Aleksandr Lyubasyuk MS4

ISP Coordinator: David Roth MD, PhD

Development of an Organotypic Slice Culture Protocol and Proof of Cultured Sample Viability

Rationale: Ischemia reperfusion injury is a major cause of morbidity and mortality worldwide. A better understanding of the cardioprotective effects of anesthetics is needed to open up opportunities for research in a variety of medical fields such as internal medicine, geriatrics, cardiology, surgery and anesthesiology. Furthermore, a better understanding of the mechanism of pre- and post- conditioning of anesthetics may lead to the development of novel cardioprotective therapeutics for older patients who are in need of cardiac surgery. This project takes a novel approach towards the study of cardiac tissue in vivo. Previously, human samples were obtained from patient surgeries. This approach presented the scientific community with several problems. First, the samples were scarce and difficult to obtain. Second, the experiments and the time available was very limited due to the viability of the tissue. New approaches include the culturing of cardiac myocytes. However, the lack of the microenvironment present in isolated cardiac cells makes it difficult to compare experimental results with those performed on adult heart tissue. Our approach involves using a vibratome to create slices of cardiac tissue with a preserved microenvironment to establish baseline parameters for mouse heart, with the hopes of replicating these experiments in human tissue. The ability to culture and study in vitro slices of human heart tissue will increase research output from difficult to obtain and limited human tissue samples.

Once a consistent protocol for the harvesting, slicing and culturing of the mouse heart tissue is developed, histology and a cell viability test will be performed to prove viability of the heart tissue during various time points during culture. Then these slices will be used to study myocardial ischemia/reperfusion (I/R) injury. The discovery of preconditioning in the 1980's and postconditioning in 2003 revealed the presence of an endogenous protective mechanism to increase ischemic tolerance in the heart.[1,2] The molecules and organelles involved in pre and postconditioning can be organized into prosurvival signaling cascades known as the reperfusion injury salvage kinase (RISK) pathway and the survivor activating factor enhancement (SAFE) pathways.[2] Many endogenous ligands and pharmaceuticals including volatile anesthetics have been shown to produce pre and postconditioning in the heart. However, recent work has shown that the potential for cardiac protective stimuli is limited in the clinical setting by factors such as age and diseases related to aging. Age dependence of these pre/post conditioning effects is a

highly relevant issue because the majority of patients that may benefit from a cardioprotective response are aged.[3]

Caveolae and Caveolin-3 (Cav-3) are prime suspects for the modulation of anesthetic-induced cardiac protection in aged hearts. Caveolae are flask like invaginations of the plasma membrane enriched in cholesterol and caveolin proteins. [4] Three caveolin protein subtypes are expressed, Cav-1, 2 and 3. However, Cav-3 is the predominant subtype expressed in striated muscles and is responsible for caveolae formation in cardiac myocytes. [5] Caveolins function as chaperones and scaffolds via a scaffolding domain recruiting signaling molecules to caveolae to provide direct temporal and spatial regulation of signal transduction. [6, 7] Ischemic preconditioning modulates the microenvironment of caveolae to enrich for proteins that promote cardiac protection including eNOS and the glucose transporter 4 (GLUT-4) that translocate to caveolae after preconditioning.[8] Molecules in the prosurvival signaling cascades associated with pre and postconditioning interact with caveolae and caveolins. [9, 10]

Project objectives:

1. To develop a protocol for the harvesting, slicing with a vibratome and culturing of mouse heart tissue similar to those described in recent literature[11,12]
2. To use histology and a cell viability test described in current literature[13] to prove viability of heart tissue during various time points during culture
3. To culture aged mouse heart tissue, maintain their viability and treat the experimental group with the Adenovirus Cav-3 vector and the control group with a similar dose of a null GFP expressing Adenovirus.
4. To assess the Cav-3 content, caveolar structure and isoflurane induced cardiac protection from the mouse heart tissue cultures.

Methods: Using recently described techniques, 300 µm slices were obtained from harvested mouse cardiac tissue using a vibratome.

Days before harvesting tissue, 20X Modified Tyrode's Solution (MTS) without glucose and without 2,3 butanedione-monoxime (BDM) was made, refrigerated and stored. Then a 1L aliquot of MTS with glucose and BDM was made up for transporting tissue, and for oxygen perfusion with 95% Oxygen/5% CO2 for 10 minutes prior to vibratome cutting. 1L aliquots of MTS with BDM was prepared to make glucose free agarose. Also a 1L aliquot of MTS with glucose only was made for incubation before staining/cryosectioning. All 1L aliquots were titrated to a pH of 7.4 and filtered. Culture media was also made up using 500ml DMEM with

50ml + 5ml Pen Strep for mouse tissue (Alternatively Medium 199 and ITS supplement was used for human tissue).

On the days of harvesting mouse cardiac tissue, the vibratome was first decontaminated with rubbing alcohol followed with a UC lamp for 1-2 hours. Premade glucose free MTS was used to make 2-3 50ml tubes of 4% low MP agarose. This was kept in a 37°C water bath until use. The vibratome and tray was filled with ICE and labeled petri dishes were placed on ice. Extracted heart tissue samples were stored in pre-gassed MTS during transport and cutting in ice-cold MTS.

Prior to slicing, tissue was embedded in 4% low melting agarose dissolved in glucose free MTS at 37°C. A 0.5 inch piece of a 50ml conical tube was used as a mold, which was then bound to the vibratome sample holder with Super Bond. The sample was quickly covered with ice-cold pre-gassed MTS.

During slicing, samples were cut into 300 um thick tissue at 4°C and oxygenated. Vibratome parameters were set at 0.07mm/s advance rate (minimum setting) with a 60Hz vibration and 1mm amplitude (maximum setting). Whenever possible, samples were cut into 8mm width x 10 mm length samples with longitudinal orientation of fibers. Minimum area was to be approximately 5x5mm. Samples were stored in ice-cold MTS for 30min before use.

For each mouse tissue sample $5x5x5ml^3$ produced 8-12 suitable sections. The first and last 3 sections are usually poor quality and used for calibration. 4 slices were used for culturing: The first slice was used for histology which was embedded in OTC, cryosectioned and stained with H&E, a actinin/vimentin, PI and FarRed similar to methods described in the current literature.[11,12,13] The second slice was used for 4 Succinate dehydrogenase enzyme activity test (MTT activity test). The last two slices were used for a second time point following culture, or in case the first two slices failed. The next 4 slices were used for immediate use. 1 slice for histology (same as above). 1 slice for the MTT activity test. Remaining slices were used as backup controls. All 8 sections were placed in culture media within 2 hours in the incubator to minimize cell stress.

Cultured slices were placed on a liquid-air interface using semi-porous tissue culture inserts PICMORG50, Millipore USA. Tissue culture inserts were placed in a 6 well culture plate Cellstar, GBO, Germany with 1ml culture solution composed of 500ml DMEM with 50ml + 5ml Pen Strep (mouse). Incubator was set at 37°C in humidified air with 5% CO2. Culture medium was exchanged daily. The protocol I developed for the isolation and culture of mouse heart

tissue is described in Figure 1. Current work in the PI's laboratory uses techniques that have been built upon the groundwork and experience of my work. The methods used to slice and culture porcine and human tissue slice cultures in the laboratory are depicted in Figure 2.

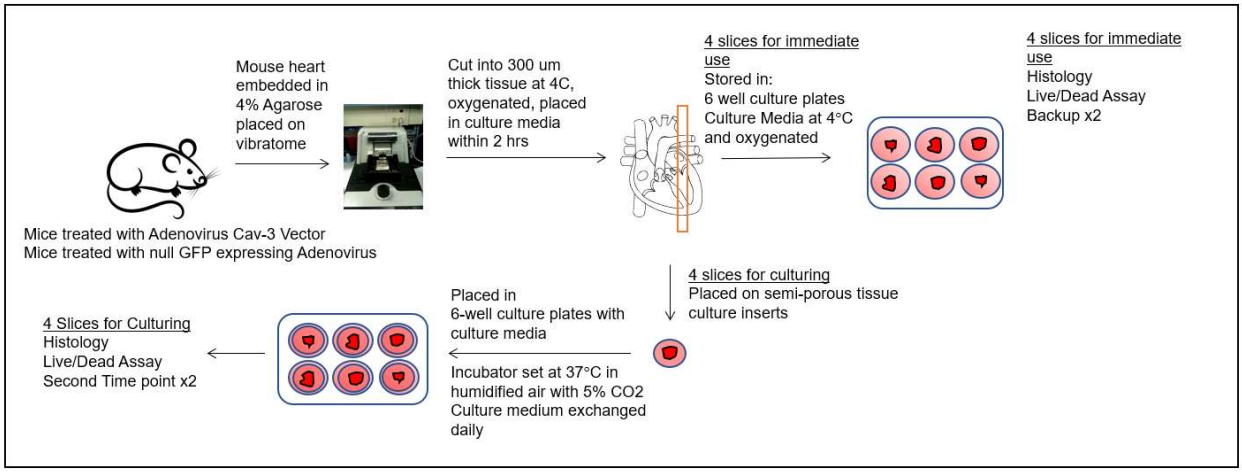

Figure 1. Diagram of Organotypic Slice Culture Protocol.

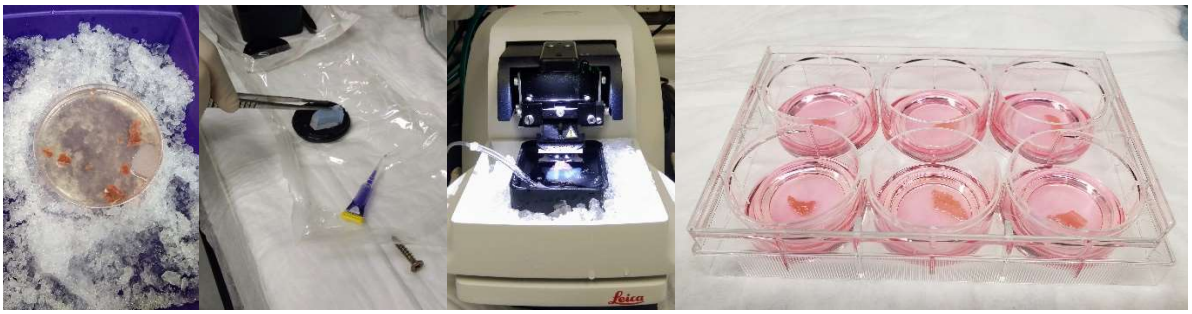

Figure 2. Organotypic Slice Culture Protocol performed with porcine cardiac tissue. From Left to right: 1. Harvested cardiac tissue stored in MTS with glucose and BDM for oxygen perfusion with 95% Oxygen/5% CO_2 for 10 minutes prior to vibratome cutting. 2. A 4% Agarose mold was glued onto the vibratome stage for backing during vibratome slicing. 3. The vibratome with the cardiac tissue against the agarose mold in a 4°C ice bath and oxygenated. 4. Cultured slices were placed on a liquid-air interface using semi-porous tissue culture inserts for further analysis.

Achievements and Limitations:

Project achievements included the development of a protocol for efficient harvesting, slicing with a vibratome and culturing of cardiac heart tissue slices. Tissue viability was confirmed with a Live-Dead cell viability assay as described in current literature[11]. However, attempts to use an LDH activity assay were unsuccessful with inconsistent results. Histological analysis was able to be performed on the H&E slices. However, the images which were taken of the 16µm sections

stained with alpha actinin/vimentin, PI and FarRed were not able to be analyzed due to artifact. In future experiments artifact could be minimized with the use of a confocal microscope which was not available during my allotted 2 months for research. Finally, the last objective to assess the Cav-3 content, caveolar structure and isoflurane induced cardiac protection was not able to be accomplished due to time and technical constraints of the protocol. The major limitation of this project was the time constraints involved with troubleshooting the many critical variables associated with the objectives of the work.

The major lessons learned from this project was the importance of planning for necessary training, the timing of experiments, the ordering of supplies and budgeting for failed/repeat experiments. Strategies learned for future projects to improve chances of success include, completion of onboarding prior to the allotted research time, performing inventory of lab supplies to ensure that all ingredients are present for required solutions/buffers/culture medium and ordering the correct supplies if necessary, prior to starting experiments. Finally, the last lesson was to seek out feedback often. For example, if fewer images had been taken prior to seeking out feedback, the time spent obtaining images with inferior equipment could have been used to obtain access to a confocal microscope. The laboratory has been able to build upon my seminal work of establishing the vibratome sectioning and culture of live cardiac tissue in the laboratory and has continued perfecting my protocols after verifying viability and baseline parameters of cultured slices of mouse, rat and pig heart tissue, and is now transitioning to replicating these experiments with human heart tissues obtained from left ventricular assist device placement surgeries at UCSD.

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