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Static and Dynamic in vitro Testing of Estrogen Coated Balloons

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STATIC AND DYNAMIC IN VITRO TESTING OF ESTROGEN COATED BALLOONS

A Thesis
Presented to
The Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Bioengineering

by
Adam Christopher Metzger
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Accepted by:
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Dr. JeoungSoo Lee
Abstract

Cardiovascular disease is the leading cause of death in the United States. Atherosclerosis is the main contributing factor for CVD. Current treatment methods for atherosclerosis include balloon angioplasty and stent implantation, which both have drawbacks associated with them. Balloon angioplasty shows restenosis in 3-6 months after deployment in 25-50% of patients. Bare metal stents have all the risks associated with implanting a medical device in a blood contacting area along with restenosis due to neointimal hyperplasia and stent fracture. In coronary arteries, drug-eluting stents (DES) have been shown to be very safe and effective with a very low percentage of major adverse cardiac events when implanted properly, but they require double antiplatelet therapy for a minimum of 1 year and have proven less effective in peripheral arteries.

In peripheral arteries stents have not been as effective because of the increased stresses put on the arteries by muscle contractions and movement. Most notably the superficial femoral artery (SFA) travels through the hunter’s or adductor canal in the quadriceps. The stress from quadriceps contractions can lead to stent fracture and ultimately dissection of the artery. To try and solve this problem drug coated balloons have tried to combine the positive qualities of DES for localized anti-proliferative drug delivery with angioplasty balloons that do not leave foreign materials in the body. Estrogen is one such anti-proliferative drug that has been shown to inhibit smooth muscle cell proliferation in vitro and in vivo.

To study the safety and efficacy of an estrogen-coated balloon in vitro static studies were performed. These studies used static weight simulations to
approximate an estrogen-coated balloon expansion. Cells were then analyzed to examine the simulation’s effect on cell proliferation, apoptosis, hypertrophy, and phenotype. The studies statistically supported that estrogen treated balloon angioplasty simulation had lower cell proliferation than uncoated balloon angioplasty simulation and the control. The estrogen treated balloon angioplasty simulation also showed no statistical significant differences in the amount of apoptosis, hypertrophy, or phenotypic change when compared to uncoated balloon angioplasty simulation.

A dynamic vascular flow model was designed to replicate an interventional cardiologist’s path from balloon insertion to deployment in the SFA. This model will be used to analyze coating techniques and drug lost from the balloon surface while the balloon is in transit to the site of deployment. The flow model has the ability to be modified to simulate varying amounts of vessel stenosis.
Acknowledgements

I would like to acknowledge the Clemson University Department of Bioengineering as well as several individuals who have contributed to the expansion of my academic knowledge and research skills. Firstly, I would like to thank Dr. Martine LaBerge, my advisor, who has guided me to becoming a successful graduate student. I would also like to thank my committee members for their feedback and support that helped to shape my project. I want to extend special thanks to Jayesh Betala for teaching me how to research properly and always being a constant support throughout the entirety of my work. Finally, I would like to thank everyone who has supported this project including Chadd Clark, Andrew Hill, Xin Xie, Pooja Panigrahi, Cassie Gregory, Dr. Bruce Gao, my family and Clemson Machining and Technical services.
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CHAPTER I

Introduction and Background:

1.1 Atherosclerosis:

Coronary artery disease (CAD) is the most common form of heart disease in the United States, affecting greater than 16 million people [1]. CAD forms as a result of atherosclerosis, where plaque forms in the arteries causing the artery to narrow. Atherosclerosis is a disease that occurs over time with fibrofatty plaque growing in the vascular lumens, obstructing blood flow, and weakening the underlying media [2]. Atherosclerosis can begin in the first decade of a patient’s life and is clinically silent while it grows [2]. Atherosclerosis is a chronic inflammatory response of the arterial wall that is initiated when the endothelium is injured [2]. The major consequences of atherosclerosis are cerebral infarction, myocardial infarction, and peripheral vascular disease [2]. The plaque found in an atherosclerotic vessel usually consists of a necrotic center that contains cholesterol crystals, foam cells, cell debris, and calcium [2]. The vascular media and a fibrous cap that protrudes into the vessel lumen encapsulate the necrotic center. The fibrous cap consists of macrophages, foam cells, collagen, vascular smooth muscle cells, and elastin among other things [2].
The environmental risk factors of atherosclerosis include hypertension, hemodynamics, viruses, smoking, lipid rich diets, toxins, and immune reactions [2]. Genetics also plays a large role in the formation of stenotic vessels with high levels of LDL, reduced levels of HDL, diabetes, and obesity all being risk factors mainly attributed to a patient’s genes [2]. Atherosclerosis can be reversible if the patient begins a diet and exercise routine along with smoking cessation (if applicable) and treatment for any diseases that increase the risk of atherosclerosis [2].

Atherosclerosis formation begins as a fatty streak, which consists predominantly of lipid-filled macrophages, vascular smooth muscle cells, and T-lymphocytes [3,4]. Fatty streaks are thought to form by the retention of lipoproteins in the endothelial and the lipoproteins undergoing modification (thought to be caused by oxidation of the lipoprotein) once there [5]. Monocytes then begin to adhere to the endothelium where the modified lipoproteins are and via chemotaxis signal more lymphocytes to the area. Once monocytes accumulate in the
subendothelial space, they differentiate into macrophages. The macrophages begin to uptake excess lipids and form foam cells [5].

The accumulation of foam cells and other lymphocytes will continue to grow over time and thus enter the tunica media of the vessel where vascular smooth muscle cells (VSMCs) reside [5]. This amount of vascular injury leads to VSMCs to migrating out of the tunica media [6]. The migration of VSMCs out of the tunica media leads to fatty streaks becoming fibrofatty lesions [5]. Once outside of their normal location, VSMCs change from the contractile phenotype to the synthetic phenotype. VSMCs in the synthetic phenotype begin to proliferate rapidly and lay down a vast network of extracellular matrix [6].

1.2 Estrogen:

It has been shown that premenopausal women have a lower rate of atherosclerosis than men of the same age [5]. There have been numerous human and animal studies that have documented a statistically significant gender difference in the occurrence of CVD [5]. In the Framingham Study, Kannel and collaborators [7] showed that below the age of 60 men develop CVD at two times the rate of women [5]. The same study also showed that postmenopausal women have almost twice the incidence of CVD when compared to premenopausal women [5]. These findings lead to the proposal that estrogen plays a key role in preventing atherosclerosis formation.

There have been studies that suggest that since the mortality rates for women in their sixties or seventies are still significantly lower than that of men of
the same age, that the hypothesis that estrogen protects women from CVD is false [8]. Since atherosclerosis is a slow progressing disease, it makes since that if estrogen exhibited a protective effect against plaque formation a swift rise in CVD rates would not be seen very soon after menopause [5]. Also estrogen production in the ovaries declines slowly over a 15-20 year period [5]. The fact that menopause is judged to begin when menstruation ends [7], the classification of going from premenopausal to postmenopausal does not take into account this gradual estrogen decline.

The studies that claimed estrogen was not involved in the decreased CVD in women are also refuted by the studies conducted with postmenopausal women and animals receiving estrogen replacement therapy [5]. Adams and collaborators performed multiple studies documenting the protective effects of estrogen on primates. In one study, primates were subjected to an ovariectomy and fed an atherogenic diet, with half of the primates receiving estrogen treatments. The primates that were not given the estrogen had twice as much atherosclerosis formation when compared to the estrogen treated primates [9]. In humans, Stampfer and Colditz performed a meta-analysis that estimated the relative risk for patients receiving estrogen replacement therapy was 0.56 (95% confidence interval) when compared with patients not receiving the treatment [10]

There have also been multiple in vivo animal studies on multiple different species that demonstrate the formation of neointimal hyperplasia that occurs after balloon expansion in the vessel is diminished in the presence of estrogen, while without estrogen there is a marked increase [6]. 17β-estradiol has been shown to
have many favorable cardio-protective attributes that include: inhibition of vascular smooth muscle cells migration and proliferation, relaxation of the coronary vessels through endothelial NO synthase activity, and reduction of monocyte and platelet aggregation in the vessel [6].

The cardio-protective effects of estrogen are induced by altering mitogen-activated protein kinase (MAPK) cascades [6]. The specific pathways that estrogen interferes with involve MAPK p38 and p42/44 [6]. The p38 MAPK pathways are involved in the mitogenic and chemotactic processes carried out by an array of different cell types [6].

It has also been shown that males carry the same estrogen receptor on their vascular smooth muscle cells as females [11]. While Espinosa et al showed there was a decreased response to estrogen treatments in male rats, when compared to females of the same age, they still showed that there was an anti-proliferative effect and, through western blot, proved the existence of the estrogen receptor in male vascular smooth muscle cells [11].

1.3 Balloon Angioplasty:

Dr. Charles Dotter performed the first balloon angioplasty in 1964. He invented the technique and procedure for an 82-year old patient with severe peripheral vascular disease who refused amputation of her gangrenous leg [12]. Dr. Dotter used a guide wire and coaxial Teflon catheters to dilate the stenosis of the superficial femoral artery [12]. The artery stayed open for the remaining two and a half years of the patient’s life [12].
It was not until 1977 that balloon angioplasty was used on a waking patient (as is done today) in a coronary artery [12]. Balloon angioplasty has drastically improved from its early beginnings. Doctors used to perform the procedure without the use of guide wires and the balloons had low burst pressures and were quite large compared to today’s models [13]. Because of the hindrances of early angioplasty technology and the complicated geometry and nature of vessels containing stenosis, only 10% of the patient population was able to get this procedure [13].

Balloon angioplasty is usually done today by inserting a deflated balloon on a catheter into the femoral or radial artery on a guide wire and inflating the balloon repeatedly in the area of stenosis. When the balloon inflates, it depresses the plaque against the artery, which reopens the artery [1].

Figure 2: Diagram showing a balloon angioplasty procedure [14].
Balloon angioplasty increases the flow through the formerly occluded artery, but there is a restenosis rate of 25-50% within the first six months after the procedure [13].

1.4 Neointimal Hyperplasia:

Restenosis of vessels after balloon angioplasty normally occurs as the result of neointimal hyperplasia. When the balloon is inflated the walls of the vessel become damaged and neointimal hyperplasia is the injury and immune response to the vascular injury. Growth factors, cytokines, metalloproteases, and upregulation of genes, among other factors, cause smooth muscle cell proliferation, activation of platelets and macrophages, remodeling of the extracellular matrix, and smooth muscle cell migration into the vascular lumen [15]. Once the vascular smooth muscle cells have entered the artery lumen, they begin to change their phenotypes from a contractile morphology to a synthetic one [15]. Once in the synthetic phenotype, vascular smooth muscle cells begin to proliferate and spread extracellular matrix [15]. The most effective manner or altering the postoperative natural healing process is interfering with molecular cell division [15].
1.5 Peripheral Vascular Disease:

Peripheral vascular (arterial) disease (PVD or PAD) occurs when there is an occlusion in a vessel that is not a coronary artery, in the brain, or the aortic arch [16]. While most peripheral occlusions occur in the lower extremities, there are also many cases involving the renal arteries, among others. PVD patients normally present with claudication (described as pain, numbness or soreness felt during or after walking), which is a symptom of critical limb ischemia (CLI) [16]. CLI in the femoro-popliteal area normally presents with long, diffuse lesions and occlusions [16]. If PVD is left untreated the usual outcome is necrosis of the downstream tissue lead to the need for amputation [16].

1.6 Drug-Eluting Stents:

When drug-eluting stents (DES) were first explored, the main concern was lowering the rate of stent thrombosis in bare metal stents, which were at high rates of 20-25% [15]. To combat these rates anticoagulant coated stents were developed. Heparin-coated stents represent the first step to loading medication on to stents [15]. These stents failed however, as they were developed at the same time as improvements to stenting techniques and stenting technology, as well as the introduction of effective antiplatelet drugs. These advancements in stent placement technology and drug therapy yielded the same stent thrombus rate improvement at a lower price [17].
Stopping cell division required the use an anti-proliferative drug, but there was the challenge of having the drug remain localized in a dynamic environment. The body’s blood flows in a closed loop throughout the body at a high rate of speed, so getting the drug to stay in the small area of blood vessel desired could not be achieved by current delivery methods. The previous work on heparin-coated stents provided the answer to this problem, but to make this technique work there were many potential pitfalls (Table 1) that had to be avoided [15].

<table>
<thead>
<tr>
<th>Pitfalls</th>
<th>Variables</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bio and blood compatibility</td>
<td>Physiochemical properties of polymer and drug</td>
</tr>
<tr>
<td>Limited surface area</td>
<td>Drug potency; total amount of drug</td>
</tr>
<tr>
<td>Maintain drug properties after coating</td>
<td>Degree of cross-linking</td>
</tr>
<tr>
<td>Heterogeneous underlying tissue</td>
<td>Drug solubility</td>
</tr>
<tr>
<td>characteristics</td>
<td></td>
</tr>
<tr>
<td>Sterilization and stent expansion</td>
<td>Polymer and drug elasticity</td>
</tr>
<tr>
<td>Inflammation</td>
<td>Porosity of the polymer, molecular weight of the polymer, thickness of</td>
</tr>
<tr>
<td></td>
<td>the coating, degree and mode of degradation; drug toxicity; local drug</td>
</tr>
<tr>
<td></td>
<td>concentration (per mm²); drug solubility</td>
</tr>
</tbody>
</table>

Table 1: Table describing the pitfalls and variables associated with drug-eluting stents [15].

The two main drugs used in drug eluting stents are Sirolimus (also known as rapamycin) and Paclitaxel. Sirolimus is a macrolide antibiotic that has anti-inflammatory, immunosuppressive, and anti-proliferative properties that make it very useful in preventing neointimal hyperplasia by inhibiting the proliferative and
migratory response of smooth muscle cells at the site of stent deployment [18]. The drug inhibits the transition from the G1 phase to the S phase in the cell cycle, thus blocking proliferation without inducing cell death [18]. Paclitaxel is a mitotic inhibitor that stops proliferation by stabilizing cell’s microtubules [18]. This stabilizing of the microtubules does not allow them to breakdown, which is necessary for cell division [18].

While drug-eluting stents have greatly improved atherosclerosis treatment, there are still areas where they are not as useful and in some cases dangerous. In peripheral areas where high stresses are put on the arteries, and thus also any stents implanted in these areas, there is a risk of stent fracture [19]. The superficial femoral artery (SFA) travels through the Hunter’s (aka Adductor) canal. This runs through the quadriceps and experiences high loading and movement during normal daily activities [19]. There are many reported cases of stent fractures in this area [19]. When the stent fractures it can lead to either to occlusion of the stented site, or, in severe cases, the stent wires can pierce the vessel wall and lead to dissection of the artery [19].

1.7 Drug-Coated Balloons:

The shortfalls of stenting (with drug-eluting or bare metals stents) have lead to resurgence in research on drug-coated balloons (DCB) [20]. Using drug-coated balloons, the physician can maintain the anti-proliferative effects of drug-eluting stents without the limitations associated with leaving a stent in the vasculature. Drug-coated balloons can be used to treat areas where DES do not perform well,
such as, small vessels, where scaffolding obstructs major side branches, bifurcated lesions, or long diffuse calcified lesions [20]. Another advantage over DES is not leaving polymer implants that have the possibility of causing chronic inflammation and late thrombosis [21]. DCB can also be used concurrently with stents. DCB have the potential to repair in stent restenosis (ISR) that is seen in bare metal stents and some DES, as well as can be used for drug delivery while expanding a bare metal stent [20].

There are also additional potential advantages to DCB when compared to DES (Table 2) including eliminating the need for sustained double anti-platelet therapy, drug transfer to the entire vessel wall instead of just around stent struts, rapid release of high concentrations of drug, and no stent left in the vessel that can alter blood flow patterns [20]. The one challenge that DCB cannot overcome is the acute vessel recoil that occurs after balloon deployment because there is no stent framework to hold the vessel fully open [20].

<table>
<thead>
<tr>
<th></th>
<th>Drug-Eluting Stents</th>
<th>Drug Coated Balloons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platform of Delivery</td>
<td>Stent scaffolding</td>
<td>Balloon</td>
</tr>
<tr>
<td>Retention</td>
<td>Polymer based</td>
<td>Embedded/Imprinted</td>
</tr>
<tr>
<td>Drug Dose</td>
<td>Low: &lt;100 to 200μg</td>
<td>High: 300 to 600μg</td>
</tr>
<tr>
<td>Release Kinetics</td>
<td>Slow and controlled</td>
<td>Fast release</td>
</tr>
<tr>
<td>Distribution</td>
<td>Strut-based vascular penetration</td>
<td>Balloon surface homogenous distribution</td>
</tr>
<tr>
<td>Advantages</td>
<td>Mechanical Support Abluminal trapping</td>
<td>Leave no implant</td>
</tr>
<tr>
<td></td>
<td>Less drug spillage into circulation</td>
<td>Larger surface area</td>
</tr>
<tr>
<td></td>
<td>Proven efficacy in many</td>
<td>Less drug localization in the vessel wall</td>
</tr>
<tr>
<td></td>
<td>Accessible to complex</td>
<td></td>
</tr>
</tbody>
</table>
indications
No acute recoil tackled dissection
lesions and long segments
May not require prolonged DAPT

Table 2: Comparison of Drug-Eluting Stents and Drug Coated Balloons [Adapted from 20].

For a drug-coated balloon to be effective, there are many biological and technical considerations that must be met (Table 3).

<table>
<thead>
<tr>
<th>Drug Features</th>
<th>Coating Features</th>
<th>Delivery Features</th>
<th>Catheter Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical structure of the drug</td>
<td>Homogenous coating thickness</td>
<td>Rapid drug transfer (&lt;60s)</td>
<td>Optimal vessel wall contact on delivery</td>
</tr>
<tr>
<td>Sustained biological effect at the nM level</td>
<td>Homogenous drug concentration throughout surface</td>
<td>Homogenous and precise drug transfer</td>
<td>Minimal drug loss during catheter transit and inflation</td>
</tr>
<tr>
<td></td>
<td>Controlled drug concentration within a predetermined nominal range</td>
<td>Efficient (high transfer, low loss ratio)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Coating fragmentation into submicron particles upon inflation</td>
<td>Rapid dissolution of the coating on vessel contact</td>
<td></td>
</tr>
</tbody>
</table>

Table 3: Considerations to make a DCB successful [Adapted from 22].

The delivery system used for DCB is very important to their actual efficacy at treating atherosclerotic vessels. If the drug and balloon meet all the consideration in Table 3, a poor catheter delivery design will ruin the balloon. The balloons have to
be able to keep the drug attached to it during transport and then rapidly release the drug upon deployment [22]. Current technologies have followed two main strategies to ensure there are enough drug particles remaining on the balloon to achieve the anti-proliferative effects desired [17]. The two techniques are to either place a sheath around the balloon that is slid back once the deployment site is reached, and the other is to load a higher concentration of drug than required with the expectation of drug being lost in transit [17].

1.8 Research Aims:

This study was aimed at testing the efficacy of estrogen treatment on vascular smooth muscle cells in balloon angioplasty conditions. The three objectives for this project were to (1) analyze the antiproliferative effects of estrogen on VSMCs, (2) examine the reaction of VSMCs to a simulation of an estrogen treated balloon expansion, and (3) design a dynamic vascular flow model to test drug retention on a balloon in transit to deployment site.
CHAPTER II

Materials & Methods:

2.1 Cell Characterization:

Vascular smooth muscle cells were isolated from the aorta of a 6-8 week old female Sprague-Dawley rat. All experiments were conducted on VSMCs between passage 3 and passage 6 before use [23]. The cells were characterized to confirm that they were VSMCs. Western Blot was used to confirm the phenotype of VSMCs through characterization of myosin heavy chain and α-actin. The cells were also immunostained with Phalloidin to confirm the presence of α-actin and the myosin heavy chain was tagged with GFP [24].

2.2 Estrogen Studies:

VSMCs were seeded in a collagen-coated 24-well plate with 2*10^4 VSMCs per well. After 24 hours in Dulbecco’s modified Eagle medium (DMEM) (Gibco, Life technologies, CA, USA) with 10% fetal bovine serum (FBS) (Corning Inc, NY) 1% antibiotic-antimicrobial (Ab-Am) (Corning Inc, NY, USA) solution, without phenol red, the VSMCs were rinsed with 1x sterile Dulbecco’s phosphate buffer solution (DPBS) (Corning Inc, NY, USA) and then growth arrested in DMEM solution with no FBS for 48 hours. VSMCs were then rinsed in 1x DPBS and then placed in fresh DMEM with no FBS. VSMCs were then treated with 20 μM 17-β estradiol (Sigma Aldrich, MO, USA) for 5 (n=4), 15 (n=4), and 30 (n=4) minutes, with solvent controls containing an equal amount of dimethyl sulfoxide (DMSO) (Thermo Fisher, MA,
USA) as the treatment group (n=4), and with DMEM solution with no FBS control (n=4). After treatment the wells were rinsed with 1x DPBS and then placed in DMEM solution with 1% FBS. VSMC proliferation was analyzed using methylthiazol tetrazolium (MTT) (Sigma Aldrich, MO, USA) assay after 24 hours.

2.3 MTT Assay:

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was dissolved in 1x DPBS at a concentration of 2mg/mL. VSMCs were rinsed with 1x DPBS and then placed in 1ml of DMEM solution with no FBS. 240μl of MTT was added to the wells and the solution incubated for 4 hours. After incubation, the MTT-DMEM solution was removed and 1mL of DMSO was added to the wells. Using a plate reader, the absorbance was then read at 570nm.

2.4 Static Contact Study:

For static studies, 0.15-inch thick silicone sheeting (Specialty manufacturing Inc, MI, USA) were sonicated in deionized water for 30 minutes and then autoclaved. The silicone sheeting was then placed at the bottom of each well in a 12-well plate. The sheets were then coated in with a 1.5ml of 100μg/ml collagen type 1 (Purecol® 3mg/ml, Advanced Biomatrix, CA, USA) solution for at least 48 hours in a 37°C incubator. The sheets were then rinsed with 1x DPBS and wells were filled with DMEM with 10% FBS and then seeded the wells with $2 \times 10^5$ VSMCs per well. After 24 hours, the wells were rinsed in 1x DPBS. Six of the seeded sheets were placed in DMEM with 5% FBS while three wells placed in a 20.25% iopromide 370
(Ultravist®-370mg/l/ml, Bayer Medical, Germany) solution with 40μM 17-β estradiol in DMEM with 5% FBS and dissolved in deionized water. Iopromide is used as an effective solvent for lipophilic drug dissolution, allows for easier coating of the balloon, and promotes smooth muscle cell inhibition [22]. The Iopromide concentration used came from previous studies done by another lab member that has yet to be published. A polycarbonate indenter was then used as a platform to apply weight to the cells for mimicking the pressure VSMCs feel during balloon angioplasty inflation (Figure 4). Polyethylene Terephthalate (PET) sheet was placed on the end of the indenter to represent the material of the balloon. An indenter loaded with a 500g (4.9N) weight was placed in each of three wells for one minute. The remaining three wells had an indenter with a PET sheet only without 500g) placed on the cells for one minute. All the experiments were repeated at least 3 times for 24 and 72 hour results that were examined by MTT assay, Tunel apoptosis assay (Promega) and hypertrophy studies. For the 72 hour studies all of the wells were rinsed with 1x DPBS and then filled with DMEM with 5% FBS after 24 hours.

![Figure 4: On left: SolidWorks representation of polycarbonate indenter. On right: SolidWorks representation of indenter on VSMC seeded silicone sheet in a 12-well plate. The indenter consists of](image)
a cylinder piece (diameter 20mm and height 30mm) and a disc piece (10 mm thick with 50mm diameter and 20.5mm diameter slot for cylinder piece to fit in that is 5mm deep)

For the static weight study we used a simulation performed by Rogers and collaborators detailing the forces that VSMCs are subjected to during balloon angioplasty [25]. Using a 2-dimensional finite element analysis (FEA), they took a cross section of an artery and showed the forces that a balloon places on a vessel when it is inflated at 8atm pressure. The FEA analysis showed that VSMCs experience between 0.004 and 0.04 N/mm$^2$ depending on their position relative balloon (as shown in Figure 16) [25]. Using these values we calculated that to simulate this pressure on VSMCs in a well plate (well bottom area of 3.83 cm$^2$) we would need to put 156 to 1560g of weight on the cells. We used 500g (plus the 32.78g weight of the two indenter pieces combined) as our weight to closely simulate the average forces that would be experienced when a balloon is inflated at 8atm.
2.5 Tunel Apoptosis Assay:

VSMCs were rinsed in 1x DPBS and then fixed in 4% formaldehyde solution (Sigma Aldrich MO, USA) for 20 minutes. VSMCs were rinsed with 1x DPBS for 5 minutes. VSMCs were permeabilized in a 0.2% Triton X-100 (Thermofisher, MA, USA) solution in DPBS for 5 minutes. VSMCs were rinsed with 1x DPBS for 5 minutes. VSMCs were then equilibrated in 100μl of Equilibration Buffer for 5 minutes. The Equilibration Buffer was removed and 50μl of Terminal Deoxynucleotidyl Transferase, Recombinant, enzyme (rTdT) incubation buffer was added. The rTdT incubation buffer consisted of 45μl of Equilibration Buffer, 5μl of
Nucleotide Mix, and 1μL of rTdT Enzyme. VSMCs were then covered in aluminum foil and incubated at 37°C for 60 minutes. 20X saline sodium citrate (SSC) was diluted in deionized water to 1X SSC and the cells were immersed in the solution for 15 minutes to terminate the reaction. VSMCs were rinsed with 1x DPBS three times for 5 minutes each. All the cells were then stained with DAPI. The silicone sheets were then mounted onto slides using Vectashield (Vector laboratories CA, USA) and the edges were sealed and examined using a fluorescence microscope. Four pictures were taken for each sample and a ratio between apoptotic cells and all cells were analyzed using ImageJ (NIH). The ratio was compared between treatment and control group for 24 and 72-hour studies.

2.6 Cell Hypertrophy:

VSMCs were rinsed in 1x DPBS and then fixed in 4% formaldehyde for 20 minutes. VSMCs were rinsed with 1x DPBS for 5 minutes. Then the cells were permeabilized in 0.2% Triton X-100 solution in PBS for 5 minutes. VSMCs were rinsed with 1x DPBS for 5 minutes. The cells were then stained with 25μg/ml Phalloidin (500ug/ml. Sigma Aldrich, MO, USA) and nuclei were stained with DAPI. The silicone sheets were then mounted onto slides using Vectashield, edges were sealed and examined using a fluorescence microscope. Four pictures were taken for each sample and the cells were measured using ImageJ for area and length of major and minor axes. These were then compared between treatment and control for 24 and 72-hour studies.
2.7 Drug Coating Study:

Sheets of PET were coated with three different solutions and examined under fluorescence microscopy. The first coating was made by dissolving a 10mg/ml 17-β estradiol-10% Iopromide mixture in 100% ethanol as a solvent. The Iopromide concentration used came from previous studies done by another lab member that has yet to be published. The solution was then pipetted onto the PET and allowed to dissolve. This same procedure was repeated with acetone as the solvent and also with 10mg/ml estrogen dissolved in 100% ethanol. The coated PET sheets were examined using fluorescence microscopy of the autofluorescent estrogen to determine which coating left the most homogenous distribution of drug on the PET.

2.8 Statistical Analysis:

For statistical analysis student’s two-tailed T-test was performed using GraphPad Prism (GraphPad Software Inc, CA, USA) with p<0.05 indicating statistical significance.
CHAPTER III

Results:

Once the cells were isolated from the rat aorta they were characterized to determine if they were VSMCs by Western Blot and Immunostaining Myosin Heavy Chain (Figure 5) and α-actin (Figure 6)

![Figure 6: Expression of Myosin Heavy Chain in smooth muscle cells shown by Western Blot and Immunostaining (length of scale bar is 400nm)](image)

![Figure 7: Expression of α-actin in smooth muscle cells shown by Western Blot and Immunostaining (length of scale bar is 400nm)](image)

In the treatment of VSMCs with 20 μM 17-β estradiol (Figure 7) we had n=16 for each treatment and control. The findings of the experiment showed statistical significance with p<0.05.
Figure 8: Percentage of viable cells after treatment with 20 μM 17-β estradiol at differing time points when compared to control

The static weight studies performed simulated the pressure VSMCs feel during balloon expansion. In both the 24 and 72-hour studies, statistically significant differences were observed with p=0.0013 between treated treatment and the untreated control (simulation of normal balloon angioplasty) at 24 hours (Figure 8) and p=0.0001 at 72 hours (Figure 9). The comparison between treatment and the indenter control (only weight of indenter placed on cells) also showed
statistical significance at 24 and 72 hours (p=0.0003 and p=0.01 respectively).

![Graph showing cell viability at 24 hours](image1)

**Figure 9**: MTT assay results for cell viability after 24 hours (p<0.05 for both Treated compared to Control (Untreated) and Treated compared to Indenter Control)

![Graph showing cell viability at 72 hours](image2)

**Figure 10**: MTT assay results for cell viability after 72 hours (p<0.05 for both Treated compared to Control (Untreated) and Treated compared to Indenter Control)
In the TUNEL Apoptotic assays, over 1000 cells in the treatment were compared to control groups to determine any statistically significant difference in the amount of apoptosis at 24 and 72 hours (Figure 10 and 11 respectively). Neither were proven significantly different with p=0.1814 in the 24-hour study and p=0.2125 in the 72-hour study. Cell count analysis was performed in ImageJ with the images from the fluorescence microscope (Figure 12).

Figure 11: The percentage of cells that were apoptotic compared to total cells in treated simulation (treatment) (n=1060) and untreated simulation (control) (n=1490)

Figure 12: The percentage of cells that were apoptotic compared to total cells in treated simulation (treatment) (n=1180) and untreated simulation (control) (n=1304)
Figure 13: Fluorescent microscopy images showing DAPI stained nuclei (top left), GFP tagged apoptotic cells (top right), and an overlay of the two.

The hypertrophic studies were completed with looking at more than 125 different cells in each group and examining their area. The analysis showed no statistical difference with $p=0.0823$ for the 24-hour study (Figure 13) and $p=0.0806$ for the 72-hour study (Figure 14). The treated and untreated group did both show consistency in the average cell area between the 24 and 72-hour studies. The study of major and minor axes (Figure 15 and 16) were used to determine if VSMCs were
in contractile or synthetic phenotype, with aspect ratios (length of major axis/length of minor axis) closer to 1 indicating more synthetic phenotype cells.

**Figure 14:** Study to compare average cell area and look for cell hypertrophy between treated simulation (treatment) (n=151) and untreated simulation (control) (n=188)

**Figure 15:** Study to compare average cell area and look for cell hypertrophy between treated simulation (treatment) (n=125) and untreated simulation (control) (n=150)
Images from the fluorescence microscope (Figure 17) were analyzed in ImageJ to measure the cell area and lengths of major and minor axes.
Figure 18: Fluorescence microscopy photo of VSMCs with actin filaments tagged with Phalloidin and nuclei stained with DAPI.
Figure 19: Fluorescence microscopy pictures of different coating techniques.

The representation of different coating solvents and solutions (Figure 18) was done with light microscopy (left column), fluorescent microscopy (middle column) and overlays (right column) to examine homogenous estrogen coating of the PET sheets. The figure shows estrogen and iopromide dissolved in 100% ethanol (top row) and acetone (2nd row) as well as, estrogen alone dissolved in 100% ethanol (3rd row) and an uncoated sheet (bottom row).
CHAPTER IV

Discussion:

Both Western Blot and Immunostaining show the presence of α-actin and myosin heavy chain. Their presence confirms that the cells are VSMCs.

MTT assay measures the amount that is converted to formazan crystals by living cells [26]. This conversion occurs within the cell's mitochondria, thus giving us a measure of the cell viability [26].

In 24-hour study of the antiproliferative effects of 17-β estradiol on VSMCs we can see that at 5 minutes in 20 μM 17-β estradiol there are already almost a 20% decrease in the number of cells (81.77% viability). This number also does not lower significantly at 15 or even 30 minutes (76.02% and 77.4% respectively) showing that the 17-β estradiol has an antiproliferative effect on its own and in a short period of time. This data has been shown to be statistically significant (p<0.05) with a sample size of n=16 used for each data point.

The results of our 24 and 72-hour studies (shown in figures 8 and 9 respectively) indicate that both the cells that were exposed to a simulation of treated balloon expansion and the cells exposed to a simulation of untreated balloon expansion incurred cell detachment leading to a lower number of viable cells when compared to the indenter control group (cells placed only under the weight of the indenter). However, 72 hours after balloon expansion simulation was completed, the untreated treatment group had proliferated to a much higher of number viable cells when compared to the control, while the cells that had undergone simulation of
an estrogen-Iopromide treated balloon expansion, was still less than the indenter control. The marked increase in the amount of cells in the untreated group show that without antiproliferative drugs, VSMCs respond to the injury incurred by balloon expansion with an increased rate of cell proliferation. *In vivo* this quick proliferation of cells would lead to restenosis of the vessel through neointimal hyperplasia. While this study does not show the vascular recoil response that is normally associated with balloon angioplasty and causes early partial reocclusion of the vessel, it does give a clear indication that balloons coated with anti-proliferative drug will help stem neointimal hyperplasia.

The DeadEnd™ Fluorometric TUNEL assay detects and quantifies apoptotic cells within a cell population by measuring the fragmented DNA of apoptotic cells [27]. This assay uses rTdT to label fragmented DNA with fluorescein-12-dUTP at the 3’-OH ends. The labeled DNA can then be observed using fluorescence microscopy and then the number of apoptotic cells can be compared to the total number of cells (non-apoptotic cells tagged with DAPI). The apoptotic assay shows no statistical difference in the percentage of apoptotic cells when comparing treated balloon expansion simulation to untreated at 24 and 72 hours (p-values= 0.1814 and 0.2125 respectively). This tells us that our estrogen-Iopromide treatment causes no more apoptosis than untreated balloon angioplasty.

For hypertrophy study we were looking to ensure that the estrogen-Iopromide treatment did not cause any increase cell growth as compared to untreated balloon angioplasty. The Phalloidin binds to the polymerized actin in the cells that helps determine area of the cell and major and minor axes. Using 12
pictures from each sample group, the cells were measured using Image J (NIH) to find the cell area along with the lengths of the major and minor axes. The results displayed in figures 13 and 14 showed that there was no statistical difference in the area of the cells at 24 hours (p=0.0823) or at 72 hours (p=0.0806). VSMCs that have transitioned from the contractile phenotype to the synthetic phenotype are normally more rounded and have an aspect ratio closer to 1. The aspect ratio comparison shows that the cells that were in the treated group at 24 hours had an aspect ratio closer to one, while untreated group had a higher aspect ratio hence showing more contractility than treated group. However, after 72 hours the treated group had a higher aspect ratio that was closer to untreated control group.

The fluorescence microscopy pictures of our three coating techniques showed that the 17-β estradiol-Iopromide mixture dissolved in 100% ethanol gave the most uniform distribution of drug on the PET sheets. The estrogen auto-fluoresces at the same wavelength as DAPI at an emission wavelength of 461nm.
CHAPTER V

Conclusions and Recommendations:

The use of antiproliferative agents to aid in interventional cardiology has been well documented to solve many of the problems associated with balloon angioplasty in the coronary and peripheral arteries. While uncoated have not been as successful at keeping a vessel open, the addition of antiproliferative agents can stem the neointimal hyperplasia that causes reocclusion. Drug-coated balloons as of yet have not been able to solve the vascular recoil after balloon deflation, and as such are not yet the best option for most cases to use alone. As of now, drug-coated balloons are shown to be effective to fight in-stent restenosis, deployment of bare metal stents and to open the lumen of areas where it is dangerous and/or difficult to place stents for longer periods than uncoated balloons. The estrogen-Iopromide coating we developed has shown signs that it can compete with alternatives such as Paclitaxel and other drugs used on balloon on the market in Europe and undergoing FDA testing in the United States. These studies have shown that our coating is effective at stemming VSMC proliferation at low doses and short release times without cause anymore apoptosis or cell hypertrophy than uncoated balloons.

For further exploration of the effects on estrogen alone, studies will be carried out with more time after treatment before analysis. One and two week studies will help to show how long the antiproliferative effects of estrogen are felt.
To test a DCB and determine how much drug is lost and if a sheath should be used to protect a DCB we designed a novel dynamic flow model to simulate the pathway an interventional cardiologist would traverse when deploying a DCB in the superficial femoral artery.

The superficial femoral artery is one of the top areas that interventional cardiologists and vascular surgeons are looking at the use of drug-coated balloons [9]. The SFA is an area where atherosclerosis occurs frequently and the high stresses placed on the artery make it difficult to stent without complications [9]. For our dynamic flow model design we will have all the arteries that are involved in balloon deployment in the SFA. The balloon will be inserted in the iliac artery and be guided up to where the thoracic aorta splits into the iliac arteries. From there, the balloon traverses the opposite iliac artery and then continues to the femoral bifurcation. It will then enter the superficial femoral artery. To ensure a proper simulation of the rate of blood flow in these areas we have used Bernoulli’s energy balance equation as well as taking into account the conservation of mass. We have sized all of our tubing to mimic that of the vessels used. These sizes include inner diameters: thoracic aorta at 10mm, common iliac artery at 8mm, and the superficial femoral artery at 6mm.
For our experiments, we will use a Cole-Parmer peristaltic pump (Cole-Parmer, IL, USA) to circulate the blood analog. We will use this device to achieve the 400-600 mL/min flow rate normally seen in the SFA. All of the artery analogs in the model will empty into a catch pan where the pump will recirculate it. This will ensure that any drug lost in transit to the deployment site will be captured in the cycle and we can then analyze the amount lost through HPLC. The tubing we chose to mimic the iliac arteries and SFA are made of clear PVC. When the tubing is left in acetone it will harden and shrink. After initial testing on smooth untreated tubing (simulating a healthy person) to ensure our experiment works, we will begin to try scenarios that simulate stenosed arteries. This will assure us that the DCB will be able to perform in the environment they will be subjected to in patients.
Based on the results gathered from the dynamic flow model we will be able to determine if the coating techniques used are the correct ones, how much drug is lost with different coating techniques, the coatings stability in an environment similar to blood, and if a sheath is necessary to prevent the majority of the drug being lost before balloon deployment.
References:

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