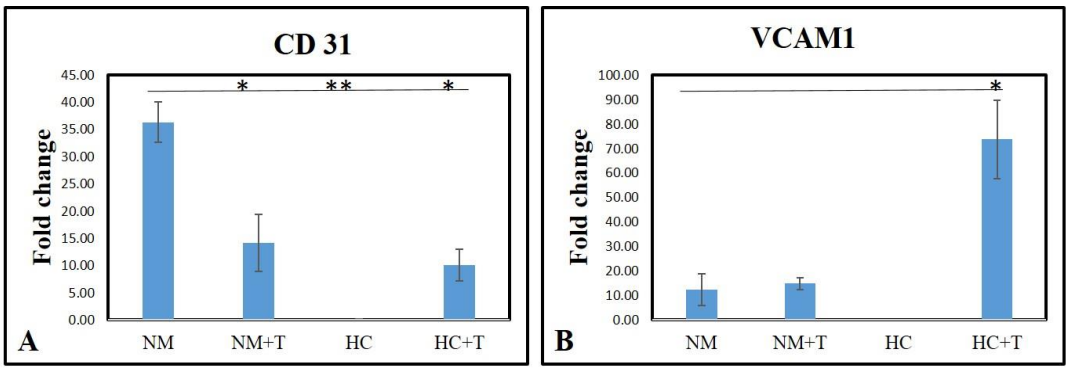

Figure 36. Combination effect of HC and TNF-1α on relative gene expressions of CD31 & VCAM-1 in EC. The relative gene expression of (A) CD 31; and (B) VCAM-1 in normal medium (NM), medium with TNF-1α (NM+T), medium with high cholesterol (HC) & medium with high cholesterol & TNF-1α (HC+T), normalized to the control ADMSCs is represented graphically. Fold change is indicated relative to GAPDH expression on each day of analysis using the $2^{-\Delta\Delta Ct}$ method. Error bars represent standard error. (n=6). *P<0.05,**P<0.01

MCP1 expression was negligible in normal medium, medium with high cholesterol and medium with high cholesterol and TNF-1α **(Figure 37.)**.But even with minimal expression, there was a significant change when the scaffolds were exposed to normal medium with TNF-1α.

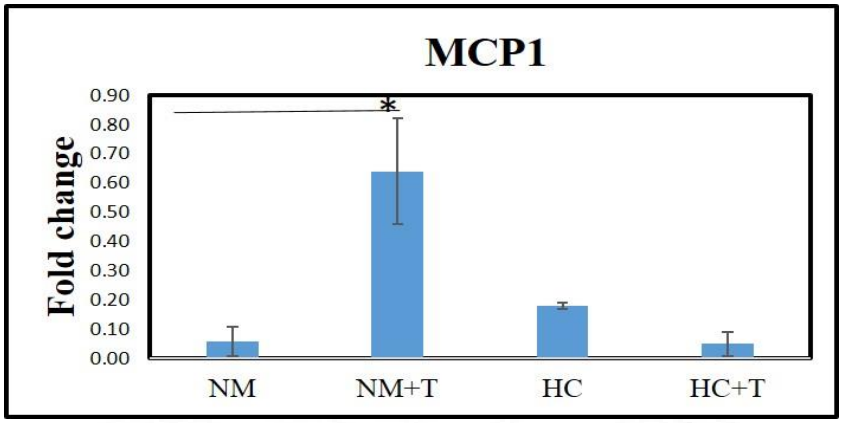

Figure 37. Combination effect of HC and TNF-1α on relative gene expression of MCP1 in EC. The relative gene expression of MCP1 in normal medium (NM), medium with TNF-1α (NM+T), medium with high cholesterol (HC) & medium with high cholesterol & TNF-1α (HC+T), normalized to the control ADMSCs is represented graphically. Fold change is quantified relative to GAPDH expression on each day of analysis using the $2^{-\Delta\Delta Ct}$ method. Error bars represent standard error. (n=6). *P<0.05

Varied levels of expressions were observed on exposure to normal medium with and without TNF-1α. CD31, eNOS and vWF demonstrated an elevated expression in normal medium while tPA, PAI, MCP1 and VCAM-1 showed a downregulated expression in normal medium. But with the addition of TNF-1α in normal medium, eNOS, vWF, tPA and CD31 were downregulated while PAI and VCAM-1 were seen upregulated.MCP1 was minimally expressed even with the addition of TNF-1α.

In summary, on comparing the different gene expressions after exposure to high glucose in the presence and absence of TNF-1α, a difference is seen in the case of each molecule. The expressions of CD31, vWF, tPA, PAI, MCP1 and VCAM-1 were less in high glucose medium as compared to that in normal medium. However, eNOS showed an up-regulated expression. But with the addition of TNF-1α in high glucose medium, eNOS, tPA, PAI and MCP1 were down-regulated while the membrane proteins CD31 and VCAM1 were up-regulated.

The combination effect of treating cholesterol and TNF-1α in molecular expressions were also observed. The eNOS, tPA, CD 31, MCP1 and VCAM-1 showed a downregulated expression in high cholesterol medium as compared to that in normal medium. But vWF and PAI showed a slightly upregulated expression. With the addition of TNF-1α in high cholesterol medium, all genes such as eNOS, vWF, tPA, PAI and MCP1 were down-regulated while the membrane proteins CD31 and VCAM-1 were expressed more on addition of TNF-1α to high cholesterol medium.

CHAPTER 5

5. Discussion

The important results of the study, illustrated in chapter 4, are discussed in this chapter. The findings are correlated with recently published literature in the relevant field of study and interpretations are made wherever possible. The limitations of the study are also discussed.

The study relates to development of functional blood vessel for applications in atherosclerosis research. One of the most important components in functional blood vessel is cell. In the context of functional blood vessel, most important cell is endothelial cell. The smooth muscle cells are important for supporting proper functioning of endothelial cells. Another important requirement is pliable conduit to provide shape to blood vessel. The tubular structure should withstand the pressure of shear stress other than supporting cell adhesion and growth. Biodegradable PCL is considered suitable for fabricating pliable conduits and can resist the hemodynamic forces. The bilayered tubular scaffold permits culture of endothelial cell and smooth muscle cells in two separate layers. The porosity of luminal layer permits communication between endothelial and smooth muscle cell layers. Coating with fibrin matrix permits better adhesion and growth of both cell types and the maintenance of their phenotypes.

The first part of this study focused on deriving partially differentiated human EPCs and SMPCs with indicators of functional phenotypes. Next was fabrication of tubular scaffold, growing the derived EPCs and SMPCs and establishing basic functional markers. In the third part of the study, the focus was demonstrating quantifiable response to three different pathological factors in a short period of exposure.

5.1. Derivation of EPCs and SMPCs from hADMSCs

Construction of a small diameter blood vessel, with a functional endothelium and mechanical properties similar to natural blood vessels, using autologous cells

remains a challenge. Different approaches to utilize mature cells for engineering a blood vessel include the use of human aortic smooth muscle cells (HASMCs) or coculture of vascular smooth muscle cells (SMCs) with vascular endothelial cell (ECs) or HASMCs with human umbilical vein endothelial cells (HUVECs). However, these cell types exhibit reduced expansion and unfavorable mechanical properties. The ideal source of cells is yet to be identified. Adipose derived mesenchymal stem cells isolated from human adipose tissue is a favourable choice. They are known for their ability to differentiate into multiple cell lineages, including osteocytes, chondrocytes, smooth muscle cells and endothelial cells. Mesenchymal and Tissue Stem Cell Committee of The International Society for Cellular Therapy has suggested the following characteristics that the MSCs should possess: a) They must adhere to plastic while being maintained in standard culture conditions b) They must be able to differentiate into osteogenic, adipogenic, and chondrogenic lineages c) They must express the surface markers CD105, CD73, and CD90 and not express CD45, CD34, CD14 or CD11b, CD79a, or CD19, or HLA-II surface molecules (Badimon et al., 2015). The results obtained in this study suggest that the cells isolated were indeed MSCs from human adipose tissue of patients admitted for coronary artery bypass graft since the isolated cells expressed all the characteristics typical of MSCs namely the plastic adherence, spindle shaped morphology, multipotency and stemness as evidenced by the trilineage differentiation and flow cytometry results respectively.

The multipotency of hADMSCs is exploited further for differentiating the isolated cells to endothelial progenitor cells and smooth muscle progenitor cells. In a study conducted to identify the optimal culture conditions to support the proliferation of human macrovascular endothelial cells, the most effective nutrient medium in augmenting cell proliferation was found to be MCDB 131. Compared to the more commonly used M199 medium, MCDB 131 resulted in a 2.3-fold increase in cell proliferation (Terramani et al., 2000). The coating of culture wares to promote cell adhesion is a well-established technique. Human ADMSCs are also known to maintain good proliferation rate on coated surfaces. Biomimetic fibrin composite

consisting of fibrin, fibronectin, and gelatin along with growth factors was found to be supportive in the proliferation and differentiation of circulating EPC into EC (Sreerekha and Krishnan, 2006). Therefore, an analogous approach was used in this study to differentiate sub cultured hADMSCs to EPCs by seeding the cells in a matrix composed of fibrin, fibronectin, gelatin and VEGF (Murphy et al., 2014). For the lineage commitment of hADMSCs to EPCs, a specific niche composed of fibrin and VEGF has been designed as culture matrix. Fibrin which is used in the matrix is clinically used as a hemostatic glue and in wound repair. Fibrin provides an adequate environment for cell attachment, growth and migration and it is found to be effective as a scaffold for vascular, skin and nerve tissue engineering (Sreerekha et al., 2006). The components of a fibrin-based niche consists of fibrinogen and thrombin, which are isolated from human blood plasma. Fibrin interacts with integrins and cadherins to promote cell attachment and provides focal contacts for cell proliferation and differentiation.

A number of studies have shown that adult stem cells differentiate into ECs in the presence of added soluble growth factors. For the optimal growth of endothelial cells, usually endothelial cell growth supplement (ECGS) is added to endothelial cell medium. But ECGS alone did not promote expression of endothelial specific markers. Studies have established that proliferation and viability of endothelial progenitor cells was best in a defined medium containing the highest number of individual growth factors (Leopold et al., 2019). VEGF is a known stimulus of the endothelial differentiation of MSCs *in vitro* and addition of VEGF significantly increased the expression of eNOS and vWF within the stem cells. VEGF, through VEGF receptors that are restricted to the endothelial lineage, participate in endothelial differentiation. Separate studies demonstrated expression of EC specific makers, CD31 and vWF, by ADMSCs stimulated with VEGF and insulin-like growth factor (IGF) (Fischer et al., 2009). Though IGF is less potent than VEGF, it is a potential component of EC differentiation medium (Wang et al., 2018). Epithelial growth factor (EGF) has proven to be a potent contributor to EC migration, maturation, and vessel formation and studies indicated that EGF, when used in

combination with VEGF, increased the latter's expression by a feedback loop. Culture medium supplemented with mitogens such as heparin sulphate, L-ascorbic acid and L-glutamine augmented the proliferation of endothelial progenitor cells. So the EPC induction medium, used in this study, containing MCDB 131 with 10% FBS supplemented with VEGF, IGF and EGF and all the required mitogens proved to be beneficial in the differentiation of hADMSCs to EPCs. The growth factor induction was given for a limited time period of two days after which the growth factors were withdrawn. Growth factors are important in increasing proliferation and survival in MSCs. It would be beneficial if the growth factors could prolong proliferation of MSCs for several population doublings, before they are differentiated into the desired tissue. So inducing with growth factors for limited time period can be considered as one of the strategies in the differentiation studies (Rodrigues et al., 2010)

The GF induced cells showed distinct morphological features, as compared to hADMSCs, and some of the molecular markers were up regulated. However, even after longer culture periods of 8-10 days, the cells remained in the elongated, slender morphology. Further, the relative expressions of all molecular markers, as compared to hADMSC, were not significantly up regulated. However, there was sufficient change towards endothelial lineage. Therefore, it was proposed to further stimulate the GF induced cells followed by hypoxic environment. Hypoxia is the most popular approach in promoting EC differentiation via regulated VEGF signaling. Effects of hypoxia are usually mediated by hypoxia-inducible factors (HIFs) namely HIF-1a and HIF-2a, which regulate tissue oxygen tension. Recent studies revealed that expression of HIF-1a and HIF-1ß are required for the normal development of the heart, blood vessels and blood cells. Adult stem cells live in hypoxic conditions in vivo. When compared to other tissues, lower oxygen concentrations are observed in adipose tissue indicating that ADMSCs too live in a hypoxic environment. Significant neovascularization occurred in an *in vivo* mouse hind limb ischemia model after treatment with ADMSC (Moon et al., 2006), pointing to the fact that the stem cells differentiate into endothelial cells, presumably in hypoxic conditions.

When stimulated by hypoxia, ADMSCs express a 5-fold increase in VEGF production and this VEGF produced directly impacts the surrounding cells. ADMSCs co-cultured with endothelial cells in hypoxic conditions produced VEGF which stimulates increased capillary formation by the endothelial cells in vitro (Abdollahi et al., 2011). These experimental evidences substantiated the usage of hypoxic condition for differentiating ADMSCs to EPCs.

The cells seeded in the specific matrix and induced with a cocktail of growth factors namely VEGF, IGF and EGF and later with hypoxic induction showed a marked difference in the cell morphology. The GF induced cells showed slender, elongated and angiogenic-like morphology. After giving hypoxic environment the cells showed a cobble stone morphology, characteristic of endothelial cells and was a promising observation. However, other than the up regulation of PAI, no other molecular markers were up-regulated as an effect of hypoxia. Therefore, additional biomimetic stimulation was found necessary.

Mechanical stimulus can elicit stem cell-EC differentiation process. In an *in vivo* scenario, ECs are constantly subjected to shear stress exerted by the blood flow. So it is reasonable to speculate that shear stress might be crucial for *in vitro* differentiation of ECs. For applying shear stress, a bioreactor is commonly employed. In this study, a perfusion pump bioreactor system is used for applying shear stress in which a pump and a scaffold chamber are joined together by tubing. A synergistic relationship between shear stress and growth factors has been established for stem cell-EC differentiation. When placenta-derived stem cells were cultured in endothelial growth medium (EGM), containing growth factors, for 3 days and thereafter stimulated with shear stress for 24 h, stem cells were found to differentiate into functional ECs (Xu et al., 2019). Moreover, a recent study has shown that under a combination of shear stress and hypoxia, eNOS phosphorylation was increased to >2-fold at 60 min, followed by an increase in NO production (Sera et al., 2019). In this study the hADMSCs stimulated with growth factors and hypoxia showed significant effect post shear stress exposure, establishing differentiation into endothelial progenitor cells. Since study reports claim that high-level shear stress

could increase EC differentiation from MSCs, a graded flow rate from 20ml/min to 100ml/min was given with the help of the bioreactor which gave a shear stress from 11.6 dynes/cm^2 to 58.2 dynes/cm^2, which is similar to an arterial level of shear stress. The gene expression in differentiated endothelial cells after the exposure to shear force was assessed by quantitative real time PCR. The results showed the significant expression of endothelial specific genes namely CD31, VCAM-1, vWF and eNOS upon activation with shear stress in the differentiated cells. Undifferentiated hADMSCs was taken as the control. These results were compared with the gene expression results after each stage namely, growth factor (GF) induction, growth factor and hypoxic induction and static cultures. Shear stress activates several pathways through endothelial surface molecules, CD31 being one among them. Shear stress also phosphorylate eNOS at the site of serine-1179 which directly results in eNOS activation and enhanced NO production, as discussed above. The results obtained in this study with respect to the expression of CD 31 and eNOS substantiate these reports. Reports suggest the presence of Wiebel –Palade bodies, the EC-specific, electron-dense granules storing von Willebrand Factor (vWF) in cells exposed to arterial shear stress, compared with those in conventional static culture (Ballermann et al., 1998). The gene expression of vWF under dynamic culture analyzed in this study also showed a similar result. This study used VCAM-1 as marker for hADMSC to EPC differentiation. The up-regulated expression of VCAM-1 confirms the differentiation into EPCs. In the context of atherosclerosis research using the EPC grown blood vessel, VCAM-1 is an important molecule to be studied. Other studies report a decreased expression of the adhesion molecule VCAM-1(Ando et al., 1995) on exposure to arterial level of shear stress. Therefore, up/down regulation of VACM-1 expressions after long term exposure to the risk factors and shear stress may be useful. The tPA expression was not found comparable to the expressions of other markers. Cyclic strain is known to increase t-PA expression (Iba and Sumpio, 1992). In this study the shear stress applied was using laminar flow, which may be the reason for not achieving higher expressions of t-PA after mechanical stimulation. Whereas, several fold up regulated expressions of other markers such as CD31, VCAM-1, PAI, eNOS and vWF were obtained, tPA

expression was not very high. It is not clear if it is the quiescent expression of tPA in normal conditions. Conversely, the expression of anti-fibrinolytic molecule PAI showed a remarkable up-regulation after induction with both growth factors and hypoxia, but showed comparable expressions in both static and dynamic culture. Gene expression of proinflammatory molecule MCP-1 was also analyzed after each stage of induction. Low shear stress induces NF-κB activation which in turn up-regulates MCP-1(Aoki et al., 2016). The results in this study showed the initial up-regulation of MCP-1 after growth factor induction alone which was further down regulated after applying shear stress. The observation indicates that shear stress is an important parameter that regulates EPC into a non-inflammatory phenotype.

Several studies are conducted with different strategies adopted for the differentiation of hADMSCs to ECs. But the tri-level induction protocol, with the synergistic use of growth factor cocktail, hypoxia and shear stress which were applied in this study has not been reported previously. Up regulation of all 6 specific endothelial markers in the cells induced sequentially with GF, hypoxia and shear stress, as compared to normal hADMSC cultured for similar duration confirms the differentiation of stem cells into EPCs. Also, the analysis of cells after each step indicated that all 3 steps are contributing to the differentiation.

The differentiated cells showed uptake of acLDL and CD31, similar to the positive control- human umbilical vein endothelial cells (HUVEC), confirming the lineage commitment. VE-Cadherin, a reliable endothelial marker often used to validate endothelial differentiation, was expressed in the induced cells against the undifferentiated MSCs showing almost no specific staining for these markers. The NO release by the EPC is another confirmatory indication of hADMSC differentiation into EPCs. The basal expressions of all molecular markers after the tri-level induction confirmed the differentiation of hADMSCs to EPCs. The differentiated cells, even after indicating endothelial specific characteristics, are called endothelial progenitor cells because the full functionality of the cells was not established. The up regulated expressions of all markers are found to be apt for quantifying the effects of risk factors. Therefore, the EPCs differentiated from

hADMSCs were employed in the construction of tissue engineered blood vessel. The behavior of EPC which were directly seeded on scaffold for shear stress based induction also could be different from the cells grown in the lumen of SMC seeded scaffold.

Smooth muscle is essential for constructing the medial layer of blood vessel. The SMCs in the medial layer provides not only mechanical strength, it also interacts with EC layer and regulate the function in a quiescent state. The differentiation of hADMSCs to SMC may be established using different molecular markers. For differentiating hADMSCs to smooth muscle cells (SMCs), the reported studies employed different soluble growth factors such as transforming growth factor β-1 (TGFβ1), transforming growth factor β-3 (TGFβ3), angiotensin II (ANG), sphingosylphosphorylcholine (SPC), ascorbic acid, bone morphogenetic protein 4 (BMP4) and platelet-derived growth factor. MCDB131 medium with 1% fetal bovine serum (FBS) and minimum essential medium with 10% FBS have also been used for differentiation of ADMSCs to SMCs (Harris et al., 2011). TGF-β1 is an important cytokine that is involved in SMC differentiation *in vitro* and *in vivo*. In previous studies, several cells were induced by TGF-β1 to differentiate into cells with an SMC-like phenotype and function (Xu et al., 2013). Bone morphogenetic protein 4 (BMP4) is a member of the TGF-β1 superfamily of secretory signaling molecules involved in embryonic development. TGF-β1 and BMP4 either alone or in combination with culture medium are used for SMC differentiation.The platelet-derived growth factor (PDGF) family is composed of disulphide-bonded homodimers of four polypeptide chains, PDGF-AA, -BB, -CC and -DD, and one heterodimeric protein, PDGF-AB. Based on the previous works, an inductive medium consisting of MCDB 131, 1% FBS, platelet growth factor (PGF), BMP4 and the required mitogens such as L-glutamine and L-ascorbic acid was designed for hADMSC differentiation to SMCs. The presence of PDGF in the PGF cocktail prepared in –house is well established. As in the case of EPC differentiation, a specific matrix composed of fibrin, fibronectin, gelatin and PGF was used for the lineage commitment of hADMSCs to SMCs. Initially PGF and BMP4, in different concentrations, were used

alone and in combination for the differentiation. Finally, a particular concentration of BMP4 and PGF used in combination induced hADMSCs to smooth muscle cell lineage. This concentration of growth factors was fixed for further induction studies. The inductive medium with adequate concentrations of PGF and BMP4 elicited the desired morphological changes in the induced cells even after repeating the experiments for different time periods. The induced cells showed the characteristic 'hill and valley morphology' of SMCs with the uninduced cells showing a spindle morphology. The common molecular markers used in the literature to assess for SMCs are alpha smooth muscle actin (α SMA), SM22, calponin, caldesmon, and myosin heavy chain 11 (MHC11). Alpha smooth muscle actin and SM22 are early markers of developing smooth muscle. They are not specific to a smooth muscle cell lineage and have been shown to be transcribed in pluripotent stem cells. Calponin, caldesmon and MHC are intermediate to late markers of SMC differentiation and are more specific to a SMC lineage. The smooth muscle lineage commitment of the sub cultured hADMSCs was assessed by immunocytochemistry, flow cytometry and quantitative real time PCR. SMC specific proteins namely α SMA, calponin and MHC11 were detected by immunofluorescence staining. In parallel, α SMA and calponin were also analyzed in HUASMC, which was used as the positive control. The results showed that the expression of α SMA and calponin in differentiated cells were similar to that in HUASMC. Expression of MHC11 in induced cells were compared with hADMSCs cells. The staining of induced cells showed the expression of MHC11 in differentiated cells with the undifferentiated MSCs showing almost no specific staining for this marker. The receptor for PDGF, PDGF R α was also assessed in differentiated cells. Staining results showed the presence of the receptor in the differentiated cells when compared with the undifferentiated stem cells. Numerous studies have established a role for PDGF in both cell proliferation and cell migration (Grako et al., 1999). The presence of PDGF Rα shows the proliferative nature of the induced cells. As discussed above, while αSMA is considered to be an early marker of developing smooth muscle cells, calponin and MHC11 are widely accepted as late markers of SMC differentiation and are more specific to the SMC lineage. It was demonstrated that BMP4 and PGF induced the expression of these

three proteins in hADMSCs as evidenced by immunofluorescence staining indicating the differentiation of hADMSCs towards the SMC phenotype. To determine the percentage of SM differentiated cells in the hADMSCs, the expression levels of αSMA was analyzed by flow cytometry. It was observed that PGF and BMP4 enhanced the expression of αSMA. The gene expression profile analyzed by quantitative real time PCR further confirmed the differentiation of hADMSCs into SMCs. Differentiated ADMSCs expressed minimal levels of αSMA and calponin, while significantly high level expression of the late marker MHC11 was noticed. This confirms the hypothesis that hADMSCs are capable of differentiating to SMCs when induced by BMP4 and PGF.As discussed earlier, GFs are needed for stem cells to differentiate into a specific lineage. But if the growth factors do not induce an early differentiation, it would have been better. To avoid the early differentiation, a dose and time dependent strategy can be used. The presence of MHC11, termed as a mature marker, indicates the contractile phenotype of the differentiated SMCs. Since the functionality of the cells are not fully studied, the differentiated SMCs are termed as smooth muscle progenitor cells. The results of the present study suggest that hADMSCs can be induced to smooth muscle lineage by a novel dose and time dependent induction with BMP4 and PGF.

Thus both EPCs and SMPCs were in the best functional phenotypes and were found suitable in the construction of functional blood vessels.

5.2. Construction and Validation of TEVG

For construction of functional blood vessel by growing EPCs in the lumen and SMPCs in the ablumen, selection of a suitable scaffold is critical. In addition to mechanical properties, the fabricated scaffold should provide a smooth and porous lumen for EPC growth in monolayer, whereas a fibrous layer of scaffold is required for growing SMPC to achieve a supple medial layer. Poly-ε-caprolactone (PCL), FDA approved biodegradable polymer, has excellent biomechanical features, slow degradation rate and very good biocompatibility. The ability to fabricate bilayered PCL graft by solvent casting (inner layer) and electrospinning (outer layer) make this

the most suitable polymer for construction of blood vessel-like tissue *in vitro*. Solvent casting/particulate leaching is a commonly used technique, and resulted in the formation of a porous PCL scaffold with a high degree of interconnectivity between the intimal layer and medial layers. Polyethylene glycol (PEG), which is a biocompatible material, was used as porogen while fabricating PCL tubular scaffolds. Studies have established that PCL scaffolds incorporated with PEG having molecular weight 3400, resulted in scaffolds with narrower pore size distribution and had 78% pores in the 12-24 μ range (Columbus et al., 2014). PEG concentration is also important as it influences the porosity, hydrophilicity, crystallinity and mechanical strength within scaffold systems. Previous study standardized that scaffolds with PEG: PCL ratio of 1:1 had better structural integrity, highest porosity and favorable mechanical strength for blood vessel construction (Soumya et al., 2014). Electrospinning is a widespread method to produce nanofiber based, 3-dimensional scaffolds from different polymers. Electrospun PCL nanofibers have shown high adaptability, high porosity, pore-interconnectivity, and large surface areas, that allow cells to adhere, proliferate, and grow and also structural similarity with the fibrous structure of the extracellular matrix of blood vessels (Park et al., 2019). The surface morphology of the constructed scaffold was analyzed by SEM and the images showed a smooth porous lumen with a pore size that is receptive for endothelial cell seeding (endothelial size being 8-12μ) and a continuous nanofibrous outer structure for seeding smooth muscle cells indicating that the techniques were successful in fabricating a scaffold with desired qualities.

To effectively recapitulate the architecture of the native blood vessel, both inner layer of ECs that are a primary modulator of vessel health, and the outer layer of SMCs that provide tensile strength are required to be grown successfully. Since vascular cells are anchorage-dependent and cell adhesion is poor on PCL surfaces due to its hydrophobicity, fibrin coating was given for the scaffold to promote cell attachment. Fibrin, as a natural scaffold for cell migration and healing, plays a pivotal role in tissue engineering of vascular grafts. As discussed previously, fibrin interacts with integrins and cadherins and helps in cell attachment and differential

gene expression in cells. In the present study, differentiated endothelial progenitor cells were seeded in the lumen of the bilayered PCL scaffolds coated with fibrin matrix. The cell seeded scaffolds were connected to a bioreactor system to apply shear force to progress the maturation of the differentiated cells. The gene expression of the shear induced cells was analyzed and the interpretation was described in detail in 5.1. The lumen of the cell seeded grafts was observed by SEM to analyze the endothelium coverage. SEM result was in compliance with the current state of science establishing that fibrin coated PCL supports cell adherence and growth. Cell alignment assessed by actin staining showed enhanced cell spreading. These results proved that fibrin coating changed the PCL surface from hydrophobic to hydrophilic and this change encouraged the cell attachment and cellular infiltration into the intra fiber space of PCL. Nitric oxide (NO) released by endothelial cells is a strong indicator of existence of functional monolayer in the lumen. It is known that NO is produced under the influence of shear stress. Both the upregulated eNOS expression and the release of NO also confirms that the shear stress is available and the EPCs respond to the forces of flow. NO release play important roles; in regulating vasodilation, inhibiting thrombosis, proliferation and inflammation. The measurement of nitric oxide released by the EPC seeded scaffold at different time periods did not show a significant increase with time, implying that a similar continuous functional EC monolayer exist in the lumen of the scaffold from 4 d to 14 d. NO production is dependent on the number of functional cells; therefore, it is concluded that in 4 days the monolayer covered the scaffold surface sufficiently and it is not required to grow them for longer period to achieve EPC proliferation.

The presence of smooth muscle cells in the outer surface enhances the functionality of artificially constructed blood vessels, if the SMCs gain their quiescent contractile phenotype. hADMSCs were seeded on the outer surface of the fibrin coated PCL scaffold and were induced to smooth muscle progenitor cells using the same GF induction protocol standardized in TCPS. Analysis of the cell DNA content after extracting from seeded scaffolds showed a significant difference within 3 days. During further growth, the cells seemed to have penetrated to the scaffold space due

to fibrous and porous morphology of the scaffold. The cells were probably stabilized in the interstitial space and all the DNA may not have extracted well. This may be the reason for comparable DNA content after growing SMC for 7d or 14d.

Mature SMCs can undergo reversible changes of phenotype (phenotype modulation or switching) from normal, "contractile" SMC phenotype toward "synthetic" or "proliferative" phenotype in response to changes in the local environment. Previous studies of smooth muscle differentiation and phenotype modulation indicate that SMC express the contractile differentiated phenotype when the cells are not in a proliferating state (Rodríguez et al., 2006). MHC11 expression by the differentiated SMPCs indicated the contractile nature of the cells and the proliferation assay results reiterated the fact that the cells remained in contractile phenotype even after culturing the cells in the scaffold for a longer period. The minimal transcriptional expression of the extracellular matrix proteins, elastin and collagen, also supported the contractile nature of the differentiated cells. Maintaining a well-differentiated contractile SMC phenotype is one strategy to minimize the development of intimal hyperplasia (Beamish et al., 2010). Studies have shown that cells grown on fibrin scaffolds produce ECM rich in collagen. Quantification of extractable collagen from the cell seeded hybrid PCL-fibrin scaffold at different time periods in the present study showed significant quantity of extractable collagen. The quantified collagen that was extracted from the scaffold correlated well with the DNA content; both followed same pattern at each period of analysis. However, the fact that the collagen has not increased significantly after 15d culture indicated controlled expression of collagen which is essential in maintaining the pliability of the scaffold. A number of factors can affect the mechanical properties of engineered tissues, ECM proteins being one among them. The results of this study established that tensile stress and modulus were significantly higher in cell seeded scaffolds than in bare scaffolds. Hence, it may be assumed that there was sufficient ECM deposition for improving the tensile properties. Therefore, a tissue engineered graft can be constructed using a bilayered hybrid PCL scaffold with functional endothelial cells inside the lumen and smooth muscle cells outside the scaffold with adequate mechanical properties, a

strategy that can be translated for clinical implantation. An important observation of this study is that when EPCs are seeded in the luminal surface of the fibrin coated PCL scaffold, and exposed to shear stress, tPA up regulation was <1. But when EPCs were seeded on SMC layer (constructed TEVG) and exposed to shear stress in normal medium >3.0 fold up regulation of tPA was found. Similarly, CD31 and vWF expressions in normal medium were also significantly up regulated when ECs were seeded on SMC grown scaffold as compared to the ECs that were grown on fibrin. These results demonstrate that the SMCs in the medial layer influences the EC phenotype.

5.3. Molecular changes in EPC on exposure to risk factors

Endothelial dysfunction marks the beginning of an atherosclerotic plaque. Due to its crucial role, it is necessary to find an ideal model to study the molecular changes involved in endothelial dysfunction. Though several animal models are developed to study the disease, such as transgenic mice, these models frequently fail to recapitulate the human condition. 2D culture models can be used for studying the disease but they lack functional human primary cells and shear stress cannot be applied on cells during cultures. So the developed tissue engineered graft can be used to determine quantifiable molecular level changes in endothelial cells upon exposure to the selected most commonly known risk factors. An *in vitro* 3D tissue lined with endothelial cells is of particular use since the model allows EC to get exposed to shear stress and the risk factors simultaneously. Only when subtle molecular changes that could occur during preliminary stages of exposure to risk factors can be measured, more significant changes to the endothelium may be expected in long term. Therefore, commonly known risk factors namely glucose and cholesterol are selected for studying markers of endothelial dysfunction. Hypercholesterolemia is considered one of the main risk factors for the development of atherosclerosis. Hyperglycemia is associated with chronic endothelium inflammation and endothelial dysfunction. *In vitro* HUVEC models are used for the identification of new biological markers associated with hyperlipidemic-induced damage and also to study the molecular mechanisms involved in EC damage induced by high glucose

concentrations (Medina-Leyte et al., 2020).Since hyperglycemia and hypercholesterolemia causes a reduction in NO synthesis, NO release after exposure of the cell seeded scaffolds to high glucose and high cholesterol medium was assessed. NO release, on comparing with normal medium, showed no significant change when exposed to high glucose or high cholesterol medium. This can be attributed to the short term exposure of the cells to the risk factors since endothelial dysfunction is a chronic long term defect, which occurs due to the continuous exposure to risk factors. Or otherwise, in *in vivo* conditions along with glucose other factors are responsible for the reduced NO release. Therefore, the increased NO release or the increased eNOS expression in EC exposed to high glucose may or may not have clinical significance. The observation suggested that high glucose by itself is not affecting eNOS or NO production.

When endothelial cells were exposed to high concentrations of glucose or cholesterol, the EC quiescence is lost, and the cells acquire new phenotypes. Changes in gene expressions were consistent and quantifiable. In high glucose medium, anti-thrombotic marker eNOS was up regulated while pro-thrombotic marker vWF did not show any significant expression; however, the anti fibrinolytic molecule PAI was up regulated. On other hand, pro- fibrinolytic molecule tPA was down regulated in both high glucose/cholesterol medium. These results are not favorable, however, if it affects the EC function or not cannot be judged from the results obtained in this study alone. Adhesion molecules CD31 and VCAM-1 also showed a similar pattern, both molecules were down regulated in the presence of HG and HC. Relative expression of CD31 and VCAM-1 were almost absent in the presence of HC. In the presence of HG, CD31 downregulation was insignificant whereas in the presence of HC it was significantly down regulated.

A different pattern of expression was seen in the presence of TNF-1α. eNOS, vWF tPA and CD31 were down regulated, whereas PAI was up regulated. VCAM-1 was comparable with that in normal medium. On exposure to the inflammatory cytokine TNF-1α and high glucose containing medium together, a remarkable difference seen was in the expression of VCAM-1. From the 10 fold up regulation of VCAM-1 in normal medium, with TNF-1α and HC, there was 40 fold up regulation, w.r.t normal

hADMSC. All other gene expressions were comparable in normal medium and in HG-TNF-1α combination. On exposure to high cholesterol and TNF-1α, all genes were showing a downregulation except VCAM-1 which showed a significant up-regulation. The increase in relative gene expression reached up to 80-fold. Accelerated expression of VCAM-1 in the presence of combinations of HG/HC with TNF-1α suggests that in clinical scenario, inflammatory cytokine and either of the 2 (HG/HC) risk factors may induce significantly up-regulated VCAM-1 expression. Even though no independent effect of glucose or cholesterol on the inflammatory protein could be detected in this study, the highly significant combination effect of the inflammatory cytokine with either HG or HC on VCAM-1 suggests that the risk of acquiring endothelial dysfunction is high when such combinations persist in circulation.

During inflammatory responses, the ligands binding to VCAM-1 on the surface of activated endothelial cells first initiate the activation of calcium fluxes followed by downstream activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 2, leading to ROS and H_2O_2 generation(Cook-Mills et al., 2011; Marchese et al., 2012). Further the matrix metalloproteinases and protein kinase Cα activation (PKCα) facilitate VCAM-1–dependent leukocyte transendothelial migration (Deem et al., 2007; Deem and Cook-Mills, 2004).VCAM-1 also stimulates the formation of actin stress fibers and finally, this signal transduction pathway leads to gap junctional weakening and facilitates leukocyte transendothelial migration under inflammation conditions (Wittchen, 2009). Therefore, the significant upregulation of VCAM-1 in EC upon exposure to combination of TNF-1α and HG/HC seems to be an important and novel observation.

It may be noted that HG or HC alone did not cause any change in the expression of the inflammatory protein. However, both HG and HC independently up-/down-regulated the expressions of pro-/anti- thrombotic and pro-/anti- fibrinolytic molecules. Whether such quantifiable changes in the molecular markers of EC can be clinically significant or not is unclear from this study. However, the prothrombotic and/or antifibrinolytic phenotypes could lead to endothelial dysfunction. Overall, it is established that in the constructed blood vessel the ECs are healthy and respond to

single or dual factor variability in the circulating medium and the subtle changes at the molecular level are quantifiable. The HG medium up-regulated the relative expression of eNOS but high cholesterol drastically down-regulated the eNOS expression at mRNA level; however the NO production was not significantly affected. Therefore, concept of using artificially constructed bilayered blood vessel with functional endothelium and smooth muscle layer is found valuable in identifying the molecular level changes in the cell phenotype upon exposing EC to single or multiple risk factors.

5.4. Limitations of the study

The study has some limitations. To study endothelial dysfunction, effect of only single concentration of glucose and cholesterol was chosen.The risk factors were exposed only for a short period of 4 days and effect of only a single inflammatory molecule was studied. Effect of variable shear stress and changes in protein expressions were not dealt in this study.

CHAPTER 6

6. Summary and Conclusion

The goal of this study was to develop a model system for conducting atherosclerosis research at the molecular level. The study conceptualized that an artificially constructed blood vessel using human endothelial cells and smooth muscle cells could be a most appropriate approach for developing a biomimetic test system. The most suitable cell source selected was freshly differentiated EPCs and SMPCs derived from human ADMSCs. For creating biomimetic test model this study targeted functional cells in the lumen of the constructed blood vessel tissue to be an important requirement. It was also proposed that endothelial layer in the endothelium alone may not be sufficient for maintaining a quiescent phenotype; therefore, SMC seeded medial layer was also constructed. Another important requirement for achieving functional endothelium was also met by providing shear stress continuously during blood vessel construction and testing. The basal characteristics of the endothelium and medial layer was established before the construct is used for analyzing the effects of risk factors. Proper function of the endothelium reflects the release of NO, upon exposure to shear stress which was quantified using standard assay. The response to risk factors was identified by quantifying the relative expressions of specific molecular markers at mRNA level. The risk factors selected to validate the suitability of the developed model were physiologically relevant and easily miscible molecules that were added in the culture medium enabling continuous exposure to the cells in the lumen.

Artificially constructed functional small diameter blood vessels hold promise for application in vessel replacement surgeries. Therefore, well advanced principles and methods of vascular tissue engineering available in the literature were adopted to easily achieve the goal of the study. The use of healthy human cells is critical for developing the model test system. On the other hand construction of small diameter blood vessel with autologous cells can result in immune-compatible tissue for vessel replacement. Considering both applications in mind, the major focus of the study was

derivation of ECs and SMCs from ADMSCs isolated from patients undergoing coronary by-pass surgery. Once the quiescent functions of blood vessel constructed using ECs and SMCs derived from patient ADMSC is established, there is scope for its use as vascular graft in vessel replacement surgeries also. The current study achieved development of functional tissue engineered vascular graft and established its utility in the atherosclerosis research. The results of the study are summarized below and the conclusions are drawn. Also the future prospects are proposed.

6.1. Summary

The proliferation potential, stemness and purity of hADMSCs isolated from patients undergoing coronary artery bypass grafting was proven, confirming the feasibility of utilizing patient derived stem cells for both testing and therapeutic purposes. A novel method for differentiating hADMSCs to EPCs was established by combining the effects of growth factors, hypoxia and shear stress, sequentially. A previously standardized biomimetic fibrin based matrix was used for EPC differentiation. Optimum concentration of growth factors for endothelial lineage commitment was determined and the optimum time period for hypoxic induction was also determined. The proliferation and endothelial lineage commitment was demonstrated at mRNA and protein level using endothelial specific markers. Expression of EPC markers at each step of induction was demonstrated by quantifying the relative expressions of the specific markers. The influence of dynamic culture over static culture, the predominant effect of shear stress in the expression of EC markers and functionality of the differentiated ECs was proven. Effect of shear stress on the differentiation of ADMSC to quiescent EPC was seen in the relative expression of MCP-1.

For differentiating hADMSCs to SMPCs, a biomimetic fibrin based matrix was standardized. A novel method was developed for SMPC differentiation by standardizing the optimum concentrations of growth factors. The lineage commitment of SMPCs and the contractile phenotype of the differentiated cells were demonstrated by quantifying the relative expressions of specific markers at mRNA and protein level. The function of SMPCs was established by demonstrating ECM

deposition under static culture which seemed to influence mechanical properties of scaffold. A functional tissue engineered vascular graft was constructed by growing SMPC on the outer surface and EPC in the lumen under dynamic culture conditions. Coating of scaffolds with fibrin matrix and cells seeding techniques in the scaffold was standardized. Morphology of ECs in the lumen of the scaffold and cell stability after shear stress was established. Under shear stress, basal level expressions of target genes were determined by standardizing the test protocols. The influence of SMC layer on the maintenance of EC phenotype reflected on the relative expressions of various genes, such as tPA, vWF and CD31.

The study successfully constructed TEVG by growing human vascular cells on biocompatible and mechanically stable scaffold and established the specific cellular functions under shear stress. The use of a bilayered degradable scaffold with smooth lumen and fibrous ablumen facilitated construction of biomimetic endothelium. Smoothness of the endothelium matters for frictionless fluid flow and steady shear rate and stress. The fibrous external surface helped 3-dimensional growth of SMC. The human cell- grown TEVG produced an opportunity to study the effect of cytokines and life-style related risk factors using a biomimetic model system. The sensitivity of EC layer to the risk factors offer major prospects of detecting the effect of various risk factors on the functional EC-specific molecular markers.

The viability of using the constructed tissue engineered graft as a model to study endothelial dysfunction was demonstrated by exposing EC seeded grafts to selective risk factors namely high glucose and high cholesterol in the presence and absence of an inflammatory cytokine. Nitric oxide release after risk factor exposure proved the function of the EC seeded grafts. On exposure of ECs to high glucose and high cholesterol, changes in relative gene expressions of all 7 molecular markers as compared to the exposure to normal medium were observed.

Similarly, on exposure of the cell seeded scaffolds to TNF-1α added in the normal medium, and medium containing high glucose/ high cholesterol resulted up/down regulation of gene expressions. Addition of TNF-1α in normal medium resulted in

the downregulation of EC specific genes eNOS, vWF and CD31 whereas PAI and vCAM1 showed upregulation. tPA was down regulated in the presence of added TNF-1α. MCP-1 showed minimal expression in the presence and absence of TNF-1α. The presence of TNF-1α in high glucose and high cholesterol medium resulted in significant upregulation in the gene expression of VCAM-1.

Endothelial dysfunction is known to be associated with a state of inflammation. Exposure to high glucose or high cholesterol itself resulted in noticeable change in expressions of EC genes, especially eNOS, CD31 and vWF. With the addition of the inflammatory cytokine TNF-1α, the change in gene expressions were more remarkable. Addition of TNF-1α resulted in higher gene expressions of adhesion molecules CD 31 and VCAM-1 indicating the proinflammatory state of the developed model.

The specific study objectives achieved are as follows:

1. Multipotent hADMSCs from patient tissue has been isolated to pure and proliferating population.
2. hADMSCs isolated were differentiated to endothelial progenitor cells in the specific biomimetic fibrin matrix.
3. A tri-level induction protocol was established for the differentiation of hADMSCs to endothelial progenitor cells.
4. Effect of each stage of induction on the endothelial lineage commitment was demonstrated.
5. Differentiation of hADMSCs to endothelial lineage was confirmed by specific markers at the mRNA level and protein level.
6. hADMSCs isolated were differentiated to smooth muscle progenitor cells in the specific biomimetic fibrin matrix.
7. Concentration of growth factors required for the differentiation of hADMSCs to SMPCs was determined.
8. Differentiation of hADMSCs to smooth muscle lineage was confirmed by specific markers at the mRNA level and protein level.

9. Tissue engineered vascular graft was constructed using PCL-fibrin hybrid scaffold and hADMSC derived EPCs and SMPCs.
10. Influence of static SMPC culture and dynamic EPC culture on TEVG properties were identified.
11. The NO release established adequate function of constructed TEVG.
12. Established shear stress as a major contributing factor for regulating EC markers and function.
13. Established growth of SMPC on electrospun ablumen of PCL-fibrin hybrid scaffold through cell proliferation studies.
14. ECM deposition by SMPC on the graft was established by the expression of ECM proteins.
15. Established the effect of ECM proteins on the tensile properties of the graft.
16. Established subtle changes to ECs upon exposure to high glucose and high cholesterol.
17. Demonstrated independent and combinatorial effect of high glucose/ cholesterol / TNF-1α on the regulation of relative gene expressions of specific EC markers.
18. Established that TNF-1α, when combined with either high glucose or high cholesterol remarkably up regulates inflammatory proteins

6.2. Conclusions

1. Differentiated human EPCs and SMPCs were derived from hADMSCs and determined their phenotypes.
2. An even EC layer was established in the lumen of porous and smooth PCL scaffold.
3. The sensitivity of endothelial phenotype to shear stress is established.
4. The sensitivity of EC layer to the cytokine and life-style related risk factors are demonstrated
5. The study hypothesis was proven by validating the utility of artificially constructed blood vessel as an *in vitro* model system for quantifying the molecular markers relevant in atherosclerosis research.

6.3. Future Prospects

In the context that the constructed blood vessel was found suitable for analyzing the subtle changes in EC at the molecular level, the model test system has many applications. The model promises scope for atherosclerosis research more effectively. There are many aspects that were not addressed in the current study which may be taken up:

1. Effect of graded concentrations of glucose and cholesterol may be studied
2. Combined effect of cholesterol and glucose may be studied
3. Effect of other risk factors may be studied
4. Effects at the protein level and dysfunction of EC may be studied
5. Effect of EC dysfunction on underlying SMC may be studied
6. Suitability of developed TEVG in vessel replacements may be explored

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LIST OF PUBLICATIONS

Original papers

- Ragaseema VM, Soumya Columbus, **Renu Ramesh** and Lissy K. Krishnan. Potential of tissue engineered blood vessel as model to study effect of flow and wall thickness on cellular communication. Current Tissue Engineering 2014; doi : 10.2174/2211542002666131209233849
- Subha S, **Renu Ramesh**, K. Jayakumar, Lissy K Krishnan. In vitro differentiation of adipose-derived mesenchymal stem cells into cardiac tissue-linked progenitor cell cluster using fibrin matrix based niche Cardiol Cardiovasc Med 2020;4(6):646-678
- **Renu Ramesh**, Jayakumar K and Lissy K Krishnan. Synergistic effect of inducible factors on the differentiation of human-adipose derived stem cells to vascular cells Archives of Clinical and Biomedical Research 2020;4 (6):632-656

Presentations and Conference Proceedings

- **Renu Ramesh**, Subha S and Lissy K. Krishnan. Autologous Blood Vessel from Human ADMSCs, 2017. TERMIS 2017 – AM chapter, Charlotte, North Carolina, USA **(Poster)**.
- **Renu Ramesh**, Subha S and Lissy K Krishnan Induction of human adipose derived stem cells to endothelial cells and smooth muscle cells, 2017. National Seminar on Frontiers in Biotechnology, University of Kerala, Kariavattom campus, Trivandrum, India **(Poster-Best Poster award)**.

APPENDIX

PBS (1000ml) pH 7.4

NaCl	-8g
KCl	-0.2g
Na_2HPO_4	-1.44g
KH_2PO_4	-0.24g

Added distilled water to 1000ml, filtered and stored at RT.

HBSS (1000ml) pH 7.4

NaCl	-8g
KCl	-0.4g
Na_2PO_4	-0.0482g
KH_2PO_4	-0.06g

Added distilled water to 1000ml, filtered, autoclaved and stored at 4°C.

SFM

DMEM LG	-50ml
Antibiotics (10X)	-500µl

Filtered and stored at 4°C.

URKUND

Document Information

Analyzed document	thesis Renu.doc (D75918587)
Submitted	7/2/2020 11:44:00 AM
Submitted by	Anugya Bhatt
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W	URL: https://www.researchgate.net/publication/6319434_Functional_tissue-engineered_bloo ... Fetched: 12/23/2019 5:20:28 PM	1
J	**Stem cells for tissue engineered vascular bypass grafts** URL: 6ef8b07a-e1f9-420a-9d64-fd2da00ecd7e Fetched: 3/12/2019 8:36:00 PM	2
W	URL: https://worldwidescience.org/topicpages/v/vascular+mural+cells.html Fetched: 3/8/2020 9:30:58 PM	1
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RESEARCH AREA

Currently my research area focuses on exploring the differentiation potential of human adipose derived mesenchymal stem cells (hADMSCs) to construct an autologous blood vessel using the principles of tissue engineering. The study involves the construction of a tissue engineered vascular graft (TEVG) using hADMSC derived vascular cells. The study also explores the suitability of using artificially constructed blood vessel for studying the effect of high glucose/cholesterol, in the absence and presence of inflammatory cytokine-TNF-1α circulated in medium, on the phenotype of endothelial cell lining the lumen. The study extensively makes use of techniques such as flow cytometry, immunocytochemistry and quantitative real time PCR to analyse the expression of specific proteins and the gene expression pattern by the differentiated cells.

WORK EXPERIENCE

Sl. No.	Position	Institute	Employer	Duration
1	PhD Scholar	Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Trivandrum, INDIA	Dr Lissy K Krishnan Scientist 'G', Senior Grade	6 years 27/01/2014-30/06/2020
2	Project Scientist	Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Trivandrum, INDIA	Dr Lissy K Krishnan Scientist 'G', Senior Grade	3 years 04/06/2010-31/01/2013
3	Project Fellow	Regional Cancer Centre, Trivandrum, INDIA	Dr Jayasree, HOD, Dept. of Pathology	2 years, 2 months (21/11/2005-31/01/2008)
4	Project Fellow	Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Trivandrum, INDIA	Dr.T.Anoop Kumar, Scientist 'E', Molecular Medicine	6 months (14/10/2004-31/03/2005)

SKILLS AND EXPERTISE

- Genetic biomarker profiling using qRT-PCR analysis.
- Culture and maintenance of Primary mammalian cells (endothelial cells, human MSCs).
- Isolation of primary endothelial cells from Human umbilical cord vein.
- Isolation of mesenchymal stem cells (MSCs) from human adipose tissue.
- Immunocytochemistry.
- Molecular biology techniques (RNA isolation, cDNA synthesis, qRT-PCR- BIORAD); sample preparation for Flow cytometry; Protein estimation by Lowry's method, SDS PAGE, Agarose gel electrophoresis.
- Planning and conducting animal experiments (rabbit).
- Scaffold fabrication by solvent cast and electrospinning techniques.

CONFERENCE PRESENTATIONS

- Presented a paper on Correlative study of Cytological methods and PCR in detecting Human Papilloma Virus in cervical cancer at **National Conference on Biotechnology and Molecular Medicine at AIMS, Kochi.**
- Exposure to international cancer research projects like "Relation between Human Papilloma Virus and Epithelial Cancers in Latin American countries, Pakistan and Bangladesh" by virtue of a short stint of association with Kagoshima University, Japan.
- Attended workshop on flow cytometry at the **10th TCS Annual Meeting &Workshop on Application of Flow cytometry in Health & Disease, held at SCTIMST (28-31 October, 2017).**

- Presented poster on "Autologous Blood Vessel from Human ADMSCs" at the **TERMIS 2017 – AM chapter held at Charlotte, North Carolina, USA (3-6 December, 2017).**
- Attended one day seminar on **Dissemination of ICMR-DBT National Guidelines for Stem Cell Research at AIMS, Kochi (February 22, 2018).**
- Presented poster on "Induction of human adipose derived stem cells to endothelial cells and smooth muscle cells" at **National Seminar on Frontiers in Biotechnology held at Kariavattom campus, University of Kerala (1-3 March, 2017).**

HONOURS AND AWARDS

- Best Poster Award for the poster entitled "INDUCTION OF HUMAN ADIPOSE DERIVED STEM CELLS TO ENDOTHELIAL CELLS AND SMOOTH MUSCLE CELLS" presented at **National Seminar on Frontiers in Biotechnology held at Kariavattom campus (1-3 March, 2017).**

PUBLICATIONS

- Ragaseema VM, Soumya Columbus, **Renu Ramesh** and Lissy K. Krishnan. Potential of tissue engineered blood vessel as model to study effect of flow and wall thickness on cellular communication Current Tissue Engineering, 2014, 3, 39-46
- Subha S, **Renu Ramesh**, K. Jayakumar, Lissy K Krishnan. In vitro differentiation of adipose-derived mesenchymal stem cells into cardiac tissue-linked progenitor cell cluster using fibrin matrix based niche Cardiol Cardiovasc Med 2020;4(6):646-678
- **Renu Ramesh**, Jayakumar K and Lissy K Krishnan. Synergistic effect of inducible factors on the differentiation of human-adipose derived stem cells to vascular cells Archives of Clinical and Biomedical Research 2020;4 (6):632-656

REFERENCES

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