The Effect of ECM Stiffness on Ovarian Follicle Development

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Introduction

A diminishing ovarian follicular reserve is normal for women throughout their functional reproductive life; however, the cause for this decline is unknown. Constant remodeling of extracellular matrix (ECM) mechanical properties initiates signaling cascades via mechanotransduction that significantly influence ovarian follicle development and could potentially dictate the exhaustion of the remnant follicle pool. A three-dimensional cell culture system was implemented to examine the effects of ECM stiffness on ovarian follicle development by modulating ovarian hydrogel concentrations. Our findings indicate that increasing ECM stiffness may prematurely trigger follicle activation causing a decrease in the immature follicle population.

Materials and Methods

Derivation of Ovarian Hydrogel

An ovarian microenvironment was simulated using tissue-specific hydrogels derived from decellularized porcine ovaries. The ovaries were treated with several chemical reagents including triton X-100, sodium deoxycholate, and peracetic acid to remove genetic material. Decellularized tissues were lyophilized and ground into fine particles for pepsin and HCl digestion. The pH of the ECM digest was neutralized using NaOH and buffered with PBS. Physical crosslinking of hydrogels occurred at 37°C within 15-20 minutes.

In vitro Ovarian Culture

Two concentrations (2 mg/mL and 5 mg/mL) of ovarian hydrogels were used to test differing ECM stiffness on follicle development. Newborn mCherry mouse ovaries were microdissected and cultured for 7 days on top of the hydrogels in Waymouth's MB 7521 media. After day 7 culture, the ovaries were imaged using confocal microscopy and quantified using Volocity software to determine the total number of viable oocytes. The ovaries were fixed and serial sectioned for histological analysis using a periodic-acid Schiff (PAS) stain.

Decellularized Ovarian Tissue and Hydrogel Characterization

Decellularized ovarian tissue was characterized using native ovarian tissue as a baseline. Histological analyses were performed using DAPI and H&E staining. Collagen and glycosaminoglycan (GAG) presence was quantified using hydroxyproline and GAG assays. Residual DNA content was measured by agarose gel electrophoresis and PicoGreen dsDNA assays. Ovarian hydrogel viscoelastic properties were assessed through rheology testing (time, frequency, and strain sweeps).

Results/Discussion

Rheology testing of the two ovarian hydrogel concentrations confirmed that the 5 mg/mL peak storage (G') and loss (G'') moduli were approximately double the 2 mg/mL gel concentration. The increase in viscoelasticity resulted in a decrease in the total number of oocytes between the two gel concentrations (2 mg/ml ~1850 oocytes; 5 mg/mL ~1300 oocytes) suggesting that modulating ECM stiffness has a significant impact on follicle viability. As a control, ovaries from day 7 wild-type mice were microdissected for oocyte quantification (~1900 oocytes) and showing similar viability to the 2 mg/mL hydrogel ovary culture. PAS stained sections confirmed oocyte presence and morphology.

Conclusion

The three dimensional culture was aided by the ability to effectively tune mechanical properties of the ovarian hydrogels with varying concentrations. It was evident that stiffening the hydrogel substrate directly correlated to a diminishing oocyte population. Future studies will focus on modulating ECM stiffness with known cross-linking reagents to further analyze the effects of localized mechanical stimuli on follicle development.

Effects of Dystrophin Loss on the Biophysical Properties of Skeletal Muscle ECM

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Background: Duchenne muscular dystrophy (DMD) is caused by the loss of the protein dystrophin, leading to a disruption of the linkage between the cell cytoskeleton and extracellular matrix (ECM). Without this connection, the sarcolemma is susceptible to damage, leading to progressive deterioration of muscle cell integrity and function. Previous therapeutic strategies for DMD have focused on introducing new stem cells to diseased muscle, or altering the muscle cell itself, both with limited success. Given the important role of skeletal muscle ECM in directing resident cell function, ECM "health" may need to be addressed to improve the success of cell-targeted therapies. However, little is known about ECM biophysical properties in DMD. **Objective:** To investigate the effects of dystrophin loss on the composition, architecture, and mechanical properties of skeletal muscle ECM. **Approach:** Transverse cryosections (30 & 50 μm) from the quadriceps of male, 20 week-old wild-type (C57BL/6) and *mdx* mice were decellularized in 1% SDS, under constant rotation. To analyze ECM architecture and composition, 30 μm sections were stained with H&E and immunofluorescence analysis was performed with antibodies for the following ECM components: laminin, fibronectin, collagen I, III, IV, and VI. Changes in ECM compliance and adhesion were assessed using atomic force microscopy (AFM), performed on 50 μm sections. AFM measurements were collected in contact mode in PBS and force-vs-indentation curves were generated from an average of 10 points/sample, each repeated 10 times. To evaluate ECM stiffness, the Young's modulus was calculated for each curve using the Hertz-Sneddon model. The overall adhesive properties of the ECM were determined as the peak rupture force during the retraction of the AFM probe away from the test sample surface. **Results:** The ECM from *mdx* mice demonstrated an increased abundance of all ECM components, in addition to alterations in the localization and distribution of collagen I, III, and fibronectin, relative to wild-type controls. ECM from the *mdx* mice demonstrated decreased compliance relative to the WT mice, indicated by an increase in the average Young's modulus (WT: 2.9 ± 1.2 kPa vs. *mdx*: 7.2 ± 1.0 kPa). In addition, a decrease in the average adhesive force was observed in the *mdx* ECM, as compared to that from WT controls (WT: 3.0 ± 1.1 nN vs. *mdx*: 1.1 ± 0.3 nN). **Conclusions & Significance:** ECM alterations observed in *mdx* mice, relative to wild-type controls, suggest an unfavorable microenvironment for muscle regeneration and may contribute to the poor regenerative capacity observed in dystrophic muscle. Future studies are needed to examine the influence of such biophysical changes on muscle stem cell function. In addition, we plan to utilize functionalized AFM tips to probe for specific ECM components, allowing us to determine which factors may have the greatest influence on ECM stiffness and adhesive characteristics.

Shrinking Collagen Scaffolds to Modulate Mechanical Properties

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Type I collagen has ample use as a biomaterial in the field of regenerative medicine. Biomaterials made of purified collagen, however, have limited mechanical properties. We developed a method to modulate the mechanical properties of collagen scaffolds by increasing collagen density using shrinking technology.

Using the Hofmeister series of salts, we have assessed the effect of incubating disc-shaped collagen scaffolds in 90% saturated salts by measuring the surface area macroscopically. $(NH_4)_2SO_4$ and $BaSO_4$ increased the scaffold surface area, whereas NH_4Cl, Na_2SO_4, NaCl, $MgSO_4$, $CaSO_4$, $Ca(ClO_4)_2$, $BaCl_2$, $MgCl_2$, $Mg(ClO_4)_2$, $CaCl_2$, and $Ba(ClO_4)_2$ led to a decrease compared to control scaffolds. Largest shrinkage was observed with calcium chloride, barium perchlorate, magnesium chloride, and magnesium perchlorate. Scanning electron micrographs showed that pore sizes were reduced after treatment in these solutions, and that the collagen fibrils were less ordered in sheet structures. For these salt solutions, shrinkage was investigated at various salt concentrations, pH and initial collagen concentration in scaffolds using the response surface methodology.

In further studies, we focused on shrinkage using $CaCl_2$ and scaffolds were stabilized in the shrunken state by EDC/NHS chemical crosslinking. As assessed with a Piuma nanoindenter, scaffolds shrunken with 4 M $CaCl_2$ showed an approximately 10x increased stiffness compared to non-shrunken scaffolds treated with water or 4 M NaCl. In a dry state, $CaCl_2$ shrunken scaffolds were rather brittle, although calcium was not present in scaffolds after washing, as was assessed by XRD and FT-IR measurements. Chemical crosslinking (EDC/NHS) resulted in a larger decrease in amine groups in $CaCl_2$ shrunken scaffolds compared to water treated scaffolds, indicating more effective crosslinking.

We further studied the *in vivo* response to collagen scaffolds treated with $CaCl_2$, NaCl and water. In a rat subcutaneous implantation model, $CaCl_2$ shrunken scaffolds showed little degradation up to 90 days, whereas NaCl and water treated scaffolds were deformed and initial pore structure was less preserved. In addition, less cell infiltration was observed in $CaCl_2$ shrunken scaffolds.

Taken together, we here present a methodology to modulate mechanical characteristics of collagen scaffolds without the incorporation of additional components. These scaffolds may be used to evaluate the effect of mechanical characteristics on cell behaviour and *in vivo* response.

Biologic Scaffold Treatment for Volumetric Muscle Loss: Results of a Thirteen Patient Cohort Study

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Volumetric muscle loss is a severe and debilitating problem with significant clinical and economic consequences. Such injuries can occur as the result of traumatic injury, excessive exercise, tumor ablation, or degenerative disease. It is estimated that 35-55% of all sports injuries and 53% of battlefield extremity injuries involve damage to soft tissue and myofibers. This results in approximately 4.5 million reconstructive surgical procedures annually which contributes to billions of dollars in health care expenses. Current standards of care include physical therapy or orthotics, which do not correct underlying strength deficits, and surgical tendon transfers or muscle transfers, which often result in donor site morbidity and fall short of restoring function.

The results of a thirteen patient cohort study are described herein and involve a regenerative medicine approach for treatment of volumetric muscle loss. Acellular bioscaffolds composed of mammalian extracellular matrix (ECM) were implanted in thirteen patients with VML representing seven different anatomic sites and who had exhausted all available standard-of-care options. Immunolabeling of ultrasound guided tissue biopsies and MRI or CT imaging were performed to evaluate the cellular remodeling response and the macroscopic three dimensional formation of new muscle tissue, respectively. Force production, range of motion, and functional task performance were quantified, and electrodiagnostic testing was conducted to evaluate the extent of innervation.

In vivo remodeling of ECM bioscaffolds was associated with mobilization of perivascular stem cells; formation of new, vascularized, innervated skeletal muscle within the implantation site; increased force production; and improved functional task performance when compared to pre-operative performance. Implantation of acellular bioscaffolds derived from ECM can promote site-appropriate formation of functional skeletal muscle in patients with volumetric muscle loss.

Automated Porcine Lung Decellularization System for Pulmonary Tissue Engineering

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Background: The decellurization and recellularization of whole organs continues to show promise as a potential solution to meet shortfalls in the supply of transplantable organs. Efficient nonclinical and clinical development of this approach requires the availability of biological scaffolds that can be produced in a repeatable and scalable manner while maintaining sufficient underlying structural, mechanical, and compositional features to enable successful outcomes. Our ultimate aim is to use decellularized porcine lung scaffolds reseeded with human pulmonary endothelial and epithelial cells to generate lungs suitable for human transplant.

Methods: A fully automated, whole-lung decellularization system was developed consisting of a bioreactor chamber connected aseptically to reservoirs containing decellularizing detergents and wash buffers. A customized software control system managed the delivery of decellularization solutions to the bioreactor and the vasculature of the lung and managed the decontamination of waste. In-process effluents from the bioreactor were monitored for DNA and protein. Resultant scaffolds were analyzed to assess gross and microscopic anatomy, biochemical composition, vascular and airway integrity, mechanical strength, and cell attachment.

Results: Decellularized scaffolds produced using this automated system were devoid of porcine cells but retained the gross anatomical characteristics of the native organ. Major structural components such as collagens, elastin, and sulfated glycosaminoglycans were retained after decellularization as determined by quantitative biochemical assays and were not significantly different than native lungs. The microvasculature of decellularized porcine lungs conducted blood with minimal cross-over from the vascular compartment into the airway and with clearly visible vascular arborization. Decellularization did not affect static mechanical properties but did affect dynamic material properties. Initial recellularization studies using primary human-derived pulmonary artery endothelial cells seeded into isolated scaffold lobes showed distribution of cells throughout the scaffolds. Additionally, the cells adhered to endothelial basement membranes, demonstrated an elongated morphology, and created lumenal structures within 24 hours.

Conclusions: Our automated decellularization system produced scaffolds that retained many of the physical, chemical, and biological properties of native lung and are apt for supporting the growth of seeded cells. These data provide additional support for the use of decellularized lung scaffolds as a basis for pulmonary engineering.

3D hydrogel as a model to understand breast cancer metastasis

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Abstract

For decades, cancer biologists relied on two-dimensional (2D) monolayer cell culture platforms and/or in vivo animal models xenografts to investigate the complex mechanisms of tumorigenesis, angiogenesis, invasion and metastasis. Using these complementary systems, researchers have gained improved understanding of cancer biology and have developed many efficacious anti-cancer treatment methods. However, both monolayer cultures and xenografts have inherent limitations. Because of the lack of spatial cues needed to establish proper cell-cell contacts and cell-matrix interactions, 2D cell cultures are physiologically irrelevant and experimentally unreliable. Although studies based on animal models predict more pathologically relevant outcomes, the presence of many uncontrollable variables associated with these models makes it challenging to determine the impact of specific factors on tumour progression or to identify the therapeutic efficacies of novel personalised medicine. Moreover, testing therapeutic agents in animal xenografts is expensive, tedious and time consuming, thus further delaying the translation of new technologies from bench to bedside.

In this study we have used a three-dimensional (3D) hydrogel system that better mimics the topography and mechanical properties of the breast tissue, and also have used components of the breast extracellular matrix, thus recreating the native tissue microenvironment *in vitro* to study breast cancer metastasis, which is clinically the most challenging and lethal aspect of breast cancer.

Modified Gelatin methacrylate (GelMA) hydrogels of modulus comparable to that of breast tissue were prepared, on which breast cancer cell lines MDA MB 231, BT474 and MCF7 were cultured, later which proliferated into spheroids. A comparative gene expression analysis revealed that cells growing in the hydrogels expressed increased levels of genes implicated in the events of metastatic progression to that of cells cultured on 2D cell cultured plates. The cells cultured in GelMA hydrogels showed increased invasiveness and spheroid formation efficiency in vitro, and increased metastatic potential *in vivo*. Microarray analysis revealed a significant increase in expression of genes involved in cell-cell and cell-matrix interactions and tissue remodelling, cancer inflammation, and the Wnt, HIF1, NF-κB, PI3K/Akt signalling pathways - all of which are implicated in metastasis. Thus, culturing breast cancer cells in 3D hydrogels that mimic the *in vivo* tumour-like microenvironment enhances their metastatic potential. This system could serve as a comprehensive *in vitro* model to investigate the manifold mechanisms of breast cancer metastasis.

Effect of urea and thiourea on structure-function properties in xenogeneic scaffold generation

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Introduction: Solubilization-based antigen removal has been shown to significantly reduce hydrophilic and lipophilic antigens in bovine pericardium (BP) that could elicit host immune rejection.[1,2] Effective lipophilic antigen removal by promoting lipophile solubilization has previously been achieved using chaotropic solution.[2,3] However, a limitation of using a high concentration urea/thiourea solution was substantially altered tissue gross morphology.[2,3] This work aims to determine whether reduced urea/thiourea concentrations maintain BP structure-function properties.

Materials and Methods: BP samples were treated as previously described for hydrophile solubilization,[1,2] followed by lipophile solubilization in a gradient of urea/thiourea concentrations (0M/0M, 4M/1M, 6M/1.5M, or 8M/2M).[2,3]

To determine structural and functional integrity of BP samples, quantitative biochemistry (for collagen, elastin, glycosaminoglycan (GAG) content), histological analysis under polarized light (for collagen alignment), and tensile testing (for elastic modulus, ultimate tensile strength (UTS) and strain at UTS) were performed.

Results and Discussion: Significant reduction of BP collagen, elastin, and GAG content was observed with any urea/thiourea treatment (except collagen at 4M/1M, which was still linked to native BP levels). All urea/thiourea concentrations significantly decreased collagen alignment. Significant alteration of tensile properties (i.e., decreased elastic modulus and UTS, and increased strain at UTS) resulted from any urea/thiourea treatment. Together, these data support the loss of BP structure-function properties with 4M/1M, 6M/1.5M, and 8M/2M urea/thiourea concentrations.

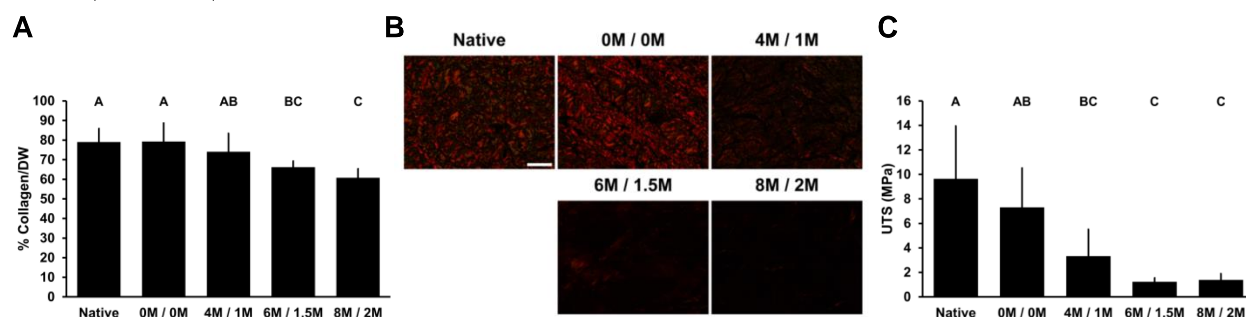

Fig 1. Collagen content per dry weight (A), collagen alignment (B), and ultimate tensile strength (C) of BP treated with a gradient of urea/thiourea concentrations. n=6/group. Scale bar=100 µm. Mean±SD. $p<0.05$.

Conclusion: Loss of BP structure-function properties following lipophilic antigen removal using 4M/1M, 6M/1.5M, and 8M/2M concentrations of urea/thiourea preclude their use in functional xenogeneic scaffold generation. Association of tissue damage with a gradient of urea and thiourea concentrations empirically supports molecular dynamics simulations describing direct urea-protein interactions.[4] Rather than the proposed indirect mechanism of solubilization via urea-mediated water network disruption, direct urea-protein binding and subsequent denaturation suggests that no urea/thiourea concentration is likely to simultaneously reduce tissue antigenicity and maintain structural and functional integrity. Indeed, 4M/1M urea/thiourea achieves less antigen removal than higher concentration solutions,[3] yet still alters structure-function properties. Therefore, chaotropes urea and thiourea may be unsuitable for use in functional xenogeneic scaffold generation for tissue engineering and regenerative medicine.

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Microvesicles within ECM Bioscaffolds as a Modulator of Cell Behavior

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Bioscaffolds composed of extracellular matrix (ECM) are prepared by decellularization of source tissues such as dermis, small intestinal submucosa (SIS), and urinary bladder (UBM). These bioscaffolds induce a variety of biologic effects in vivo including angiogenesis, modulation of macrophage phenotype, proliferation and mobilization of stem/progenitor cells, among others. These bioscaffolds also affect cell behavior in vitro. The proposed mechanisms for these induced cell responses include contact with select ECM ligands, release of ECM embedded growth factors/cytokines, chemokines, creation, exposure, and/or release of cryptic peptides from ECM parent molecules, and physical/mechanical properties of the matrix itself.

Microvesicles (MVs) are nano-sized extracellular vesicles created by mammalian and non-mammalian cells which can mediate cell to cell communication and affect cell behavior. MVs transport a variety of signaling and effector molecules including microRNAs, mRNAs, and proteins. We hypothesize that MVs withstand the tissue decellularization process and are present within ECM bioscaffolds, thus having the potential to affect cell behavior.

Porcine UBM, SIS, and dermis were digested with collagenase or proteinase K for 24 hours. The concentration of free nucleic acid and MVs-bound nucleic acid was determined. Electron microscopy, western blots and RNA sequencing techniques were used to identify MVs and their contents. In addition, functional assays including cell migration were investigated. Results show clear evidence of MVs within ECM bioscaffolds and the ability of these MVs to affect cell morphology and behavior.

In summary, the described work identifies the presence of MVs within naturally occurring bioscaffolds composed of ECM and suggests a novel mechanism by which ECM bioscaffolds affect cell behavior both in vitro and in vivo.

Development of Biologic Scaffolds from Human Glioma Tumors as an Organotypic Model to Study Disease Pathogenesis.

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Advancements in tissue engineering and regenerative medicine have led to the development of extracellular matrix (ECM)-based scaffolds that closely reflect the biological, structural and biochemical environment of native tissue ECM. Although much attention has been given to the therapeutic applications of scaffold-based systems in regenerative medicine, their utility in the establishment of *in vitro* physiological models to study disease pathogenesis is also of interest. Applying this strategy toward cancer research, (decellularized) matrix from human tumors provides an ideal *in vitro* microenvironment that closely mimics the ECM composition and biochemical property of native tumors.

The objectives of this study were to develop biologic scaffolds from non-tumorigenic brain and human glioma tissue for direct comparison of biochemical composition and *in vitro* analysis of neural stem cell migration and differentiation. Optimization of decellularization protocols was established for surgically resected non-tumorigenic brain tissue and human glioma tumor tissue. These protocols were designed to preserve the ultrastructure and biochemical ECM composition of the native source tissue. Following pepsin solubilization, biochemical analysis identified ECM proteins differentially expressed in decellularized glioma ECM compared to non-tumorigenic brain ECM. Results show a distinct molecular readout of glioma specific ECM components that are reflective of the tumor heterogeneity and pathological grade of the glioma tumor. Furthermore, retention and increased deposition of collagen and sulfated glycosaminoglycans occurred within tumor ECM, both hallmarks of neoplastic progression in many forms of cancer. The bioactive components retained in glioma ECM scaffolds are neurotrophic as evident by the increased formation of neurite extensions in differentiating neural stem cells, and increased migration when compared to the non-tumorigenic brain ECM, observations which may have important implications for neural stem cell based therapies currently proposed to treat glioblastoma.

The research described herein is the first reported ECM hydrogel specifically derived from human glioma tissue. This study provides evidence that decellularized glioma extracellular matrices are promising biologic scaffolds for improved modeling of the tumor microenvironment. The implications of this work extend beyond an in-depth understanding of the glioma microenvironment, and may offer insights into the identification of novel candidate tissue biomarkers and potential molecular targets for therapeutic intervention. Furthermore, the methods described can be applied to other tumor types and demonstrate the utility of tissue engineering concepts to advance tumor biology research.

Restoring Mucosal Barrier Function and Mediating Inflammation with an Extracellular Matrix Hydrogel: Potential Therapy for Ulcerative Colitis

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Ulcerative colitis (UC) affects over 700,000 individuals in the United States and current therapies are inadequate. Mucosal repair, combined with inflammation reduction and restoration of epithelial barrier function, is essential to effective treatment of UC. Extracellular matrix hydrogels (ECMH) have shown immunomodulatory activity and enhancement of stem/progenitor cell proliferation in in-vitro assays, but it is unknown whether ECMH can facilitate mucosal repair via these mechanisms in UC patients. The present study presents a proactive approach to UC therapy, which is a distinct departure from the immunosuppressive (defensive approach) and surgical (salvage approach) methods currently used to treat UC. The objective of the present study was to determine the efficacy of ECMH for restoration of colonic mucosa in-vitro and in a rat model of UC.

The effect of ECMH on transepithelial electrical resistance (TEER) and tight junction protein (E-cadherin) was assessed with monolayers of colonic epithelial cells. The adherence strength of ECMH to colonic mucosa was tested in-vitro. The efficacy of ECMH for treating colitis was tested in rats by administering 5% dextran sodium sulfate (DSS) in drinking water for 7d. Retention time of the ECMH following enema delivery was evaluated qualitatively using FITC-labeled ECMH and quantitatively with ^{14}C-labeled ECMH. Rats were then divided into groups based on treatment (ECMH, vehicle, no enema) and were sacrificed at 7d and 14d post-DSS (n=14/group/time point). Outcomes included clinical response, barrier function (measured by serum concentration of TRITC-dextran that was enterically administered 4h prior to sacrifice), colon length, gross and histologic scores, and ex-vivo secreted cytokine profile.

The adhesion strength of ECMH to healthy colonic mucosa was approximately 600 Pa. Accelerator mass spectroscopy showed that approximately 50% of the total of 40mg of ^{14}C-ECMH was retained following 2h, and 10% of the initial ECMH volume retained as long as 24h post-enema. Similarly, FITC-ECMH was visualized throughout the colitic colon for 12-24h. In-vitro, treating damaged colonic epithelial cells with ECMH resulted in expedited restoration of barrier function with increased electrical resistance and recovery of E-cadherin levels.

The present study showed that ECMH is mucoadhesive and remains adherent to the colon for >24 hours. Results of the present study suggest that an ECM hydrogel is a potential therapeutic option for individuals with UC.

Natural/Synthetic Hybrid Scaffolds for Tissue-Engineered Organ

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Poly(D,L-lactide-co-glycolide) (PLGA), a biodegradable synthetic polymer with great mechanical property, is widely used in tissue engineering applications. However, hydrophobicity and insufficient active factors of naive PLGA can make difficult to construct neo-organ. So, small intestine submucosa (SIS), demineralized bone particle (DBP) and collagen (extracted from duck feet, DC) are applied to fabricate hybrid scaffolds in order to improve the limited properties. Also, as gellan gum (GG) has a weak mechanical property, scaffold formation is considerably challengeable issue. GG/PLGA hybrid scaffold is fabricated in order to enhancing mechanical property of GG, by mixing PLGA. Cartilage, bone and intervertebral disc (IVD) are targeted in this study and physicochemical properties such as surface properties, compression strength, wettability, *in vitro* degradation as well as biological properties like cell viability, attachment, proliferation, mRNA expression and *in vitro/in vivo* histochemistry evaluation. Compared with PLGA scaffold, SIS/PLGA hybrid scaffolds show significant calcification as well as peri-implant fibrosis being altered the healing characteristics of SIS. A variety of growth factors, cytokines and ECM of SIS can promote bone formation. DBP/PLGA scaffold provide proper environment for enhancement of IVD phenotype and biological behavior. DC/PLGA scaffold can improve the cell bioactivities and osteogenesis and the increase of DC contents can affect the formation of cytoplasm actively, mineralization and distribution of calcification widely. GG/PLGA scaffolds show the best cell proliferation and active extracellular matrix secretion due to pore size and exceptional connections between pores. We can conclude that natural/synthetic hybrid scaffolds from SIS, DBP, DC, GG and PLGA provide an excellent substrate for behavior of cells with improved scaffold characteristics and can be a promising environment. This research was supported by the Bio & Medical Technology Development Program of the NRF funded by the Korean government (MEST) (NRF-2012M3A9C6050204), BK21 PLUS and Technology Commercialization Support Program (KMIFAFF 814005-03-1-HD020).

Self-assembled and Three-dimensional Multilayered Electrospun Nanofibrous Scaffold for Biomedical Applications

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In the field of tissue engineering, there are many advantages to mimic the extracellular matrix (ECM) that can be found in nature. To mimic nature ECM structures, nanofibrous scaffolds were fabricated using electrospinning techniques. Conventional electrospun scaffold showed the several limitations including limited cellular infiltration and two-dimensional (2-D) sheet-like structures. Herein, this advanced novel technique was developed to create three-dimensional (3-D) self-assembled and multilayered nanofibrous structures that can mimic patterns of nature tissues. Simple additional process which is a preparation of salt-added polymer solutions is the key idea. The remnant solvent which contains salts in a polymer solution can organize numerous-layer of electruspun structures due to the electrostatic repulsion between adjacent fibers and deposited mats. This novel technology demonstrated great potential as a drug delivery system with fast rate of drug adsorption, sequential and localized transgene expressing by using adeno-associated viral vector, and physical guidance to create multilayred cellular distribution *in vivo*. This advanced platform technology covers a variety of tissue engineering and regenerative medicine applications.

[1] G Jin, M Shin, SH Kim, H Lee, JH Jang, *Angewandte Chemie International Edition*, **54**, 7787 (2015)

Acellular Fish Skin Graft's Structure and Bioactivity is Better Preserved Compared to Mammalian Derived Scaffolds due to Less Harsh Processing

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Introduction: Acellular fish skin grafts are remarkably similar to human skin in its basic structure.1 yet the fish skin graft is fundamentally different from other mammalian derived scaffolds due to structural preservation and lipid preservation. While mammalian scaffolds require harsh chemical processing to reduce disease transmission risk (including viral and prion transmitted diseases), such risk from the Atlantic cod (Gadus morhua) to humans is nonexistent. Therefore, fish skin graft is subjected to gentle processing that preserves its structure and its bioactive compounds2, including omega-3 polyunsaturated fatty acids (PUFAs). Previous studies have shown that omega-3 fatty acids have anti-viral3 and anti-bacterial4,5 properties and also act as regulators of inflammation.6 Double blind randomized clinical trials have shown that acellular fish skin grafts promote significantly faster healing when compared to porcine small-intestinal derived scaffolds.7 A variety of other studies on the fish skin grafts, including acute wounds, oral wounds, burn wounds and dura replacement,8 have been performed with promising results. The acellular fish skin is currently being used in a regulatory approved and patented wound treatment product being marketed in the US and in Europe under the brand name Kerecis Omega3.**Objectives**: We set out to evaluate the following biological properties in fish skin and mammalian derived scaffolds: micro-structure, bacterial barrier, hemostatic properties, omega-3 PUFA content and cellular infiltration.

Methods: The scaffold structure was examined with scanning electron microscopy (SEM). NIH 3T3 fibroblasts were seeded onto a defined area of scaffold and cultured for 7-14 days. The scaffolds were stained with either hematoxylin and eosin (H&E) or fluorescent markers. Two chamber model was used to test bacterial barrier properties, with sterile broth in one of two chambers and broth with bacteria in the other. Effect on blood clotting was tested with the Lee White test. Lipids were extracted from the fish skin graft and omega-3 content examined with gas chromatography.

Results: Micro-structure of the fish skin grafts is highly porous, generally 10-100 μm in diameter while the micro structure of other biological scaffolds examined were denser and less porous. The acellular fish skin grafts possess superior ability to support three-dimensional ingrowth of cells when compared to human amnion/chorion membrane ($P<0.0001$). The material also acts as a barrier to bacterial invasion for over 48 hours in a two-chamber model at 37 °C. The fish skin graft had significantly faster aggregation effect compared to bovine pericardium collagen matrix ($p \leq 0.05$). The acellular fish skin graft contains EPA and DHA omega-3 fatty acids.

Conclusion: The importance of the structural preservation in biological scaffolds was demonstrated with cell ingrowth studies. Based on these histologic findings fish skin derived graft showed significant ability to support three-dimensional cell infiltration compared to human amnion/chorion membrane. The native omega-3 PUFAs content of the fish skin graft might play a key role in its ability to resist bacterial invasion. These results also show that structural preservation and the biomechanical properties of the fish skin graft provides a supportive environment for cellular infiltration.

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Electrospun Clay: Three-dimensional, macroscopic, and macroporous electrospun sponges with high moldability and flexibility for stem cell and adeno-associated viral vector delivery

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Electrospun nanofibers have been utilized as a powerful tool in numerous biomedical applications including scaffolds for regenerative medicines due to their advantageous characteristics such as biomimicry and capability to deliver cells. However, the electrospun scaffolds have been criticized from known hurdles including fixed shapes as thin sheet-like shapes, small pore sizes and necessity of major surgical procedures to be implanted. Herein, in order to overcome the aforementioned limitations, the novel concepts of electrospun nanofibers, electrospun clay as moldable electrospun scaffolds is presented. By the core/sheath electrospinning technique with the polystyrene (PS) and the poly (ε-caprolactone) (PCL) followed by selective leaching procedure, three-dimensional, macroporous and highly moldable nanofibrous structure was fabricated. The scaffolds could be shaped into a variety of shapes with various shaping methods including molding, rolling and manual shaping without compromise of nanofibrous structures on the outer surface and inside of the scaffolds. In order to investigate the potential of the scaffolds as tissue engineering scaffolds, various cell types including cell lines and stem cells were seeded on the scaffolds. Cells could infiltrate into the whole scaffolds *in vitro* and *in vivo* experiments, which is clearly advanced from the infiltration aspects of cells on the conventional electrospun scaffolds. The scaffolds were also utilized as a depot for gene delivery, which the adeno-associated viral (AAV) vectors were adhered on the scaffolds. Using AAV-containing macroporous electrospun clay, gene delivery efficiency was increased 4-times over the conventional electrospun scaffolds.

Additionally, advanced researches applied to cell and gene delivery systems based on fabrication of electrospun clay would be introduced. Furthermore, by co-utilization of other material groups including hydrogels and additives the macro- and microscopic properties of the scaffolds could be modified or enhanced. Additives such as carbon nanotubes (CNT), and polydopamine were blended to core PCL solution to enhance the mechanical (tensile) property of the electrospun clay. In conclusion, the presented fabrication method is a platform technology to produce macroscopic, mobile and flexible nanofibrous matrices whose mechanical and chemical properties could be precisely modified. The nanofibrous and biodegradable scaffolds with any desirable three-dimensional shapes with tunable mechanical strengths have great potential to contribute to tissue engineering and regenerative medicine.

Omics analyses of Islet-1+ clusters identify ECM proteins for biomaterial functionalization

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Islet-1 (Isl1) has been previously described as a cardiac progenitor cell (CPC) nuclear marker in the mammalian heart. Our group previously described extracellular matrix (ECM) proteins within and surrounding morphologically unique clusters of Isl1+ cells in mouse and human hearts. In 3D cell cultures, protein coatings more than doubled the number of mESC-derived Flk1+ CPCs when compared with controls. In this study, we sought to better understand the mechanisms of cell differentiation and proliferation in cardiac Isl1+ clusters using gene transcription and proteomics analyses. We identified 3 distinct Isl1+ clusters in the atria of 1st (1T) and 2nd (2T) trimester human hearts: cluster 1 (C1) containing a mixture of Isl1+ cells, the sinoatrial node (SAN), and cluster 3 (C3) containing only Isl1+ cells. LCM was used to isolate the 3 Isl1+ clusters from FFPE hearts. We identified no Isl1+ cells in the SAN of 2T human hearts, leaving 6 groups for omics comparison. NGS analysis showed that 1T C1 versus SAN (383), 1T SAN versus C3 (472), and C1 1T versus 2T (312) had the most significant statistical transcription differences amongst the clusters, followed by 1T C1 versus C3 (69), C3 1T versus 2T (29), and 2T C1 versus C3 (0). Proteomics analyses confirmed NGS results showing that 1T C1 and C3 were similar to each other and significantly different than the SAN. Gene Ontology Consortium web portal and Ingenuity Pathway Analysis were employed for further NGS and proteomics assessment, which showed that C1 and C3 are neuronal clusters innervating the atria of the heart. Canonical pathways such as “Axonal Guidance Signaling”, “Cardiac β-adrenergic Signaling”, “Calcium Signaling”, “Cardiomyocyte Differentiation via BMP Receptors” and “Factors Promoting Cardiogenesis in Vertebrates” were statistically activated in the different clusters. Proteomics data identified members of the collagen, fibrillin, and laminin families, as well as proteins such as perlecan and nidogen-1, as potential regulators of Isl1+ clusters. Protein-associated cell integrin binding sites are currently being investigated as promoters of cardiac or neural cell proliferation and survival under hypoxic conditions. In other studies, identified ECM proteins have been investigated for their in vivo affect post myocardial infarction in mouse and rat models.

Tissue Generation with Acellular Dermal Collagen Matrices: Clinical Comparison of Human and Fetal Bovine Matrices

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Since 2012, our center has treated over 320 patients with various bioengineered collagen scaffolds as a dermal regenerative strategy: a human-derived dermal matrix (HDM; AlloDerm LifeCell), a bilayered collagen wound matrix (BWM; Integra Dermal Regeneration Template; Integra Lifesciences), and a fetal bovine acellular dermal matrix (FBADM; PriMatrix, TEI Biosciences).

Selection of a bioengineered collagen scaffold is been based on a number of factors including wound location, bacterial burden, wound size and wound complexities. For example, BWM is typically utilized on minimally colonized, full-thickness wounds with fascia, tendon, and/or muscle. For diabetic foot wounds, deep venous stasis wounds, wounds with exposed periosteum, and fasciotomy wounds, we utilize native acellular dermal matrices, such as HDM and FBADM (200 HDM cases, 85 FBDAM cases).

Both HDM and FBADM are derived from native dermal tissues. However, differences in source tissue species (cadaver skin versus fetal bovine dermis) and processing techniques generate distinct product compositions and structural tissue features. These varying product features have been reported to influence product integration and the host tissue response.[1-3] Accordingly, while we observe that both HDM and FBADM are associated with reduction in pain and a relative increase in healing trajectory, we also observe differences in how these matrices appear clinically and integrate with host tissue over time.

Here, we present our findings on the gross characteristics of HDM and FBADM matrices over a one month follow-up period post-application. We demonstrate that while both products adhere directly to the wound bed; FBDAM typically engrafts more completely without as much (any) non-viable graft to remove at the two and four week follow-ups. We also note that a greater thickness of the FBADM fills with vascularized tissue compared to host tissue integration of HDM. Furthermore, re-epithelization appears to occur below the HDM, while epithelial migration occurs along a grossly indistinct border with the FBADM. These different clinical responses have lead us to use HDM as a bridge to split thickness skin graft (STSG) or epidermal bullae grafting (EBG); while we have used FBADM a more as a stand- alone product, although it supports STSG and EBG as well at one month.[4-5]

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Peripheral Nerve-Specific Extracellular Matrix Hydrogel Supports Repair After Peripheral Nerve Injury

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Introduction: Peripheral nerve injury commonly results in loss of neuromuscular function, often resulting in significant impact upon both quality of life and cost of care for patients. An easily injectable material in an off the shelf formulation which speeds and/or improves return to function would significantly improve quality of life for affected patients and result in a significant reduction of cost associated with chronic care related to their condition. We believe that a peripheral nerve specific extracellular matrix hydrogel (PNS-ECM) will provide a tissue-specific microenvironment which is conducive to nerve repair, including: nerve specific growth factors that are chemotactic signals for Schwann cells, and promote neurite outgrown.
Materials and Methods: Porcine sciatic nerve was decellularized to generate PNS-ECM using a previously described method(1). PNS-ECM was compared in vitro to small-intestinal submucosa matrix (SIS) and urinary bladder matrix (UBM). Experiments included nerve specific growth factor ELISAs, neurite outgrowth, and Schwann cell migration assays.). Neurite outgrowth performed using primary spinal cord neurons collected from E14 embryonic rat pups. Media was supplemented with solubilized PNS-ECM (125, 250, or 500 µg/ml), pepsin (50 µg/ml, negative control), or nerve growth factor (NGF 50 pg/ml, positive control). Schwann cell migration was performed using a boyden chamber with serum supplemented media with 50 or 100 pg/mg NGF as a positive control and negative controls were serum free media with pepsin and ECM supplemented on cell side only. The PNS-ECM was then used as a lumen filler during the repair of critical length gap nerve injury in a rodent sciatic nerve. Animals were sacrificed at days 7, 14, 28, and 90 and the nerves and gastrocnemius were harvested. Nerves were investigated for the rate of outgrowth of Schwann cells and axons across a 15 mm nerve gap. Slides of the nerve were stained for axons (β-tubulin III) and Schwann cells (GFAP). Muscles sections were stained with collagen V and muscle fiber diameter was measured.
Results and Discussion: PNS-ECM has a similar nerve specific growth factor profile to healthy nerve tissue, including high levels of nerve growth factor (NGF; PNS-ECM 6.1±0.6 pg/mg, healthy nerve 6.0±0.8 pg/mg), brain derived neurotrophic factor (BDNF; PNS-ECM 8.8±1.1 pg/mg, healthy nerve 10.8±0.6 pg/mg), and ciliary neurotrophic factor (CNTF; PNS-ECM 10.2±0.5 pg/mg, healthy nerve 10.2±0.4 pg/mg). SIS and UBM were dissimilar to native nerve except for BDNF in SIS and NFG in UBM. Outgrowth of neurites supplemented with solubilized PNS-ECM (250 and 500 µg/ml) were as much as 40% longer than negative control and the application of PNS-ECM showed a dose dependent effect that at 500 µg/ml promoted 80% of positive control. Solubilized PNS-ECM (1000 and 500 µg/ml) also produced significantly enhanced Schwann cell migration (1.46±0.37 and 1.28±0.36; normalized to positive control, serum supplemented media) compared to negative controls (solubilized pepsin 0.60±0.11 and PNS-ECM on cell side only 0.53±0.01).. PNS-ECM groups have significantly less atrophy at 28 and 90 days (48±2, 68±1) than saline control (59±2, 75±1).
Conclusions: *In vitro* experiments show chemotactic bioactivity of the PNS-ECM degradation products. Preliminary animal results suggest that treatment with PNS-ECM gel may improve outcomes metrics after repair of a critical length defect.

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Human Tumor-Derived Extracellular Matrix Recapitulates Tumor Vasculature compared with Human Normal-Derived Colon Matrix in a Three-Dimensional Model.

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The extracellular matrix (ECM) forms a dynamic an*d* complex scaffold that is composed of a variety of proteins and is specific to each tissue type. This matrix provides structural support, signaling cues, and influences the functions of cells *in vivo*. In particular, the ECM plays an essential role in vascularization and tumor angiogenesis. It is increasingly evident that two-dimensional models do not recreate the complex ECM structures or the cell-ECM interactions found *in vivo*. To study vascularization, recent studies have employed three-dimensional (3D) models. However, these models utilize a single ECM component, such as fibrin or collagen, and therefore, do not accurately mimic the matrix environment found *in vivo.* To overcome this discrepancy, matrix bioengineering techniques are being utilized to recreate the ECM composition *in vitro*. The aim of this study was to reproduce the both the normal and the tumor colon microenvironment and to compare how these different microenvironments support vascularization and tumor growth. We have successfully decellularized both normal human colon, as well as a human metastasized colon tumor and generated an ECM hydrogel that can act as a 3D scaffold for co-culture of endothelial progenitor cells and fibroblasts. A comparison of the protein compositions of the two matrices by mass spectrometry revealed a set of proteins present in the tumor ECM that was not present in the normal colon ECM, suggesting that some of these proteins may be involved in tumor progression. Moreover, a comparison of the vasculature formed using each of the ECMs showed statistically significant differences in the vessel diameter, vessel length, the number of branch points, the vessel percentage and the fractal dimension. Additionally, we studied the metabolism of the tumor and endothelial cells using Fluorescence Lifetime Imaging Microscopy (FLIM), and observed that cells seeded in the normal ECM are less glycolitic than in the tumor ECM. These results highlight the importance of using native tissue specific matrices in vascularization and tumor models, which better, mimic and recapitulate the complexity of the microenvironment *in vivo.*

Epithelial Regrowth Prevents Reconstructive Remodeling of the Muscle Wall in the Porcine Esophagus after Replacement with Biomatrix

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Objective

Early epithelialisation may prevent reconstructive healing of the esophageal muscle wall in a procine modell where 3 cm of the thoracic esophagus is replaced by a bridging graft composed of a silicone stented Biodesign® mesh.

Methodology

In previous studies, a porcine model for studying esophageal regeneration after resection of intrathoracic esophagus using a silicone stented Biodesign mesh ,has been developed.
A series of six piglets have been operated with the method, and they lived 20 days prior to sacrifice for histological analysis.
The histological analysis showed a thick layer of smooth-muscle cells in the bridging area, which begins to organize itself in two perpendicular layers in the center. The analysis implies a regenerative healing response.
To prevent stent migration, wich has been show to be crucial for non-stricture remodeling, six piglets were operated with a more rigid stent and sacrificed after 35 days.

Result

After 20 days, the reconstructive remodelling of the muscle wall depended on whether the stent was in situ or lost at sacrifice.
Inversly, the mucosa had healed over most of the bidging area in the animals where the stent was lost, but not in the animals where the stent was in place. Instead the luminal side of the bridging area was composed of inflammatory cells, including nitrophils and significantly more of macrophage type two especially near the bioprosthetic mesh.
The histologic analysis after 35 days showed a thicker epihelium and less small muscle cells and macrophage type two in the in the bridging area, compared to the 20 days study.

Conclusion/discussion

In the field of tissue enginering, a combination of both in vitro and in vivo methods can be used in order to replace missing tissue after malformations, damage and resections. The aim is to acheive a fully functioning replacement of the part that it missing. Studies of esophageal tissue engineering has previously used both decelluriarised matrices with or without seeding of cells.
Some authors have acheived an increased reepethilalisation of the replaced part of the esophagus, using seeded cells or copper ions. In these studies faster reepithelialization has been interpreted as an improved regenerative outcome.
In regenerative work done on amphibian limbs, the reformation of a severed limb is dependent of a delayed epithelialisation, as an ionic leak over the damaged area is needed to promote the regenerative respons.
In our model, the reepithelialization of the briging area seems to counteract the accumulation and organisation of muscle cells in the esophageal wall. This implies that methods acheiving faster repithelialization might leed to a poorer functional end result.
Further studies are needed to conclude where the regeneration of esophageal tissue are dependent on ionic leaqks in this model.

Effect of donor age on extracellular matrix composition in xenogeneic scaffold generation

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Introduction: Xenogeneic tissue scaffolds represent a potential solution for patients requiring lifelong vascular grafts. As structure-function properties are critical in functional tissue engineering applications, age-associated differences in xenogeneic tissue composition are important factors to consider when selecting a source tissue. This study aims to compare the extracellular matrix (ECM) composition of adult and calf bovine carotid artery.

Materials and Methods: Adult and calf bovine carotid artery samples (n=3 per group) were processed for biochemical ECM content as previously described.[1,2] 5 mm diameter discs were weighed, frozen at -20°C overnight, lyophilized for 48 hours, and weighed again. Collagen and glycosaminoglycan (GAG) content per dry weight (DW) were quantified using commercially available kits.

Results and Discussion: Water content of adult carotid was not significantly different from that of calf carotid (**Fig 1A**). However, significant differences in collagen and GAG content between adult and calf carotid were observed. Carotid collagen content of adult samples was significantly greater than that of calf samples (**Fig 1B**). Carotid GAG content of adult samples was significantly less than that found in calf samples (**Fig 1C**). These age-related differences in carotid ECM composition are in agreement with known differences in cardiovascular physiology. Adult animals generally possess higher blood pressure levels than their young,[3] which may explain the need for greater collagen content in the carotid artery.

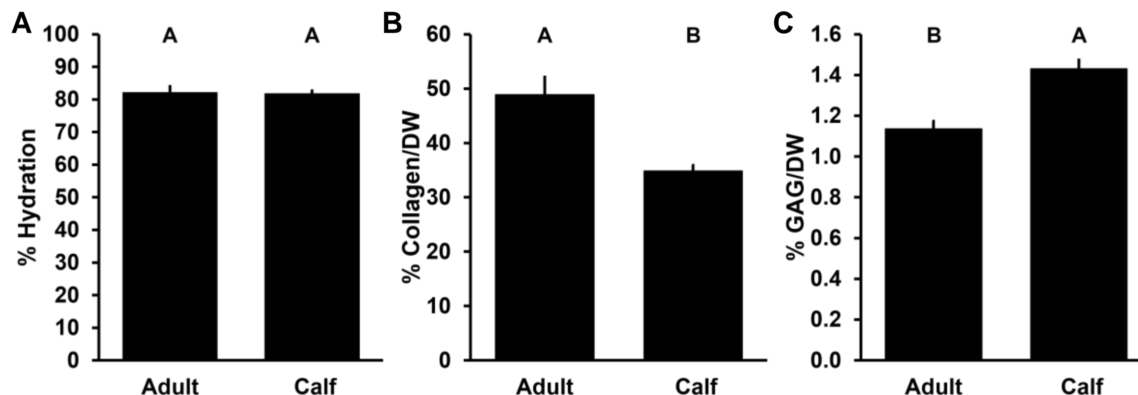

Fig 1. Water (A), collagen (B), and GAG (C) content of adult and calf carotid arteries. n=3/group. Mean±SD. $p<0.05$.

Conclusion: Differences in ECM composition exist between adult and calf carotid artery and may be attributed to age-dependent differences in cardiovascular physiology. Towards development of functional xenogeneic tissue scaffolds for tissue engineering and regenerative medicine applications, these differences in ECM biochemical composition may be critical factors in patient outcome, underscoring the importance of their consideration during source tissue selection.

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Toward Whole Liver Engineering: Liver Extracellular Matrix Promotes the Phenotype and Function of Human iPSC-derived Hepatocytes and Endothelial Cells

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Whole organ engineering offers an alternative to traditional liver transplantation that may address the critical shortage of donor organs and eliminate the need for recipient immunosuppression. Through perfusion decellularization of livers, biocompatible xenogeneic extracellular matrix (ECM) scaffolds can be produced that retain the liver's structural complexity. Subsequent recellularization of these scaffolds requires hepatocytes and liver sinusoidal endothelial cells (LSECs). However, these two cell types are notoriously difficult to culture *in vitro*—displaying a rapid loss of phenotype and function as well as limited proliferation—which restricts their application in liver engineering. We have previously shown that liver ECM can be used to enhance primary rat LSEC and hepatocyte stability in 2D culture (Sellaro *et al.* 2007; Loneker *et al.* 2016). In moving toward clinical translation, utilization of patient-derived cells for recellularization of liver scaffolds will minimize or eliminate graft rejection. One of the most promising cell types for this purpose is induced pluripotent stem cells (iPSCs). It has been shown that iPSCs can be differentiated into endothelial cells and hepatocytes (Adams *et al.* 2013; Takayama *et al.* 2012; Takebe *et al.* 2013). However, they have yet to be evaluated for liver scaffold re-endothelialization. To better understand the microenvironment necessary for the regulation and differentiation of LSECs, an *ex vivo* whole-organ culture system was used to investigate the application of human induced pluripotent stem cell-derived endothelial cells (iPSC-ECs; Cellular Dynamics International, Madison, WI) as a clinically-relevant source of cells for reconstitution of the sinusoids of liver scaffolds. Decellularized whole rat liver ECM scaffolds were mounted in a custom bioreactor and iPSC-ECs were seeded into both the portal vein and hepatic veins. Tissue sections stained with hematoxylin and eosin showed that iPSC-ECs attached throughout the 3D liver scaffolds. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) of tissue sections showed that iPSC-ECs were viable after 7 days of *ex vivo* culture in our perfusion-bioreactor. In preliminary work using human iPSC-derived hepatocytes (iPSC-Heps; Cellular Dynamics International), cells grown in culture vessels coated with pepsin-digested porcine liver ECM displayed better retention of hepatocyte morphology than vessels coated with collagen. When porcine liver ECM was used as a coating or as a media supplement, iPSC-Heps exhibited significantly more albumin and urea production than un-supplemented cells. Overall, the data suggests that liver ECM, even in a solubilized form, promotes hepatocyte phenotypic stability in culture.

Mechanical modulation of a human plasma-based skin scaffold via reactive multi-arm polyethylene glycols

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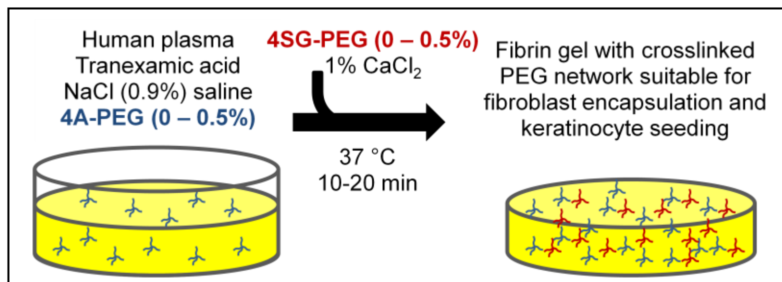
Aim of Research



Over the past decade, an autologous human plasma-based dermoepidermal skin equivalent designed by our group has been clinically applied to treat burns and surgical wounds; however, poor mechanical properties including fragility during handling, high degradation rates, and shrinking during culture and implantation persist and demand creative solutions. The described research project aims to better understand the material properties of these scaffolds and cell-scaffold interactions through mechanical modulation using versatile polyethylene glycols (PEGs). Amine reactive multi-arm PEG platforms have previously been investigated as tissue adhesives, surgical sealants, and hemostatic agents, and have demonstrated excellent strength as network gels. The incorporation of a biologically reactive biodegradable PEG network into this clinically relevant skin scaffold should improve the aforementioned mechanical deficiencies and demonstrate new methods of scaffold preparation, processing, and handling.

Methodology and Important Findings

Autologous human plasma-based hydrogel scaffolds were prepared by combining citrated human plasma of known fibrinogen concentration with tranexamic acid, normal saline solution, and calcium chloride. Gelation occurs within 12 minutes of $CaCl_2$ addition. Amine reactive succinimidyl glutarate terminated 4-arm PEG (4SG-PEG) and amine terminated 4-arm PEG (4A-PEG), each with a MW of 10 kDa, were used to modulate mechanical properties. When mixed in a 1:1 ratio in 1X PBS at a total polymer concentration of 6% (w/v), a network gel formed within 5 minutes. The addition of the same concentration of 4SG-PEG at to the plasma protocol resulted in irreversible inhibition of plasma gelation, whereas the addition of 4A-PEG resulted in delayed gelation proportional to the concentration added. An *in vitro* method was optimized to form a PEG network in the plasma hydrogel by introducing the two PEGs in low concentrations (0-5%) in a 1:1 ratio (Figure). Preliminary rheological tests on plasma hydrogels without PEG were made. A stress sweep identified the linear viscoelastic region of the hydrogel from which a functional stress was selected for future frequency oscillation tests.

Significance

A method was developed to modulate the material properties of autologous human plasma-based hydrogel scaffolds by incorporating a matrix of biodegradable multi-arm PEGs. Amine reactive succinimidyl glutarate terminated 4-arm PEG (4SG-PEG) and amine terminated 4-arm PEG (4A-PEG) reacted covalently with fibrinogen, plasma proteins, and each other to form a PEG-fibrin network hydrogel. Amine-reactive 4SG-PEG was shown to react with the amine terminus of fibrinogen, preventing cleavage and fibrin formation. This finding is supported by literature describing thrombin cleavage of fibrinogen at the N-terminus. After 24 hours, plasma proteins leeched from the human plasma-based fibrin gel into solution, but the addition of the PEG network significantly reduced protein leeching. This suggests covalent and physical interactions between the PEG network and the plasma contents are present and is predicted to significantly improve cell proliferation. Studies on swelling, degradation, gel time, material strength, cell proliferation, and cytotoxicity are planned. The presented polymer system has known biocompatibility, hydrolytic degradability, and tunable mechanical properties that could widely impact biological scaffolds.

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Mitral valve tissue engineering – a model for investigating valve degeneration

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Background and Objectives: Mitral valve prolapse is the most common cause of regurgitation referred for surgical repair or replacement of the mitral valve in the western world, but currently available substitutes do not adequately comply with the performance and flow pattern requirements of the left ventricle. Our goal is to develop a tissue engineered mitral valve possessing appropriate composition, structure and function with “ideal” characteristics: large orifice, rapid opening and closure, no rigid support, maintenance of mitral annulus – papillary muscle continuity, extended durability, biocompatibility, and also, easy to produce and construct.

Methods: An extracellular matrix-based scaffold was developed based on the native porcine mitral valve, as starting material, and a technique to remove the porcine cells without damaging the matrix components. In order to stabilize the matrix components and slow down their degradation, scaffolds were treated with penta-galloyl glucose (PGG), a well-characterized polyphenol with high affinity for collagen and elastin. Scaffolds were seeded with human fibroblasts and incubated in the presence of TGF-beta1.

Results: The insoluble extracellular matrix components, crucial for maintaining the valve shape and function, were well preserved in leaflets and chordae after cell removal. Biaxial testing showed similar mechanical characteristics of the PGG- treated scaffold to the fresh tissue. Cell-seeded scaffold conditioned in the bioreactor showed good viability. Cells seeded on PGG-treated scaffolds presented reduced matrix metalloprotease activity.

Conclusions: A mitral valve scaffold was developed, that presented the biochemical composition essential for the valve highly dynamic mechanical demands, as well as its durability. PGG could avoid weakening of the leaflets and chordae under mechanical and biochemical stress and also prevent activation of fibroblasts.

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ELECTRODIAGNOSTIC EVALUATION OF INDIVIDUALS IMPLANTED WITH EXTRACELLULAR MATRIX FOR THE TREATMENT OF VOLUMETRIC MUSCLE INJURY

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Background: While skeletal muscle has a great capacity for repair, VML (comprising a muscle compartment defect of >20%) often leads to irrecoverable loss of tissue. VML is generally caused by trauma, including battlefield injuries, degenerative disease, or tissue resection for removal of neoplasms. The permanent loss of tissue impairs long-term function and results in significant disability. Unfortunately, procedures such muscle transfer and amputation, often utilized as a last resort in the treatment of VML, result in similarly high morbidity rates and substantial functional deficits. Recently, the use of biologic scaffolds composed of extracellular matrix (ECM) has been evaluated as a new treatment option for VML. Our group recently demonstrated positive outcomes in five patients treated with an ECM scaffold for VML, yet variability in functional improvements and strength were observed[1]. It is likely that restoration of nerves and the formation of new motor endplates within the ECM may contribute to the variability among subjects, however it is not clear whether the new tissue formed in these subjects is innervated and how this may relate to functional outcomes.
Objective: The purpose of the present study was to quantify nerve conduction study (NCS) and electromyography (EMG) changes following ECM bioscaffold placement in individuals with VML. We also explored the ability of pre-surgical NCS and EMG to be used as a tool to help identify candidates that are likely to display improvements post-surgically.
Methods: Eight subjects (including 5 subjects from the previous study) with a history of chronic VML were recruited to participate in this study. Prior to surgery, strength of the affected region was measured using a hand-held dynamometer, and electrophysiologic evaluation was conducted on the affected limb with standard method of NCS and EMG. Patients also completed a pre-physical therapy program to maximize their strength and function prior to surgery. During surgery, scar tissue debridement and selective tenolysis were performed, followed by surgical placement of ECM bioscaffold at the site of VML. Following surgery, all patients again participated in a physical therapy program. Electrophysiologic evaluation and strength measurements were repeated six months after surgery.
Results: Seven out of eight subjects presented with a pre-operative electrodiagnosis of incomplete mononeuropathy within the site of VML. After ECM treatment, five of eight subjects showed improvements in NCS amplitude or needle EMG parameters. The presence of electrical activity within the scaffold remodeling site was concomitant with clinical improvement in muscle strength.
Conclusions & Significance: Electrodiagnostic data provides objective evidence of physiological improvements in muscle function following ECM placement at sites of VML. The data also support the conclusion that muscle judged to be denervated at baseline, as determined by NCS or EMG, are unlikely to respond to ECM placement. Future studies are warranted to further investigate the potential of needle EMG as a predictor of successful outcomes following ECM treatment for VML.

References:

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Degrees Expected: B.S
Expected Year of Graduation/ Program Completion: 2016
Major or Field of Study: Chemical Engineering
Academic Level: Undergraduate Student

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Title: *In Vitro* Evaluation of Calcium Peroxide Release from Composite Poly(lactic-*co*-glycolic acid) Microsphere Scaffolds

Category: Materials Science, Polymer Science, Chemical Engineering

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Bone tissue engineering is the study of how the intersection of cells, biomaterials, and bioactive factors can restore normal bone function after surgical, degenerative, or traumatic bone loss. The objective of this project was to investigate the potential of a materials-only approach for guided bone regeneration.

Many bone tissue engineering therapies involve the use full-length proteins as bioactive agents. However, calcium-releasing materials have been shown as effective at inducing osteogenesis. In this study, the capabilities of composite poly(lactic-*co*-glycolic acid) (PLGA) and calcium peroxide (CaO_2) sintered microsphere scaffolds were investigated as an alternative to current bone repair strategies. We hypothesize that the

hydrolytic degradation of composite PLGA/CaO_2 3-dimensional (3D) scaffolds would result in a measurable *in vitro* release of CaO_2 over 28 days.

Scaffold Fabrication: 0%, 0.5%, and 1% 85:15 PLGA/CaO_2 microspheres were fabricated via the single emulsion technique. 3D scaffolds were created by sintering loaded microspheres in a 10 x 5 mm cylindrical stainless steel mold.

Calcium Release: The release of Ca^{2+} ions was determined by submerging scaffolds in 1 ml of calcium-free phosphate buffered solution with 500 μL samples taken and replaced at 1, 2, 4, 8 and 12 hours, and 1, 2, 3, 4, 5, 6, 7, 9, 11, 13, 15, 17, 19, 21 and 28 days. Quantities of Ca^{2+} were measured using the Calcium (CPC) Liquicolor kit.

Scaffold Imaging: Composite microspheres were analyzed qualitatively for surface integrity using a scanning electron microscope (SEM).

We report that the calcium release for 0.5% and 1% PLGA/CaO_2 increased in a time-dependent manner. However, 1% PLGA/CaO_2 scaffolds released a significantly greater amount of calcium ions over time. As expected, there was no calcium release for 0% scaffolds. The *in vitro* release kinetics reveal the structure-function relationship between the materials, which can serve as a correlation to *in vivo* release. Furthermore, SEM images suggest that in comparison to pristine scaffolds, CaO_2 loading did not cause morphological changes on the microsphere surfaces. For future studies, we predicted that the observed calcium release could influence the osteogenic differentiation of stem cells without other supplemental factors, as noted in recent studies.

Emerging Implications of Ineffective Biologic Scaffold Decellularization upon the Host Response

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The clinical outcome of therapies employing biologic scaffolds derived from decellularized tissues for tissue repair depends on many factors, including those which affect the type of immune response to the implanted construct. An association has been established between components of the innate immune response, such as macrophage phenotype, and the clinical outcome of these technologies. Changes in macrophage phenotype in response to these biologic scaffolds have been shown to be dependent upon the presence of antigenic cellular remnants that remain following the decellularization process. The objective of the present study was to assess the effect of specific cellular components on the host response to implanted biologic scaffolds and evaluate the role of toll-like receptors (TLR) in this process.

To test the hypothesis, purified collagen scaffolds were inoculated with DNA, mitochondria and cell membrane fragments at three different concentrations. Primary bone marrow-derived mouse macrophages were cultured on these inoculated scaffolds and the change in macrophage phenotype was assessed by staining for F4/80 (pan macrophage), iNOS (M1 macrophage) and Fizz1 (M2 marker). The same scaffolds were also implanted for 14 days in the rat abdominal wall and macrophage phenotype in vivo assessed using CD68 (pan macrophage), CD86 (M1) and CD206 (M2). In addition the involvement of TLR 9 in mediating this response was investigated by exposing wild-type and TLR-deficient macrophages to the scaffolds in vitro and measuring TNF alpha expression by ELISA.

All three cellular components stimulated a pro-inflammatory response in a dose dependent manner and corresponding increases in TNF alpha expression. The strongest effects were seen with cell membrane fragments followed by mitochondria and DNA, however, each component was able to stimulate a Proinflammatory response independently. These results show that effective decellularization is essential for successful constructive remodeling of these biologic scaffolds to occur and that reliance on a single factor for demonstrating successful decellularization does not infer a positive constructive remodeling response will occur since other cellular components could have significant influence on host response.

Biological Scaffolds for Regenerative Medicine – Ninth Symposium (Napa Valley, CA)
Date of submission: December 15th, 2015 (Restrictions – 3,000 characters)
Date of conference: April 28th-30th, 2016

Title: Extracellular Matrix Hydrogel Promotes Tissue Remodeling, Arteriogenesis, and Perfusion in a Rat Hindlimb Ischemia Model

Authors: Jessica. L. Ungerleider, Todd. D. Johnson, Melissa J. Hernandez, Dean. I. Elhag, Rebecca L. Braden, Monika Dzieciatkowska, Kent G. Osborn, Kirk C. Hansen, Ehtisham Mahmud, and Karen L. Christman

The prevalence of peripheral artery disease (PAD) is increasing and can lead to critical limb ischemia (CLI), ultimately increasing the risk of potential limb amputation. Currently, there are no therapies for PAD to effectively treat all of the underlying pathologies, including reduced tissue perfusion and muscle atrophy. This study aimed to examine acellular extracellular matrix based hydrogels as potential therapies for treating PAD. We tested the efficacy of using a tissue-specific injectable hydrogel, derived from decellularized porcine skeletal muscle (SKM), compared to a new human umbilical cord derived matrix (hUC) hydrogel. The latter could have greater potential for tissue regeneration due to its young tissue source age. In a rodent hindlimb ischemia model, both hydrogels were injected 1-week post-surgery, and perfusion was regularly monitored with laser speckle contrast analysis (LASCA) for 35 days post-injection. Immunohistochemistry and histology were used to assess neovascularization and muscle remodeling. Quantitative proteomic analysis showed that both SKM and hUC contained complex, tissue-specific compositions (Figure 1). Significant improvements in hindlimb tissue perfusion and perfusion kinetics were observed with both biomaterials (Figure 2). End point histology indicated this was a result of arteriogenesis, rather than angiogenesis (Figure 3A), and verified the materials were biocompatible. Furthermore, muscle fiber analysis showed the tissue specific matrix (SKM)-injected animals had muscle fiber area and circularity most closely resembling healthy contralateral muscle (Figure 3B,C). These results show the efficacy of an injectable ECM hydrogel alone as a potential therapy for treating patients with PAD. They also suggest that non-tissue specific responses such as vascularization can be stimulated with a non-tissue specific ECM hydrogel, but a tissue specific ECM hydrogel may better influence muscle regeneration and remodeling.

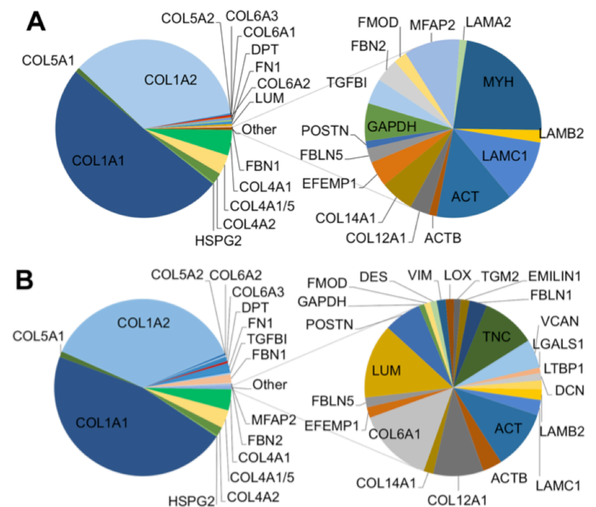

Figure 1: Targeted quantitative mass spectrometry analysis of extracellular matrix (ECM) components indicates complex tissue-specific composition of SKM (A) and hUC (B).

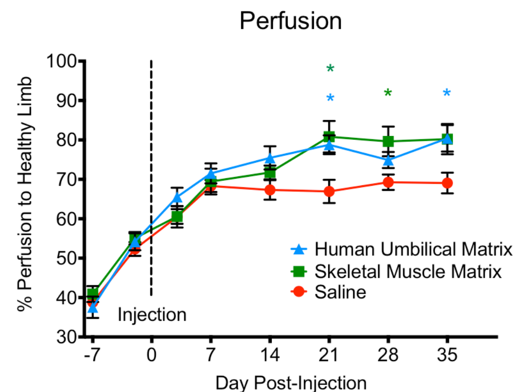

*Figure 2: SKM and hUC both significantly improved perfusion after 21 days over saline control. *P<0.05 as determined by one-way ANOVA.*

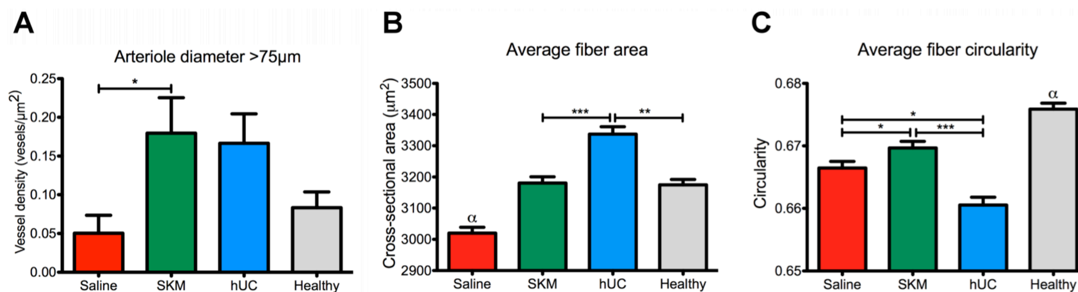

*Figure 3: (A) Vascular remodeling analysis at day 35 post-injection shows that SKM-injected animals have significantly more arterioles of diameter greater than 75 µm. Muscle remodeling analysis shows that animals injected with SKM have both muscle fiber area (B) and fiber circularity (C) most closely resembling healthy muscle. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, αP<0.001 compared to all other groups.*

Application of Urinary Bladder Matrix (UBM) in the Treatment Algorithms for Traumatic and Combat Casualty Extremity Wound Care

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Introduction: Due to the destructive nature of crush, avulsion, and low- or high-energy wounding mechanisms, massive soft tissue defects are encountered at ever increasing rates. Treatment of wounds secondary to severe civilian trauma as well as war-related trauma requires extensive surgical care algorithms with complex salvage techniques to accomplish stable, definitive wound coverage. Commonly, exposed at-risk structures, such as tendons, nerves, blood vessels, and bone, require the creation of durable, vascularized coverage to facilitate healing. The goal is to provide a viable wound environment to achieve successful wound control and/or coverage in order to allow the patient to begin rehabilitation. This clinical case series reports on the regenerative potential of urinary bladder matrix (UBM) scaffolds in the reconstruction of traumatic and combat extremity wounds.
Methods: A retrospective review of reconstruction cases employing UBM for the treatment of traumatic and combat casualty extremity wounds was performed. All cases reviewed were performed at Walter Reed National Military Medical Center, Bethesda, MD between 2010 and 2014. UBM is an acellular, non-cross-linked, resorbable extracellular matrix (ECM) biologic scaffold, available in powder and sheet forms. The data collected and analyzed during the retrospective review included: patient demographics, injury sites, number and type of UBM treatments, complications, and overall tissue remodeling outcome.
Results: This clinical case series details a number of examples in which UBM was utilized to treat various traumatic and combat extremity wounds. A total of 51 cases with severe extremity wounds were treated with UBM. Use of UBM was successful in 86% (44/51) of these cases in directly stimulating a viable granulation wound bed in preparation for either re-epithelialization, secondary dermal regeneration template (DRT) placement, skin grafting, and/or flap coverage. UBM failures occurred in 14% (7/51) of cases which was determined to be most commonly associated with material losses secondary to frictional forces/shearing, debridement of early remodeled tissue, superficial wound infections, or inadequate neovascularization requiring more advanced wound care therapies measures (e.g. hyperbaric ozygen).
Conclusions: Wound care modalities and regenerative therapies including ECMs and biologic scaffolds such as UBM can play an important role in the surgical care algorithms for improving outcomes and achieving definitive stable wound coverage for extremity and other related soft tissue injuries. This work is one of the largest clinical series illustrating the clinical application of UBM to traumatic extremity injury reconstruction. Application of UBM to severe soft tissue defects or hypovascular wounds can directly aid in facilitating definitive soft tissue reconstruction via establishment of neo-vascularized wound beds acceptable for second stage wound and skin coverage options within traumatic and combat-related extremity wounds.

Detergent Decellularization Methods Affect the Surface Molecular Functionality of Biologic Scaffolds

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Generation of extracellular matrix (ECM) biologic scaffolds for tissue engineering and regenerative medicine applications commonly involves the use of detergents to solubilize cell membranes and dissociate DNA from proteins. The deleterious effects of detergents upon ECM structure, growth factor content and protein denaturation are well known. However, the effect of detergent treatment upon the surface ligand landscape of biologic scaffolds has not previously been characterized. The objective of this study was to determine the effect of different detergents upon the surface molecular functionality of a representative tissue, specifically the basement membrane complex (BMC) of porcine urinary bladder.

The BMC and underlying lamina propria were isolated and harvested from porcine urinary bladders. Six different treatments were utilized comprising: 3% Triton-X 100, 4% sodium deoxycholate, 1% and 0.1% sodium dodecyl sulfate (SDS) and 8 mM 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) and a non-detergent agent, 0.1% peracetic acid. BMC samples were analyzed by time of flight secondary ion mass spectroscopy (ToF-SIMS), a powerful and sensitive surface analytical technique, to provide information on the composition, structure, orientation and spatial distribution of molecules present on the surface of the treated bladders. Positive and negative ion ToF-SIMS spectra were obtained from 100 x 100 µm spots per treatment group ($n \geq 7$ from ≥ 3 replicate samples) on the basement membrane side with an ION-TOF 5 instrument using a 25 keV Bi3+ analysis beam. Total ion dose was maintained under 10^{12} ions/cm^2. Primary human urothelial cells (HUCs) were seeded upon the basement membrane surface of detergent treated bladders; bladders treated with water only served as a control. HUCs were cultured for 7 days prior to fixation; cell phenotype was visualized with fluorescent probes for F-actin and 4',6-diamidino-2-phenylindole (DAPI) and immuolabeled E-Cadherin. Apoptosis was investigated using the Tunel assay.

Principle component analysis (PCA) of negative ion spectra separated detergent treatments by the presence of phosphates and other peaks observed in reference spectra for detergents. Highly loading peaks confirmed PCA results with characteristic peaks of residual sodium deoxycholate, Triton-X 100 and SDS identified. Viability, proliferation and phenotype of HUCs, demonstrated by F-actin and DAPI, was maintained on all bladders except those treated with SDS. Additionally, significantly more apoptosis was observed on 1% and 0.1% SDS scaffolds and PAA treated scaffolds. HUCs cultured on water treated bladders expressed E-Cadherin, localized to intracellular borders in stratified cell layers whereas HUCs cultured on detergent treated bladders showed moderate, diffuse E-Cadherin expression.

These results demonstrate the suitability of ToF-SIMS to characterize the surface molecular composition of the BMC of porcine urinary bladders treated with detergents commonly used for decellularization. Residual detergent fragments from SDS, Triton-X 100 and sodium deoxycholate were identified in the spectra of the BMCs. Treatment of bladders with 0.1% and 1% SDS affected both the phenotypic characteristics and apoptosis of HUCs seeded onto the basement membrane complex. An understanding of the effects of detergent exposure on BMC surface molecular functionality will facilitate a rational strategy for successful in vitro and in vivo recellularization techniques.

Techniques for Harvesting and Decellularizing Neurovascularized Muscle to Replace Autologous Free Flaps: A Comparison Between Immersion and Perfusion Decellularization

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ABSTRACT:

OBJECTIVES: Decellularized musculoskeletal tissues may be recellularized with recipient-specific cells to provide "autologous" allografts, thereby reducing donor site morbidity, eliminating immunosuppression, and overcoming critical shortages of viable donor tissues. Decellularized dermal and nerve grafts are commercially available, but no alternatives exist for *decellularized neurovascularized muscle (NVM)*. Ott, et al. have made great strides in whole-limb de-/recellularization. We propose a synergistic approach by focusing on individual musculoskeletal tissues, in order to optimize techniques specific to each tissue type. Furthermore, regenerated muscle allografts would offer far broader utility than whole faces or limbs. We harvest quadriceps NVM and compare two methods of decellularization (perfusion and immersion), by detailed characterization of the remaining extracellular matrix (ECM) integrity.

METHODS: Vastus muscle groups (medialis, intermedius and lateralis) were harvested in both rat and rabbit species, in situ on the femur, bilaterally for either immersion (R.) or perfusion (L.) decellularization. Muscles for perfusion were harvested together with their intact neurovascular supply, to include proximally the infrarenal aorta and inferior vena cava, which were cannulated for perfusion. Tissue biopsies were taken before and after decellularization by each technique (using SDS + Triton-X), and were characterized for comparison:

Sampling: Neurovascular bundle (NVB) slices were sampled proximally around the cannulae. Muscles were biopsied radially (from deep to superficial) from a transverse slice at each muscle's thickest portion.

Histology: 10 μm transverse sections of muscle and NVB were stained using H&E, Masson's trichrome, and myosin staining to assess for condensed nuclei and any intact muscle fibers.

Immunohistochemistry: 10 μm transverse sections of muscle and NVB were stained for endothelial and myogenic antigens, elastin, chondroitin sulfate, hyaluronic acid, fibronectin and laminin.

Collagen integrity: Samples of muscle and NVB were assessed for premature degradation or crosslinking: (1) Collagenase assay for degradation rate of ECM collagen; (2) Differential scanning calorimetry (DSC) for phase transition and denaturation temperatures of ECM structural proteins.

DNA quantification: PicoGreen® assay to quantify DNA remaining (<50 ng / mg tissue is decellularized).

RESULTS: Immersion decellularization inadequately decellularized the inner portions of muscles, but was adequate for neurovascular components. Perfusion decellularization using Ott, et al.'s whole-organ protocol initially generated increasing perfusion pressures (assumed due to rapid de-endothelialization). The SDS concentration and flow rate were lowered to minimize this, and complete decellularization of both muscle and NVBs were achieved. ECM components (fibronectin, laminin, etc.) withstood both methods.

SIGNIFICANCE: Perfusion decellularization yields more complete decellularization of large volume composite tissues than immersion. Using this approach, intact muscle groups can be harvested and decellularized with their neurovascular supply. We are currently working on recellularizing these NVM scaffolds, to ultimately provide a highly relevant clinical alternative to autologous free tissue transfers and their associated morbidities.

[2,937 char / 441 words]

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Mauricio Villegas[2]

Daniela Lopera[3]

1,2,3 Tissue Engineering and Cell Therapy Group from the Medicine Faculty at the Antioquia University in Medellin Colombia South America.

Objective.

To evaluate L-PRF (Platelet Rich Fibrin) matrix membrane interaction with autologous and allogenic human mucosal fibroblasts and adipose derived mesenchimal stem cells for dental applications.

Methodology.

L-PRF was first developed in France by Choukroun et al 2001, for specific use in oral and maxillofacial surgery.This technique requires neither anticoagulant nor bovine thrombin (nor any other gelling agent). The PRF protocol is very simple: A blood sample is taken without anticoagulant in 10-mL tubes which are immediately centrifuged at 3000 rpm for 10 minutes. A fibrin clot is then obtained in the middle of the tube, just between the red corpuscles at the bottom and acellular plasma at the top. The PRF protocol makes it possible to collect a fibrin clot charged with serum and platelets. By driving out the fluids trapped in the fibrin matrix, it is posible to obtain a very resistant fibrin membrane. The 3-dimensional organization will give great elasticity to the fibrin matrix. The biochemical analysis of the PRF composition indicates that this biomaterial consists of an intimate assembly of cytokines, glycanic chains, and structural glycoproteins enmeshed within a slowly polymerized fibrin network. These biochemical components have well known synergetic effects on healing processes.

Important findings.

A protocol was standardized to adhere fresh and thawed human autologous and allogenic mucosal fibroblasts, and mesenchimal stem cells derived from adipose tissue to the L-PRF surface.

It was verified the surface adherence of those cells to the L-PRF matrix by Hematoxilin/Eosin, fluorecence and SEM.

Three L-PRF with human autologoous mucosal fibroblasts were applied to patients with gingival problems.

Results and perspectives.

To date, we have been very successfull results with our three clinical applications.
The possibility of utilize autologous adipose derived mesenchimal stem cells and L-PRF autologous membranes to treat diabetic skin ulcers is a very important issue.