

**2007 Summer Internship Program**  
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1

**QUANTIFYING STRAIN AND PRESSURE-VOLUME CHARACTERISTICS OF THE RAT BLADDER USING A NOVEL EX VIVO TESTING DEVICE**

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The bladder is a contractile organ layered with smooth muscle responsible for storing and voiding urine, yet its normal physiological loading state during filling is not yet fully understood. Pathological conditions such as after spinal cord injury have profound effects on the tissue structure leading to dysfunction of the bladder.<sup>1,2,3</sup> These effects cannot be understood without first studying the healthy bladder's mechanical behavior. Current mechanical tests of the bladder involve uniaxial stretching of bladder strips<sup>4</sup> and biaxial stretching of square specimens<sup>1</sup> rather than mechanical testing of the entire bladder. By closely mimicking physiological conditions during testing of the entire rat bladder using a device designed to measure strain and pressure while controlling bladder influx volume, we aim to develop a more biologically accurate understanding of the bladder's stretching behavior during normal filling.

Bladders were excised from euthanized female Sprague Dawley rats between 2-3 months old. The urethras were catheterized and the ureters were ligated to allow controlled inflow/outflow. Carbon markers were arrayed with adhesive on the posterior surfaces of the bladders in a grid of 12-15 points. The ex vivo testing device included a transparent custom test bath, a pressure transducer and Bridge 8 amplifier (World Precision Instruments, Inc), two Charged Couple Device (CCD) cameras (Edmund Industrial Optics), a Harvard Pico Plus Syringe Pump (Harvard Apparatus), a Dell PC, a data acquisition card (National Instruments, Inc.) and an Isotemp hot plate (Fisher Scientific). The bladders were suspended in Krebs solution in the bath kept at 37°C by the hot plate. The syringe pump manipulated a 14.57 mm syringe at a rate of 0.04 mL/min to fill the bladders via the urethra catheters to a target volume of 1 mL. The internal pressure of the bladder during filling was measured by amplifying the signal from a transducer located at a Y-connector between the pump and bladder. The pressure and volume data were transcribed by a Labview program which also recorded still images at 0.01 mL intervals from the two cameras focused on the bladder. To analyze the data, SigmaScan Pro was used to produce an XY-coordinate system utilized by MATLAB to track the coordinates of the marker array in each frame. Another MATLAB program employed direct linear transformation (DLT) to extrapolate three-dimensional coordinates from the set of two-dimensional coordinates obtained from the images.<sup>5</sup> A Mathcad script then calculated strain data from the marker positions in both the longitudinal and circumferential directions.

Strain data suggests that the smooth muscle exhibits anisotropic stretch and is much more compliant in the longitudinal direction than in that of the circumference of the bladder. Furthermore, the tissue nearer to the neck and urethra of the bladder are less compliant than the rat bladder's posterior wall, superior to the urethra and ureters. Further analysis of these strain data will form the foundation for the development of a constitutive model of the bladder.

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2

**CONTROLLED FACTOR DELIVERY FROM A BIODEGRADABLE ELASTOMER FOR TISSUE ENGINEERING AND REGENERATIVE MEDICINE APPLICATIONS**

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The use of biodegradable, elastomeric scaffolds for soft tissue repair and regeneration is an attractive approach to the fields of tissue engineering and regenerative medicine. Utilizing such scaffolds for spatial control of gene expression would allow researchers to direct subpopulations of cells on a scaffold down different pathways in a spatially-defined manner. In addition, local, sustained delivery of factors to targeted areas would contribute to tissue regeneration and neovascularization. Insulin-like growth factor-1 (IGF-1) has been shown to aid in stem-cell mediated muscle regeneration through recruitment of satellite cells<sup>1</sup>. More recently, IGF-1 has been implicated in repair of infarcted myocardium<sup>2</sup>. The goals of this study were 1) to achieve spatial and temporal control of gene expression on a biodegradable poly(ester

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urethane)urea (PEUU) and 2) to achieve controlled, sustained release of IGF-1 from a 3-dimensional PEUU scaffold for repair of infarcted myocardium.

PEUU was synthesized in a two-step solution polymerization using polycaprolactone diol, butyldiisocyanate, and putrescine as previously described<sup>3</sup>. For gene expression studies, RSL1 ligand inducer molecule was mixed into PEUU solutions prior to casting films. B16 cells transfected with receptor and GFP reporter genes were cultured on PEUU films with varying amounts of RSL1 ligand and assessed visually for GFP expression. To determine release kinetics, degradation media were collected over a 28-day period, and RSL1 concentration was determined by UV spectrometry comparing degradation media to a calibration curve of known concentrations. Towards controlled IGF-1 release, scaffolds were processed by a thermally induced phase separation (TIPS) method following polymer synthesis as previously described<sup>4</sup>. IGF-1 (250 ng/mL) was dispersed throughout the scaffold during fabrication. TIPS with and without IGF-1 (control group) were placed in Balb/c3T3 basal media (DMEM) or MG-63 basal media (MEM) for degradation studies. Degradation solutions were collected over a 28-day period and were transferred to Balb/c3T3 or MG-63 cells on tissue culture polystyrene. Cell number was quantified via MTT assay (an assay for mitochondrial activity) four to five days post-degradation solution treatment.

RSL1 was continually released from PEUU films over a 28 day period *in vitro*. Cells plated directly onto PEUU films containing varying concentrations of ligand expressed GFP in a dose-dependent manner. Therefore, it has been concluded that inducer molecules can be incorporated into PEUU and released in a sustained, controlled manner *in vitro* to control gene expression in a dose-dependent manner. Degradation solutions from TIPS scaffolds containing IGF-1 resulted in increased cell proliferation in culture when compared to degradation solutions from scaffolds without IGF-1. Subsequently, it was concluded that IGF-1 is released in a sustained and controlled manner from TIPS scaffolds and that released IGF-1 is bioactive. Current ongoing studies are examining the spatial control of gene expression on TIPS scaffolds and the *in vivo* effects of TIPS scaffolds containing IGF-1 in a small animal infarct model.

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**3**  
**CHANGES IN GENE EXPRESSION OF PASSAGED BONE MARROW DERIVED CELLS IN CULTURE**

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Functional tissue engineering (FTE) efforts to regenerate musculoskeletal tissues may require large numbers of cells, often obtained through cell passage. Bone marrow derived cells (BMDCs) are an ideal source for FTE because they are auto-transplantable, easily expandable, and have the ability to differentiate into various lineages<sup>1</sup>. While changes in gene expression throughout passage have been documented in chondrocytes and medial collateral ligament (MCL) fibroblasts, they have not yet been quantified in BMDCs<sup>2</sup>. Specifically, one study showed a drastic decrease in the RNA expression of the major matrix constituents of articular cartilage (collagen type II and superficial zone protein) by chondrocytes in early passages, suggesting that passage of chondrocytes is not ideal for cartilage FTE.

Preliminary results obtained in our research center indicated that rat MCL fibroblasts may be a good source for FTE approaches due to the increase in collagen type I expression and relative consistence in collagen type III expression throughout various passages. Furthermore, preliminary studies seemed to suggest that rabbit BMDCs have a fibroblast phenotype as well; as shown by an elongated cell morphology and high collagen type I gene expression at passage 1 (P1). The objective of this study was to quantify gene expression through passage in rat BMDCs. Based upon preliminary studies that showed a quantitative increase of collagen type I expression of MCL cells, we hypothesized that BMDCs would do the same.

Bone marrow derived cells of Long Evans rats (n=3) were isolated from the femur with a syringe and deposited on a petri dish in 10 ml of Dulbecco's Modified Eagle's Medium (DMEM). The same rats from the MCL study were used, thus allowing a one to one comparison to be made. After 2 days, adherent cells were observed and the media was changed to remove any non-adherent cells. When cells reached

confluency, they were trypsinized (P1). From that point on, ascorbic acid (50mg/ml) was added to the medium and cells were re-plated at 30,000 per well in 2 cm<sup>2</sup> wells in 24 well plates for one more passage. Collagen type I, collagen type III and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expressions were quantified by real-time RT-PCR and the abundance of each gene was calculated. Results for collagen type I and collagen type III were both normalized to GAPDH abundance. A one-way ANOVA to compare gene expression across passage was performed with significance set at  $\alpha=0.05$ .

The preliminary results from previous studies showed that MCL fibroblasts might be a good cell source for FTE approaches. If BMDCs express large amounts of collagen type I and collagen type III remains relatively constant, in addition to being auto-transplantable and easily expandable, then this will be the first implication that BMDCs may be a more appropriate cell source for FTE approaches to regenerate musculoskeletal tissues. However, the gene expression of other important molecules such as TGF- $\beta$ 1, Fibronectin, MMP-13, and TIMP1 should be measured and evaluated to further determine if BMDCs are an appropriate cell source. Future work may include the use of BMDCs as a source for cell seeding of biological or artificial scaffolds and their *in vivo* application.

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#### **4** **THE EFFECTS OF CLAMPING ON DETERMINING THE MECHANICAL PROPERTIES OF THE MEDIAL COLLATERAL LIGAMENT AND THE PATELLAR TENDON DURING TENSILE TESTING: A PRELIMINARY STUDY**

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The medial collateral ligament (MCL) and patellar tendon (PT) have been commonly used to study ligament and tendon biomechanics through uniaxial tensile testing. Often the bones (which the MCL or PT connect) are clamped during testing; however these tissues are large enough in some animals to cut into a dog bone shape and measure mechanical properties

using soft tissue clamps. Since the mechanical properties of the tissue are a measure of tissue quality, they should be independent of the clamping method and small changes in tissue size. However, previous studies in our research center have shown that the PT displayed a significant decrease in mechanical properties with soft tissue clamping compared to bone-to-bone clamping methods.<sup>1</sup> One explanation may be that when cutting PT tissue, individual fibers or crosslinks may be disrupted. Thus we hypothesized that the measured mechanical properties for the MCL, which possesses a highly aligned collagen fiber structure, should be independent of the clamping method, regardless of whether bone-to-bone or soft tissue clamping methods were used. The objective of this preliminary study was to 1) evaluate the effects of using soft tissue clamping methods on the mechanical properties of the MCL by comparing to historical data using a bone-to-bone clamping method and 2) compare the results from the MCL to those of the PT mentioned earlier.

To test this hypothesis, MCLs (n=3) were taken from 2 rabbits and the dimensions were measured with calipers after cutting the tissue into a dog-bone shape (aspect ratio ~ 8mm). The cross-sectional area (CSA) was measured using a laser micrometer system.<sup>2</sup> An Instron<sup>TM</sup> materials testing machine was used for uniaxial tensile testing. Markers were placed on the tissue's midsubstance to track strain using a Dynamic Motion Analysis System<sup>TM</sup>. To determine the mechanical properties of the MCL, stress (load/CSA) and strain  $((l - l_0) / l_0)$  were calculated to create a stress-strain curve. From the stress-strain curve, the mechanical properties of the tissue, i.e. tangent modulus (slope of the linear portion of the curve) and ultimate tensile strength (UTS) (maximum stress) could be determined. An unpaired t-test was used to compare the mechanical properties of the MCL using soft tissue clamping to historical bone-to-bone clamping data with significance set to  $p < 0.05$ . The same comparison was made for historical PT data as well.

When comparing soft tissue clamping (n=13) to that of bone-to-bone clamping (n=16) for the PT, a 60% decrease in the tangent modulus (581±199 MPa vs. 1,506±523 MPa, respectively,  $p < 0.05$ ) and a 35% decrease in UTS (47.1±19.4 MPa vs. 71.9±9.2 MPa, respectively,  $p < 0.05$ ) was observed.<sup>1</sup> For the MCL, early results indicate no differences in the tangent modulus could be detected between the soft tissue (n=3) and the bone-to-bone (n=16) clamping methods (832±74MPa vs. 936±284 MPa, respectively).<sup>3</sup> Since both MCL specimens failed at or near the soft tissue clamps, the UTS values could not be compared for the MCL. Due to the limited sample size, more testing of the MCL using soft tissue clamps will be performed to confirm these early results.

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**5**  
**USE OF A BIOSCAFFOLD TO IMPROVE HEALING OF A PATELLAR TENDON DEFECT FOLLOWING GRAFT HARVEST FOR ACL RECONSTRUCTION: A BIOCHEMICAL ANALYSIS**

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Bone-patellar tendon-bone autografts are often used for anterior cruciate ligament reconstruction. However, the untreated healing patellar tendon (PT) is structurally and mechanically inferior to the native tissue. Furthermore, adhesions to the underlying fat pad are often observed. Functional tissue engineering methods are being employed to aid the healing response, such as the use of bioscaffolds. Porcine small intestine submucosa (SIS) is an extracellular matrix derived bioscaffold that consists primarily of collagen type I, but also contains growth factors. SIS also degrades rapidly in-vivo and prevents cell infiltration from its luminal side (which is useful for reducing adhesions to the fat pad). Recent research has shown that when SIS is applied to a medial collateral ligament (MCL) defect, the healing MCL exhibits better structural and biochemical properties as well as larger fibril diameters and lower collagen type V content when compared to the non-treated group<sup>1</sup>. Elevated levels of collagen type V has been shown to limit collagen fibril diameter and cause poor mechanical properties<sup>1</sup>.

In twenty New Zealand White rabbits, the central third of the PT was removed from the right hind leg<sup>2</sup>. The rabbits were divided into two groups of ten with one group receiving SIS treatment while the other was non-treated. In both groups, the left hind leg served as a sham control. After 12 weeks, gross morphological results showed that the SIS-treated group had fully filled in the defect and adhesions to the fat pad were greatly reduced while the non-treated group showed a concavity in the defect and multiple adhesions to the fat pad. The SIS-treated group also had a 68% greater cross-sectional area than the non-treated group. Structurally, the SIS-treated group exhibited a 98% greater stiffness and a

113% greater ultimate load when compared to the non-treated group.

A preliminary study has been initiated to determine the biochemical properties of the neo-PT tissue. The study has three groups: SIS-treated (n=3), non-treated (n=3), and sham control (n=3). The tissue was first dried and weighed. The tissue was then degraded using 1M acetic acid and pepsin at a 30:1 dry weight to pepsin ratio. A BCA assay was performed to determine total protein content. This data serves to normalize the collagen content. We plan to run a Sircol assay to determine total soluble collagen content and then SDS-PAGE to compare the ratios of collagen type V to type I and collagen type III to type I.

We expect to find that both of these ratios will be smaller in the SIS-treated group compared to the non-treated group. Smaller ratios of collagen type V to type I and type III to I suggest a better healing response because the neo-PT tissue would more closely resemble native tissue. From the results of this preliminary study, we will be able to run a power analysis to determine the number of rabbits needed to perform a full study. If the results are then proven favorable, the next study would use goats for a more clinically relevant model of the human knee.

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**6**

**EFFECT OF DRAG-REDUCING POLYMERS ON TRANSESTERIFICATION.**

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Enzyme-catalyzed transesterification is a solution for several problems encountered in polymer synthesis. One major advantage is that enzyme-catalyzed reactions are much more environmentally friendly, and are more selective. The enzymes used are lipases, which are generally known for their function to degrade lipids. Lipases are also able to combine alcohols and esters in anhydrous conditions.

The polymerization reaction of divinyl adipate (DVA) and 1,3-butanediol (BD) is a polycondensation reaction. The enzyme-catalyzed reaction can be split into three parts: an initial, very rapid transesterification, followed by intermediate polymerization with a significant increase in molecular weight, and the final very slow polymerization reaching the limiting molecular weight. The goal of the project is to try and overcome the mass-transfer limitations encountered in the last step of the reaction, so that larger polymers can be synthesized without the decrease in reaction rate. Drag-reducing polymers can be used to overcome these limitations.

Reactions are carried out utilizing 70mmol each of DVA and BD, combining them to form a heterogeneous mixture in a glass vial. A layer of molecular sieves is added to the vial, which is then stored in a desiccator until use. The DVA/BD mixture is preheated in an oil bath to 60°C, and the water-jacketed glass reactor is also preheated to 60°C using a circulating pump. After both reach 60°C, the DVA/BD mixture is poured into the glass reactor. The optimum concentration of Chirazyme L2-C2, 2% w/w (0.4032g), is weighed out and added to the glass reactor. Drag-reducing polymer (DRP) is then added in various concentrations. The system is then sealed. Mixer speeds are set for 300, 500, and 800rpm; power consumption of the motor is recorded regularly to indirectly test increasing viscosity of the polymer. Gel-Permeation Chromatography is then used to analyze the polydispersity index (PDI) and the maximum mass of the polymers.

Preliminary data has shown that 2% w/w Chirazyme is the ideal concentration for reducing PDI, and that increasing amounts of DRP's will decrease the PDI and increase the maximum mass of the polymer achieved. We predict that further experimentation and results will confirm one concentration of Chirazyme to reduce the mass-transfer

limitation the best, so that larger polymers can be synthesized. The results also show that enzyme-catalyzed reactions are successful enough to potentially be used more regularly, having the added benefit of being friendly to the environment.

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**7**

**CULTURING TRACHEAL EPITHELIAL CELLS ON ECM, STRUCTURAL TESTING OF PORCINE TRACHEAL ECM, AND EVALUATING PARTIALLY DECELLULARIZED TRACHEAL XENOGRAFTS IN CANINES**

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The default mechanism for healing in the trachea is the formation of scar tissue and eventual stricture. Avoiding this undesirable outcome can only be achieved if rapid angiogenesis and successive reepithelialization occur before inflammation and fibrosis cause stricture.<sup>1</sup> This race is further complicated by the maintenance of the lateral rigidity and longitudinal flexibility of the graft.<sup>2</sup>

A possible solution to promote angiogenesis and reepithelialization is the use of naturally occurring extracellular matrix (ECM) scaffolds. ECM scaffolds have been shown to promote the formation of functional tissue in a variety of body systems, including musculotendinous and gastrointestinal tissues.<sup>3</sup>

A previous study examined if ECM scaffolds derived from the porcine urinary bladder matrix (UBM) and trachea (TECM) could promote constructive remodeling in partial tracheal defects. It was shown that the remodeled ECM scaffolds consisted of dense, organized collagenous tissue with immature epithelium and no cartilage formation. In light of this previous study and the other problems facing tracheal grafts, the current study aimed to: 1) determine the feasibility of culturing tracheal epithelial cells on ECM 2) characterize the structural behavior of decellularized porcine tracheal ECM, and 3) evaluate the effectiveness of hydrated, partially decellularized porcine tracheal xenografts in a canine model.

Mice tracheas were enzymatically digested and the entire population of cells was seeded on TECM, UBM, and collagen coated membrane that was glued to well inserts. These well inserts gave the option of creating an air-liquid

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interface by removing the media from the well insert, yet still leaving media in the bottom well. The media used was conducive to growing epithelial cells.

Four porcine tracheas were tested to compare the structural properties after various decellularization treatments. One native trachea was used as a control, then trachea decellularized in 3% Triton X-100, 3% SDS, and 3% Triton X-100, then lyophilized, were used. The tracheas were mounted in a perfusion system and the diameter was measured using a laser.

Two adult porcine tracheas were harvested and immediately placed into 3% sodium docedyl sulfate (SDS), 1% penicillin/streptomycin, and 1% fungizone. The tracheas were kept in the SDS solution for 1 hour then disinfected for 1 hour in a 0.1% peracetic acid (PAA). Next, they were washed in sterile phosphate buffered saline (PBS), and finally rinsed in sterile saline. Into 2 adult mongrel dogs, 3 cm long full circumferential sections of the porcine trachea were implanted.

Both tracheal xenografts were partially decellularized. This was verified with H&E and DAPI histologic staining, which showed complete removal of epithelial cells while the chondrocytes and cells of the glandular structures remained present. However, on postoperative day 11 one dog died from tracheal stricture and the second dog was euthanized on day 13. Preliminary structural data will be available shortly, as will results from cell seeding.

The problem of finding a tracheal graft is a difficult one that has many interdependent aspects. It is evident that the tracheal epithelial cell growth, structural integrity, and overall limitations of tracheal grafts must be better understood before further trials can be performed.

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**8**

**CCT-ETA AND PIN DOWNREGULATION DURING SCARLESS WOUND HEALING IMPLICATE NITRIC OXIDE SIGNALING AS A POTENTIALLY IMPORTANT MECHANISM**

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Adult mammalian tissues heal injury with scar formation. While scarring seals a wound, it can also compromise essential body functions, such as movement, respiration, and speech. In contrast, mammalian fetal tissue can heal *without* scar. We have used expressomic techniques to identify genes differentially regulated during fetal wound healing. Several of these genes have been found to implicate nitric oxide (NO) signaling as a potentially important mechanism.

Incisional wounds were made in fetal and adult New Zealand white rabbits. Twelve hours post-injury, the wounds were excised and total RNA was isolated from both wound and unwounded control tissue. RNA from wound and control samples was evaluated by differential display and microarray analysis. Multiple differentially expressed candidate genes were identified; two genes found to be downregulated in fetal wounds are the protein inhibitor of nitric oxide synthase (PIN) and the eta subunit of the chaperonin containing T-complex polypeptide (CCT-eta), which is itself an inhibitor of soluble guanylyl cyclase (sGC), the major intracellular signal transducer for nitric oxide. These findings suggested that increased NO signal may be important in scarless wound healing.

To confirm that PIN is downregulated in fetal wound healing, a quantitative real-time RT-PCR assay was designed and validated. Use of this assay confirmed that PIN is specifically downregulated in healing fetal wounds. Since no specific sequence information existed for rabbit sGC, primers have been designed using anticipated homology to mouse sequences to generate an RT-PCR product. These amplicons have been subcloned, propagated and sequenced to yield a partial cDNA sequence for rabbit sGC. With this additional sequence information, we are designing a quantitative real-time RT-PCR assay for sGC subunits as well.

In addition to examining fetal integumentary wound healing, these assays will be used to investigate both adult skin and mucosal wound healing. In addition, the cDNA probes generated will be used to examine both fetal and adult healing wounds by *in situ* hybridization, thereby further clarifying which cell types may critically employ nitric oxide signaling during wound healing. These experiments will hopefully begin to delineate the importance of NO dependent pathways during fetal wound healing.

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**9**  
**BIOMECHANICAL PROPERTIES OF BLOOD BASED BIOPLASTICS**

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Tissue engineering is an interdisciplinary field whose objective is regenerating devastated or missing biological tissue.<sup>i</sup> Traditionally, autografts and allografts are implanted into the damaged sections of the body to induce and guide wound healing. Now, scaffolds being made out of native biological materials show great promise for tissue engineering. One such family of materials includes blood-based products that can be isolated and molded into bioplastic graft substitutes. They can be engineered with a range of biomechanical properties, from soft and pliant to hard. In addition to providing mechanical support and delivering growth factors throughout damaged tissue, they degrade over time by proteolysis to eventually be replaced by new healthy tissue.

As part of the development process, biomechanical testing of these native bioplastics is required to determine appropriate material compositions and processing parameters for matching the plastic graft substitutes to the target application, including both soft and hard tissues. Because tissue substitutes are intended for treating a variety of tissue regenerating problems, the composition of the graft substitutes must have variable controllability.<sup>ii</sup> Occasionally, inserts may require initial compression strength for load bearing applications such as the replacement of degenerative vertebral discs. Non-load bearing applications, like those used for the treatment of skull calvarial defects, have to resist compression of surrounding soft tissue. The biomechanical testing of the native products will also assure both manufacturing consistency and quality control.

A large range of bioplastics were developed by adding a plasticizer to a blood based material. This process was then repeated to make a sequence of mixtures with only

one variable composite element. After each combination was incubated, the samples were cut into slices of varying thicknesses. They were then subjected to mechanical compression testing using the Bose EnduraTEC 3200. Ramp-to-failure tests were performed on the samples while both the load and displacement were closely monitored. The results varied depending upon which constituent of the original mixture was changed.

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**10**  
**NON-INVASIVE IN VIVO TRACKING OF SCAFFOLD DEGRADATION USING QUANTUM DOTS**

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Currently, tracking and evaluating the remodeling process of an *in vivo* scaffold typically requires the sacrifice of the animal subject. This projects aims to develop a non-invasive labeling system that allows *in vivo* tracking and quantification of scaffold degradation via fluorescent imaging of infra-red emitting quantum dots (QDs). In these experiments, we use the loss of QD fluorescence over time to measure the rate of remodeling of subcutaneously implanted fibrin-based constructs. Co-labeled with Alexa 647 fibrin, fibrin constructs were made with 605 and 800 carboxyl QDs. The results show that the rate at which 655 and 800 QDs fluorescence declined was proportional to the fading fluorescence of the Alexa 647 fibrin as the body broke down and absorbed the construct. Thus, this indicates that QDs can be used to detect the remodeling process that occurs *in vivo*. Such real-time measurements will enable a wide range of investigations in tissue engineering and regenerative medicine.

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**11**

**THE EFFECTS OF STORAGE TIME ON HEMOLYSIS, MECHANICAL FRAGILITY, AND VISCOELASTICITY OF BOVINE BLOOD**

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With the advent of artificial organs, promoting effective blood circulation throughout a system becomes a high priority. Circulating blood must be able to nourish surrounding tissue by passing through microscopic capillaries and withstand high shear stresses generated by blood-contacting devices. The deformability of red blood cells [RBCs] directly influences their ability to enter and pass smallest capillaries under these conditions. During storage, blood experiences biochemical and cellular alterations that may be lethal or sublethal to RBCs and thereby reduce their deformability. Sublethal damage of blood cells is an often overlooked factor in complications associated with blood transfusion and circulatory assist devices as well.

In this study, we investigated the rheological alterations in bovine blood over a storage period of six days. Since deformability is a function of viscoelasticity, we aimed to measure both the viscosity and elasticity of whole blood each day using a Vilastic-3 viscoelastic analyzer. We also measured the viscosity of the plasma portion of blood. Lethal damage to RBCs induces hemolysis and ruptured cells expel their hemoglobin contents into blood. Therefore, another aim was to measure the concentration of free hemoglobin in the plasma each storage day. In contrast to hemolysis, sublethal impairment to individual cells induced by storage may increase their mechanical fragility. After inducing a standard mechanical stress to whole blood, we measured the hemoglobin content in the plasma. A greater hemoglobin concentration indicated more RBCs were ruptured, correlating with increased RBC mechanical fragility. Except for mechanical fragility, all parameters were determined several times on the first day and every day over six days following blood collection.

Fresh blood was acquired from slaughter and treated with an anticoagulant/glucose medium and an antibiotic. The blood was stored in six separate plastic transfusion bags at 12°C. To measure the hemoglobin content, whole blood was centrifuged to separate the plasma. Plasma free hemoglobin content was measured using a calibrated spectrophotometer. In order to assess the mechanical fragility, standard vacutainers with whole blood along with five steel shots were placed on an automated rocker for one hour to induce mechanical trauma. A spectrophotometer measured the plasma free hemoglobin content. Viscosity and elasticity were measured using the aforementioned viscoelastometer analyzer.

Initial results indicate that blood experiences minimal hemolysis while stored. The plasma free hemoglobin of each day's sample remained relatively constant. However, the viscosity of the whole blood increased over the six day period. The mechanical fragility of the RBCs also increased, indicating the cells experience sublethal damage while stored. In summary, our results yielded evidence of significant sublethal blood damage that contributes to decreased deformability of bovine RBCs while in storage.

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**12**

**ISOLATION AND LABELING OF FAT-DERIVED STEM CELLS FOR IN VIVO TRACKING**

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Through the use of fat (i.e., adipose tissue), regenerative medicine and tissue engineering will be able to further evolve and technologically advance medicine. Because

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of its large abundance and accessibility, adipose tissue is one of the most prominent and reliable sources of adult stem cells [1]. Adipose-derived adult stem cells (ASCs) are a mesenchymal population of adult stem cells extracted from discarded or extraneous adipose tissue. ASCs can be isolated from liposuction by enzymatic digestion and differential centrifugation [2].

When compared with bone-marrow derived stem cells, adipose-derived stem cells demonstrate equal potential to differentiate into cells and tissues of mesoderm origin, such as fat, cartilage, bone and skeletal muscle; however, the easy and repeatable access to subcutaneous adipose tissue and the simple isolation procedure show a clear advantage. Regenerative medicine applications of ASCs include soft tissue regeneration, as well as bone and cartilage repair. Breast reconstruction after mastectomy by injection of allogenic ASCs mixed with biodegradable scaffolds is a promising new approach. A mouse model of breast reconstruction was established in our laboratory, but improved *in vivo* cell tracking methods are needed.

In this study, human ASCs were isolated from a female patient, and a mutant replication-defective herpes simplex virus (HSV) virus with green fluorescent protein (GFP) expression cassette on UL41 locus [3] was utilized to infect the ASCs. GFP was expressed homogeneously and the majority of the cells were infected. The infected cells have continued to express GFP for two weeks without a significant decrease of GFP expression; therefore, replication-defective HSV virus is a suitable label marker for tracking ASCs. *In vivo* animal studies are necessary for validation of *in vitro* data.

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**13**  
**THE LOCAL BIOPHYSICAL ENVIRONMENT MAY**  
**MEDIATE THE REMODELING MECHANISMS OF**  
**THE ABDOMINAL AORTIC WALL DURING**  
**ANEURYSM DISEASE**

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Abdominal aortic aneurysms (AAA) affect over 200,000 people each year in the US [1]. From a basic engineering standpoint, AAA rupture occurs when wall stress acting on the vessel exceeds the wall strength; however, pathophysiology of rupture remains unknown. Previous studies from our group have shown that both wall stress and strength vary spatially over a given AAA [2]. This spatial variation in mechanical properties may imply that there is a spatial variation in the cellular and non-cellular mediators of this pathogenesis. We hypothesize that once formed, the wall of an AAA progressively weakens as a result of increasingly discordant remodeling mechanisms, and these are mediated by the local biophysical environment. The purpose of this work was to analyze pairs of AAA wall samples for wall thickness, RNA expression, and collagen and elastin content. An understanding of the biophysical mechanisms involved in tissue remodeling is critical for tissue engineering.

Samples of anterior, medial AAA wall were harvested in pairs at the time of elective surgical repair from patients who had undergone abdominal CT angiography within the past 12 months. CT scans were used to generate 3D reconstructions of the abdominal aorta with intraluminal thrombus (ILT) from the renal arteries to the bifurcation of the common iliacs. ILT thickness was assessed from the reconstruction, and finite element modeling was used to determine the wall stress distribution of each aneurysm. Different pairs of samples were used to assess wall thickness, collagen and elastin content, and gene expression changes. For wall thickness measurements, pairs of AAA wall were excised, fixed in 2% paraformaldehyde for four hours at 4°C, followed by an overnight incubation in 30% sucrose at 4°C. The tissue samples were then frozen in isopentane chilled to liquid nitrogen temperature. Tissue was embedded in paraffin and sectioned as needed. Ten micron sections were stained with H&E. Images were taken at 4X and 10X with light microscopy, and Scion Image (Scion Corp.) was utilized to measure the total wall and tunica media thicknesses. Other paired samples were excised and analyzed with the Sircol™ (Accurate) assay for soluble collagen content, hydroxyproline for total collagen content, and the Fastin™ (Accurate) for total elastin content. Lastly, a final pair was excised and homogenized in Trizol® (Invitrogen), followed by total RNA isolation with a Qiagen RNeasy kit. TaqMan QPCR assays were used to determine gene expression in the sample.

RNA results showed a consistent increase in collagen expression in high stress compared to low stress samples. Collagen and elastin assays should confirm our initial gene expression findings. Thicknesses are being assessed.

The local biophysical environment may play a role in the repair and remodeling of AAA wall. A better

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understanding of mechanopathobiology is critical if we are to make continued progress in tissue engineering.

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**14**

**Adrenal Cortical Cell Proliferation and Function on Laminin-Coated Adrenal Extracellular Matrix**

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**Introduction:** Adrenal insufficiency is a progressive and life threatening disorder, caused by an inability of the adrenal glands to produce and secrete adequate hormone levels. Treatment involves exogenous hormone replacement, yet patients remain at high risk for acute adrenal crisis.

The extracellular matrix (ECM), composed of structural proteins, bioactive molecules, and growth factors, has been used as a biologic scaffold in tissue engineering.<sup>1</sup> Laminin, an ECM glycoprotein, is an essential component of the basement membrane and promotes cell migration, adhesion and differentiated cell phenotypes.<sup>2</sup> Previous work in our lab demonstrated that decellularization of native ECM results in decreased laminin staining.<sup>3</sup>

We hypothesized that decellularized adrenal ECM is an ideal scaffold for adrenocortical cell growth in a tissue-engineered adrenal gland. Our study examined the effects of laminin-coated ECM on adrenocortical cell proliferation and physiologic function.

The purpose of this study was to: 1.) determine a method to coat adrenal ECM with laminin, 2.) culture primary porcine and human adrenocortical cells on laminin-coated and uncoated adrenal ECM, 3.) compare cell proliferation and physiological function of adrenocortical cells on laminin-coated and uncoated adrenal ECM.

**Materials and Methods:** Porcine adrenal glands were harvested, snap frozen in liquid nitrogen, and stored at -80°C. Tissue slices were thawed in PBS at 25°C then decellularized in Trypsin/EDTA, Triton X-100, sodium deoxycholate, and peracetic acid. ECM was stored in sterile water at 4°C.

Human fetal and porcine adult adrenocortical cells were harvested from intact adrenal glands, digested with

collagenase/dispase and DNase1, filtered through 100 µM mesh, centrifuged, and resuspended in fresh medium.

Adrenal ECM was cut into 2 mm<sup>3</sup> slices. ECM samples were incubated for 2 hours at 37°C in PBS (1X) or 1:125 laminin (Sigma-Aldrich, Inc.) in PBS. Primary adrenocortical cells were seeded onto laminin-coated and uncoated ECM samples and cultured at 37°C in 1:1 Ham's F12/DMEM with 10% FBS, 5% horse serum, 1X penicillin/streptomycin/fungizone.

Cortisol levels were measured at 24 and 48 hours after ACTH stimulation at 100 mU/mL. Cell proliferation was calculated using the ViaLight Plus Cell Proliferation and Cytotoxicity BioAssay Kit (Cambrex Bio Science). Cell-ECM constructs were examined with Hematoxylin and eosin (H&E), 4', 6-diamidino-2-phenylindole (DAPI) fluorescent nuclear stains, and scanning electron microscopy (SEM).

**Results:** Laminin coating of ECM was confirmed with immunofluorescence. DAPI and H&E demonstrated viable adrenocortical cells on both laminin-coated and uncoated ECM constructs. Cortisol secretion 48 hours after ACTH stimulation was comparable on laminin-coated and uncoated ECM in porcine (135 and 88.66±40.12 vs. 182±75.97 and 134.66±92.19 µg/dL) and human (69.33±14.84 vs. 32.66±34.29 µg/dL) ECM constructs. ATP assay demonstrated no significant difference between laminin-coated and uncoated ECM in porcine (2608.607±649.749 and 1060.02 vs. 2203.33±1138.71 and 2619.393±914.426 RFL) or human (5186.937 vs. 3569.439±2292.35 RFL) adrenocortical cell proliferation.

**Conclusions:** Both laminin-coated and uncoated ECM facilitated adrenocortical cell proliferation and physiological function. Uncoated ECM actually was associated with greater cell proliferation and cortisol secretion when compared to laminin-coated ECM. This study shows that decellularized adrenal ECM alone is an effective bioscaffold to support adrenocortical cells in an *in vitro* tissue engineered adrenal gland.

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15

**DEVELOPMENT OF A 3D IN VITRO AGAROSE-BASED MODEL FOR QUANTIFICATION OF AXONAL ELONGATION WITHIN A NERVE GUIDE**

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Across peripheral nerve gaps where direct end-to-end suturing of the distal and proximal stumps is not possible due to excessive tensile strain, nerve regeneration is severely hindered. Tissue engineering techniques have been shown to have success in promoting nerve regeneration across short distances (<10mm). However, such techniques have not proven to be more efficacious than the preferred clinical treatment of insertion of a nerve autograft in the case of short nerve gaps. Both autograft and tissue engineering techniques have not proven efficacious in the case of long nerve gaps (>15mm). Thus, the need to find a suitable tissue engineering strategy for initiating and sustaining nerve regeneration is immense. The use of biodegradable nerve conduits, (e.g., hollow tubes which are sutured to both ends of a severed nerve), have been used clinically to provide protection and a pathway for regenerating nerve tissue. Such conduits have been constructed using numerous biodegradable polymers. Fabrication of conduits which are both mechanically strong, resisting compression and suture tearing, while suitably porous to allow diffusion of nutrients into the luminal environment, is necessary. Studies have shown that agarose hydrogels allow for neurite extension at low concentrations and that neuronal membranes bear no agarose receptor proteins.<sup>1,2</sup>

This study attempts to develop a agarose hydrogel based model which allows for 3D neurite extension in model neurons, (e.g., dorsal root ganglion cells), to quantify extension and direction of neurite outgrowth in porous poly(caprolactone) (PCL) conduits.<sup>3</sup> Agarose hydrogels were constructed of 0.5% and 1% (w/v) and used to maintain a vertical orientation of PCL conduits within the gel and placed at 37° C to ascertain the gel's ability to maintain the PCL conduit in the vertical position. PCL conduits were fabricated using a mandrel-coating technique where a poly(vinyl alcohol) coated glass mandrel was briefly submerged into a PCL/solvent solution (12.5% w/v) with the addition of sodium chloride (NaCl) to create porosity.<sup>4</sup> To fabricate PCL conduits with varying wall thicknesses of 200µm and 600µm, the use of the solvents tetrahydrofuran, dichloromethane, and ethyl acetate was tested. Varying the NaCl content of the guides to obtain 70% and 80% porosity and the number of glass mandrel immersions into the PCL/solvent solution was done and the wall thickness of the resulting nerve guides was calculated using light microscopy. Scanning electron microscopy was also used to image PCL nerve guides.

Thus far, both the 0.5% and 1% agarose hydrogels are able to maintain PCL conduits in a vertical orientation, as the gels both possess sufficient strength. However, it is observed that the 1% hydrogel is stiffer than the 0.5% hydrogel, and allows for a easy insertion of PCL conduits. Both gels are optically clear. Consistent fabrication of PCL conduits with a wall thickness of 200µm has been achieved using a 80% porous ethyl acetate based solution, and a 600µm wall thickness has been consistently produced using the same ethyl acetate solution, only with more submersions.

These results are necessary fundamental studies prior to proceeding in a rat sciatic nerve defect model.

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16

**EFFECTS OF THE TNF-α AND IL-8 CYTOKINES ON INTERVERTEBRAL DISC CELL MATRIX HOMEOSTASIS**

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Human intervertebral discs consist of the gelatinous nucleus pulposus (NP) center surrounded by the more rigid annulus fibrosus (AF). The discs provide the spine with the flexibility needed for several degrees of motion. However, as the discs age, the level of matrix proteins decreases appreciably, and their structure alters significantly, leading to varying degrees of degeneration (1). Previous studies have demonstrated that exposure of disc cells to cytokines, including tumor necrosis factor-alpha (TNF-α) and interleukin-1 (IL-1), inhibit the syntheses of several matrix proteins, which may contribute to the degenerative process (2). Gene microarray analysis of TNF-α stimulated human NP cells revealed high induction of a number of cytokines and

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suppression of disc matrix protein genes (3). Of the proinflammatory cytokines, interleukin-8 (IL-8) is of particular interest because it is drastically induced by TNF- $\alpha$ . Moreover activated macrophages, which have been detected in ruptured disc AF tissue, can stimulate disc AF cells to produce high levels of IL-8 in vitro (4). Based on these observations, we hypothesized that the exposure of intervertebral disc cells to TNF- $\alpha$  induces IL-8 expression that in turn mediates the catabolic cascades that lead to a loss of matrix proteins.

We are testing this hypothesis by exposing human NP cells to TNF- $\alpha$  and IL-8 and measuring the expression of genes involved in matrix homeostasis, including selected matrix protein genes (cartilage intermediate layer protein, collagen I, collagen II, and aggrecan), proinflammatory cytokines (interleukin-1-alpha, interleukin-1-beta, interleukin-6 and interleukin-8), and matrix degrading enzymes (metalloproteinase-3, 13). Human NP cells were cultured in monolayer and exposed to TNF- $\alpha$  in intervals of 0, 4, 10, 24, 48, and 72 hours, at a concentration of 5 ng/mL. Total RNA was isolated from these cells following TNF- $\alpha$  exposure. Primers for the selected genes were designed, purified and validated. Gene expression levels of these gene targets in TNF- $\alpha$  treated cells and untreated control cells are currently being investigated using quantitative real time reverse-transcription polymerase chain reactions (RT-PCR) and Enzyme Linked Immunosorbent Assay (ELISA). The levels of expression of these gene targets are also being explored in cells cultured in monolayer and 3-dimensional alginate beads left exposed to IL-8 for 24 hours at concentrations of 0, 1, 10, 20, 50, and 100 ng/mL.

The information gathered in this study will be used to determine how TNF- $\alpha$ , in close conjunction with IL-8, modulates disc matrix homeostasis, providing additional insights into disc degeneration, with the goal of opening new pathways for research and therapy.

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**17**

**THE QUEST FOR SYSTEMIC LUPUS ERYTHEMATOSUS BIOMARKERS: CONTRIBUTIONS FROM THE ALTERNATIVE PATHWAY OF COMPLEMENT ACTIVATION**

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Systemic Lupus Erythematosus (SLE) is regarded as the prototypical autoimmune disease, with a clinically diverse presentation and still unknown etiopathophysiology (ref. 1). Given this fact, diagnosis and treatment can pose a significant challenge to the physician. The role of biomarkers has become paramount in understanding, diagnosing and treating SLE. Recent advances in immunoassays detecting cell-bound complement product C4d and complement inhibitory receptor CR1 (ref. 2) have allowed us to expand the clinical toolkit of diagnostic and disease-monitoring biomarkers for SLE. These recent findings, coupled with the mounting evidence that the alternative pathway is involved in autoimmune and inflammatory diseases (ref 3.), compels us to investigate the cell-bound products of this pathway for SLE biomarkers.

We hypothesize that patients with SLE will have abnormal levels of erythrocyte-bound Factor Bb (E-fBb) as compared to healthy controls. To test this hypothesis, retrospective data from healthy controls, patients with SLE and patients with other inflammatory diseases will be compiled for cross-sectional and longitudinal analysis. The cross-sectional cohort will be compared using non-parametric tests in STATA to determine whether the levels of E-fBb are different between the three populations. The SLE population will then be analyzed for possible correlations between clinical activity and E-fBb levels. Longitudinal data will also be compiled for the SLE patients based on E-fBb levels of 1.5 or greater specific mean fluorescence and evaluated for clinical features worthy of case reports.

The populations were determined to be different by the Kruskal-Wallis equality of populations test. The Wilcoxon rank-sum test of distributions showed a difference between controls and SLE, but no difference between SLE patients and patients with other diseases. Using the Spearman Correlation test, no correlations were found for clinical activity measures and E-fBb levels. Two patterns emerged from the longitudinal data: the first was a positive relationship between E-fBb and E-C4d levels; and the second was a positive relationship between E-fBb and E-CR1 levels. Two case reports were conducted on patients representing each of these patterns. Of note, renal involvement was a clinical factor for both of these

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SLE patients and relationships were seen between other biomarkers of disease activity and E-FBb levels.

In conclusion, although no correlations between clinical activity and E-fBb levels were found in the SLE population there may be cause for further analysis with consideration of demographic variables or that involves a larger number of patients. The longitudinal case reports indicate a potential relationship for renal involvement with E-fBb that warrants further examination.

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**A PHYSIOLOGICALLY-BASED PHARMACOKINETIC MODEL FOR THE ANTITUMOR AGENT-GEMCITABINE AND THE DEOXYCITIDINE DEAMINASE INHIBITOR TETRAHYDROURIDINE**

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Gemcitabine (2'-difluoro-deoxycytidine, dFdC), a pyrimidine nucleoside analogue, is a chemotherapeutic that has proven effective in solid tumor models and has been used successfully in treating ovarian, breast, non-small cell lung, and pancreatic cancers [1]. The drug is activated intracellularly through saturable phosphorylation pathways to its triphosphate form (dFdCTP), with the rate-limiting step being the initial phosphorylation by deoxycytidine kinase. dFdCTP is the primary active metabolite that blocks DNA synthesis and results in apoptosis when incorporated into DNA. Simultaneously, dFdC is converted intracellularly to the inactive metabolite difluorodeoxyuridine (dFdU) by deoxycytidine deaminase; this mechanism is not saturable.

dFdC is currently administered intravenously (IV) due to limited bioavailability of oral formulations. One method for increasing dFdC plasma exposure is to co-administer tetrahyouridine (THU), an inhibitor of cytidine

deaminase. This study focused on modeling dFdC and THU dynamics and interactions in order to improve dFdC bioavailability following oral administration (PO). Five studies were performed in severe combined immune deficient (SCID) mice [2]: 100 mg/kg IV dFdC, 100 mg/kg PO dFdC, 100 mg/kg IV dFdC + 100 mg/kg IV THU, 100 mg/kg IV THU + 100 mg/kg PO dFdC, and finally 100 mg/kg PO dFdC + 100 mg/kg PO THU.

Physiologically-based pharmacokinetic (PBPK) models of dFdC and THU plasma dynamics following either IV or PO drug dosing were constructed. The following compartments were modeled: plasma, liver, kidney, lung, stomach, gut, spleen, rapidly equilibrating tissue, and slowly equilibrating tissue. Tissue compartments were modeled as perfusion-limited, except for the slowly equilibrating tissue compartment, which required an additional subcompartment due to an increased half-life compared to the other tissues. Primary metabolism for both dFdC and THU occurs in the kidney and liver, with concurrent excretion in the kidney necessitating the inclusion of elimination parameters in those compartments. All parameters were estimated by minimizing the weighted sum of squared errors between model predictions and actual experimental data; weights were the inverse of the standard deviation of the measured concentrations at each time point. The models were developed by first fitting the IV data, locking those parameters, and then fitting the oral absorption dynamics to the concentration measurements resulting from PO administration. This sequential parameter estimation decreases the number of parameters to fit at each step, thereby improving estimation accuracy.

Two physiologically-based models were constructed, for dFdC and THU, respectively. The dFdC model returns accurate predictions for each organ where experimental data was available; THU PBPK modeling is ongoing at present. The model-calculated percentage of dose that was excreted in the urine was 15% for IV dFdC, 1.6 % for PO dFdC, and 37.5% for IV THU and IV or PO dFdC. These match percentages collected from previous studies [2]. The long term objective is to couple the dFdC and THU models, thereby allowing predictions of THU effects on dFdC metabolism and dosing.

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**CALCIUM AND FORCE TRANSIENTS IN ENGINEERED CARDIAC TISSUE**

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Cardiac tissue engineering strategies hold great potential as a rescue therapy after myocardial injury<sup>1</sup>. Current approaches to treating myocardial dysfunction particularly after infarction remain unsatisfactory. Engineered cardiac tissue [ECT] may represent an attractive therapeutic modality<sup>2</sup>. However, at this time several aspects of ECT function need to be better defined. Intracellular calcium concentration ( $[Ca^{2+}]_i$ ) is a central regulator of cardiac contractility, as its presence in the myocyte allows formation of crossbridges, which are necessary for force generation. Alterations in  $[Ca^{2+}]_i$  have been implicated in many forms of heart disease<sup>3</sup>.

The goal of this study was to compare force production and  $[Ca^{2+}]_i$  transients in ECT with the age-matched native cardiac tissue [NCT]. Cells were isolated from neonatal rat hearts, mixed with collagen and Matri-gel, and formed into cylinder constructs using a TissueTrain system. After eight days in culture the ECT was attached to a force transducer and a static hook in a superperfusion bath at 37° C and paced at 2-6 Hz. The ECT was then loaded with the dual excitation, single emission fluorescent calcium dye Fura-2. The ratio of the emissions (*R*) at two excitation wavelengths is directly correlated to the  $[Ca^{2+}]_i$ . Similar experiments were then carried out on acutely isolated left ventricular papillary muscles from nine day old rat pups, which served as the NCT. Data reported are means ± SEM.

Experiments were done in 5 ECTs and 7 NCTs. The change in the ratio from baseline  $[Ca^{2+}]_i$  to peak developed  $[Ca^{2+}]_i$  was not different between the ECT and NCT (ECT:  $R_{dev} = 0.56 \pm 0.11$ , NCT:  $R_{dev} = 0.88 \pm 0.19$ ,  $P = 0.23$ ). While this is not indicative of quantitatively equal  $[Ca^{2+}]_i$  transients, it proves that ECT exhibits robust  $[Ca^{2+}]_i$  transients. Nonetheless, force generation in ECT was statistically much lower than in NCT (ECT:  $0.03 \pm 0.01$  mN/mm<sup>2</sup>; NCT:  $4.14 \pm 0.67$  mN/mm<sup>2</sup>,  $P < 0.01$ ). Additionally, the force transient temporal pattern was faster in ECT ( $T_{rise}$ : ECT =  $56 \pm 7$  ms, NCT =  $79 \pm 4$  ms,  $P < 0.01$ ;  $T_{relax}$ : ECT =  $124 \pm 19$  ms, NCT =  $205 \pm 12$  ms,  $P < 0.01$ ). The temporal pattern for the  $[Ca^{2+}]_i$  transients were also faster in ECT than in NCT ( $T_{rise}$ : ECT =  $28 \pm 2$  ms, NCT =  $35 \pm 2$  ms,  $P = 0.04$ ;  $T_{relax}$ : ECT =  $254 \pm 16$  ms, NCT =  $303 \pm 13$  ms,  $P = 0.04$ ). Unlike ECT, NCT did not exhibit stable force production above 3Hz. The ECT's faster temporal pattern seen in the  $[Ca^{2+}]_i$  and force transients may have been responsible for this.

Despite the encouraging result that ECT exhibited calcium transients that were relatively similar to NCT, they generated considerably less force when compared to NCT. This may be attributable to two factors; the low level of cardiomyocyte-like cells in the ECT construct and therefore decreased mechanical coupling between cells (data not shown), and the fast temporal rate of the calcium transients. Nevertheless, with further optimization, ECT may prove to be a viable therapeutic approach to help treat cardiovascular disease.

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