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Engineered heart slices for electrophysiological and contractile studies

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ABSTRACT

A major consideration in the design of engineered cardiac tissues for the faithful representation of physiological behavior is the recapitulation of the complex topography and biochemistry of native tissue. In this study we present engineered heart slices (EHS), which consist of neonatal rat ventricular cells (NRVCs) seeded onto thin slices of decellularized cardiac tissue that retain important aspects of native extracellular matrix (ECM). To form EHS, rat or pig ventricular tissue was sectioned into 300 μm-thick, 5 to 16 mm-diameter disks, which were subsequently decellularized using detergents, spread on coverslips, and seeded with NRVCs. The organized fiber structure of the ECM remained after decellularization and promoted cell elongation and alignment, resulting in an anisotropic, functional tissue that could be electrically paced. Contraction decreased at higher pacing rates, and optical mapping revealed electrical conduction that was anisotropic with a ratio of approximately 2.0, rate-dependent shortening of the action potential and slowing of conduction, and slowing of conduction by the sodium channel blocker lidocaine. Reentrant arrhythmias could also be pace-induced and terminated. EHS constitute an attractive *in vitro* cardiac tissue in which cardiac cells are cultured on thin slices of decellularized cardiac ECM that provide important biochemical, structural, and mechanical cues absent in traditional cell cultures.

1. Introduction

The field of cardiac tissue engineering has made steady progress over the past two decades. It is fueled not only by the prospect of myocardial repair [1,2] but also by the opportunity to develop new functional *in vitro* models that are physiologically relevant [1–3]. Various strategies have been employed in the fabrication of functional cardiac preparations that mimic native myocardium [1,2]. However, significant challenges remain in the ability of present engineered tissues to recapitulate the complex biochemical and biomechanical environment in native myocardium.

Decellularization of whole organs has provided a natural scaffold that can be repopulated with a variety of cell types. Decellularized myocardial matrix has been successfully obtained by whole-heart perfusion [4,5] or through treatment of mm-thick sections of myocardium [6], and shown to provide a biocompatible substrate for cellular attachment while largely preserving matrix composition, organization, and mechanical properties. Cardiomyocytes cultured on this native matrix survive [7,8], contract [6], and respond to electrical stimulation [4,9,10]. Thus, decellularized myocardium is proving advantageous for the maintenance of cardiomyocytes and is receiving increasing attention as a scaffolding material for cardiac tissue engineering. Despite this progress, recellularization of three-dimensional native tissue remains a challenging proposition, particularly in the whole organ. On the other hand, recellularization of smaller and thinner tissues should be easier to achieve. With this approach in mind, our objective was to develop a physiological tissue system that supports cardiomyocyte survival and organization at confluent densities, contracts, and exhibits electrical conduction, anisotropic properties, and tissue-level arrhythmias.

2. Methods

An extended description of the methods is available in the Supplementary material.

2.1. Preparation of decellularized slices

For slices made from rat extracellular matrix (ECM), adult rat hearts were suspended on a Langendorff perfusion setup and rinsed with distilled deionized water (ddH_2O) to remove blood and assist in cell lysis via osmotic pressure. After removal of the atria, the ventricles were embedded in 4% low gelling temperature agarose and sectioned into 300 μm-thick slices using a vibratome (7000smz, Campden Instruments, Lafayette, IN) before being cut with a hollow punch to the desired

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diameter (5–8 mm). For slices made from pig ECM, cylindrical plugs of myocardial tissue were punched out of the excised left ventricle of hearts from slaughterhouse pigs using a 14 mm or a 16 mm-diameter hollow punch. Plugs were stored at −80 °C for a minimum of 16 h. To obtain thin sections for decellularization, plugs were partially thawed, and an epicardial portion ~1 cm thick was cut out, embedded in agarose, and sliced 300 μm thick using a vibratome. In this report, "native" slices refer to tissue slices prepared in this way and stored in PBS instead of undergoing decellularization.

Slices from rat and pig ECM were decellularized using SDS and Triton X-100 in a procedure modified from Ott et al. [4]. All solutions contained 1% antibiotic-antimycotic and 1% penicillin/streptomycin. The decellularized slices were carefully spread on sterilized plastic 5 mm-diameter coverslips (for rat ECM), or 14 mm-diameter coverslips (for pig ECM), and the outer perimeter of each slice was hooked around the edges of the coverslip. Coverslips with affixed slices were placed in 15 mm-diameter wells of standard 24-well culture plates and kept in sterile PBS or HBSS until use.

2.2. Seeding of decellularized slices with neonatal rat ventricular cells (NRVCs)

All animal procedures were performed in compliance with guidelines set by the Johns Hopkins Committee on Animal Care and Use and all federal and state laws and regulations. Two million NRVCs were seeded in each well of a 24-well culture plate containing a single decellularized slice, to form an engineered heart slice (EHS). Day 0 of culture was defined to begin at the time of NRVC plating. After 18 h, EHS were washed with warm PBS, and fresh medium with 10% serum was added. On day 2, serum in the culture medium was reduced to 2% to inhibit non-cardiomyocyte proliferation, and cells were fed with 2% serum every other day thereafter.

2.3. Tissue characterization

Native and decellularized slices of pig ECM were dessicated, weighed, and digested in Tris EDTA containing 1% SDS and 1% Proteinase K by volume. Since the decellularized slices weighed much less than the native slices, ten decellularized slices were combined for more accurate weighing and DNA quantification. After digestion, DNA was isolated and quantified by measuring absorbance in a spectrophotometer (NanoDrop 1000, NanoDrop products, Wilmington, DE).

For mechanical testing, 30 mm-diameter, 300 μm-thick slices were prepared from pig left ventricle as described earlier, and a 1 cm-wide strip was cut from the center of each slice along the fiber direction. Native and decellularized samples were mounted on an EnduraTEC ELF 3200 Series instrument (Bose ElectroForce, Eden Prairie, MN) by clamping each end, leaving a 1 cm initial length between the clamps. Samples were stretched uniaxially to 40% strain, in increments of 5% strain.

Slices of native and decellularized pig ECM were imaged by second harmonic generation (SHG). Native and decellularized slices from pig ECM were also frozen at −20 °C in optimal cutting temperature media, and 10 μm-thick cross-sections were cut every millimeter with a cryostat. These sections were imaged using a phase contrast microscope, and the cross-sectional thickness was measured using a custom MATLAB script.

Live cells in EHS made from pig ECM were imaged after staining using a viability/cytotoxicity kit 7 days after plating. Live cells were also imaged in combination with SHG to visualize cell locations relative to the matrix. For this, cells were additionally stained with either cell-impermeant ethidium homodimer-1 to image dead cell nuclei or cell-permeant DRAQ5 to image live cell nuclei. A z-stack of images was created, and the intensity of calcein-AM, DRAQ5 and SHG at each z-level, normalized to the maximum intensity for each stain, was plotted using a custom MATLAB script.

F-actin, DNA, collagen I, collagen III, and laminin in fixed native and decellularized slices of ECM, as well as cardiac troponin I (cTnI), α-actinin, connexin 43 (Cx43), and nuclei in fixed EHS, were stained using standard fixation and staining techniques and imaged using a confocal microscope. Nuclear elongation and alignment in EHS were analyzed using a custom MATLAB script.

2.4. Contraction measurements

During culture, the spontaneous beating condition of EHS made with pig ECM was recorded daily. For quantitative measurements of contractility, EHS from pig ECM were placed on a 37 °C heated stage in a 35 mm dish filled with Tyrode's solution 5 days after seeding. A section of the EHS was unhooked from the coverslip so that it could move freely. The EHS was paced at 1–5 Hz, and the free region was imaged by a CCD camera. A custom MATLAB script was used to segment the image and calculate the mean displacement of the edge of the EHS over time, which was used as a surrogate for contractility to measure the force-frequency relationship of the EHS.

2.5. Electrophysiological studies

Most EHS were optically mapped 5–8 days after seeding. Some EHS made from pig ECM were mapped 21 days after seeding. EHS from rat ECM were placed in Tyrode's solution in a 35 mm dish and stained with 10 μM of the voltage-sensitive dye di-4-ANEPPS for 10 min. This solution was then removed from the dish, and replaced with Tyrode's solution containing 10 μM of the contraction inhibitor blebbistatin. At least 10 min after adding blebbistatin, the EHS were field stimulated and optically mapped using a CMOS camera (MiCAM Ultima-L, SciMedia).

EHS from pig ECM was optically mapped using a custom optical fiber system and protocol described previously [11]. Briefly, EHS were placed in the mapping chamber and treated with 20 μM di-4-ANEPPS and 10 μM blebbistatin in Tyrode's solution for 10 min. Tyrode's solution (35 ± 1 °C) containing 10 μM blebbistatin was then continuously perfused through the chamber for the duration of each experiment to inhibit motion artifacts. The stimulus threshold voltage was determined at 2 Hz, and a voltage 10% higher was used for experiments. EHS were paced at 2 Hz, then in increments of 1 Hz up to 5 Hz, and then in smaller increments until loss of capture. Some EHS were challenged with stepwise increasing doses of lidocaine ranging from 90 μM to 360 μM and paced at increasing rates at each drug level.

Mapping data was analyzed using custom MATLAB scripts. Data was linearly detrended and low-pass filtered with a cutoff frequency of 32 Hz. Automated filters were applied to remove excessively noisy or low-signal channels, and this was sometimes supplemented by manual removal of channels with poor signal. Activation maps were constructed using a 5-point derivative to identify the time of maximum upstroke rate of the action potential, and fit in x-y-t space to an ellipsoidal cone (Supplementary Fig. 1). Conduction velocities (CV) in the longitudinal and transverse directions were calculated as vectors (position, direction, and magnitude) starting at the vertex of the cone with magnitudes equal to the reciprocal slope of the cone along the major and minor axes, respectively. Action potential durations at 30 and 80 percent repolarization (APD_{30} and APD_{80}) were also calculated from the optical voltage signal.

2.6. Statistics

All data are presented as mean ± SD, except when stated otherwise. Anisotropy ratio and nuclear elongation were log transformed to make them more normally distributed since they were right-tailed. They were expressed as the log-transformed mean as well as the interval of the log-transformed mean plus or minus one SD, after inverse transformation back into linear space (see Supplementary Material for more details). Paired t-test was used for statistical significance between experimental groups, except when data were normalized and then compared to control (Figs. 6D and 8C,D), in which case an unequal variance t-test was used to compare them to 1. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Preparation of thin decellularized slices of ECM

Slices of rat ECM were prepared by starting with Langendorff perfusion of whole rat hearts with ddH_2O (Fig. 1A). Ventricular tissue was excised, embedded in agarose (Fig. 1B) and sectioned into 5-8 mm-diameter, 300 μm-thick slices (Fig. 1C). Following treatment by SDS and Triton X-100, the sectioned slices became transparent (Fig. 1D). Slices of ECM were also prepared from left ventricles of pig hearts (Fig. 1E). The tissue was frozen, cut as 14 or 16 mm-diameter plugs, and a 1 cm-deep epicardial portion was cut out and embedded in agarose in a 35 mm dish (Fig. 1F). These tissue plugs were then sectioned into 300 μm-thick slices (Fig. 1G) and decellularized by SDS and Triton X-100, becoming nearly transparent (Fig. 1H). Slices of decellularized ECM did not maintain their shape when lifted out of liquid. To allow for easy handling and cell seeding, each slice was carefully spread over a plastic coverslip and hooked onto the edges of the coverslip (Fig. 1H).

Because the slices were only 300 μm-thick, they were decellularized after only 3.5 h in detergents, and then washed overnight in PBS or HBSS. Imaging of collagen using SHG showed that fiber alignment and structure was maintained after decellularization (Fig. 2A,B). Images of phalloidin staining for F-actin and DAPI counterstaining for nuclei showed cells were present in native tissue slices (Fig. 2C) and absent in decellularized slices (Fig. 2D). Cryosections of the ECM were visualized before and after decellularization using phase contrast imaging (Fig. 2E,F) and showed that the thickness of the ECM decreased from 301 ± 36 μm (n = 4) prior to decellularization to 61 ± 17 μm (n = 3) post-decellularization. DNA content decreased from 2.0 ± 0.6 (n = 8) to 0.12 ± 0.10 (n = 4) μg/mg initial dry weight, and dry weight decreased from 10.2 ± 3.5 (n = 8) to 0.9 ± 0.2 mg (n = 4). Staining for ECM components collagen I, collagen III, and laminin showed retention of