A STUDY OF STRENGTH AND VASOACTIVITY IN A TISSUE ENGINEERED VASCULAR MEDIA

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A STUDY OF STRENGTH AND VASOACTIVITY IN A TISSUE ENGINEERED VASCULAR MEDIA

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LIST OF ABBREVIATIONS

2D	Two Dimensional
3D	Three Dimensional
5-HT	5- Hydroxytryptamine (Serotonin)
ACA	ε-Amino Caproic Acid
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
BGS	Bovine Growth Serum
BHT-920	5,6,7,8-tetrahydro-6-(2-propenyl)-4H-thiazolo[4,5-d]azepine-2-amine
BSA	Bovine Serum Albumin
CABG	Coronary Artery Bypass Grafting
cAMP	Cyclic Adenosine Monophosphate
cDNA	Complementary Deoxyribonucleic Acid
COX	Cyclo-oxygenase
cpm	Counts per Minute
CVD	Cardiovascular Disease
DAG	Diacylglycerol
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
ECM	Extracellular Matrix
EDHF	Endothelium Derived Hyperpolarizing Factor

ePTFE	Expanded Polytetrafluoroethylene
FBS	Fetal Bovine Serum
GAG	Glycosaminoglycan
HYAFF	Esterified Hyaluronic Acid
IP_3	Inositol Triphospate
MAGP-1	Microfibril-Associated Glycoprotein 1
MLC	Myosin Light Chain
MLCK	Myosin Light Chain Kinase
MMP	Matrix Metalloproteinase
NO	Nitric Oxide
P4HB	Poly-4-Hydroxybutyrate
PAF	Platelet Activating Factor
PAH	Poly(Allylamine Hydrochloride)
PBS	Phosphate Buffered Saline
PCI	Percutaneous Coronary Intervention
PCL	Poly(ε-Caprolactone)
PCR	Polymerase Chain Reaction
PEG	Poly(Ethylene Glycerol)
PEUU	Poly(Ester Urethane) Urea
PDGF	Platelet Derived Growth Factor
PGA	Polyglycolic Acid
PHA	Polyhydroxyalkanoate
PLA	Poly-L-Lactide
PLLA	Poly-L-Lactic Acid
PLMA-ECA	Poly(Lactide-*Co*-β-Malic Acid) with Extended Carboxyl Arms
PMBU	Poly(2-Methacryloyloxyethyl Phosphocholine-Co-Methacryloyloxyethyl Butylurethane)

POC	Poly(1,8-Octanediol Citrate)
PSS	Poly(Styrene Sulfonate)
PVDF	Polyvinylidene Difluoride
RASMC	Rat Aortic Smooth Muscle Cell
RIPA	Radio-Immunoprecipitation Assay
RNA	Ribonucleic Acid
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Standard Error of the Mean
SIS	Small Intestinal Submucosa
SLRP	Small Leucine Rich Repeat Proteoglycan
SMC	Smooth Muscle Cell
SNP	Sodium Nitroprusside
TEVM	Tissue Engineered Vascular Media
TGF-β1	Transforming Growth Factor Beta-1
TIMP	Tissue Inhibitors of Matrix Metalloproteinases
TNF-α	Tumor Necrosis Factor Alpha
TBS	Tris Buffered Saline
TBS-T	Tris Buffered Saline with Tween

SUMMARY

To be successful a tissue engineered small diameter blood vessel must be non-immunogenic, non-thrombogenic, have mechanical properties similar to native vessel and be vasoactive. The vascular media is responsible for the mechanical properties and the vasoactivity of the vessel. The collagen hydrogel approach has been long used and has many advantages, but has not yet achieved the mechanical integrity needed for implantation. No collagen-based tissue engineered vascular media has been shown to be vasoactive using culture techniques required to achieve the cell numbers needed to make a vascular graft. To study collagen synthesis, two model systems were used. Cells were seeded on top of an adsorbed collagen I or fibrin layer. Alternatively the cells were encapsulated in a collagen or fibrin hydrogel. Collagen I, decorin and biglycan synthesis was affected by both matrix type and presentation. After two weeks in culture the smooth muscle cells produce more type I collagen in the collagen based hydrogels then in the fibrin hydrogels and was used for further studies. The collagen based tissue engineered vascular media produced a consistent vasoactive response between two and eight weeks of culture. The smooth muscle cells have functional endothelin, kinin, adrenergic, serotonergic and purinergic receptors. The application of cyclic strain improves both the tissue strength and the contractile response. Use of transforming growth factor-β improved tissue strength, but reduced the contractile response. Transforming growth factor- β actually promoted a more contractile cell phenotype, but a stronger contractile force was required to overcome the thick compact collagen hydrogel and elicit a measurable contraction. This work adds to what is known about collagen-based tissue engineered vascular medias by identifying means of improving not only strength but vasoactivity. The trade-offs found between these two important characteristics are relevant to all tissue engineered medias.

CHAPTER 1: INTRODUCTION

Tissue engineering and regenerative medicine aims to repair, replace or enhance tissue function. The development of a small diameter blood vessel has long been considered the 'holy grail' of tissue engineering due to the clinical need for sufficient amounts of suitable tissue. Despite medical advances that are less invasive such as angioplasty and stents, bypass grafting remains the gold standard and the procedure of choice for certain conditions. Small diameter grafts are needed for coronary artery bypass grafting as well as for bypass grafts for peripheral vessels such as the femoropopliteal artery. Persons needing bypass grafts do not always have sufficient amounts of healthy blood vessels that are can be harvested for bypass surgery. Synthetics that have been used clinically for large vessel bypass occlude when used for small vessel bypass.

A successful tissue engineered vascular graft must be non-thrombogenic, non-immunogenic, and vasoactive, be able to arterialize, and have material properties such to withstand arterial pressures. Blood vessel tissue engineering is advancing and there are some successful clinical applications, but most of these successes have been in pediatric applications on the low pressure side [1-7]. Clinical trials are also being completed for use as an arteriovenous shunt [8]. No small diameter grafts have progressed to the clinic. All but the most recent trials have used biodegradable polymers, and none of the new polymers have been tested in small diameter applications and it is unknown if they will remain patent. Natural biopolymers are considered a better choice for small diameter application in this regard, but the mechanical properties are often lacking. To this end this work focuses on the tissue engineering of the medial layer. This layer imparts the mechanical properties of the blood vessel and is responsible for the blood vessel contraction and dilation required for regulation of blood flow.

Mechanical integrity is essential for a vascular graft; vasoactivity, while not as important, is still considered a desirable trait. Only a few groups have demonstrated the capability of their tissue engineered blood vessels to respond to vasoactive agonists and antagonists. While there is work on how different stimulation affects smooth muscle cell phenotype there has not been work to show if this change in phenotype results in any functional improvement.

The central hypothesis is that a collagen I as a TEVM scaffold can promote collagen production required for strength and a vasoactive response. Further the TEVM strength and vasoactive function could be improved through mechanical or biochemical stimulation. This hypothesis will be tested by pursuing the following aims:

Specific Aim 1: Investigate the effects of matrix type and 2D/3D presentation on synthesis of type I collagen and the associated proteoglycans decorin and biglycan.

Collagen I synthesis and deposition is important for tissue strength. Decorin and biglycan play important roles in collagen fibrillogenesis in different tissues. The hypothesis was that both matrix type and method of presentation would affect collagen, decorin and biglycan synthesis. The effects of collagen type I and fibrin on smooth muscle cell synthesis of these proteins is presented in Chapter 4.

Specific Aim 2: Characterize the contractile response of the tissue engineered vascular media.

A contractile response to vasoactive agonists and antagonists has not yet been shown in a collagen-based tissue engineered vascular media. The hypothesis was that adult smooth muscle cells in collagen would present multiple classes of vasoactive receptors and could exhibit a contractile response. As part of this study the effects of tissue culture time and cell passage number were explored. These results are presented in Chapter 5.

Specific Aim 3: Investigate the effects of the application of cyclic strain after cessation of gel compaction on tissue strength and vasoactive function.

Previously the Nerem lab has shown that cyclic strain can improve tissue strength, significant gel compaction and not significant increase change in cell phenotype as assessed by smooth muscle α-actin. The hypothesis for this specific aim is that by waiting until gel compaction ceased before applying strain the gel would better adhere to the silicone sleeve, eliminating compaction. Further, the change would produce improved strength and improved vasoactive function. The results of this aim are presented in Chapter 6.

Specific Aim 4: Investigate the role of TGF-β and the Smad signaling pathway on tissue strength and function.

Transforming growth factor-β (TGF-β) has been shown to promote a more contractile cell phenotype and is used by multiple groups to enhance extracellular matrix production. It was hypothesized that the contractile response might be influenced by the Smad pathway and that TGF-β could improve both tissue strength and vasoactive function. To do this tissue engineered vascular media were cultured with either TGF-β or tumor necrosis factor-α (TNF- α). The results are shown in Chapter 7.

An additional study is presented looking at vasoactive function after cryopreservation. Cryopreservation may be a way to provide 'off-the-shelf' availability of tissue engineered organs. Previously our lab had shown that both vitrification and freezing resulted in similar viability rates. Both methods resulted in enhanced tissue strength. For this study the effects of these cryopreservation methods on vasoactive function were investigated. The results are shown in Chapter 8.

This chapter is followed by a background section, 5 research chapters as indicated by the aims discussed above and a discussion and conclusions section. The research chapters are divided based on the research questions and methods used. Chapters 4 and 6

are similar to manuscripts. Portions of chapters 5, 7 and 8 will be combined with other chapters for publication.

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CHAPTER 2: BACKGROUND

Cardiovascular Disease

Cardiovascular disease (CVD) is a major clinical and economic issue as well as a leading cause of death in developed nations. CVD affects 80.7 million Americans and is attributed to 36.3% of all deaths in the United States in 2004. Direct and indirect costs are estimated to reach $448.5 billion in 2008. Atherosclerosis is the most common form of CVD and leads to narrowing of the arteries which can lead to reduced function or amputation of limbs, stroke, or myocardial infarction depending on location. Coronary artery disease is the most prevalent CVD accounting for 52% of all CVD [1, 2]. Coronary stenosis can be treated by coronary artery bypass grafting (CABG) or by percutaneous coronary intervention (PCI), commonly known as angioplasty; PCI can be completed with or without a stent. While PCI has become an alternative, CABG is still considered the "gold-standard" of treatment and is the preferred treatment options for patients with left main or triple-vessel coronary artery disease with reduced left ventricular function.[3-5]. Additionally PCI treatment requires significantly more repeated procedures than CABG; while drug eluting stents have reduced restenosis they have not eliminated it or the need for repeated surgery [3, 6].

There are several choices of material for bypass graft conduits. Autologous venous grafts, most commonly from the saphenous vein were the material of choice at the advent of CABG surgery. More recently autologous arterial grafts, especially from the internal mammary artery, have gained popularity in CABG procedures due to the lower incidence of intimal thickening [7-9]. One issue with the autologous tissue grafts is that patients with atherosclerosis do not always have sufficient amounts of healthy tissue. There are several non-autologous tissue options such as use of allogeneic umbilical or

saphenous vein or xenogeneic bovine internal thoracic artery. Most tissues are made nonviable through use of glutaraldehyde fixation. While these are an option the patency rates are low ranging from 65% for cryopreserved saphenous vein grafts to less than 16% for the xenografts [7]. Synthetic material such as Dacron has been used successfully to replace larger diameter vessels (>6 mm); however, these materials have not be successful for small diameter (diameter ~3mm) bypass procedures due to occlusion. Much work is being done to improve patency of new biomaterials; however, there has been little success to date [7, 10-12].

Artery Anatomy and Physiology

The arteries are larger vessels (>0.1 mm diameter) that transport blood away from the heart and to the tissues. An artery is more than just an inert pipe; it is able to sense and respond to hemodynamic changes to regulate blood flow. There are three distinct layers to an artery that work together to accomplish this task: the tunica intima, tunica media and tunica adventitia, shown in Figure 1. The layers are composed of different cell types and matrix components to provide different functions. While all arteries contain each of the layers, the thickness and extracellular matrix (ECM) composition can vary slightly depending on the distance from the heart. The intimal layer is the innermost layer; it is composed of a confluent monolayer of endothelial cells and is separated from the other layers by a basal lamina of type VI collagen, laminin, elastin and proteoglycans. The endothelial layer creates a non-thrombogenic lining that can act as a selective barrier. The endothelial cells also play an important role in sensing hemodynamic changes and regulating vascular tone. The middle layer, or medial layer, is composed of smooth muscle cells within a matrix of types I and III collagen, elastin and proteoglycans. This is usually the thickest of the three layers and provides the strength, compliance and vasoactivity of the artery. The collagen is aligned circumferentially and provides the

tensile strength required to withstand arterial pressure. The elastin network is the elastic component providing compliance and recovery after each pressure pulse. Smooth muscle cells synthesize matrix; they are contractile and critical to vasoconstriction. The adventitia is the outermost layer of connective tissue; it consists of fibroblasts in a loose network of type I collagen. The adventitia stabilized the artery by anchoring it to the surrounding connective tissue. In larger blood vessels the nerves and vasa vasorum, the small vessels that provide nutrients for the arterial wall, are found in the adventitial layer [13, 14].

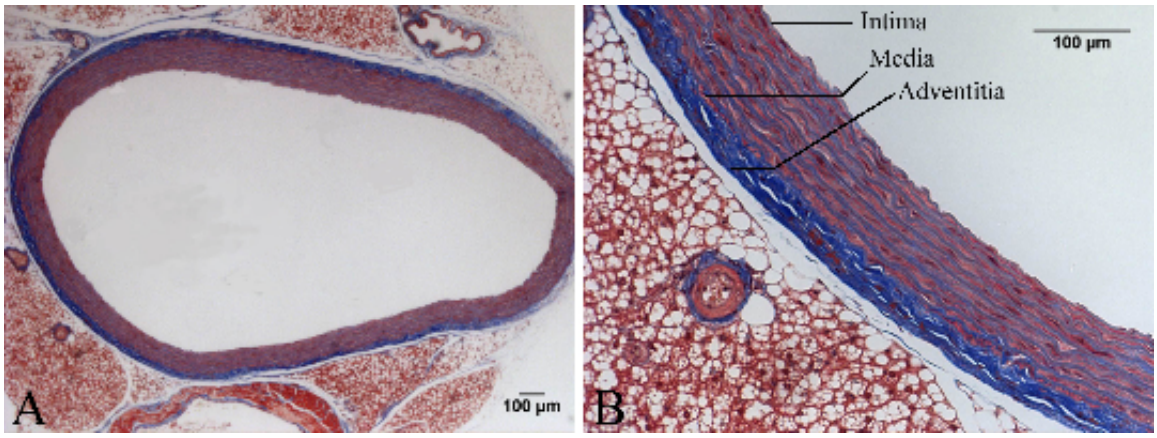

Figure 1: Cross-section of rat muscular artery. Sections are stained with Masson's Trichrome. Collagen fibers are stained blue, nuclei are stained purple, and the cytoplasm, keratin and muscle fibers are stained red. The three layers are shown in the magnified section (B).

The size, composition, and even presence of the three layers vary in the different blood vessels based on functional requirements. Table 1 shows a side by side comparison of the composition of arterial and pulmonary blood vessels. All blood vessels have an intima layer consisting of a basement membrane and a confluent monolayer of endothelial cells. Their function to maintain selective permeability and non-thrombogenicity are important for every size vessel. The importance of the medial layer and its' components varies by vessel. Elastin's role in recovery of strain is especially necessary in the aorta, closest to the heart. The aorta can expand by approximately 10%

and contract to act as a pulse dampener for the system. Large amounts of elastin are needed to accomplish this; elastic arteries contain approximately 40% elastin. Muscular arteries and the smaller resistance vessels contain a lesser amount of elastin due to the smaller strains. Arterioles and veins contain little to no elastin due to the small amount of strain that these vessels see. Greater pressures require greater amounts of collagen to provide tensile strength. The muscular arteries have the largest amount of collagen. The smooth muscle cell content varies based on the need for blood pressure regulation. The smooth muscle cells contract and relax to change the diameter of the blood vessel to control flow. This is especially important in the muscular arteries. Some smooth muscle cells or pericytes are found in and capillaries; the contractile function of the cell is important for maintaining blood flow in the small vessels. The anchoring and scaffolding for vasa vasorum is most important for the larger vessels [13, 14]. The coronary and femoropopliteal arteries are muscular arteries.

Table 1: Composition of Arteries and Veins

Arterial Vessels	Pulmonary Vessels
Elastic Artery • Endothelial layer • Thick medial layer • Plentiful elastin • Thick adventitia	Large Vein • Endothelial layer • Medial layer • Little elastin • Adventitia
Muscular Artery • Endothelial layer • Thick medial layer • More collagen then elastin • Adventitia	Medium-sized Vein • Endothelial layer • Medial layer • Adventitia
Arteriole • Endothelial layer • Medial layer mostly smooth muscle cells • No adventitia	Venule • Endothelial layer • No medial layer • Thin adventitia
Continuous Capillary • Endothelial layer • Occasional pericytes	Fenestrated Capillary • Endothelial layer • Occasional pericytes

Arterial Hemodynamics

Blood vessels are subjected to multiple forces due to the pulsatile nature of blood flow. The blood vessels both shear and normal stresses. As blood flows across the endothelium, frictional forces cause shear stress on the arterial wall (τ_w). The τ_w is proportional to the shear rate at the wall and the fluid dynamic viscosity. For many blood vessels detailed measurements of the velocity at the wall must be taken to determine wall shear stress. For some *in vivo* and *in vitro* situations the wall shear stress can be approximated as:

$$\tau_w = 4\mu Q/\pi R^3$$

where μ is the dynamic viscosity, Q is the volumetric flow rate and R is the tube diameter. This approximation assumes steady laminar flow of a Newtonian fluid through a rigid pipe. It is not appropriate areas of branching or tapering, non-circular cross-sections or areas with pulsatile flows. It cannot be used in small diameter vessels where blood does not behave as a Newtonian fluid. It is a good approximation for smaller arteries. Arteries can adapt to regulate pressure and flow; typically a mean wall shear stress of approximately 15 dynes/cm^2 is maintained. Wall shear stress provides important cues to endothelial cells and can affect endothelial cell alignment. Areas of plaque formation often correspond to oscillating or stagnant flow [15-18].

The blood pressure creates a hoop stress (σ_h), the circumferential stress in the blood vessel walls. For a thin walled tube the hoop stress is approximated as:

$$\sigma_h = PRt^{-1}$$

where P is pressure, R is the radius and t is the wall thickness. The wall stress increases with blood pressure and vessel diameter and decreases with increased wall thickness. The mean pressure found in arteries is approximately 100 mmHg; veins see mean pressures of approximately 10 mmHg. The blood vessels will adapt to sustained changes in blood pressure. Hypertension leads to smooth muscle cell hypertrophy, leading to a thicker

medial layer. This increase in wall thickness serves to decrease the pressure seen by the smooth muscle cells in the wall [15, 18, 19]. Hoop stress is the best studied stress in native and engineered blood vessels; however, work has been done to investigate the effects of longitudinal or axial stress and strain [20, 21].

Vasoactivity

Regulation of vascular tone, the smooth muscle tension, is important for regulation of blood pressure and flow. In mature blood vessels smooth muscle cells contain a number of proteins involved in contraction, many which have smooth muscle cell isoforms such as smooth muscle α-actin, myosin heavy chains, myosin light chains, α-tropomyosin, calponin, smooth muscle 22α, h-caldesmon, γ-vinculin and α- and β-*meta*-vinculin [22]. The contractile machinery consists of sarcomeric units of actin and myosin filaments [14].

The smooth muscle cells maintain a partially contracted state at all times; this minimum blood vessel tension is called the tonic or basal tone. The increase or decrease in tone is regulated via sensitivity to and concentration of Ca^{2+}. The change in intracellular free Ca^{2+} necessary for contraction is on the order of 1 μM [23]. Following an increase in cytosolic Ca^{2+}, the calcium ions bind to calmodulin which is associated with the enzyme myosin light chain kinase (MLCK). MLCK catalyzes the phosphorylation on myosin light-chain (MLC) which is associated with the head of each myosin molecule. This phosphorylation increases myosin's interaction with filamentous actin producing contraction. Relaxation is caused by the dephosphorylation of myosin by myosin light chain phosphatase [24].

Vascular tone has complex regulation that requires both intrinsic and extrinsic factors. This section is meant to be a review of the most common agonists and antagonists but is not all inclusive. The primary mechanisms of intrinsic and extrinsic

regulation are listed in Table 2. Intrinsic regulation consists of factors such as the endothelial cell secreted nitric oxide and the autacoid histamine that act locally. Autoregulation of flow and functional and reactive hyperaemia are mediated exclusively by intrinsic regulation. Extrinsic regulation involves mechanisms such as the hormone adrenaline and regulation by vasomotor and vasodilator nerves.

Table 2: Intrinsic and Extrinsic Mechanisms of Vascular Tone Regulation [14]

Intrinsic Regulation	• Bayliss myogenic response • Vasoactive metabolites • Endothelial secretions • Autacoids – local vasoactive paracrine secretions • Temperature
Extrinsic Regulation	• Vasomotor nerves • Circulating hormones

Intrinsic Regulation

Bayliss Myogenic Response

Intrinsic regulation is a local regulation designed to meet the needs of the organ. The Bayliss myogenic response is the contraction of the smooth muscle cells in response to changes in arterial pressure. This response contributes to basal tone and stabilizes local blood flow. The Bayliss myogenic response is mediated by depolarization which increases the number of open Ca^{2+} channels raising cytosolic Ca^{2+} which activates the contractile machinery. Regulation of the constriction is thought to be due to increased shear stress in the narrowed vessel which causes the endothelial cells to increase synthesis of nitric oxide.

Vasoactive Metabolites

Vasoactive metabolites secreted by skeletal muscle, myocardium or active brain regions can cause local increases in blood flow. Adensosine, interstitial K^+, phosphate ions as well as CO_2 and O_2 levels can all affect local vascular tone. The type of tissue dictates the sensitivity and type of response that occurs. Many of these reactions are due to responses to exercise where oxygen and nutrient needs change and increased flow is needed for removal of byproducts such as lactic acid.

Endothelial Secretions

Endothelial cells secrete the vasodilators nitric oxide (NO), endothelium derived hyperpolarizing factor (EDHF) and prostacyclin, as well as the vasoconstrictor endothelin. Nitric oxide is important for regulating basal tone including the reduction in tone during pregnancy, and causing dilation in response during inflammation and due to increased flow during exercise. NO is regulated primarily by shear stress although circulating insulin and oestrogen also play a role. Basal tone is also regulated by the vasoconstrictor endothelin. Endothelin binds to the ET_A and ET_B receptors in arterial and venous smooth muscle cells causing constriction that can last 2-3 hours. Endothelin-1 has been shown to be one of the most potent vasopressors [25]. The activation of the receptors raises cytosolic Ca^{2+} through activation of the receptor operated Ca^{2+} channels and through the phospholipase C-IP_3-DAG pathway.

Autacoids

Local vasoactive paracrine secretions, or autacoids, are primarily secreted in response in pathological events such as inflammation and bleeding. A brief list of some of the important autacoids and their primary functions are listed in Table 3.

Table 3: The Autacoids [14]

Autacoid	Function
Histamine	Mediates Inflammation
Bradykinin	Mediates Inflammation
Serotonin	Mediates Inflammation Arrests Bleeding
Prostaglandins	Mediates Inflammation Contributes to Reactive Hyperaemia
Thromboxane	Arrests Bleeding
Leukotrienes	Mediates Inflammatory Response
Platelet Activating Factor	Mediates Inflammation Mediates Bronchoconstriction

Histamine

Histamine is produced by the decarboxylation of histidine; it can cause constriction or dilation depending on which receptors are present in the vessel. Binding of histamine to the H1 receptors in the veins causes activation of the phospholipase C-IP_3 pathway which causes constriction. Conversely, binding of histamine to the H2 receptor activates the adenlyate cyclase-cAMP pathway which results in vascular relaxation in the arterioles.

Bradykinin

Bradykinin is generated by the enzyme kallikrein which is activated during inflammation. Bradykinin activates the bradykinin receptors B1 and B2. Bradykinin is considered a vasodilator, but can cause constriction. A biphasic response to bradykinin has been seen [26-28]. Activation of the B1 receptor results in vasodilation while activation of the B2 receptor results in constriction [26]. NO, EDHF and prostacyclin mediate dilatory effect of bradykinin.

Serotonin

Serotonin (5-hydroxytryptamine, 5-HT) is a vasoconstrictor that is produced from tryptophan in platelets, agentaffin cells in the intestine and some central neurons. The

main function of serotonins is to arrest bleeding, although it also plays a role in regulation of local blood flow in the intestine.

Prostaglandin

Prostaglandin is eicosanoid; it is produced from the fatty acid arachidonic acid through action of cyclo-oxygenase (COX). Endothelial cells, leukocytes, fibroblasts and macrophages all produce prostaglandins. Different prostaglandins have different effects and vascular tone. The E series of prostaglandins and prostacyclin are vasodilators while the F series of prostaglandins are mostly vasoconstrictors.

Thromboxane

Thromboxane is also an eicosanoid. Thromboxane A_2 is a vasoconstrictor and thrombotic agent that is synthesized by COX within platelets. Its' primary role is to help arrest bleeding. The function of aspirin as an antithrombotic agent occurs by blocking inhibiting COX and reducing thromboxane content.

Leukotrienes

Leukotrienes are vasoconstrictors produced by leukocytes from arachidonic acid by the enzyme lipoxygenase. They mediate the inflammatory response. It is a vasoconstrictor and also increases venular permeability.

Platelet Activating Factor

Platelet activating factor (PAF) is a lipid that is produced by activated inflammatory cells. PAF is involved in regulation of inflammation and mediates bronchoconstriction.

Extrinsic Regulation

Extrinsic regulation originates outside of the organ to meet the needs of the body. It is comprised of neurotransmitters secreted by the vasomotor nerves and circulating hormones.

Neurotransmitters

Vasoactive neurotransmitters are secreted by the vasomotor nerves. There are three kinds of autonomic vasomotor nerves: the sympathetic vasoconstrictor nerves, the parasympathetic vasodilator nerves and the sympathetic vasodilator nerves. The sympathetic vasoconstrictor nerves are the most prevalent and have important physiological roles. The parasympathetic vasodilator nerves are not found in all tissues. They are responsible for increasing blood flow when required for organ function. There are limited sympathetic vasodilator nerves in the body. One function of these nerves is vasodilation in skin that occurs along with sweating. There are more roles for these nerves in non-primates. Several of the more common vasoactive neurotransmitters are described.

Norepinephrine

Norepinephrine, also known as noradrenaline, is both a neurotransmitter and a circulating hormone. It is synthesized by the sympathetic vasoconstrictor nerves and quickly diffuses across the junctional gap to bind to the α-adrenoceptors α_1 and α_2. The α_1 receptor is found on most blood vessels while the α_2 receptor is found on resistance vessels. The effect of norepinephrine is mediated by many vasodilator metabolites and autacoids. Angiotensin II facilitates transmitter release and amplifies the constriction.

ATP

Purine ATP is synthesized locally by the sympathetic vasoconstrictor nerves and is released along with norepinephrine. ATP stimulates post junctional P_{2x} purinergic receptors which activate non-selective cation channels.

Neuropeptide Y

Neuropeptide Y is synthesized in the postganglionic cell body of sympathetic vasoconstrictor nerves and is stored with norepinephrine. It is released in response to high frequency stimulation that occurs during times of stress. Neuropeptide Y produces a slower and more prolonged response than ATP; it also causes increased sensitivity to norepinephrine.

Acetylcholine

Acetylcholine is secreted by the parasympathetic vasodilator nerves. It produces dilation through cholinergic stimulation of the endothelial cell to produce NO; acetylcholine also interacts directly with the smooth muscle cells by activating the M_3 muscarinic receptor.

Circulating Hormones

There are several hormones that affect blood vessel tone. Generally the effects of the hormones are less than that of the vasomotor nerves. Table 4 shows some of the more potent vasoactive hormones and the response that they elicit. Some of the hormones are discussed in more detail.

Table 4: Circulating Hormones and Their Vasoactive Response [14]

Hormone	Response
Adrenaline	Primarily Vasoconstriction
Norepinephrine	Primarily Vasoconstriction
Angiotensin II	Vasoconstriction
Vasopressin	Primarily Vasoconstriction
Insulin	Vasodilator
Oestrogens	Vasodilator
Relaxin	Vasodilator
Thyroxine	Vasodilator

Adrenaline and Norepinephrine

Adrenaline is a methylated form of norepinephrine. Both hormones are secreted from the adrenal gland and cause vasoconstriction due to activation of the α-adrenoceptors. While the response to adrenaline is most often vasoconstriction, vasodilation can occur in skeletal muscle, myocardium and liver through activation of the β-adrenoceptors.

Angiotensin II

While not its' primary role, Angiotensin II has a direct effect on pressure regulation through vasoconstriction. Angiotensin II interacts directly with smooth muscle cells through activation of the angiotensin receptors. It also has an indirect effect by stimulating norepinephrine production through activation of angiotensin receptors on the sympathetic terminals.

Vasopressin

Vasopressin acts as a vasoconstrictor mainly during pathological events and acts to maintain blood pressure and volume through other means under normal conditions. It is produced by the mangnocellular neurons in the hypothalamus. Secretion is stimulated by a decrease in blood pressure and volume such as during hemorrhage. While

vasopressin is a potent vasoconstrictor in most tissues, it causes vasodilation of the cerebral and coronary arteries through stimulation of NO.

Temperature

Responses to temperature can be intrinsic or extrinsic. Change of vascular tone is important for regulation of tone in the blood vessels of the skin in order to help maintain core body temperature. Body temperature is maintained by balancing internal heat production with external heat loss; changes in cutaneous vascular tone help to regulate heat loss. There is an intrinsic response to local changes in temperature such as a person puts their hand in ice water. There are also extrinsic responses as a result of core temperature changes. Cutaneous blood vessels contain an abundance of α_2-adrenoreceptors which interact with the sympathetic transmitter norepinephrine. Norepinephrine is discussed in more detail in the extrinsic regulation section.

The Vascular Smooth Muscle Cell in Culture

Vascular smooth muscle cells are often defined as having either a 'contractile' or 'synthetic' phenotype. There is some evidence that in a mature vessel there are some cells that seem to be of a more synthetic type while the majority is in the quiescent 'contractile' phenotype. The reality is that there are not two distinct phenotypes but a gradient. Quiescent cells can synthesize extracellular matrix while retaining their contractile apparatus.

In a mature blood vessel the majority of the smooth muscle cells will be quiescent; they will not proliferate nor synthesize much extracellular matrix. After isolation and placement into culture, the smooth muscle cells initially appear long and spindly; however, after a few passages their morphology becomes more fibroblastic (see Figure 2). Along with the change in morphology is a change to a more synthetic

phenotype and the contractile apparatus is significantly down regulated. This spontaneous phenotypic modulation happens after 6-8 days in culture when cells are kept in subconfluent conditions [29].

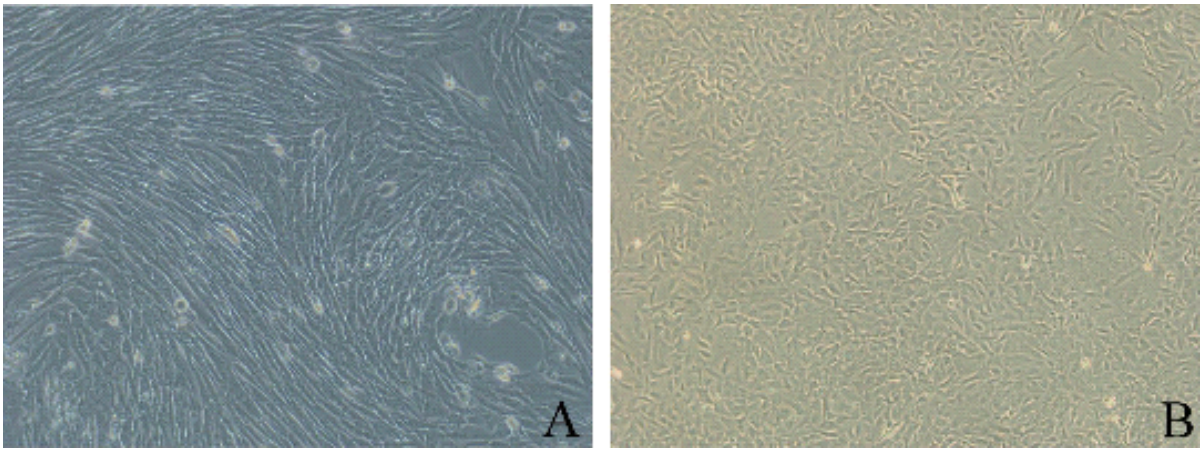

Figure 2: Vascular Smooth Muscle Cells in Culture. SMCs at P0 (A) have a long spindly contractile smooth muscle cell morphology. SMCs at P7 (B) have a more fibroblastic morphology associated with a more synthetic cell phenotype.

Phenotype modulation is thought of as irreversible when seeding at low densities and passaging multiple times, techniques that are often used and required to generate cells for most tissue engineering applications [29]. More recent work has shown that synthetic adult smooth muscle cells can convert to a contractile phenotype after serum starvation [30, 31]. In vascular tissue engineering different biochemical and mechanical stimulation has been shown to alter smooth muscle cell phenotype, but little work has been done to determine if this has an effect on the contractile response [32, 33].

Tissue Engineered Small Diameter Blood Vessel

Tissue engineering is the combination of cells, scaffold and mechanical and biochemical cues to create an engineered tissue. Tissue engineering approaches have attempted to create substitutes for one or more of these layers; these range from the use of

biopolymer scaffolds to biodegradable scaffolds with varying cell sources, mechanical and biochemical stimulation. A review of these approaches follows. Nanotechnology is being investigated for tissue engineering applications and is being considered for vascular tissue engineering, especially with respects to endothelialization. This work is still in its infancy with respect to vascular tissue engineering, and has been suggested but not practically used for creation of vascular tissue. For information on research into nanotechnology for tissue engineering please see the following reviews: [34-36].

Biopolymer Scaffolds

The first biological blood vessel substitutes were created by Weinberg and Bell using type I collagen hydrogels to create all three layers of the blood vessel [37]. Since that time collagen I hydrogels have been explored by the Nerem lab and others as a scaffold choice for tissue engineering. The smooth muscle cells can be easily encapsulated with the hydrogel and will compact and remodel the gel. Endothelial cells can be seeded and will form confluent monolayers on the lumen [38]. The collagen gels are quite weak; Weinberg and Bell used Dacron support sleeves to achieve sufficient strength. For small diameter applications synthetic materials have not remained patent, so other methods of improving the strength of the collagen gels have been investigated. Several cross-linking agents have been investigated to improve strength. Most cross-linking agents are cytotoxic, so Berglund et al investigated the used of chemical cross-linking of a collagen support sleeve. An acellular collagen gel was created and dehydrated. Dehydrothermal, ultraviolet, and glutaraldehyde were used to cross-link the sleeves. All methods increased strength and collagen gel stiffness while retaining high cell viability [39], Only a few cross-linking techniques are non-cytotoxic and can be directly applied to the cell seeded hydrogels. Methylacrylamide photo-cross-linking has been used to improve shear modulus 12 fold with high cell viability [40]. Enzymatic glycation by transglutaminase has been shown to improve burst pressure of collagen

tubes by 35% [41]; collagen hydrogels populated with cell transfected to overexpress lysyl oxidase were almost twice as strong as those with non-transfected cells [42]. Non-enzymatic glycation has also been used to cross-link and improve collagen gel strength; when applied after cell mediated compaction has ceased ribose and glucose both increased the tissue strength. Ribose resulted in better cross-linking than glucose [43, 44]. When cyclic strain was applied for 5 weeks to the glycated tissue cyclic, elastin deposition was found after. Elastin deposition in engineered tissue made from adult SMCs had not been previously reported. Incorporation of elastin into a tissue engineered blood vessel remains a challenge; elastin scaffolds have been isolated from mature vessels and incorporated into the collagen based engineered tissue. The elastin scaffolds not only improved the elastic modulus of the tissue but also greatly enhanced the tensile strength of the tissue [45]. In order to incorporate elastin into the scaffold a freeze dried collagen-elastin scaffold was created from a 1:1 collagen – elastin suspension. Different cross-linking methods were assessed. Smooth muscle cells were seeded into the scaffolds by filtration. Yield stresses of 4-50 kPa were obtained using the various cross-linking and culture conditions [46, 47].

While type 1 collagen is the most abundant protein in the body and blood vessel and is important for tensile strength, it may not be the best choice of scaffold material. The extracellular matrix has a profound effect on smooth muscle cell proliferation and phenotype. One review on the role of matrix in tissue engineering is that of Vogel and Baneyx [48]. Fibrin has been used successfully as an alternative material to the collagen hydrogels. As with collagen cells can be encapsulated in fibrin hydrogels. Smooth muscle cells encapsulated in fibrin have been shown to synthesize more collagen then cells in collagen hydrogels [49, 50]. Under similar culture conditions the tensile strength of the fibrin hydrogels was greater than that of the collagen hydrogels [50]. Fibrin based tissue engineered blood vessels have also demonstrated a vasoactive response to specific agonists [51-54], and have been tested *in vivo* [52]. To improve the mechanical properties

of the fibrin graft a second acellular fibrin layer has been added. This improved the tensile strength and caused a reduction in measured vasoactive response [53]. Fibrin gel compaction and strength can also be affected by the concentration of thrombin used for fibrillogenesis [55]. The combination of collagen I and fibrin has been investigated. The tensile strength was found to correlate highly with the degree of gel compaction with the composite collagen-fibrin hydrogel being the strongest [56]. Varying the concentrations of the proteins can also affect the material properties; tensile strength correlated to gel compaction so higher concentrations were not always the optimal choice [57]. Fibronectin-fibrinogen scaffolds have been studied; these grafts have demonstrated burst pressures approaching 200 mmHg [58]. While fibrin based TEVM has demonstrated substantial strength, ε-amino caproic acid (ACA) is needed to prevent proteolytic degradation. If the fibrin is not slowly degraded and replaced with ECM prior to implantation, the TEVM could fail *in vivo*.

A chitosan based engineered tissue with a chitosan core and chitosan-gelatin outer layer was used; and a burst pressure over 4000 mmHg was achieved [59]. The use of a collagen mimetic combined with chitosan using a different fabrication process has recently been described with slightly lower burst pressures [60].

Esterified hyaluronic acid (HYAFF) has also been used as a scaffold for blood vessel tissue engineering. Initial studies showed that cells could attach to HYAFF and a tubular configuration could be created [61]. Further studies using perfusion or rotation cell seeding to promote cell infiltration improved the tissue strength; measured tensile strength of both seeding methods was in the MPa range [62]. One benefit to this material is that it has been approved for human use for skin and cartilage applications. Hyaluronic acid may also have additional benefits of promoting elastin synthesis and recruiting and retaining an endothelial cell layer [63, 64].

Cell Sheets

In cell sheet TEVM, the cells produce the matrix. Smooth muscle cells or fibroblasts are cultured until they are post confluent and have created their own tissue. The sheets are then wrapped around an inner mandrel to create a tube and cultured longer to promote adhesion of the layers. All three layers can be created by wrapping the fibroblast layer around the smooth muscle layer and then seeding endothelial cells into the lumen [38]. This approach results in burst pressures of over 2000 mmHg, similar to native vessel [65]. The vasoactive response of this engineered tissue has also been shown to be similar to native vessel [66-69]. This approach has been successful and is now being tested in clinical trials [70, 71]. Mechanical testing of an engineered blood vessel using fibroblast sheets only has been shown to have burst pressures comparable to artery and compliance between artery and vein [72]. The creation of tissue engineered blood vessels using the cell sheet approach requires long cultures times which can be problematic if autologous cells are required. These TEVM are being tested clinically, although not in a small diameter application [73].

Synthetic Scaffolds

The most widely used polymer is polyglycolic acid (PGA). Because it is rapidly resorbed *in vitro* culture is required prior to implantation. SMCs cultured in PGA have been shown to synthesize and deposit elastin [74]. Cell seeded PGA engineered blood vessels have been shown to be vasoactive and have burst pressures over 2000 mmHg after 7 weeks in culture [75], while these vessels exhibit significant strength compared to most of the hydrogels, the strength and burst pressure is still below native and elastin deposition was not seen [76]. While the burst pressures are similar to native tissue, direct comparison of mechanical properties found that the engineered tissue was not as strong as the native tissues, even though collagen densities were similar [76], instead collagen fiber diameters and alignment were different [77].

Copolymers or multilayered polymers have been used, many using PGA for reinforcement. Many of these were tested as acellular grafts in a variety of animal models with all grafts remaining patent throughout the test. All grafts contained combinations of two or more of the following polymers: PGA, polyhydroxyalkanoate (PHA), polyglactin, poly-L-lactide (PLA), poly(ε-caprolactone) (PCL), poly-L-lactic acid (PLLA), polyglycolide fiber, poly(allylamine hydrochloride) (PAH), poly(styrene sulfonate) (PSS), poly-4-hydroxybutyrate (P4HB), poly(ester urethane)urea (PEUU), and poly(2-methacryloyloxyethyl phosphorylcholine-co-methacryloyloxyethyl butylurethane) (PMBU). Table 5 shows the combinations of polymers used successfully in animal models and an occasional human patient. While there has been success using these polymers as blood vessel grafts, it is still unknown if they would remain patent in a small diameter application.

Table 5: Polymers Tested *In Vivo*

Polymer	Authors
PGA-PHA	Shum-Tim [78]
PGA-polyglactin	Shinoka [79]
PGA-PLA-PCL	Watanabe [80] Matsumura [81] Shin'oka [82]
PGA/PLA	Isomatsu [83]
PLA-PCL-PLA	Matsumura [84] Shin'oka [85]
PLA-PCL-polyglycolide	Matsumura [86]
PGA/collagen-PLLA	Yokota [87]
PCL	Pektok [88]
PGA/collagen-PCL-PLLA	Torikai [89]
PGA-PCL-PLA	Brennan [90]
PGA-P4GB	Mettler [91]
Umbilical Vein-PAH-PSS	Kerdjoudj [92]
PEUU-PMBU	Hong [93]

Other copolymers have been tested *in vitro*. PGA-P4HB grafts were made by coating PGA with a thin layer of P4HB. Myofibroblasts were seeded onto the inner layer

first followed by endothelial cells. The engineered blood vessels were then cultured using a pulse duplicator system. After 28 days in culture burst pressures over 300 mmHg were measured [94]. These grafts have now been tested *in vivo* with seeded endothelial progenitor and mesenchymal stem cells [91]. Poly(D,L-lactide)-7 co(1,3-trimethylene carbonate) dipped in collagen to create a collagen 'microsponge' inside the pores has been investigated as a potential polymer for small diameter blood vessel tissue engineering. The mechanical properties after seeding and culture with smooth muscle cells for 7 days was shown to be similar to native artery [95]. A multi-layered tissue engineered blood vessel was constructed to mimic the architecture of native vessel. A sheet of smooth muscle cell seeded PGA was wrapped around a mandrel followed by a SMC seeded sheet of PCL then a sheet of fibroblast seeded PGA. Finally endothelial cells were seeded on the lumen and the tissue was placed into a culture into a pulsatile bioreactor. Tensile strengths similar to native tissue were seen; measured values were greater than 800 kPa [96].

Poly(ethylene glycerol) (PEG) has been used as a biodegradable polymer hydrogel. Diacrylate PEG can be rapidly polymerized using ultraviolet light with little cell damage. The material has tunable properties producing strengths of 40-100 kPa [97].

More recently elastomeric biodegradable polymers have been used for tissue engineering of blood vessels. Poly(1,8-octanediol citrate) (POC) has been used along with a pulsatile bioreactor [98]. Poly(glycerol sebacate) can be fabricated in to a tubular form. Smooth muscle cells are seeded using gentle perfusion after which time endothelial progenitor cells were seeded onto the lumen. After 8 weeks of culture the pressure-diameter responses were similar to native tissue and there was detectable elastin present. Tensile strength was approximately 25 kPa [99]. Poly(ester urethane) urea (PEUU) was fabricated in tubular form and seeded with muscle derived stem cells using a rotational vacuum seeding technique. After 7 days in culture the measured tensile strength was approximately 2.5 kPa [100].

A copolymer of poly(lactide-*co*-β-malic acid) with extended carboxyl arms (PLMA-ECA) has been suggested as a scaffold for blood vessel tissue engineering due to the low adherence of platelets and high cell affinity [101].

There is some debate of the need for cell seeding in the biodegradable polymers. The references in Table 5 include studies both with cell seeding and without. One study has shown that expanded polytetrafluoroethylene (ePTFE) seeded with endothelial cells has lower platelet adherence than that of the unseeded controls when tested in an *ex vivo* shunt [102]. It is likely that other polymers will also require endothelial cell seeding in order to remain patent. Some of the polymers discussed are being used clinically in pediatric applications. For a review see Shinoka and Breuer [103]. For small diameter application these materials may not be successful. Polymers that remain patent in large diameter applications may not in small diameter vessels. Degradation byproducts may also alter the local environment and lead to alterations in cell function or cell death [104, 105].

Decellularized Scaffolds

Acellular matrices that can be repopulated by autologous cell *in vivo* have been explored; this approach could lead to off-the-shelf availability of graft material. Immune acceptance and cell source would not be a problem. Small intestinal submucosa (SIS) has been tested for use as a vascular graft since 1989 [106]. Only one of 9 dogs tested died of thrombosis when SIS was used as a large diameter vascular graft. After 90 days the grafts arterialized with a functional endothelial layer and a smooth muscle populated medial layer [107]. SIS grafts have been shown to have excellent mechanical properties both pre- and post-implantation with burst pressures greater than 3000 mmHg [108, 109]. The SIS grafts have also been tested successfully *in vivo* in a growth model showing that they are not only able to arterialize but also to adapt and grow in a pediatric model [110]. A similar study using acellular small intestine excluding the mucosa layer had similar

results. All the grafts grew, but one of seven did not remain patent [111]. In a similar vein, the intestinal collagen layer of the small intestine was also used as a vascular graft. This layer alone had poor patency rates until a small layer of bovine collagen was applied with heparin. These grafts exhibited burst pressures greater than 900 mmHg and remained patent. Cells populated the graft and the grafts exhibited a vasoactive response when explanted after 90 days. This is the only study where a small diameter graft (4 mm) was used as a graft for a rabbit carotid artery [112].

Decellularized porcine ureters have been suggested as a scaffold material for vascular tissue engineering. The decellularized ureters had similar mechanical properties as saphenous veins. They had significantly less immune response than cellularized controls in a subcutaneous implant [113].

While SIS provides a more natural environment than many of the other approaches, the use of decellularized vascular tissue was investigated as well. Decellularized, heparin treated segments of porcine carotid artery were shown to have similar mechanical properties to that of native vessels. When tested *in vivo* as a xenogeneic graft, a confluent endothelial cell monolayer developed within 2 months [114]. Decellularized fetal pig aorta has been shown to have similar mechanical properties of fresh artery; endothelial cells cultured on the lumen remained viable and created a confluent monolayer [115]. Decellularization methods can affect cell viability and mechanical integrity [116, 117]. Decellularization method is important as some can affect collagen spacing and proteoglycan density which would impact the mechanical integrity [118]. Still the use of decellularized aortic segments show burst pressures of over 1000 mmHg; endothelial cells will form a confluent monolayer on the surface and will produce vasoactive agents in response to agonists [119].

Most of the approaches with decellularized tissues are used to provide a more natural extracellular environment and to avoid potential thrombosis found that occurs when polymers are used in small diameter blood vessel applications. There are some

studies that combine the decellularized matrix with a polymeric coating in the hopes of improving patency. Recently human umbilical arteries were decellularized and coated with PAH/PSS and tested in rabbits; all remained patent [92].

The Body as a Bioreactor

In order to form a TEVM with autologous cells in a relatively short amount of time, the use of the body as a bioreactor to create a blood vessel like structure was developed [120]. The body's foreign body response forms granulation tissue around a silastic mold resulting in a blood vessel like structure with myofibroblasts and a monolayer of mesothelium after only 2 weeks in culture in rats. The tissue is removed from the tubing and everted so that the mesothelium is facing the lumen. The engineered tissue was then placed into the carotid artery of the same animal and remained patent for the 4 month trial [120]. The myofibroblasts responded to KCl, phenylephrine, and acetylcholine after 3 weeks implantation; after 4 months an adventitia layer with vasa vasorum was seen [121]. Canine studies were performed using a variety of tubing materials. Histologically there was no difference in the granulation tissue, although the thickness varied. The tissue not grown on mesh had burst strengths over 2500 mmHg. When implanted, 8 out of 11 grafts remained patent for 6.5 months; these grafts did not respond to vasoactive drugs [122]. Use of the multilayered tubes was tested. PLA was coated with hyaluronic acid followed by one or more coatings including matrigel, elastin, tropoelastin, laminin, collagen I and collagen III. All materials resulted in a foreign body response. Matrigel resulted in the most significant cell response [123]. This approach is promising due to its short culture time and use of autologous cells. Off-the-shelf availability will likely be needed for some patients; this approach would not be able to meet that need.

Mechanical Stimulation

The most common form of mechanical stimulation is the application of cyclic strain. The application of cyclic strain has been used to provide a more physiological environment for tissue engineered blood vessel culture; cyclic strain of the engineered tissue has been shown to increase tissue strength [47, 94, 98, 124-128], improve smooth muscle cell alignment [124, 129] and promote a more contractile cell phenotype [33, 75, 129]. ECM synthesis and deposition is altered with cyclic strain, although the results are mixed due to different test conditions including but not limited to differences in cell type, scaffold choice, culture medium and strain profile. Several studies show increased collagen after cyclic strain [75, 94, 97, 128, 130]; this includes a study that saw differences in collagen content but no difference in elastin deposition when cyclic strain was applied to the PEG hydrogels [97]. This is counter to a study with cross-linked collagen hydrogels that had increased elastin deposition but no difference in collagen content [125]. MMP activity is affected by cyclic strain. An increase in MMP-2 activity was seen in collagen gels with human cells [131, 132]. While the increased MMP activity is of concern due to the decreases in strength seen at least in the short term, MMP activity is essential for remodeling. When non-specific MMP activity is inhibited cyclic strain no longer resulted in improved mechanical properties [131]; however, without inhibition the collagen based TEVM were strained for longer time periods the MMP activity resulted in reduced strength after 8 days in culture [132]. Studies have shown that strain amplitude and rate may influence the mechanical properties [133-136] and collagen deposition [134, 135] in engineered tissue. High strain amplitudes of 10-15% have been shown to improve strength and collagen as compared to static with stepwise incremental strain increases having the best results [134]. Another study showed that pulse rate could affect collagen content and MMP-1 content [130]. Conversely a different study found that lower strain rates to be preferable for strength [98]. Differences may very well be due to cell type,

matrix type and the method for applying strain. Higher strain rates have led to increased collagen deposition and increased levels of tissue inhibitor of metalloproteinases type 1 (TIMP-1) both of which would positively impact tensile strength [136].

Fluid shear stress is known to affect endothelial cells (for a review see [137, 138]). Several of the pulsatile bioreactors also incorporate flow through the lumen. Most of the tissues using hydrogel scaffolds cannot be subjected to flow and cyclic strain at the same time due to insufficient mechanical properties, although recently fibrin based medial equivalents were subjected to pulsatile luminal flow with pressures up to 200 mmHg [139]. Many polymer scaffolds have been subjected to both flow and strain [47, 75, 94, 96-98]. Laminar flow alone does have an impact on collagen deposition which should affect tissue strength [140]. The combination of shear stress and cyclic circumferential strain may have different effects than cyclic strain alone. At this time the effects cannot be separated. Newer systems are combining cyclic circumferential strain with perfusion and shear [141, 142], electrical stimulation [143, 144], and longitudinal extension [145, 146]. Use of perfusion with pulsatile pressure was compared to a rotational bioreactor; the rotational bioreactor was shown to increase the ECM deposition and tensile strength of tissue engineered blood vessels compared to those cultured in the perfusion bioreactor [62]; this suggests that application of cyclic strain may not be necessary for a viable tissue engineered vascular graft. This is supported by *in vivo* and clinical studies that utilize cell secreted or biodegradable polymer scaffolds.

Biochemical Stimulation

Various forms of biochemical stimulation have been used to improve the properties of tissue engineered blood vessels. Growth factors such as transforming growth factor β (TGF- β) are a widely used for their effects on ECM synthesis (for reviews of growth factors in tissue engineering see [147, 148]). Ascorbic acid or sodium

ascorbate is often used to promote ECM production [70, 75, 149-151]. Retinoic acid has also been shown to increase ECM synthesis [151].

Addition of exogenous TGF- β and platelet derived growth factor (PDGF) has been shown to increase cell mediated gel compaction while heparin has been shown to decrease compaction [152]. There is strong correlation between gel compaction and tissue strength. PDGF and TGF- β are both been shown to affect smooth muscle cell phenotype. Addition of PDGF leads to reduction in smooth muscle α-actin while TGF- β leads to an increase [33]. The effects of TGF- β alone or in combination with other factors on tissue strength and ECM synthesis have been investigated. TGF-β has been shown to increase collagen production [153-155], increase strength [50, 155], increase elastin deposition [155], and promote a more contractile cell phenotype [33]. The addition of TGF-β along with insulin was also shown to improve the contractile response of the engineered tissue [54] and increase both collagen and elastin deposition [156]. This combination improve collagen synthesis and tissue strength more than TGF-β alone; the addition of plasmin further enhances collagen synthesis and strength [157]. The combination of TGF-β, insulin, and aprotinin also resulted in improvements in strength over TGF-β and insulin alone [54]. The method of TGF-β presentation can also have an effect. TGF-β tethered to the matrix was shown to increase matrix production compared to TGF-β provided in culture medium [153].

Extracellular Matrix

This section seeks not to review all of the components of the medial extracellular matrix, but the matrix materials that are important to this work.

Collagen

Collagen is the most abundant protein in blood vessels and throughout the body. It has a triple helical protein with an α-chain secondary structure made up of $(Gly\text{-}X\text{-}Y)_n$ repeats where X and Y are often proline or hydroxyproline. Within the triple helix, every backbone carbonyl and amide group interacts to form hydrogen bonds. In collagen types I, II, III, V and XI, the collagen monomers self-associate into periodic cross-striated fibers. Collagen is first synthesized as soluble triple-helical procollagen; enzymatic cleavage of the terminal propeptides by procollagen metalloproteinases is required to convert procollagen to collagen. Without this conversion collagen fibers will not form; with the conversion the fibers will self assemble. The fibers are then cross-linked via lysyl-oxidase. These fibers provide tensile strength for the tissues they are in, and as such the organization of the fibers is very important [158-160]. Collagen hydrogels can be easily created; neutralization of acid solubolized collagen will result in fibrillogenesis forming a gel at high enough concentrations. The collagen fiber diameter can be influenced by temperature with thinner fibers occurring at higher temperatures [161]. While collagen is attractive for use as a hydrogel based on it abundance and function, there are some issues. Alignment of the fibers in the tissue engineered blood vessel is important for strength and is not always easy to obtain [162]. Another concern is that when cells are entrapped in a collagen hydrogel, they exhibit little ECM synthesis [163]. This fact has led to the investigation of alternative matrix materials for creation of biopolymer tissue engineered blood vessels.

Fibrin

Fibrin is the structural protein in blood clots and is important to wound healing. Prior to clotting, fibrin can be found in its monomeric form, fibrinogen. Fibrinogen is enzymatically cleaved by thrombin; after this cleavage the molecules self-associate to form linear fibrils. These fibers are eventually degraded by plasminogen [164]. This

process can be resisted through use of the inhibitor ε-amino caproic acid. Fibrin has been used as a hydrogel for blood vessel tissue engineering; in this matrix smooth muscle cells have been shown to synthesis more collagen than when entrapped in Type I collagen gels [165].

Small Leucine Rich Repeat Proteoglycans

This section reviews a class of proteoglycans. These are some of the smallest proteoglycans; there are at least 9 members of the small leucine rich repeat proteoglycan (SLRP) family. Domain III of these proteins contains 6-10 repeats of a 24-amino acid residue sequence, LxxLxLxxNxLSxL, where L is leucine, isoleucine, or valine and S is serine or threonine. There are three classes of SLRPs based on protein and genomic organization. Class I consists of decorin and biglycan, two of the smallest proteoglycans with much homology and similar functions. Class II includes fibromodulin, lumican, and keratocan. Class III includes epiphican and osteoglycin [166-168].

Decorin

Decorin is widely distributed in connective tissues throughout the body and plays an important role in collagen fiber formation. In the blood vessel it is found in the adventitial layer and to a lesser extent in the medial layer [169]. Decorin binds and interacts with a number of extracellular matrix proteins including collagen I, II, III, VI, XII, and XIV [170-176], tropoelastin [177], and fibronectin [178, 179], thrombospondin [180], and tenascin-X [181]. Through interactions with these proteins, decorin may influence cellular phenotype and adhesion [182]. The model structure of decorin is an arch shape with a single glycosaminoglycan (GAG) chain. The inner concave surface has a size and shape that can accommodate one collagen triple helix which could allow decorin to act as a spacer during collagen fiber assembly [183]. The binding of the d-band in each D period of collagen has been confirmed experimentally to occur at the leucine

rich repeats found in the arch of decorin [184, 185].

Collagen fiber diameter and packing is affected by decorin and affects fiber and tissue strength. On the single collagen fiber level it has been shown that when decorin is present during fibrillogenesis, a stronger fiber is formed [186]. At the tissue level, a decorin knock-out model showed that lack of decorin resulted in abnormal collagen fiber size and alignment and in skin fragility [187]. When decorin is incorporated into a collagen hydrogel it has been shown to influence gel compaction. Compaction was shown to be delayed in cellularized gels exposed to exogenous or overexpressed decorin [188]. A second study showed that overexpression of decorin led to the increased compaction of the collagen hydrogel while exogenously applied decorin did not have the same effect. The transfected cells that overexpressed decorin also synthesized more collagen than empty vector controls [189]. Decorin knockout cells also increase gel compaction, most likely due to the dramatic increase in biglycan expression [190]. Increased synthesis of collagens I and V was also seen when exogenous decorin was added to a three dimensional culture of dermal fibroblasts in their own secreted matrix. No difference in collagen fiber diameter was found in the synthesized collagen [191].

Matrix metalloproteinase (MMP) activity has been shown to be influenced by decorin. This influence is important for engineered tissue since too much activity could result in loss of mechanical integrity while too little hinder remodeling. When fibroblasts overexpressed decorin, MMP-1 and MMP-3 were down-regulated while MMP-2 was up-regulated. Levels of tissue inhibitors of MMP (TIMP) as well as several cytokines were also affected by decorin.[192] Other studies confirm the influence of decorin on MMP secretion; however, the effects appear to be dependent on the ECM environment or to cell type [193, 194].

Decorin is also known to interact with TGF-β. Initial studies showed that decorin inactivates TGF-β [195-197]; however, more recent studies show that there is only selective inhibition [198, 199]. Binding of collagen and TGF-β is non-competitive,

suggesting separate binding sites [200]. This finding along with the finding of inverse correlation between decorin expression and TGF-β mediated stimulation of collagen hydrogel compaction has led to the hypothesis that decorin is involved in sequestering TGF-β rather than inactivating it [201]. This may be supported by the finding that TGF-β tethered to cell seeded polymer vascular constructs resulted in greater matrix production than when exogenous TGF-β was provided [202]. Additionally, MMP degradation of decorin can release TGF-β that is bound in the matrix [203]. Decorin also interacts and regulates the function of tumor necrosis factor-α [204], platelet derived growth factor [205], fibroblast growth factor-2 [206], and insulin-like growth factor I [207]. Decorin plays a role in wound healing, although the exact relationship is yet to be established. Expression of decorin is altered during wound healing and scar formation [208-219], and decorin deficiency impairs healing [220].

Along with its roles regarding matrix synthesis and stability, decorin also has effects on cell cycle, cell survival and angiogenesis. Decorin has been shown to suppress growth [221-225] through up-regulation of p21 [226]. It has been shown to promote cell survival by inhibiting apoptosis [227, 228]. Decorin effects on angiogenesis can be inhibitory [220, 229, 230] or promoting [227, 231, 232].

Biglycan

Biglycan is homologous to decorin; the main differences in structure are the absence of one oligosaccharide chain and the presence of 2 dermatan or chondroitin sulfate chains. Biglycan interacts many of the same extracellular matrix proteins and growth factors, including collagen I, II, III, and VI [170, 176, 233], TGF-β [234, 235], and TNF-α [204]. Some of the differences may be attributed to proteoglycan distribution, biglycan is localized on the cell surface or in the pericellular matrix [236]; it is also synthesized by such as endothelial cells that do not constitutively synthesize decorin [237] . Biglycan knockouts lead to improper collagen fibril formation and organization

much like decorin, but biglycan primarily affects tissues of the musculoskeletal system [238, 239]. This along with its abundance in this tissue has resulted in most research being done in these tissues. Biglycan does play a role in the cardiovascular system though. Knocking out biglycan led aortic dissection and rupture has been reported in male mice [240]. Bigylcan is also known thought to play important roles in cardiac tissue in both physiologic and pathologic conditions [241]. Pathologically, increased biglycan expression by smooth muscle cells is associated with the formation of atherosclerotic plaques [242]. Physiologically biglycan is found in native vessels; it has a similar localization pattern as decorin with light medial staining and strong adventitial staining [243]. Biglycan binds to both tropoelastin and microfibril-associated glycoprotein 1 (MAGP-1) forming a ternary complex and may play a role in elastinogenesis [177].

Biglycan has been shown to influence cell proliferation; it has been found to enhance cell proliferation and migration [244]. A good review for more information on biglycan is [245].

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CHAPTER 3: GENERAL METHODS

Introduction

This chapter provides descriptions of common methods used in multiple chapters. These methods are intended to go into more depth than what is found in the individual chapters, but may be generalized slightly when subtle modifications can be made. Any deviations to these methods are described in the chapters.

Cell Isolation and Culture

Smooth muscle cells from the thoracic portion of the aorta of normal adult Sprague-Dawley rats were isolated previously [1]. Briefly, rat aortic smooth muscle cells (RASMC) were isolated using the facilitated migration method which combines enzymatic digestion and migration techniques. Cells were cultured using standard culture conditions of 37°C and 5% CO_2. Cells were cultured and expanded in medium consisting of Dulbecco's modified eagle medium (DMEM), 10% fetal bovine serum (FBS), 1% L-glutamine and 1% penicillin-streptomycin. Experimental work was completed using cells between passages 6 and 9 unless otherwise noted.

Tissue Engineered Vascular Media Fabrication

Cell seeded collagen hydrogels are used in all experiments; this describes the general method for making a tissue engineered vascular media (TEVM). Deviations from this general method are sometimes required for specific experiments and are described in detail in the respective chapters.

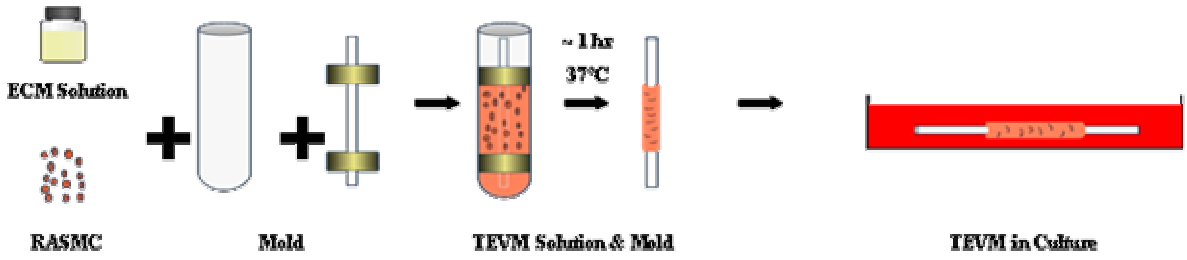

Figure 3: TEVM Fabrication. Typically collagen and RASMC are mixed and placed in a tubular mold. After the solution gels the hydrogel is removed from the outer mold and placed into culture.

TEVM Formation

Prior to TEVM fabrication lyophilized type 1 bovine collagen (MP Biomedical) is solubolized in 0.02 N glacial acetic acid. To create the TEVM, RASMCs are trypsinized, counted. The appropriate amount of RASMC solution is removed, centrifuged and resuspended in culture medium so that there will be a final concentration of 1 x 10^6 cells/ml. In a separate tube collagen is added to 5X DMEM and sodium hydroxide to neutralize the pH; the amounts are such to have a final concentration of 2 mg collagen/ml collagen and a final concentration of 1X DMEM. The cell solution is then added to the collagen solution and the TEVM solution is then placed in a tubular mold and incubated at 37°C (see Figure 3). After the solution has polymerized, approximately one hour, the TEVM are removed from the outer molds and placed into a dish with culture medium. The gels are cut away from the stoppers to promote circumferential alignment of the cells and matrix [2]. The TEVM are cultured using standard culture conditions described previously. Two different serums were used for construct culture in this work. See Appendix A for more information.

Contractility Testing

To test contractility ring sections were placed onto hooks in a physiological organ bath shown in Figure 4; each bath contains 5 ml Krebs Hensleit solution with continuous gaseous perfusion of 95% O2, 5% CO2 at 37°C [3, 4]. The hooks are connected to a force transducer and force changes are captured using a digital data acquisition system (Gould Instrument Systems, Valley View, OH). The hook height is adjustable so that a preload can be applied to the rings. An array of baths is setup to test multiple ring segments at one time; the current setup contains 12 organ baths. One bank of baths including the large reservoir containing the Krebs Hensleit solution is shown in Figure 4.

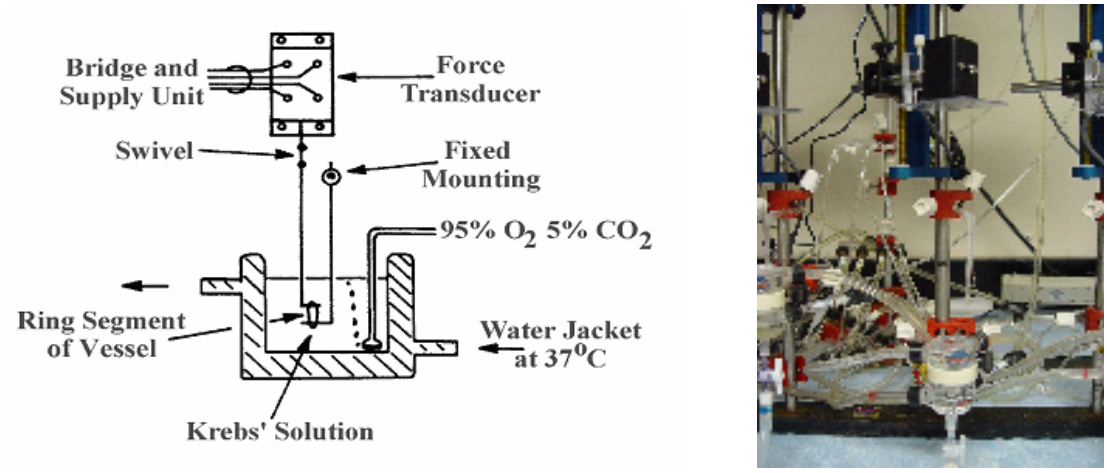

Figure 4: Individual Organ Bath for Contractility Testing. Ring segments are loaded on wire hooks in a physiological organ bath containing Krebs Hensleit solution that is continuously gassed and maintained at 37°C. Tubing allows for solution to drain and be refilled. The change in force due to contraction or relaxation is measured with a force transducer.

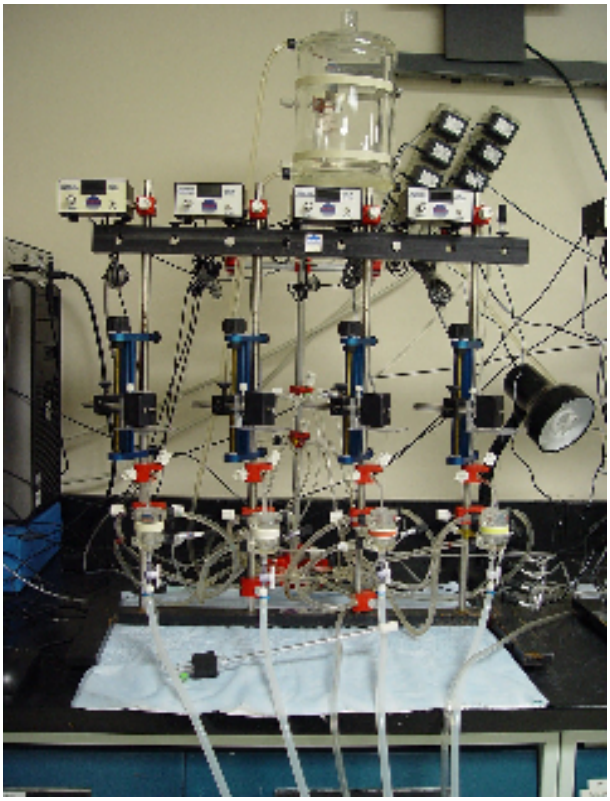

Figure 5: Contractility Ring Test Set Up. One reservoir is used for one bank of 4 baths.

The TEVM were cut into 2 mm rig segments. The segments were preloaded to 0.15 g and allowed to equilibrate for 1 hour followed by stimulation with two doses of the nonspecific agonist KCl (60 mM, 120 mM). Then the rings were subjected to different agonists and antagonists. Unless specified in the individual chapters the method depicted in Figure 6 was used. Rings were subjected to the agonist endothelin-1 (10^{-8} M, 10^{-7} M) followed by the antagonist papaverine (10^{-5} M, 10^{-4} M), bradykinin (10^{-5} M, 10^{-4} M) and then a second application of papaverine (Sigma, St. Louis, MO). The maximum change in force was recorded. Nominal stress was calculated by dividing the force by the cross-sectional area for the unloaded ring segment. Cell number was determined by using the Hoechst DNA assay and assuming 7.6 pg DNA per cell; this was used to calculate the force per cell.

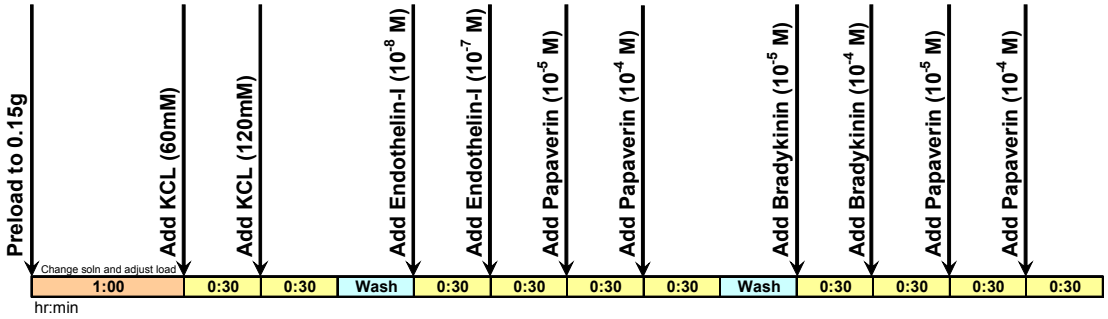

Figure 6: Contractility Test Timeline. This is the procedure used for the majority of the testing. Sections are preloaded and equilibrated for 1 hour. The ring sections are allowed to equilibrate for 30 minutes after each drug dose. Washes are completed between drugs or drug pairings.

Histology and Immunohistochemistry

Samples were rinsed in PBS and placed into 4% formalin at room temperature. After twenty four hours the samples were rinsed in PBS and transferred to 70% alcohol at 4°C until processing. The samples were paraffin embedded; 7 μm sections were cut for histology and immunohistochemistry (IHC). Samples were deparaffinized prior to staining. Histological stains such as Masson's trichrome and hematoxylin and eosin (H&E) were performed using standard histology protocols.

IHC protocols may vary based on primary antibody; any deviations from this protocol will be noted in the chapter where used. After deparaffinization, samples were blocked in phosphate buffered saline (PBS) with10% serum for 20 minutes at room temperature. The samples were then incubated with the primary antibody in PBS with 1.5% serum for one hour at 37°C. The samples were rinsed three times in PBS prior to incubation with the secondary antibody. The secondary antibody was used in a PBS buffer with 1.5% serum and Hoechst dye; samples were incubated for 45 minutes at room temperature. The slides were rinsed with PBS three times, coverslipped and imaged.

Gelatin Zymography

Samples were rinsed in PBS and placed in RIPA buffer (Sigma). Hydrogels were homogenized using an ultrasonicator and all samples were stored at -80°C until use. Prior to protein quantification, samples were centrifuged at 12,000×g for 10 minutes; only the soluble protein in the supernatant was used. The protein content was determined using the modified Lowry method (Pierce) using albumin standards for the standard curve.

Gel electrophoresis was performed using Novex 10% gelatin Zymogram gels (Invitrogen). Equal amounts of protein in loading buffer were added to each lane. The samples were not boiled and were run in nonreducing conditions. Electrophoresis was run at 125 V for 90 minutes at room temperature. After electrophoresis the gel was placed in renaturing buffer (Invitrogen) for 30 minutes at room temperature with gentle agitation. This removes the SDS and allows the proteins to renature. The solution was decanted and developing buffer (Invitrogen) was added for 30 minutes at room temperature with gentle agitation. Fresh developing buffer was then added and the gel was incubated overnight at 37°C. The gel was stained with colloidal blue (Invitrogen) for 3-6 hours and then destained in dI H_2O with gentle agitation overnight; the water was changed three times during this time. The gelatin stains blue; areas of MMP activity where gelatin has been degraded do not stain and appear clear. The gel was then imaged using the LiCor Odyssey imaging system and images were converted to gray scale. An example of a scanned zymogram gel is shown in Figure 7. As shown blue molecular weight ladders can be visualized using this system.

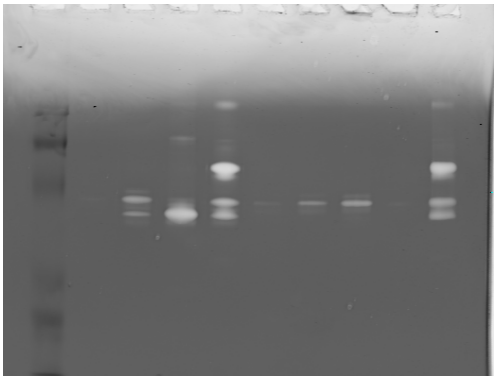

Figure 7: Stained Zymogram Gel. Along with experimental samples is a molecular weight ladder (lane 1) and an MMP 2/9 standard (lanes 5 and 10). The proteins are separated by weight using SDS-PAGE under nonreducing conditions. Zymogen degrades the gelatin leaving unstained bands corresponding to the active MMP.

Statistical Analysis

Data is expressed as mean ± standard error of the mean. Statistical analysis was completed using a 95% confidence interval. Tests with two parameters were completed using the Student t-test. For three or more parameters one way ANOVA was used; post hoc testing was performed using the Student t-test.

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CHAPTER 4: THE EFFECTS OF MATRIX TYPE AND PRESENTATION ON SYNTHESIS OF COLLAGEN TYPE I AND THE ASSOSCIATED PROTEOGLYCANS DECORIN AND BIGLYCAN

Introduction

Type I collagen is important for the mechanical integrity engineered tissue. While collagen can be added exogenously, the cell synthesized collagen in far superior in strength due to improved organization and cross-linking. Decorin and biglycan play key roles in collagen fibrillogenesis. These two homologous proteoglycans are part of the family of small leucine rich repeat proteoglycans (SLRP) [1, 2]. Knocking out decorin or biglycan results in irregularly sized collagen fibrils and fragility in skin [3] and orthopedic tissues [4, 5] respectively.

Decorin is critical in extracellular matrix (ECM) assembly; it interacts with matrix materials such as collagen types I, II, III, VI, XII, and XIV [6-12], tropoelastin [13], and fibronectin [14, 15]. Decorin affects extracellular matrix production through interactions with transforming growth factor-β (TGF-β). Once thought to inactivate TGF-β [16-18], decorin is now thought to selectively inhibit [19-21] TGF-β by sequestering it in the extracellular matrix [22]. Decorin can bind to both collagen and TGF-β [23]; TGF-β can then be release cleavage by MMP-2 [24]. It also interacts and regulates the function of tumor necrosis factor-α [25], platelet derived growth factor [26], fibroblast growth factor-2 [27], and insulin-like growth factor I [28]. Decorin plays a role in tissue remodeling; it can modulate matrix metalloproteinase (MMP) activity, although the response varies greatly by tissue [29-31]. MMP activity is important for remodeling of tissue engineered blood vessels. Non-selective inhibition of MMP activity results in diminished remodeling and reduced strength [32]; however, excessive MMP activity can lead to excessive extracellular matrix degradation and a reduction in tissue strength [33]. Decorin also

plays an important role in angiogenesis. It has been shown to both inhibit [34-36], and promote angiogenesis [37-39].

Biglycan also interacts with collagen I, II, III, and VI [6, 12, 40]. It may play a role in the mechanical integrity of the blood vessel; biglycan deficiency resulted in abnormal collagen fibrils in mice resulting in reduced strength and aortic dissection [41]. Decreased biglycan expression is also associated with abdominal aortic aneurysm [42]. Elastinogenesis is affected by biglycan. Biglycan binds to both tropoelastin and microfibril-associated glycoprotein 1 (MAGP-1) forming a ternary complex and may play a role in elastinogenesis [13]. Conversely, the glycosaminoglycan chains (GAG) have been shown to have an inhibitory role on elastin production [43]. Biglycan can also enhance proliferation and migration [44].

Decorin and biglycan expression play important roles in compaction of collagen hydrogels, which is important for many tissue engineering applications. Decorin expression has been shown to affect both collagen [45, 46] and fibrin [47] hydrogel compaction. When decorin is knocked out biglycan is up-regulated and collagen gel compaction also increases [48]. Expression of both decorin and biglycan are up-regulated during wound healing [49-52]. Deficiency of decorin reduces the wound healing response [35].

This study looks at how the expression of type I collagen, decorin and biglycan is affected by both matrix type and architecture. Culture of cells on an adsorbed protein versus within a hydrogel is known to affect multiple cell types, influencing cell phenotype and gene expression [53-61]. Differences are also seen when cells are cultured on top of 2D and 3D matrices [62]. Collagen I and fibrin were chosen as matrix materials due to their prevalence in vascular tissue engineering.

Materials and Methods

Cell Isolation and Culture

Smooth muscle cells were isolated and cultured as described previously.[63] Briefly, rat aortic smooth muscle cells (RASMC) were isolated from the thoracic portion of the aorta of normal adult Sprague-Dawley rats using the facilitated migration method which combines enzymatic digestion and migration techniques. Cells were cultured and expanded in medium consisting of Dulbecco's modified eagle medium (DMEM), 10% fetal bovine serum (FBS), 1% L-glutamine and 1% penicillin-streptomycin using standard culture conditions of 37°C and 5% CO_2. Cells were used between passages 6 and 9.

Fabrication of Monolayer (2D) and Hydrogel (3D) Culture Environments

Two dimensional monolayer cultures conditions were obtained seeding rat SMC on adsorbed protein slides. SMC were cultured on either Type 1 dermal bovine collagen (Mediatech Cellgro, Kansas City, MO) or fibrinogen (Sigma). Glass slides were coated with 1 ml of 0.05 mg/ml protein in phosphate buffered saline (PBS) for 1 hour. The excess solution was then gently removed and the SMCs were seeded onto the slides at a density of 20,000 cells/cm^2. After 30 minutes, 30 ml of cell culture medium were added to a dish containing two slides. The culture medium for the fibrin coated slides was supplemented with 2 mg/ml ε-amino-caproic acid (ACA) (Sigma) to prevent enzymatic degradation of the fibrin. Cells were cultured for 2 days prior to experimentation. Figure 8 depicts the 2D setup.

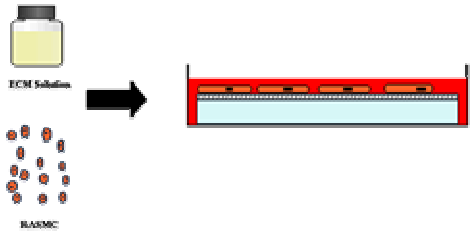

Figure 8: 2D Culture - Cells on ECM Coated Slide. Collagen or fibrinogen is adsorbed onto a glass slide. RASMC are seeded on the slides.

Three dimensional culture conditions were created by encapsulating RASMC in collagen or fibrin hydrogels. All hydrogels contain 2 mg/ml type I collagen or fibrin and 1 million cells/ml. Collagen hydrogels were made by neutralizing the pH of acid solubolized bovine dermal collagen and combining it with rat aortic smooth muscle cells and culture medium. Fibrin gels were made by combining fibrinogen, rat smooth muscle cells, thrombin (0.1 U/mg fibrinogen) and culture medium containing ACA. Two milliliters of the protein/cell solution was placed into a well in a 12 well plate and incubated at 37°C for 1 hour to allow the solutions to gel at which time additional culture medium was added. The next day the gels were transferred to a 6 well plate and 7 ml of culture medium was added. Medium for the fibrin hydrogels contained 2 mg/ml ACA. The hydrogels were sampled at 2 and 14 days for experimental work. Figure 9 depicts hydrogel fabrication.

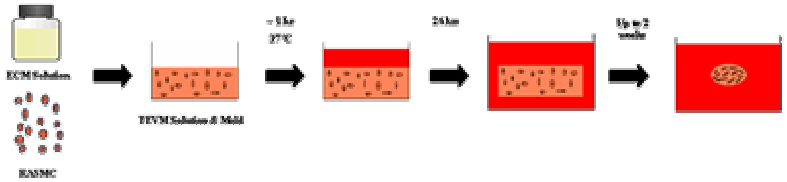

Figure 9: 3D Culture - Hydrogel Fabrication and Culture. RASMC are encapsulated in collagen or fibrin. After the ECM forms a gel medium is added. The hydrogels are liberated from the walls.

RNA Isolation and qRT-PCR

The RNA from monolayers was isolated using the RNeasy kit (Qiagen, Valencia, CA). The slides were rinsed twice with PBS prior to cell lysis; the samples were further homogenized using a Qiashredder (Qiagen). Due to the large amount of protein in the hydrogels, the RNeasy Lipid Tissue kit (Qiagen) was used to isolate suitable amounts of high quality RNA from the hydrogels. The hydrogels were rinsed in PBS, placed in lysis buffer and homogenized using a sonicator. For all samples the optional DNase digest was completed during all isolations per the RNeasy kit instructions using RNase-free DNase Set (Qiagen).

The cDNA was reverse transcribed from the isolated RNA using Superscript III First Strand cDNA synthesis kit (Invitrogen) using oligo(dT) as the primer.

Quantitative real time polymerase chain reaction (qRT-PCR) was used to study gene expression. Primers were designed using Primer Express software (Applied Biosystems) and are listed in Table 6. Collagen I expression was assessed by detecting the expression of the α1 subunit. SYBR green master mix (Applied Biosystems) was used for detection and the PCR reaction was run on a Step One Plus Real-Time PCR System (Applied Biosystems). Gene expression was determined using a relative standard curve and normalized to 18s.

Table 6: PCR Primers

Gene	Forward	Reverse
Biglycan	TGCTCCCGGTTGGCC	GGACTAGGATGTCAGGCTGTCG
Collagen I	GGTGGAGATTCTGGCTTTTGC	GAATGTAGGGCTTTGTCCGG
Decorin	TGAAAATGGAGCCTTGCAGG	TGAAGGGTCTCGGATACA
18s	TAGAGCTAATACATGCCGACGG	TCTGATAAATGCACGCGTCC

Western Blot

Samples were rinsed in PBS, lysed in RIPA buffer with proteinase inhibitors (Sigma) and hydrogels were homogenized. When necessary the samples where condensed using ethanol precipitation. In order to detect decorin and biglycan the samples were treated with 0.3 U/ml chondroitinase ABC for 2.5 hours at 37°C. Equal amounts of protein were mixed with loading buffer and the samples were boiled for 5 minutes at 95°C prior to loading. SDS-PAGE completed using 10% Tris-Glycine gels (Invitrogen) followed by protein transfer to a PVDF membrane (BioRad). A prestained molecular weight ladder (BioRad) was used to determine molecular weight; acellular homogenates or fresh medium were used as negative controls. The membrane was blocked in near IR blocking buffer (Rockwell) and then exposed to the primary antibodies in Tris-buffered saline with 0.1% Tween 20 (TBS-T). The decorin antibodies (LF-113) and biglycan antibodies (LF-159) were generously donated by Larry Fisher [64]. Secondary antibodies with fluorochrome in the near infrared spectrum (Rockwell) were used for detection. Blots were imaged using the LiCor Odyssey Infrared imaging system.

Collagen Synthesis

Collagen synthesis was determined using ^{3}H proline. The cells and hydrogels were incubated in medium containing 15 μCi/ml ^{3}H proline for 3 hours. The specimens were rinsed in PBS. Cells were trypsinized and collected. The hydrogels incubated in Proteinase K at 54°C overnight. Radioactivity was determined by counts per minute (cpm). Dead cell hydrogels were used for controls. The cpm of the dead cell hydrogels were subtracted from the cpm of the 2 and 14 day hydrogels. The cpm was normalized to DNA content which was determined by fluorescence of Hoechst 33258 dye binding. Calf thymus DNA was used as the standard curve for quantification.

MMP Activity

MMP activity was assessed by gelatin zymography. Samples were rinsed in PBS, lysed in RIPA buffer and hydrogels were homogenized using a sonicator. Samples were centrifuged at 12,000×g for 10 minutes to remove insoluble protein. The modified Lowry method (Pierce) was used for protein quantification. Equal amounts of protein were loaded into each lane of a Novex 10% Zymogram (gelatin) gel (Invitrogen). An MMP-2/9 standard (Millipore) was used for comparison. SDS-PAGE was run under non-reducing conditions after which the gel was placed in renaturing buffer (Invitrogen) followed by incubation overnight at 37°C in developing buffer (Invitrogen). The gel was stained with colloidal blue (Invitrogen) and imaged using the LiCor Odyssey imagine system; images were converted to gray scale.

Histology and Immunohistochemistry

Samples were rinsed in PBS and placed into 4% formalin at room temperature. After twenty four hours the samples were rinsed in PBS and transferred to 70% alcohol at 4°C until processing. The samples were paraffin embedded; 7 μm sections were cut for histology and immunohistochemistry (IHC). Masson's trichrome staining was performed on deparaffinized sections. For decorin and biglycan detection sections were incubated in chondroitinase ABC at 37°C for 1 hour. Antibodies for decorin (LF-113) and biglycan (LF-159) were generously donated by Larry Fisher.

Statistical Analysis

Data is expressed as mean ± standard error of the mean. Statistical analysis was completed using a 95% confidence interval. Tests with two parameters were completed using the Student t-test. For three or more parameters one way ANOVA was used; post hoc testing was performed using the Student t-test.

Results

Gene Expression

Monolayers and disks were cultured for two days prior to isolating the RNA. Differences in gene expression were found for collagen, decorin and biglycan; expression is affected by both matrix type and presentation. The results are shown in Figure 10. In monolayers, cell cultured on fibrin expressed significantly higher amounts of collagen I, decorin and biglycan than with cells cultured on collagen. After 2 days of culture in disks, the cells within the fibrin matrices still produced more collagen I than the cells within the collagen matrices. There were no differences in gene expression for decorin and biglycan under these conditions. By 14 days no significant differences are seen with respect to the matrix material. The effects of 2D and 3D culture were significant for decorin and biglycan expression. The cells cultured on adsorbed fibrin had similar levels of decorin expression as those encapsulated within fibrin gels. Once encapsulated in collagen the smooth muscle cells up-regulated decorin to the level seen with cells encapsulated in fibrin. Biglycan was significantly down-regulated in both matrixes compared to cells cultured on adsorbed proteins. Cells within both types of matrix produced similar low levels of biglycan. There were no significant differences between the hydrogels of the same matrix materials at 2 and 14 days in culture. There is an insignificant increase in collagen expression within the collagen gels that may become significant with extended culture.

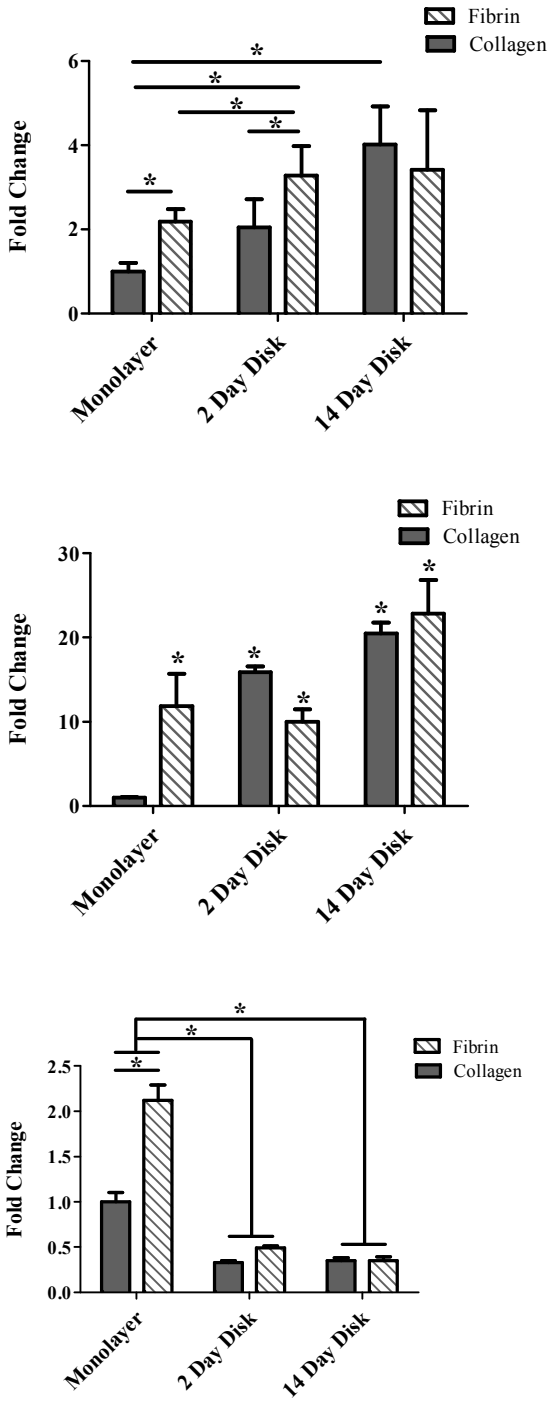

Figure 10: Effects of Matrix Type and Configuration on Gene Expression. Collagen (top), decorin (middle), and biglycan (bottom) are shown. * = p≤0.05 as compared to collagen monolayers or as depicted. Gene expression is affected by both matrix type and 2D/3D configurations. Only collagen expression is affected by time in culture in the hydrogels

Collagen Synthesis

Due to the use of type I collagen on the slides and for the hydrogels, radiolabeling was used for collagen detection instead of western blotting; the results are shown in Figure 11. There was only one instance in which a significant difference between fibrin and collagen matrices was seen; at two days there is a significantly greater amount of collagen synthesized in fibrin disks versus collagen disks. This is due in part to a reduction in collagen synthesis after SMCs are encapsulated in collagen hydrogels. No significant differences were seen between monolayers and disks using either matrix after 2 days in culture. After 14 days in culture collagen synthesis is increased in both collagen and fibrin gels compared to their monolayer and two day counterparts. One reason for the increased collagen synthesis is that by 14 days in culture the cells start to grow on top of the collagen or fibrin hydrogel, as shown in Figure 12.

Protein Expression

Western blotting was completed to confirm that the gene expression changes resulted in differences in protein expression. Western blots of decorin and biglycan from the cell lysates and hydrogel homogenates are shown in Figure 13. Decorin is being secreted and retained in both the collagen and fibrin hydrogels. Biglycan expression was low and was only seen in the collagen hydrogels after 14 days. Acellular controls were used and there was no detectable decorin or biglycan.

Western blots of the spent medium are shown in Figure 14. While there was more decorin in the collagen gels, there is more decorin in the spent medium from the fibrin gels. Biglycan was found in the spent medium from the fibrin disks, suggesting that the biglycan is not being retained in the fibrin disks.

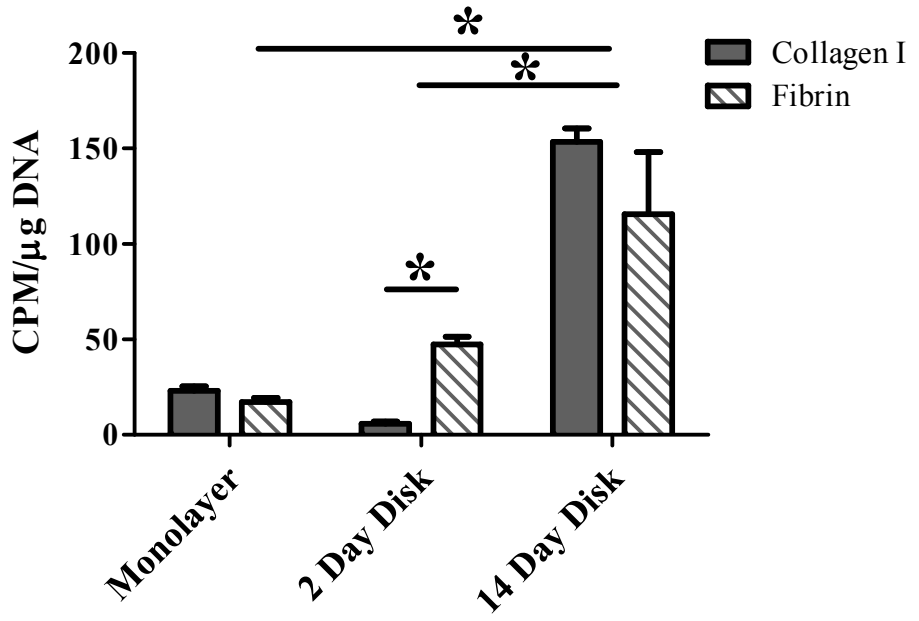

Figure 11: Effects of Matrix Type and Presentation on Collagen Synthesis. Collagen synthesis is assessed using tritiated proline incorporation. Results are normalized to DNA content. Cells encapsulated in fibrin initially produce more collagen. After 2 weeks in culture more collagen is synthesized in both matrices and no difference is seen between collagen and fibrin.

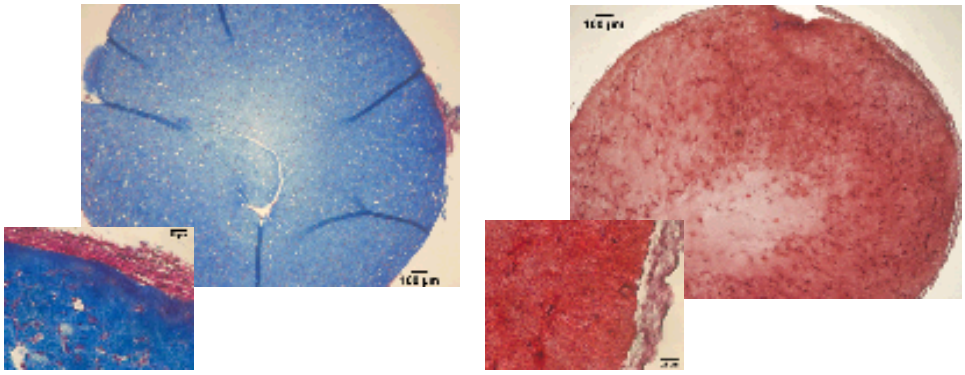

Figure 12: Masson's Trichrome Staining of 14 Day Collagen (Left) and Fibrin (Right) Disks. Collagen is stained blue. A dense 'cell capsule' is seen on both the collagen and fibrin hydrogels.

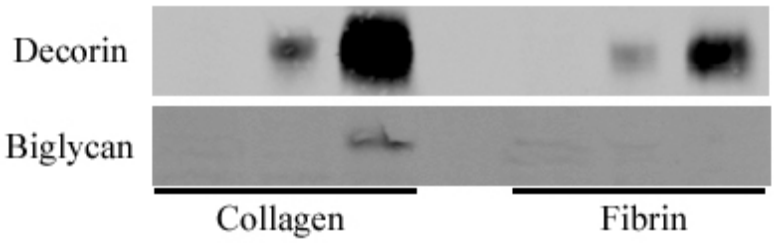

Figure 13: Western Blots of Decorin and Biglycan. Samples from left to right: monolayer, 2 day disk, 14 day disk for both collagen and fibrin. Acellular controls are not shown. More decorin and biglycan is found in the collagen disks than in the fibrin disks. Little proteoglycan is found in the cell lysates.

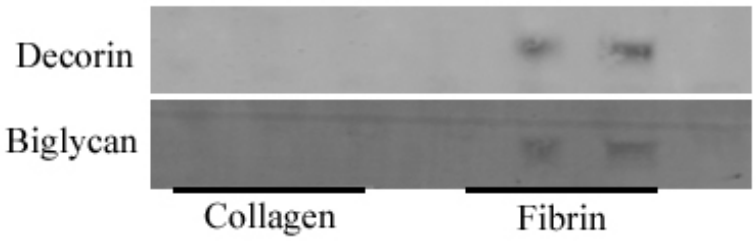

Figure 14: Western Blot of Decorin from Spent Medium. Samples from left to right: monolayer, 2 day disk, 14 day disk for both collagen and fibrin. Last lane is fresh medium control. More decorin and biglycan is seen in the spent medium from the fibrin disks than the collagen disks.

Cellularity

Cellularity of the constructs was determined using the Hoechst DNA assay. The results were the scaled for volume and are shown in Figure 15. The monolayers have fewer cells than the hydrogels, although fewer cells were used initially. The collagen disks contain more cells than the fibrin disks. The difference can also be seen in the slightly larger cell capsule on the collagen hydrogels versus the fibrin. There is little difference in cell number between the disks which suggests an error in the data as there is a lack of a cell capsule at day 2. This data is based on the results from the collagen synthesis work. It is valuable as a normalizing measure for collagen synthesis but was not

completed as rigorously as would be for determining DNA content, especially as pertains to the different culture types.

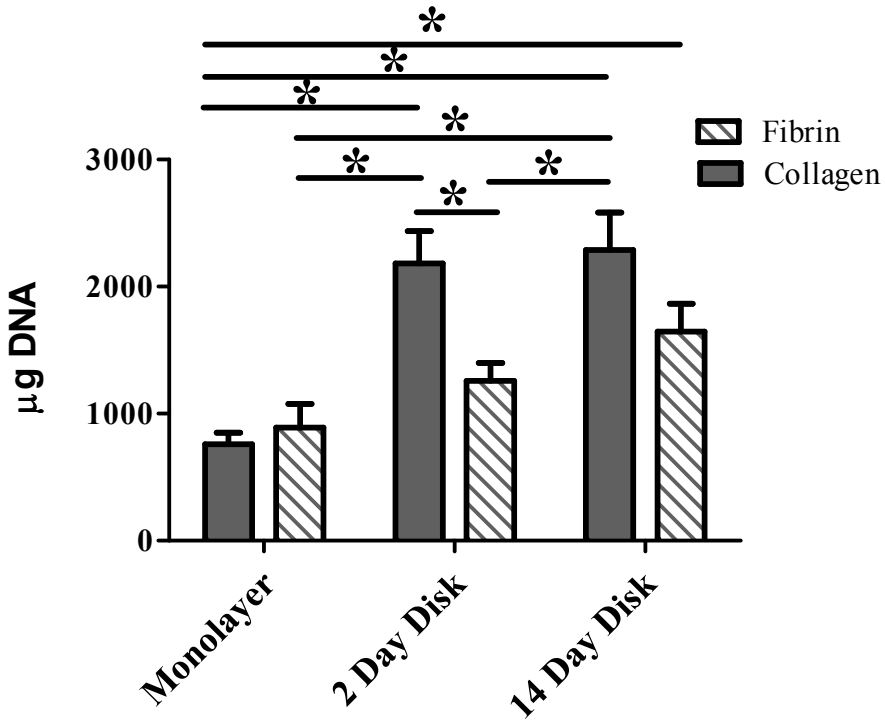

Figure 15: Cellularity of the Monolayers and Disks. * is p≤0.05. The monolayers have significantly less cells than the disks. Collagen disks contain more cells than the fibrin disks.

MMP Activity

Gel zymography was performed to determine MMP activity. MMP activity increased in the hydrogels. Latent and active MMP-2 is seen at 68 kDa and 62 kDa respectively. In the collagen hydrogel there is a distinct shift from the latent to the active form of MMP-2 when placed in the hydrogels as seen in Figure 16. Encapsulation in fibrin did not result in a detectable activation of MMP-2. Spent medium samples were also analyzed; only the latent form of MMP-2 was detected, see Figure 17.

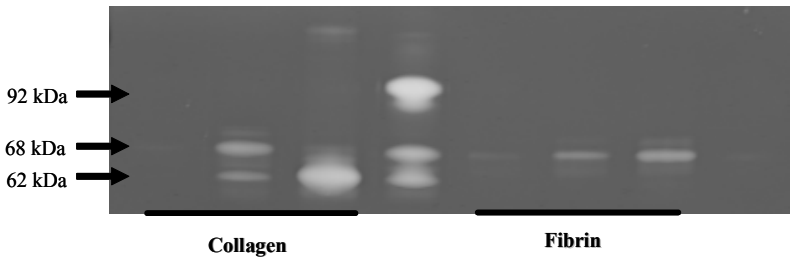

Figure 16: Effects of Matrix Type and Presentation on MMP Activity. Samples from left to right: monolayer, 2 day disk, 14 day disk for both collagen and fibrin. The collagen and fibrin samples are separated by an MMP 2/9 standard. The last lane is an acellular control. Acellular collagen is shown, but both have been have same results. Latent MMP-2 is found in all samples. MMP-2 is activated in the collagen hydrogels.

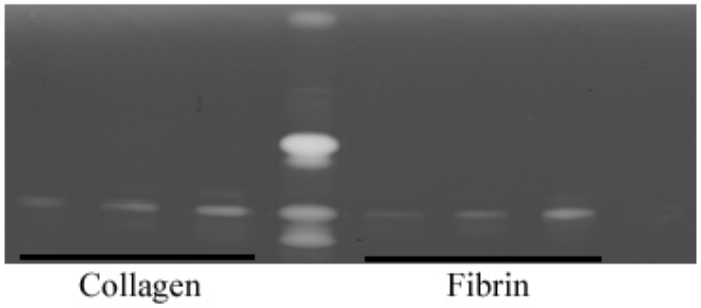

Figure 17: Zymogram of Spent Medium. Samples from left to right: monolayer, 2 day disk, 14 day disk for both collagen and fibrin. The collagen and fibrin samples are separated by an MMP 2/9 standard. The last lane is fresh medium control. Latent MMP-2 is found in all samples.

Discussion

There are several limitations to this study. Hydrogels are studied at 2 and 14 days of culture while the monolayers are only tested at 2 days. The monolayers could not be tested at 14 days as the cells grow on top of each other and can detach from the glass slide leading to a very different 3D model. By day 14 both the collagen and the fibrin hydrogels had formed small 'cell capsules' which also leads to a different microenvironment for the cells then the 2 day hydrogels. Only collagen and fibrin were chosen as they are popular biopolymer choices for tissue engineering. Certainly there are other matrix materials that could be of interest. The monolayer studies were only done

with adsorbed collagen and fibrin. Neither the hydrogels nor the monolayers provide an environment much like the *in vivo* condition. Use of higher cell concentrations could be an interesting study with different results. The 'cell sheet' method may also provide insight. A comparison to tissue culture treated plastic may also be of interest. Radiolabeling was done for detection of collagen and comparison to the PCR data. This method uses tritiated proline which is incorporated into all collagen types and a few other proteins, as well as collagen I.

Collagen I synthesis is important for the mechanical integrity of engineered tissues such as blood vessels. The expression of decorin and biglycan are of interest due to their role in collagen fibrillogenesis [12, 65-69] and hydrogel compaction [21, 45-48]. The 2D and 3D environments are known to have different effects on cells as is the extracellular matrix. The three genes studied were all regulated differently with respect to matrix type and presentation. In all cases cells grown on adsorbed fibrin had higher levels of gene expression than those grown on adsorbed collagen. Fibrin is the structural protein in blood clots and plays a key role in wound healing; both decorin and biglycan are known to be up-regulated after injury [49-52]. Once encapsulated within the hydrogels, no significant differences in gene expression were seen in cells cultured within the two different matrices. Differences exist between cells in 2D and 3D culture. Cells within collagen hydrogels express decorin at levels similar to cells exposed to fibrin, significantly higher than cells cultured on adsorbed collagen. Biglycan is down-regulated in cells in 2D culture versus cells in 3D culture. Within the hydrogel biglycan is expressed at similar levels for both matrix types. While the increase of decorin may suggest a wound healing response, the decrease in biglycan is inconsistent with what is seen after arterial injury. The additional culture time had no significant affect on gene expression on cells within the hydrogels. The presence of the cell capsule does seem to have some affect, and may have an affect on other genes of interest. Time may need to be

considered when using these smaller biopolymer hydrogels as model systems for tissue engineering applications.

Collagen synthesis has been shown to be greater in fibrin hydrogels then in collagen hydrogels [70]. This study has shown that collagen I gene expression is not significantly different in cells within these two matrix materials. Both collagen I gene expression and proline incorporation show elevated collagen synthesis in cells within fibrin versus collagen at day 2 in this experiment; however, by day 14 the difference no longer exists. The change in synthesis is most likely due the different microenvironment within the cell dense layer that is not present at day two. The work by Grassl et al [70] never discusses a cell capsule. This difference may explain the different outcomes. The concentration of ACA in the medium appeared to influence collagen synthesis in the Grassl work. This study used a lower fibrin concentration and a higher ACA concentration which may affect collagen synthesis in the fibrin hydrogels. The culture conditions that were used by this group are not expected to deter cell proliferation on the ECM surface; however, differences in serum or ECM lots may influence the cell capsule formation. Conversely, the Grassl work uses a higher concentration of neonatal RASMC in a smaller hydrogel which may form a cell capsule earlier. This could lead to a different microenvironment with a greater cell dense region that may also influence collagen synthesis.

Decorin is known to be retained within fibrin and collagen hydrogels [38, 45, 47]. In this study we show that decorin retention in collagen gels is superior to retention in fibrin gels. There was significantly more decorin within the collagen hydrogels than the fibrin hydrogels and more decorin in the spent medium from the fibrin hydrogels than the collagen.

MMP studies show that cells encapsulated in collagen activate MMP-2 which degrades both collagen and decorin [24]. The presence of large amounts of collagen most likely triggers this remodeling response, where as there is only small amounts of collagen

comparatively within the fibrin gels. Proteolytic degradation is still a problem with fibrin hydrogels. ACA must be added to the culture medium or the cells will degrade the matrix more rapidly then they can lay down new matrix.

This study highlights the importance of considering the effects of monolayer versus 3 dimensional culture. Many biological studies are completed with cells in monolayers which is not how most cells are found *in vivo.* The results from these studies may not directly relate to tissue engineering research where cells are cultured in 3 dimensional environments. Collagen, fibrin, or other matrix hydrogels can be used as model systems for the study of matrix biology and for tissue engineering. Few differences were seen in the disks between two and fourteen days; the disk shaped gels require less material and short culture times. This would be an ideal model for study of biochemical treatments such as transforming growth factor-β.

Collagen synthesis is important for tissue strength. This study shows that the smooth muscle cells produce equal amounts, if not more, collagen in collagen hydrogels than in fibrin hydrogels. Collagen gels also do not require the supplementation of enzyme inhibitors as do fibrin. The activation of MMP-2 could be problematic, but MMP activity has been shown to be important for remodeling and engineered tissue strength [32]. These facts led to the choice of collagen for future studies.

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CHAPTER 5: VASOACTIVITY OF A COLLAGEN BASED TEVM

Introduction

Tissue engineering of small diameter blood vessels has long been considered the 'Holy Grail' of tissue engineering due to the need for a material for bypass grafting that will remain patent.[1] An engineered blood vessel must be non-thrombogenic, have similar mechanical properties and be vasoactive. While much work has focused on the first two requirements, there has been little investigation of the vasoactive response. One reason may be that vascular smooth muscle cells in culture revert to a more synthetic phenotype, losing much of their contractile proteins and reducing their capacity to respond to vasoconstrictors and vasodilators [2, 3]. Even with these concerns, contractile responses have been shown with tissue engineered vascular media (TEVM) made with umbilical cord derived [4-7], neonatal [8, 9], and adult smooth muscle cells (SMC) [10-13]. While some of these TEVM come close, none match the response of native blood vessels.

Regulation of vascular tone is regulated by both intrinsic and extrinsic factors to meet the needs of local tissue and the body as a whole. In addition to the numerous classes of receptors, many receptors have multiple subtypes that can actually elicit different vasoactive responses. Adrenaline causes vasoconstriction when it acts as an α-adrenoceptor agonist and vasoconstriction when it is an agonist of the β-adrenoceptor [14]. Likewise, bradykinin is known as a vasoconstrictor, but the bradykinin B2 receptor can trigger contraction in veins [15, 16]. A biphasic response has been seen in veins and arterioles [15, 17, 18].

Endothelin-1 is one of the most potent agonists known [19]; it is used to study the contractile response in most of the reported studies along with bradykinin. In this study

we are investigating the vasoactive response of adult rat SMCs. We study the response over time and tested for the responses to different classes of vasopressors and vasodilators.

Materials and Methods

Cell Isolation

Smooth muscle cells from the thoracic portion of the aorta of normal adult Sprague-Dawley rats were isolated as previously described [20]. Briefly, rat aortic smooth muscle cells (RASMC) were isolated using the facilitated migration method which combines enzymatic digestion and migration techniques. Cells were cultured using standard culture conditions of 37°C and 5% CO_2. Cells were cultured and expanded in medium consisting of Dulbecco's modified eagle medium (DMEM), 10% fetal bovine serum (FBS), 1% L-glutamine and 1% penicillin-streptomycin. Experimental work was completed using cells between passages 6 and 10 unless otherwise noted.

Porcine aortic smooth muscle cells were isolated and expanded using the same facilitated migration technique. Smooth muscle cells were isolated from the abdominal aorta of neonatal Yorkshire piglets. Cells were used between passages 5 and 8.

Human aortic smooth muscle cells were purchased from Lonza. Lonza's smooth muscle cell growth medium-2 was used for cell expansion. Cells were used between passages 5 and 7.

Tissue Engineered Vascular Media Fabrication

Tissue engineered vascular media (TEVM) were made as described previously.[20] Rat aortic smooth muscle cells were used unless otherwise noted. The smooth muscle cells were incorporated into a type 1 collagen hydrogel for final concentrations of 1 million cells/ml and 2 mg/ml collagen. The collagen-cell solution was

placed into the tubular mold and allowed to polymerize. The hydrogel was then removed from the mold and placed into culture in medium consisting of Dulbecco's Modified Eagle Medium (DMEM, Mediatech Cellgro), 10% bovine growth serum (Hyclone, Logan, UT), 1% L-glutamine and 1% penicillin–streptomycin (Gibco, Grand Island, NY) unless noted. Culture medium was changed weekly.

Contractility Testing

TEVM were cut into 2 mm long rings that were mounted on hooks in a physiological organ bath of 5 ml Krebs Henseleit solution with continuous of 95% O2, 5% CO2 at 37°C [21-24]. The segments were preloaded to 0.15 g; the rings were first stimulated with the nonspecific agonist KCl (60 mM). Unless noted, the ring segments were then subjected to endothelin-1 (10^{-8} M, 10^{-7} M) or bradykinin (10^{-5} M, 10^{-4} M); both were followed by the antagonist papaverine (10^{-4} M) (Sigma, St. Louis, MO). Changes in tension due to chemical stimulation were measured using a digital data acquisition system (Gould Instrument Systems, Valley View, OH). The maximum change in force was recorded. Nominal stress was calculated by dividing the force by the cross-sectional area for the unloaded ring segment.

Also used in this study were phenylephrine, 5,6,7,8-tetrahydro-6-(2-propenyl)-4H-thiazolo[4,5-d]azepine-2-amine dihydrochloride (BHT-920), isoproterenol, ATP disodium salt, 5-hydroxytryptamine (5-HT), and sodium nitroprusside (SNP). All were purchased from Sigma.

Statistical Analysis

All graphs are shown as mean ± SEM. Statistical analysis was completed using a 95% confidence interval. Tests of two parameters were completed using a t-test. Tests of three or more parameters were completed using one way ANOVA with a post hoc t-test.

Results

Time Course

Up to now contractility in collagen hydrogels has not yet been demonstrated by either our group or any others except with a high concentration of low passage cells.[13] The response was measured weekly between 2 and 8 weeks in culture (Figure 18). While there are no statistical differences in contractility between the weeks, the response to endothelin-1 seems to peak around week 3 while bradykinin appears to provoke the maximum response at 4 weeks. There is significant batch to batch variability; if this variability could be reduced statistical differences might be seen or a more consistent contractile response might be found. To better understand the variability, the contractile response of the passage number of the TEVM in the time course was investigated and is shown in Figure 19. The cells used for this study were between passage 6 and passage 10; there is no significant difference in the contractile response as a function of passage. Differences due to batch are significant.

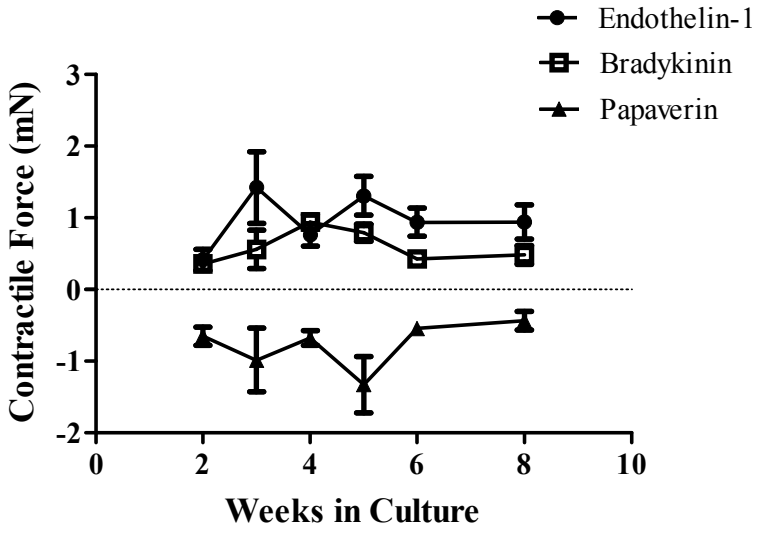

Figure 18: Contractile Force as a Function of Culture Time. No significant differences in response are found.

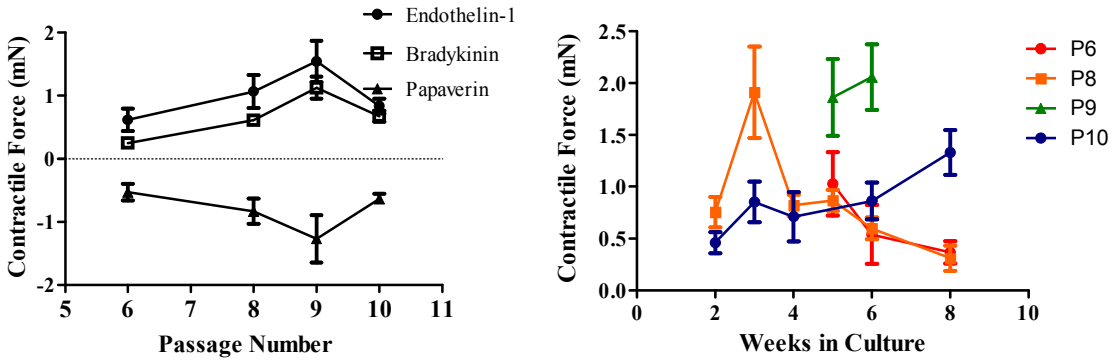

Figure 19: Contractile Force as a Function of Cell Passage. Shown is the data combined (left) and by week in culture (right). No differences due to passage number are found.

While the most significant variation was batch to batch, there was still some variability within the batches. For a subset of the rings tested the ring location from the TEVM was noted. The ring locations are noted in Figure 20, and the results are shown in Figure 21. The center rings produce a greater contractile force in response to endothelin-1 than the end segments. This is true only for endothelin-1 and not for bradykinin. There is a similar trend with papaverine, but this is most likely due to the fact that the initial contractile force is higher and may not actually be an enhanced response to the antagonist. The results thus may be slightly misleading. While there is a significant difference, the difference does not always hold. In at least one batch the end segments actually produced the greatest response to both agonists. The ring location was not noted for every test, so there is significantly less data than for the time course or the passage number study.

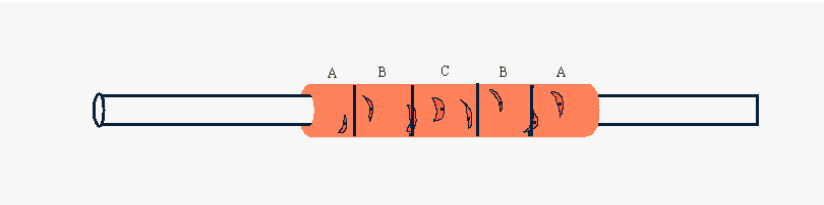

Figure 20: TEVM Sections. Locations noted are the end segments (A), the midsections (B), and the center (C).

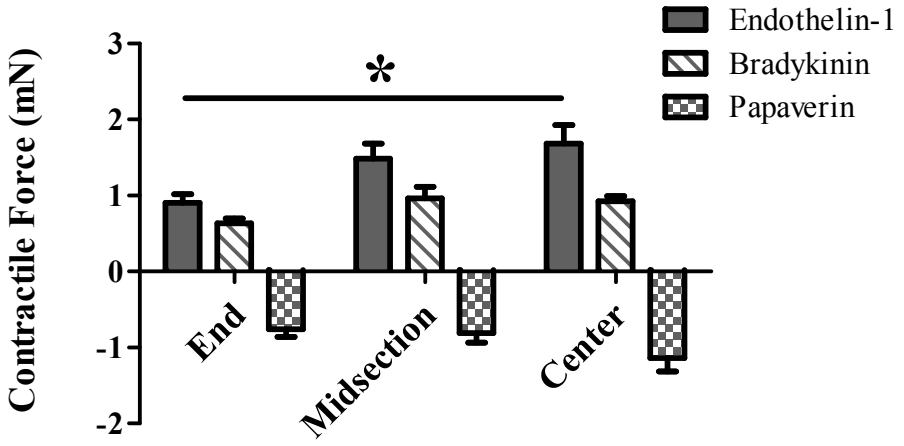

Figure 21: Contractile Force of Ring Segments Based on Position in TEVM. There are significant differences in the response of the rings from the ends versus the rings from the center of the TEVM.

Response to Different Agonists and Antagonists

To get a better understanding of which receptors were present and functional, several agonists and antagonists were chosen. Representative responses to the chosen agonists and antagonists are shown in Figures 22 through 31. Unless noted the bars shown are 10 mg in the y-direction and 1 minute in the x-direction. Arrows indicate the dosage of the drug in mM. All rings were stimulated with the non-specific agonist KCl prior to application of other drugs. A representative response to KCl is shown in Figure 22.

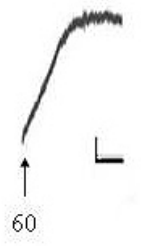

Figure 22: Representative Response to KCl

To look at responses to endothelial cell secretions, responses to endothelin-1 and SNP were measured. Endothelin-1, shown in Figure 23, was the most potent vasoconstrictor tested. Endothelin-1 is an agonist of the endothelin receptors, ET_A and ET_B. SNP, a nitric oxide donor, was used to try to elicit a relaxation response. The rings that relaxed did so very slowly, in some cases more slowly than untreated rings. A representative response to SNP when relaxation was seen is shown in Figure 24. It is probable that this is not a receptor mediated response, but a naturally occurring non-receptor mediated relaxation. A representative force trace showing lack of response is shown in Figure 25.

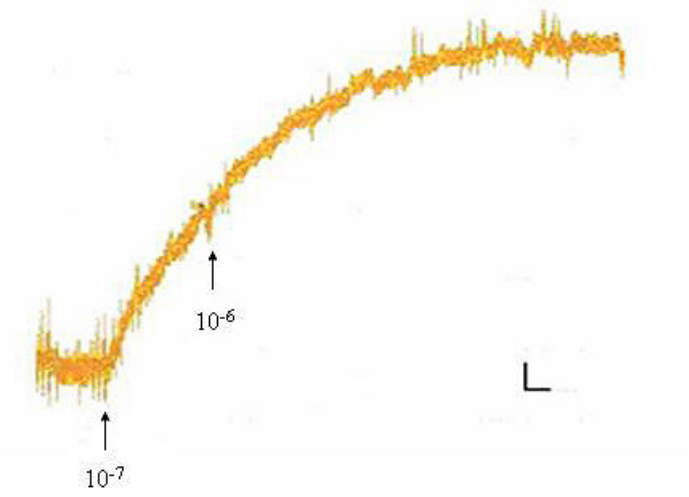

Figure 23: Representative Response to Endothelin-1

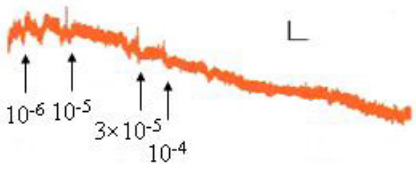

Figure 24: Representative Response of SNP When a Response was Present

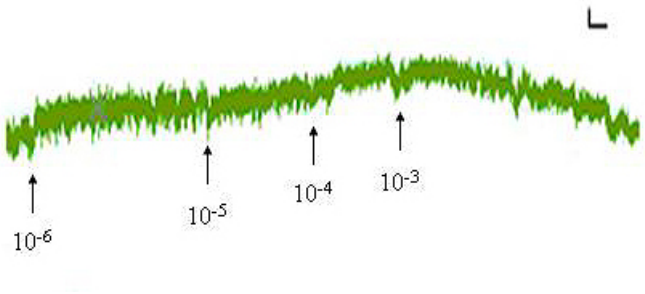

Figure 25: The Lack of Response to SNP

The response to bradykinin is shown in Figure 26. Bradykinin interacts with the bradykinin receptors B_1 and B_2. Bradykinin can elicit a contractile or dilatory response depending on the receptors involved. In this model of the media, bradykinin elicits a contractile response.

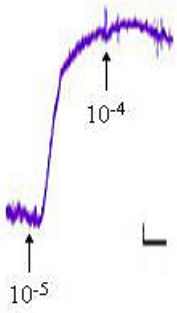

Figure 26: Representative Response to Bradykinin

There are four adrenergic receptors: α_1, α_2, β_1 and β_2. Phenylephrine is an α_1 agonist; the response to phenylephrine is shown in Figure 27. The response appears to be dose dependent; a contractile response is seen with the higher doses of phenylephrine. BHT-920 is an α_2 agonist; the data is shown in Figure 28. In each case an excellent contractile response to endothelin-1 was observed after the last dose of BHT-920 demonstrating that the vessels were vasoactive. There may have been a small drug-induced relaxation from baseline. This may be receptor mediated or may be a non-

specific tissue relaxation. To test for the presence of β receptors, isoproterenol was given. A dose responsive relaxation is seen (see Figure 29).

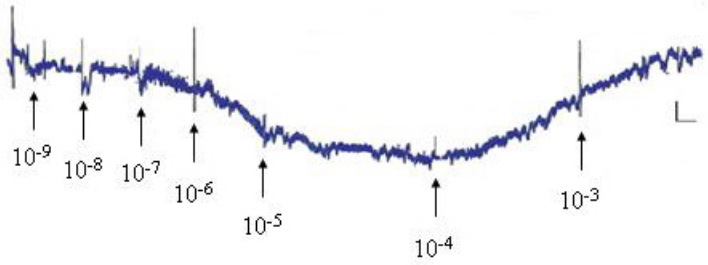

Figure 27: Representative Response to Phenylephrine

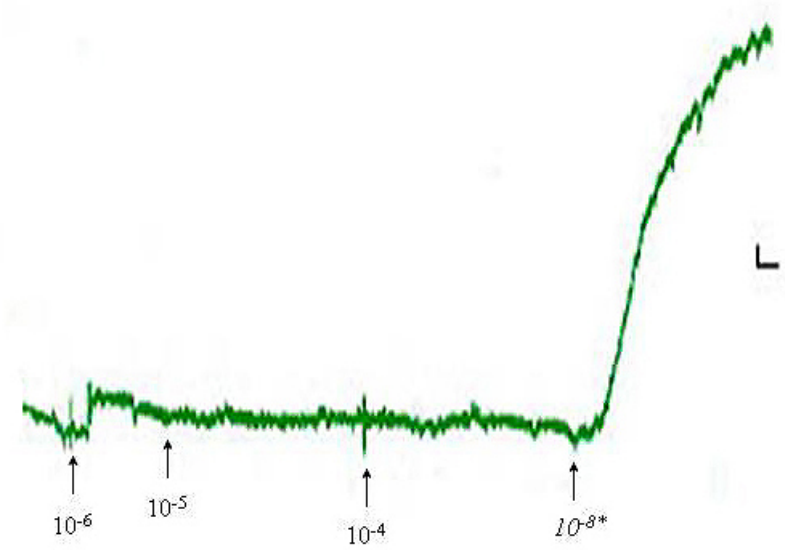

Figure 28: Representative Response to BHT-920 Followed by a Dose of Endothelin-1(*)

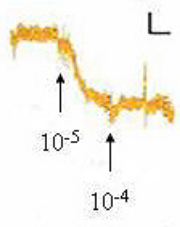

Figure 29: Representative Response to Isoproterenol

The presence of purinergic receptors was confirmed using ATP. Contraction was evident after ATP was given, although the contractions were short lived with the smaller doses (see Figure 30).

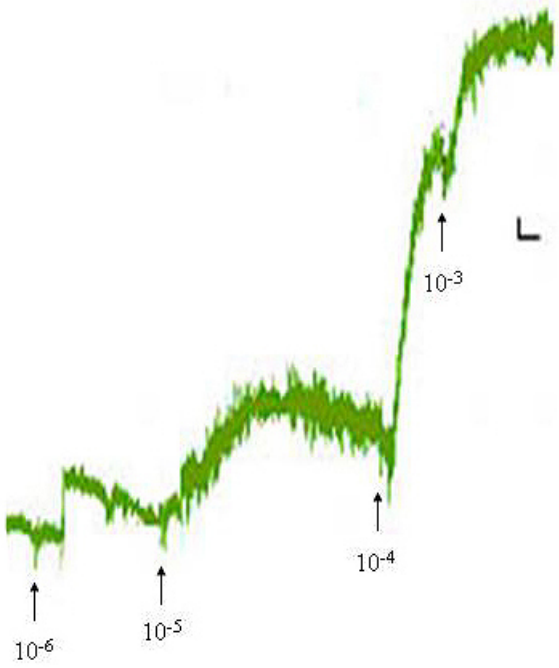

Figure 30: Representative Response to ATP

5-HT, also known as serotonin, was used to look for the serotonergic receptors. There appears to be a biphasic response, with the highest dose resulting in the highest contraction (see Figure 31).

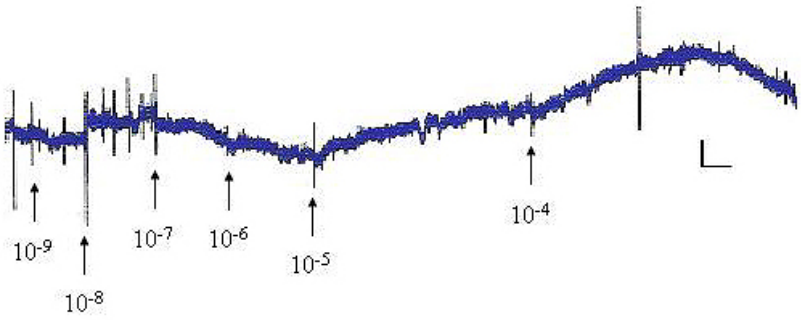

Figure 31: Representative Response to 5-HT

A summary of the results of the drugs tested is found in Table 7. Most of the drugs elicited a vasoactive response.

Table 7: Summary of Responses to Drugs

Drug	Receptor	Response
KCl	Non-specific	Contraction
Endothelin-1	ET_A and ET_B	Contraction
SNP	NO	No response
Bradykinin	Kinin B_1 and B_2	Contraction
Phenylephrine	α1-adrenergic	Biphasic
BHT-920	α2-adrenergic	No response
Isoproterenol	β-adrenergic	Dilation
ATP	Purinergic	Contraction
5-HT	Serotonergic	Fair Contraction

Contractility and Species

Contractility testing was completed on TEVMs made with human, rat and pig SMC. Only the vasoconstrictors endothelin-1 and bradykinin were tested. All species responded to the agonists; no significant differences in the amplitude of the response were seen between species, this is shown in Figure 32.

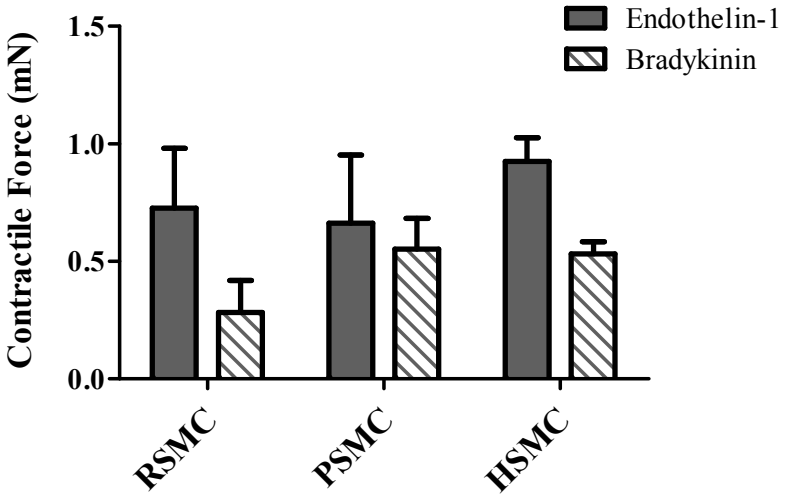

Figure 32: Contractile Force due to Species Difference. There are no significant differences in contraction between species.

Discussion

The majority of the work was done with rat smooth muscle cells; however, some studies were performed between species. The response to endothelin-1 and bradykinin was found to be similar across the species tested, but that may not be the same for the other drugs tested. While rat smooth muscle cells are easy to obtain and grow and are used often, they are very different from the human cells that would most likely be required for an implantable graft or for a pharmacological model. Cells in this study were used up to passage 10 with rats. This is much higher than would be used with human cells. While no differences in vasoactive response were seen with passage with rat cells, this may not be the case when human cells are used.

For this study only force data is being presented since the wall thickness and cell content was not measured for all samples. Traditionally the contractile response is reported as force or as a percentage compared to the maximum response to ATP. While this is sufficient to study the response, stress is a more appropriate measure for comparison of samples with different wall thickness or lengths.

The majority of the work in this and the following chapters focuses on the response to endothelin-1, bradykinin, and papaverine. While other agonists and antagonists were investigated, the number of replicates is small. This thus allows for a discussion of the presence of function, but not for a complete assessment of the presence of certain receptors or the magnitude of the response with much confidence.

The TEVMs maintained a consistent vasoactive response between 2 and 8 weeks, consistent with a similar study of the cell sheet TEVMs [6]. The cell passage number had no effect on the response within the range tested. There are spatial effects to the TEVM contractility. While not consistent, the ring segments in the center of the graft in general produced a larger contractile force than those close to the ends. Creation of a longer TEVM may allow for more rings with less variability.

While not completely characterized, the cells in the TEVMs have functional endothelin, bradykinin, adrenergic, purinergic and serotonergic receptors. The contractile response to bradykinin shows the presence of the B_2 bradykinin receptor. The response to the α_2-adrenergic receptor agonist was lacking, as was a clear response to SNP. The TEVM can dilate as shown with isoproterenol, so the receptors may be lacking. It is possible that culturing with endothelial cells may affect the presentation and function of the receptors, but cell-sheet TEVMs without endothelial cells have responded to SNP [6].

In comparison with the literature, this study has more extensively tested vasoactivity in response to both vasopressors and vasodilators than most. The cell sheet TEVMs model is the best characterized. It has been shown to be a good model for pharmacological studies, employing a variety of agonists and antagonists [6]. Several studies of the endothelin receptors have also been completed [10, 25]. The PGA-based TEVMs have shown responses to prostaglandin $F_{2\alpha}$, serotonin and endothelin-1 [11, 12, 26]. Fibrin based TEVMs have shown responses to KCl, norepinephrine and the thromboxane A_2 mimetic U-46619 [5, 7-9]. Only one study has been done with collagen; it used high numbers of lower passage cells. While it is a TEVM, only small rings were made to investigate the role of RhoA in the regulation of contractility. The agonists tested were K^+, bradykinin, histamine and endothelin [13]. This work shows that a larger panel of vasopressors and vasodilators are required to show a fully functional TEVM. For example, Swartz et al. [5] have demonstrated that their fibrin based TEVM are responsive to norepinephrine. Norepinephrine is an α-adrenoceptor agonist, it is possible that as in the present work the response is due to the α_1-adrenoceptor alone and that the α_2-adrenoceptor may not elicit a response.

While there was a measurable response to most agonists, it was less then native tissue and less than found with TEVM made using the cell sheet method [6]. Compared to the other collagen TEVM, the effects of bradykinin and endothelin-1 were different. Bi et al. saw a more potent reaction to bradykinin, with endothelin-1 producing the weakest

response [13]. This study saw the strongest response to endothelin-1, with bradykinin eliciting a smaller response. There are multiple factors that might play a role including cell density, cell source and cell expansion methods as well as the order in which the drugs were used. The TEVMs in this study have a rather large sparsely populated collagen inner layer with a highly cellular cell layer around this. As for cell source, this work has shown no difference in response between adult human and rat cells. The cell-sheet work used human umbilical vein smooth muscle cells, which may influence the vasoactive response [27]. There may be ways to improve the vasoactive response. Cyclic strain, transforming growth factor-β (TGF-β) and platelet derived growth factor [20] have been shown to influence smooth muscle cell phenotype and may result in a stronger contraction. The combination of TGF-β and insulin has been shown to improve contractile force [9].

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CHAPTER 6: CYCLIC STRAIN IMPROVES TEVM MECHANICAL PROPERTIES AND VASOACTIVITY

Introduction

Autologous blood vessels, veins or arteries, are the "gold standard" for both coronary and peripheral arterial bypass grafting. However, many patients do not have veins suitable for grafting [1]. This has resulted in research into tissue engineered blood vessels (TEBV) as a potential alternative source of vascular grafts for patients. Experimental and clinical vein grafting indicates that it is not necessary to implant a fully developed artery as veins undergo arterialization upon transplantation [2]; therefore, our ultimate clinical and commercial target for blood vessel engineering should be a construct that can be sutured, is non-thrombogenic and non-immunogenic, able to withstand arterial pressures, compliant enough to form non-hyperplastic anastomoses, and vasoactive [1, 3, 4].

The medial layer of the blood vessel is responsible for the mechanical integrity and vasoactivity of blood vessels and is an important are of focus for tissue engineering. The application of cyclic strain has been used to provide a more physiological environment for tissue engineered vascular media (TEVM) culture; cyclic strain of TEVM has been shown to increase tissue strength [5-12], increased ECM deposition [6, 11-14] and improved smooth muscle cell alignment [5, 15]. Only a few studies have shown an increase in contractile protein expression in TEVM [13, 15, 16]; however, studies stretching monolayers of cells adhered to silicone sleeves have also shown improvements in contractile protein expression with the application of stretch [17-19].

Vasoactivity is a desirable trait for a TEVM; however, it may be difficult to achieve in a commercially viable TEVM. One issue with a TEVM is that, to create

enough tissue for a graft, large numbers of cells are needed. To this end much of the work has used primary cells cultured out several passages. After a short time in culture these cells shift to a more synthetic phenotype, losing much of their contractile proteins and reducing their capacity to respond to vasoconstrictors and vasodilators [20]. Even with these concerns, contractile responses have been shown with a TEVM made with umbilical cord derived [21-24], neonatal [25, 26], and adult smooth muscle cells (SMC) [13, 27, 28] as well as bone marrow progenitor cells [24]. Cyclic strain may improve the vasoactive response of the TEVM through improvements in cell alignment and phenotype.

Typically TEVM are subjected to cyclic strain very early in culture; this significantly increases compaction of a hydrogel-based TEVM [5, 16]. A concern with this is that longitudinal compaction suggests incomplete adherence of the tissue to the silicone sleeve which is important for application and recovery of strain. To overcome the issue with compaction of the hydrogels one group has investigated the use of collagen cross-linking after hydrogel compaction ceased [6]. This study aims to build upon our previous work investigating how cyclic strain affects TEVM strength by improving the gel adherence to the sleeve to better control the applied strain and then study effects of strain on the mechanical properties and vasoactivity.

Materials and Methods

Tissue Engineered Vascular Media Fabrication

Rat aortic smooth muscle cells (SMC) were isolated previously from Sprague-Dawley rats weighing ~300-350 g [16]. The cells were isolated using a facilitated migration method which combines enzymatic digestion with cell migration from the partially digested tissue. The cells were expanded and used between passages 6 and 9. The TEVM were fabricated as described previously [5]. Briefly, prior to TEVM

fabrication the silicon sleeves were etched with 10 N sulfuric acid, rinsed and sterilized. The sleeves were coated in a 1 mg/ml type 1 bovine collagen (Mediatech Cellgro, Kansas City, MO) solution to aid gel adherence; the sleeves were placed onto glass mandrels used in the tubular molds. TEVM were created by neutralizing acid solubolized type 1 bovine collagen (MP Biomedicals, Solon, OH) and adding SMCs for final concentrations of 1 million cells/ml and 2 mg/ml collagen. The collagen-cell solution was placed into the tubular mold and incubated at 37°C. After polymerization, the hydrogel on the inner mandrel was then removed from the outer mold and placed into culture medium consisting of Dulbecco's Modified Eagle Medium (DMEM, Mediatech Cellgro), 10% bovine growth serum (Hyclone, Logan, UT), 1% L-glutamine and 1% penicillin – streptomycin (Gibco, Grand Island, NY). The gel was cut away from the stoppers and cultured statically for two weeks unless otherwise noted. This was done to allow for gel compaction and adherence of the gel to the silicone sleeve. Culture medium was changed weekly.

Compaction Assessment

Hydrogels were made as described with the exception that 2 ml of solution was placed in a well dish to create a disk shape. The gels were gently detached from the sides of the dishes and cultured in an unconstrained condition. At 2, 7, 14, 21, 28, 35 and 42 days disks were removed from culture and the volume assessed using volumetric displacement. A 10 ml volumetric flask was filled with DMEM for better visualization. The disks were rinsed in PBS and excess fluid was gently removed using a kimwipe. The disk was then added to the flask and the excess liquid removed so that the volume was reduced to the original volume. The displaced volume represents the compacted volume of the construct. The volumetric flask conforms to ASTM E 237 and the volume was assessed as described in ASTM E 542.

Cyclic Strain

Cyclic strain was applied to the collagen TEVM using a system previously developed in our lab [5]. Unlike previous studies, TEVM were cultured for two weeks to allow compaction to cease prior to the application of strain. The tubular constructs on the silicone sleeves were mounted on hollow mandrels in the strain bioreactor containing 300 ml of culture medium. The lumen and reservoir were filled with culture medium. Two 0.2 μm filters were placed in the vents in the lid to allow for oxygen exchange. The bioreactor was placed in a CO_2 incubator at 37°C, and connected to a filtered regulated air supply. The pneumatic pressure was controlled in order to produce 10% circumferential strain at a rate of 1 Hz. TEVM were cyclically strained for 5 days; statically cultured controls were used for comparison. At the conclusion of testing, the TEVM were gently removed from the silicon sleeves prior to testing.

Tensile Testing

TEVM were cut into 5 mm rings; black beads of approximately 300 μm were affixed to one side of the rings in order to measure strain. The segments were mounted onto hooks in a phosphate buffered saline (PBS) bath on an Instron single column test system (Instron). The rings were preloaded to 0.3 mN and precycled for five cycles to approximately 20% of the yield strain. The rings were then loaded to failure at a constant strain rate of 0.2 mm/s.

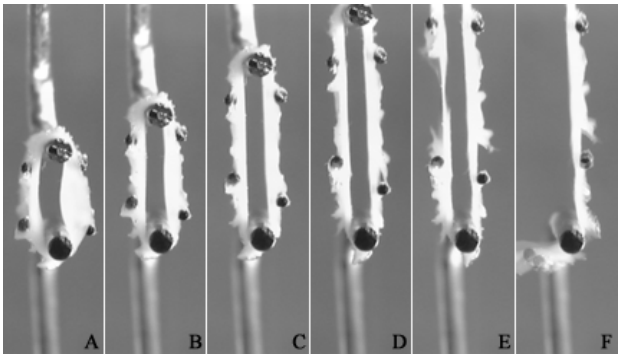

Figure 33: Uniaxial Tensile Test of Ring Sample. Shows frames from 6 timepoints in the test.

Matrox Inspector 8 was used to capture and analyze images taken during the test. Wall thickness was measured in order to determine cross-sectional area and calculate stress. Bead displacement was tracked so that local strain could be calculated (see Figure 33). The recorded force and displacement data were then converted to stress and strain where the ultimate tensile strength, yield strength and elastic modulus could then be determined.

Contractility Testing

TEVM were cut into 2 mm long rings that were mounted on hooks in a physiological organ bath of 5 ml Krebs Henseleit solution with continuous gaseous perfusion of 95% O2, 5% CO2 at 37°C [29-32]. The segments were preloaded to 0.15 g for 60 minutes; the rings were first stimulated with the nonspecific agonist KCl (120 mM). Responses to the vasoconstrictor endothelin-1 10^{-8} M, 10^{-7} M) or bradykinin (10^{-5} M, 10^{-4} M) and followed by the antagonist papaverine (10^{-5} M, 10^{-4} M) (Sigma, St. Louis, MO). Changes in tension due to chemical stimulation were measured using a digital data acquisition system (Gould Instrument Systems, Valley View, OH). The maximum change in force was recorded. Nominal stress was calculated by dividing the force by the cross-sectional area for the unloaded ring segment.

MMP Activity Assay

To determine the MMP activity, gelatin zymography was performed. Ring segments were rinsed in PBS, placed in RIPA buffer (Sigma) and homogenized using an ultrasonicator. Samples were stored at -80°C until use. Prior to protein quantification, samples were centrifuged at 12,000×g for 10 minutes. The protein content of the supernatant was determined using the modified Lowry method (Pierce) with albumin standards for comparison.

Gel electrophoresis was performed using Novex 10% Zymogram (gelatin) gels (Invitrogen). Equal amounts of protein in loading buffer were added to each lane. MMP 2/9 standards (Millipore) were used for determination of molecular weight; acellular hydrogels or fresh medium were used for controls. After electrophoresis the gel was placed in renaturing buffer (Invitrogen) followed by overnight incubation at 37°C in developing buffer (Invitrogen). The gel was stained with colloidal blue (Invitrogen) for 3-6 hours and then destained in water. The gel was then imaged using the LiCor Odyssey imaging system and images were converted to gray scale.

DNA Quantification

Segments of the tissue were taken, rinsed in PBS and lyophilized using a Freezone freeze dry system (Labconco). The tissue was digested in 0.5 mg/ml Proteinase K in phosphate buffer with EDTA at 60°C. The samples were loaded in triplicate into a 96 well plate along with DNA standards diluted from calf thymus DNA (Sigma) stock solution. The samples were incubated for 15 minutes at room temperature with 0.1 μg/ml Hoechst 33258 dye in 10 mM Tris-HCl buffer with 1 mM EDTA and 0.2 M NaCl. The plate was read on a flurometer using an excitation wavelength of 365 nm and emission wavelength of 458 nm. DNA quantity was determined by comparing to the standard curve made from calf thymus DNA (Sigma). The DNA content was converted to cell number assuming 7.6 pg DNA/cell [33]. The results were normalized to dry weight to account for differences in amount of tissue.

Collagen Quantification

TEVM sections were digested in 0.5 mg/ml Proteinase K. Digested samples and collagen I standards were added to 0.8 mM direct Sirius red dye in 0.5 M acetic acid. The samples were centrifuged at 10,000×g for 15 minutes and the supernatant loaded into a 96 well plate in triplicate. The plate was read on a spectrophotometer at 540 nm

wavelength. The collagen content was determined by comparison the standard curve and normalized to dry weight to account for differences in amount of tissue.

Histology and Immunohistochemistry

Samples were fixed in 4% formalin, paraffin embedded and sectioned. The 7μm thick sections were deparaffinized and stained with Masson's Trichrome. Smooth muscle alpha actin was studied employing antibodies and immunofluorescent microscopy. Slides stained with Masson's trichrome were imaged using a standard light microscope. Fluorescently labeled slides were imaged using a Zeiss confocal microscope.

Statistical Analysis

All graphs are shown as mean ± SEM. Statistical analysis was assessed using a 95% confidence interval. Tests of two parameters were assessed using a Student's t-test. Tests of three or more parameters were assessed using one way ANOVA with a post hoc t-test.

Results

Gel Compaction

Volumetric displacement measurements of disk shaped hydrogels were performed to determine the duration of compaction with our TEVM. Disks were chosen since the starting volume could be better controlled. More than 50% of the gel compaction occurred during the first 2 days of culture; however, measurable compaction was observed for 2 weeks (Figure 34). Girten et al. showed that in a similar TEVM measurable compaction continued until 2 weeks in culture. The volumetric data showed similar results to Girten et al. where tubular sections were used [34].

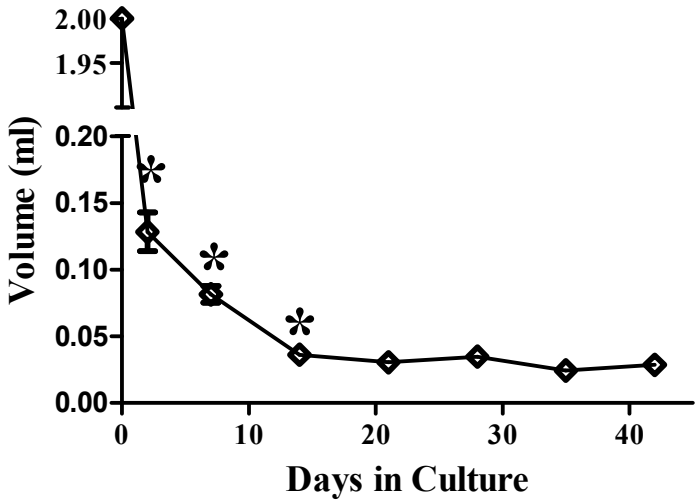

Figure 34: Volumetric Compaction Over Time * = p≤0.05 when compared to the previous timepoint. There is significant compaction up to 2 weeks in culture.

In previous studies, the TEVMs were subjected to cyclic strain after just 2 days in static culture. Under these conditions significant changes in longitudinal compaction are seen suggesting incomplete adherence to the silicone sleeve that [5, 16]. Since adherence to the silicone sleeve is important for controlling strain amplitude of the tissue, the initial culture time was increased to 2 weeks to allow for the construct compaction to cease before strain was applied. By waiting two weeks instead of two days, the TEVMs were more firmly attached to the silicone sleeves. This was seen first in the improved handling. The younger constructs had to be handled very carefully and could be easily detached from the sleeves while mounting them in the bioreactor. The two week old constructs were not as easily dislodged.

The firmer attachment also resulted in a difference in gel compaction. Unlike the two day old constructs, no longitudinal compaction was visible as seen in Figure 35. While no changes in length were visible, radial compaction still increased. The wall thickness of the cyclically strained TEVM was slightly thinner than that of the statically cultured controls, 0.41 mm and 0.48 mm respectively (see Figure 36).

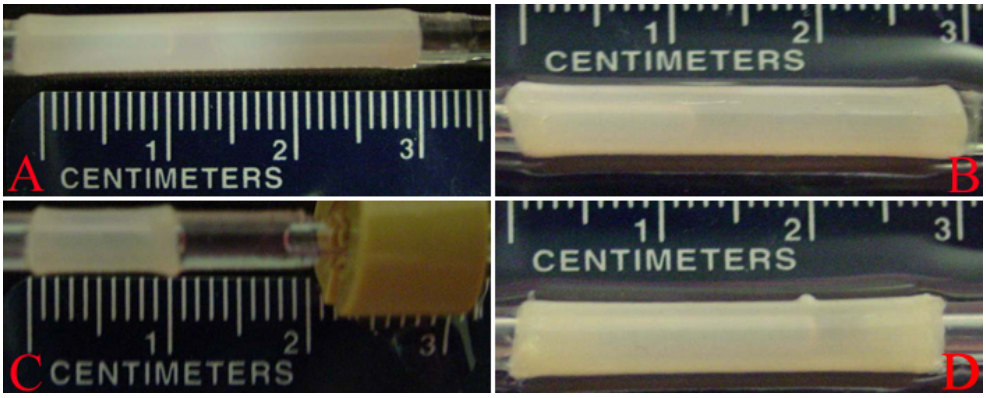

Figure 35: Compaction of TEVM. The culture conditions were statically cultured for 2 days (A), statically cultured for 2 weeks (B), 5 days of cyclic strain applied after 2 days (C), and 5 days of cyclic strain applied after 2 weeks (D). Delaying application of strain allows for better adherence and eliminates the longitudinal compaction (D) seen when TEVM are exposed to strain at 2 days (C).

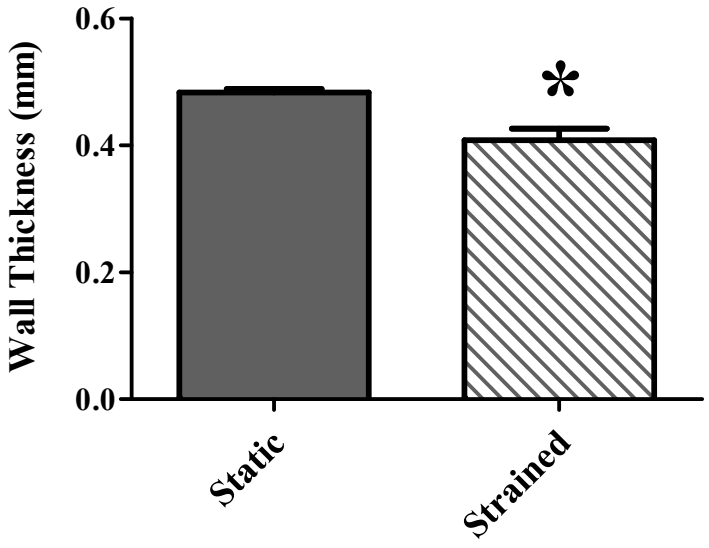

Figure 36: Wall Thickness TEVM. TEVM were cultured for 2 weeks statically followed by 5 days of cyclic strain or additional static culture. Strained samples have a thinner wall than the strained samples.

Effect of Cyclic Strain on Vasoactivity

Preliminary physiology studies were performed and showed a consistent contractile response from 2 to 8 weeks in static culture. This contributed to the choice of 5 days of cyclic strain after the initial 2 weeks of static culture. Upon completing the 5 days of strain or static culture, the TEVMs were cut into rings and contractility testing was performed on the segments. The contractility data is presented three ways (Figure 37), each of which gives slightly different information. First it is presented as the maximum recorded force. This is traditionally the way contractility data has been presented and is acceptable for comparing homogeneous samples. Nominal stress was calculated by dividing the measured force by the unloaded cross-sectional area. This allows for better comparisons across heterogeneous samples and gives us properties of the tissue. The force per cell is also calculated by dividing the force by the number of cells determined by the Hoechst DNA assay. This assessment shows differences in contractile forces that the cells are exerting, but differences in cell number and mechanical properties of the tissue can confound the results. The results of this study show that the application of cyclic strain resulted in a significant increase in contractile stress in response to endothelin-1 as well as an increased relaxation in response to papaverine. The response to bradykinin was not significantly different after application of strain. The improved response to papaverine may be due to the improvement in contraction since the relaxation force cannot exceed the load on the ring. The same trends are present regardless of analysis method; however, the statistical significance of the responses to endothelin-1 and papaverine are lost when normalized to cell number.

Effects of Cyclic Strain on Mechanical Properties

Uniaxial tensile testing was completed to assess the mechanical properties of the strained and statically cultured TEVM. The mechanical stimulation enhanced the strength of the engineered tissue in this study by more than 20%, without the large gel compaction

seen previously. The modulus of elasticity was also higher although not significantly (see Figure 38). The increase is due in part, but not entirely, to the thinner wall. The average forces are also higher suggesting that there are other factors involved. Representative stress-extension curves are shown in Figure 39. Extention is shown instead of strain as the strain measurements taken are only valid for the linear portion of the curve.

MMP Activity

Gelatin zymography showed little gelatinase activity other than that of MMP-2. All samples showed the presence of MMP-2. In Figure 40, the 72 kDa band corresponds to latent MMP-2 and the 68 kDa band corresponds to activated MMP-2. The homogenates contained both latent and active MMP-2 and the strained samples had more active MMP-2 than the static controls. The spent medium from the static controls contained more MMP which is surprising since the medium to construct volume is consistent between the two experimental groups. While there might be differences in latent MMP-2 in the spent medium, neither sample contained significant amounts of activated MMP-2.

TEVM Composition

Masson's trichrome staining was used to look at collagen content as well as overall general tissue composition. This staining demonstrated a more compact hydrogel layer and more circumferentially aligned cells in the strained TEVM, (see Figure 41). The cell layer in the strained TEVM had more collagen staining than the statically cultured TEVM. The increased collagen content in the strained TEVM was confirmed with the Sirius red assay (see Figure 42). Unlike previous studies, using this regimen DNA quantification showed no difference in the amount of DNA per dry weight (Figure 43).

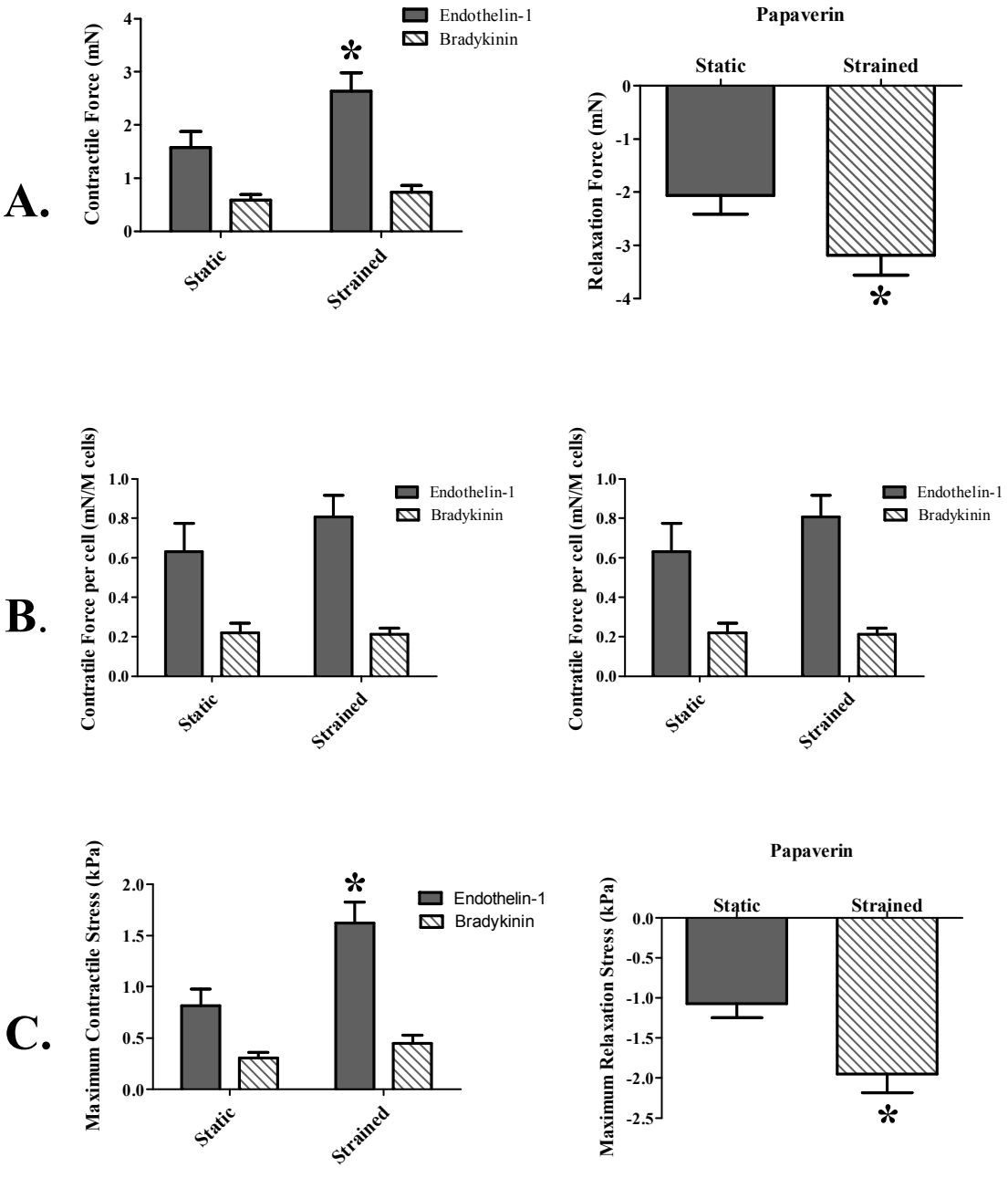

Figure 37: Contraction and Relaxation Data for Static and Strained Samples. The data is presented as maximum measured force (A), as well as the calculated values for force per cell (B), and nominal stress (C). The trends remain the same for all analysis methods, although the statistical significance is affected by the normalization to cell number. Strained samples have a greater contraction in response to endothelin-1 than the static controls. No significant differences in response to bradykinin are seen.

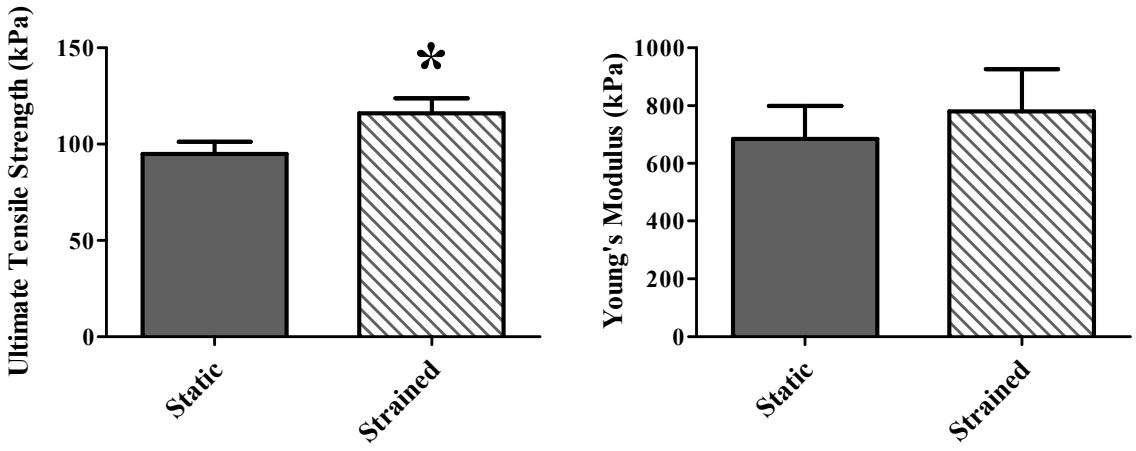

Figure 38: Mechanical Properties of TEVM Subjected to Cyclic Strain and the Static Controls. The strained TEVM are stronger than the static controls.

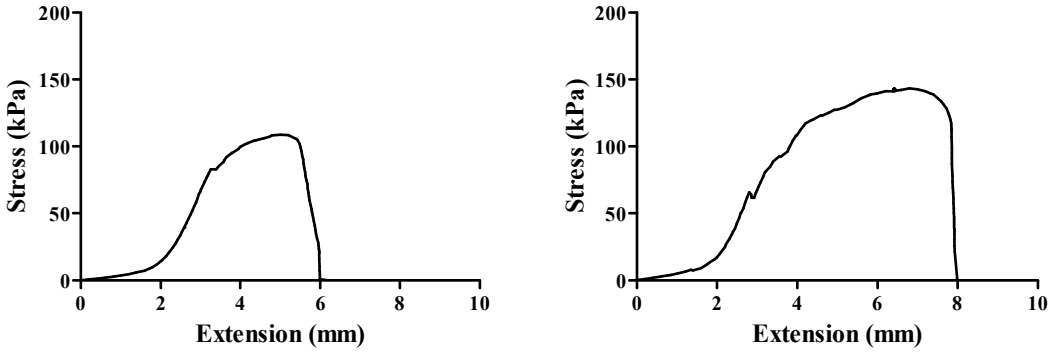

Figure 39: Representative Stress-Extension Curves for Static and Strained Samples. Static is shown on the left. Strained is shown on the right.

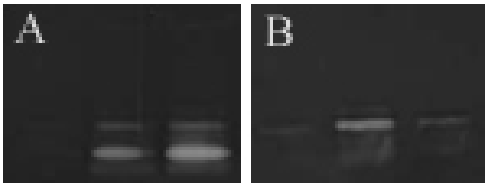

Figure 40: Zyomograms. Samples shown are for tissue homogenates (A) and spent medium (B). The samples run in each case are an acellular or medium only control (lane 1), statically cultured (lane 2), and cyclically strained (lane 3). There is more active and latent MMP-2 in the strained TEVMs.

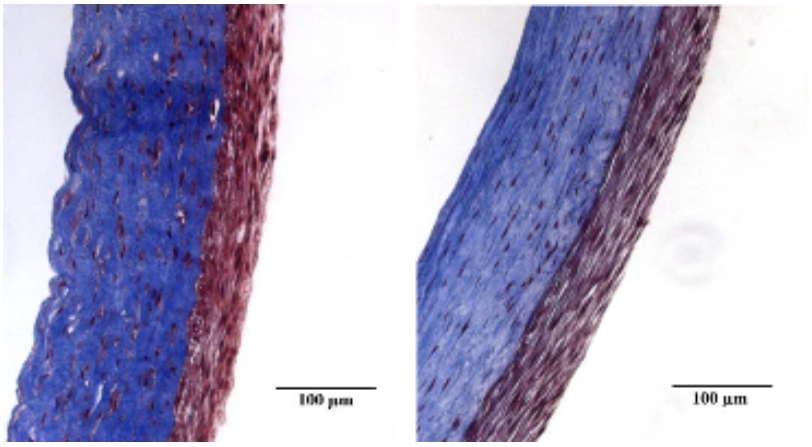

Figure 41: Masson's Trichrome. Statically cultured (Left) and cyclically strained (Right). Collagen is stained blue.

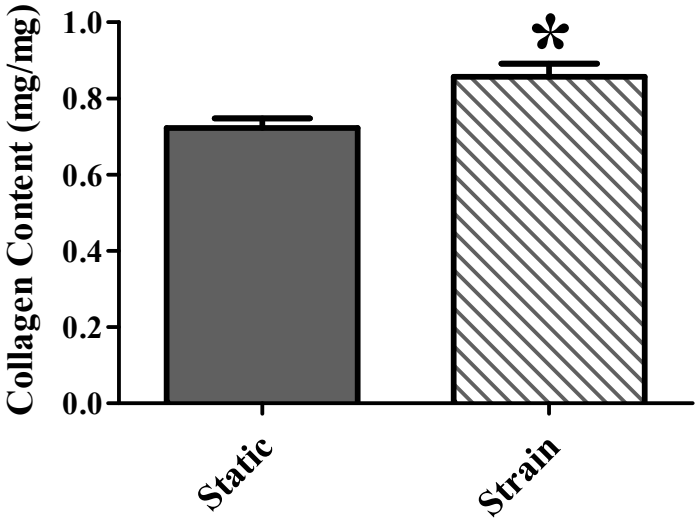

Figure 42: Collagen Content as Assessed by Sirius Red. Collagen content is greater in the strained TEVMs than in the static controls.

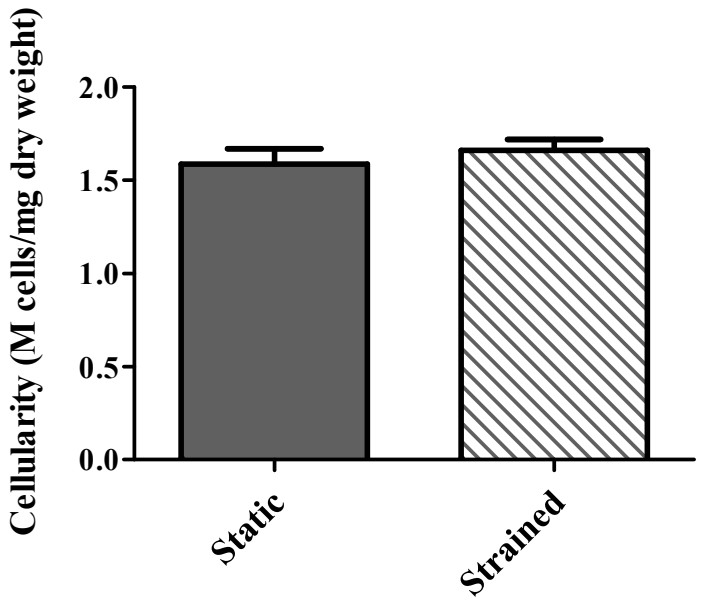

Figure 43: Cellularity of Static and Strained TEVMs. There are no significant differences in the cellularity of the static and strained TEVMs.

The TEVM were stained for smooth muscle α-actin. All of the TEVM had positively stained samples; however, the strained samples had more staining (see Figure 44), most of which was located in the cell capsule rather than in the encapsulated cells (see Figure 45).

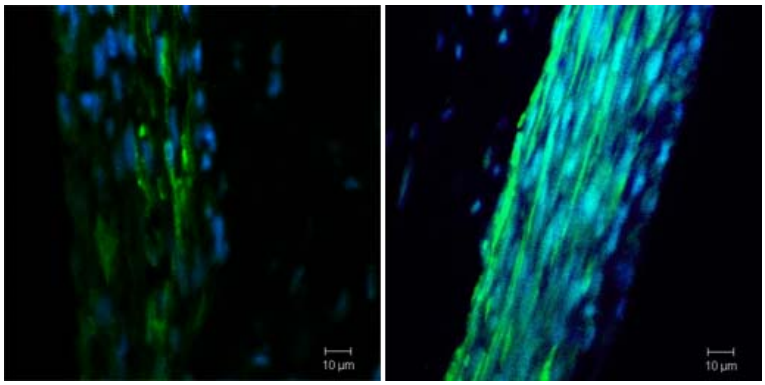

Figure 44: Alpha Actin Staining of Cell Capsule. Smooth muscle alpha actin is stained green; DNA is stained blue. There is more alpha actin in the strained TEVMS than the static controls.

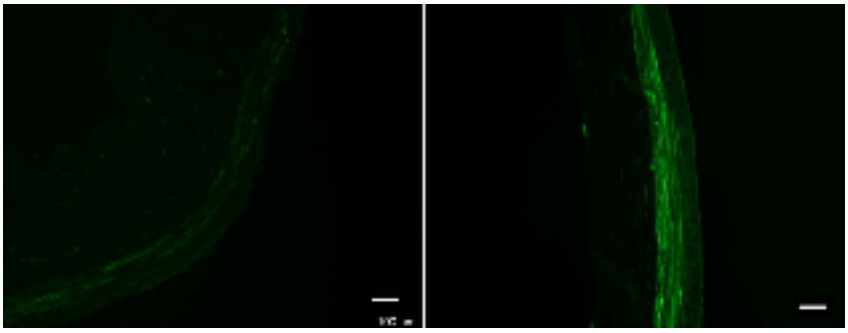

Figure 45: Alpha Actin Staining of TEVM. Static culture (left) has significantly less alpha actin than the strained TEVM (right). The majority of the staining is seen in the cell capsule, not in the encapsulated cells.

Discussion

One limitation of this study is the choice of cell type. While rat smooth muscle cells are easy to obtain and grow and are used often, they are very different from the human cells that would be required for an implantable graft. Rat cells continue to proliferate to a much higher passages than other cell types including human. TEVMs have been made with rat SMCs up to passage 30 [35]. Human cells produce more MMP, and additional collagenase types, than rat cells and can have different reactions to cyclic strain [36]. This can affect remodeling and may result in different optimal culture conditions.

The choice of the collagen hydrogel method for TEVM creation has inherent limitations. Smooth muscle cells will contract the collagen fibers of the hydrogel leading to a stronger, stiffer matrix. The blood vessel wall has a basal tone; the smooth muscle cells are never in a completely relaxed state. This nature of the smooth muscle cell requires that TEVM be culture on the glass mandrels or similar mold so that a basal tone can be established. The tubular geometry will be lost within days due to cells contracting the gel to try and maintain this partially contracted state. The collagen gels are very weak, porous and exhibit significant creep without cross-linking. In order to apply cyclic strain

to the hydrogels they must be attached to a non-porous sleeve for application of cyclic strain. Without adherence to an elastic sleeve, no significant strain would be possible due to rupture. Even if possible at a low strain, recovery of strain is unlikely during the cycles. Since an inner mandrel or sleeve is needed for both static and dynamic culture, the nutrients and oxygen are supplied on the outside of the TEVM and not at the lumen which would be more consistent with the *in vivo* condition where nutrients come from the lumen or *vasa vasorum*. The smooth muscle cells do not proliferate much inside the collagen gels, but will proliferate on the gels leading to a cell capsule starting after approximately 2 weeks in static culture, thus creating a non-homogeneous tissue composition.

Disk shaped constructs were used rather than tubular for initial gel compaction studies. The method used for making the gels requires that some of the starting material be cut away resulting in high variation in starting material. The cells also start contracting the gels early, even before the gels were removed from the molds, thus adding further error to the starting volume. The unconstrained disks do compact to a greater degree than that of the constrained tubes [16]. The duration of compaction was thought to be the same and the results agree with other published work [34]. Using this method the gels adhered better to the sleeves so that no longitudinal compaction could occur.

The strain profile was chosen based on previous work from our lab [5, 16]; however, to determine the effects of waiting until compaction ceased before applying strain, the strain rate and amplitude needed to be the same as used previously. The 10% strain amplitude is physiological, but very aggressive and only seen in the larger, more elastic vessels.

The only mechanical properties assessed were ultimate tensile strength and elastic modulus. While these parameters are important, these assessments are not sufficient. The tensile test is uniaxial and does not assess the mechanical properties in the longitudinal direction of the tissue which also influence burst pressures. Testing viscoelastic

properties such as creep is important; too much creep could lead to aneurysm. Fatigue testing may also be important to assure that the tissue can withstand the *in vivo* hemodynamics without failure.

Only two drugs were tested to look at the physiological contractile response. There are multiple receptors involved in the regulation of vascular tone which may or may not be affected by cyclic strain. The calculation of nominal stress is not ideal. For future studies the measurement of the cross-sectional area under load could be completed so that the actual stress could then be calculated. While not ideal, it is preferable to the other methods that have been tried such as force per dry weight.

An ideal tissue engineered vascular media would not only be able to withstand blood vessel hemodynamics, but would also be vasoactive. The application of cyclic strain is often used in TEVM culture to mimic *in vivo* conditions and help develop a tissue more suitable for implantation. Previous studies from our lab have shown that cyclic strain increases compaction of collagen hydrogels suggesting incomplete adhesion to the silicone sleeve used to confer strain [5, 16]. To overcome this issue, Isenberg et al. cultured collagen based TEVM statically until compaction ceased. This was followed by cross-linking by using glycation to assure there was no gel compaction [6]. The results from the first study with glycation were very encouraging, including elastin deposition in long term culture; however, the use of glycation increases necessary culture time and may result in undesirable effects due to the advanced glycation endproducts. Later Syedain et al. also delayed application of strain until after compaction ceased. In this instance fibrin scaffolds were used and no cross-linking was used [37].

While vasoactivity is a desirable trait in a TEVM, there is only one study that shows contractility using a collagen hydrogel model [38]. This model was used to study contraction so cells were used at low passage and at a very high cell to collagen concentration which would be difficult to maintain for the larger tissues needed for grafting. Studies have been performed to look at how mechanical stimulation affects

SMC phenotype, primarily by looking at smooth muscle α-actin. The change in phenotype has been associated with an increase in cell contraction. The measured contractile response of the tissue is more complex than the force from a single cell. It involves the integration of the cells and tissue; the cells have to work together to compact the tissue. Differences in tissue stiffness, cellularity and cell orientation may all affect the actual measured results as such a functional test is necessary to determine actual changes in the performance of the tissue.

Cyclic strain has been shown to increase the amount of smooth muscle α-actin, caldesmon and in some cases calponin and myosin heavy and light chains, all responsible for cellular contraction [13, 15-19]. The previous work from our lab found an increase in smooth muscle α-actin associated with strain, but only with the addition of TGF-β to the culture medium. The increase in α-actin expression was 1.7 times the unstrained control, similar to what is shown in this study where strain improves the contractile strength by two fold in response to endothelin-1. This study was able to demonstrate that strain does not only qualitatively improve α-actin expression, but quantitatively shows a functional improvement in TEVM with relatively high passage SMC. The α-actin staining was almost exclusively found in the cell dense capsule of the TEVM, with little to no staining of the cells encapsulated in the collagen hydrogel. The cell-cell contact or cell density may be important in generation of measurable force.

Cyclic strain has been shown to improve the strength of engineered tissue in multiple tissues. As expected the strained TEVM were significantly stronger than the statically cultured controls, although the increase was not as large in magnitude as other studies including those from our lab have shown. Seliktar et al. demonstrated a 57% increase in TEVM strength after 4 days of strain [5]. While cyclic strain had more of an effect previously, the TEVM in this study are approximately 10 times stronger than what was previously published. The differences in strength are due to the increased culture time (19 versus 6 days) and the different serum source. The diminished improvement in

strength by strain may be due to the reduction in compaction, the serum or the additional day in dynamic culture.

While some of the improvement to strength in this study is due to increased radial compaction of the collagen gel, the measured force was also higher. The increased collagen deposition in the strained TEVM also plays a part. This is consistent with some of the literature as the impact of cyclic strain on collagen production is mixed. Several studies show increased collagen after cyclic strain [11-14]. Hahn et al. noted the increase in collagen, but no difference in elastin deposition. This is counter to what Isenberg et al. saw with cross-linked collagen TEVM where increased elastin deposition but no difference in collagen content was observed [6]. All of the cited studies tested the tissue after long term cyclic distention of 5 weeks or more. The current study saw an increase in collagen content after only 5 days in culture. Long term culture may further improve the collagen content and strength. A recent study by Syedain et al looked at the effects of cyclic strain over time and found that porcine valvular interstitial cells (VIC) in a fibrin hydrogel led to greater collagen contents at higher strains or with incremental strain profiles [37]. Interestingly with these VIC/fibrin gels the static gels had greater collagen content after 1 week. By two weeks the strained samples contained more collagen; this difference increased with further culture. This suggests that collagen synthesis varies over time.

One result of this study is that an increase in MMP activity that is seen, much like what was seen previously with human smooth muscle cells [39-41]. This was not seen with rat cells in work done previously in our lab [5]. In studies with rat cells the ultimate tensile strength continued to improve with cyclic strain, a 265% increase compared to controls after 8 days of strain. In this study the cells behaved more like the human cells where strain resulted in activated MMP-2. Differences may be due to the differences in compaction or differences in serum. While the increased MMP activity is of concern due to the decreases in strength seen at least in the short term, MMP activity is essential for

remodeling. When non-specific MMP activity is inhibited, cyclic strain no longer resulted in improved mechanical properties [39]; however, without inhibition the collagen based TEVMs eventually decreased in strength due to the MMP activity [40]. Preliminary work showed that, with this strain regime, the strength of the strained TEVMs started to decrease by day 6. It is unknown whether longer culture times would result in continued degradation of the tissue mechanical properties or eventually result in improved remodeling and a superior tissue. It is possible that like Syedain et al. an initial decrease in strength and collagen content will be seen prior to increases in both. The differences in cell type and matrix material may affect the outcomes. As shown in Chapter 4 significant amounts of MMP-2 activity is seen in the collagen hydrogels while the fibrin hydrogels have little activated MMP.

This study has shown improvements in mechanical properties and physiological contractile response of the collagen based TEVM. While these improvements are significant, they are far from optimized. Studies have shown that different strain amplitudes and rates may be preferable for improved mechanical properties [37, 42-44] and collagen production [37, 43]. Syedain et al. found 10-15% strain led to improved strength and collagen as compared to static, with stepwise incremental strain increases having the best results. Conversely, Joshi and Webb [42] and Boerboom et al. [43] found that lower strain rates to be preferable for strength. Differences may very well be due to cell type, matrix type and the method for applying strain. Solan et al found that higher strain rates led to increased collagen deposition and increased levels of tissue inhibitor of metalloproteinases type 1 (TIMP-1) [44].

While improvements are seen, the strengths reported are significantly lower than that of native vessel and what would be required for *in vivo* testing. Further improvements may be made through longer culture times, biochemical stimulation such as platelet derived growth factor or transforming growth factor-β, or collagen cross-linking.

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CHAPTER 7: BIOCHEMICAL STIMULATION AFFECTS TEVM STRENGTH AND VASOACTIVITY

Introduction

Since the time of Weinberg and Bell [1], cell seeded hydrogels have been utilized for the tissue engineering of blood vessels. While this method has its advantages, the resulting tissue still lacks the strength required for *in vivo* testing and has a weak contractile response. Several groups have studied the effect of transforming growth factor β (TGF- β) alone or in combination with other biochemical stimulation on tissue strength, collagen synthesis and cell phenotype and reactivity. In a TEVM TGF-β has been shown to increase collagen production [2-4], increase strength [4, 5], increase elastin deposition [4, 6], and promote a more contractile cell phenotype [7]. The addition of TGF-β along with insulin was also shown to improve the contractile response of the TEVM [8].

TGF-β is a member of a superfamily of highly plieotropic polypeptides that includes the bone morphogenetic proteins, avidins and inhibins. Three mammalian forms of TGF-β exist, TGF-β_1, TGF-β_2, and TGF-β_3. This family of proteins has been found to regulate extracellular matrix production, cell proliferation, apoptosis, differentiation and migration. Vascular cells including endothelial cells and smooth muscle cells (SMC) express TGF-β_1. In monolayer culture, TGF-β_1 has been shown to influence SMC proliferation in a dose dependent manner and increase synthesis of elastin, fibrillin-1 and collagens I, III, V, VI and VIII [9-14]. It has also been shown to increase lysyl oxidase enzyme activity which cross-links collagen [15]. Cell phenotype has also been shown to be affected by TGF-β [16-18].

TGF-β is part of the Smad signaling pathway shown in part in Figure 46. It is synthesized in an inactive form where the latency-associated peptide (LAP) is cleaved

intracellularly to generate the mature polypeptide. The TGF-β domain will then bind to the cleaved LAP to form a dimeric complex called the small latent complex which can bind to other members of the latent TGF-β binding protein family to form an inactive larger latent complex. The latent TGF-β can be sequestered within the pericellular matrix to create a store for later use. The regulation and activation of TFG-β is quite complex. For a review of TFG-β signaling see Massagué and Chen and Massagué and Wotton [19, 20]. For a review of the role of TGF- β in the vascular system see Ghosh et al [21].

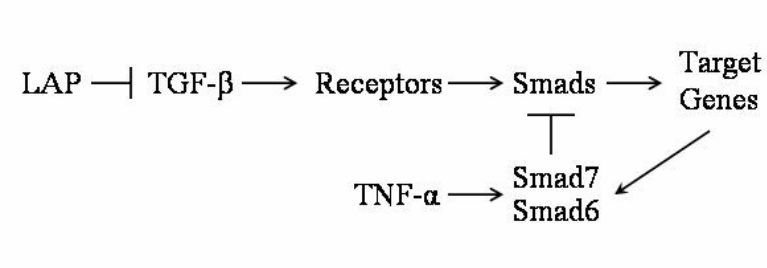

Figure 46: The TGF-Beta/Smad signaling Pathway. Adapted from Massagué and Chen [19].

Previous work from our lab has shown TGF- β to promote a more contractile cell phenotype in a collagen based TEVM [22]. As an extension of this work we are investigating its effect on tissue strength and on contractile response. Results presented in Chapter 5 suggest that there is a soluble factor that enhances the contractile response. To investigate whether TGF- β may play a role, the response to tumor necrosis factor α (TNF- α) was studied alone and in combination with TGF- β. TNF- α acts as an inhibitor in the Smad pathway and has been shown to promote a less contractile cell phenotype.

Materials and Methods

Tissue Engineered Vascular Media Fabrication

Tissue engineered vascular medias (TEVMs) were made as described previously.[7] Rat aortic smooth muscle cells from passage 6 to 9 were incorporated into

a type 1 collagen hydrogel for final concentrations of 1 million cells/ml and 2 mg/ml collagen. The collagen-cell solution was placed into the tubular mold and allowed to polymerize. The hydrogel was then removed from the mold and placed into culture in medium consisting of Dulbecco's Modified Eagle Medium (DMEM, Mediatech Cellgro), 10% bovine growth serum (Hyclone, Logan, UT), 1% L-glutamine and 1% penicillin–streptomycin (Gibco, Grand Island, NY). Medium was supplemented with 2 ng/ml TGF-β1 (Sigma), 10 ng/ml TNF-α (Sigma) or both; controls with no added growth factors were maintained. Culture medium was changed three times a week to assure that growth factor concentration remained high.

Contractility Testing

TEVM were cut into 2 mm long rings that were mounted on hooks in a physiological organ bath of 5 ml Krebs Henseleit solution with continuous of 95% O2, 5% CO2 at 37°C.[23-26] The segments were preloaded to 0.15 g; the rings were first stimulated with the nonspecific agonist KCl (120 mM). Responses to the vasoconstrictor endothelin-1 (10^{-7} M) or bradykinin (10^{-4} M) and followed by the antagonist papaverine (10^{-4} M) (Sigma, St. Louis, MO). Changes in tension due to chemical stimulation were measured using a digital data acquisition system (Gould Instrument Systems, Valley View, OH). The maximum change in force was recorded. Nominal stress was calculated by dividing the force by the cross-sectional area for the unloaded ring segment.

Tensile Testing

TEVM were cut into 5 mm rings; black beads of approximately 300 μm were affixed to one side of the rings in order to measure strain. The segments were mounted onto hooks in a phosphate buffered saline (PBS) bath on an Instron single column test system (Instron,). The rings were preloaded to 0.3 mN and precycled for five cycles to approximately 20% of the yield strain. The rings were then loaded to failure at a constant

strain rate of 0.2 mm/s. Matrox Inspector 8 was used to capture and analyze images taken during the test. Wall thickness was measured in order to determine cross-sectional area and calculate stress. Bead displacement was tracked so that local strain could be calculated. The recorded force and displacement data were then converted to stress and strain where the ultimate tensile strength, yield strength and elastic modulus could then be determined.

MMP Activity

MMP activity was assessed using gelatin zymography. Ring segments were rinsed in PBS, placed in RIPA buffer (Sigma) and homogenized using an ultrasonicator. Samples were stored at -80°C until use. Prior to protein quantification, samples were centrifuged at 12,000×g for 10 minutes. The protein content of the supernatant was determined using the modified Lowry method (Pierce) with albumin standards for comparison.

Gel electrophoresis was performed using Novex 10% Zymogram (gelatin) gels (Invitrogen). Equal amounts of protein in loading buffer were added to each lane. MMP 2/9 standards (Chemicon) were used for comparison; acellular hydrogels or fresh medium were used for controls. After electrophoresis the gel was placed in renaturing buffer (Invitrogen) followed by overnight incubation at 37°C in developing buffer (Invitrogen). The gel was stained with colloidal blue (Invitrogen) for 3-6 hours and then destained in dI H_2O. The gel was then imaged using the LiCor Odyssey imaging system and images were converted to gray scale.

DNA Quantification

Segments of the tissue were taken, rinsed in PBS and lyophilized with a Freezone freeze dry system (Labconco) overnight. The dry weights were recorded. The tissue was then digested by incubating with 0.5 mg/ml Proteinase K in phosphate buffer with EDTA

for 12-16 hours at 60°C or until no tissue was visible. The samples were loaded in triplicate into a 96 well plate along with DNA standards diluted from calf thymus DNA (Sigma) stock solution. The samples were then incubated for 15 minutes at room temperature with 0.1 μg/ml Hoechst 33258 dye in 10 mM Tris-HCl buffer with 1 mM EDTA and 0.2 M NaCl. The plate was then read on a flurometer using an excitation wavelength of 365 nm and emission wavelength of 458 nm. DNA quantity was determined by comparing to the standard curve. The results were normalized to dry weight to account for differences in amount of tissue.

Collagen Quantification

Segments were taken and were digested in Proteinase K as described previously for DNA quantification. Samples were loaded in triplicate in a 96 well plate along with collagen standards made from Type I bovine collagen (MP Biomedical). The samples were then incubated for 30 minutes in a solution of 0.8 mM direct Sirius red dye in 0.5 M acetic acid. The samples were then centrifuged at 10,000×g for 15 minutes; the supernatant was loaded into a 96 well plate in triplicate. The plate was then read on a spectrophotometer at 540 nm wavelength. The collagen content was determined by the standard curve and normalized to dry weight to account for differences in amount of tissue.

Histology and Immunohistochemistry

Samples were rinsed in PBS and placed into 4% formalin at room temperature. After twenty four hours the samples were rinsed in PBS and transferred to 70% alcohol at 4°C until processing. The samples were paraffin embedded; 7 μm sections were cut for histology and immunohistochemistry (IHC). Masson's trichrome staining was performed on deparaffinized sections as was IHC for smooth muscle α-actin.

Statistical Analysis

All graphs are shown as mean ± SEM. Statistical analysis was completed using a 95% confidence interval. Tests of two parameters were completed using a t-test. Tests of three or more parameters were completed using one way ANOVA; post hoc testing was completed using a t-test.

Results

Gel Compaction

The different biochemical treatments resulted in large visible differences in gel compaction, see Figure 47. The TEVM treated with TGF-β alone or in combination with TNF-α resulted in significant gel compaction. The majority of the visible compaction occurred within the first week of culture. Much of the compaction was longitudinal resulting in differences in wall thickness as well, see Figure 48.

Mechanical Properties

The mechanical properties of the TEVM were assessed using uniaxial tensile testing. The biochemical treatments had significant impact on the mechanical properties of the TEVM. Results are shown in Figure 49. Even with the thicker cross-sectional area, the treatment with TGF-β resulted in a 56% increase in strength compared to control. Treatment with TNF-α, which resulted in the thinnest wall, resulted in a 77% decrease in strength. Combining the two growth factors still improves strength, but not to the same extent as TGF-β alone. The modulus of elasticity follows the same general trend as the strength although only the TNF-α treated group was significantly different. Representative stress-extension curves for the treatments are shown in Figure 50.

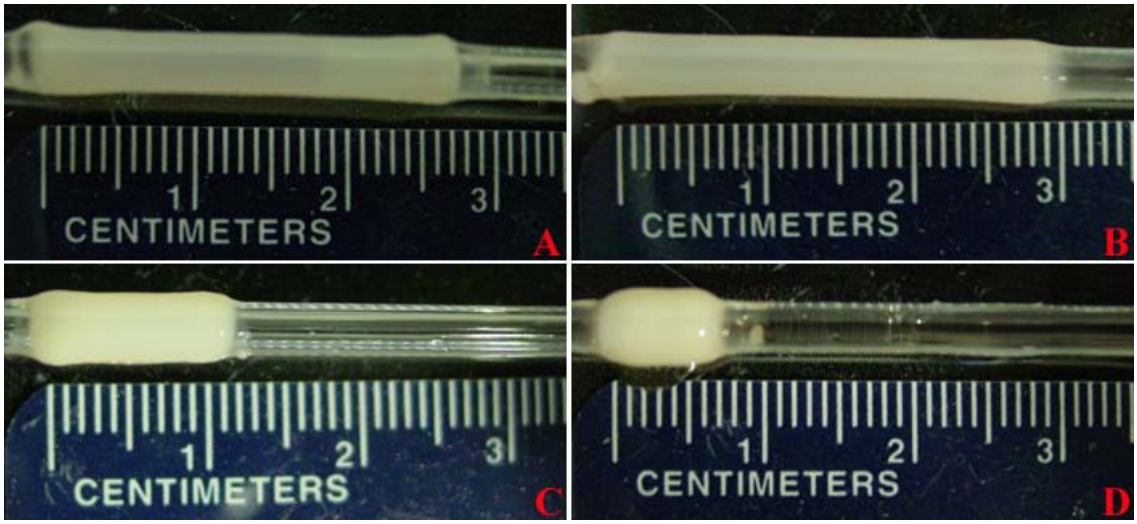

Figure 47: Compaction due to Biochemical Stimulation. TEVMs are shown after 3 weeks of culture with no additives (A), TNF-α (B), TGF-β (C), and the combination of TNF-α and TGF-β (D). The addition of TGF-β alone or in combination with TNF-α leads to increased longitudinal compaction.

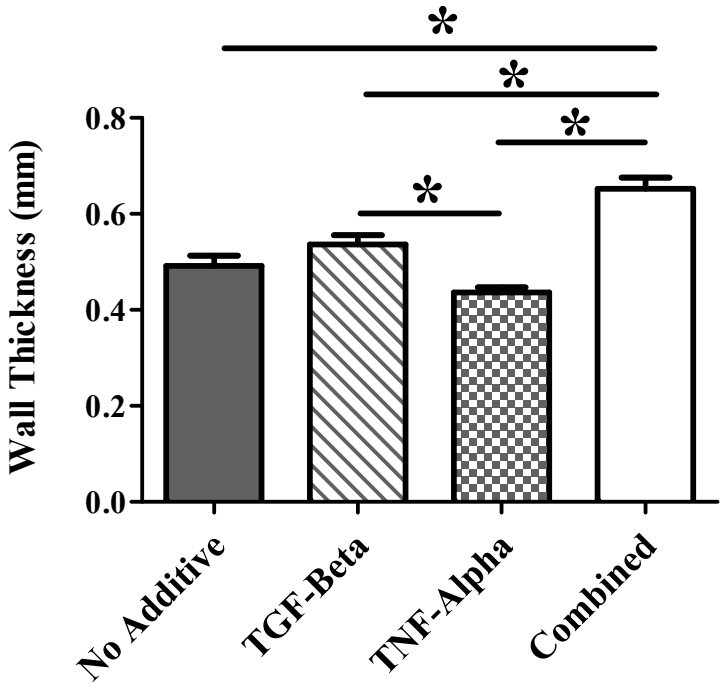

Figure 48: Wall Thickness of TEVMs Exposed to Different Biochemical Stimuli. * = p≤0.05 as depicted. The combined growth factor treatment resulted in a significantly thicker wall as compared to all other treatments. The difference in wall thickness between the TGF-β and TNF-α treated TEVMs was also significant.

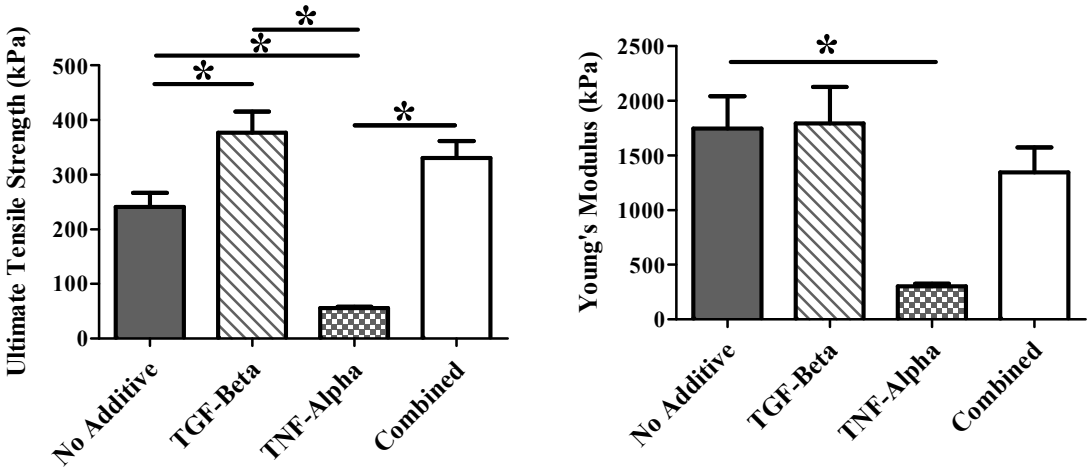

Figure 49: Mechanical Properties of TEVMs Subjected to Biochemical Treatment. * = p<0.05 as depicted. TEVMs treated with TGF-β alone had the greatest ultimate tensile strength. Those treated with TNF-α had the least strength.

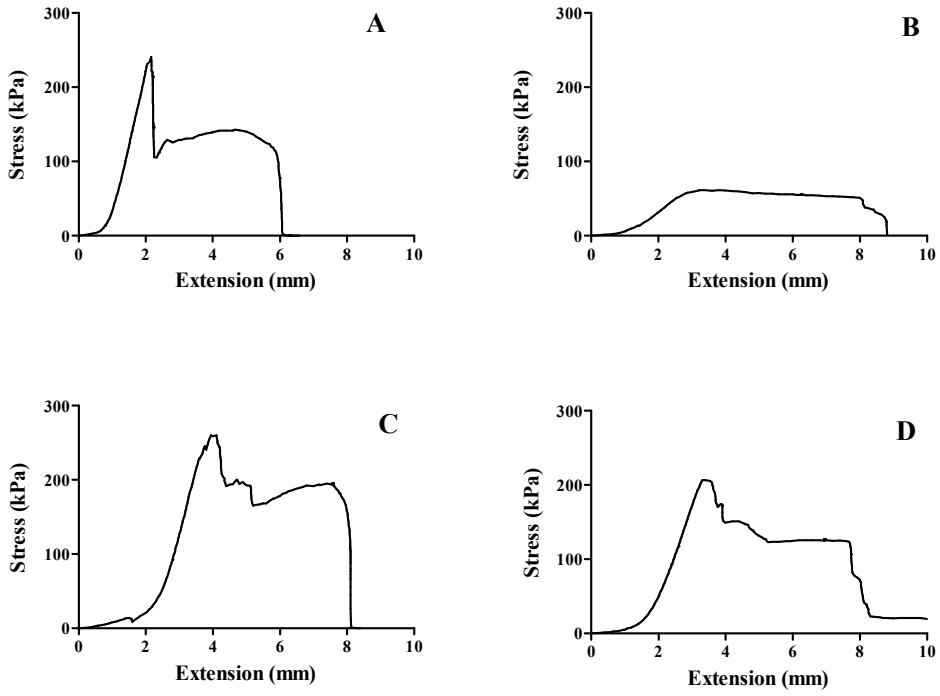

Figure 50: Representative Stress-Extension Curves. Shown are the untreated control (A), TNF-α treated (B), TGF-β treated (C) and the combined treatments of TNF-α and TGF-β.

Cell Phenotype/Contractile Protein Expression

TGF-β and TNF-α have been shown to influence smooth muscle cell phenotype. IHC was completed on ring sections to look at smooth muscle α-actin, myosin heavy chain and calponin expression. Only α-actin was detectable (see Figure 51); TEVM exposed to TGF-β expressed the highest levels of α-actin, followed by the control TEVM. TEVM given TNF-α alone or in combination with TGF-β did not contain significant amounts of α-actin.

Vasoactivity

All biochemical treatments resulted in a significant decrease in contractile force and stress in response to both vasoconstrictors, with no significant difference between the any of the treated groups (see Figure 52). When looking at contractile force per cell, the difference between TGF-β and TNF-α treatment also becomes significant. The fact that the TEVMs treated with TNF-α had a higher contractile response than those treated with TGF-β does not mean that the actual force exerted by the cells was stronger in the TNF-α treated TEVMs than in the TGF-β treated TEVMs. The force measured is that of the tissue, not of the cells. The cells have to compress the hydrogel layer in order for a contraction to be measured. In the case of the TGF-β treated TEVMs the thick, dense collagen layer requires more force from the cells to compact the tissue, resulting in a lower tissue contraction.

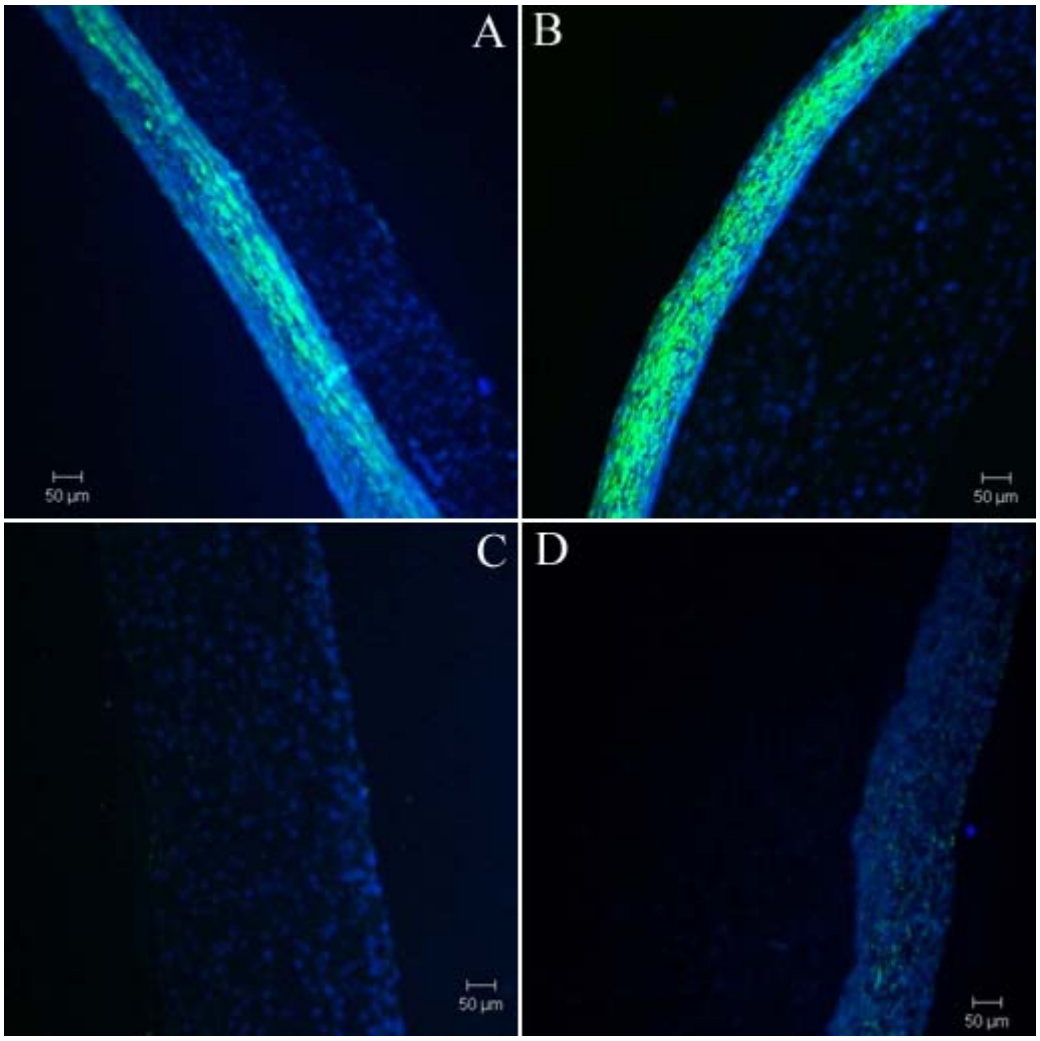

Figure 51: Alpha Actin Staining. Alpha actin is stained green, nuclei blue. The sections shown are of the non-treated control (A), TNF-α (B), TGF-β (C), and the combination of TNF-α and TGF-β (D.) The TEVMs treated with TGF-β stained the strongest for smooth muscle alpha actin. Those treated with TNF-α alone or in combination with TGF-β had very weak alpha actin staining.

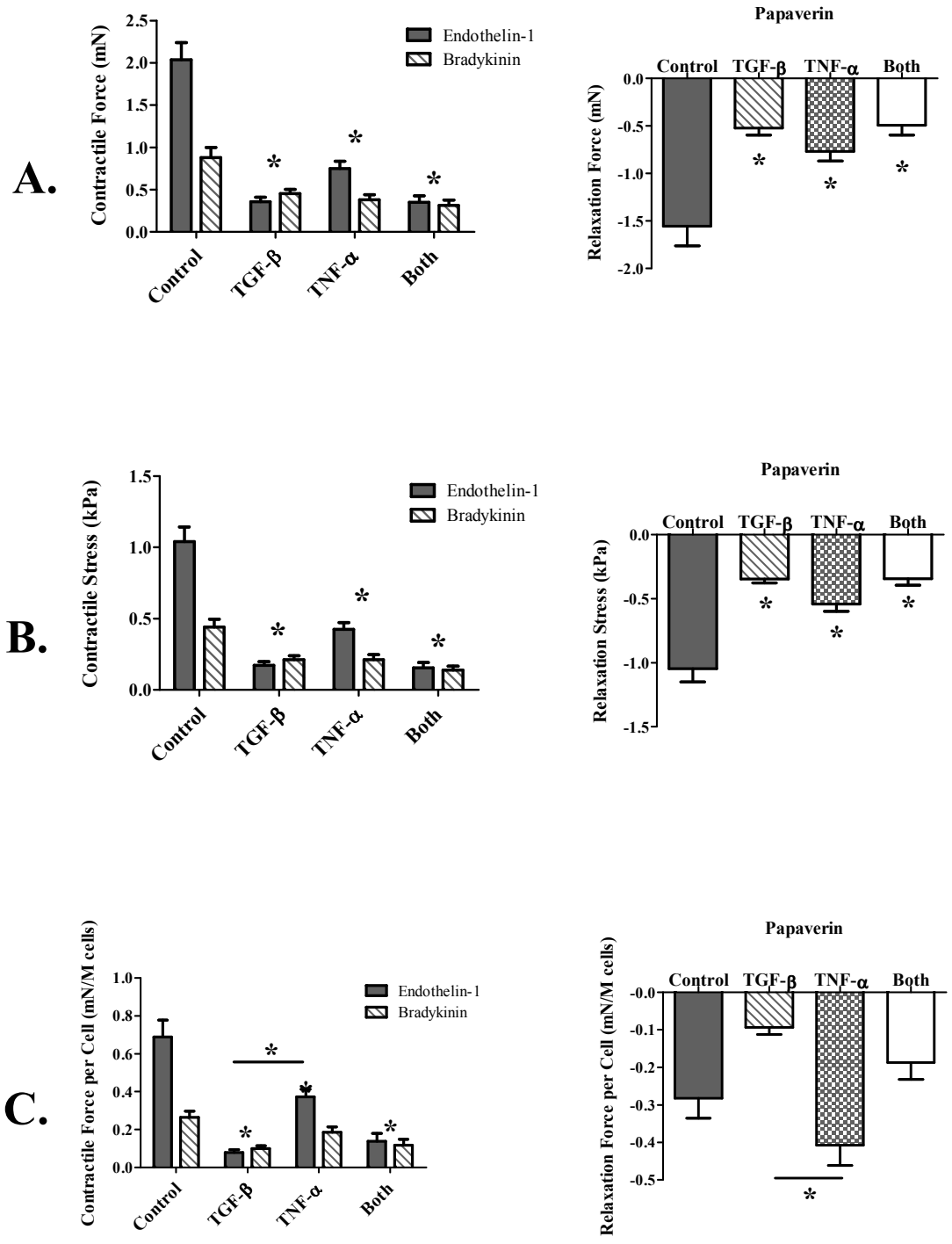

Figure 52: Vasoactive Response of TEVM Subjected to Biochemical Treatment. * = p≤0.05 as compared to control or as depicted. Data shown as measured force (A), and the calculated results for nominal stress (B), and force per cell (C). Trends generally remain the same; however, due to the differences in cellularity larger differences are seen and statistical significance is affected. All treatments resulted in a significant decrease in contractile strength as compared to the untreated controls.

MMP Activity

Gelatin zymography was performed to see qualitative differences in MMP activity, results are shown in Figure 53. While several MMPs have gelatinase activity, MMP-2 activity was significantly higher than other MMPs. Other bands could only be seen when MMP-2 was overloaded. Both the latent and active form of MMP-2 can be detected using gelatin zymography. The 72 kDa bands correspond to latent and 68 kDa bands correspond to active MMP-2. All homogenates contained latent and active MMP-2; the two groups that were exposed to TGF-β showed increased MMP-2 activity, with the combination of TGF-β and TNF-α resulting in the most activation. The MMP-2 activity levels correspond to the degree of hydrogel compaction with the combined treatment having significant MMP-2 activity and gel compaction. Spent medium samples did not contain significant amounts of activated MMP-2.

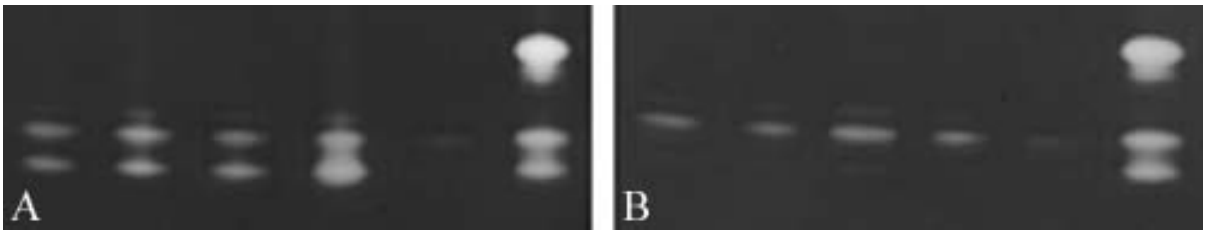

Figure 53: Gelatin Zymography for Biochemically Treated Homogenates and Spent Medium. The zymogram show samples from tissue homogenates (A), and spent medium (B). The samples run were no additive controls (lane 1), TGF-β treated (lane 2), TNF-α treated (lane 3), combined treatments (lane 4), acellular or fresh medium controls (lane 5), and MMP 2/9 standards (lane 6). The TEVMs treated with TGF-β alone or in combination with TNF-α had higher levels of MMP-2 activity.

TEVM Composition

There is a noticeable difference in the wall thickness and the collagen density in the hydrogel portion of the TEVMs. The TEVMs subjected to TGF-β alone or with TNF-α had thick dense collagen hydrogels, while the TNF- α treated TEVMs had a thinner wall with a thinner cell capsule layer, as shown in Figure 54.

There are differences in cellularity between the treatment groups. The combination of TGF-β and TNF-α results in the lowest cell number, which is significantly lower than the untreated control. The addition of TNF-α alone or in combination with TGF-β reduces the number of cells compared to TGF-β treated TEVMs. Results are shown in Figure 55.

Sirius red collagen assay shows no difference in the collagen content per dry weight of any of the TEVMs (see Figure 56).

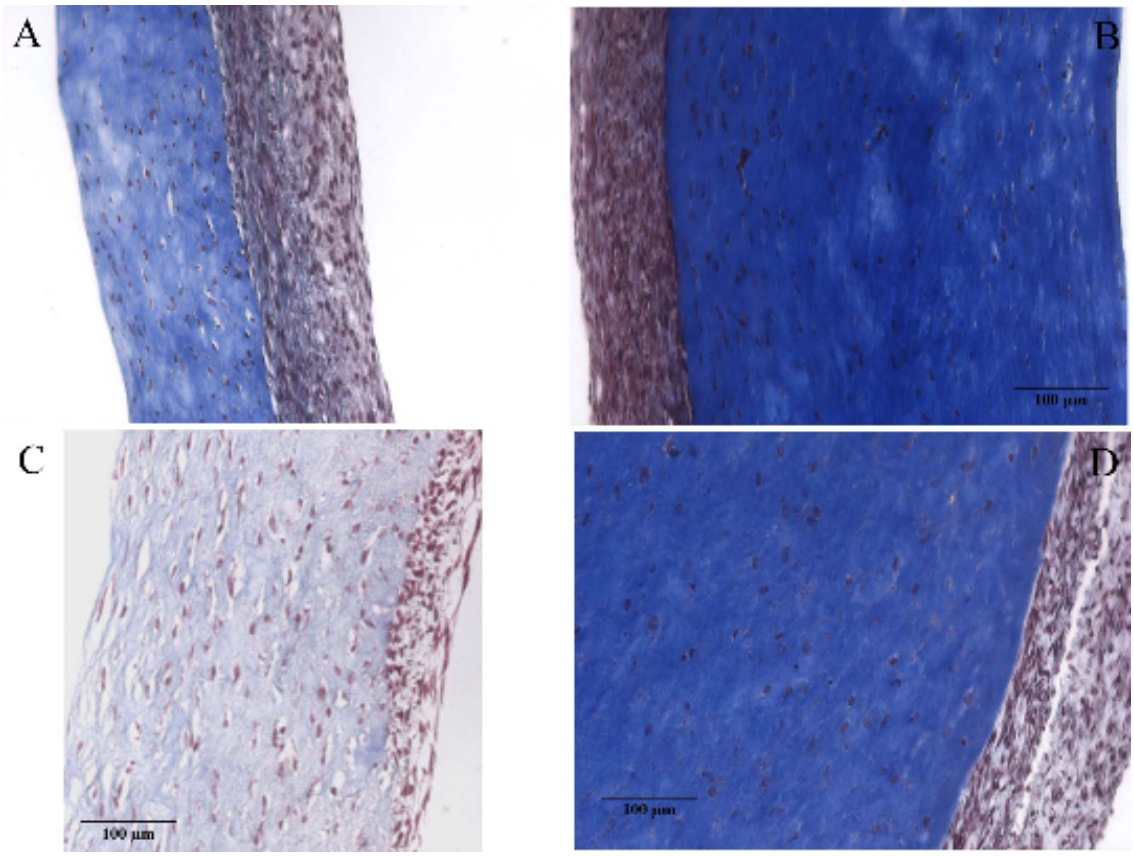

Figure 54: Masson's Trichrome Staining of Biochemically Treated TEVM. Collagen is stained blue. No additive control (A), TGF-β treated (B), TNF-α treated (C), and combined treatment (D) samples are shown. The wall thicknesses and collagen densities vary greatly between samples.

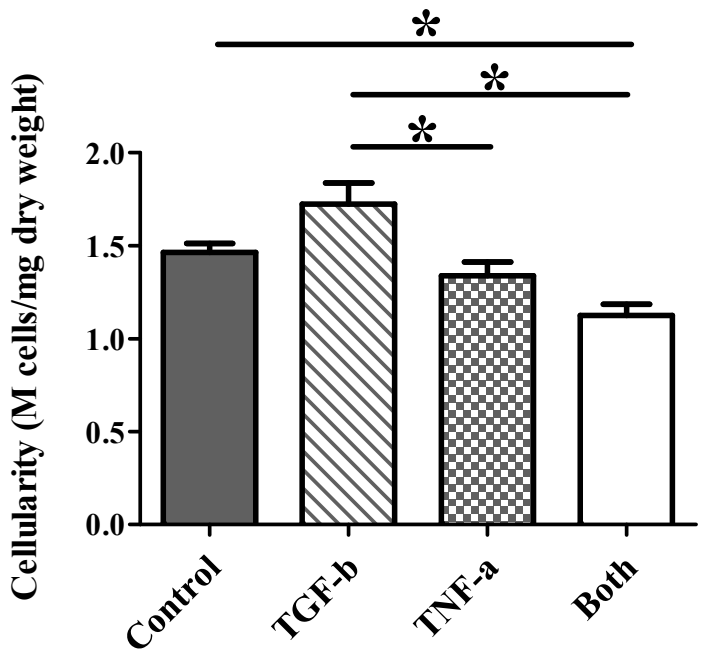

Figure 55: DNA Content for TEVM Subjected to Biochemical Treatment. * = p≤0.05 as depicted. The combined growth factor treatment resulted in the lowest number of cells. TGF-β treated TEVM contained the highest number of cells.

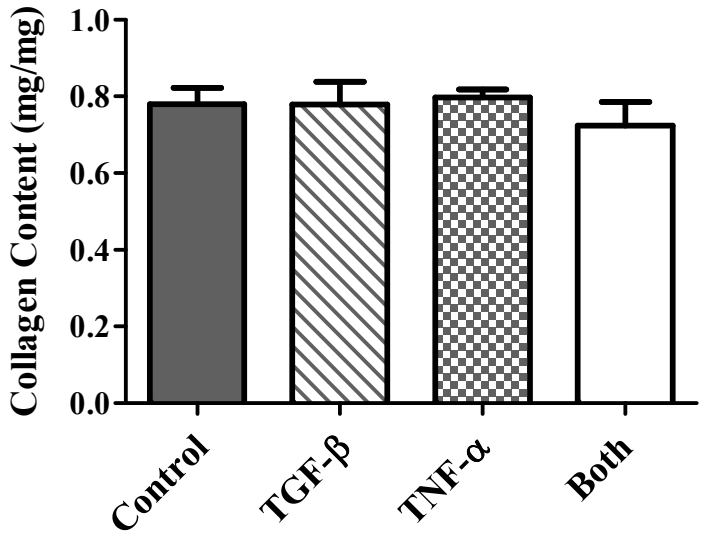

Figure 56: Collagen Content of Biochemically Treated TEVM. No significant differences were seen after 3 weeks of static culture.

Discussion

This study only looked at one concentration of TGF-β and TNF-α. There may be different levels that result in better mechanical and contractile responses. The culture medium was changed 3 times per week. This was done so as not to deplete the growth factors; however a more consistent time frame such as every 2 to 3 days may have made a difference in the results.

By looking at both TGF-β and TNF-α, it is possible to get an idea of whether these factors may play a part in TEVM mechanical and contractile properties. TGF-β does have a biphasic response so an increase in strength or contraction may not be seen by adding additional TGF-β to the culture medium. To better understand if TGF-β plays a role in contraction it should have been added to the culture medium where no contractile function was seen would have given additional information. In both cases where TGF-β was added to the culture medium there was increased gel compaction. When combined with TNF-α, the TEVM compaction was so significant that it was difficult to cut into ring segments and only one or two per construct could be tested. Total collagen content was assessed using the Sirius Red assay. Newly synthesized collagen may not be detectable amidst the large amount of collagen present in the TEVM.

The addition of TGF-β and TNF-α had significant effects on the TEVMs. Most noticeable was the increased compaction, most of which occurred in the first week. In both of the culture conditions where TGF-β was added to the culture medium there was a significant increase in compaction, much of this in the longitudinal direction. The combination of TGF-β and TNF-α resulted in a dramatic decrease in TEVM length. When taken in context with the wall thickness data, it is difficult to determine if the overall volume changed or if only the direction of compaction was altered. A previous study in our lab did not find differences in volume associated with the addition of TGF-β; however, the TEVM were adhered to silicone sleeves and the total culture time was only

6 days [7]. Neidert et al also did not notice differences in compaction with TGF-β. In this case the compaction was determined by measuring the height of the hemisphere shaped gels that were adhered to the bottom of a dish [27]. It is also possible that differences in serum or collagen lot could contribute to the changes. While none of these studies saw differences in compaction, it has been shown that TGF-β can influence hydrogel compaction [28, 29].

As expected, TGF-β increased the strength of the tissue and TNF-α reduced the strength. The addition of TGF-β resulted in more than a 1.5 fold increase in strength after 3 weeks of culture. This is compared to a 12 fold increase that was reported by Grassl et al [5]. While the magnitude wasn't the same, the Grassl work used neonatal smooth muscle cells at four times the concentration. Neonatal SMC have been shown to produce more collagen than adult cells which would impact the results [30].

The improvements in strength are almost exclusively due to the increased compaction. There were no differences in collagen deposition seen, this may be real or this may not be detectable differences using the Sirius red assay within this culture time. While TGF-β is known to regulate ECM synthesis, it has not always been shown to improve collagen synthesis in a TEVM. Grassl et al showed that TGF-β and insulin resulted in increased collagen synthesis, whereas the addition of TGF-β alone gave variable results [5]. It may also be that a different concentration is required.

The addition of TGF-β and TNF-α impacted the level of MMP-2 activity. The TEVMs treated with TGF-β alone or in combination with TNF-α resulted in an increase in MMP-2 activity. This is consistent with what was seen by Ross et al. where the addition of TGF-β resulted in increased MMP-2 expression. This may also have an effect on the TEVM strength. It has been shown that an increase in MMP-2 activity can negatively impact the strength of a collagen-based TEVM. The increase in MMP-2 activity resulted in a reduction in strength within 8 days [31]. In this study the TEVM were cultured for 3 weeks. Conversely in Ross et al the increase in MMP-2 and MMP-1

expression did not result in any loss of strength in the fibrin based TEVMs. As seen in Chapter 6, the MMP-2 activity also correlates with the degree of compaction. The addition of TNF-α resulted in the highest level of MMP-2 activity and the most longitudinal compaction.

The addition of TNF-α alone or in combination with TGF-β resulted in a drastic reduction in α-actin expression as compared to untreated controls, consistent with the literature. The addition of TGF-β resulted in increased smooth muscle α-actin expression as compared to the control as seen with immunofluorescence staining. This is consistent with previous work that showed that TGF-β increased α-actin expression by approximately 25%. Even with the increased α-actin expression, the actual contractile response of all treatments was reduced by more than 65% to almost undetectable levels. The contraction of the TNF-α was actually greater than that of TGF-β. While the measured force was reduced, that does not mean that the force generated by each cell was actually reduced. The TGF-β treated TEVMs were much more compact with a thicker wall and stronger matrix. This would mean that the cells would need to generate more force to move the tissue.

The collagen compaction that occurred due to the addition of the biochemical agents was significant and impacted the strength and contractile function. While some compaction is desired in order to increase collagen density and improve TEVM strength, too much longitudinal compaction is not ideal. A graft must be of a certain length and must be reproducible, and longitudinal compaction is highly variable. As seen with the cyclic strain work in Chapter 6, the cessation of longitudinal compaction does not necessarily result in reduction in strength. By controlling this length, the effects of TGF-β on cell phenotype may result in a measurable improvement in contractile response. To control the length, the TEVMs could be cultured on a silicone sleeve as done previously, or the growth factors could be added after compaction ceases. The combination of insulin

and TGF-β could be further studied as it has been shown to improve the strength, the collagen production, and the vasoactive response of fibrin based TEVM [8, 27].

The results seen here suggest that TGF-β is not the factor in the culture medium that resulted in measurable TEVM contraction. Finding that factor, or factors, is of interest to the tissue engineering community. This could be achieved by using a proteomics approach comparing the serum that result in TEVM with and without a measurable vasoactive response.

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CHAPTER 8: EFFECTS OF CRYOPRESERVATION ON VASOACTIVITY

Introduction

Tissue engineering of small diameter blood vessels has long been considered the 'Holy Grail' of tissue engineering due to the need for bypass grafting that will remain patent. An ideal tissue engineered blood vessel will be non-thrombogenic, non-immunogenic, possess sufficient mechanical properties to withstand blood pressure and be vasoactive. To be commercially viable, the graft material must have 'off-the-shelf' availability as most patients cannot wait the weeks or months that it takes to create tissue engineered substitutes. Cryopreservation could provide a solution for this need.

The two methods of cryopreservation available are freezing and vitrification. Freezing of cells or tissue is done using a slow cooling method, often in the presence of cryoprotectants. With freezing preservation is assisted by ice formation as the cryoprotectant concentration is gradually increased as ice crystals form. Even with controlled-rate freezing, ice damage can occur causing both cellular and extracellular matrix damage [1, 2]. Vitrification is an 'ice-free' preservation method where fast cooling rates and cryoprotectants are chosen to prevent ice formation and to transition the liquid into a glass state. With vitrification higher cryoprotectant concentrations are needed which can be deleterious to the cells and thus these need to be chosen carefully [3].

For cryopreservation to be considered successful, the preserved tissue would need to be viable and maintain the properties of the fresh tissue. For a blood vessel the mechanical properties and contractile function are two important properties that should be maintained. While results vary due to differences in cryopreservation methods, studies show that most cryopreservation methods do not negatively impact the mechanical properties of native tissue [3, 4]. Tissue engineered blood vessels behave similarly and

some even gain strength after rewarming [4-6]. Studies of vasoactive function after cryopreservation of native vessels have shown that freezing results in diminished function, but that function is maintained with vitrification [1, 7]. Only one study has been done with engineered tissue. With both methods the response was diminished as compared to fresh tissue. Vitrification was far superior to freezing and retained significantly more vasoactive function; vitrification also resulted in higher cell viability then freezing [4]. The difference in functional response was much more dramatic then the difference in viability suggesting that vasoactive response is a more stringent assessment of cryopreservation success in a tissue engineered blood vessel.

Previously our lab has studied the effects of cryopreservation on mechanical properties and the viability of a tissue engineered vascular media [5]. This study looks at the effects of freezing and vitrification on the vasoactivity of the tissue.

Materials and Methods

Tissue Engineered Vascular Media Fabrication

Tissue engineered vascular medias (TEVMs) were made as described previously [8]. Rat aortic smooth muscle cells from passage 6 to 9 were incorporated into a type 1 collagen hydrogel for final concentrations of 1 million cells/ml and 2 mg/ml collagen. The collagen-cell solution was placed into the tubular mold and allowed to polymerize. An etched collagen I coated sleeve was used in the mold for constructs to be subjected to cyclic strain as well as to the static control counterparts. The hydrogel was then removed from the mold and placed into culture in medium consisting of Dulbecco's Modified Eagle Medium (DMEM, Mediatech Cellgro), 10% bovine growth serum (Hyclone), 1% L-glutamine and 1% penicillin –streptomycin (Gibco, Grand Island, NY). Culture medium was changed weekly.

Cyclic Strain

Cyclic strain was applied to the collagen TEVMs using a system previously developed in our lab [8]. The TEVM were cultured for two weeks prior to being subjected to cyclic strain to allow for better adherence of the tissue to the silicone sleeve. The tubular constructs on the silicone sleeves were mounted on hollow mandrels in the strain bioreactor containing 300 ml of culture medium. The lumen and reservoir were filled with culture medium. Two 0.2 μm filters were placed in the vents in the lid to allow for oxygen exchange. The bioreactor was placed in a CO_2 incubator at 37°C, and connected to a filtered regulated air supply. The pneumatic pressure was controlled in order to produce 10% circumferential strain at a rate of 1 Hz. TEVMs were strained for 5 days. Statically cultured controls were created for comparison. At the conclusion of testing, the TEVMs were gently removed from the silicon sleeves prior to testing.

Contractility Testing

TEVMs were cut into 2 mm long rings that were mounted on hooks in a physiological organ bath of 5 ml Krebs Henseleit solution with continuous perfusion of 95% O2, 5% CO2 at 37°C.[9, 10] The segments were preloaded to 0.15 g, and the rings were first stimulated with the nonspecific agonist KCl (120 mM). Responses to the vasoconstrictor endothelin-1 (10^{-8},10^{-7} M) or bradykinin (10^{-5},10^{-4} M) were measured. Each agonist was followed by the antagonist papaverine (10^{-4} M) (Sigma, St. Louis, MO). Changes in tension due to chemical stimulation were measured using a digital data acquisition system (Gould Instrument Systems, Valley View, OH). The maximum change in force was recorded. Nominal stress was calculated by dividing the force by the cross-sectional area for the unloaded ring segment.

Controlled-Rate Freezing

Freezing of the TEVM rings was done using a commercial procedure.[9] The ring segments were placed into Hepes-Buffered DMEM with 1M dimethyl sulfoxide (DMSO), 2.5% chondroitin sulfate and 10% fetal calf serum at 4C. The segments were then placed vials and cooled to -80°C at 1°C/min. The vials were then transferred to liquid nitrogen and stored for a minimum of 12 hours.

To thaw, the vials were placed in a 37°C waterbath until ice was no longer visible. DMSO was then eluted by placing the samples in DMEM containing mannitol at decreasing concentrations of 0.5 M, 0.25 M and 0 M.

Vitrification

TEVM rings were vitrified using VS55 vitrification solution containing 2.2 M propylene glycol, 3.1 M formamide and 3.1 M DMSO [1, 11]. Precooled (4°C) VS55 was introduced to the samples in 6 steps of 15 minutes each, gradually increasing the VS55 concentration. At the final concentration of 55%, isopentane was added to the air-liquid interface to prevent condensation. The samples were cooled rapidly (43°C/min) to -100°C followed by slow cooling (3°C/min) to -135°C. The TEVMs were stored at 135°C for a minimum of 12 hours.

Rewarming was completed in two stages. First the samples were slowly rewarmed (30°C/min) to -100°C using convection. This was followed by rapid rewarming (225°C/min) to 4°C. The VS55 concentration was decreased to 0 M in seven sequential steps.

Statistical Analysis

All graphs are shown as mean ± SEM. Statistical analysis was assessed using a 95% confidence interval. Tests of two parameters were assessed using a Student's t-test.

Tests of three or more parameters were assessed using one way ANOVA with a post hoc t-test.

Results

Vasoactivity After Cryopreservation

TEVMs were cultured statically for two weeks followed by 5 days of 10% cyclic strain at 1 Hz. The TEVMs were cut into 2 mm ring segments. At least one ring from each tube was either cryopreserved by vitrification, cryopreserved by freezing, or not preserved ('fresh'). Cryopreserved samples were rewarmed after a minimum of 12 hours of storage. After rewarming, the specimen rings underwent physiological testing for contractile function. The contractile and relaxation data is being shown both as measured force and nominal stress (see Figure 57). Both freezing and vitrification had a negative effect on contractile response to endothelin-1; the contraction of the frozen samples was 14-20% of the fresh samples, while the contraction of the vitrified samples was 7-17% of fresh. The response to bradykinin was not as affected by the preservation, especially in the frozen samples. Freezing resulted in 42-43% functional response while vitrification resulted in 20-27%. The contractile response to bradykinin may appear to be less affected by freezing than endothelin-1 since the response in fresh tissue is more than half of that to endothelin-1. While there appears to be subtle trends, there is no significant difference in function between vitrified and frozen TEVMs. Application of strain had no effect on the function of the cryopreserved specimen. The statically cultured TEVMs retained 17-20% of endothelin-1 function and 27-43% of bradykinin function, while the strained samples retained 7-14% of endothelin-1 function and 20-42% of bradykinin function. The percentages are misleading here as well since the fresh dynamic samples produced twice the force as the statically cultured rings. Neither freezing nor vitrification was able to preserve any substantial function for this tissue using the current protocols.

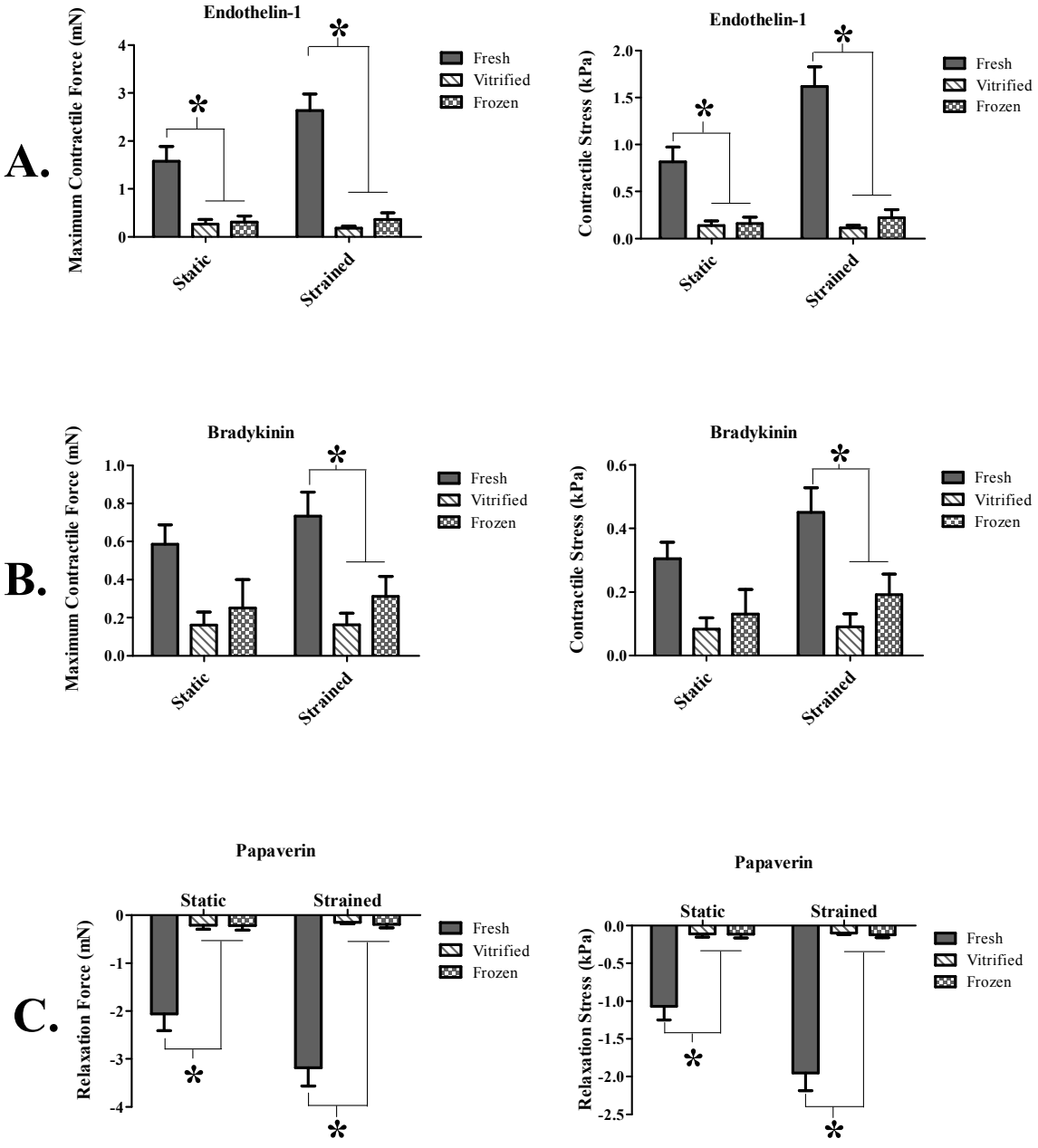

Figure 57: Contractility of Fresh and Cryopreserved TEVMs. Data is presented as measured force (left) or calculated nominal stress (right). Results are shown for the agonists endothelin-1 (A) and bradykinin (B) and the antagonist papaverine (C). Both cryopreservation methods resulted in significant decreases in contractile force. No differences were found between freezing and vitrification or between static and strained TEVMs.

Vasoactivity After Modified Vitrification Protocol

To try to improve function after vitrification, the rewarming temperature was reduced. For this study all TEVM were cultured statically for 3 weeks prior to testing and the silicone sleeve was not used, resulting in a slightly thicker tissue. TEVMs were cut into 2 mm rings with at least one ring from each tubular construct going into each test group. Rings were either tested fresh or vitrified. Vitrified rings were rewarmed at either -10°C or 4°C and tested for contractile function. The decreased rewarming temperature had no effect on the contractile response after cryopreservation. Both methods resulted in significant deterioration of the contractile response as shown in Figure 58. No relaxation data is shown since it was not tested on more than 75% of the vitrified samples due to a lack of response to the agonist. The bradykinin data for the ring segments rewarmed at 4°C may be skewed slightly as one batch was not tested due to poor performance. The nominal stress presented is lower than in the cyclic strain study. No sleeve was used for this study, changing the thickness of the tube which can affect remodeling and growth as well as stress due to the increased wall thickness.

Discussion

The one freezing method was used may not be optimized for this tissue. Since vitrification has been shown to be superior for many applications including other tissue engineered blood vessels modifications to the freezing protocol were not undertaken. The vitrification method is certainly not optimized. Only one modification to the technique was investigated: the final temperature in the two step rewarming procedure. There are many variables to investigate in both the cooling and rewarming stages as it is unknown when the damage occurs. The vitrification cocktail as well as cooling and rewarming rates and temperatures can be investigated.

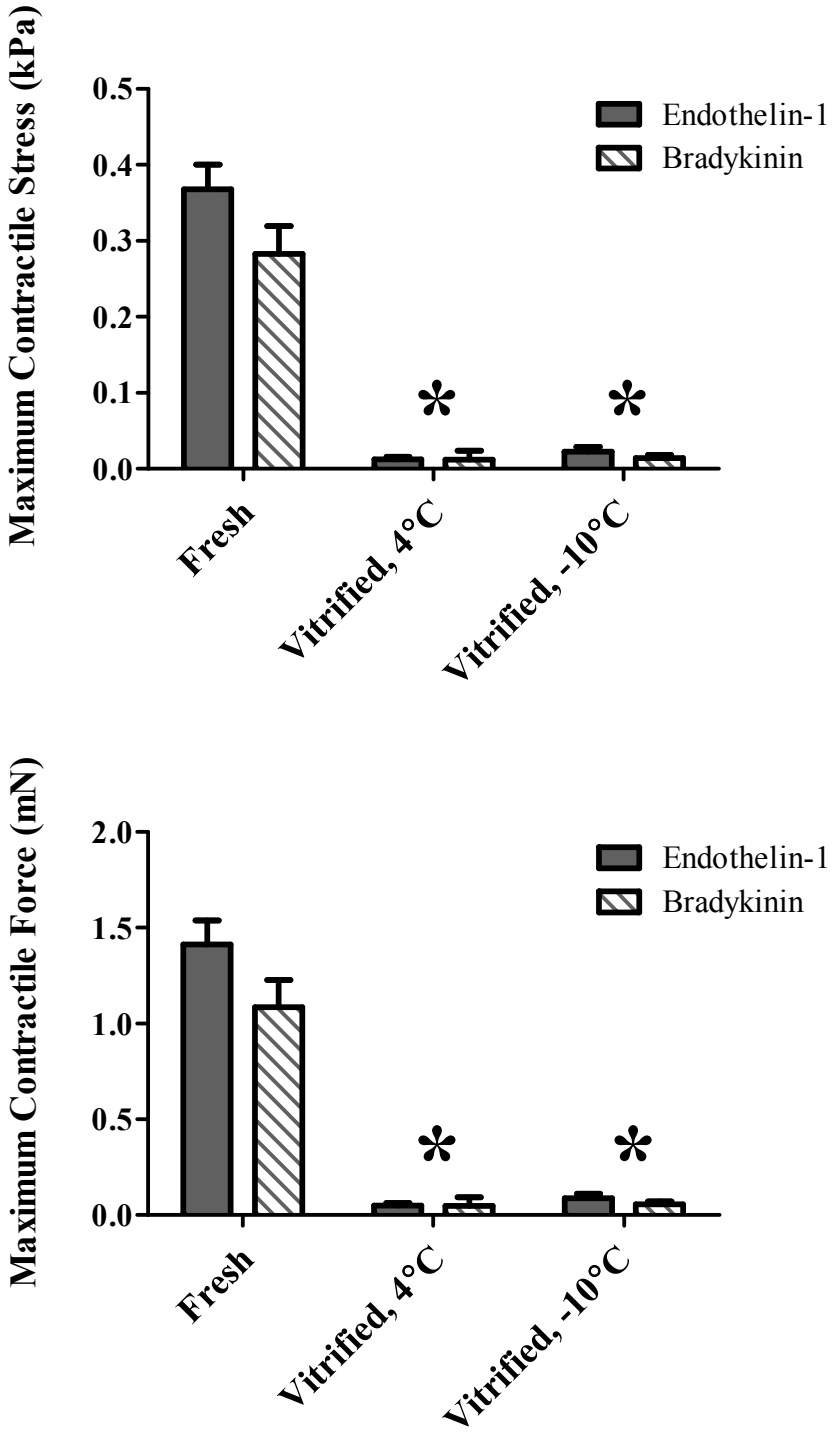

Figure 58: Contractility of Ring Segments Before and After Vitrification. Data presented as measured force (top) and calculated nominal stress (bottom). No improvements were seen with the modified protocol.

The contractile response was the only assessment used on the majority of the samples. Early testing showed that viability was lower than with other TEVMs from our lab and others using the same protocols. It is unknown whether viability was improved with the change in the rewarming protocol; the decrease in contractile response may be related to the decreased viability, damage to cell-cell junctions, damage to the receptors, or to the combination of these factors.

For tissue engineered blood vessels to be commercially viable, they would need to have 'off the shelf' availability; cryopreservation may fill that need. In order for cryopreservation to be considered successful, the preserved tissue must remain viable, maintain function and mechanical integrity. Assessment of these requirements after cryopreservation has been undertaken by a small number of groups; in most cases vitrification results performs better than conventional cryopreservation (freezing). A summary of this data is found in Table 8. For a review of vitrification of engineered tissue see Kulkeshova et al [3]. Previously our lab showed vitrification to have comparable viability to freezing of a collagen-elastin hybrid TEVM [5]. Viability can be greatly influenced by the method used. Neidert et al. showed viability of approximately 40% to >90% by varying the cryoprotectant choice and concentration as well as the end temperature [6]. Baicu et al. has shown a 10% improvement in viability after vitrification just by varying the rewarming rate [10]. The ultimate tensile strength of the tissue engineered blood vessels has been assessed before and after cryopreservation. Cryopreservation by all but the unoptimized freezing protocols not only maintained the mechanical properties but often enhanced the strength [4-6]. These results are consistent with results for native tissue. Thakrar et al. showed no difference in the viscoelastic properties of human vascular grafts after vitrification [11]. Venkatasubramanian et al. shows that freezing affects the stress-strain relationship, especially in the toe region of the curve [12]. A study with a valve leaflet showed no significant difference in maximum stress, but the valves did exhibit a different modulus [13].

Table 8: Assessments of TEVM and native vessels after Cryopreservation. An * indicates native tissue. • indicates work done in our lab. Ranges are due to multiple methods used or multiple contractile agents.

	Viability		Strength		Contractile Response	
Author	**Frozen**	**Vitrified**	**Frozen**	**Vitrified**	**Frozen**	**Vitrified**
*Song et al. [1]	n/a	n/a	n/a	n/a	15-29%	83-87%
Neidert et al. [6]	40->90%	n/a	40-200%	n/a	n/a	n/a
•Elder et al. [5]	65%	67%	130%	200%	n/a	n/a
*Baicu et al. [7]	n/a	n/a	n/a	n/a	n/a	30-50%
Dahl et al. [4]	50%	70%	140%	190%	10.7%	>82%
*Brockbank et al. [14]	n/a	n/a	n/a	n/a	n/a	25->80%
*Hoenicka et al. [15]	58%	22%	n/a	n/a	23-28%	2.5-4.5%
*Baicu et al. [10]	n/a	80-90%	n/a	n/a	n/a	30-70%
•Current Study	n/a	n/a	n/a	n/a	14-43%	7-27%

Since the effects of cryopreservation on viability, ice formation and mechanical properties were studied previously in our lab for a similar TEVM [5], this study focused on the contractile response after cryopreservation. Only one other study has looked at function of an engineered tissue after cryopreservation and few studies have looked at function of native tissue after cryopreservation. Dahl et al. used the same freezing and vitrification methods as were used in this study on their PGA based TEVM [4]. Vitrification was shown to preserve over 80% of the functional response of fresh tissue as compared to 10% for frozen samples. The differences in function correlate to viability with vitrified samples possessing significantly more metabolic activity. Vitrification also has been shown to preserve function in native tissue. Song et al. demonstrated that veins could retain over 80% of the contractile function after vitrification which was significantly better than the frozen vein segments [1]. While vitrification often results in a more functional tissue, human umbilical vein performed better after freezing rather than after vitrification even though the same vitrification method was used. The cell viability

was better with freezing than with vitrification which clearly plays a role [15]. Compared to all of the reports in the literature, the segments tested in this study had severely diminished contractile response after both freezing and vitrification with function after freezing being slightly but not significantly better. Some of the differences may have to do with the different agonists chosen for functional testing. The work in the chapter and the cited literature suggests that there are some receptor pathways are affected by cryopreservation. In all cases the retention of functional response correlates with the viability, suggesting that the reduction in function is due in part to the cell viability. Unfortunately we cannot confirm that for this study as viability testing was not done except in preliminary work. While the previous study in our lab shows viability at or above 65%; the TEVMs in this study are similar but not exactly the same as the previously published work. In Elder et al. the TEVM is a collagen-elastin hybrid cultured in fetal bovine serum [5], resulting in tissue where the SMCs are found evenly dispersed in the collagen hydrogel in relatively low density. The TEVM in this study were made with collagen only and cultured in bovine growth serum. These conditions resulted in a bi-layered TEVM with the hydrogel layer having evenly dispersed cells. The SMC proliferate on top of the collagen hydrogel creating a cell 'capsule' that is cell dense. Freezing and vitrification may affect the tissues differently resulting in a different viability. The preliminary testing suggests that the viability is reduced in this tissue. This is surprising since the tissue tested by Dahl et al. is more cell dense and still retained 70% viability after vitrification and over 80% contractile function [4]. Native artery has also been successfully vitrified with the vitrified tissue assessed both *in vitro* and *in vivo* [1, 16-18]. All of the cited work showing functional data after preservation has used the same or similar vitrification protocols with varied results. There may not be one optimal procedure for all tissue, and there may be a few key parameters that need to be adjusted for successful preservation of tissues of different cell type, cell density and thickness.

To attempt to improve the post-cryopreservation function, the end temperature after the rapid rewarming stage was decreased. This is the temperature at which the cryoprotectant concentration is decreased through sequential steps. This made no improvement in function after vitrification. There are many other variables such as choice of cryoprotectant and its concentration, cooling and rewarming rates to name a few. Investigation into the cell viability and ice formation in the tissue could guide the changes. The TEVMs tested here still do not have the strength required for *in vivo* testing nor have they demonstrated the appropriate suture retention strengths or viscoelastic properties. Modifications to the method that would result in improved mechanical properties may also impact the way the cryopreservation techniques affect the tissue.

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CHAPTER 9: DISCUSSION AND CONCLUSIONS

Discussion

Taken as a whole this work provides insight into the use of the tissue engineered vascular media (TEVM) as a model system and gives insight into the vasoactive response and factors that influence the magnitude of this response. Matrix type and presentation was shown affect collagen I, decorin and biglycan synthesis and deposition. The collagen-based TEVM was shown to be vasoactive using high passage smooth muscle cells (SMC). The contractile force measured is influenced by many factors, while looking at shifts in cell phenotype may yield insights, functional testing is required. Modulation to a more contractile cell phenotype may not result in increased contraction of the tissue due to influence by the matrix. Both cyclic strain and transforming growth factor-β (TGF-β) were shown to increase TEVM strength and promote a more contractile phenotype. Vasoactivity was affected by both types of stimulation, but in different ways. Cyclic strain improved vasoactivity while TGF-β decreased the contractile force of the TEVM. This work shows the influence of both cell phenotype and cell/matrix interactions on the vasoactivity of the tissue.

TEVM as a Model System

The TEVM can be used as a model system, both in disk and tubular form. In Chapter 4, disk shaped TEVMs were used for study of gene expression and protein synthesis. The use of hydrogels as a model system is beneficial for the study of matrix biology. Many studies are done using monolayer cultures on tissue culture plastic or adsorbed matrix material. This study shows that some genes and proteins are affected by matrix, by the 3D configuration, or by both. This system is a good choice for studying effects of different matrix materials or different biochemical stimulation as small

amounts of material and cells and short culture times are needed. It is beneficial for matrix biology as smooth muscle cells, and many other cell types, live in a 3D environment. The architecture of the extracellular matrix (ECM) can also have effects on cells like endothelial cells that are found in monolayers *in vivo* [1]. For tissue engineering applications this model can be used a method of screening for different biochemical stimuli. For use in tissue engineering, longer culture times may be required as some of the regulation of genes changed with increased culture time. The disk shaped TEVM can be used to look at gene expression and protein synthesis and incorporation as shown in Chapter 4. It can also be used to look at compaction, as shown in Chapter 6. The disks are limited in this capacity as they compact more than constrained tissue and can only be used to look at total volume changes. The differences in volume between disks and tubes should be comparable; however, constraint and other factors can affect the direction of compaction as seen in Chapter 7. There different biochemical treatment resulted in tissue with different lengths and wall thicknesses. The volumetric assay with disks could be used here though to determine whether the compaction is in different directions, longitudinal versus radial, or if the total volumes are different.

Use of collagen hydrogel and cell sheet TEVMs has already been demonstrated [2, 3]. This work has shown that even cells that were not cultured using the methods employed by both groups can be used in hydrogels culture. This work also used a lower cell density, but longer culture times and longer tissues. Differences were seen between the work by Bi et al. [2] which used collagen hydrogels and this study. The differences may be due to the culture techniques, cell densities and culture times or collagen and serum lots.

Vasoactivity

This study is important as it shows a vasoactive response to cells that were not isolated using techniques to maintain a contractile phenotype and without serum

starvation. As compared to much of the work, this study has looked at the vasoactivity in response to more vasopressors and vasodilators then most. The cell sheet TEVM have been the best characterized. It has been shown to be a good model for pharmacological studies, looking at a variety of agonists and antagonists [3]. Several studies of the endothelin receptors have also been completed [4, 5]. Comparisons to other studies are difficult due to the wide choices in agonists and antagonists and the methods for presenting the contractility data. The most common way to present the data is as grams force or as a % of the maximum contractile force to ATP. Table 9 attempts to compare the results of this work with the other published work. The results shown are data presented in Chapters 5 and 6; it is the average of the maximum change in force for any given drug. It excludes the work in Chapter 7 where media change frequency was higher. Only work from static samples is shown. Results from phenylephrine are used to compare to norepinephrine. This should be a good approximation as phenylephrine is an α_1-adrenergic agonist and norepinephrine is an α-adrenergic agonist. The TEVM had no response to an α_2-adrenergic agonist, so the responses should be similar.

It is difficult to compare the results from this study to those done with PGA scaffolds. If the assumption is made that the ring segments in Dahl et al. [6] are also 2 mm then the response is significantly lower. This could be due to differences in cell density, or the fact that the PGA TEVMs are cultured under pulsatile flow for 7 weeks. Compared to the cell sheet TEVM, the response is similar if you consider the differences in length. The results are also comparable to contractility of the fibrin TEVM. Surprisingly the largest differences are to the other collagen hydrogel TEVM. These reacted more strongly to bradykinin, and less to endothelin-1. Difference could be due to the addition of endothelial cells or different culture times, cell densities, cell isolation techniques, or differences in medium. This study used 10% BGS; the Bi study used 20% FBS with ascorbic acid, insulin, TGF-β_1 and aprotinin.

Table 9: Comparison of TEVM Vasoactivity with Literature. * Work with other agonists that are presented as % E_{max} is not shown. ** Ranges are due to different factors being studied. * Norepinephrine is being compared to phenylephrine results.**

Published Work	Agonist	Published Result	In This Study
PGA Scaffolds			
Niklason et al. [8]	Prostaglandin $F_{2\alpha}$ Serotonin Endothelin-1	0.17 g / 2mm response response	n/a response response
Dahl et al. [6]	Endothelin-1	~0.8 g	0.16 g / 2 mm
Cell Sheet TEVM			
L'Heureux et al. [3]*	Histamine Bradykinin ATP	182 mg / 5-7 mm 104 mg / 5-7 mm 80 mg / 5-7 mm	n/a 60 mg / 2 mm 62 mg / 2 mm
Laflamme et al. [4]*	Endothelin-1 ATP	0.7 g / 5 mm 0.3 g / 5 mm	0.16 g / 2 mm 0.06 g / 2 mm
Laflamme et al. [9]*	Endothelin-1	100% KCl at 10^{-8}M	250% KCl
Fibrin Hydrogels			
Swartz et al. [10]	KCl U-46619 Norepinephrine	~60 g/g dry weight 21 g/g 9 g/g	47 g/g n/a 11 g/g***
Yao et al. [11]**	KCl NE U-46619	5-75 g/g dry weight 5-15 g/g 0-40 g/g	47 g/g 11 g/g*** n/a
Liu et al. [12]**	KCl Norepinephrine	0.60 N/g dry weight 0.20 N/g	0.46 N/g 0.11 N/g***
Yao et al. [7]	KCl KCl	0.8 – 1.2 mN 0.05-1.15 kPa	0.46 mN 0.40 kPa
Collagen Hydrogel			
Bi et al. [2]	K+ Bradykinin Histamine Endothelin-1	44.1 mg / 1mm 90.9 mg / 1mm 91.9 mg / 1 mm 8.6 mg / 1 mm	n/a 60 mg / 2 mm n/a 161 mg / 2 mm

The varied methods of presenting the data make comparisons difficult. Grams force and force per dry weight are good choices if there is no interest in comparisons or if the tissues being studied are homogeneous. Grams force can be greatly affected by the geometry of the ring segments, especially with differences in length. While the normalization to dry weight can account for differences in length, different tissues can have very different dry weight for segments with similar lengths and wall thicknesses. Differences in compaction and ECM synthesis would affect this and are common between different cell types. The most correct way to present the contractile data would be actual stress. This work has presented the contractile response as nominal stress.

While not as accurate, it is still better than what has been used. Only one study has presented the data as stress [7]; since the method for calculating was not described it is assumed to also be nominal stress.

For these studies bovine growth serum was used instead of fetal bovine serum (FBS). The choice of serum was found to have a significant effect on the contractile response of the TEVM made with either rat or human SMC, Figure 59. TEVMs with rat aortic SMC were cultured in medium containing 10% fetal bovine serum (FBS) had almost no measurable function. A slight but statistically insignificant increase in contractile force was seen when the medium change frequency was increased in the cultures containing 10% bovine growth serum (BGS). The same phenomenon was seen with human aortic SMC. Human SMC were cultured in either smooth muscle growth medium 2 (SMGM-2) from Lonza which contains 5% FBS, or medium with 5% or 10% BGS. The SMGM-2 medium resulted in TEVM with a weak contractile response; while the 5% BGS resulted in a response similar to the rat SMC. The human TEVM cultured in 10% BGS could not be tested; the wall was too thin to handle without damage.

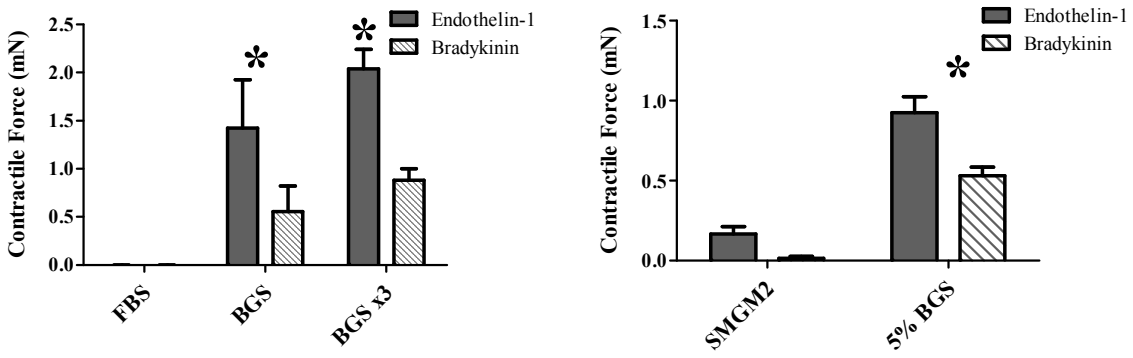

Figure 59: Differences in Contractile Response due to Serum. TEVM with rat SMC (Left) and with human cells (Right). * is p≤0.05 compared to the medium containing FBS. The use of BGS significantly increased the contractile force in response to both drugs. BGS resulted in increased contraction of both rat and human SMC. Increasing the medium change frequency from once a week to 3 times a week increases the contractile force, although not significantly.

Further evidence for a soluble factor in the culture medium that can be exploited is the difference in smooth muscle α-actin staining that is seen when the medium change frequency is changed. The increase in contractile apparatus would result in a greater contractile force, assuming no major differences in the wall thickness, collagen density or tissue strength. A comparison of α-actin staining is shown in Figure 60.

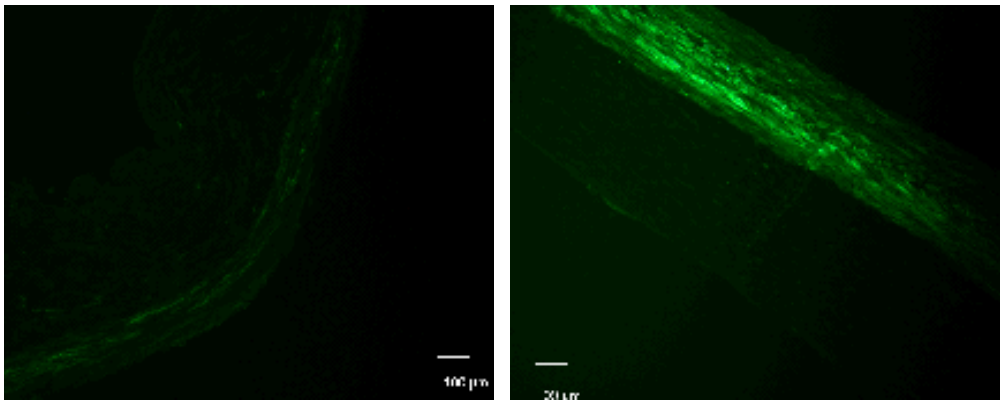

Figure 60: Effects of Medium Change Frequency on Smooth Muscle Alpha Actin Expression. Increasing the medium change frequency from once a week (left) to three times a week (left) resulted in an increase in alpha actin staining.

Mechanical and Biochemical Stimulation

Both cyclic strain and biochemical stimulation impacted the strength, vasoactivity and composition of the TEVM. A comparison of the two types of treatment is presented in Table 10. The data are presented as percent change from the value of the control TEVMs. Media change frequency affected the properties of the controls in each case. Both types of stimulation improved the strength of the tissue over the controls, but had opposing effects on the contractile stress.

Both cyclic strain and TGF-β promoted a more contractile cell phenotype, but TGF-β resulted in significant compaction leading to a thick dense collagen layer. To overcome thickness alone a larger contractile force would be needed to elicit a measurable tissue contraction. Combination of TGF-β and cyclic strain has been shown

to effect cell phenotype [13]. If the longitudinal compaction could be controlled, the contractile strength and tensile strength could be further improved.

Table 10: Change in Properties from Control TEVM. Results are shown as a percent change from the static or untreated controls. * = p≤0.05

Treatment	Strength (kPa)	Vasoactivity (kPa)	Wall Thickness (mm)	Cellularity (M cells/mg)	Collagen (mg/mg)
Cyclic Strain	23%*	99%*	-16%*	4.6%	19%*
TGF-β	56%*	-83%*	9%	18%	-0.2%

Cryopreservation

Studies of vasoactive function after cryopreservation of native vessels have shown that freezing results in diminished function, but that function is maintained with vitrification [14, 15]. Similar results were seen with a PGA based TEVM [6]. While vitrification often results in a more functional tissue, human umbilical vein performed better after freezing rather than after vitrification even though the same vitrification method was used. The cell viability was better with freezing than with vitrification which clearly plays a role [16].

Importance of the Cell Capsule

The importance of the cell capsule is highlighted throughout this work. In Chapter 4, cell capsules were seen on both collagen and fibrin gels. The collagen I gene expression varied as a function of time in the hydrogels, most likely due to the absence of a cell capsule and 2 days and presence at 14 days of culture. The collagen and other ECM that the cells produce affect the TEVM strength. The cell capsule is responsible for much of the TEVM strength. Figure 61 shows a representative force displacement curve where

there are two distinct areas, one corresponding to when the cell capsule is the primary load bearing component and one after the cell capsule fails and the hydrogel is loaded. The picture on the right shows a ring segment where the cell capsule has failed and the hydrogel layer is still intact.

The cell capsule is also responsible for much of the vasoactivity of the TEVM. As shown in Figure 62, most of the α-actin staining is found in the cell capsule. Little to no staining is found in the cells within the collagen hydrogel.

Masson's trichrome staining of a TEVM is also shown in Figure 62. The cell layer is densely populated and contains cell synthesized collagen which improves the TEVM strength. Cells may produce more collagen while encapsulated in fibrin rather than collagen [17], but the SMCs produce more collagen when grown on top of collagen then fibrin. The trend existed in the work shown in Chapter 4; however, significance is found when the monolayers are cultured on higher concentrations of collagen. The results shown in Figure 63 are from cells grown in monolayer culture on top of 1 mg/ml collagen or fibrin. This is essentially a thin layer of hydrogel. At this concentration the collagen is not likely in monomeric form; a collagen hydrogel can be created. Cell produced more collagen when cultured on 1 mg/ml matrix than on the 50 μg/ml matrix. The difference between the collagen production on the two surfaces was amplified with the higher concentration substrate.

Importance of Decorin and Biglycan

While decorin and biglycan were only discussed in Chapter 4, they are proteoglycans worth studying in tissue engineering. These are two homologous proteoglycans that are part of the family of small leucine rich repeat proteoglycans (SLRP). Both SLRPs play a role in collagen fibrillogenesis. Knocking out these proteoglycans results in irregularly sized collagen fibrils and fragility in skin (decorin) [18] and orthopedic tissues (biglycan) [19, 20].

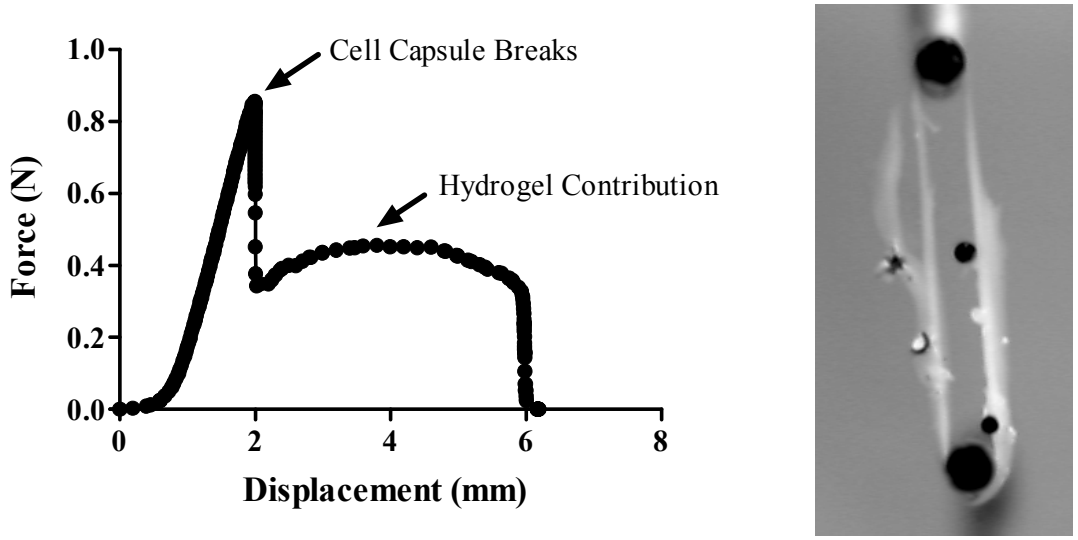

Figure 61: The Cell Capsule and Tensile Strength. The force – displacement graph is shown on the left. Note the two distinct regions of the graph that correspond to the cell capsule and the collagen hydrogel after rupture of the capsule. A picture of a ring after the failure of the cell capsule is shown on the right.

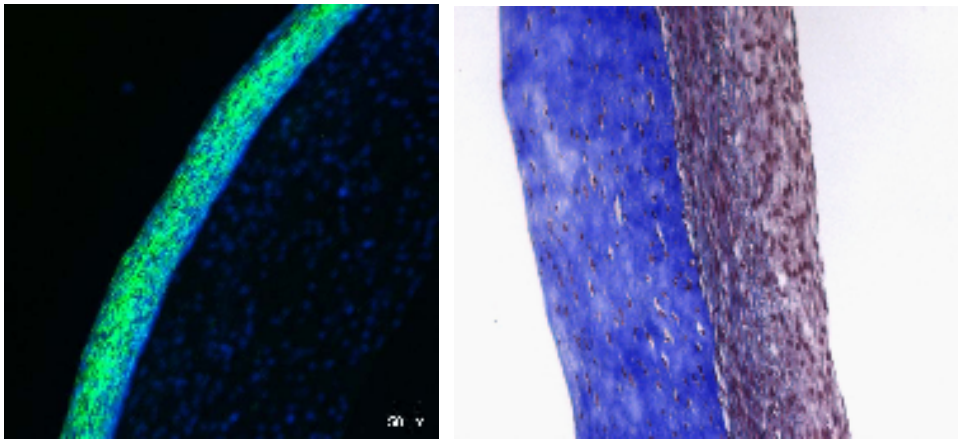

Figure 62: Histology of the TEVM. Alpha actin staining of the TGF-β treated TEVM is shown (Left). Alpha actin is stained green; nuclei are stained blue. Note that the alpha actin staining is almost exclusively in the cell capsule, not in the hydrogel. Masson's Trichrome staining of untreated TEVM is shown (Right). Note the significant amounts of collagen seen in the cell capsule.

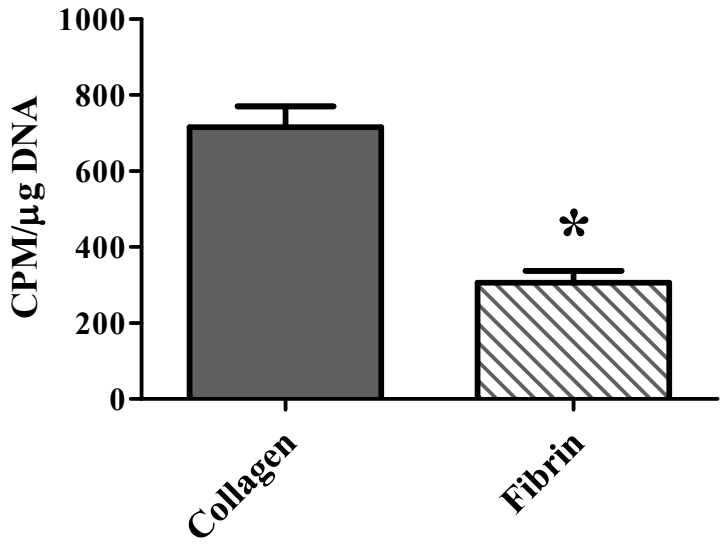

Figure 63: Proline Incorporation of Cells Grown on 1 mg/ml Collagen or Fibrin. Collagen synthesis is greater in cells grown on collagen I than those grown on fibrin.

Both decorin and biglycan bind to tropoelastin; biglycan binds to both tropoelastin and microfibril-associated glycoprotein 1 (MAGP-1) forming a ternary complex and may play a role in elastinogenesis [21]. Both proteoglycans also bind to transforming growth factor-β (TGF-β). The role of decorin has been better studied. Once thought to inactivate TGF-β [22-24], decorin is now thought to sequester and modulate the activity of TGF-β [25-27]. Decorin is also known to modulate MMP activity, although the response and the specific MMPs affected varies greatly by tissue [28-30].

Decorin and biglycan expression play important roles in compaction of collagen hydrogels, which is important for tissue engineering. Decorin expression has been shown to have a direct effect on hydrogel compaction [31]. Transfected cells where decorin was either upregulated [32] or knocked out [33] led to significant increases in hydrogel compaction. In the case of the decorin knock out the increased compaction may be related to an increase in biglycan synthesis. Both studies highlighted the different effects of exogenous and endogenous decorin on gel compaction.

Cyclic strain [34], TGF- β and TNF- α [35] are all known to influence the synthesis of decorin and biglycan in monolayers. The application of these factors to the TEVM also affected the synthesis of decorin, see Figure 64. Biglycan levels were very low and are not shown. The results of cyclic strain are consistent with those shown with cyclic stretch; decorin is not as abundant in the strained TEVM. The results do not directly compare to those shown with monolayer cultures. The addition of TGF- β or TNF- α caused a decrease in decorin, but not to the same extent as seen with monolayer cultures. The combination of TGF- β and TNF- α caused an increase in decorin, contrary to the drastic decrease seen in monolayers.

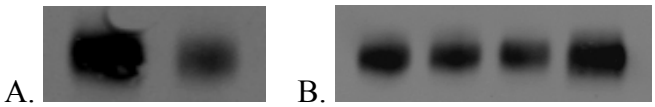

Figure 64: Western Blots for Decorin in Mechanically and Biochemically Treated TEVM. Comparisons of static and strained TEVM are shown (Right). The strained samples contain significantly less decorin than the static controls. The biochemically treated TEVM are also shown (B). From left to right: untreated control, TGF-β, TNF-α, and combined TGF-β and TNF-α.

Future Recommendations

There are several directions that this work can be taken. The main areas are: using the TEVM as a model system for matrix biology, further investigations of vasoactivity and the factors that influence it, application of cyclic strain, and new TEVM fabrication methods.

TEVM as a Model System

The disk shaped TEVM provide a nice 3D environment for studying matrix biology. The effects of growth factors and other soluble factors could easily be studied. One factor of interest is TGF-β. While the effects on TEVM strength and vasoactivity were studied, the effects on gene expression were not. TGF-β is known to influence

decorin and ECM expression. This model could also be used to study effects of cell density and other matrix materials such as SIS, Matrigel, and hyaluronic acid. The expression of collagen type I, decorin and biglycan were studied due to their importance in tissue strength; however, multiple genes could easily be studied from the same sample. Use of the Qiagen RNeasy lipid kid resulted in very high yields of good quality RNA which would allow for a large number of genes to be explored using one sample. Along with gene expression the model system can be used to study protein synthesis and retention in the hydrogels as well as gel compaction. One note is as follows. The disks can be used to look at overall volume changes but cannot determine the direction of compaction. Additionally some of the constraints used in TEVM fabrication such as the silicone sleeve may also impact the final gel compaction.

TEVM and Vasoactivity

The importance of serum was discussed previously. Clearly there is a soluble factor (or factors) that are involved in the improvement of cell contraction. Finding these factors would be of great interest, especially as the field moves toward the use of a defined culture medium. A proteomics approach to look at differences between medium that produces functional and non-functional tissue would help to identify these potential factors. Understanding how these factors are working would also be of interest. It is possible that the differences in function are due to differences in the phenotype of the cell and the amount and type of contractile machinery contained in the cells. It is also possible that the differences may be due to the presence or absence of certain receptors.

There may be ways to improve the vasoactive response. Mechanical and biochemical stimuli [13] have been shown to influence smooth muscle cell phenotype and may result in a stronger contraction. This work has shown that cyclic strain can lead to improved function. The addition of TGF-β resulted in a more contractile phenotype; if the longitudinal gel compaction could be eliminated, improvements in vasoactivity

possibly could be seen. There are certainly other biochemical factors that may also be of interest.

Mechanical Stimulation

This study has shown improvements in the mechanical properties and the physiological contractile response of the collagen-based TEVMs. While these improvements are significant, they are far from optimized. Studies have shown that different strain amplitudes and rates may be preferable for improved mechanical properties [36-39] and collagen production [37, 38]. Work with valvular interstitial cells has shown that incremental increases of strain amplitude may yield superior results for strength [37]. None of these studies have looked at the effects of the strain parameters on vasoactivity. Additionally, if the mechanical properties can be improved prior to application of strain, the silicone sleeve could be removed and pressure could be applied directly to the TEVM. This would work best with the incremental strain regime as lower strains and pressures would be used initially. To accomplish this, the bioreactor would need to be modified slightly to allow for control of flow through the lumen and would require instrumentation to measure the TEVM circumferential strain in a sterile manner while the TEVMs were in the bioreactor.

Biochemical Stimulation

This study looked at the effects of TGF-β on TEVM strength and vasoactive function. The large gel compaction was beneficial for TEVM strength, but detrimental to TEVM vasoactivity. Additionally, longitudinal compaction is highly variable and not desirable. If the longitudinal compaction could be eliminated, different results may be seen with regards to strength and vasoactivity. To accomplish this, the TEVM could be cultured on silicone sleeves or TGF-β could be added after 2 weeks in culture, when cell mediated compaction ceases. There are other factors that may be of interest alone or in

combination with TGF-β. The combination of insulin and TGF-β could be further studied as it has been shown to improve the strength, the collagen production, and the vasoactive response of fibrin based TEVM [11, 40]. Ascorbic acid or sodium ascorbate is often used to promote ECM production [8, 41-44]. Retinoic acid has also been shown to increase ECM synthesis [44]. These factors alone or in combination could yield desirable results. Additionally, the combination of biochemical and mechanical stimulation is of interest. An interactive effect on cell phenotype has been shown [13]. Applying the correct stimulation at the correct time could lead to large improvements in tissue strength and function.

Cryopreservation

One item lacking in this work is cell viability after cryopreservation. Native and engineered blood vessels that were vitrified using the same protocol have retained significant vasoactive function after being rewarmed [6, 14]. It is important to understand if the differences are linked to cell viability such as was seen with the human umbilical cord vein [16]. It is possible that vasoactivity is a more stringent test of cryopreservation success, or we may find that if viability can be maintained, vasoactivity will also be retained. One difference between the composition of TEVM in this study versus native and the PGA-based TEVM is the presence of the hydrogel layer. Compared to the other tissues, this layer is not densely populated with cells. A similar TEVM from our lab consisting of a cell-populated collagen hydrogel with an elastin scaffold has been vitrified and found to have over 65% cell viability upon rewarming [45]. This suggests that if the problem is with viability it is not entirely due to the collagen hydrogel layer. There are multiple factors that can be modified to improve the viability including cooling and rewarming rates and choice of cryoprotectant.

Strategies for further improving TEVM composition, mechanical properties and function

As discussed previously, the cell capsule is an important feature of the TEVM in these studies. The majority of the strength and the vasoactivity comes from the cells and matrix in this cell dense layer. The collagen hydrogel itself is fairly weak and does not promote cell proliferation or extracellular matrix production within the gel. The most successful strategies for creating a TEVM with mechanical properties similar to native tissue have relied on the cells creating their own matrix. Cell sheet TEVMs have burst pressures greater than 2000 mmHg [46]. Cell seeded PGA engineered blood vessels have been shown to be vasoactive and have burst pressures over 2000 mmHg after 7 weeks in culture. At this time most of the PGA has been degraded and replaced with cell synthesized ECM [8]. The use of fibrin gels is also moving in this direction. By allowing the fibrin to be degraded in a slow, controlled manner the cells can replace the fibrin with secreted ECM.

The use of collagen hydrogels for tissue engineering has many benefits over fibrin and synthetic materials. While cells may not like to be encapsulated in type I collagen, they are proliferative and secrete matrix when cultured on top of collagen I. A possible new strategy for using collagen hydrogels would be to create a thin, cell dense collagen gel. The SMCs would quickly form a 'cell capsule' and lay down significant amounts of collagen and other matrix. The collagen layer could be minimized to be like an internal elastic lamina, or may be degraded by the cells. This approach is beneficial over fibrin, as no supplementation would be needed to slow down the proteolytic degradation. Unlike PGA there is no concern over any undegraded polymer. Compared to cell sheets, the culture time could be shorter since the matrix would not have to be wrapped and the layers would already be integrated. This also reduces the risk of possible delamination that could occur with the cell sheet TEVMs.

Conclusions

This work has shown that a tissue engineered vascular media can be used as a model system for study of matrix biology. Differences in gene expression were seen in response to both matrix material and to matrix presentation (2D or 3D culture). For study of matrix biology, shorter timeframes should be used as cells can proliferate on the surfaces of the hydrogels and create a 'cell capsule' where the matrix is cell secreted and not as well defined. For studies regarding tissue engineering, longer cultures may be of interest as tissues require longer culture times. Some of the gene expression observed was affected by culture time.

Both cyclic strain and TGF-β improved tissue strength; however, the stimulation used in this work was not sufficient to increase the strength to the physiological levels needed for implantation. The viscoelastic properties were not studied; the hydrogels without cross-linking are known to creep. It is likely that the mechanical properties are such that the grafts would fail due to aneurysm or damage due to fatigue, even if the graft strength could withstand blood pressure. Cyclic strain improved tissue strength due to increased radial compaction and increased collagen deposition. TGF-β improved strength primarily through increased compaction. It is possible that there was additional collagen deposition that could not be measured. The presence of additional cross-linking was not investigated which could also play a role.

The presence of endothelin, kinin, adrenergic, serotonergic and purinergic receptors was demonstrated in these collagenous TEVMs. While multiple receptors where shown to be present and functional, not all are fully characterized. For example, endothelin-1 and ATP bind to more than one receptor, and it is possible than only one of the endothelin or purinergic receptors is present and functional. This possibility was demonstrated with the adrenergic receptors where a contractile force was measured in response to an α_1-adrenergic agonist but not to an α_2-adrenergic agonist. Not all receptors

were present and functional; relaxation was not seen in response to SNP, a nitric oxide donor. The presence of a vasoactive response is especially interesting in this case as cells were isolated and expanded using techniques that have been shown to cause a phenotypic shift toward a synthetic phenotype which some believe cannot be reversed [47]. Serum starvation has been shown to cause cultured smooth muscle cells to shift to a more contractile phenotype [48, 49]. The application of cyclic strain was shown not only to affect cell phenotype but also to improve the function of the TEVM. The application of TGF-β is a reminder that contractility is due to integration of cells and matrix. Improvements in strength can create a tissue that cannot be easily contracted by the smooth muscle cells, even when a shift towards a more contractile phenotype is seen.

This work as a whole has shown the importance of the cell-matrix interaction. The matrix type and presentation can affect ECM synthesis. The interaction of cell and matrix can affect the tissue vasoactivity and the mechanical properties. For example, increased compaction of the exogenously supplied ECM can improve the tissue strength while reducing the measured contractile strength. The studies presented here provide insights into future directions such as future matrix biology studies and strategies for creating a TEVM.

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APPENDIX A: SUPPLEMENTAL RESULTS

APPENDIX A1: FBS VS. BGS

Introduction

Importance of serum is well known in cell biology and many purchased cell lines come with 'qualified' serum. Serum lot matching is important and many labs will even screen lots. In order to reduce cost, bovine growth serum (BGS) was compared with the fetal bovine serum (FBS) lot that was being used. BGS is obtained from calves which results in reduced cost and improved availability. There is a claim of less lot to lot variability than with FBS due to choice of farms and supplementation of the BGS. The serum was tested for cell culture and for construct culture.

Results

Preliminary cell proliferation studies were completed. Equal numbers of rat smooth muscle cells were plated sparsely. After 8 days the cells were trypsinized and counted using the Coulter Counter. The cells cultured in FBS had proliferated 26 fold, while the cells cultured in BGS had proliferated 45 fold.

TEVM were cultured in medium containing either 10% FBS or 10% BGS serum. Initially medium was changed weekly. By the second week in culture noticeable differences in the medium color were noted indicating higher metabolism, which may be due to an increased number of cells in the TEVM. An additional condition was added where BGS medium was changed twice a week. The results are shown in Figure 65. At one week in culture the TEVM had similar strengths, although the strength of the TEVM cultured in BGS medium was slightly higher (8 kPa versus 5.5 kPa). The TEVM that were cultured in FBS maintained a relatively constant tensile strength over the 4 weeks of culture; however, there is a small increase after 4 weeks in culture which may be the start

of an upward trend of strength. The TEVM cultured in BGS increased in strength up to 3 weeks in cultured where the tensile strength leveled off even with more frequent media changes. At 7 days the TEVM in BGS were almost twice as strong as those cultured in FBS. By 14 days the strength of the TEVM BGS medium was 5 times that of the TEVM in FBS. Changing the medium more frequently increased the strength further to more than double the TEVM with weekly changes of BGS medium. The plateau seen with BGS may be overcome with additional medium changes or may be due to problems of nutrient diffusion due to the large cell capsule at 4 weeks.

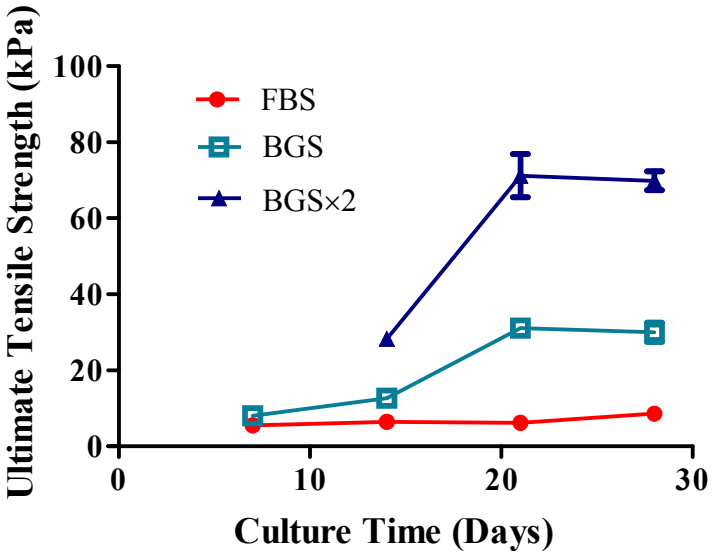

Figure 65: Serum Effects on UTS Over Time. TEVMs cultured in BGS are stronger than those cultured in FBS at most timepoints. Increasing the change frequency of the BGS medium also increased the TEVM strength.

The study was repeated with TEVM that were cultured for 3 weeks. The FBS medium was supplemented with 50 ug/ml ascorbic acid, 3 ng/ml cupric sulfate and 1% non-essential amino acids; the BGS medium was not supplemented. The results for the tensile testing are shown in Figure 66. Once again the strength of the TEVM cultured in

BGS was more than double that of those cultured in FBS. There were no differences in wall thickness associated with the serum, see Figure 67.

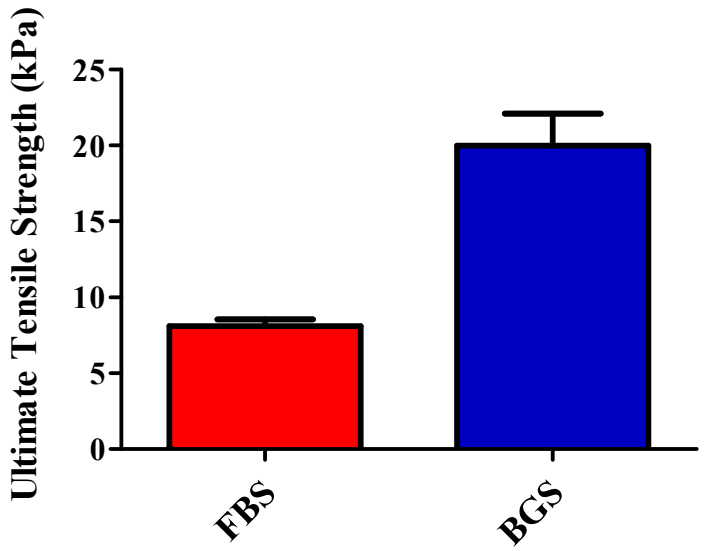

Figure 66: Serum Effects on Ultimate Tensile Strength at Four Weeks. BGS increased the TEVM strength versus FBS medium.

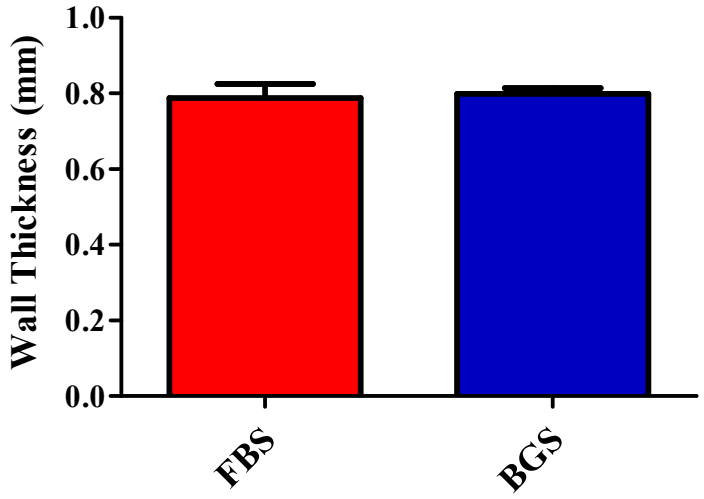

Figure 67: Serum Effects on Wall Thickness. No differences in wall thickness were seen between the two serums.

As discussed in Chapter 9, there were significant effects on vasoactive response. For completeness the figure is presented here as well in Figure 68. This study was done with rat cells cultured for 3 weeks. The same phenomenon was seen with human smooth muscle cells. Human cells were cultured in smooth muscle growth medium-2 (SMGM-2) (Lonza) initially. This medium contains 5% FBS with insulin, EGF and FGF. No response was recorded in response to the agonists. When the human TEVM were cultured in 10% BGS, the walls were so thin that the TEVM could not be handled without damage, thus there are no contractility results. When 5% BGS was used, a robust contractile response was seen similar to that of the rat TEVM.

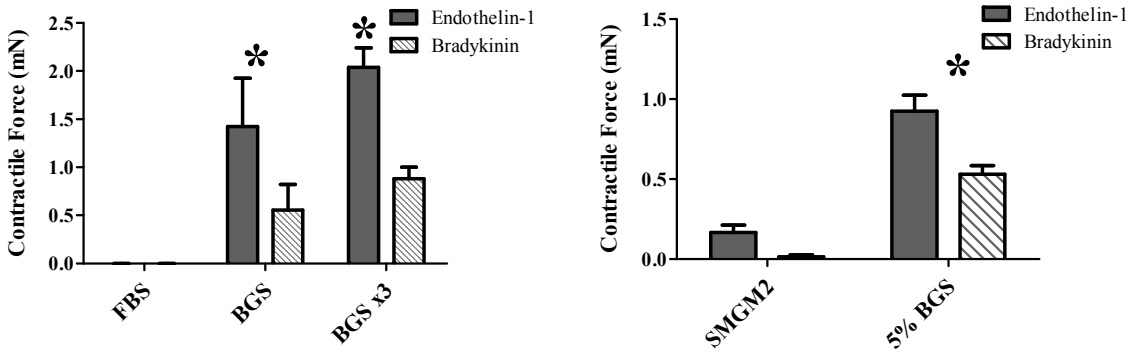

Figure 68: Serum Effects on Vasoactivity. Work was completed with rat cells (Left) and human cells (right). BGS medium leads to increased contractile force in response to both drugs tested. This is true for TEVMs made with both rat and human SMC.

Discussion

Serum choice is important for tissue engineering. BGS medium was chosen for TEVM culture due to the increases in strength and the presence of vasoactive response. With all serum the cell to medium ratio is important, but the results are more dramatic with BGS. The construct numbers in a dish, the size of the constructs and the medium change frequency should be rigorously maintained to reduce variability. The cost of BGS

was approximately ⅓ that of FBS. Even with the increased media changes, the cost is similar for the two serums.

The results presented here are not meant to suggest that BGS is better than FBS, although it is true that BGS is better for some tissue engineering applications than some lots of FBS. Other groups have shown a vasoactive response using medium containing FBS. It is possible that this is due to differences in cell type, source or choice of scaffold. It is also possible that the lot of FBS that is being used promotes a more contractile cell phenotype. Comparisons of strength are difficult due to scaffolding differences and the addition of other growth factors. It is interesting to note that there is a soluble factor (or factors) that can promote a more contractile smooth muscle cell. Finding this factor would be beneficial so that lot screening isn't necessary, or so a defined medium could be used rather than serum.

One thing to note in these studies is that the strengths presented in this appendix are significantly less than what is presented in the main chapters. Just as some groups screen serum lots, others screen collagen lots. During the course of this work the collagen lots changed and resulted in significantly stronger tissues. Even with the different collagen lots, the strength difference between TEVM cultured in BGS versus FBS still exists. The differences in strength also are seen with TEVM made with different species. Both rat and porcine TEVM are stronger when cultured in BGS.

APPENDIX A2: EFFECTS OF EXOGENOUS DECORIN AND BIGLYCAN

Introduction

Decorin and biglycan are homologous small leucine rich repeat proteoglycans (SLRP). The structure of decorin and biglycan is very similar. Both core proteins have a horseshoe configuration; decorin has one more oligosaccharide chain than biglycan. Decorin has one dermatan sulfate or chondroitin sulfate glycosaminoglycan (GAG) chain. Both decorin and biglycan interact with extracellular matrix (ECM) and growth factors by binding to the core protein [1]. Both SLRPs play a role in collagen fibrillogenesis. Knocking out these proteoglycans results in irregularly sized collagen fibrils and fragility in skin (decorin) [2] and orthopedic tissues (biglycan) [3, 4]. More severe consequences are not seen since the other SLRPs are upregulated and compensate for part of the lost function. Even dual knockouts of both decorin and biglycan result in additional fragility of the orthopedic tissues [5, 6]. Differences in function between decorin and biglycan are in part due to differences in proteoglycan localization. Decorin is found bound to collagen in the ECM as well as in the pericellular matrix; biglycan is primarily found on the cell surface and in the pericellular matrix [7, 8].

Both decorin and biglycan bind to tropoelastin; biglycan binds to both tropoelastin and microfibril-associated glycoprotein 1 (MAGP-1) forming a ternary complex and may play a role in elastinogenesis [9]. Both proteoglycans also bind to transforming growth factor-β (TGF-β). The role of decorin has been better studied. Once thought to inactivate TGF-β [10-12], decorin is now thought to sequester and modulate the activity of TGF-β [13-15]. Decorin is also known to modulate MMP activity, although the response and the specific MMPs affected varies greatly by tissue [16-18].

Decorin and biglycan expression play important roles in compaction of collagen hydrogels, which is important for tissue engineering. Decorin expression has been shown to have a direct effect on hydrogel compaction [19]. Transfected cells where decorin was either upregulated [20] or knocked out [21] led to significant increases in hydrogel compaction. In the case of the decorin knock out the increased compaction may be related to an increase in biglycan synthesis. Addition of exogenous decorin did not affect gel compaction in either case. While the addition of exogenous decorin did not result in differences in compaction, it was hypothesized that it might have other influences that could affect TEVM strength. There is one study that has shown that synthesis of collagens I and V increased with both overexpression of decorin and addition of exogenous decorin into the secreted matrix [22].

The effects of exogenous decorin on TEVM strength and collagen synthesis follows. The results are broken into two sections: 'pre-' and 'post-' 2006. Around this time the collagen lot changed and the effects changed. In the 'post-2006' work effects due to addition of biglycan were also investigated.

Results

Pre-2006

To determine the most effective way to deliver decorin, a study was completed to investigate the addition of decorin to the culture medium or to the TEVM during fabrication. TEVMs were made using the method described in Chapter 5, using porcine aortic smooth muscle cells isolated from neonatal pigs. For TEVM exposed to decorin in the culture medium, the decorin was added fresh with every weekly media change. For decorin in the hydrogel, decorin was only added to the gel at that time. For both circumstances 0.5 μg/ml decorin was used. The results are shown in Figure 69. By four weeks in culture there were significant differences in strength. Adding decorin to the

medium did improve the strength versus the untreated controls, but not to the same level as the TEVMs where decorin was added to the hydrogel alone. This application method was chosen both for the improvements in strength and the lower cost.

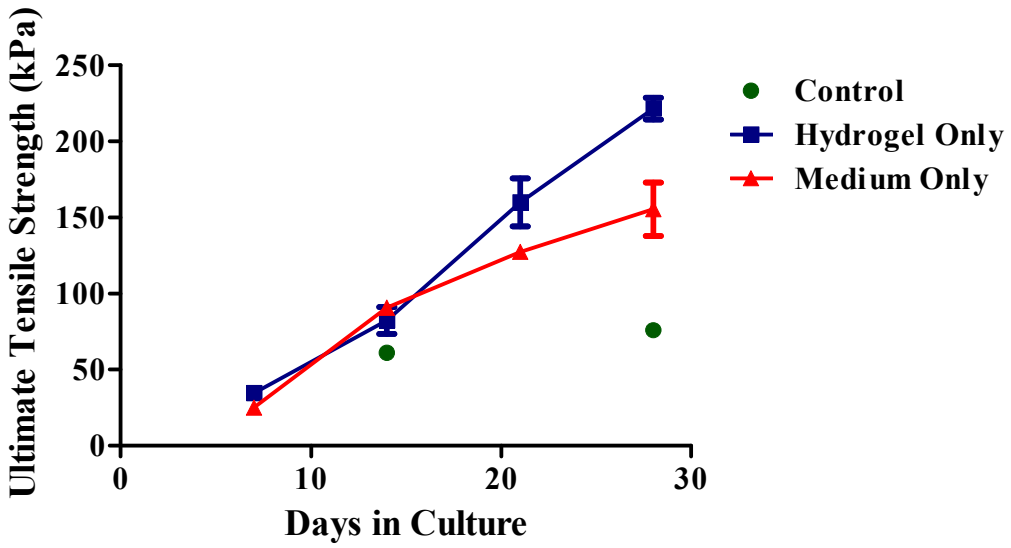

Figure 69: Effects of Method of Decorin Delivery on TEVM Strength. Adding decorin increased the TEVM strength. Adding it to the hydrogel during fabrication only led to the greatest increase in strength.

The effect of decorin dose was then explored. Six different dosages were used and the TEVM were cultured for 4 weeks at which time tensile testing was completed. As shown in Figure 70, the strength was affected by dosage. The strength improved from 1.8 to 4.7 fold as compared to the untreated controls. All of the dosages except for 0.25 μg/ml resulted in significant improvements in strength over the untreated controls. There were no significant differences in strength between 0.15, 0.25 and 0.5 μg/ml dosages or between 0.01 and 0.075 μg/ml. All other doses were statistically different from each other.

Hydrogel compaction was compared over a 6 week timeframe using the method described in Chapter 6. Disk shaped gels were made with 0 or 10 ng/ml decorin, see Figure 71. No differences in gel compaction were seen at any timepoint.

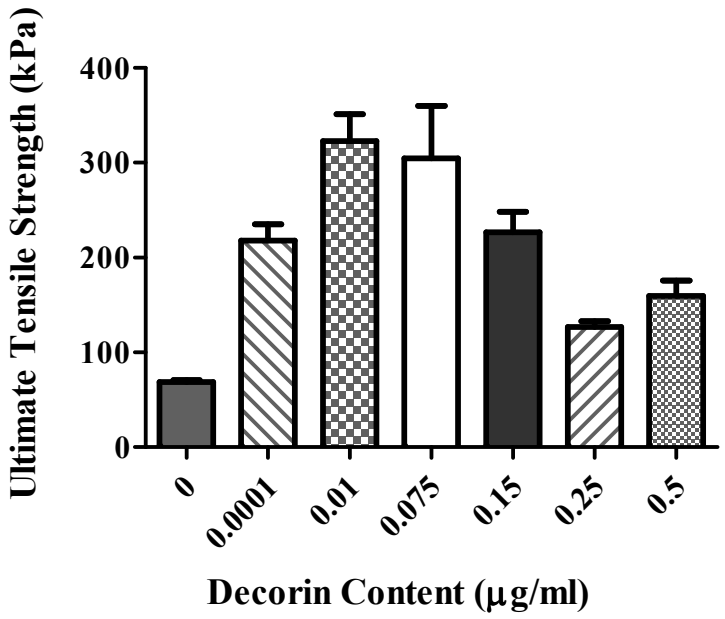

Figure 70: The Effect of Decorin Concentration on TEVM Strength. Decorin concentration influenced the TEVM strength. The maximum strength appears near 10 ng/ml.

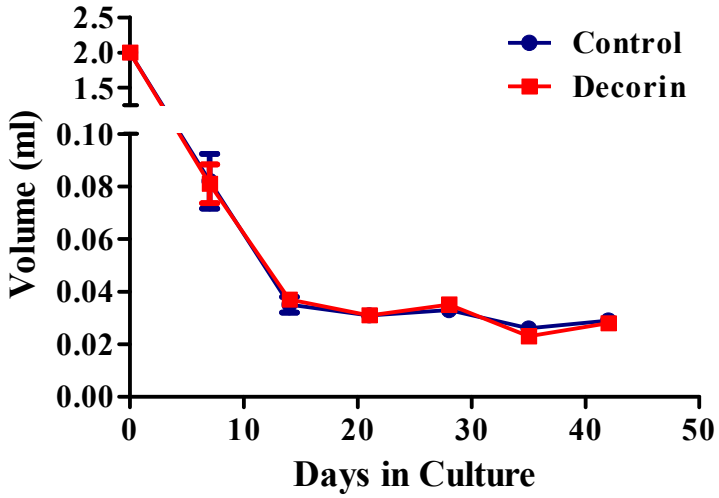

Figure 71: Effect of Decorin on Hydrogel Compaction. No differences in hydrogel compaction are seen when exogenous decorin is added to the hydrogels.

Tubular shaped TEVMs were made with 0 or 10 ng/ml decorin. The TEVMs were cultured for 4 weeks at which time the cellularity, collagen content and GAG content were measured. The cellularity of the gels was measured using the Hoechst DNA assay. Collagen content was assessed using the Sirius Red assay. Both of these methods are described in Chapter 6. GAG content was determined by using the DMMB assay. The results are shown in Table 11. There are no significant differences in cell or GAG between hydrogels with or without exogenous decorin. The collagen content is significant for a 90% confidence interval. It is possible that with additional samples significance to 95% would have been seen. This could not be completed due to the change in collagen lot.

Table 11: Effects of Decorin on TEVM Composition

Treatment	DNA Content (µg/mg)	Collagen Content (µg/mg)	GAG Content (µg/mg)
Control	10.17 ± 2.57	24.67 ± 3.88	34.70 ± 4.06
Decorin	12.30 ± 1.35	46.67 ± 11.25	32.01 ± 2.53
p-value	$p>0.1$	$p<0.1$	$p>0.1$

Qualitative comparisons were also completed by using histology. Masson's trichrome was done to look at collagen content and localization. Saffronin-O staining was done to look at GAG content and localization. Masson's trichrome stains collagen blue and nuclei purple, see Figure 72. Saffronin-O stains GAG red and nuclei purple, see Figure 73. Both pictures show a partial-cross section of the TEVM wall. The lumen is to the right and the cell capsule is found on the left. No differences are seen in the collagen hydrogels. Both have cells evenly dispersed throughout. The cell capsules of the decorin hydrogels appear slightly more compact and have darker staining for collagen. The GAG staining appears to be darker in spots, but the cell capsule appears slightly more compact which may account for this difference.

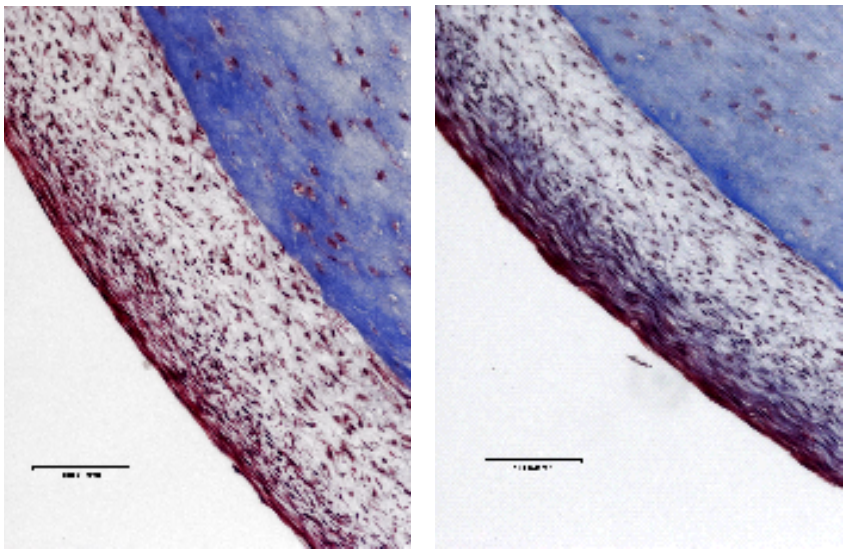

Figure 72: Masson's Trichrome. Shown are Controls (Left) and Decorin Containing (Right) TEVM. More collagen is seen in the cell capsule of the decorin containing TEVM.

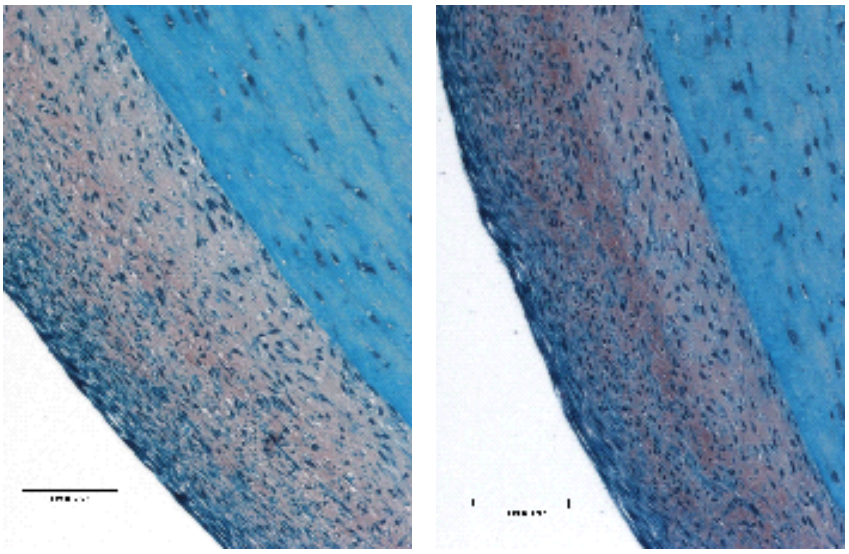

Figure 73: Saffronin-O Staining. Shown are Controls (Left) and Decorin Containing (Right) TEVM. Both TEVMs have significant amounts of GAG in the cell capsule region.

Post-2006

After the collagen lot was changed, the effect of decorin could no longer be seen. This was not due to a decrease in strength of the TEVM where exogenous decorin was added, but due to an increase in strength of the controls. Results in Figure 74 are for porcine TEVMs cultured for 4 weeks in medium containing 10% BGS. The increase in strength was seen regardless of cell type (rat or pig) or serum (BGS or FBS).

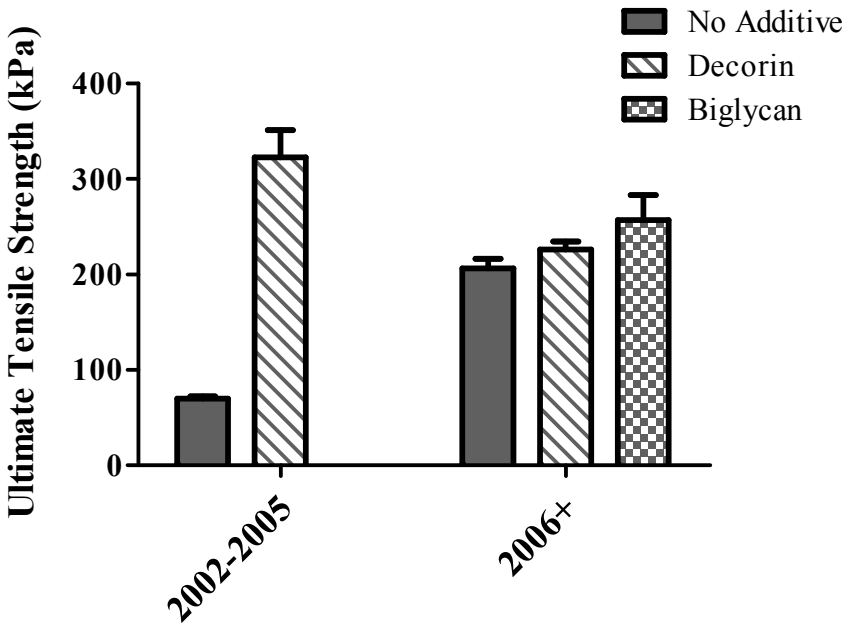

Figure 74: Effects of Decorin and Biglycan on TEVM Strength Before and After 2006. After 2006 no significant differences in strength are seen with the addition of decorin. This is mainly due to the significant increase in the strength of the control TEVMs.

It is unknown what exactly changed to elicit this effect. The current collagen lots are not pure. Transmission electron microscopy (TEM) was completed on TEVM without added decorin. Cupromeronic blue was used to stain GAG, see Figure 75. There is significant staining of GAG in these sections, most appears to be associated with the collagen fibrils. Unfortunately, no collagen from previous lots was available for

comparison. The difference may also be related to the endogenously synthesized decorin. As seen in Chapter 5, the SMCs produce significantly more decorin when placed in the collagen gels. Once again it is unknown how this compares to SMC encapsulated in the previous lot of collagen.

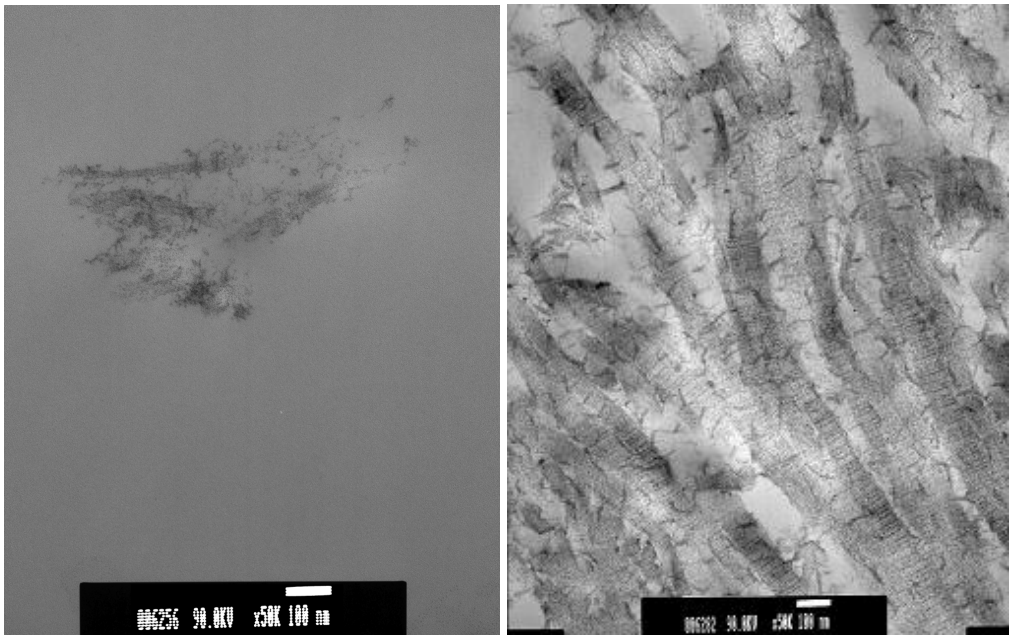

Figure 75: TEM of Collagen Layer of TEVM. Acellular Collagen Gel (Left) and Collagen Layer of 2 Week Old Rat TEVM (Right). GAG Staining is Visible in Both Pictures.

Western blots were completed to look at collagen and biglycan in the collagen hydrogels, see Figure 76. There is some slight banding at 40 kDa for both decorin and biglycan; this is the expected location of the core protein. A large band of biglycan is seen around 20 kDa.

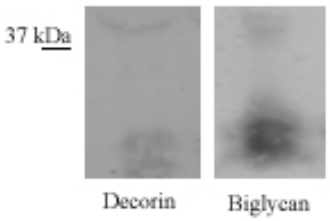

Figure 76: Western Blots for Decorin and Biglycan in Collagen Hydrogels. There is staining for decorin and biglycan in the collagen hydrogels.

Even though there weren't significant differences anymore, it was hypothesized that exogenous decorin or biglycan might affect collagen synthesis and degradation. The addition was expected to have an impact on decorin and biglycan synthesis as well. The synthesis of collagen I, decorin and biglycan were determined by PCR. Rat SMC were cultured in 2D or 3D as described in Chapter 5. Preliminary studies looked at different concentrations and denatured decorin and biglycan. No statistically significant differences were seen for concentration. Denatured proteoglycan resulted in reduced or similar gene expression (data not shown). Exogenous decorin or biglycan were added to the collagen hydrogels for 25 ng/ml concentrations; results are shown in Figure 77.

There are some statistically significant differences found with monolayer culture. Exogenous decorin increases decorin and biglycan gene expression. Biglycan increases collagen gene expression. Once in 3D culture, biglycan continues to up-regulate collagen, but only for a short time frame. By 14 weeks in culture collagen gene expression is the same between all culture groups.

Discussion

From this data it is clear that there are certain conditions in which the addition of exogenous decorin could lead to an increase in tissue strength. This increase in strength is not due to changes in gel compaction. This is consistent with the literature which showed significant differences in gel compaction with changes in endogenously synthesized decorin but not with exogenous decorin [19-21]. There appears to be an increase in collagen synthesis, but it is not as much as was seen with the addition of cyclic strain. In 4 weeks of static culture there was an almost 5 fold increase in strength, but only a 2 fold increase in collagen content which was fairly variable. With cyclic strain, 5 days of strain led to a 1.2 fold increase in strength with a 1.2 fold increase in collagen content. There are other mechanisms involved that are of interest.

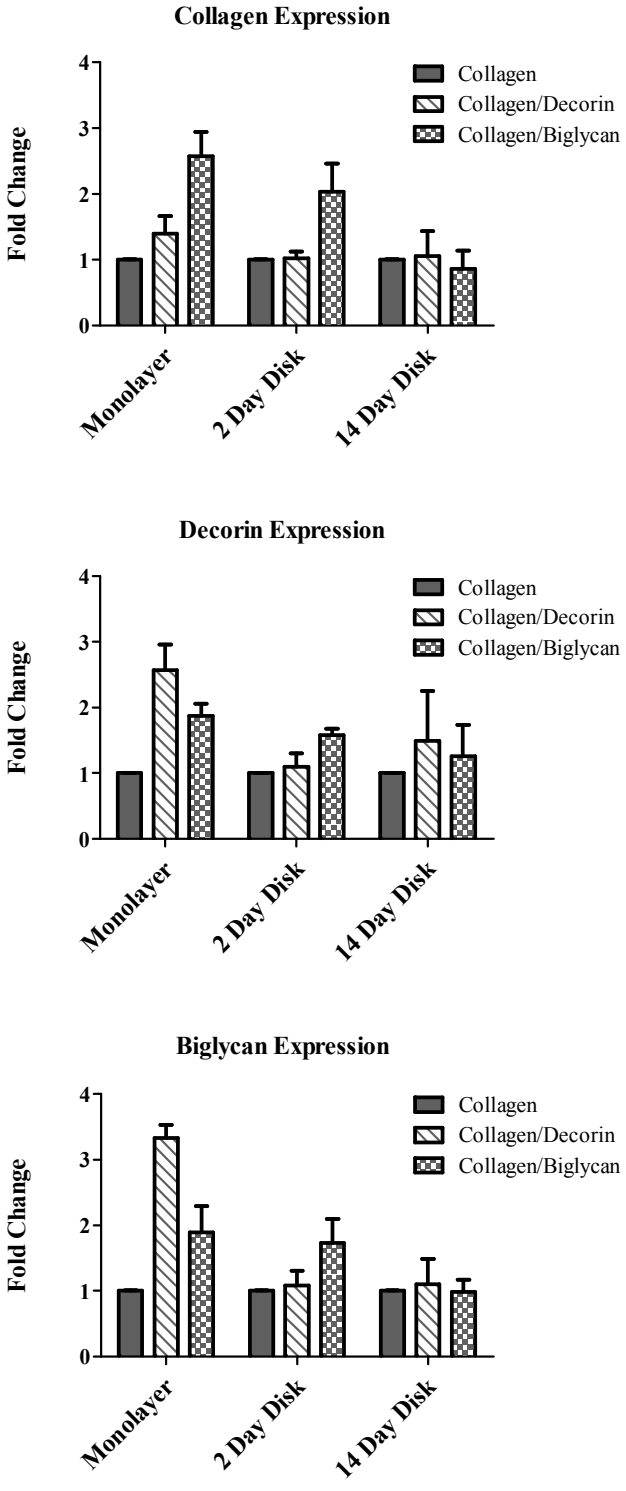

Figure 77: Effects of Decorin and Biglycan on Gene Expression. Shows Results of Exogenous Decorin or Biglycan with Monolayers or Disks. Results are shown for Collagen (Top), Decorin (Middle), and Biglycan (Bottom) Gene Expression. There are some differences in gene expression. All is lost by 14 days of hydrogel culture.

After the collagen lot change, exogenously added proteoglycan became less interesting. While both exogenous decorin and biglycan influenced gene synthesis of monolayer culture, neither significantly affected gene expression of collagen hydrogels. Biglycan leads to up-regulation of collagen, but the effect does not last. It is possible that with continued dosage it might be retained.

If additional work is done with exogenous decorin or biglycan, it is recommended that these proteoglycans be obtained by isolating them from cells that are transfected to overexpress them. Isolating decorin and biglycan from tissue can damage the proteoglycan and lead to inconsistent and incorrect results [23]. The decorin and biglycan in this study were purchased from Sigma and were isolated from bovine collagen. The source of these proteoglycans does not appear to be the cause of the change. Multiple lots were used in both sets of experiments, and the shift in results appears to be affecting the controls.

Proteoglycans are an important part of the extracellular matrix and are often overlooked, especially in tissue engineering. Decorin and biglycan play important roles in collagen fibrillogenesis. They also influence cell mediated collagen gel compaction. Both of these roles are important for tissue strength.

Acknowledgement

Sample preparation and TEM were completed at the Integrated Microscopy & Microanalytical Facility at Emory University.

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APPENDIX B: PRODUCT LISTS

Antibodies

The following is a list of antibodies that have been successfully used for immunostaining, western blotting, or flow cytometry. Note that the LiCor antibodies can also be purchased through VWR from Rockwell Immunochemical (different product #s).

Table 12: Primary Antibodies

Antigen	Description	Uses	Supplier	Product Number
α-smooth muscle actin	Mouse monoclonal	IHC	Sigma	F 3777
β-actin	Mouse monoclonal	WB	abcam	ab8226
BRDU	Mouse monoclonal	FC, IHC	Sigma	B 2531
Biglycan, mouse	Polyclonal antisera	IHC, WB	NIH, Larry Fisher	LF-113
Biglycan, bovine	Polyclonal antisera	WB	NIH, Larry Fisher	LF-96
Calponin	Goat polyclonal	IHC, FC	Santa Cruz	sc-16604
Decorin, mouse	Polyclonal antisera	IHC, WB	NIH, Larry Fisher	LF-159
Decorin, bovine	Polyclonal antisera	WB	NIH, Larry Fisher	LF-94
Myosin heavy chain	Monoclonal to full length MHC	IHC, FC	Santa Cruz	sc-6956

Table 13: Secondary Antibodies

Antigen	Description	Supplier	Product Number	Notes
Donkey anti-goat	800CW	LiCor	926-32213	1:30,000
Donkey anti-mouse	680	Alexa Fluor	A10043	Cross reacts with rabbit
Donkey anti-mouse	680	LiCor	926-32222	1:30,000
Donkey anti-rabbit	Cy5	Jackson Immuno Research	711-175-152	
Donkey anti-rabbit	Rhodamine	Jackson Immuno Research	711-025-152	
Donkey anti-rabbit	800CW	LiCor	926-32213	1:30,000
Goat anti-mouse	FITC	Jackson Immuno Research	115-095-062	
Goat anti-mouse	Rhodamine	Jackson Immuno Research	115-025-062	

Western Blotting And Zymography Products

The following are the products used for gelatin zymography and SDS-PAGE.

Table 14: Products for Zymography and SDS PAGE.

Description	Supplier	Part Number
Lysis		
RIPA Buffer	Sigma	R 0278
Proteinase Inhibitor Cocktail	Sigma	P 2714
Enzymatic Digestion		
Proteinase K	Sigma	P 2308
Chondroitinase ABC	Sigma	C 2905
Protein Quantification		
Modified Lowry Kit	Pierce	23240
Pre-cast Gels		
10% Tris-Glycine Gel	Invitrogen	EC60752BOX
10% Zymography Gelatin Gel	Invitrogen	EC6175BOX
Standards		
Kaleidoscope Prestained Standards	BioRad	161-0375
Precision Plus All Blue Standards	BioRad	161-0373
MMP-2/9 Standard	Chemicon	CC073
Buffers		
Tris-Glycine-SDS	BioRad	161-0732
Tris-Glycine	BioRad	161-0734
Renaturing Buffer	Invitrogen	LC2670
Developing Buffer	Invitrogen	LC2671
Staining		
Colloidal Blue Staining Kit	Invitrogen	LC6025
Blocking		
Near IR Blocking Buffer	VWR/Rockwell	RLMB-070

TEVM Fabrication and Culture Products

The following is a list of the products used for creation and culture of TEVM.

Table 15: Products for Construct Fabrication and Culture.

Description	Supplier	Part Number
Serum		
Bovine Growth Serum	Hyclone	SH3-542
Cells		
Aortic SMC, Human	Lonza	CC-2571
SMGM-2 Bullet Kit	Lonza	CC-3181
Scaffolds		
Collagen I	VWR/MP Biomedical	IC15002683
Fibronectin	Sigma	F1141
Thrombin	Sigma	T 7513
Biochemical Stimulation		
Biglycan	Sigma	B 8041
Decorin	Sigma	D 8428
TGF-β_1	Sigma	T 7039
TNF-α	R&D Systems	210-TS-010

PCR Products

Products for performing qRT-PCR.

Table 16: Products for qRT-PCR.

Description	Supplier	Part Number
RNA Isolation		
Qiashredder	Qiagen	79654
RNeasy Mini Kit	Qiagen	74106
RNeasy Lipid Kit	Qiagen	74804
DNase kit	Qiagen	79254
cDNA synthesis		
Superscript III First Strand	Invitrogen	18080-051
Primers		
Custom Primers	Invitrogen	
Master Mix		
Power SYBR Green	Applied Biosystems	4367659

Miscellaneous Products and Reagents

The following are other miscellaneous products and reagents that have been used for other biochemical assays or staining.

Table 17: Miscellaneous Products.

Description	Supplier	Product Number
BRDU	Boehringer Mannheim	1296 736
Cupromeronic Blue	USBio	C8210
DNA, Calf Thymus	Sigma	D 4522
Proline, ^{3}H	Perkin Elmer Life	Net483250UC