Human Tissue Engineered Model of Myocardial Ischemia-Reperfusion Injury

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ABSTRACT

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Timely reperfusion after a myocardial infarction is necessary to salvage the ischemic region; however, reperfusion itself is a major contributor to the final tissue damage. Currently, there is no clinically relevant therapy available to reduce ischemia-reperfusion injury. While many drugs have shown promise in reducing ischemia-reperfusion injury in preclinical studies, none of these drugs have demonstrated benefit in large clinical trials. Part of the failure to translate therapies can be attributed to the reliance on small animal models for preclinical studies. While animal models encapsulate the complexity of the systemic *in vivo* environment, they do not fully recapitulate human cardiac physiology.

In this thesis, we utilized cardiac tissue engineering methods in conjunction with cardiomyocytes derived from human induced pluripotent stem cells, to establish a biomimetic human tissue-engineered model of ischemia-reperfusion injury. The resulting cardiac constructs were subjected to simulated ischemia or ischemia-reperfusion injury *in vitro*. We demonstrated that the presence of reperfusion injury can be detected and distinguished from ischemic injury. Furthermore, we demonstrated that we were able to detect changes in reperfusion injury in our model following ischemic preconditioning, modification of reperfusion conditions, and addition of cardioprotective therapeutics. This work establishes the utility of the human tissue model in studying ischemia-reperfusion injury and the potential of the human tissue platform to help translate therapeutic strategies into the clinical setting.

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List of Acronyms

hiPS	Human induced pluripotent stem cell
CM	Cardiomyocyte
MI	Myocardial infarction
IRI	Ischemia-reperfusion injury
MPTP	Mitochondrial permeability transition pore
PCI	Percutaneous coronary intervention
LDH	Lactate dehydrogenase
AK	Adenylate kinase
Norm	Normoxia
Rep	Reperfusion
Isch	Ischemia
PreC	Preconditioning
CsA	Cyclosporine A
NAC	N-acetyl-L-cysteine
NHE	Sodium Hydrogen Exchanger
NBC	Sodium Bicarbonate Cotransporter
MCT	Monocarboxylate transporter (lactate-proton cotransporter)
RISK	Reperfusion Injury Survival Kinase pathway
SAFE	Survival Activating Factor Enhancement pathway

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Preface

The work presented in this written dissertation focused on the development of a human tissue engineered model of ischemia-reperfusion injury. In addition to the presented work, I assisted in the development of a cardiac tissue engineering platform to promote maturation of cardiomyocytes derived from human induced pluripotent stem cells and characterization of the resulting cardiac construct. This work was published in *Nature*:

Ronaldson-Bouchard K, Ma SP, Yeager K, Chen T, et al (2018) Advanced maturation of human cardiac tissue grown from pluripotent stem cells. Nature 556:239–243 . doi: 10.1038/s41586-018-0016-3

During my work on developing cardiac tissue engineering platforms, I utilized the technologies to improve upon the current state of the art in *in vitro* models of ischemia-reperfusion injury. I published a first-author review article in *Regenerative Engineering and Translational Medicine:*

Chen T, Vunjak-Novakovic G (2018) In Vitro Models of Ischemia-Reperfusion Injury. Regen Eng Transl Med. doi: 10.1007/s40883-018-0056-0

Specific Aims

Timely reperfusion of ischemic heart is required to limit the damage caused by myocardial infarction (MI). However, it is recognized that reperfusion of the ischemic region paradoxically leads to additional ischemia-reperfusion injury (IRI). IRI is a major and potentially preventable contributor to the final damage after an MI; however, there is no effective clinically relevant therapy available at this time. Thus, there is a great interest in the discovery of a pharmacological therapy for IRI that can be introduced at the time of reperfusion. Despite a multitude of preclinical success in animal models, all the drugs that have made it to large clinical trials have failed to demonstrate a clinical benefit[1–3].

Criticisms have been levied on the specifics of the clinical trials [4] and the broader drug development process [5]. It is clear that more work needs to be done between preclinical studies and clinical trials. Currently, much of the preclinical testing is done in small animal models, which provide a cost-effective, whole-organism *in vivo* background to work in, but also introduce multiple confounding factors that may obfuscate the effects of drugs [6]. While animal studies are critical to the drug development process, it is evident that more attention needs to be paid to *in vitro* and reductionist models of IRI. *In vitro* models are important in that the direct effect of reperfusion and the drug on the cardiomyocytes can be observed. The various factors can be controlled for to better assess the effectiveness of a particular drug. Currently, most *in vitro* testing is performed on animal-derived cardiomyocytes, but these models do not demonstrate the best clinical predictivity because of the physiological differences between animals and humans [7]. In general, there is a great interest in developing human tissue platforms for pharmacological screening and models of disease for improved preclinical assessments [8, 9].

In this thesis, we developed a simple human cardiac tissue model of ischemia-reperfusion injury. Cardiomyocytes derived from human induced pluripotent stem cells (hIPS-CMs) were incorporated into tissue engineered cardiac constructs and were subjected to conditions leading to their maturation to provide a more physiological tissue response in the context of ischemia-reperfusion. The ischemia-reperfusion injury (IRI) in the system was validated by differentiating the ischemic and reperfusion injury. Finally, treatments against IRI were tested in the cardiac constructs, and the results were compared to current knowledge and understanding of these treatments in the preclinical and clinical setting. Developing a human model of IRI allowed for better understanding of IRI to develop treatments against this potentially preventable damage.

Aim 1: Design a Human Tissue-Engineered Bioreactor Model of Ischemia-Reperfusion Injury

We developed a bioreactor that supported the formation of a tissue engineered cardiac construct. The bioreactor promoted maturation of the incorporated hIPS-CMs by mechanical conditioning providing passive tension and allowance for auxotonic contractions. The bioreactor was designed for easy manufacturing and manipulation, and towards higher throughput testing of the resulting cardiac constructs.

Aim 2: Validate Ischemia-Reperfusion Injury in the Tissue Engineered Cardiac Construct

The effectiveness of the bioengineered cardiac construct used as a human model for IRI was validated by demonstrating the presence of reperfusion injury. To determine this, we demonstrated increased cell death in constructs subjugated to hypoxia-reoxygenation over normoxic constructs and increased cell death in constructs subjugated to hypoxia-reoxygenation over hypoxia alone. We also tested the effectiveness of ischemic preconditioning in decreasing IRI, and the response of immature constructs to simulated ischemia-reperfusion.

Aim 3: Establish and Evaluate Therapeutic Strategies for Reducing IRI During Reperfusion

We tested strategies to reduce IRI by modifying reperfusion conditions and introducing pharmacological agents. These strategies targeted effectors of IRI based on the current understanding of the pathophysiology of IRI. In addition, we compared these results to published testing done in other preclinical studies and clinical trials.

Background and Significance

Ischemia-Reperfusion Injury is a Critical Therapeutic Target

Cardiovascular disease is a major cause of mortality in the world [10]. Myocardial infarction (MI) occurs when coronary blood flow is restricted by occlusion, thus preventing oxygen and nutrient delivery to the downstream ischemic region. The only available therapy to limit damage to the ischemic region following an MI is reperfusion of the occluded artery and reestablishment of nutrient delivery. The time lapse between occlusion and reperfusion is critical for preventing irreversible damage of the myocardium [11]. Paradoxically, reperfusion of the ischemic region can lead to an increase in myocardial damage and in the final infarct size. Ischemia-reperfusion injury (IRI) involves the death of cells upon reperfusion that were still viable at the end of ischemic insult. IRI is recognized as a major contributor to the final damage after an MI, but it is also a potentially preventable source of damage. Reducing IRI will be a major step in managing cardiovascular disease (**Figure 1**).

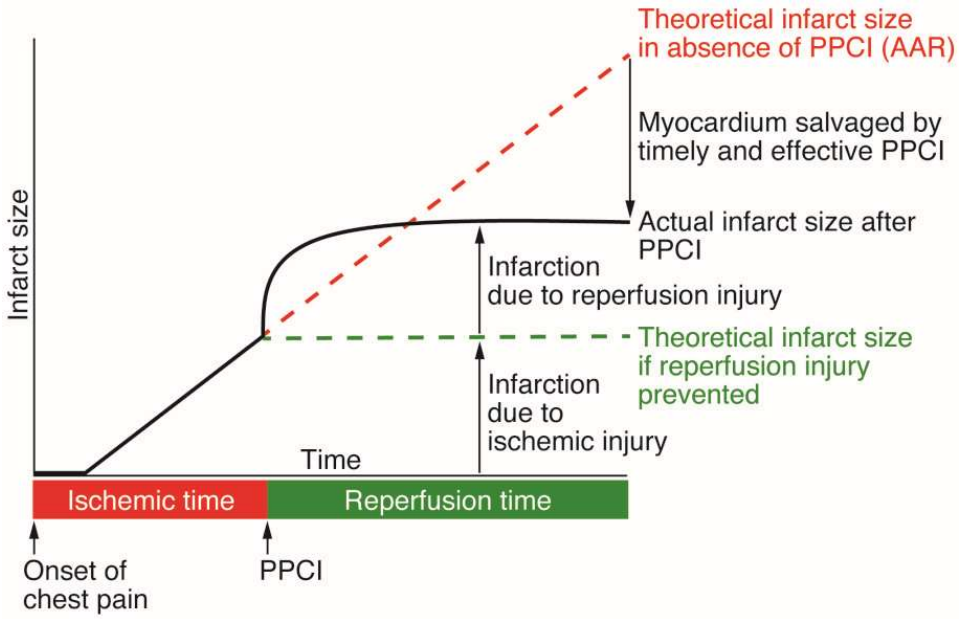


Figure 1: Timeline of ischemia-reperfusion injury. Cell death due to ischemic injury increases with ischemic time (red line). Reperfusion by primary percutaneous coronary intervention (PPCI) halts the progression of ischemic injury (green line) but causes additional damage that leads to a larger final infarct size than predicted by ischemic injury alone (black line). This additional reperfusion injury is potentially preventable and limiting it through therapeutics would lead to better clinical outcomes. Adapted from Hausenloy D, et al. [12]

It was first demonstrated by Murray and colleagues in 1986 that ischemic preconditioning of the heart can protect against IRI [13]. Ischemic preconditioning exposes the heart to short bouts of ischemia and reperfusion prior to the actual ischemic insult. However, this is not a clinically relevant therapy in the setting of an acute MI. To date, there is no clinically relevant therapy available to treat IRI. Researchers have experimented with ischemic postconditioning, exposing the heart to short periods of ischemia after the ischemic insult but before reperfusion, and remote ischemic conditioning, exposing a distant limb to short periods of ischemia before reperfusion. These conditioning methods have demonstrated success in animal studies [14]; however, ischemic postconditioning has so far shown no benefit in clinical trials [15].

Remote ischemic conditioning has shown promise in early clinical trials, but more studies are still needed [16]. Thus, the development of a pharmacologic agent that can be introduce at the time of reperfusion is of great interest.

Establishing New Models of Preclinical Testing

Many drugs have shown promising results in animal models; however, all of the drugs that have made it to large clinical trials have failed to demonstrate clinical benefits [1–3]. Some criticisms have been levied on the specifics of these clinical trials [4], and the drug development process in general [5]. It is clear that more predictive testing needs to be done during translation of preclinical data into clinical trials. Currently, most of the preclinical testing is done in small animal models, which provide a cost-effective *in vivo* background to work in, but introduce multiple confounding factors that may obfuscate the effects of drugs [6]. While animal models remain critical for drug development, it may be worth considering human cell-based *in vitro* and reductionist models of IRI. The *in vitro* models are important in that the direct effect of reperfusion and the drug on the cardiomyocytes can be observed, and any external factors can be isolated for and individually controlled.

There is a growing notion that current preclinical models of IRI fail to translate to the clinical setting [17]. An entirely new approach to preclinical studies of IRI may be needed that would utilize controllable models with sufficient power to predict the human pathophysiology of ischemia and reperfusion. In particular, human tissue models have the potential to be powerful tools to facilitate translation into the clinical setting.

Pathophysiology of Ischemia-Reperfusion Injury

Any improvements in the identification of therapeutic targets and development of effective drugs for myocardial infarction patients can only be based on understanding the progression and basic mechanisms that drive ischemia-reperfusion injury. In general, myocardial reperfusion injury encompasses more than just cardiomyocyte death. Reperfusion-induced arrhythmias and myocardial stunning after reperfusion are reversible factors of reperfusion injury. On the other hand, microvascular obstruction, the failure of reperfusion to reestablish blood flow in the underlying tissue, is irreversible and is an independent indicator of mortality.[18] However, we are primarily concerned with the pathways that lead to lethal reperfusion injury of cardiomyocytes. It is thought that the confluence of intracellular calcium accumulation, reactive oxygen species generation, and mitochondria membrane permeabilization seen in reperfusion drives cardiomyocyte death (**Figure 2**).

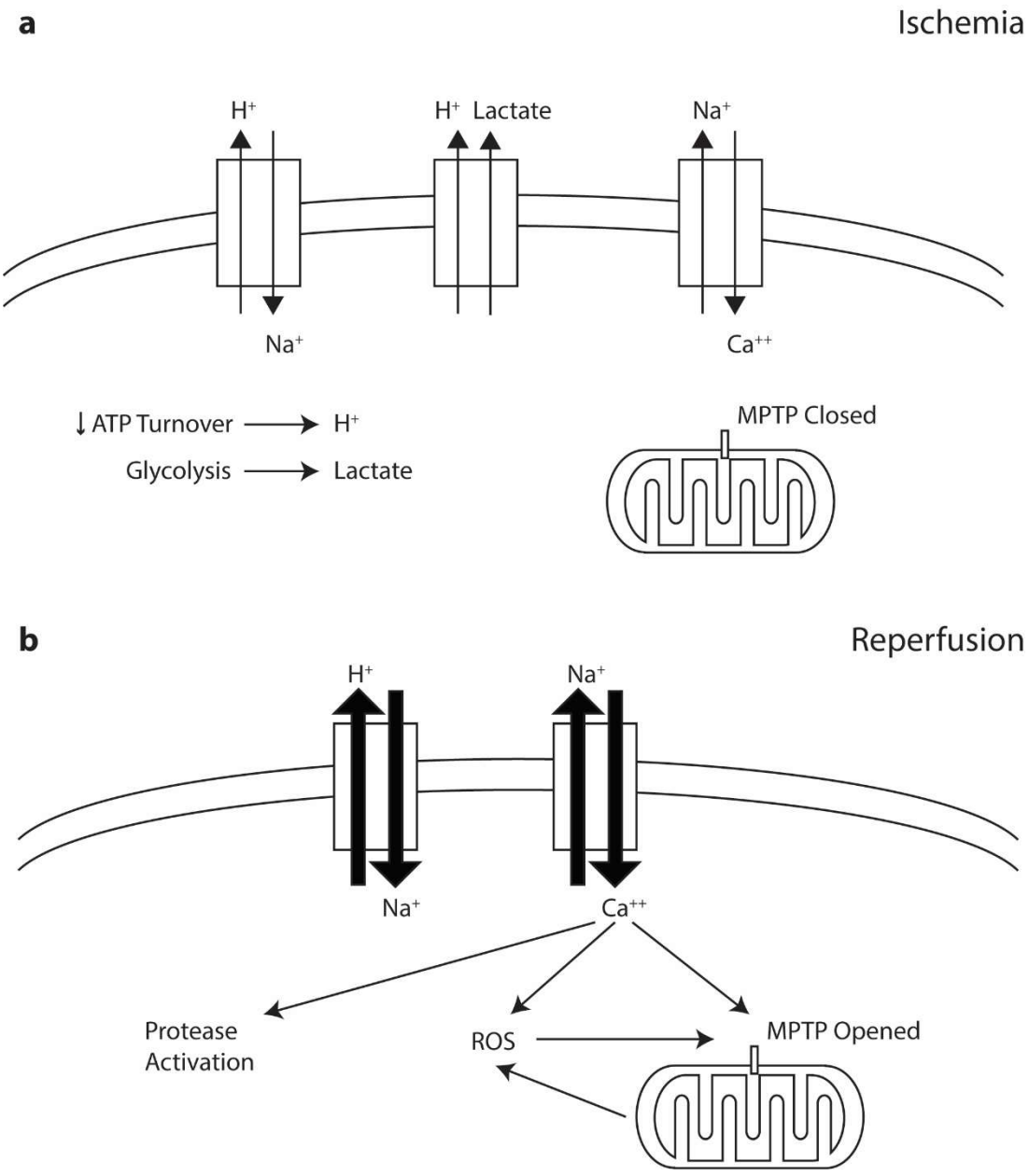


Figure 2 Pathophysiology of ischemia-reperfusion injury (a) During ischemia, the lack of oxygen drives cells from aerobic respiration to anaerobic glycolysis for ATP production. The subsequent decrease in ATP turnover leads to intracellular acidosis. Lactate from glycolysis helps to buffer the accumulated protons, but the stagnation of blood flow prevents the removal of lactate. Cardiomyocytes attempt to reestablish normal intracellular pH through the sodium-hydrogen exchanger. The subsequent accumulation of intracellular sodium drives the sodium-calcium

exchanger in reverse and causes intracellular accumulation of calcium. (b) During reperfusion, the rapid normalization of extracellular pH exacerbates the conditions seen in ischemia. The large proton gradient increases proton efflux and results in intracellular calcium overload. Reoxygenation also leads to increased reactive oxygen species (ROS) generation. Intracellular calcium overload and rapid ROS generation contribute to opening of the mitochondrial permeability transition pore (MPTP), decoupling of oxidative phosphorylation, and eventually cell death.

Ischemia

MI occurs when a dislodged plaque clogs an artery and occludes blood flow to the downstream ischemic region. This occlusion prevents nutrient and oxygen delivery, and the removal of metabolic by-products. During ischemia, the lack of oxygen causes cardiomyocytes to switch from aerobic respiration to anaerobic glycolysis for their primary ATP production. Because glycolysis is far less efficient at producing ATP than mitochondrial oxidative phosphorylation, this transition disrupts ATP turnover. The subsequent decrease in ATP production with an increase in ATP hydrolysis results in increased proton production and intracellular acidification [19]. Glycolysis also results in the production of lactate, which helps restore cytosolic NAD^+ stores to support continued ATP generation from glycolysis. Lactate production also helps to buffer intracellular pH by extruding protons through the lactate-proton cotransporter [20]. Importantly, the lack of blood flow during ischemia results in extracellular accumulation of lactate that prevents further efflux of lactate. The failure to remove lactate inhibits glycolytic ATP production and contributes to continued intracellular acidosis [21].

The ischemic cardiomyocytes attempt to reestablish a normal intracellular pH by utilizing the sodium-hydrogen exchanger to excrete protons and accumulate sodium [22]. The lack of ATP prevents the sodium-potassium ATPase from excreting sodium. Instead, the high intracellular sodium concentration

drives the sodium-calcium exchanger in reverse in an attempt to normalize intracellular sodium concentrations, which results in intracellular accumulation of calcium (**Figure 2A**) [23].

Reperfusion

Reperfusion is achieved by unblocking the artery through percutaneous coronary intervention (PCI) or thrombolytic therapy. The restoration of blood flow salvages the ischemic region by reestablishing oxygen and nutrient delivery, and by removing metabolic by-products; however, reperfusion also directly leads to cell death. Lethal reperfusion injury is the death of cardiomyocytes upon reperfusion that were otherwise viable at the end of the ischemic period.

Reperfusion restores extracellular ionic balance and normalizes the extracellular pH. The rapid normalization of the extracellular pH creates a large proton gradient that exacerbates the conditions seen in ischemia. The sodium-hydrogen exchanger accelerates excretion of hydrogen to restore physiological levels of intracellular pH. In turn, the rapid normalization of intracellular pH leads to an even greater increase in the intracellular accumulation of calcium through the sodium-calcium exchanger [24]. The high intracellular calcium concentration helps to activate calpains, proteases that target intracellular proteins [25]. Reoxygenation also leads to an increase in the generation of reactive oxygen species (ROS) that contribute to cell injury [26].

Intracellular calcium overload and the generation of reactive oxygen species (ROS) lead to opening of the mitochondria permeability transition pore (MPTP). The opening of the MPTP is thought to be the critical step in IRI, because it causes a loss of mitochondrial membrane potential, decoupling of the electron transport chain, and eventually the failure of ATP production. It also allows water to enter, causing swelling of the mitochondrial membrane. If the mitochondrial membrane is ruptured, cell death signaling proteins are released into the cytosol, resulting in loss of viability (**Figure 2B**) [27, 28].

Despite the identification of this major mechanism of reperfusion injury, the exact progression and mechanisms of IRI are still not completely defined. Inhibition of MPTP opening by targeting its regulator, cyclophilin D, has proven promising in animal studies [29]. However, the use of cyclosporine A, an inhibitor of cyclophilin D, has not been successful in clinical trials [1, 30]. It is unclear if the failure of cyclosporine A in clinical trials is due to overemphasizing the importance of the MPTP and some other factors related to the clinical setting [4]. Researchers have also found success in limiting IRI by targeting other pathways, such as the unfolded protein response or histone deacetylation [31, 32]. It is possible that there are more unknown therapeutic targets. It is clear that the field needs to further develop our understanding of the mechanisms driving IRI to develop effective therapeutic modalities. A human *in vitro* model would provide an ideal platform to robustly study the important pathways of IRI.

Current Standard of Preclinical Testing

To understand the failure of translating drugs from the preclinical to clinical setting, we have examined the current state of preclinical testing in IRI.

Animal Models of Ischemia-Reperfusion Injury

Currently, researchers primarily rely on animal models when testing new therapeutics for IRI. Ischemia-reperfusion in animals is typically simulated by using a suture to occlude the left descending coronary artery for the designated ischemic time, and then releasing the suture to allow for reperfusion [33]. This approach best captures the process of hypoxia-reoxygenation but does not fully simulate the clinical setting, where an atherosclerotic artery is clogged by a dislodged plaque and then reperfused by PCI. Animal models are useful in that they allow for examination of the interactions between various cell types during reperfusion. For example, it has been found that the adhesion of neutrophils to the endothelium can be modified, and it decreased IRI [34]. These interactions involve paracrine signaling through a multitude of molecules, such as TNF-α and nitric oxide [35], and are difficult to accurately simulate *in vitro*. These other cell types play important roles and must be considered when studying IRI.

While animal models are very helpful, they fail to fully emulate human physiology. Pigs are the closest analogues to humans because they have a comparable heart size and heart rate to humans, lack the protective coronary collateral blood flow found in dogs [36], and do not have the innate resistance to MI found in primates [37]. However, large animals are difficult and expensive to work with. Thus, most researchers rely on rodents for their *in vivo* work. Compared to humans, rodent hearts have a much higher intrinsic beating rate [38], different myosin isoform predominance, higher cardiac basal metabolism [39], and different electrophysiology of the heart [40]. While the relative importance of these differences is undefined, they influence the response to IRI. The fact that the therapeutic demonstrating success in

animal studies have not made the transition to clinical utility indicates that these animal models are not adequate

Ex vivo Model of Ischemia-Reperfusion Injury

Researchers have also explored the use of isolated perfused hearts, which maintain the physiological simulation of ischemia-reperfusion but allow for more control over the conditions. Their effectiveness has been demonstrated by reducing infarct size using various cardioprotective agents [41]. However, *ex vivo* models – similar to animal models – do not recapitulate human physiology, and motivate the need for development of new preclinical testing platforms.

In vitro Models of Ischemia-Reperfusion Injury

An alternative to animal models is to use *in vitro* models based on isolated cardiomyocytes. While these models do not fully recapitulate the complexity of the heart, they allow for examination of the direct effect of drugs on cardiomyocytes, with better manipulation and control of the various confounding factors found in animal models. *In vitro* models could provide a platform to rigorously study the important pathways of IRI and test the candidate therapeutic options in settings that are more predictive of the pathophysiology of ischemia-reperfusion than animal models.

Modeling of Ischemic Conditions *In Vitro*

In vitro models examine IRI in a non-physiological setting, and ischemia and reperfusion injury can be simulated by manipulating the cellular environment. In addition to providing a hypoxic environment, simulation of the ischemic state must also encompass other factors, including high extracellular potassium, acidosis, nutrient deprivation, and waste accumulation [42].

In vitro studies have attempted to simulate ischemic conditions by culturing cells in physiological salt solutions that are free of metabolic substrates, supplemented with additional potassium to achieve high extracellular potassium, and titrated to an acidic pH [43]. Additionally, lactate is added to simulate its accumulation due to anaerobic glycolysis. Some studies have also added 2-deoxy-d-glucose, a nonmetabolizable glucose analogue, into the ischemic solution to inhibit glycolysis and further shutdown cellular metabolism [44]. It is unknown if the non-metabolizable 2-deoxy-d-glucose phosphate interferes with the reperfusion process, and its effect needs to be further studied before being included in a simulated IRI model. The precise concentrations and compositions of the salt solutions varied across the different studies reviewed; however, a potassium concentration of 12 mM, lactate concentration of 20 mM, and pH of 6.5 are most commonly used [43, 45]. The cells were placed in a gas-tight chamber, which is then flushed with anoxic gas (95% N2, 5% CO2) to achieve a hypoxic environment.

Another method to achieve a hypoxic state has depended on the pelleting of cells with a minimal amount of media under a layer of mineral oil that hindered oxygen diffusion to create a hypoxic environment [46]. The key idea behind this model is to achieve ischemia through a more physiological means, with gradual reduction of nutrients and accumulation of wastes. However, this method is more variable and the pelleting through centrifugation can lead to mechanical damage to the cells. In our system, we utilize aspects of this method through volume restriction of the cardiac constructs during simulated ischemia.

Metabolic inhibition through compounds, such as cyanide, to decrease oxygen consumption has also been explored [47]. These chemicals may be hard to wash out or may have irreversible effects that can disrupt a physiologically accurate reperfusion process. Overall, we chose to utilize a salt solution simulating ischemic conditions and a hypoxic chamber to maximize control over the *in vitro* model.

The necessary ischemic time *in vitro* has also not been precisely determined. In animal experiments, ischemic times are relatively short and well defined. Thirty minutes is all that is needed to achieve an

ischemic state within the heart tissue [33, 48]. However, isolated cardiomyocytes cultured *in vitro* are at different levels of maturity and oxygen dependency than those in intact mature tissue. The embryonic heart is more resistant to hypoxia than the adult heart, and has improved recovery after an ischemic insult [49]. As a result, much longer ischemic times, ranging from 90 minutes [50] to 9 hours [33], are needed to demonstrate a decrease in cell viability. The necessary duration of ischemia is dependent on the cell source used, but it can also vary within the cell types used. One study had to adjust the ischemic time to two to five hours [32].

Further complicating the matter, it has been found *in vivo* that after a certain length of ischemia, preconditioning no longer has cardioprotective effects with respect to the final infarct size [13]. This time dependence of the effectiveness of ischemic conditioning must be accounted for when assessing the potential of a therapeutic. The European Society of Cardiology Working Group Cellular Biology of the Heart has recommended that the combined ischemic and reperfusion times should be selected to result in 50% cell death [6]. Each *in vitro* model must therefore independently establish an ischemic time that causes enough cell death, but is not too long to affect possible preconditioning or drug benefits.

Simulating Reperfusion *in vitro*

Reperfusion was also simulated through non-physiological means *in vitro*. In clinical settings, the aim of reperfusion therapy is to restore nutrient and oxygen delivery and to remove the accumulated metabolic by-products. Reperfusion is typically simulated *in vitro* by replacing the ischemic solution to remove metabolic waste, adding normal culture media for nutrient delivery, and removing the system from the hypoxic chamber to return the environmental levels of oxygen to normoxia [51, 52].

The composition of culture media added to simulate reperfusion must be carefully selected. Most *in vitro* studies utilize commercially available basal media supplemented with serum. However, it is unclear if

normal cell culture media best simulates physiological conditions. The reperfusion solution needs to provide nutrients to the cells and normalize extracellular pH and ion balance in order to cause intracellular calcium overload and allow for the generation of ROS. Cell culture media provide nutrients in the forms of glucose in the basal media, and lipids in the serum. Cardiomyocytes are more dependent on lipids for metabolism [53]; however, serum supplementation may be problematic. The exact composition of serum is unknown and there are lot-to-lot variations, which introduces variability that may obfuscate the effect of the therapeutic of interest. Serum also contains antioxidants that may ameliorate the oxidative stress seen in IRI. Serum-free media are utilized for cardiomyocytes derived from human iPS cells [54] and a chemically defined reperfusion solution can help establish a consistent response to IRI.

It is also important to re-establish the physiological extracellular calcium concentration (1-2 mM) [55] because intracellular calcium overload is one of the important effectors of reperfusion damage [42]. Certain types of basal media contain less calcium, and it has been recognized that physiological calcium levels are required for normal function of cardiomyocytes [56].

Isolated Cardiomyocytes as Models of Ischemia-Reperfusion Injury

In vitro models of IRI primarily rely on isolated cardiomyocytes derived from animals, immortalized cell lines, or human pluripotent stem cells. The source of cardiomyocytes affects their response to IRI, and thus, their predictive power for pharmacologic testing.

Most of the *in vitro* work has utilized cardiomyocytes derived from animal sources. Primary cardiomyocytes have been isolated for study from a variety of animal types, including rats, pigs, and rabbits, and from animals of different levels of maturity. Cardiomyocytes derived from adult animals are more relevant for the studies of IRI because the immature cardiomyocytes derived from neonatal animals are more resistant to hypoxia than adult human cardiomyocytes [49]. This resilience causes neonatal

hearts to respond very differently to IRI. For example, ischemic preconditioning does not protect neonatal hearts [57], and neonatal cardiomyocyte mitochondria are not as sensitive to IRI-induced membrane permeabilization [58]. These physiological differences are significant, and they need to be accounted for when comparing preclinical data. Therefore, therapeutics should be tested in adult-derived cardiomyocytes whenever possible.

Researchers have also explored the use of cell lines, such as immortalized mouse atrial HL-1 cells or rat H9c2 cardiomyoblasts. These cell lines are more cost effective than primary cardiomyocytes, and have demonstrated similar drug responses to IRI as primary cardiomyocytes [33, 59]. However, these cell lines do not recapitulate the cardiac physiology because they are dividing, unlike mature cardiomyocytes, and they display differences in their cell death pathways and bioenergetics [60, 61]. Because mitochondria are important effectors of IRI, caution must be exercised when extrapolating results obtained with cell lines to the *in vivo* situation.

Human cardiomyocytes would be ideal for *in vitro* models of IRI because they can provide a more biomimetic platform to conduct studies by avoiding the problem of interspecies comparisons. Animal and adult human cardiomyocytes differ in many aspects, such as the beating rate, myosin isoform predominance, ATP utilization, and electrophysiological properties [7, 62]. These differences can affect the cell response to IRI and would make it difficult to extrapolate results from animals to humans. The challenge is that human primary cardiomyocytes are impossible to work with because they are terminally differentiated, have limited proliferative potential, and are very difficult to obtain.

In an attempt to provide models based on adult human cardiomyocytes, researchers have developed a proliferating human cardiomyocyte cell line (AC16) by fusing primary cells from human ventricular tissue with transformed fibroblasts [63]. These cells are differentiated following transfer into mitogen-depleted media that stops their proliferation, and they express cardiac-specific proteins and maintain cardiac

nuclear and mitochondrial DNA. AC16 cardiomyocytes have been used and validated in an *in vitro* study that examined the effect of asiatic acid on IRI [64]. The results were consistent with other studies in a rat model and an *in vitro* H9c2 cardiomyoblast model that also demonstrated asiatic acid attenuation of IRI [65, 66]. Still, there are concerns about the physiologic relevance of transformed and fused cell lines.

The most attractive cell source for biomimetic studies of IRI are cardiomyocytes derived from human induced pluripotent stem cells (hiPS-CMs) or embryonic stem cells (hESC-CMs) (**Table 1**). It has been demonstrated that human stem cell derived cardiomyocytes can be cultured and differentiated in a defined and reproducible manner [54], and they have been used in IRI studies with some success.

Hsieh et al found that the simulation of IRI in hESC-CMs caused an increase in the qPCR ratio of anti-apoptotic BCL-2 to the apoptotic BAX gene following a pretreatment with sodium nitrite [67]. Paloczi et al demonstrated the cardioprotective effect of exogenous nitric oxide supplementation in simulated IRI experiments on hESC-CMs derived from embryoid bodies (EB) [52]. However, these studies failed to specifically separate the cardiomyocytes from the other cells in the EBs, and instead relied on a GFP expression to identify cardiac regions and quantify cell death. The limited range of methods that these studies employed makes it unclear if hESC-CMs are a suitable cell source to study IRI.

The establishment of hIPSCs as a source of human cardiomyocytes has allowed researchers to avoid the ethical concerns of using hESCs and to also derive patient-specific cell lines. Specifically, hiPS-CMs allow for studies of patient-specific disease backgrounds [68]. There is a great interest and some success in utilizing hIPS-CMs in pharmacological screening [69, 70], but very little work has been done in the setting of reperfusion injury. In one of the few IRI studies done using hIPS-CMs, Wei et al. demonstrated their utility by confirming the cardioprotective effects of the herbal medicine, Danshen, *in vitro* [71]. These results were consistent with previous studies in animal models demonstrating the therapeutic benefit of Danshen [48]. However, hIPS-CMs are relatively immature and more closely resemble fetal than adult

cardiomyocytes [72]. As known, the fetal phenotype and bioenergetics are associated with non-physiologic responses to reperfusion injury, and can pose challenges to predictive testing of drug efficacy and safety.

Table 1 Cardiomyocyte sources for in vitro models of Ischemia-Reperfusion Injury

Cardiomyocyte Source	Ease of use	Predictive Utility	Cell Source Issue
Animal cell source			
Isolated primary cardiomyocytes			
Adult cardiomyocytes	Low	Medium	Interspecies physiolocal differences
Neonatal cardiomyocytes	Low	Low-Medium	Immature phenotype, Interspecies physiological differences
Immortalized cell lines (e.g. HL-1, H9c2)	High	Low	Proliferating cell line, Interspecies physiolocal differences
Human cell source			
Transformed cell line (e.g. AC16)	High	Low	Proliferating cell line
Cardiomyocytes derived from Pluripotent Stem Cells			
hESC-CMs	Medium	Low-Medium	Immature phenotype
hIPS-CMs	Medium	Low-Medium	Immature phenotype

Organ on Chip Models

Because the fetal nature of hIPS-CMs limits their use in IRI studies, tissue engineering provides an intriguing avenue for maturing these cells into a more adult phenotype. Previous studies have demonstrated that incorporating hIPS-CMs into tissue engineered heart constructs has helped mature their gene expression profile, ultrastructure, metabolic and electrophysiological properties [56, 73–77]. However, these cells still fall short of adult cardiomyocyte function.

One study utilized an engineered heart tissue model of IRI [78], but unfortunately, the tissue constructs were based on neonatal rat cardiomyocytes. Nevertheless, it is instructive to look into how tissue engineering can help advance the IRI research. The study examined how hypoxia affected electrical conduction in the tissue and used protein expression to characterize reperfusion injury. Cyclosporine A, akin to animal studies [79], showed cardioprotective effects in this system. This study helped establish tissue engineering as a useful tool for studying IRI and testing of therapeutic options.

Current State of the Art in Cardiac Tissue Engineering

In order to develop a human tissue engineered model of IRI, it was first necessary to assess current developments in cardiac tissue engineering to determine the appropriate model **(Table 2**). The initial focus of cardiac tissue engineering was to develop a cardiac patch that could support the postinfarct region and improve heart function. Cell therapy by injecting cardiomyocytes has a notoriously low retention rate [80], and the patch would theoretically improve cell retention and provide direct mechanical support. To achieve this goal, it would be necessary to construct a thick tissue that can sufficiently bridge the postinfarct area. Making a thick tissue is challenging because cell survival is limited by the oxygen diffusion limit (~100-200 μm) [81]. Many cardiac tissue engineering approaches have sought to overcome this by incorporating a vascular network to perfuse the tissue bulk and establish a thicker cardiac tissue [82–84]. Other efforts have focused on generating large tissues that can cover the entire infarct area. In particular, the Bursac group has established methods to mature hiPS-CMs in thin (approximately 100 μm) patches up to 4 x 4 cm in size [74]. They cultured cardiomyocytes around polydimethylsiloxane (PDMS) posts to promote functional alignment of the cells, and to achieve higher force generation and faster conduction velocity [85].

A more recent development in cardiac tissue engineering has been a focus on generating *in vitro* models. Previous knowledge and methods aimed at improving cardiomyocyte maturation for implantation have been turned to achieve better predictive pharmacological testing and models of disease. Unlike the criteria for a cardiac patch, these models should be small, easy to manufacture, and simple, depending on the application. In the case of a model of IRI, the exclusion of endothelial cells and microvasculature may be prudent in initial models to isolate the study of IRI to just the response of the cardiomyocytes. The need to simplify the construct and limit its size offers greater freedom in designing a bioreactor for

maturation of hiPS-CMs. Various groups have developed differing cardiac microtissue systems to promote cardiomyocyte maturation.

The Parker group has established a method to generate muscular thin films (MTF), where cardiomyocytes are seeded onto a 2D cantilever [77]. The force generated by cardiomyocytes can then be optically measured by deflection of the cantilever. They have used the platform to demonstrate physiologic responses to isoproterenol, and as a disease model to detect deficiencies in force generation in Barth syndrome [86]. However, the MTF chips are more 2D than 3D in nature, which could limit their predictive power. Furthermore, the cardiomyocytes are seeded in the cantilever and the surrounding non-mobile regions. This creates two different populations of cardiomyocytes that can make it difficult to utilize other assays besides force generation analysis.

The Healy group established a cardiac microtissue system where cardiomyocytes were packed into a narrow channel between two perfused microchannels [87]. The narrow channel was 100-200 μm wide, and promoted alignment of the cardiomyocytes, while the perfused side channels provided constant nutrient delivery. The cardiac microtissue was stress-shielded from the perfused channels by a strip of narrow microchannels that still allowed for nutrient exchange. The researchers were able to maintain tissue viability and determine drug responses using optical contraction analysis of the cardiac tissues. However, the use of a perfusable system makes it complex to setup and perform experiments in a high-throughput manner. Furthermore, the cardiac tissue is not anchored to any points that would provide passive tension to the system or allow for optical force generation analysis.

The Radisic group established a cardiac microtissue system where they seeded cardiomyocytes in a collagen gel around a suture [73]. The resulting biowire featured cardiomyocytes that aligned along the suture. A ramped electrical stimulation was utilized in this model to further mature cardiomyocytes. These cardiac constructs demonstrated physiological calcium handling, and the ramped electrical stimulation

regime resulted in enhanced gene expression, ultrastructure organization, and electrophysiological properties compared to controls. However, the constructs were also not anchored to any points that would provide passive tension to the system or allow for optical force generation analysis.

The most common format for engineered cardiac tissue has been the seeding of a cardiomyocyte-hydrogel mixture between two flexible pillars. The hydrogel allows the cells to remodel and condense it down to a compact cellular structure. The pillars provide passive tension to the construct to promote alignment and maturation, and their flexibility allows for physiological auxotonic contractions by the constructs for mechanical conditioning [88, 89]. These platforms also allow for real time optical measurements of force generation by measuring pillar deflection [90, 91]. This method to generate cardiac constructs is simple, expandable, and has been successfully adapted by many groups [75, 91–93].

In particular, our group has adapted the flexible pillar format to achieve much higher levels of maturation than previously seen in hIPS-CMs [75]. Early-stage hiPS-CMs, hiPS-CMs that had just started to display spontaneous contractions, were incorporated in a hydrogel around flexible pillars and matured through electromechanical conditioning. The resulting cardiac constructs demonstrated highly organized cardiac ultrastructure with the presence of transverse tubules that had previously never been seen in hiPS-CMs. Furthermore, the constructs had adult-like gene expression, a switch to oxidative metabolism, a positive force-frequency relationship, and functional calcium handling. The high levels of structural and functional maturation seen in this tissue engineered cardiac platform established the superiority of this format and the basis for the development of the model of ischemia-reperfusion injury.

Other groups have developed their own flexible pillar designs for cardiac tissue engineering. The platform developed by the Eschenhagen group is the most similar to our published platform [94, 95]. Their constructs were cast in agarose molds around lids with PDMS posts, and these lids were then transferred for culture in normal 24 well plates. However, their constructs were formed using 10% Matrigel, which is

a not-fully characterized animal product that is undesirable for human disease models. Their electrical stimulation setup included carbon electrodes attached to the lids, and stainless-steel plates screwed into the carbon electrodes to descend into the tissue culture well. It has previously been established that carbon electrodes are optimal for electrical stimulation because their high surface area for charge injection [96]. Our published platform has demonstrated improved maturation compared to the one developed by the Eschenhagen group, and we therefore sought to adapt our published platform for the studies of IRI.

The Zimmermann group has also developed a cardiac tissue engineering platform that features a circular construct around flexible PDMS pillars [56, 97]. They were able to demonstrate that their animal product-free protocol demonstrated increased maturation compared to their original protocol. While the platform featured a circular construct instead of the linear ones found in our platforms, many of the lessons learned can be applied to further enhance our cardiac construct culture. Our published platform still demonstrated enhanced morphological maturation with presence of t-tubules [75].

The Chen group developed a flexible pillar platform that produced miniaturized cardiac constructs [91, 98]. The platforms were made of PDMS and were formed in a mold constructed by photolithography. The constructs themselves were rapidly formed by centrifuging a cell-hydrogel mixture into microwells that each featured a pair of flexible pillars. The small size of the constructs allowed for high throughput testing while utilizing a minimal number of cells per construct. However, the small size of the construct would be a limitation for studies of IRI because of limited analysis and manipulation.

The Flexcell platform is a commercially available system for generation of engineered cardiac constructs, and it has been used for study of hiPS-CM maturation [99]. To form the cardiac construct, hiPS-CMs were seeded in a hydrogel mixture between two nylon mesh tabs of a fixed distance. The nylon mesh tabs are porous to allow the cell-hydrogel mixture to infiltrate and provide anchors for passive tension to promote

alignment of the cardiomyocytes. Ruan *et al.* found that the static stress provided by the nylon mesh anchors promoted maturation over no stress.

The Flexcell system could also be adapted to stretch the cardiac constructs by inflating the underlying rubber membrane to increase the distance between the anchors [89, 100]. This mechanical stretch conditioning is different from the auxotonic contractions of flexible pillar systems, where cardiomyocyte force generation causes shortening of the construct length. Mechanical stretch represents diastolic filling of the ventricles, whereas auxotonic contractions represent systolic force generated by the cardiomyocytes to contract and pump blood.

Our published platform focused on mechanical conditioning through auxotonic contractions because we wanted to couple cardiomyocyte contractions with external electrical stimulation [75]. However, both types of mechanical conditioning are necessary to fully simulate physiological contractions. To our knowledge, no platform has successfully coupled dynamic mechanical stretch to electromechanical excitation-contraction. The Flexcell system has the additional benefit of not utilizing PDMS, which has been found to absorb drugs in a time dependent manner [101, 102].

Our lab has also developed a platform to provide mechanical stretch to engineered cardiac constructs similar to that of the Flexcell system [103]. The platform was made of PDMS using a mold constructed using photolithography, and it consisted of many microwells that feature PDMS posts for the constructs to attach to. Mechanical stretch was induced by using a pump to inflate the underlying channel to deflect and increase the distance between the PDMS posts. These cardiac constructs were much smaller in scale (less than 1 mm in length) and allowed for the simultaneous formation of many constructs for study. However, all of the constructs were cultured in the same media, and it was difficult to study and manipulate a single construct due to its size. The platform has also only been tested for use using neonatal

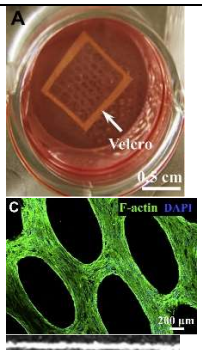
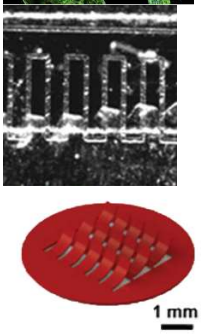
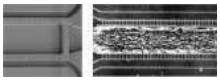
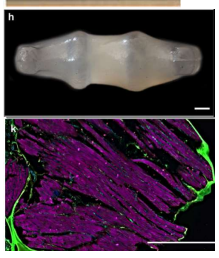
rat cardiomyocytes. We wanted to create a cardiac construct that was larger and easier to manipulate, and a platform that could provide combined electromechanical stimulation.

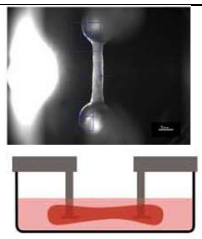
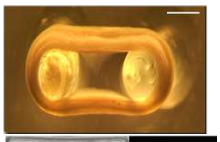
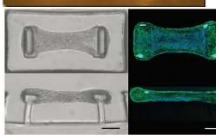
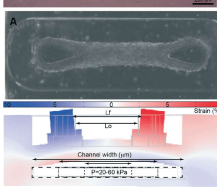
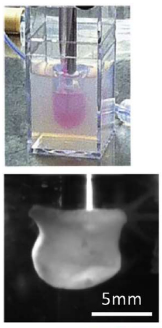
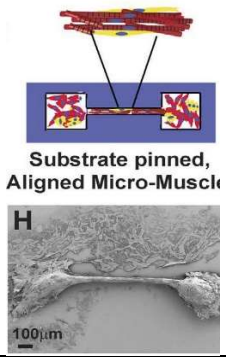
The Costa group has also demonstrated another method of coupling electrical stimulation and mechanical contractions outside of the flexible pillar system [76]. They constructed a fluid-ejecting ventricle-like chamber using hiPS-CMs formed in a hydrogel around a catheter bulb. They could have further added mechanical stretch by filling the ventricle-like structure with fluid during diastole. Their ventricle-like cardiac organoid demonstrated a more mature gene expression profile and functional maturation. Furthermore, they were able to generate pressure-volume loops and characterize changes in response to drug addition. However, we chose to go with the flexible pillar approach because it is a much simpler system that allows us to generate many constructs for analysis. The ventricular cardiac organoids are very time consuming to generate and prepare for experimentation, which would greatly reduce the throughput.

Another cardiac tissue engineering platform was developed by Huebsch *et al.* [104]. This platform relied on the attachment of the constructs to the underlying tissue culture polystyrene substrate. However, the platform still utilized PDMS, as it consisted of a PDMS stencil bonded to underlying tissue culture polystyrene, where the stencil formed the cultured cardiomyocytes into miniaturized constructs. Each construct featured two square “knob” regions with a thin “shaft” region that connected them. The shaft region promoted the alignment of cardiomyocytes with uniaxial contractions, while the knob region attached the construct to the underlying substrate and provided static stress for the construct. This platform is advantageous in that it utilizes very few cells in each miniature cardiac construct and is easy to use. However, the platform does not provide any dynamic mechanical conditioning to simulate the heart and help mature the hiPS-CMs.

Our published platform was designed to promote hiPS-CM maturation through electrical stimulation that induced dynamic mechanical conditioning of the constructs pulling the pillars. This platform demonstrated superior maturation of hiPS-CMs compared to the other platforms surveyed. We adapted this platform into a new design suitable for studies of IRI that maintained the flexible pillar design and potential for electromechanical stimulation.

Table 2: Summary of Potential Cardiac Construct Formats for Maturation of hIPS-CMs. Construct size refers to general relative size compared to other cardiac tissue engineering platforms. All "small" construct size platforms, besides the muscular thin film [77, 86], were manufactured using photolithography techniques. All platforms, besides the pneumatic microfluidic system which utilized neonatal rat cardiomyocytes [103], demonstrated enhanced maturation of hiPS-CMs.

Cardiac Construct Format	Representative Images	Construct Size	Maturation Protocol	Demonstrated Maturity	Possible Platform Limitations	References
Cardiac Patch		Large	Culture on rocking platform, structural alignment	High contractile stress, high conduction velocity, Frank-Starling relationship	Large patch not necessary for models of disease and pharmacological testing	[74]
Muscular Thin Film		Small	Auxotonic contractions	Oxidative metabolism, ultrastructural alignment, positive ionotropic response	More 2D than 3D in nature	[77, 86]
Microfluidic System		Small	Structural alignment	Ultrastructural alignment, physiologic beat frequency drug responses	No mechanical conditioning, perfusion system limits throughput	[87]
Biowire		Large	Structural alignment, electrical stimulation	Calcium handling, electrophysiology, aligned ultrastructure	No mechanical conditioning	[73]
Flexible Pillar		Large	Passive tension, auxotonic contractions, electrical stimulation	T-tubule formation, positive FFR, oxidative metabolism, positive ionotropic response, gene expression, ultrastructure	Needs to be adapted for studies of IRI	[75]

Flexible Pillar		Large	Passive tension, auxotonic contractions, possible electrical stimulation	Oxidative metabolism, aligned ultrastructure, Frank-Starling relationship	Needs to be adapted for studies of IRI	[94–96]
Flexible Pillar		Large	Passive tension, auxotonic contractions	Calcium handling, positive FFR, electrophysiology	Needs to be adapted for studies of IRI	[56]
Flexible Pillar		Small	Passive tension, auxotonic contractions, electrical stimulation	Positive ionotropic response, aligned ultrastructure	Small size limits usability	[91, 98]
Flexcell		Large	Passive tension, electrical stimulation, possible mechanical stretch	Frank-Starling relationship, negative FFR	Mechanical conditioning through mechanical stretch instead of auxotonic contractions	[89, 99, 100]
Pneumatic microfluidic system		Small	Passive tension, mechanical stretch	Ultrastructural alignment	Small size limits usability, feasibility not demonstrated with hiPS-CMs	[103]
Ventricle-like chamber		Large	Auxotonic contractions, structural alignment	Gene expression, Pressure-volume loops, physiological drug responses	Complicated setup of platform limits usability and throughput	[76]
Micro-Heart Muscle (µHM) arrays		Small	Passive Tension	Frank-Starling Relationship, positive ionotropic response, negative FFR	Small size limits usability, no mechanical conditioning	[104]

Significance

In this thesis, we developed a human tissue engineered model of IRI to address shortcomings in current preclinical studies of IRI. None of the drugs studied and found to be effective in *in vivo* or *in vitro* models have improved outcomes in clinical trials, and this is partly because most of the preclinical studies are done in animals. As discussed, pigs are the ideal animal of choice, but are not widely utilized because of cost and experimental difficulties. Instead, most studies are done in rodents, which do not recapitulate the human cardiac physiology in critical ways. While animal studies are currently the best in simulating the ischemia-reperfusion process, their deficits in emulating humans are becoming apparent.

Studies of isolated cardiomyocytes are suitable for examining the direct effects of drugs on cells. There are many studies on animal cardiomyocytes, but they have limited potential for translation to human cardiomyocytes. On the other hand, there is very little research done on human cardiomyocytes. The main and most promising source of human cardiomyocytes are human iPS cells. hiPS-CMs are abundant, easily derivable, and can be patient-specific. Unfortunately, the cardiomyocytes derived from hIPS are fetal in nature. Fetal cells are rather resistant to hypoxia, and this can be an issue when attempting to simulate IRI. Tissue engineering techniques have been demonstrated to help mature hiPS-CMs and can help in making a physiologically relevant model of IRI. A human tissue engineered model of heart muscle can recapitulate ischemia-reperfusion injury *in vitro* and enable comprehensive studies of cardioprotective drugs.

Results

Aim 1: Design a Human Tissue-Engineered Bioreactor Model of Ischemia-Reperfusion Injury

Tissue Engineering Platform

To develop a human engineered model of IRI, we first had to develop a bioreactor to support the maturation of hiPS-CMs in the cardiac construct. We had previously demonstrated the formation of an engineered cardiac construct with superior maturity compared to existing developments in the cardiac tissue engineering field [75]. In this published study, we demonstrated that tissue engineered cardiac constructs should be formed from early-stage hiPS-CMs (soon after the development of spontaneous contractions) and subjected to an intensity-training regimen to best promote cardiomyocyte maturation. The construct training regimen was achieved through electromechanical conditioning, where mechanical conditioning was provided by culture on two flexible pillars that provided passive tension and allowed for auxotonic contractions, and electrical stimulation was provided through attached carbon rods. In addition, the electrical stimulation frequency was gradually increased to simulate the development of the fetal heart, and this intensity electrical stimulation regimen had previously demonstrated enhanced maturation of hiPS-CMs compared to constant or no stimulation [73]. We saw that the constructs derived from this platform demonstrated adult-like gene expression, highly organized ultrastructure with the presence of transverse tubules, mature functional response with a positive force-frequency relationship, and increased oxidative metabolism [75].

Adapting the platform

Because of the advanced maturity that was demonstrated, we wanted to utilize this platform for our studies of ischemia-reperfusion. However, we identified that we would need to modify and adapt the platform to better suit our needs. To simulate ischemia-reperfusion, we wanted to culture the constructs in a minimal amount of solution during ischemia to simulate stagnation of blood flow and accumulation of wastes, and then add a large amount of normal culture media during reperfusion to restore extracellular nutrient, ion, and pH balance. This would allow us to quickly change culture conditions between ischemia and reperfusion. The setup of the published platform prevented this because all constructs in a single platform (up to 12) were exposed to the same large media bath. This also meant that we could not differentially control the conditions or drug exposure for each individual construct, and the intracellular enzyme release assays that we wished to utilize for cell death were a summation of all the constructs in a single platform rather than a single construct. In the platform used for our studies, we aimed to design a bioreactor that featured a small well for construct formation that sat inside a larger space for normal media addition (**Figure 3A**). During ischemia, we could add 120 µL of the ischemic solution to restrict the media volume, and then reperfuse the ischemic construct by adding 2 mL of normal culture media. This design allowed us to rapidly change conditions between ischemia and reperfusion with minimal manipulation required. Furthermore, each construct was contained in its own individual well (up to 4 per platform), which allowed us to analyze each construct separately from the others on the same platform.

Our adapted platform also allowed us to seed and culture the constructs in the same bioreactor. In the published platform, we had originally intended to seed and culture the constructs in the same bioreactor; however, we found that the culture wells only held approximately 0.5 mL of media. The media had to be frequently changed to maintain construct viability. A perfusion system for changing the media can be difficult to setup and can introduce multiple points where platform sterility can be compromised. Instead,

the bottom of the culture bioreactor was removed, and the entire bioreactor was placed in approximately 30 mL of media for adequate nutrient supplementation. Because the bottom of the culture bioreactor was removed, we could no longer seed and form constructs on the platform. Instead, a separate seeding platform was created to allow the constructs to form and condense, and the constructs were then transferred into the culture bioreactor. We wanted to modify the platform to seed and culture the constructs in the same bioreactor to simplify our workflow and avoid having to transfer the constructs between bioreactors. We provided enough room for greater than 2 mL of media per construct to avoid issues with inadequate media supplementation.

In adapting the platform for our studies of IRI, we wanted to maintain the flexible pillar design for mechanical conditioning of the constructs. In the published platform, the flexible pillars are vertically-oriented and attached to a lid that allowed the tissues to be transferred from seeding to culture bioreactor. The pillars were made out of polydimethylsiloxane (PDMS) and separately casted onto the polycarbonate lids after they were manufactured. In adapting the published bioreactor for our purposes, we decided to orient the pillars horizontally for two reasons. The horizontal pillars allowed us to have all of the pillar motion in the same viewing plane as that of a normal microscope, which would allow us to better correlate pillar deflection with force generation in the future. Vertical pillars have motion that is orthogonal to the viewing plane of a microscope. Furthermore, the switch to a horizontal pillar design allowed us to reduce the number of components that we had to manufacture. We no longer had to separately manufacture a lid and cast pillars onto it, and instead, we could directly form the flexible pillars with the rest of the bioreactor by casting the entire structure out of PDMS in a two-part mold. We maintained the flexible pillar design to provide passive tension to the constructs and allow for auxotonic contractions for maturation through mechanical conditioning.

A major difference between the two platforms was the material utilized. The published platform was primarily manufactured out of polycarbonate, but it did utilize PDMS for the flexible pillars. On the other

hand, the platform utilized in the current studies was entirely constructed out of PDMS. One issue with PDMS use is that it has been found to absorb drugs in a time dependent manner [101, 102]. However, it is to be noted that all platforms using the flexible pillar design utilize PDMS [75, 91–93, 105]. Our published platform attempted to minimize the impact of PDMS by only utilizing it in the flexible pillars while the majority of the bioreactor was made of polycarbonate. In the platform utilized for studies of IRI, we created the entire bioreactor out of PDMS as a single component. We do recognize that PDMS use is a potential confounding factor in this and future experiments on this platform. In the future, we will explore if the impact of PDMS could be reduced by creating the base of the platform out of plastic and then casting the PDMS flexible pillars onto the platform. There is currently no material that can replace PDMS, which provides the flexible and biocompatible material for flexible pillar designs, and PDMS use is a general limitation of these designs for cardiac tissue engineering.

We tried to minimize changes between the two platforms, but one component we had to remove was electrical stimulation. Electrical stimulation has demonstrated that it is beneficial for maturation of hiPS-CMs, and furthermore, it has been demonstrated that a ramped electrical stimulation regimen can be even more beneficial than a static stimulation protocol [73, 75]. The new platform supported electrical stimulation through embedded carbon rods adjacent to the construct formation well. However, pilot experiments saw that electrical stimulation caused cell damage, possibly due to unforeseen issues that arose due to the design changes that were made. The carbon electrodes were seated adjacent to the construct formation well, and the smaller space may have led to unfavorable reactions. We ultimately decided to remove electrical stimulation from the final experiments. Other groups have demonstrated enhanced maturation of hiPS-CMs on a flexible pillar format without utilizing electrical stimulation [56, 94, 95, 97]. Thus, we aimed to promote maturation through mechanical conditioning.

Similar to the published platform, the constructs were formed by seeding hiPS-CMs in a collagen-fibrinogen hydrogel around the flexible pillars (**Figure 3B**). We utilized hiPS-CMs 12-17 days after the start

of differentiation, which was around the same time frame that we had defined as early stage hiPS-CMs in our published study [75]. The constructs were cultured for two weeks in the platform before we utilized them in simulated ischemia-reperfusion experiments.

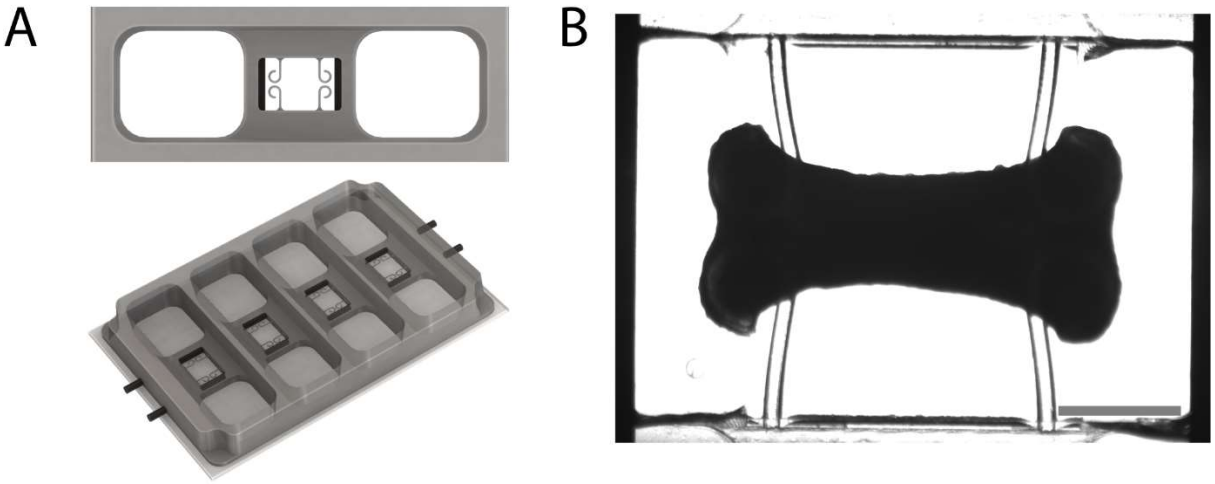


Figure 3: Bioreactor to support formation of human cardiac constructs. (A) Schematic of the bioreactor and a single well depicting the horizontal flexible pillars. The bioreactor was made of PDMS, and it allowed for the simultaneous culture of 4 separate cardiac constructs. (B) Image of a cardiac construct formed by seeding hIPS-CMs in a collagen-fibrinogen hydrogel around the pillars. Scale bar is 2 mm.

Our published platform demonstrated advanced maturation of hiPS-CMs, but we needed to adapt the platform for studying IRI. We wanted to be able to restrict media volume during ischemia and then rapidly washout the ischemic solution during reperfusion to restore normal extracellular conditions. We also wanted to be able to analyze each construct individually in its own well instead of the platform as an aggregate. In adapting the platform, we maintained the flexible pillar design for mechanical conditioning, the embedded carbon rods for electrical stimulation, and the incorporation of hiPS-CMs soon after the

start of spontaneous contractions. However, we had to ultimately remove electrical stimulation from our final experiments due to cell damage, possible due to our design changes. We also aimed to simplified the platform design by orienting the flexible pillars horizontally so that the entire bioreactor could be cast out of PDMS in a two-part mold. This had the additional benefit of orienting all motion of the construct and pillars in the same viewing plane as that of a normal microscope, which will allow us to more easily correlate pillar deflection with force generation in future studies. The new platform design allowed us to have a high success rate in cardiac construct formation.

Engineered Cardiac Construct Structure

To characterize the cardiac constructs that we generated, we examined the distribution and structure of the hiPS-CMs by immunostaining for cardiac troponin T, which is specific for cardiomyocytes [106]. We found that the cardiomyocytes were not evenly distributed throughout the construct (**Figure 4A**). Furthermore, we saw that the hiPS-CMs demonstrated the highest cellular density, alignment, and striated morphology near the edge of the construct (**Figure 4B-C**). This was expected because the edge of the construct is exposed to the highest stress and is not limited by the diffusion of nutrients and oxygen compared to the center of the construct. This indicates that in order to achieve a homogenous and dense distribution of cardiomyocytes throughout the construct, we will need to alter our bioreactor design or culture conditions. We can modify our platform mold to generate a thinner construct that will be less restricted by diffusion limitations, and also culture our platform on a rocker to provide bulk nutrient delivery to center of the construct [107]. However, the presence of aligned and striated cardiomyocytes along the edge of the construct allowed us to assess the effect of simulated ischemia-reperfusion injury in a human tissue model.

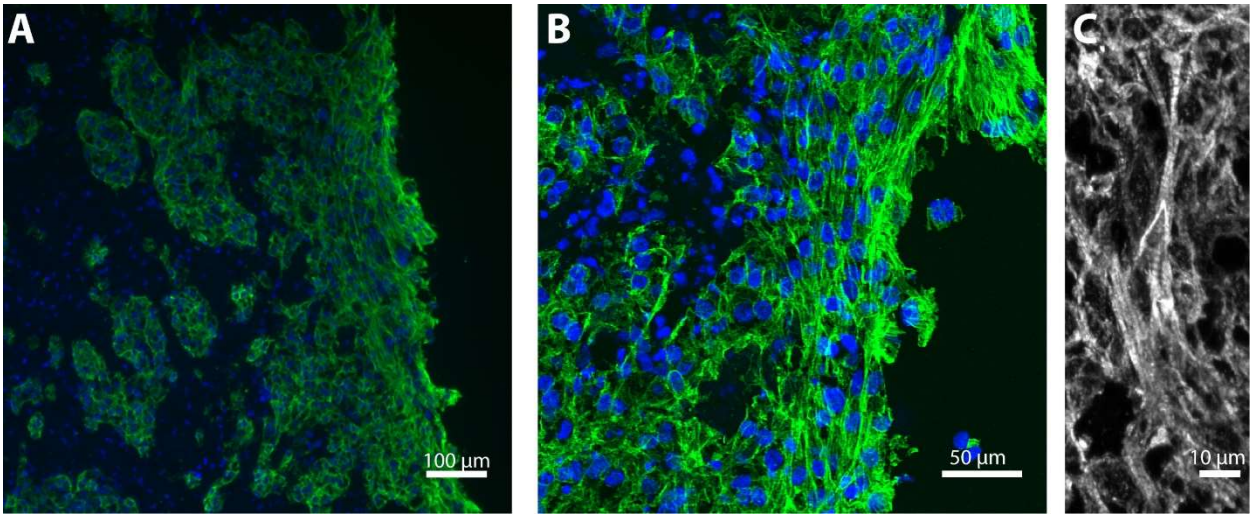


Figure 4: Immunofluorescence images detailing the cardiomyocytes in the engineered cardiac constructs. The cardiomyocytes are not homogenously distributed throughout the construct, but

they do demonstrate alignment and cross-striated morphology near the edge. (A-B) Cardiac Troponin T (green), DAPI (blue). (C) Cardiac Troponin T (white).

Response to β-adrenergic Stimulation

We assessed the functionality of the cardiac constructs by examining their responses to β-adrenergic stimulation. Cardiac constructs were exposed to serial dilutions of isoproterenol, a β-adrenergic agonist, and videos were taken for analysis. The constructs demonstrated a positive chronotropic response to isoproterenol stimulation, with an increase from 29.9 ± 10.6 beats per minute (BPM) at the baseline to 54.0 ± 7.5 BPM at 10 μM (**Figure 5A**). The chronotropic response to isoproterenol had a measured EC_{50} of 1.32 nM, which is lower than reported data for adult human ventricular tissue slices [108]. The constructs were also analyzed for their fractional area change (FAC) in response to isoproterenol stimulation as a proxy for force generation. The constructs demonstrated an increase in FAC from baseline to 100 nM, and nonlinear regression of the dose-response determined that it was significant compared to no response (**Figure 5B-C**). The increase in FAC in the constructs is indicative of a positive ionotropic response to isoproterenol. hiPS-CM monolayers have shown positive chronotropic responses to β-adrenergic stimulation, but because they are still immature, they do not show a positive ionotropic response [109]. Only hiPS-CMs cultured in tissue engineered cardiac constructs have demonstrated a positive ionotropic response, which is consistent with a more mature phenotype [75, 85, 91]. The constructs in our platform demonstrated comprehensive responses to β-adrenergic stimulation, which indicated that the platform helped to promote the maturation of hiPS-CMs.

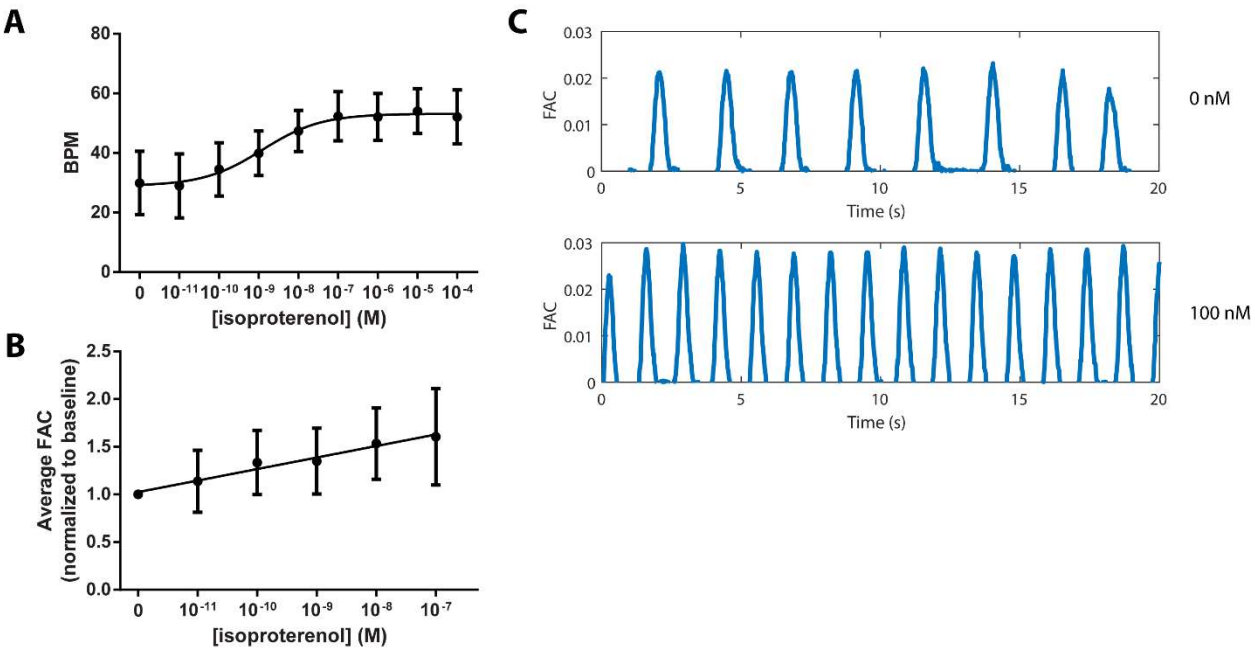


Figure 5: β-adrenergic stimulation of engineered cardiac constructs. (A) Cardiac constructs demonstrated a positive chronotropic response to isoproterenol, with an increase in beat frequency. (B) Cardiac constructs demonstrated an increase in fractional area change (FAC) with increasing isoproterenol concentration. This is correlated with an increase in force generation, and it indicates a positive ionotropic response. (C) Representative FAC traces at baseline (top) and with 100 nM isoproterenol (bottom). N = 6, data are presented as mean ± SD.

Further Characterization of Construct Maturity

We would like to further characterize construct maturity in our future studies. Some of the assays we want to utilize, such as gene expression, will require comparisons, and others are measurements of inherent construct behavior. We can assess maturation of constructs by comparison with younger constructs, age-matched constructs where the flexible pillars were cut to not provide mechanical conditioning, fetal cardiac tissue, and if possible, healthy adult cardiac tissue. Comparison with younger constructs and constructs without mechanical conditioning will allow us to asses increased maturation in the platform. Comparison with fetal or adult cardiac tissue will allow us to assess maturity in terms of cardiac development.

We would first like to examine changes in gene expression. We would like to examine genes that are associated with electrophysiology, calcium handling, and contractile proteins. We had previously demonstrated that our published platform increased expression of these genes relative to more immature constructs [75]. We can further validate these changes in gene expression by examining protein levels in western blot through immunostaining.

We would then like to assess changes in construct function. We had demonstrated that our constructs had a positive chronotropic and ionotropic response to β-adrenergic stimulation, but there are other aspects of functional behavior that we would like to assess. We would like to directly quantify force generation and contractile stress by measuring the constructs on an organ bath. More mature constructs should demonstrate increased contractile stress compared to less mature ones. Measurements of adult ventricular cardiomyocytes have demonstrated contractile stress around 51 kN/m^2 [110]. Furthermore, we can measure inherent properties of the construct on the organ bath. We want to assess changes in force in response to increasing calcium concentration and electrical stimulation frequency. Mature constructs should demonstrate a positive force-frequency relationship [111], and this is not seen in

immature hiPS-CMs [112]. Very few engineered cardiac constructs have demonstrated a positive force-frequency relationship [56, 75].

Furthermore, we would like to characterize the electrophysiology of the constructs. We accomplished this in our published platform by dissociating the cardiomyocytes from the hydrogel to demonstrate enhanced electrophysiology [75], but cardiac tissue engineering studies have also explored impaling electrodes into the constructs to directly measure readouts [56]. Immature cardiomyocytes have a higher resting membrane potential, decreased action potential amplitude, increased action potential duration, and decreased depolarization velocity compared to adult ventricular cardiomyocytes [56, 113, 114]. Furthermore, we can characterize conduction velocity in the construct to determine functional coupling of the cardiomyocytes. We have utilized a voltage-sensitive dye to optically map propagation across a construct in our published platform to measure conduction velocity [75].

Finally, we would like to characterize construct metabolic activity. Fetal cardiomyocytes are more reliant on glycolysis for metabolism, while the mature adult heart primarily relies on fatty acid oxidation [115, 116]. Thus, we wish to characterize an increase in mitochondrial oxidation that has been seen in some tissue engineered cardiac constructs [75, 88]. This can be measured by dissociating the cardiomyocytes from the constructs and measuring their energetics in the Seahorse platform [75]. Oxygen consumption rate and extracellular acidification rate can be measured to determine mitochondrial respiration and glycolysis in the constructs. We can also assess metabolism by using ^{14}C-labeled energy substrates [88]. Furthermore, we can also measure mitochondrial mass as a measure of potential cell oxidative capability. Mitochondrial mass can be easily compared between constructs by using mitochondrial fluorescent dyes, such as Mitotracker [117], or by assessing ratio of mitochondrial proteins to cytosolic proteins [118]. Mitochondrial mass can also be assessed through TEM imaging. Furthermore, we can utilize TEM imaging to measure sarcomere length or the presence of t-tubules. T-tubules are characteristic of mature cardiomyocytes, and they have so far only been seen in the hiPS-CMs of our published platform [75].

Simulating Ischemia-Reperfusion Injury

The bioreactor featured a small well for tissue seeding and culture and was surrounded by a larger reservoir for media. This feature allowed us to better simulate a myocardial infarction *in vitro* by precisely changing culture conditions between ischemia and reperfusion (**Figure 6A**). Constructs were cultured for 2 weeks in the bioreactor prior to simulated ischemia-reperfusion experiments.

To recapitulate the ischemic state, we placed the constructs in a salt solution to simulate the ischemic environment. This salt solution was devoid of metabolic substrates to mimic nutrient deprivation and had a high potassium concentration and low pH to mimic extracellular conditions seen in ischemia. Furthermore, we restricted the solution volume to just 120 µL to allow for the accumulation of wastes and further modification of the extracellular environment by the constructs to simulate the lack of blood flow. The bioreactor was placed in an anoxic environment (95% N_2, 5% CO_2) for 6 hours to induce hypoxia. We found that the constructs did not demonstrate spontaneous contractions when we removed them from the hypoxic chamber, which indicated a lack of ATP production.

To simulate reperfusion, the constructs were returned back to a normoxic environment, and normal culture media was added to the larger reservoir to fully washout the accumulated waste, replenish nutrients, and reestablish pH and ionic balance. Constructs were analyzed after 3 hours of simulated reperfusion and were compared to constructs continuously cultured under normoxic conditions (**Figure 6B**).

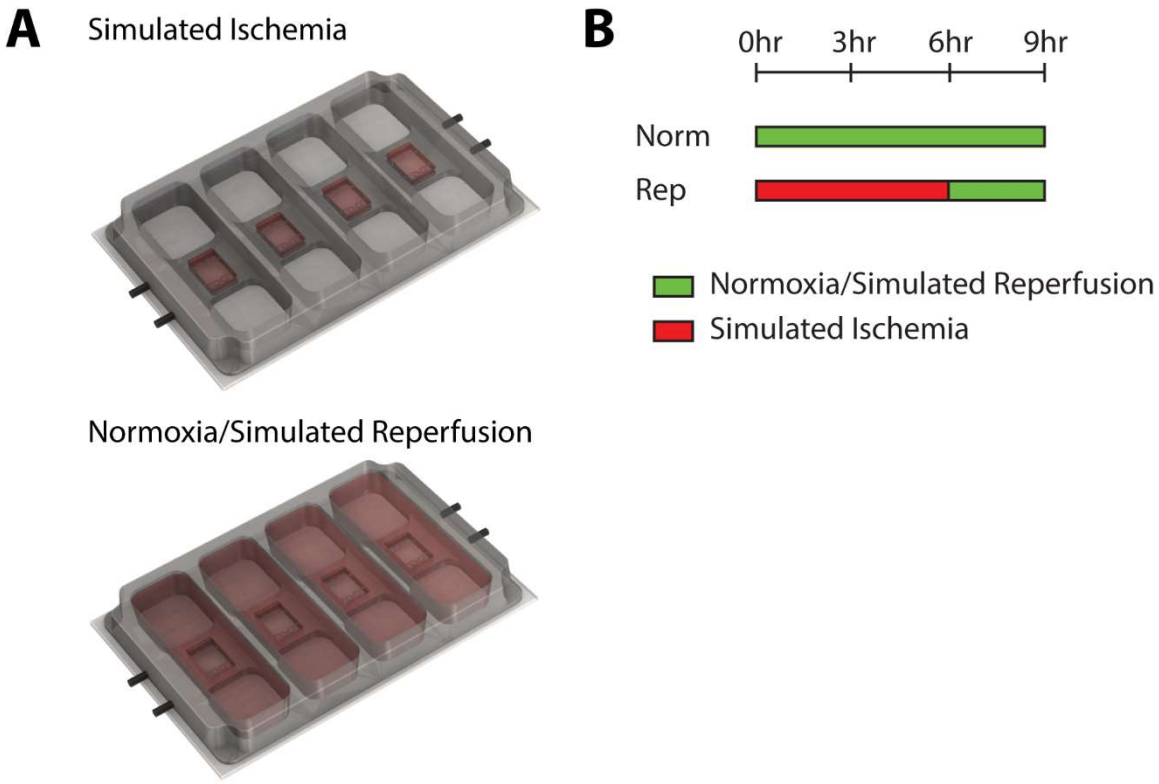


Figure 6: Experimental protocol for ischemia-reperfusion injury. (A) Schematic detailing the ischemic state and reperfusion. Media volume is restricted during simulated ischemia to promote metabolic waste accumulation in the extracellular space. (B) Constructs were placed in hypoxia for 6 hours, and then reperfused for 3 hours before assessing for reperfusion injury.

Discussion

ISCHEMIA

THE ISCHEMIC STATE

Ischemia is the restriction of blood flow that prevents oxygen and nutrient delivery to the downstream region and removal of metabolic waste. Thus, to simulate ischemia, we not only needed to create a hypoxic environment, but also an environment that was deprived of substrates to stop ATP generation and that promoted the accumulation of wastes to simulate the lack of blood flow. Furthermore, the

ischemic state also involves other modifications to the extracellular environment, such as a high extracellular potassium concentration due to ischemic cell death and a decrease in extracellular pH. We simulated ischemic conditions by placing the constructs in a substrate-free solution under hypoxia with a minimal amount of volume to simulate lack of blood flow. Under ischemic conditions, the cardiomyocytes should have a decrease in ATP turnover that leads to cessation of normal function, such as spontaneous contractions, and eventually cell death. We saw that our constructs were not beating after the ischemic period, indicating a lack of ATP production, and demonstrated an increase in cell death compared to normoxic constructs (**Figure 8**).

RECAPITULATING THE ISCHEMIC STATE

Inhibition of ATP production

To decrease ATP turnover in simulated ischemia, we needed to inhibit the ATP generating processes glycolysis and oxidative metabolism. Cardiomyocytes are primarily dependent on oxidative phosphorylation for their ATP production [53]; thus, we placed the cardiac constructs into a hypoxic environment to shut down oxidative metabolism. Without oxygen, the cardiomyocytes must instead rely on the much less efficient anaerobic glycolysis for their ATP production. Glycolysis converts glucose to pyruvate to produce ATP, with reduction of NAD^+ to NADH. NAD^+ stores need to be replenished to continue glycolysis and production of ATP. The cell can accomplish this by converting pyruvate to lactate using lactate dehydrogenase, which also oxidizes NADH to NAD^+. Overall, glycolysis causes cardiomyocytes to produce lactate. To inhibit glycolysis, we placed the constructs in a glucose-free environment to deprive them of metabolic substrates. Many other studies have further inhibited glycolysis with either the addition of lactate or 2-deoxyglucose [32, 33, 44, 45, 52, 67, 119–123] to prevent the metabolism of any leftover glucose. Lactate addition would inhibit glycolysis through the accumulation of its end product,

while 2-deoxyglucose is a direct competitive inhibitor of glycolysis. We decided to further inhibit glycolysis with the addition of lactate because it is a physiological compound, unlike 2-deoxyglucose.

Lactate Addition

The addition of lactate to the ischemic solution can be contentious because the heart can also utilize lactate for ATP production [124]. This would subvert the intended goal of lactate addition, which is to simulate its accumulation during ischemia and inhibit glycolysis to decrease cell ATP production. Lactate is metabolized through two pathways. In the first pathway, lactate is converted into pyruvate by lactate dehydrogenase. Pyruvate can then be converted by pyruvate dehydrogenase and utilized in the tricarboxylic acid cycle and oxidative phosphorylation to produce ATP [125]. However, under hypoxic conditions, the lack of oxygen should block the metabolism of lactate through this mechanism. Alternatively, pyruvate can be converted by pyruvate carboxylase into oxaloacetate, which can then be converted into glucose through the ATP-consuming gluconeogenesis pathway [126]. Gluconeogenesis primarily occurs in the liver as part of the lactic acid cycle [127], but cardiomyocytes have been found to have low expression of some of the important enzymes required for gluconeogenesis [128]. However, this pathway is ATP-consuming, and it would not be utilized in the ATP-depleted hypoxic environment. It has been seen that a cardiac construct subjected to anoxia in a lactate-only environment ceased beating, while the glucose-only construct maintained beating [88]. In the anoxic environment the cardiac constructs are placed in, the additional lactate would not be utilized as a substrate for further metabolism.

The necessity of lactate addition for simulation of ischemic conditions has not been conclusively determined. Multiple *in vitro* studies have utilized lactate addition in their respective ischemic solutions to simulate ischemic conditions [32, 33, 45, 52, 67, 120–122], but other studies have not [50, 129]. In an anoxic, glucose-free environment, cellular ATP production should eventually cease without the addition

of lactate. Lactate addition to the ischemic solution can help further decrease glycolysis, but there is a concern that lactate will instead become a metabolic substrate. Without oxygen, this should not be a concern, but there is a worry that the hypoxic chamber does not maintain a hypoxic environment that would prevent the oxidation of lactate. A leaky hypoxic chamber would allow further metabolism of lactate and defeat its purpose.

To address these concerns, we utilized relatively short simulated ischemia times of 6 hours to minimize gas leakage, flushed the chamber with anoxic gas (95% N_2, 5% CO_2 instead of 1% O_2, 94% N_2, 5% CO_2) to maintain hypoxic conditions, purged the chamber a second time one hour after the first to maintain hypoxic conditions, and continuously monitored oxygen levels using oxygen sensor spots (PreSens PSt3) to confirm hypoxia (<1% O_2). These measures have been demonstrated to maintain experimental consistency [130]. A model of oxygen consumption in cardiomyocytes found that 0.004 mM extracellular oxygen is the unsustainable concentration for maintenance of ATP for non-beating cardiomyocytes [131]. Our oxygen sensor consistently measured around 0.31% O_2 in the hypoxic chamber, which corresponds to a dissolved oxygen concentration of approximately 0.0029 mM. The limit of detection of the sensor is 0.03% oxygen with a 0.05% accuracy at low oxygen levels. This indicated that our method of inducing hypoxic conditions should have provided an environment of depleted cellular ATP.

Elevated blood lactate levels are seen in patients with acute MI. A venous blood lactate level > 1.5 mM was found to be sensitive for an acute MI [132], and patients with an arterial blood lactate levels > 4 mM had a large increase in mortality [133]. However, most of the patients had an arterial blood lactate level between 1-2 mM. On the other hand, the lactate concentration utilized in *in vitro* studies ranged from 5 – 40 mM, with a mode of 20 mM [32, 33, 45, 52, 67, 120–122]. This discrepancy between *in vitro* simulations and the clinical setting can be explained by differences between the infarcted myocardium and the circulating blood. Local levels of lactate around ischemic cardiomyocytes should be much higher than the systemic circulation due to lack of blood flow during an MI. However, it is unclear what local

extracellular concentration of lactate is appropriate. There are no measurements of local levels of lactate after a myocardial infarction, but it has been found that transient levels of blood lactate can reach up to 15-25 mM during exercise [134]. These high transient increases further indicate that the local extracellular concentration is much higher than that in the circulation. The lactate added to the ischemic solution should be a higher concentration than the systemic blood levels. We utilized a concentration of 20 mM for our ischemic solution. A concentration of 20 mM does not seem unreasonable given how high transient levels can reach, but further titration of concentrations may be informative to better understand the role of lactate in the ischemic state.

The necessity of lactate addition for simulated ischemia-reperfusion may be further examined by comparing cell injury of cardiac constructs with or without lactate addition. None of the studies reviewed have made this comparison. In particular, we would seek to determine if the addition of lactate to the ischemic solution is cardioprotective. Lactate addition should lead to increased cell death due to downregulation of glycolysis under our current experimental conditions, but if lactate is being utilized in oxidative metabolism, we should see increased cell survival. Furthermore, the use of radio-isotope labeled lactate and measurement of $^{14}CO_2$ production could be performed to measure lactate oxidation during ischemia.

The aim of simulated ischemia conditions was to inhibit ATP production in the cardiomyocytes. Oxidative phosphorylation was inhibited by culture in a hypoxic environment, while glycolysis was inhibited by culture in a glucose-free environment and by addition of the end product of glycolysis, lactate. Lactate can be utilized by the heart for ATP production, but this process requires oxidative phosphorylation, which is inhibited in a hypoxic environment.

2-deoxyglucose for Inhibition of Glycolysis

Further inhibition of glycolysis can also be achieved through the addition of 2-deoxyglucose. 2-deoxyglucose is a nonmetabolizable glucose analog that can competitively inhibit glycolysis and consume ATP stores through its phosphorylation by hexokinase. It has been included in simulations of ischemia for metabolic inhibition of isolated cardiomyocytes [44, 119, 123]. 2-deoxyglucose addition is an alternative to lactate addition for inhibition of glycolysis on top of substrate deprivation. However, we chose to not include it in our studies because it is unclear how else 2-deoxyglucose can influence cellular behavior. 2-deoxyglucose is not a physiological compound and is not washed out during reperfusion. Thus, we chose to utilize lactate addition in addition to glucose deprivation for inhibition of glycolysis during simulated ischemia.

Extracellular Potassium Concentration

A high extracellular potassium concentration is seen in an MI due to cell death [135, 136]. High extracellular potassium concentrations lead to alterations in cardiomyocyte electrophysiology that can lead to development of arrhythmias and injury [137]. The ischemic solutions that we based our solution off of only utilized 5.4 mM potassium [52, 67], which is only mildly elevated compared to normal potassium levels between 3.5-5 mM [138]. However, measurements of potassium concentration in the heart during an MI have demonstrated increases up to 11-12 mM [136]. Thus, we decided to increase the concentration of potassium to 12 mM to better simulate the expected concentration in the local extracellular space around the cardiomyocytes.

Acidic Environment

We further titrated the ischemic solution to an acidic pH to better simulate ischemic conditions. Intracellular acidification during ischemia and rapid normalization of intracellular pH is an important part of the pathophysiology of IRI. Intracellular acidification occurs due to low ATP turnover, with increased hydrolysis and decreased synthesis of ATP that leads to excess production of protons [20]. The cardiomyocytes attempt to buffer the intracellular acidification through transport of protons through the monocarboxylate transporter (MCT) and sodium hydrogen exchanger (NHE), which results in acidification of the extracellular environment. It is the rapid restoration of the extracellular pH to neutral that causes rapid normalization of intracellular pH and intracellular calcium overload.

We simulated ischemic conditions by titrating the ischemic solution to pH 6.4 based on previous studies [52, 67, 120]. A pH of 6.4 is around the intracellular pH in ischemic hearts, which has been measured to be as low as 6.2 [139]. Other studies have utilized a higher [33, 121] and lower pH [50] for their simulations, but we ultimately decided on pH of 6.4 to provide an order of magnitude difference between ischemia and reperfusion conditions. We also later attempted to utilize reperfusion with acidic media as a cardioprotective strategy. Previous studies on rat hearts utilized reperfusion with acidic media of pH 6.4 [140], and we wanted to maintain the same pH for the acidic media as the ischemic media.

There is an argument to not titrate to an acidic pH. The cardiomyocytes should acidify their environment through the natural process of lactic acidosis and decreased ATP turnover [20]. One of the concerns we had when starting this study was that we would not be able to see the cell death due to IRI. While the ischemic solution attempts to be physiologic, it inherently cannot capture the *in vivo* environment by itself. There may be interactions that we are not accounting for. Acidification of intracellular and extracellular space is an important part of the pathophysiology of IRI, and we wanted to ensure that we were able to capture this with an acidic pH during ischemia and a return to neutral pH during reperfusion. It has been demonstrated that lowering the pH in simulated ischemia leads to an increase in cell death

[50]. With further understanding of the cellular response, we can create ischemic conditions that will naturally promote intracellular acidification for a more physiologic process rather than imposing it on the cells through titration to an acidic pH. However, in our current studies, we wanted to have more control over ischemic conditions.

Buffers

The ischemic solutions used in *in vitro* studies relied on 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) or bicarbonate for their buffering capabilities [32, 33, 45, 52, 67, 120–122], and the ischemic solution we utilized used 5 mM HEPES. HEPES and bicarbonate are the most common buffers utilized in cell culture; however, HEPES has a pK_a of 7.31 with a useful range of pH 6.8-8.2. Sodium bicarbonate does have a pK_a of 6.4, but its buffering capabilities are dependent on atmospheric carbon dioxide levels and may not work as well during excessive handling under room air. Thus, we wanted to avoid bicarbonate buffering. No studies have explored the use of other buffers, such as 2-(N-morpholino) ethanesulfonic acid (MES) which has a pKa of 5.97 at 37°C with working range of pH 5.5-6.7. These other buffers may be more appropriate for maintaining the low extracellular pH seen in ischemia, but we wished to avoid them because of their current nonexistent use in *in vitro* studies.

A more important factor in simulating ischemia is that the buffering capabilities of the ischemic solution must not overwhelm the cells' ability to modify their extracellular environment. Intracellular acidification and rapid normalization during reperfusion are critical to the pathophysiology. To address this, we utilized lower concentrations of HEPES compared to the standard working range of ≥10 mM [141], the ischemic solution was titrated to an acidic pH to begin with, and a minimal amount of solution was added. These factors forced ischemic conditions onto the construct and allowed the cells to exert more control over their surrounding extracellular environment.

Ischemic Time

We utilized an ischemic time of 6 hours for experiments conducted in this thesis. The ischemic time was chosen based off a cardiac tissue engineering platform utilizing neonatal rat cardiomyocytes for study of IRI [78]. In general, *in vivo* studies use much shorter ischemic times to induce injury compared to *in vitro* studies [33, 48]. This is because the intact heart is much more mature and reliant on its blood supply than any isolated cardiomyocyte. In particular, hiPS-CMs still fall short of adult maturity and are resistant to hypoxia. The ischemic time we utilized in these studies demonstrated differences in cell death that we could explore, but there are reasons to alter the ischemic time in future studies.

It is recognized that the cardiac construct subjected to simulated ischemia-reperfusion does not represent the entire myocardial infarct. In this model, the entire construct is subjected to the same ischemic conditions and ischemic time. On the other hand, the center and border of the infarct area are subjected to different degrees of injury *in vivo*. The border zone is partially sustained by collateral circulation, is more resistant to ischemic injury, and thus, demonstrates better survival compared to the center of the infarct region. Under the current ischemic time, most of the constructs did not regain beating and are more representative of the center of the infarct area. Decreasing the ischemic time would allow us to better assess the response of cardiomyocytes in the border zone to ischemia-reperfusion and give us a better understanding of the entire myocardial infarct area.

REPERFUSION

Reperfusion serves to restore nutrients and oxygen to the cells, washout accumulated waste, and reestablish normal extracellular pH and ionic balance. In our model, reperfusion was achieved by returning the constructs back to a normoxic environment and by the addition of 2 mL of RPMI 1640 supplemented

with 1.4 mM calcium chloride (for a final calcium concentration of 1.8 mM) and 2% B27 supplement, minus antioxidants. The media helped to restore metabolic substrates and washout the ischemic solution. This media was also utilized for culture of the normoxic constructs for the duration of simulated ischemia-reperfusion to minimize culture differences between the reperfused groups and controls. The media utilized for reperfusion was very similar to that used for normal culture of cardiac constructs, but it had a higher concentration of calcium and removed antioxidants. Calcium concentration in base RPMI 1640 media is 0.42 mM, which is much lower than the 1-2 mM found in normal blood levels [55]. Additional calcium was supplemented into the reperfusion media to bring levels into the physiological range, and this would also allow for calcium overload of the cardiomyocytes during reperfusion through the sodium-calcium exchanger (NCX) [142]. Furthermore, we removed antioxidants from the reperfusion media by utilizing B27 supplement without antioxidants and by not including ascorbic acid. It has been recognized that oxidative stress and generation of reactive oxygen species is an important component of reperfusion injury [26], and we wanted to ensure that we were not artificially limiting it.

The reperfusion media we utilized was buffered using bicarbonate. The sodium-bicarbonate cotransporter (NBC) is important in the regulation of intracellular pH, and it helps to extrude acid by cotransport of sodium and bicarbonate into the cell [143, 144]. Thus, the transport of bicarbonate from the extracellular to intracellular space contributes to reperfusion injury through rapid intracellular pH normalization. Studies have attempted to isolate out the effects of bicarbonate using HEPES-buffered media [140], but we thought it was appropriate to include bicarbonate in the reperfusion media because it is prevalent in blood and physiological reperfusion. The bicarbonate concentration in the media was 23.8 mM, which is within the normal serum bicarbonate range of 22-29 mM [145]. We sought to reperfuse our cardiac constructs using modified normal culture media to minimize differences between culture and reperfusion conditions but also allow for changes during reperfusion.

We analyzed our constructs after 3 hours of reperfusion. Reperfusion times for studies of IRI vary drastically, with as short as 15 minutes [119] to as long as 17 hours [50]. Most studies analyzed their samples after 1-6 hours of reperfusion [32, 33, 45, 120, 146]. Many of the mechanisms that cause reperfusion injury happen in the short period surrounding reperfusion, but there are also many signaling pathways that are activated to cause long term changes. We decided to analyze our samples after 3 hours of reperfusion in an attempt to capture both the short and long-term changes caused by reperfusion. Further characterization of shorter and longer reperfusion times will allow us to build a better timeline of the changes caused by reperfusion on top of our current ischemia-only and reperfused constructs.

FUTURE METHODS

ELECTRICAL STIMULATION

As illustrated in the model of cardiomyocyte oxygen consumption, ATP utilization is much higher when the heart is beating [131]. Electrical stimulation has been utilized to help promote maturation, but it can also help to improve simulation of ischemia-reperfusion. Under the global hypoxia conditions utilized in our experimental setup, the cardiomyocytes in the cardiac construct can go into hibernation, stop contracting, and reduce their energy demands [147]. However, the heart does not have this luxury during an acute MI and still needs to meet contractile demands. This puts a greater stress on the *in vivo* cardiomyocytes and forces higher ATP utilization than what is seen in the *in vitro* cardiomyocytes. To help simulate this process, electrical stimulation during ischemia can help drive construct contraction to help force contractile work by the cardiac constructs.

The bioreactor platform was originally designed to incorporate electrical stimulation through carbon rods. However, technical issues lead to the removal of electrical stimulation from the final experiments because it seemed to damage cardiomyocytes and prevent formation of a cohesive contracting cardiac construct.

Our adapted platform is capable of providing electrical stimulation to the constructs, but the issues with electrical stimulation that arose will need to be solved to allow for improved maturation of the cardiac construct and improved simulation of ischemia-reperfusion.

PROPOSED BIOREACTOR TO SIMULATE OXYGEN GRADIENT IN AN MI

Currently, we subject the entire cardiac construct to an anoxic environment to simulate the infarct region. However, a complete anoxic environment is not necessarily representative of the full MI. The human heart does have some collateral coronary circulation that can provide partial oxygenation to parts of the infarct area [148]. Recapitulation of these dynamics is difficult to achieve *in vitro*. One study attempted to simulate a spatially controlled MI in a microfluidic device [149]. Cardiomyocytes were seeded in a central channel, which was surrounded by 2 perfusable channels. Only one channel was perfused with carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), which uncouples oxidative phosphorylation and ATP synthesis, in an attempt to simulate ischemia. This led to a gradient of FCCP and cell death between the two perfused channels. However, the use of a drug for metabolic inhibition to simulate ischemia is undesirable because of difficulties in washing it out to simulate reperfusion. Instead, a bioreactor that supports partial oxygenation of the cardiac construct can examine how minute changes in oxygen concentration affect the cardiomyocyte response.

We developed the concept of a bioreactor that could potentially allow for partial oxygenation of the cardiomyocytes with a functional microvasculature. The bioreactor was based on a previous platform developed by Nguyen et al. to study angiogenic sprouting of endothelial cells between two channels [150], but it features critical differences that optimize the platform for study of ischemia-reperfusion. Nguyen et al. demonstrated that the endothelial cells lining the channel could be induced to sprout into the hydrogel through a combination of proangiogenic factors and shear stress through culture on a rocker. In this platform, the hydrogel would instead by seeded with cardiomyocytes to form a cardiac parenchyma for

the endothelial cells to sprout into and form a functional microvasculature. Shear stress and flow would be introduced by culture on a rocker, and proangiogenic factors would be added to promote the formation of a functional microvasculature that connects the two channels (**Figure 7A**). The original reactor was made of PDMS, which is permeable to gases. On the other hand, this reactor was constructed out of polymethylmethacrylate (PMMA) to be impermeable to gases and allow for potential hypoxic conditions. PMMA, similar to PDMS, is cheap and transparent to allow for optical examination of the cardiac tissue. The reactor was formed by solvent bonding laser cut layers of PMMA together, but PMMA can also be formed by injection molding for large scale manufacturing (**Figure 7B**). A PMMA cap with a gasket could be screwed to one half of the reactor to block access to atmospheric oxygen, and the idea was that oxygen levels would eventually be depleted on that side to create a hypoxic environment in one channel (**Figure 7C**). This would create an oxygen gradient through the cardiac construct and allow for examination of cardiomyocyte response to varying oxygen concentrations. We were able to demonstrate that we could form two separately perfusable channels in the formed hydrogel by removal of steel rods after hydrogel casting (**Figure 7D**).

This platform of course has multiple issues that must be overcome, and it is merely a concept of potential directions to better understand the response of cardiomyocytes to ischemia-reperfusion. The current platform offers no methods to help promote maturation of the cardiomyocytes through electrical stimulation or mechanical conditioning. The method of inducing hypoxia and oxygen depletion also depends on cellular consumption of existing oxygen on one half of the reactor, which offers less control and more variability than the current method of purging environmental gas in the hypoxic chamber. It is unclear how long it would take for hypoxic conditions to take hold, but this may be helped by making the media wells smaller on that side so that there is less dissolved oxygen that needs to be consumed. However, the platform does offer certain advantages over the existing platform. The use of perfusion and endothelial cells to form a microvasculature would allow us to examine interactions between endothelial

cells and cardiomyocytes [151]. Nitric oxide is synthesized by the endothelium, increased synthesis is seen during ischemia, and it can be cardioprotective in ischemia-reperfusion [52, 152–154].

Since this thesis was the first to explore ischemia-reperfusion in a human tissue model, we decided to focus on the cardiomyocytes themselves, exclude other cell types, and focus on a simplified engineered cardiac construct. We aimed to focus on utilizing cardiac tissue engineering to promote the maturation of hIPS-CMs for simulation of ischemia-reperfusion.

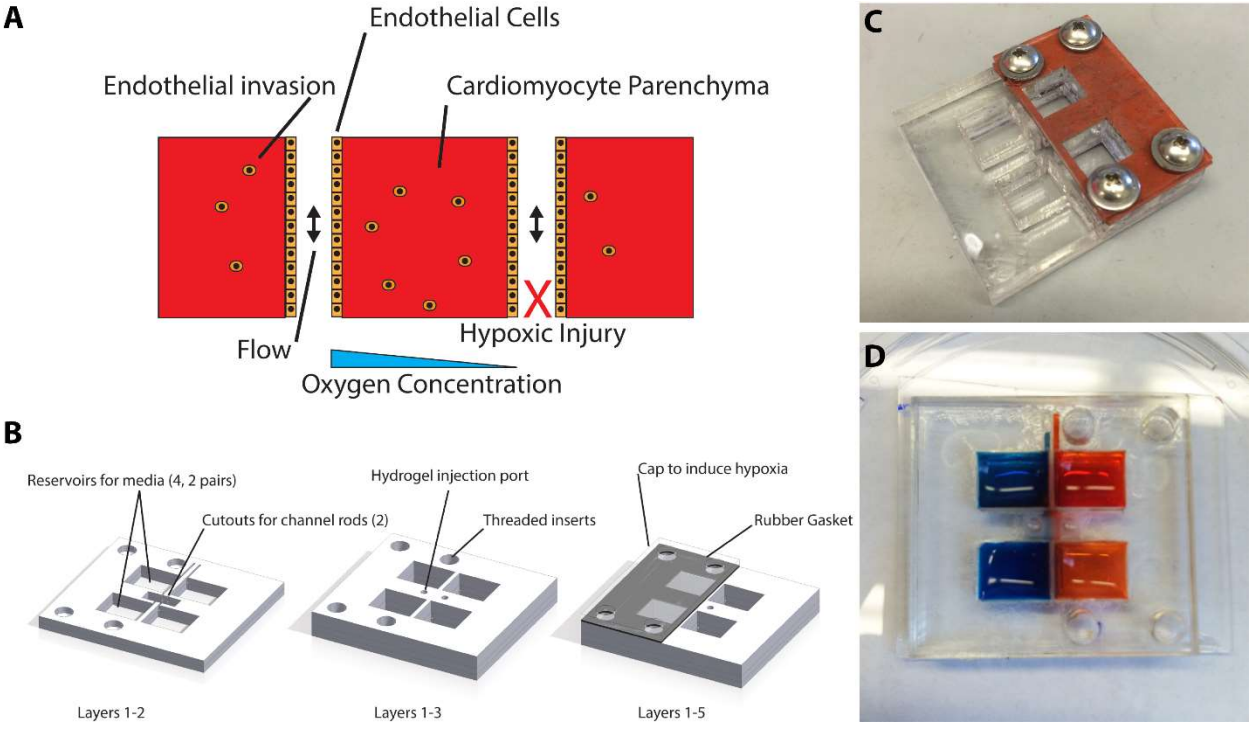


Figure 7: Proposed bioreactor to induce oxygen gradient in cardiac construct with functional microvasculature. (A) Schematic of the cardiac construct hydrogel with infiltrating endothelial cells and two perfusable channels. An oxygen gradient can be induced by restricting oxygenation of one channel. (B) Schematic of the bioreactor. Different polymethylmethacrylate (PMMA) layers are solvent-welded together to form an oxygen impermeable platform. (C) Oxygen can be

restricted by addition of a PMMA cap with gasket to one side of the bioreactor. (D) Initial testing of the bioreactor demonstrated the formation of two separate channels in the hydrogel that can be perfused through culture on a rocker.

Aim 2: Validate Ischemia-Reperfusion Injury in the Tissue Engineered Cardiac Construct

Criteria to Validate Ischemia-Reperfusion Injury

Because *in vitro* models examine IRI in non-physiological settings, they must first be validated to demonstrate that IRI can be observed. Critically, we wanted to distinguish ischemic injury from reperfusion injury. In these studies, we sought to demonstrate the presence of IRI and that we can detect changes in it by demonstrating: (1) an increase in cell death in the hypoxia-reoxygenated group over normoxic control, (2) an increase in cell death after hypoxia-reoxygenation compared to hypoxia only, and (3) a decrease in cell death after hypoxia-reoxygenation using a therapy that mediates reperfusion injury.

Distinguishing Ischemic Injury from Reperfusion Injury

We first assessed the presence of ischemia-reperfusion injury in the engineered cardiac constructs by comparing the response of ischemia-only (referred to as "*Isch*") and ischemia followed by reperfusion (referred to as "*Rep*") constructs. *Isch* constructs were subjected to 6 hours of simulated ischemia, while Rep constructs were subjected to 6 hours of simulated ischemia followed by 3 hours of simulated reperfusion. This comparison allowed us to differentially assess the effects of ischemia and reperfusion injury. The *Isch* and *Rep* groups were further compared to constructs under normal culture conditions (referred to as "*Norm*") (**Figure 8A**).

Lactate dehydrogenase (LDH) and adenylate kinase (AK) release were used to assess membrane integrity and cell death. *Isch* constructs demonstrated a significant increase in cell death over *Norm* constructs. Reperfusion led to further cell injury, which was reflected by the significant increase in cell death in *Rep* over *Isch* constructs (**Figure 8B-C**). These differences were further confirmed in measurements of cell activity to assess cell viability. The *Isch* group demonstrated a significant decrease in cell viability compared to *Norm* controls, and reperfusion lead to a further decrease in the *Rep* group (**Figure 8D**). These results demonstrated that simulated ischemia caused cell injury in the cardiac constructs, and reperfusion further increased the cell injury and decreased cell viability compared to ischemia alone.

To further characterize IRI in the constructs, we utilized JC-1 staining to assess mitochondrial membrane permeability. Opening of the MPTP with subsequent mitochondrial membrane depolarization is a critical step leading to cell death in lethal reperfusion injury. The JC-1 dye was used to assess the mitochondrial membrane permeability and the state of the MPTP. The JC-1 dye normally aggregates in the mitochondria with a peak emission at 590 nm, but depolarization of the membrane causes it to disperse into the cytosol with a peak emission at 528 nm. By comparing the relative fluorescence of the dye, it is possible to determine the relative permeability of the mitochondria membrane. In the constructs, we saw a

significant increase in mitochondrial membrane permeability in the *Isch* group over the *Norm* group. Reperfusion led to an even larger increase in mitochondrial membrane permeability, with a significant increase in *Rep* constructs over ischemia-only (**Figure 8E**). This large increase is likely due to opening of the MPTP during reperfusion, which depolarizes the mitochondrial membrane, releases its contents into the cytosol, and leads to cell death.

Reperfusion and restoration of oxygen to the infarct region also leads to the rapid generation of reactive oxygen species (ROS) and oxidative stress that contributes to reperfusion injury [155]. As expected, reperfused constructs demonstrated higher levels of ROS compared to ischemia-only and normoxic constructs (**Figure 8F**). This indicates that the generation of ROS after reperfusion could be contributing to IRI in the human tissue model, and antioxidant therapy should be explored.

Ischemia leads to lactic acidosis and a decrease in ATP turnover that leads to intracellular acidosis [20]. Furthermore, we titrated the ischemic solution to a pH of 6.4 to simulate the acidic extracellular environment seen in ischemia. Reperfusion washes out the acidic extracellular environment to restore a neutral pH, which causes a rapid normalization of intracellular pH that leads to cell injury and death. We utilized Phrodo staining to measure intracellular pH and confirmed that our ischemia-only constructs demonstrated a lower intracellular pH compared to both normoxic and reperfused constructs (**Figure 8G**). Our constructs demonstrated a normalization of intracellular pH after reperfusion, and we need to target this to reduce cell death after ischemia.

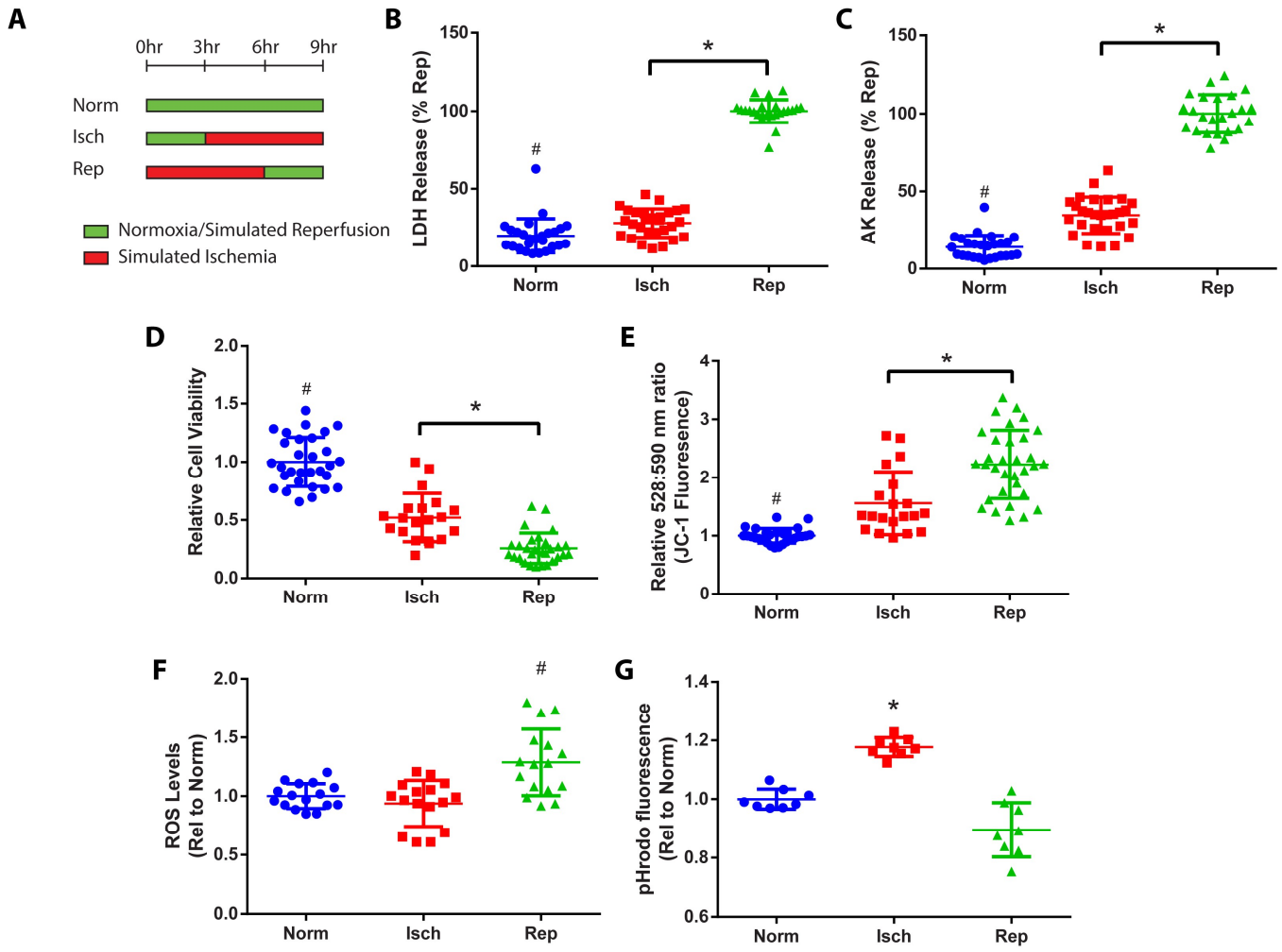


Figure 8: Comparing response of cardiac constructs to simulated ischemia only (referred to as "*Isch*") and simulated ischemia followed by reperfusion (referred to as "*Rep*"). (A) Schematic of the experimental protocol for the comparison groups. (B-C) Lactate dehydrogenase (LDH) and adenylate kinase (AK) release were used to assess cell membrane permeability and death. (D) Cell viability was determined by measuring cell activity using RealTime Glo assay. (E) Mitochondrial membrane permeability was determined by comparing emission of JC-1 dye at 528 nm and 590 nm, where increased ratio is correlated with higher permeability. (B-E) Data represents aggregated results from 5 independent experiments. (F) Reactive oxygen species (ROS) levels in cardiac constructs as measured by ROS-Glo assay. Data represents aggregated results from 4 independent experiments. (G) Measurement of intracellular pH using pHrodo dye. Higher

fluorescence signal indicates a lower intracellular pH. Data represents aggregated results from 2 independent experiments and are depicted as individual data points with mean ± SD. Statistical analysis was done using ANOVA with post-hoc Tukey's HSD, # indicates statistical significance compared to all other groups, * indicates significant difference between groups, $p < 0.05$.

Constructs were also fixed and stained to examine ultrastructural changes after ischemia and reperfusion. Normoxic constructs demonstrated aligned and striated staining for cardiac troponin T. This organization was mildly disrupted in *Isch* constructs, but the *Rep* constructs demonstrated highly disorganized structure. The *Rep* constructs did not demonstrate the aligned and striated staining seen in the *Norm* constructs. Reperfusion and intracellular calcium overload leads to activation of intracellular proteases [25, 156], and this is reflected in the disorganized ultrastructure seen in reperfused constructs.

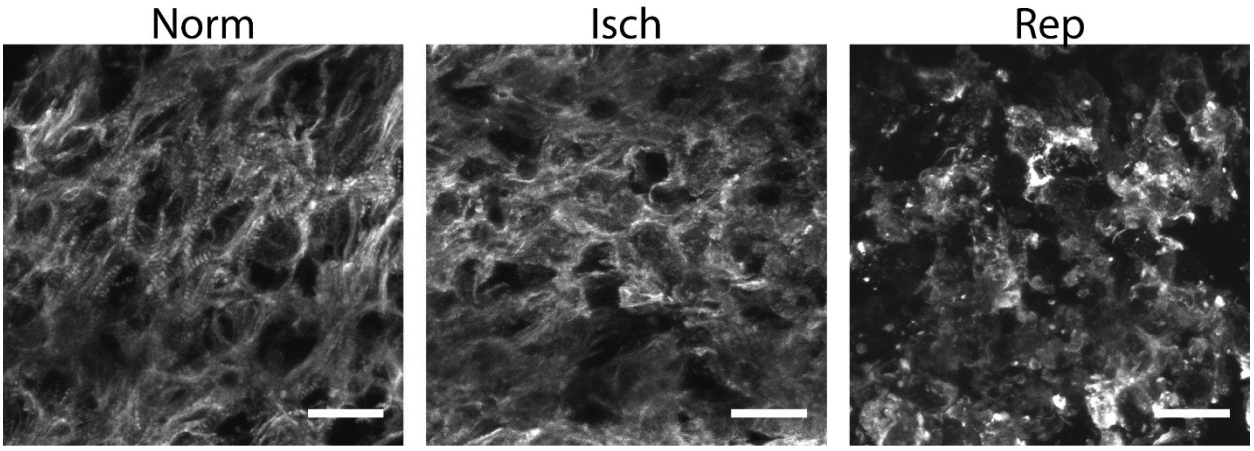


Figure 9: Representative images of cardiac construct ultrastructure (cardiac troponin T staining). Images demonstrate increased disorganization of ultrastructure after ischemia, and further disorganization and loss of striations after reperfusion. Scale bar is 20 µm.

While ischemia leads to initial activation of apoptotic pathways to cause cell death, reperfusion leads to a further large increase in apoptosis to cause even greater injury [157–160]. We assessed the activation of apoptosis in the constructs by examining the cleavage of caspase 3 into its active form through western blot analysis. As expected, *Isch* constructs demonstrated a small increase in the ratio of cleaved to total caspase 3, and *Rep* constructs demonstrated a significantly higher increase in caspase 3 cleavage (**Figure 10**). Caspase inhibition during reperfusion has helped to decrease IRI [161], but there is also a significant amount of necrosis during reperfusion that contributes to the final injury [162].

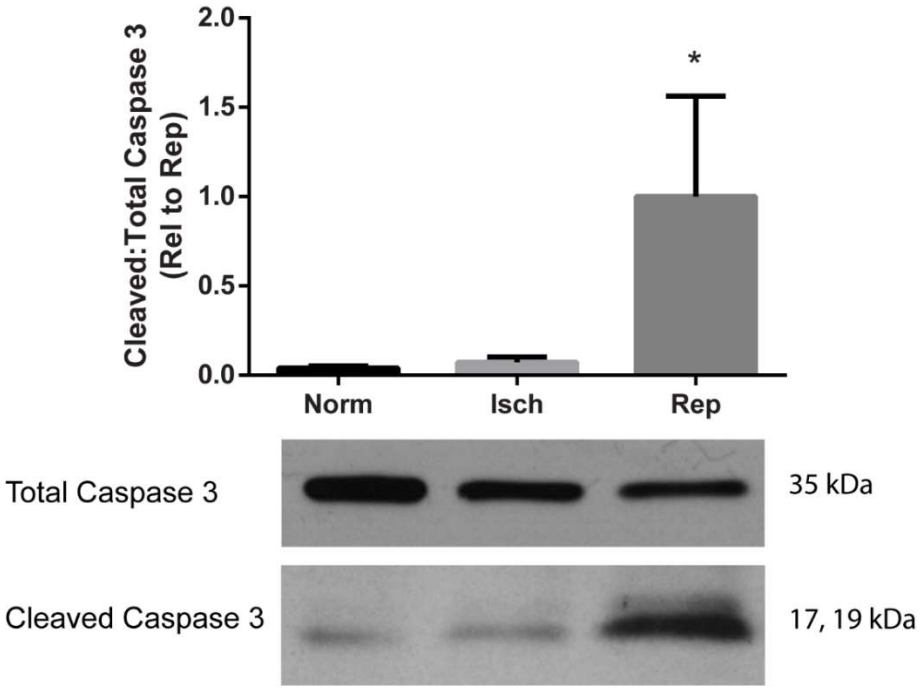


Figure 10: Western blot analysis of apoptosis in the cardiac constructs. Apoptosis was determined by activation and cleavage of caspase 3 and demonstrates an increase after reperfusion. Data is from 3 independent experiments, mean ± SD. Statistical analysis was done using ANOVA with post-hoc Tukey's HSD, * indicates significant difference compared to other groups, $p < 0.05$

The presence of reperfusion injury was established by comparing ischemia-only to ischemia-reperfusion constructs. Reperfusion lead to higher cell death, lower cell viability, and an increase in mitochondrial membrane permeability due to opening of the MPTP. We also saw disorganized cardiomyocyte ultrastructure in the reperfused constructs, and an increase in apoptosis upon reperfusion. By establishing the differential response of the constructs to ischemia and reperfusion, we can next explore cardioprotective strategies to treat IRI.

Discussion

MEASURING CELL DEATH

To validate the presence of ischemic and reperfusion injury in our platform, we needed to assess and characterize cell death. However, cell death is a difficult endpoint to measure, and every assay has deficiencies in its use. It has been recommended to analyze cell death utilizing multiple assays that are methodologically different [163]. In our studies, we utilized intracellular protein release, measurements of cell activity, and mitochondrial membrane permeability as our primary assays to analyze our primary endpoint, cell death. We saw that ischemia-only constructs demonstrated an increase in intracellular enzyme release, reduced cell activity, and increase in mitochondrial membrane permeability compared to normoxic constructs. Reperfusion lead to a further significant difference in these assays compared to the ischemia-only constructs. We were able to utilize these cell death assays to demonstrate that reperfusion lead to a further increase in construct injury over ischemia alone.

One of our primary assays to assess cell death was to measure release of the intracellular proteins lactate dehydrogenase (LDH) and adenylate kinase (AK). Release of these enzymes indicates cell membrane permeability and cell death, and their release can be measured by sampling cell media supernatant and measuring enzyme activity by addition of a substrate. Measuring enzyme release is a simple method and

can be rapidly scaled up to analyze many constructs. However, this method of quantification can also be problematic because enzyme activity can be affected by other factors. In particular for our study, we were concerned with how pH differences could affect enzyme activity. Our ischemia-only constructs were subjected to pH 6.4 ischemic solution. To measure enzyme release, we added culture media to normalize pH and volume, and then quickly sampled the media to minimize differences compared to the reperfused constructs. Critically, we saw that our ischemia-only constructs demonstrated an increase on LDH and AK release over normoxic controls, which demonstrated that these assays are able to detect cell death due to ischemic injury. Another method we attempted in order to normalize conditions was to use a lysis buffer to obtain 100% lysis of the construct for full LDH and AK measurements. However, we had issues with variable results due to variable penetration of the lysis solution into the tissue bulk. To further confirm the increase in LDH and AK release that we saw with reperfusion, we utilized other methodologically different assays to assess cell death after ischemia-reperfusion.

Another method that we could utilize to measure intracellular protein release is enzyme-linked immunosorbent assay (ELISA). ELISA would allow for the direct quantification of proteins in the cell media without being dependent on enzyme activity. In particular, ELISA to quantify release of cardiac troponin I would allow us to assess cardiomyocyte cell death after ischemia-reperfusion. ELISA is an expensive assay that is cost-prohibitive for large-scale use, but it can be utilized in future studies to confirm the initial findings in the less expensive intracellular protein release assays.

In addition to intracellular enzyme release, we sought to measure cell activity to assess cell viability after ischemia-reperfusion. Tetrazolium dye-based assays, such as the MTT assay, are widely utilized for determination of cell metabolic activity. These tetrazolium dye assays are dependent on the cell reduction of a water-soluble substrate to an insoluble product, which can then be analyzed and quantified. However, we found that these assays had many issues when used for our purposes, and the product seemed to negatively impact the viability of the cardiac constructs in pilot experiments. Instead, we utilized

RealTime-Glo, a nonlytic luminescent assay, that allowed us to detect cell reduction of the substrate with minimal manipulation of the cardiac constructs. It was an ideal assay to facilitate analysis of many constructs at a time. Using the RealTime-Glo assay, we were able to demonstrate that reperfusion led to a further decrease in construct viability compared to ischemia alone. The major concern for utilizing assays that measure cell metabolic activity to assess cell death is that there are many reasons unrelated to cell death for why metabolic activity can decrease. For our current comparisons of ischemia-only and ischemia-reperfusion this was not an issue. But these concerns do need to be accounted for once we modify reperfusion conditions in non-physiological manners to decrease IRI. Thus, we still relied on multiple assays to understand cell death in our model.

We also examined mitochondrial membrane permeabilization as an assay for cell death. Permeabilization of the mitochondrial membrane is implicated in all forms of cell death [164]. Moreover, opening of the MPTP is critical in the pathophysiology of reperfusion injury [27, 28], and thus, it was important to assess mitochondrial membrane permeability in characterizing cell death in IRI. We utilized the JC-1 dye to measure mitochondrial membrane potential and found that reperfusion led to a significant increase in the mitochondrial membrane permeability. This result indicated that IRI in our platform activated mitochondria-dependent cell death pathways. However, IRI also involves oxidative stress, activation of intracellular proteases, and other mechanisms of injury that are not necessarily dependent on mitochondria [25, 26]. It was important in our characterization of IRI to assess total cell death, and not just the mitochondria.

OXIDATIVE STRESS

After demonstrating an increase in cell death after reperfusion, we sought to further characterize IRI in our cardiac constructs by examining other aspects of the pathophysiology that contribute to injury. We

aimed to characterize oxidative stress by measuring ROS levels in our constructs. There are 2 main types of ROS that are measured in experiments, superoxide anion and hydrogen peroxide [165], and both have demonstrated increases after reperfusion [166]. However, superoxide is a less stable ROS and disproportionates into hydrogen peroxide, a more stable ROS. Thus, we chose to analyze hydrogen peroxide levels to assess cell ROS levels and oxidative stress. Furthermore, hydrogen peroxide is cell membrane permeable, which allowed us to measure ROS levels in our constructs without a need for cell lysis or microscope observation. There are many fluorescent dyes available to assess hydrogen peroxide levels, but we chose to utilize the nonlytic luminescent assay, ROS-Glo. The luminescent assay allowed us to multiplex the assay with other fluorescent readouts. By measuring construct hydrogen peroxide levels, we are able to determine that reperfused constructs had higher levels of ROS, which could be contributing to the greater injury seen in these constructs through oxidative stress.

In addition to cell ROS levels, mitochondrial ROS generation can also be characterized by utilizing mitochondrial specific dyes. MitoSOX and MitoB are fluorescent dyes that can detect mitochondrial superoxide and hydrogen peroxide levels respectively. Reactivation of the electron transport chain during reperfusion is thought to drive the production of ROS [166], and thus, characterization of mitochondrial ROS production will help us further demonstrate changes due to reperfusion. Luminescent and fluorescent assays for ROS levels can be analyzed on a plate reader for simple readings and comparisons.

We could have further characterized oxidative stress by measuring glutathione. Glutathione is an endogenous antioxidant that is produced by cells, and reduction in reduced glutathione (GSH) levels is indicative of oxidative stress [167]. Furthermore, the antioxidant, N-acetylcysteine (NAC) that we utilized in later studies functions by replenishing glutathione levels. However, assays to directly measure GSH levels require cell lysis to function, which may give us variable results due to variances in ability to completely lyse a 3D construct. Instead, measurements of free thiol levels can be an alternative method to assess GSH levels because reduced glutathione comprises the majority of free thiols in a cell [168]. The

ThiolTracker Violet dye is a fluorescent dye that can even by fixed for analysis of cell glutathione levels in future studies. Measuring GSH levels can give us a better understanding of the cell response to total oxidative stress during reperfusion.

INTRACELLULAR PH

We also measured the intracellular pH after simulated ischemia and confirmed that the constructs had a reduction in intracellular pH during ischemia. Rapid normalization of intracellular pH upon reperfusion is an important mechanism in causing IRI [42]. Intracellular pH was assessed utilizing the pHrodo dye, a fluorescent dye that has higher fluorescence with a decrease in pH, and we saw higher fluorescence in the constructs after ischemia. We later attempted to prevent the rapid normalization of intracellular pH by reperfusion with acidic media. In future studies, we wish to examine intracellular pH over the time course of reperfusion to better assess how therapeutic strategies can change the rate of intracellular pH normalization for cardioprotection.

APOPTOSIS

To further demonstrate differences between ischemic and reperfusion injury, we sought to characterize apoptosis through the cleavage and activation of caspase 3. Ischemia is thought to be primarily a necrotic cell death process, while reperfusion leads to increased necrosis and activation of apoptosis [159]. However, it is important to note that the majority of injury seen in ischemia-reperfusion injury is due to necrosis [162]. Caspase 3 is one of the executioner caspases and is responsible for enacting apoptosis [169]. Western blot allowed us to assess the cleavage and activation of caspase 3 by utilizing different antibodies for the differential comparison of the cleaved and pro-form of caspase 3. Alternatively, there are other assays to assess caspase activation through the introduction of luminescent or fluorogenic

substrates for caspase cleavage. However, we found that these assays were difficult to utilize in our model in initial studies because they depended on complete lysis of the cardiomyocytes to isolate the caspases and measure their activity. Instead, western blot analysis allowed us to utilize longer and more aggressive lysis and homogenization protocols that did not require preservation of enzyme function. We were able to demonstrate that ischemia led to very little change in caspase 3 activation, but reperfusion lead to a large increase in cleaved caspase 3 and apoptosis. It is important to note that caspase inhibition does not prevent cell death, and caspases are involved in non-lethal intracellular signaling [170, 171]. While western blot is not a high throughput analysis method, it did allow us to characterize differential activation of apoptosis in IRI.

Western blot can also be utilized to further characterize apoptosis in our cardiac constructs by differential assessment of the cytosolic and mitochondrial fractions. For example, the proapoptotic protein BAX normally resides in the cytosol, but is directed to the mitochondrial membrane upon activation of apoptosis to assist in membrane permeabilization [172]. Mitochondrial membrane permeabilization also releases proapoptotic proteins, such as cytochrome c, which are directed into the cytosol to activate caspases [173]. Thus, translocation of proteins between the cytosolic and mitochondrial fractions can be assessed by western blot in future studies to further characterize apoptosis.

In addition to caspase activation, apoptosis can also be morphologically characterized by double strand DNA breaks [174]. This characterization is most often accomplished by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). However, TUNEL staining is also very non-specific, and we found in initial studies that TUNEL staining was inappropriate for our cardiac constructs. Cardiomyocytes that had died during the formation of the construct were retained within the hydrogel, and it was impossible to distinguish TUNEL staining of these cardiomyocytes from those injured during ischemia-reperfusion.

Further morphological characterization of apoptosis can be done through electron microscopy. Apoptosis is characterized by chromatin condensation, membrane blebbing with formation of apoptotic bodies, and nuclear fragmentation [175]. Electron microscopy is a low throughput process, and it should only be utilized for secondary characterization and not for primary assays.

Early apoptosis can also be assessed by detecting the exposure of phosphatidylserine to the outer cell membrane using Annexin V. Furthermore, early apoptosis can be distinguished from late apoptosis or necrosis by also staining with a cell impermeable dye, such as propidium iodide (PI). We would expect that ischemia-only constructs have low single-staining for Annexin V and moderate staining for double staining of Annexin V and PI due to primarily necrotic cell death. Reperfusion would lead to increased single staining of Annexin V and double staining for Annexin V and PI, demonstrating an increase in apoptotic and necrotic cell death respectively. Annexin V and propidium iodide staining is typically assessed on flow cytometry, but this proved to be difficult on our human tissue platform. The constructs required long dissociation times to isolate the single cells necessary for flow cytometry. Furthermore, the wash steps for staining caused loss of some of the dead cells for incomplete analysis of the total population. We would also expect lower forward scatter and higher side scatter measurements of cardiomyocytes from reperfused constructs, which would reflect decreased cell size and increased granularity associated with apoptosis upon reperfusion. Currently, flow cytometry analysis is not an ideal tool to assess cell death in our constructs after IRI due to technical limitations and low throughput.

INTRACELLULAR CALCIUM

One aspect of IRI that we did not characterize was the accumulation of intracellular calcium seen during reperfusion [176]. This can be assessed by utilizing a calcium dye. We initially tried to assess calcium overload and differences in calcium cycling using the FLUO-4 dye, but this caused changes in construct

behavior. Normoxic constructs displayed slowed beating, while the reperfused constructs, if beating, completely stopped and demonstrated no signal. These issues were possibly due to calcium chelation by the dye. Different calcium dyes may need to be experimented with, but future studies should explore the use of genetically encoded calcium indicator cell lines, such as the GCaMP6f hIPS cell line [177]. This would allow for minimal manipulation of the cardiac constructs during reperfusion to better assess calcium overload and defects in calcium propagation. Furthermore, mitochondrial calcium overload has been implicated in the pathophysiology of IRI [178, 179]. The rho-2 AM calcium indicator localizes to mitochondria and can be utilized to assess mitochondrial calcium overload. Calcium overload can be measured through a fluorescence plate reader, and defects in calcium propagation and construct contraction can be assessed through optical video analysis.

CALPAIN ACTIVITY

Intracellular calcium overload during reperfusion also leads to the activation of calpains, calcium-dependent proteases that target intracellular proteins. Inhibition of calpain activity has demonstrated a decrease in IRI and should be a target to be further explored in future experiments [180, 181]. Calpain activity can be assessed by introducing fluorescent or luminescent substrates to measure enzyme activity, but these assays require cell lysis in order to function. Calpain activity can alternatively be assessed by examining its translocation from the cytosol to the cell membrane and its targeting and cleavage of membrane-associated cytoskeletal proteins (such as α-fodrin) through western blot [25]. Furthermore, we can measure levels of the endogenous inhibitor of calpain activation, calpastatin. Calpastatin is normally associated with calpain to inhibit it, but reperfusion leads to degradation of calpastatin [25, 182]. Thus, increased calpain activity during reperfusion can also be indirectly measured through reduced calpastatin levels.

CONTRACTILE ANALYSIS

Analysis of cardiac construct contraction was not done in this thesis because most reperfused constructs did not regain spontaneous beating. Lowering the ischemic time in future experiments will allow more reperfused constructs to regain beating in order for us to analyze their contractile properties. Contracting cardiac constructs can be tracked through optical video analysis. Increased cell death is correlated with decreased beat frequency and fractional area change. Variations in the time interval between beats and fractional area change per beat can be indicative of increased arrhythmic events. We expect cardioprotective strategies to reduce cell death and arrhythmic events.

AUTOPHAGY

One aspect of IRI that was not explored in this thesis is autophagy. Autophagy helps in cell maintenance by processing damaged organelles into autophagosomes for transport to lysosomes for degradation, and it is activated under ischemic conditions to break down cellular components to maintain energy stores [183, 184]. Stimulation of autophagy has been found to be cardioprotective in IRI [32, 129, 185], and decreased autophagic flux or clearance of autophagosomes has been seen on reperfusion [186]. However, overactivation of autophagy leads to cell death [187]. The role of autophagy in IRI is still not fully understood, and it should be characterized for better understanding of IRI and as a cardioprotective strategy in future studies.

Autophagy can be characterized in a variety of manners. Electron microscopy can be utilized to directly assess the presence of autophagosomes, but it is a very costly and low throughput process. Alternatively, monodansylcadaverine (MDC) is a fluorescent dye for autophagosomes [188], and can be detected through flow cytometry, fluorescence microscopy, or a fluorescent plate reader. Staining of microtubule-

associated protein light chain 3 (LC3) has also been used to characterized autophagy. LC3 precursors are diffusely distributed in the cytosol. Activation of autophagy causes processing of LC3 into LC3-I and then LC3-II, where LC3-II associates with the autophagosomes and demonstrates a more punctate distribution. Fluorescence microscopy can assess the rearrangement of LC3, but it may not be possible to utilize in our model due to tissue thickness. Western blot can also be utilized to assess autophagy by analysis of LC3-I conversion to LC3-II and overall LC3-II levels. Higher LC3-II levels correspond with increased formation of autophagosomes, but LC3-II is also degraded once the autophagosome fuses with the lysosome. Thus, western blot analysis of LC3-II levels alone is unable to distinguish between increased autophagy and formation of autophagosomes or decreased autophagic degradation in lysosomes.

Measuring autophagic flux is important to demonstrate that the autophagy process reaches its endpoint. Autophagic flux can be demonstrated by introducing lysosome inhibitors and assessing LC3-II levels to determine that lysosome inhibition causes an increase in LC3-II levels [189]. Another method of assessing autophagic flux involves the addition of lentiviral vectors expressing red fluorescent protein (RFP) and green fluorescent protein (GFP) tagged LC3 (RFP-GFP-LC3). The presence of combined red and green fluorescence demonstrates the formation of autophagosomes, while the acidic pH of lysosomes quenches GFP fluorescence and only leaves a red signal [32]. This method is amenable for use in our platform and can allow for more rapid analysis of autophagy in the cardiac constructs.

SUMMARY

We differentially characterized cell death in our studies due to ischemia and reperfusion by utilizing intracellular enzyme release, cell activity, and mitochondrial membrane permeability. We sought to further characterize the changes caused by reperfusion by examining oxidative stress, intracellular pH, and apoptosis. There are other aspects of IRI that we did not characterize in this thesis, but that we wish

to assess and target for cardioprotective strategies in future studies. In particular, we want to decrease ischemic times to reduce injury and allow more constructs to regain beating after reperfusion. This will allow us to assess changes in construct contractile behavior due to cardioprotective therapies. We demonstrated that reperfusion leads to a significant increase in injury compared to ischemia alone, and reperfusion injury is an important target to improve overall viability after ischemic injury.

Failure to Reperfuse the Infarct Region

Cardiac constructs subjected to simulated ischemia for the full 9 hours (referred to as "*Isch (9hr)*") were compared to constructs subjected to simulated ischemia for 6 hours (referred to as "*Isch (6hr)*") and constructs reperfused after 6 hours (referred to as "*Rep*") (**Figure 11A**). The cardiac constructs subjected to 9 hours of simulated ischemia represented a failure to reperfuse the infarcted myocardium in a timely manner. Constructs subjected to 9 hours of simulated ischemia demonstrated a significant increase in cell death compared to those under only 6 hours of ischemia, confirming that increased ischemic time leads to increased cell death (**Figure 11B-C**). The difference between the two groups corresponds to the infarcted myocardium that is potentially salvaged by reperfusion therapy. When the constructs that were reperfused were compared to constructs that were not reperfused, the significance of reperfusion injury becomes apparent. Reperfused constructs demonstrated increased cell injury compared to non-reperfused constructs, which was significant on measurements of adenylate kinase release but not on lactate dehydrogenase release. Reperfused constructs exhibit much higher cellular injury than their counterparts exposed to the same ischemic time, but this is potentially preventable damage. Reducing IRI will be important in improving clinical outcomes after an acute MI.

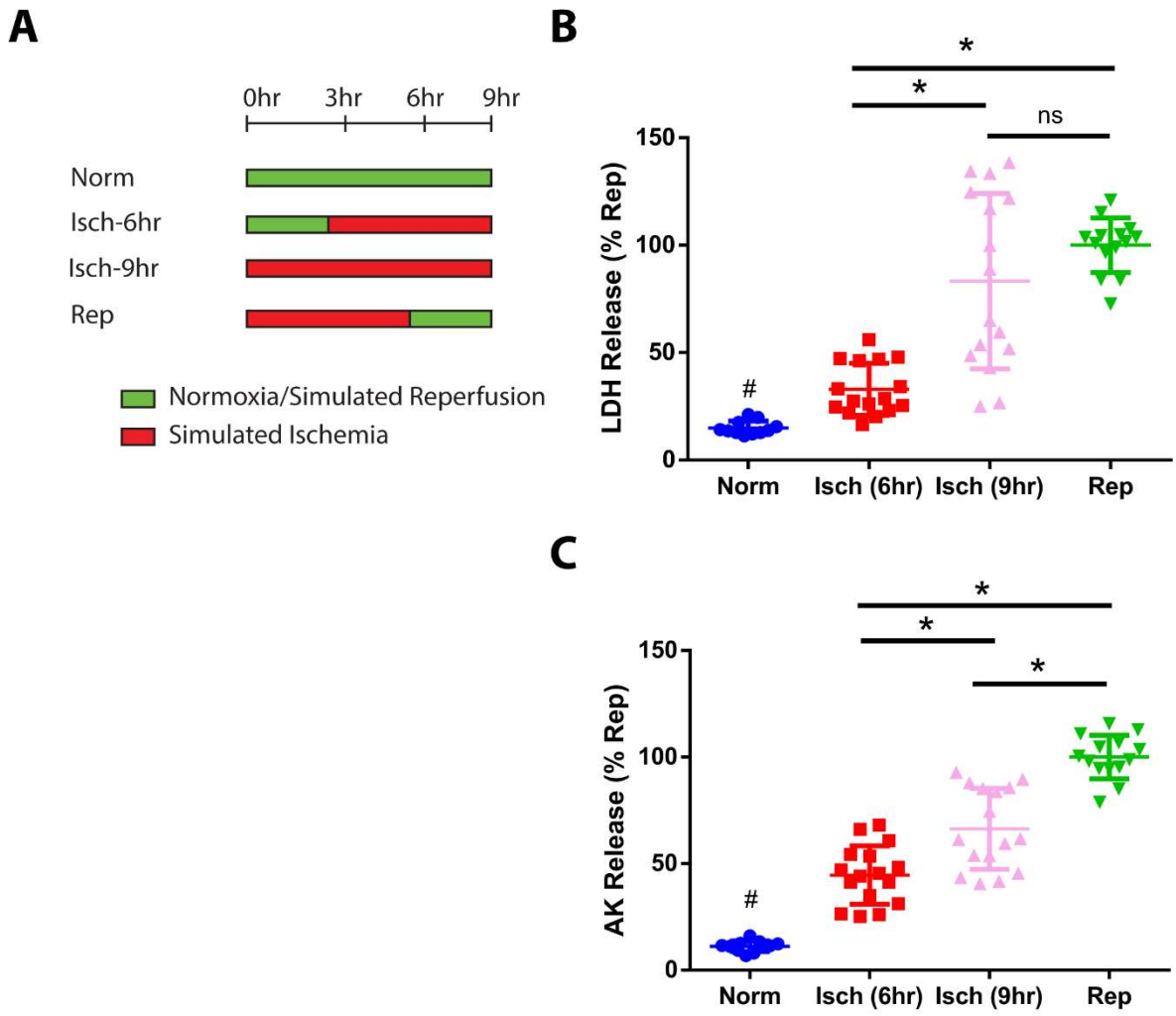


Figure 11: Cardiac constructs were subjected to 9 hours of simulated ischemia to model failure to reperfuse the infarct region. (A) Schematic of the experimental protocol for the comparison groups. (B-C) Lactate dehydrogenase (LDH) and adenylate kinase (AK) release were used to assess cell membrane permeability and death. Data represents aggregated results from 3 independent experiments, and are depicted as individual data points with mean ± SD. Statistical analysis was done using ANOVA with post-hoc Tukey's HSD, # indicates statistical significance compared to all other groups, * indicates significant difference between groups, $p < 0.05$

Discussion

In this study, we utilized our intracellular enzyme release assays to characterize cell death due to increased ischemic time. One aspect of these assays that we were concerned with was that unlike other groups tested in this thesis, the 6-hour ischemia-only group had a wash step 3 hours into the experiment to switch the constructs from normoxic conditions to simulated ischemia. The difference in intracellular enzyme release assays between the ischemia-only group and reperfused constructs could be due to this wash step that washed away any LDH or AK that was released during the first 3 hours of the experiment. To assuage these concerns, we demonstrated that ischemia-only constructs had a significant increase in intracellular enzyme release over normoxic constructs. This demonstrated that the assays were sensitive enough to detect an increase in ischemic cell death over normoxic controls despite the difference in culture time, and baseline readings would not significantly impact results. Furthermore, we compared our ischemia-only and reperfused constructs on other cell death assays and other methods to characterize reperfusion injury to confirm that we visualized a true difference in cell death on intracellular enzyme release assays.

The constructs subjected to 9 hours of simulated ischemia demonstrated an increase in LDH and AK release over 6 hours of ischemia, which further confirms the validity of utilizing intracellular enzyme release to measure cell death. An increase in ischemic time lead to a significant increase in measured cell death. Moreover, the 9-hour ischemia-only group demonstrated a difference in the intracellular enzyme release assays compared to the reperfused group despite the same culture time. This result further establishes that reperfusion leads to changes in behavior that cause injury over ischemia only.

In our future studies, we would like to characterize cell death due to ischemia and reperfusion injury as a percentage of overall cell death. This would allow us to move away from construct comparisons into a quantifiable standard of therapeutic efficacy. Our initial attempts to accomplish this relied on complete lysis of the cardiomyocytes for 100% LDH and AK release; however, we encountered many issues due to

variable penetration of the lysis reagent into the construct. It may be necessary to enzymatically dissociate the constructs to separate out the cardiomyocytes from the matrix components before lysing. We will also focus on making design modifications to the bioreactor to create a thinner construct to allow for more homogenous cell lysis.

Ischemic Preconditioning to Decrease IRI

We next sought to demonstrate the utility of the system by examining current strategies to prevent IRI and applying them to the engineered cardiac constructs. Currently, ischemic preconditioning is the only treatment known to robustly decrease IRI, and it has been demonstrated to be beneficial across various animal species [190]. Ischemic preconditioning involves subjecting the heart to brief periods of hypoxia and reperfusion that cause no cell injury prior to the actual ischemic insult.

Ischemic preconditioning has not unequivocally been demonstrated to decrease IRI in humans because it is very difficult to study in the clinical setting. Also, preconditioning must occur before the ischemic insult, and thus, cannot be studied in patients with acute MI. Small-scale studies on patients undergoing coronary artery bypass surgeries (CABG) have indicated that preconditioning decreases cardiac injury [191, 192]. Preconditioning has also been thought to be the mechanism behind reduced infarct size and better outcomes in patients with angina prior to their MI [193, 194]. In the laboratory setting, preconditioning has shown benefit in the limited models of quiescent primary human ventricular cardiomyocytes and isolated atrial trabeculae [195, 196]. An engineered human tissue model offers a better and more robust platform to test and examine ischemic preconditioning and the potential benefits of this treatment.

We simulated ischemic preconditioning by subjecting the constructs to 30 minutes of ischemia and 15 minutes of reperfusion, before the actual ischemia-reperfusion regimen of 6 hours of ischemia and 3 hours of simulated reperfusion (constructs referred to as "*PreC*") (**Figure 12A**). Preconditioned constructs demonstrated a decrease in cell death as measured by lactate dehydrogenase and adenylate kinase release (**Figure 12B-C**) and an increase in cell viability compared to the *Rep* group (**Figure 12D**). Furthermore, JC-1 staining demonstrated that preconditioning helped to partially prevent the increase in mitochondrial membrane permeability seen in the reperfused constructs (**Figure 12E**). Western blot

analysis also indicated that preconditioning helped to decrease caspase 3 cleavage and apoptosis, although the difference was not statistically significant (**Figure 13A**).

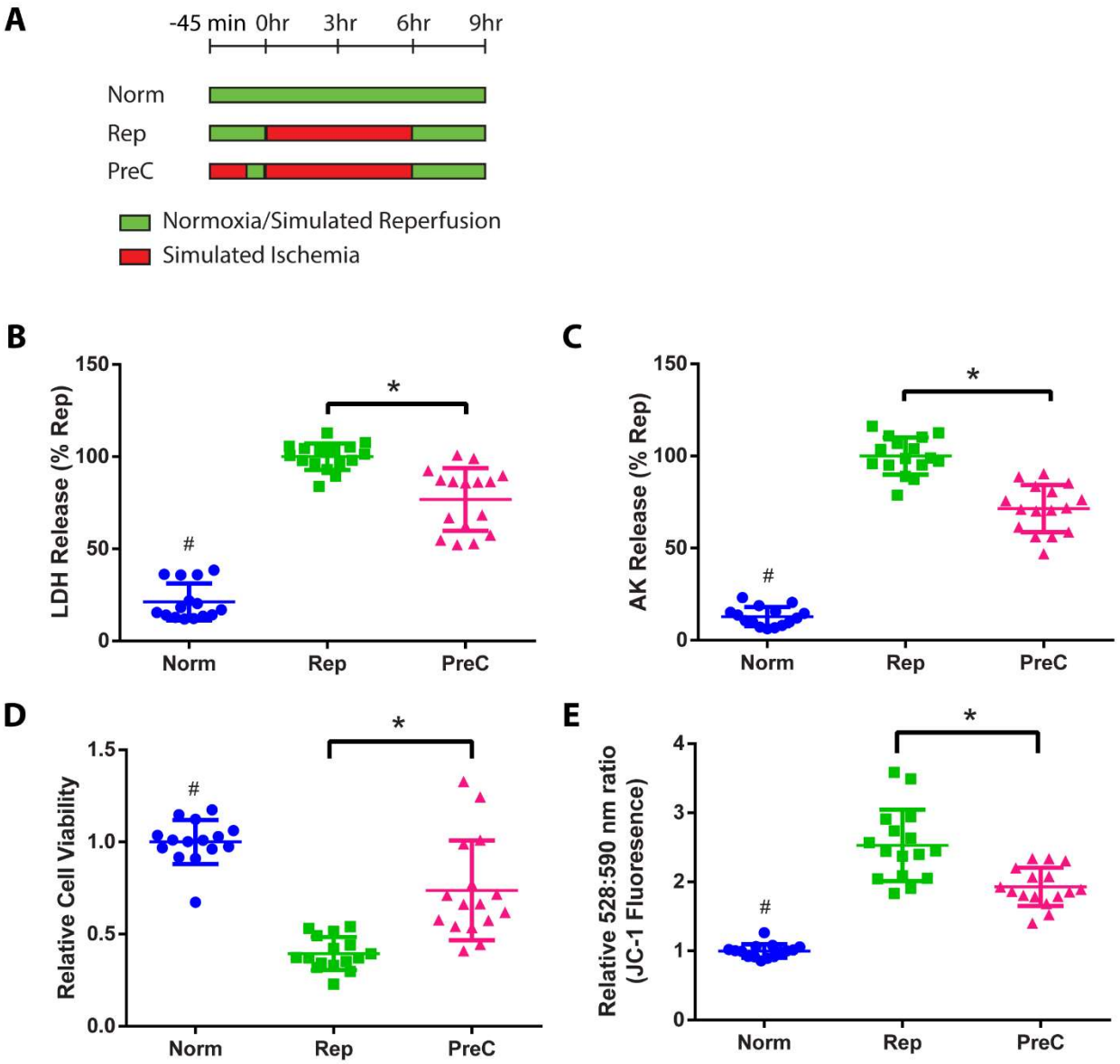


Figure 12: Ischemic preconditioning of cardiac constructs to decrease IRI (A) Schematic of the experimental protocol for comparison groups. Ischemic preconditioned constructs referred to as "*PreC*". (B-C) Lactate dehydrogenase (LDH) and adenylate kinase (AK) release were used to assess cell membrane permeability and death. (D) Cell viability was determined by measuring cell activity

using RealTime Glo assay. (E) Mitochondrial membrane permeability was determined by comparing emission of JC-1 dye at 528 nm and 590 nm, where increased ratio is correlated with higher permeability. Data represents aggregated results from 4 independent experiments and are depicted as individual data points with mean ± SD. Statistical analysis was done using ANOVA with post-hoc Tukey's HSD, # indicates statistical significance compared to all other groups, * indicates significant difference between groups, $p < 0.05$.

Ischemic preconditioning is cardioprotective through the activation of pro-survival kinases [197, 198]. Western blot analysis of the cardiac constructs determined that there was increased phosphorylation and activation of the cell survival factor Akt in the preconditioned constructs (**Figure 13B**). This difference was not statistically significant, but it does suggest that the activation of kinases in animals can also be seen in human tissue models. Reperfused constructs have lower levels of intact protein despite the addition of equal amounts of total protein into each well. This is consistent with the increased activation of caspase 3 and degradation of cardiac ultrastructure seen previously (**Figure 9**, **Figure 10**). Studies have identified several survival pathways that are activated by ischemic preconditioning [199], in studies done in animals. It would be important to identify the exact kinases and pathways in humans for clinical translation. For example, STAT3 activation and STAT5 attenuation was cardioprotective in mouse models [200], but the opposite was found in humans [201]. Deeper understanding of the exact composition of kinases that are activated for cardioprotection will be necessary in order to understand what the therapeutics need to target.

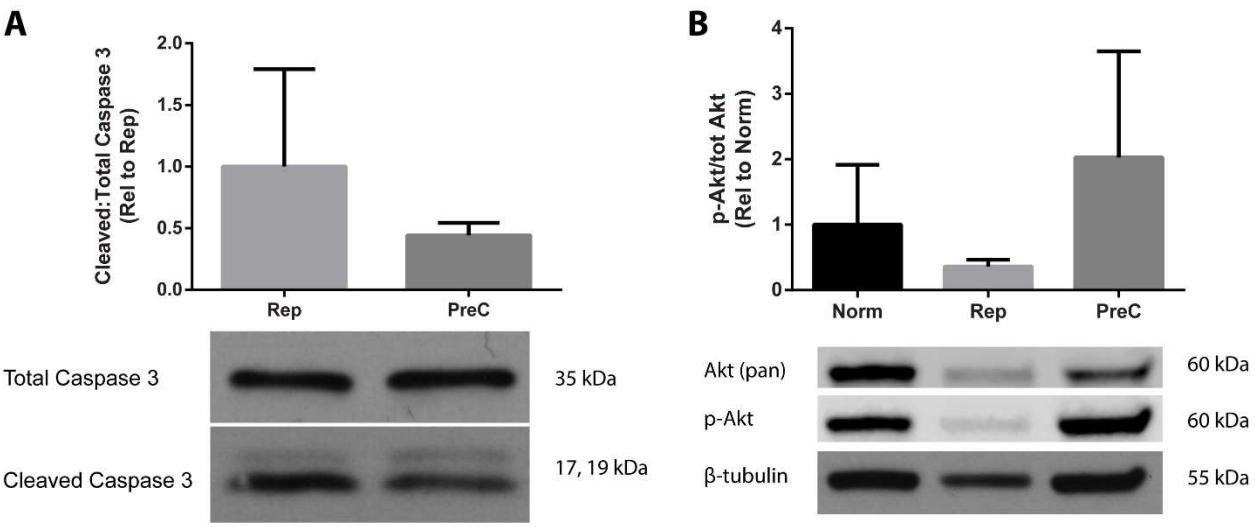


Figure 13: Western blot analysis of ischemic preconditioning in cardiac constructs. (A) Analysis of apoptosis by cleaved to total caspase 3 ratio. Results were not statistically significant by paired t-test. (B) Analysis of Akt and pro-survival kinase activation by phosphorylated to total Akt ratio. Data was not statistically significant by ANOVA. Data are from 3 independent experiments, mean ± SD.

Ischemic preconditioning reduced IRI in the engineered cardiac constructs, with a decrease in cell death and improvement in cell viability. We also saw that the preconditioned constructs displayed decreases in mitochondrial membrane permeability upon reperfusion, and these results are consistent with previous studies that demonstrated cardioprotection in ischemic preconditioning by prevention of MPTP opening [202]. Ischemic preconditioning is extremely difficult to study in humans. Overall, these results support the use of the engineered cardiac construct as a human model of ischemic preconditioning. We therefore utilized the platform to gain insights into the cardioprotective mechanisms of ischemic preconditioning.

While preconditioning is not a clinically relevant therapy in the setting of an acute MI, ischemic postconditioning and remote ischemic conditioning can potentially be. These methods activate similar

survival pathways as ischemic preconditioning to provide cardioprotection [41, 203, 204], but they have demonstrated mixed results in early clinical trials [15, 205, 206]. Larger clinical trials are needed to better understand their impact. One potential issue is that the clinical trials have had great heterogeneity in their conditioning regimens, and this is due to the undefined nature of ischemic conditioning. We hope to further use the platform to better define the important factors in conditioning regimens and turn these insights into a clinically relevant therapy.

Simulating Ischemic Preconditioning

Normally preconditioning involves multiple cycles of ischemia and reperfusion, and there is a great variance in ischemic conditioning regimens. We were concerned that the constant manipulation of the constructs with wash steps and handling outside of the incubator could be a potentially confounding factor in our comparisons; therefore, we limited preconditioning to one cycle of 30 minutes of ischemia followed by 15 minutes of reperfusion. One cycle of preconditioning has demonstrated cardioprotection in both *in vitro* and animal studies [44, 207, 208]. We utilized 30 minutes of ischemia in our preconditioning regimen to ensure that our stimulus reached a minimal threshold for cardioprotection [208], but we were concerned that the duration may be too long and lead to no change [209]. We did demonstrate decreases in cell death in our preconditioned constructs (**Figure 12**), but we do want to explore the importance of preconditioning duration and number of cycles in future studies.

Our current simulation of preconditioning involves subjecting the constructs to the same ischemic conditions as our actual ischemic insult. We did this to limit the number of variables in our experimental protocol, but it may be incorrect for physiological simulation of ischemic preconditioning. Ischemic preconditioning involves subjecting the heart to nonlethal cycles of ischemia and reperfusion, but our ischemic solution aimed to simulate the ischemic state and injury with lactate addition, high extracellular potassium, and an acidic extracellular environment in addition to substrate deprivation and hypoxia.

Lactate accumulation, high extracellular potassium, and extracellular acidification are unlikely to occur in the short ischemic time of preconditioning, and it may be more appropriate to simulate preconditioning with only substrate deprivation and hypoxia. It will be necessary to compare construct responses between these two protocols and determine if there are any changes. We want to study ischemic preconditioning in our constructs to identify the signaling changes that lead to cardioprotection and the targets for future cardioprotective therapies. Any differences in construct response to these two preconditioning protocols will help us in the effort to identify relevant stimuli.

Discussion

Preconditioning involves subjecting the myocardium to short bouts of ischemia and reperfusion that cause no damage, but this was not verified in our platform. One of the controls we need to add in the future is to confirm that the 30 minutes of ischemia and 15 minutes of reperfusion we utilized to simulate preconditioning did not cause cell death. We need to analyze constructs immediately after the preconditioning cycle and compare them to our normoxic controls on cell death assays. There is a concern that the decrease in cell death that was assessed by intracellular enzyme release and mitochondrial membrane permeability assays may be due to washing away the dead cells on the preconditioning regimen. The extra wash steps and manipulation of the preconditioned constructs could be leading to a false decrease in cell death compared to reperfused constructs. By assessing cell death immediately after the preconditioning regimen, we can determine if there is an issue with these assays. In our studies, we utilized measurements of cell activity to determine cell viability and supplement our other cell death assays. Cell activity assays are not impacted by washing away dead cells. The increased cell activity that was saw in the preconditioned constructs compared to reperfused constructs supports our result that ischemic preconditioning reduced IRI.

We further identified increased phosphorylation of Akt in our preconditioned constructs compared to reperfused constructs. This is consistent with animal and human studies which have identified activation of survival pathways as a mechanism behind cardioprotection by ischemic conditioning [41, 198, 210]. Akt activation is part of what has been termed the reperfusion injury survival kinase (RISK) pathway, and we should further assess the pathway and its importance in cardioprotection in our platform. To accomplish this, we first need to more fully characterize activation of the RISK pathway. We want to assess the activation of ERK1/2, which functions parallel to the PI3K-Akt activation, and the downstream GSK3β, which is the common target of PI3K-Akt and ERK1/2 activation. GSK3β is thought to be the end effector of the RISK pathway, where phosphorylation of GSK3β inhibits it and leads to inhibition of MPTP opening [211]. Characterization of the phosphorylation of these proteins will allow us to demonstrate activation of the RISK pathway in our preconditioned constructs.

After characterization, we want to modify the RISK pathway to assess its importance. Currently, it is unclear if GSK3β inhibition is required for cardioprotection by ischemic preconditioning. Rodent studies using transgenic GSK3β that cannot be inhibited found mixed results [212, 213], and furthermore, the RISK pathway has been found to be not necessary for cardioprotection by ischemic postconditioning in pigs [214]. The importance may be species dependent. To further characterize the RISK pathway, we want to introduce inhibitors and activators of PI3K-Akt, ERK1/2, and GSK3β. There are many inhibitors of PI3K that target and inhibit downstream activation of Akt; however, the commonly used wortmannin and LY294002 inhibit all classes of PI3K. We want to use specific inhibitors of class I PI3K to target Akt activation. Furthermore, we can also directly inhibit or activate Akt itself to assess its effects. The use of ERK1/2 inhibitors in conjunction with inhibition of PI3K-Akt will allow us to fully inhibit the RISK pathway and also assess relative importance of these two parallel pathways. Furthermore, direct inhibition of GSK3β along with inhibitors of MPTP opening will allow us to assess the importance of GSK3β and the RISK pathway in cardioprotection by ischemic preconditioning in humans.

Independently of the RISK pathway, activation of the survivor activating factor enhancement (SAFE) pathway has been demonstrated to be a target of ischemic preconditioning. The SAFE pathway involves signaling through TNFα receptor 2, janus kinase (JAK), and signal transducer and activator of transcription (STAT) to ultimately target the mitochondria and prevent MPTP opening [215]. We need to characterize if activation of this pathway is prevalent in our preconditioned constructs. Furthermore, it has been found that while activation and phosphorylation of STAT3 drives the SAFE pathway in rodents and other animals [200], it is STAT5 activation that is relevant in humans [201]. These differences between species further emphasize the need for a human tissue platform to explore the mechanisms driving ischemic preconditioning. After characterization of STAT activation, we would like to further test the importance of the SAFE pathway through inhibition of the upstream JAK or direct inhibition of STAT activity in our preconditioned constructs. We want to use these studies to establish the importance of the RISK and SAFE pathways in ischemic preconditioning to identify the relevant pathways for cardioprotection.

Responses of Immature Cardiac Construct to Simulated Ischemia-Reperfusion

We next sought to establish the importance of the bioreactor in providing a platform to mature the hiPS-CMs by examining the responses of relatively immature cardiac constructs to simulated ischemia and reperfusion. Immature cardiac constructs were tested at three days after seeding (compared to the normal two weeks after seeding), when the constructs first started to demonstrate spontaneous contractions.

The immature constructs were subjected to the same regimen of 6 hours simulated ischemia and 3 hours simulated reperfusion. The responses of the immature reperfused constructs were compared to immature preconditioned and normoxic (control) cardiac constructs. Cell membrane integrity and death were measured by analyzing lactate dehydrogenase (LDH) and adenylate kinase (AK) release (**Figure 14A-B**). Reperfused constructs demonstrated no difference compared to normoxic controls on LDH release. They did have a significant increase on AK release, but overall, the differences were much smaller than those seen in more mature constructs. Preconditioned constructs had a significantly lower LDH and AK release compared to *Rep*. Cell viability and mitochondrial membrane permeability measurements demonstrated no difference between the three groups (**Figure 14C-D**). Overall, immature cardiac constructs did not demonstrate the comprehensive responses of increased cell death, decreased cell viability, and increased mitochondrial membrane permeability to ischemia-reperfusion that were observed in their more mature counterparts (**Figure 8**).

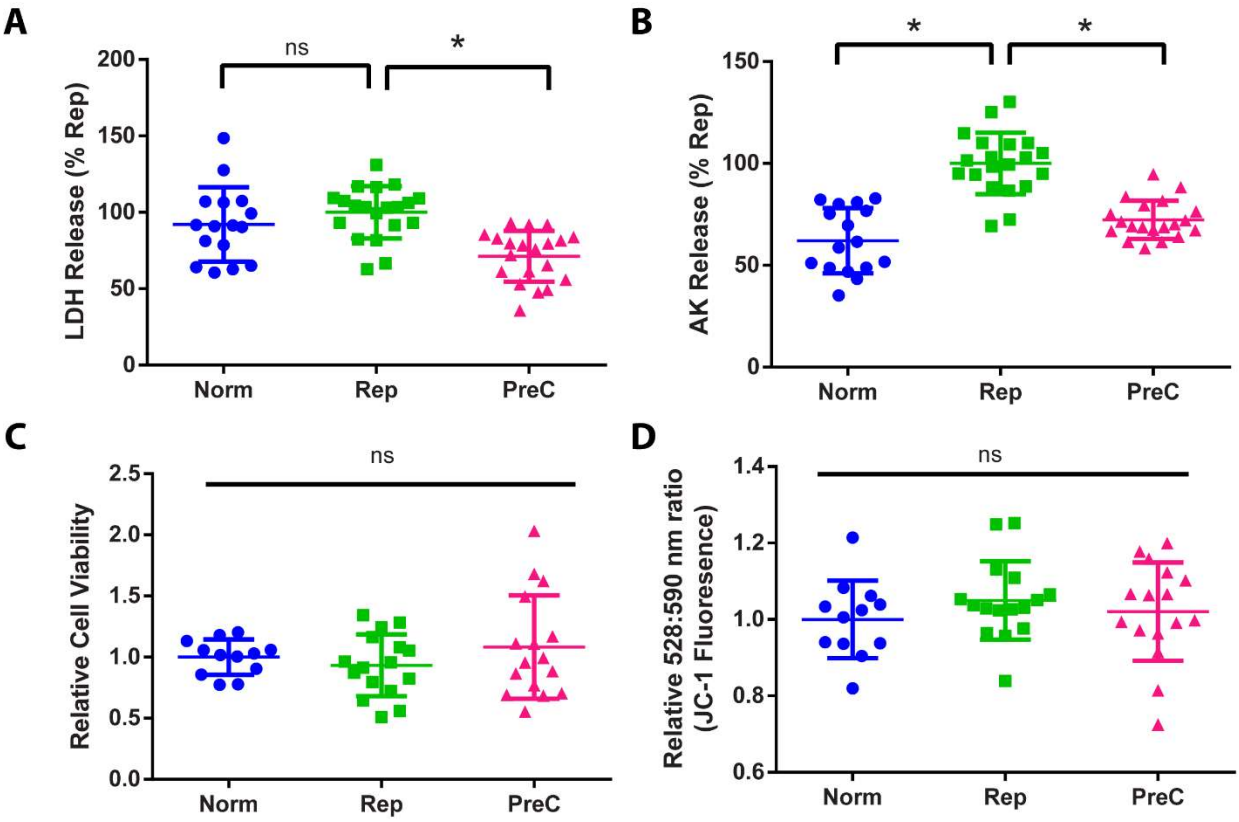


Figure 14: Response of immature cardiac constructs to simulated ischemia-reperfusion. (A-B) Lactate dehydrogenase (LDH) and adenylate kinase (AK) release were used to assess cell membrane permeability and death. (C) Cell viability was determined by measuring cell activity using RealTime Glo assay. (D) Mitochondrial membrane permeability was determined by comparing emission of JC-1 dye at 528 nm and 590 nm, where the increased ratio correlates with higher permeability. Data represents aggregated results from 3 independent experiments and are depicted as individual data points with mean ± SD. Statistical analysis was done using ANOVA with post-hoc Tukey's HSD, * indicates significant difference between groups, $p < 0.05$.

The relative resistance of the fetal heart to hypoxia is recapitulated by the lack of response to IRI in the immature reperfused constructs [49]. These results help to establish the necessity of the bioreactors to promote maturation of the hiPS-CMs and increase their susceptibility to ischemic injury. Ischemic

preconditioning was studied in immature cardiac constructs because it has previously been demonstrated that preconditioning does not protect the neonatal heart [57]. The preconditioned constructs did demonstrate a decrease in LDH and AK release compared to reperfusion, but the overall lack of difference between reperfusion and normoxic constructs makes it difficult to draw insights about preconditioning in the immature constructs. The tolerance to ischemic injury seen in the immature constructs offers a platform to further explore the mechanisms that increase susceptibility to hypoxia in the mature heart. Furthermore, study into the mechanisms of cardiomyocyte maturation that lead to the development of cardioprotective measures during ischemic preconditioning may be insightful. It has been demonstrated that the diseased myocardium leads to a more fetal phenotype of cardiomyocytes [216, 217], which makes understanding the response of immature cardiomyocytes to IRI important.

Discussion

Relatively immature constructs were subjected to simulated ischemia-reperfusion after 3 days of culture, when they had just started to display spontaneous contractions. We saw that the reperfused relatively immature constructs did not demonstrate the same increase in cell injury compared to normoxic controls as displayed by our relatively more mature constructs. We hypothesized that this difference in response is due to the relative resistance to hypoxia that has been characterized in more immature cardiomyocytes [49]. We would like to further characterize these differences by direct comparisons of relatively immature and mature constructs and testing of constructs in between the two time points. Direct comparisons can be easily performed through characterization of gene expression in qPCR and protein levels in western blot. These studies can be done in an asynchronous manner to assess the changes and developments in cardiomyocyte maturity that may be leading to susceptibility to ischemic injury. We also would want to test a construct at a midpoint time point to establish its response to ischemia-reperfusion and characterize

its relative maturity compared to the 3 day and 2-week-old constructs. This would allow us to further assess the changes in construct properties that lead to increased ischemic injury.

The lack of increased cell death in the relatively immature constructs may be because the cells are less metabolically active. They may have less energy demands and could thus better withstand ischemic stress. This is why we chose an early time point where the constructs had already started spontaneous contractions and were metabolically active. We need to directly compare metabolic activity of the relatively immature to more mature cardiac constructs through measurements of cell activity to determine if this is a cause. We could possibly determine if decreased cell activity is a cause of decreased cell death by comparing constructs subjected to electrical stimulation and blebbistatin under simulated ischemia-reperfusion. Electrical stimulation would force the cardiomyocytes to contract, while blebbistatin would inhibit contraction and reduce energy demands. However, we would first have to determine if electrical stimulation or blebbistatin causes an increase in cell death in normoxic constructs.

Furthermore, it is known that fetal cardiomyocytes are more reliant on glycolysis for metabolism, while the mature adult heart primarily relies on fatty acid oxidation [115, 116]. Oxidative phosphorylation is more efficient at producing ATP than glycolysis; therefore, decreased metabolism may underly hypoxia resistance in immature constructs. It has also been demonstrated that cardiac tissue engineering platforms can help increase oxidative metabolism [75, 88]. We need to assess changes in cardiomyocyte metabolism. We can assess cellular metabolism by dissociating the cardiomyocytes from the constructs and measuring their energetics in the Seahorse platform [75]. Oxygen consumption rate and extracellular acidification rate can be measured to determine mitochondrial respiration and glycolysis in the constructs. We can also assess metabolism by using ^{14}C-labeled energy substrates [88].

Furthermore, we want to determine if the reliance on fatty acid oxidation for cellular metabolism in mature cardiomyocytes underlies susceptibility to ischemia. It has been shown that fatty acid oxidation is

far less efficient than glucose oxidation in the use of oxygen for ATP production [218]. To test this, we would culture the constructs normally, but for a few days prior to the experiment, we would culture the constructs in either media containing glucose-only or fatty acid-only as the energy substrate. This would limit any differences at baseline, but then condition the constructs to a fatty acid rich environment. It was demonstrated that cardiac constructs cultured under fatty acid-only containing media demonstrated no change in contractile behavior after one day [88]. However, these experiments were done under 40% O_2 culture conditions, and we will need to assess how long our constructs can tolerate fatty acid-only media. We would subject the groups to the same simulated ischemia-reperfusion conditions conducted in these studies. The thought is that culture under fatty acid-only conditions would make the cardiomyocytes more dependent on oxygen due to increased oxygen consumption. We would expect that constructs cultured under fatty acid-only conditions prior to IRI would demonstrate an increase in cell death due to prior culture and reliance on a more oxygen-consuming energy substrate. To control for glycolysis, we can test constructs under lactate-only conditions. Lactate oxidation should be as oxygen efficient as glucose oxidation without the ATP production from glycolysis.

One issue in direct comparisons of the constructs as they develop over time is that it is difficult to directly compare the constructs at different time points. In our experiments, we only compared constructs seeded from the same batch of hiPS-CMs to minimize any initial differences. This made it difficult to directly compare immature with relatively more mature constructs. One way that we could alter our experimental setup to overcome these possible technical limitations is to remove the aspects of the bioreactor that assist in cardiomyocyte maturation. Mechanical conditioning by culture on the flexible pillars helps to promote maturation of the cardiac constructs. We can remove mechanical conditioning by cutting the flexible pillars so that they no longer provide passive tension after construct formation. In this manner, we can directly compare the same batch of constructs and further test the importance of mechanical conditioning for maturation in our bioreactor. Furthermore, when we reintroduce electrical stimulation

into our cardiac culture protocol, we can compare constructs that were cultured under chronic electrical stimulation to those that were not. Electromechanical stimulation can be utilized to promote maturation of hiPS-CMs, and removal of these conditioning regimens can allow us to more directly compare relatively immature and mature construct responses.

Aim 3: Establish and Evaluate Therapeutic Strategies for Reducing IRI During Reperfusion

Ischemic preconditioning of the cardiac constructs decreased IRI, but it is not a clinically relevant therapy because it must be applied prior to the ischemic insult. We sought to test therapeutic strategies that can be applied during reperfusion. These strategies entailed either modifying reperfusion conditions or addition of drugs during reperfusion to target the pathophysiology of IRI previously discussed.

Reperfusion with Acidic Media to Prevent Rapid Normalization of Intracellular pH

Our first therapeutic target was the rapid normalization of intracellular pH during reperfusion. As previously discussed, ischemia leads to a decrease in intracellular pH. Reperfusion rapidly restores normal intracellular pH, which leads to intracellular calcium overload and cell death. Previous studies have demonstrated a reduction of infarct size and IRI in animals using pharmacological inhibition of the sodium-hydrogen exchanger (NHE) [219, 220]. However, clinical studies have failed to demonstrate improved outcomes using NHE inhibitors [22, 221]. In this study, we instead aimed to leverage the enhanced control of the *in vitro* platform and modify reperfusion conditions in a non-physiologic fashion to reduce IRI. We sought to prevent the rapid normalization of intracellular pH and subsequent reperfusion injury by reperfusion with acidic media to decrease the pH gradient.

Normally, simulated reperfusion by culture media with pH of 7.4 leads to a large proton gradient between the extracellular and intracellular space (referred to as "*Rep (pH 7.4)*"), but reperfusion with a media pH of 6.4 serves to minimize this gradient and prevent intracellular calcium overload (referred to as "*Rep (pH 6.4)*") (**Figure 15A**). Phrodo staining confirmed a lower intracellular pH in tissue constructs after simulated ischemia (**Figure 8G**). Reperfusion with acidic media demonstrated a decrease in cell death, as assessed

by lactate dehydrogenase and adenylate kinase release compared to *Rep (pH 7.4)* (**Figure 15B**-**C**). However, the *rep (pH 6.4)* group did not demonstrate improvements in cell viability as measured by cell activity (**Figure 15D**). The *rep (pH 6.4)* group did have a significant decrease in mitochondrial membrane permeability compared to the *rep (pH 7.4)* group, thought it was still significantly higher than the normoxic constructs (**Figure 15E**). Western blot also determined that reperfusion with acidic media lead to a significant decrease in the cleavage and activation of caspase 3 compared to *rep* constructs (**Figure 16**).

Overall, reperfusion with acidic media decreased IRI-related cell death, partially prevented mitochondrial membrane permeabilization, and resulted in decreased apoptosis. However, reperfusion with acidic media did not improve cell viability. This was most likely due to acidic media inhibiting normal cell activity. Reperfusion with acidic media has previously demonstrated a reduction in IRI in rat cardiomyocytes and isolated hearts [140, 222], and we have shown that it is cardioprotective in humans too. These results demonstrate that we can decrease IRI in the human tissue model by modifying the reperfusion conditions. The results also indicate that inhibition of intracellular pH normalization remains a viable therapeutic target.

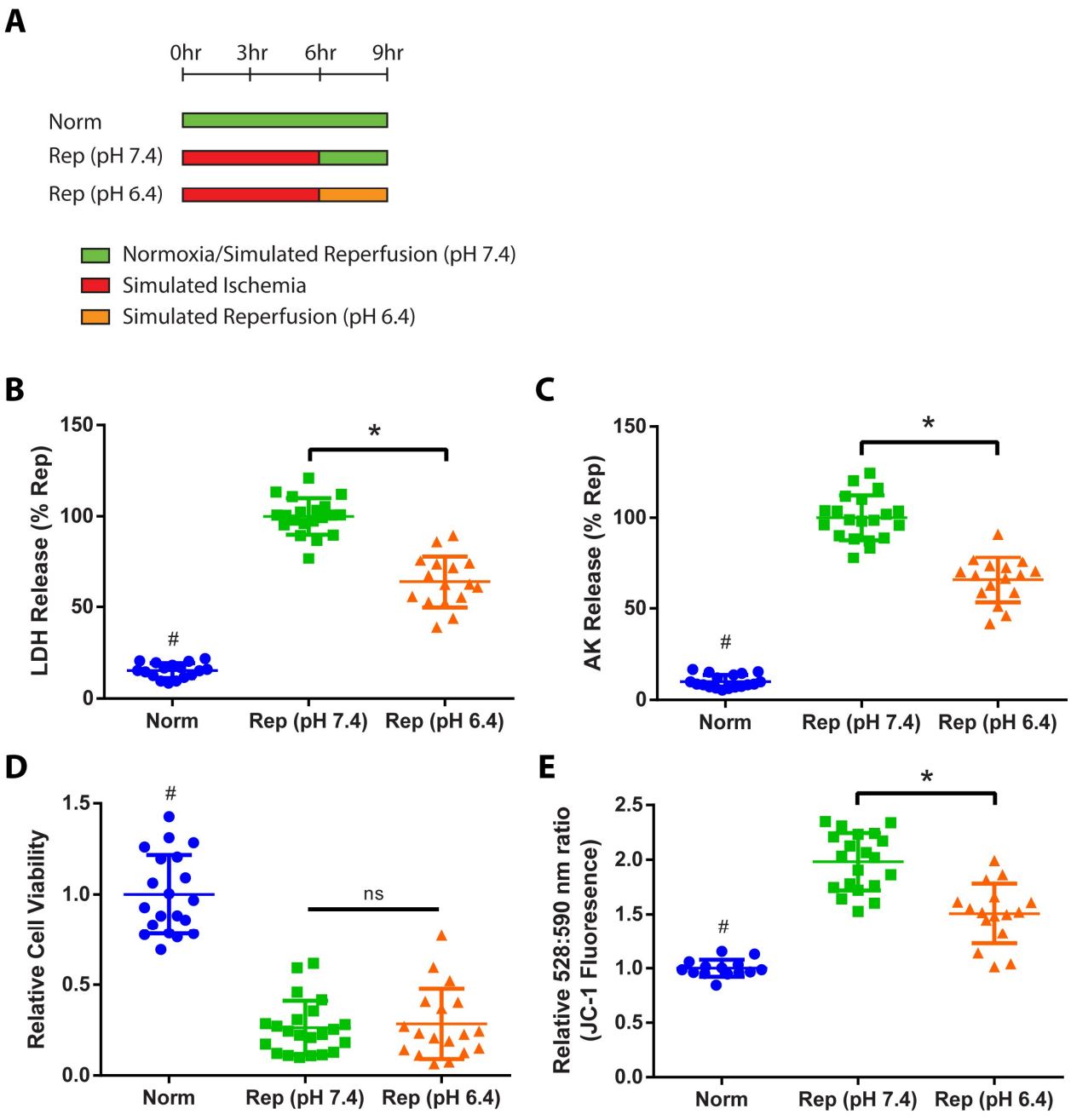


Figure 15: Reperfusion with acidic media *(Rep pH 6.4)* was compared to reperfusion using neutral pH media *(Rep pH 7.4)* to decrease reperfusion injury. (A) Schematic of the experimental protocol for the comparison groups. (B-C) Lactate dehydrogenase (LDH) and adenylate kinase (AK) release were used to assess cell membrane permeability and death. (D) Cell viability was determined by measuring cell activity using RealTime Glo assay. (E) Mitochondrial membrane

permeability was determined by comparing emission of JC-1 dye at 528 nm and 590 nm, where the increased ratio correlates with higher permeability. Data represent aggregated results from 4 independent experiments and are depicted as individual data points with mean ± SD. Statistical analysis was done using ANOVA with post-hoc Tukey's HSD, # indicates statistical significance compared to all other groups, * indicates significant difference between groups, $p < 0.05$.

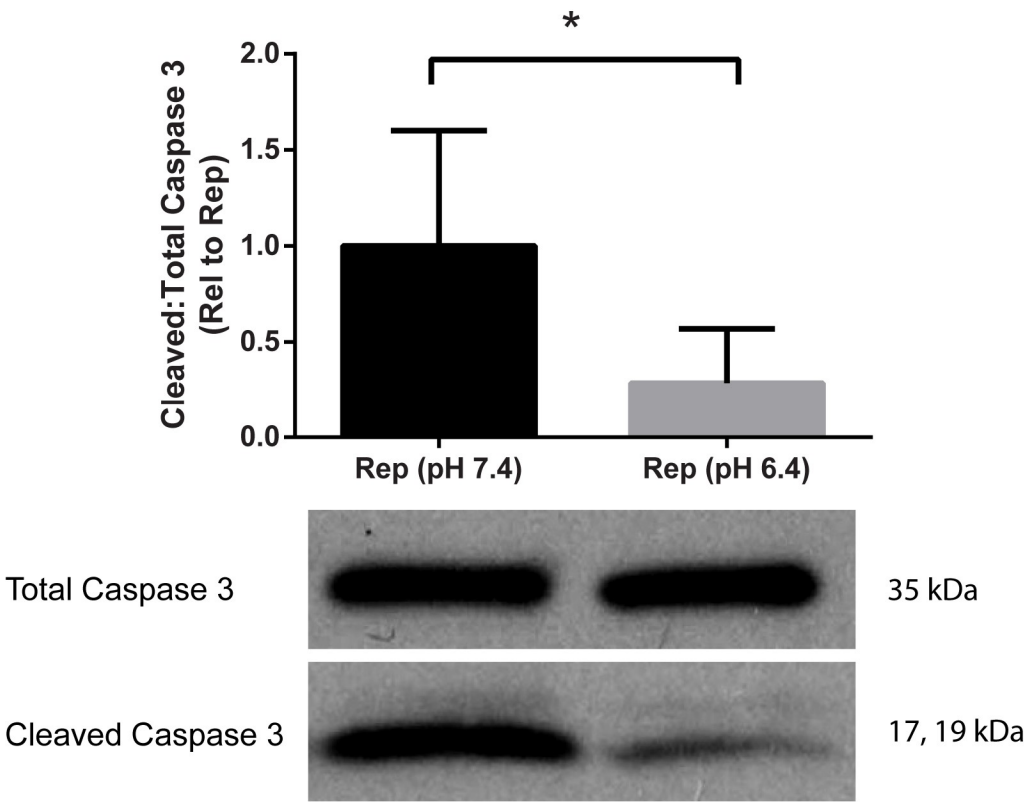


Figure 16: Western blot analysis of cardiac constructs reperfused with acidic media. Decrease in apoptosis was determined by cleaved to total caspase 3 ratio. Data is from 3 independent experiments, mean ± SD. * indicates significant difference between groups as determined by paired t-test, $p < 0.05$

Discussion

Reperfusion with acidic media demonstrated a decrease in intracellular enzyme release, but this result could potentially be erroneous. The intracellular enzyme release assays rely on measurements of enzyme activity by introducing substrates, but enzyme activity can be affected by pH. While the construct extracellular pH gradually normalized to a more neutral pH during the reperfusion time period, the extracellular pH still remained lower than that of reperfused constructs. We did not characterize how the enzyme activity changes over different pH, or the final pH at the end of the reperfusion period. These assays could be further improved by assessing if LDH or AK activity changes at the pH our experiments were performed at. We could have also normalized the extracellular pH by adding a base prior to measuring enzyme activity. We could also utilize ELISA as a direct measurement of intracellular protein release and cell membrane permeabilization. In our study, we utilized mitochondrial membrane permeability as a measurement of cell death that was not affected by extracellular pH differences. We saw that reperfusion with acidic media lead to a decrease in mitochondrial membrane permeability, which helps to validate that we saw a decrease in cell death by reperfusion with acidic media.

We can further assess cardioprotection by inhibition of rapid intracellular pH normalization through further modification of reperfusion conditions. The intracellular acid extruders are the sodium hydrogen exchanger (NHE), sodium bicarbonate cotransporter (NBC), and monocarboxylate transporter/lactate-proton cotransporter (MCT) [223]. The NHE is the primary acid extruder at low pH [224], but NBC is a significant contributor. The acidity of the media should inhibit NHE activity, but we can further modify our acidic reperfusion media to target the NBC by increasing bicarbonate concentration or by using bicarbonate-free HEPES-buffered media. Increasing bicarbonate concentration should increase acid extrusion and further exacerbate the rapid intracellular pH normalization and cell death. On the other hand, reperfusion with bicarbonate-free acidic media should further reduce cell death compared to normal acidic media. These results would further demonstrate the importance of inhibition of rapid

intracellular pH normalization for IRI reduction. The failure of NHE inhibitors to reduce IRI in clinical studies may be due to NBC activity [22, 221], and we would like to examine NHE inhibitors in conjunction with NBC inhibition through modified reperfusion conditions or pharmacological inhibitors in future studies.

Pharmacologic Testing

To further assess the utility of the engineered cardiac constructs as *in vitro* models of IRI, we explored the use of pharmacologic agents that could be added during reperfusion to decrease IRI. We previously identified important targets to treat IRI in our discussion about the pathophysiology of IRI.

Targeting the MPTP

Rationale

During ischemia, the mitochondrial permeability transition pore (MPTP), a large and nonspecific pore in the mitochondrial membrane, is closed, resulting in low permeability of the inner mitochondrial membrane [225]. Reperfusion causes opening of the MPTP and depolarization of the mitochondria, and it is a critical step in reperfusion injury [119]. Opening of the MPTP leads to uncoupling of the electron transport chain and depletion of ATP. It also allows the entry of water and solutes into the mitochondria. This leads to rupture of the mitochondria, release of its contents into the cell, and cell death. Cyclophilin D (CypD) is a regulator of the MPTP, and it has been found that knockout of CypD in animals is cardioprotective [29]. Cyclosporine A (CsA) can regulate CypD and decrease IRI by inhibiting opening of the MPTP.

Experimental Results

We assessed the efficacy of cyclosporine A to reduce IRI in the engineered cardiac constructs (**Figure 17A**). It was shown that the addition of cyclosporine A (1 μM) during reperfusion (constructs referred to as "*CsA*") was able to reduce cell death as shown by lactate dehydrogenase and adenylate kinase release and increased cell viability (**Figure 17B-D**). CsA-treated constructs also had a decrease in mitochondrial membrane permeability compared to reperfused constructs (**Figure 17E**). These results indicate that cyclosporine A can decrease IRI in the human tissue model. Cyclosporine A can prevent IRI by inhibiting

opening of the MPTP, and importantly, it was seen that cyclosporine A addition during reperfusion helped to decrease mitochondrial membrane permeability.

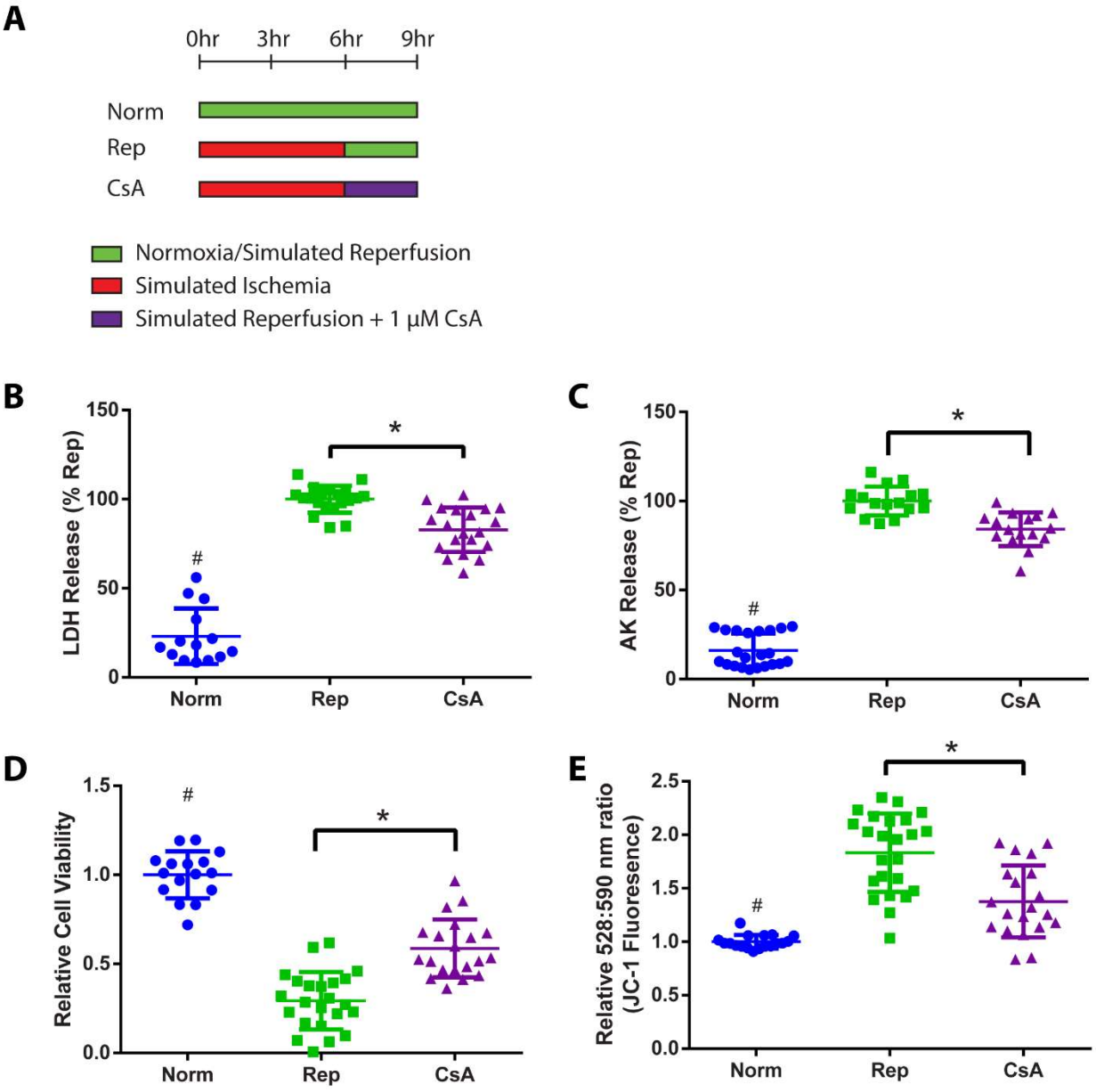


Figure 17: Evaluating the efficacy of cyclosporine A (CsA, 1 µM) in reducing reperfusion injury by targeting the MPTP. (A) Schematic detailing experimental protocol for the comparison groups. (B-C) Lactate dehydrogenase (LDH) and adenylate kinase (AK) release were used to assess cell membrane permeability and death. (D) Cell viability was determined by measuring cell activity

using RealTime Glo assay. (E) Mitochondrial membrane permeability was determined by comparing emission of JC-1 dye at 528 nm and 590 nm, where increased ratio is correlated with higher permeability. Data represents aggregated results from 4 independent experiments and are depicted as individual data points with mean ± SD. Statistical analysis was done using ANOVA with post-hoc Tukey's HSD, # indicates statistical significance compared to all other groups, * indicates significant difference between groups, $p < 0.05$.

Comparison to preclinical and clinical studies

Cyclosporine A has been demonstrated to be effective in both *in vivo* and *in vitro* models of IRI [78, 226], but there are also some preclinical studies that demonstrated no benefit [227, 228]. Early clinical trials indicated that its use during reperfusion could decrease infarct size and pathological ventricular remodeling [229, 230]. Cyclosporine A also helped reduce cardiac damage during coronary artery bypass graft (CABG) and aortic valve surgery [231, 232]. However, two recent large clinical trials, CIRCUS and CYCLE, showed that cyclosporine A administration upon reperfusion was ineffective in reducing IRI [1, 30]. Researchers have criticized the differences in formulations of cyclosporine A, greater use of cardioprotective platelet inhibitors, inappropriate use of clinical endpoints, and different selection of patients compared to the earlier clinical trial as the potential reasons for different results [4, 233]. Importantly, cyclosporine A was administered in patients up to 6-12 hours after the MI in the large clinical trials, compared to 4 hours in the early trial, which could be past the therapeutic window to salvage the ischemic myocardium. This suggests that more work still needs to be done in assessing cyclosporine A as a therapeutic. The early studies in this human *in vitro* model indicate that cyclosporine A may be effective in reducing IRI in cardiomyocytes, but it is possible that other factors in the clinical setting could be mitigating its effect.

Discussion

We saw that addition of CsA to reperfusion was able to demonstrate a decrease in in cell death and mitochondrial membrane permeability compared to reperfused constructs. CsA was dissolved in DMSO, and one of the controls we failed to add was a DMSO-only control. DMSO can be cytotoxic to cells, but the final concentration of 0.1% that we used in our study is generally regarded to be safe for cells. Furthermore, we were assessing cytoprotective effects of CsA, which is opposite of the cytotoxic effects of DMSO that are generally recognized. However, we do recognize that a vehicle-only control should be included in future studies in case of any extraneous effects. In addition, we also need to include CsA addition to normoxic constructs as a future control. This control will allow us to assess if CsA affects the constructs at baseline. While we do not anticipate these controls to affect our results, they should be included in future experiments to more fully verify the effect of CsA administration during reperfusion.

In addition, we would like to better assess the interaction of CsA with the MPTP in future studies. CsA is also an immunosuppressive drug, and it functions through inhibition of calcineurin. We would like to separate out its immunosuppressive effects by assessing response of constructs to tacrolimus, a calcineurin inhibitor with no effect on the MPTP. The addition of tacrolimus on ischemia-reperfusion has demonstrated no effect in animal studies [234], and we would like to also confirm this in our human tissue model. Furthermore, the drug auranofin has been shown to induce opening of the MPTP [235], and its addition during reperfusion should further increase reperfusion injury. CsA addition should help prevent cell death by auranofin by blocking mitochondrial membrane permeabilization.

CsA inhibits opening of the MPTP through regulation of cyclophilin D [236], and we would like to create a cyclophilin D knockout hiPS line. This would allow us to first independently verify the importance of cyclophilin D, where it has previously been demonstrated that cyclophilin D knockout in mice protected the heart against IRI [29]. In addition, CsA addition should have no effect on IRI in the cyclophilin D

knockout cell line. Furthermore, we will test the effects of NIM811, a CsA derivative that maintains inhibition of the MPTP without any of CsA immunosuppressive effects [237]. Demonstrating the effect of NIM811 in reducing IRI in our cardiac constructs and a neutral result in cyclophilin D cell lines will further confirm the interaction of CsA and the MPTP.

Targeting Oxidative Stress

Rationale

Reperfusion leads to a rapid increase in the generation of reactive oxygen species (ROS) due to reactivation of the electron transport chain [238]. The large amount of ROS damages cellular membranes and proteins and induces opening of the MPTP to contribute to lethal reperfusion injury [155, 239]. The generation of ROS also helps recruit neutrophils to the infarct region, but it is unclear if neutrophils play a pathological role in the progression of IRI [240]. It is thought that using antioxidants to target oxidative stress can help reduce reperfusion injury.

Experimental Results

The antioxidant, N-acetyl-L-cysteine (NAC, 5 mM), was added during reperfusion to test if it could reduce IRI (constructs referred to as "*NAC*") (**Figure 18A**). In the tissue engineered cardiac constructs, the administration of NAC on reperfusion significantly decreased ROS levels compared to normoxic and reperfused constructs, which confirmed its antioxidant properties (**Figure 18B**). However, NAC-treated constructs demonstrated no difference compared to reperfused constructs in measurements of cell death (**Figure 18C-D**). They also demonstrated no significant differences in cell viability or mitochondrial membrane permeabilization (**Figure 18E-F**). These results indicated that NAC can reduce ROS levels in the constructs, but it remained ineffective as a treatment for IRI in this *in vitro* model.

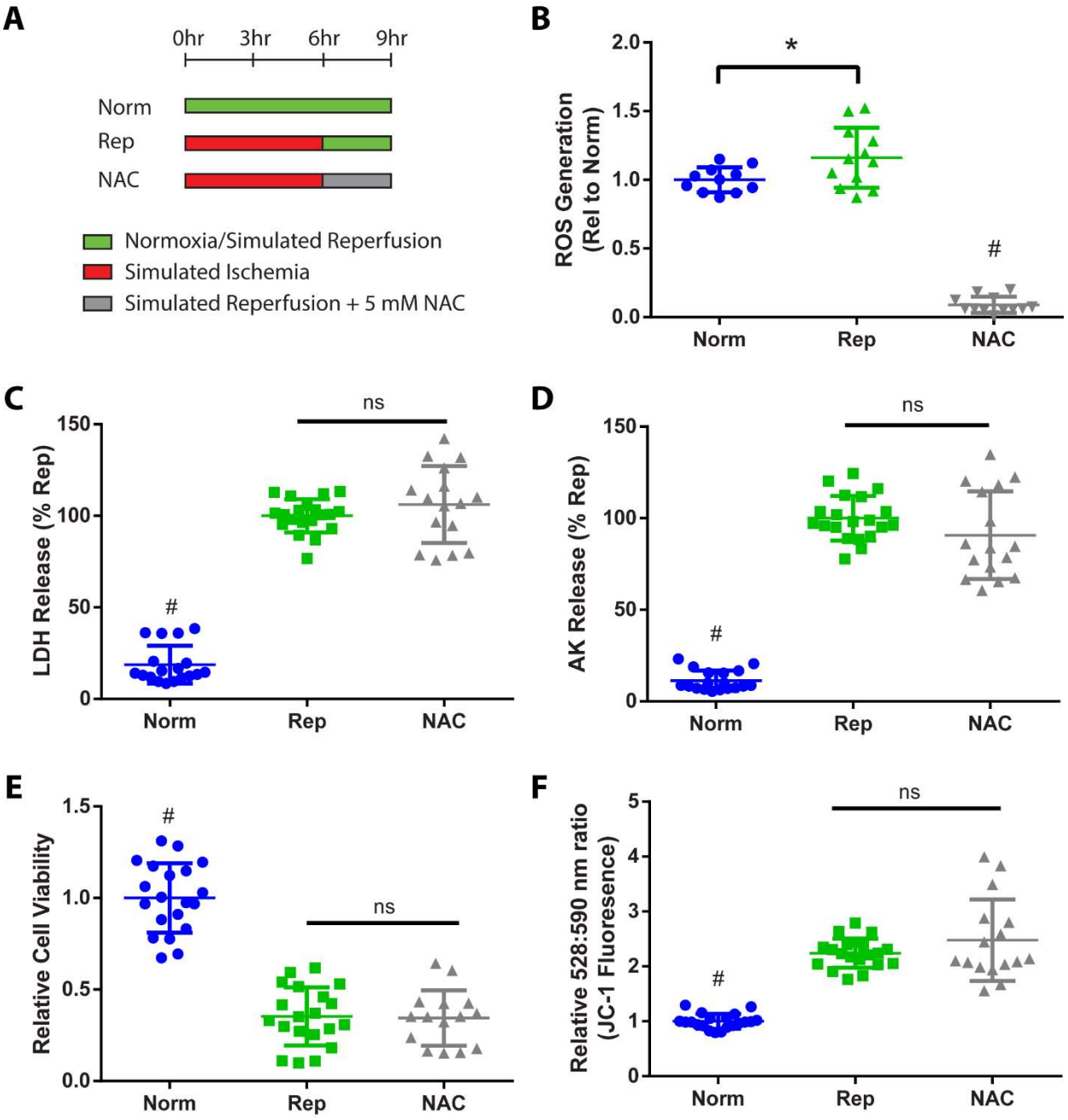


Figure 18: Evaluating the efficacy of N-acetylcysteine (NAC, 5 mM) as an antioxidant to prevent oxidative damage during reperfusion. (A) Schematic of the experimental protocol for the comparison groups. (B) Reactive oxygen species (ROS) levels in cardiac constructs as measured by ROS-Glo assay. Data represents aggregated results from 3 independent experiments. (C-D) Lactate dehydrogenase (LDH) and adenylate kinase (AK) release were used to assess cell

membrane permeability and death. (E) Cell viability was determined by measuring cell activity using RealTime Glo assay. (F) Mitochondrial membrane permeability was determined by comparing emission of JC-1 dye at 528 nm and 590 nm, where increased ratio is correlated with higher permeability. Data represents aggregated results from 4 independent experiments and are depicted as individual data points with mean ± SD. Statistical analysis was done using ANOVA with post-hoc Tukey's HSD, # indicates statistical significance compared to all other groups, * indicates significant difference between groups, $p < 0.05$.

Comparison to preclinical and clinical studies

Previous preclinical studies in dogs demonstrated mixed results for NAC. One study indicated that administration of NAC during reperfusion could decrease infarct size [241]. On the other hand, another study demonstrated no difference in infarct size, but that NAC could reduce myocardial stunning [242]. A small clinical trial, ISLAND, indicated that NAC could increase ejection fraction and decrease infarct size alongside streptokinase treatment [243]. However, there has not been any testing of NAC in a large clinical trial to conclusively demonstrate its efficacy.

NAC failed to reduce IRI in cardiac constructs despite the promise shown in the ISLAND clinical trial; however, the results from the human *in vitro* model are consistent with those of other antioxidants. The antioxidants superoxide dismutase and trimetazidine have not demonstrated clinical benefit in their respective clinical trials [244, 245]. Furthermore, it was seen that NAC can increase mortality in multisystem organ failure when administrated late in critically ill patients [246]. These results coupled with negative clinical trials for other antioxidants may have tempered the enthusiasm for NAC as a therapy for reperfusion injury and prevented its progression to a large clinical trial. Antioxidant therapy against IRI still demonstrates mixed results, indicating that it may not be suitable as a pharmacologic agent. More

recently, the specific targeting of mitochondrial ROS generation has become of interest [247]. Early results have indicated that these therapies can reduce IRI and need to be further explored [248].

Generation of reactive oxygen species and oxidative stress during reperfusion is a major mechanism in causing IRI. Here, we used a general antioxidant, N-acetyl-l-cysteine, to mitigate oxidative stress. We demonstrated that NAC does decrease overall ROS levels in the construct, but despite this reduction, NAC did not seem to inhibit cell death or improve cell viability. It also had no effect on mitochondria membrane potential and depolarization.

Discussion

The lack of benefit from the use of the antioxidant NAC was not unexpected due to mixed results seen in preclinical and clinical studies [242, 243]. Signaling through ROS is an important part of normal cell function [249], and we should have included NAC addition to normoxic constructs to establish any changes to baseline function. This would help us establish if NAC disrupts normal physiological function, which could abrogate any of its potential antioxidant benefits during reperfusion.

NAC helps to reduce oxidative stress as a ROS scavenger by replenishing endogenous glutathione. Further developments in antioxidant therapy may instead want to focus specifically on mitochondrial ROS generation. The large generation of ROS seen in reperfusion is thought to be driven by reactivation of the electron transport chain [166]. Superoxide is produced due to electron leak and partial reduction of oxygen at complexes I and III as a part of normal physiology [250]. Superoxide then quickly disproportionates into hydrogen peroxide. Damage of these complexes due to ischemia leads to a greater generation of ROS during reperfusion [251]. The burst of ROS generation can lead to opening of the MPTP, which leads to further generation of ROS and injury in a positive feedback process termed ROS-induced ROS release [225, 252, 253]. It is possible that NAC is unable to reduce oxidative stress in the mitochondria,

and we will want to specifically examine the effect of NAC on mitochondrial ROS levels with the MitoSOX or MitoB dyes. Furthermore, we will want to focus on mitochondrial targeted antioxidant agents. There are mitochondrial ROS scavengers that have been developed, such as MitoVit-E and MitoQ10 [254, 255], and we should explore whether they are able to target and reduce injury at the site of oxidative stress. Furthermore, we can use rotenone and atovaquone to directly inhibit complex I and III respectively to prevent ROS generation during reperfusion. Targeting reperfusion oxidative stress at the source of ROS generation may prove to be a more effective therapeutic strategy.

Discussion

Cardiac Tissue Engineering Platform to Study IRI

This is the first characterization of ischemia-reperfusion injury in a human tissue model. The model utilized hIPS-CMs seeded in hydrogel around flexible pillars that provided passive tension and allowed for auxotonic contractions for mechanical conditioning. hiPS-CMs are a robust source of human cardiomyocytes and can improve models by providing a patient-specific background. However, because immature cardiomyocytes are more resistant to ischemic injury, hiPS-CMs are limited in studies of IRI because of their relatively fetal phenotype. Cardiac tissue engineering has demonstrated enhanced maturation of hiPS-CMs.

The cardiac constructs in these studies demonstrated aligned and striated cardiomyocytes along the periphery. However, the cardiomyocytes were not homogenously distributed throughout the construct, and did not demonstrate some of the structural hallmarks of maturity such as transverse tubule formation. This indicates that additional bioreactor design changes may be necessary to achieve a mature structure. The cardiac constructs did demonstrate functional maturation. The constructs had positive chronotropic and ionotropic response to β-adrenergic stimulation. Positive ionotropic responses to β-adrenergic stimulation are not seen in monolayers because of their relative immaturity and is considered a sign of cardiomyocyte maturation [109]. The presence of aligned and cross-striated cardiomyocytes along the edge of the construct and a functionally mature response to β-adrenergic stimulation allowed us to utilize the platform to study ischemia-reperfusion injury.

Establishing Human Tissue Engineered Model of IRI

We were able to establish the presence of ischemia-reperfusion injury by comparing constructs exposed to ischemia only and constructs exposed to ischemia followed by reperfusion. There was an increase in cell death and decrease in cell viability after ischemia, and these changes were further accelerated by reperfusion. Critically, we saw a significant increase in mitochondria membrane permeability after reperfusion, which correlates with opening of the MPTP and cell death in the pathophysiology of IRI. Many efforts to treat IRI have focused on the opening of the MPTP as a critical therapeutic target. Reperfusion also led to the acceleration of apoptosis with increased caspase 3 cleavage and activation compared to the minimal levels in the normoxic and ischemic constructs. These results established differences in the processes leading to injury in ischemia and reperfusion, and we also established assays that can detect these changes.

We also examined the response of relatively immature constructs to simulated ischemia-reperfusion. These constructs had just started to display spontaneous contractions 3 days after formation, and they had not had the time to mature in the bioreactor. We saw that these constructs were resistant to ischemic injury. The immature constructs did not demonstrate significant differences in our assays compared to normoxic constructs and helps to establish the importance of mechanical conditioning and maturation in developing a model of IRI.

Ischemic Preconditioning Reduced IRI in the Human Tissue Engineered Model

Ischemic preconditioning has demonstrated robust and strong cardioprotection in various animal species [190], but study in humans has been difficult. Because ischemic preconditioning must occur before the ischemic insult, it cannot be tested in patients with an acute MI. Studies in patients undergoing CABG surgeries and other limited *in vitro* models have indicated cardioprotection by ischemic preconditioning

[191, 192]. In this study, we demonstrated that ischemic preconditioning helped to decrease IRI in the human tissue model. The preconditioned constructs had a decrease in cell injury, increase in cell viability, and decrease in MPTP opening.

Similar to what has been shown in animals, the preconditioned constructs had increased activation of pro-survival kinases. This indicates that ischemic preconditioning functions similarly in humans through the activation of cell survival pathways to protect the cardiomyocytes during reperfusion. Further identification of the specific activated proteins will be important because they differ between humans and animals. The more clinically relevant ischemic postconditioning and remote ischemic conditioning function through similar activation of pro-survival pathways [41, 203], and further use of this human tissue model for study of ischemic conditioning can help bring greater insights into preventing IRI.

IRI can be reduced by modification of reperfusion conditions

Ischemic preconditioning of the cardiac constructs helped to decrease IRI, but because it must occur before the ischemic insult, it is not a clinically relevant treatment. Instead, we sought to prevent IRI by modifying the reperfusion conditions. The rapid normalization of intracellular pH has been targeted in studies through the pharmacologic inhibition of the NHE to varying degrees of success [22, 219, 220]. In our study, we instead leveraged the increased control offered in an *in vitro* model and sought to prevent pH normalization by reperfusion with acidic media to decrease the pH gradient. We saw a decrease in cell death, MPTP opening, and apoptosis. While there was no difference in cell viability, this was most likely due to decreased cell activity in the acidic media. Reperfusion with acidic media demonstrated that we could decrease IRI by modifying the cellular environment during reperfusion.

One of the ideal uses of the proposed model of IRI would be for pharmacological screening of therapeutics that can be added during reperfusion to decrease IRI. Many drugs have demonstrated a decrease in infarct

size in animals, but none have shown benefit in a large clinical trial. We sought to decrease IRI by using cyclosporine A (CsA) to prevent MPTP opening and N-acetyl-l-cysteine (NAC) to reduce oxidative stress. CsA addition during reperfusion demonstrated a decrease in IRI, and critically, it decreased the mitochondria membrane permeability. This indicated that CsA was able reduce reperfusion injury by preventing opening of the MPTP. CsA usage in the CYCLE and CIRCUS clinical trials did not demonstrate better clinical outcomes [30, 256], but the trials have also been criticized for utilizing a different formulation of CsA compared to the earlier clinical trial and using cardioprotective platelet inhibitors in the patients [233]. The encouraging results of CsA in the human tissue model indicate that clinical issues may be confounding the cardioprotective effect of the drug. On the other hand, the antioxidant NAC failed to reduce IRI in the cardiac constructs despite the importance of oxidative stress in causing reperfusion injury [155, 239]. Antioxidants have shown mixed results in preclinical studies and small clinical trials [243–245]. More recent efforts have focused on specifically targeting mitochondrial ROS generation. We aim to continue using the human tissue model as a platform for pharmacologic testing of therapeutics against IRI.

Limitations

Overall, it is difficult to determine the clinical predictivity of the cardiac constructs for pharmacological screening. CsA helped to decrease IRI in the cardiac construct and in other preclinical studies, but it has not been effective in patients. This difficulty is further compounded by the fact that no therapeutic has robustly demonstrated improved clinical outcomes in patients. Ischemic preconditioning has demonstrated the most robust cardioprotection against IRI [190], but even its effect in an acute MI in humans has not been fully verified.

In this study, we demonstrated comparable results to animal models with cardioprotection by ischemic preconditioning, reperfusion with acidic media, and CsA administration. However, the human tissue engineered model has significant advantages over animal models for clinical translation. The *in vitro* model offers enhanced control of the various variables in IRI, study on a biomimetic platform of human physiology, and furthermore, hiPS-CMs offer the opportunity to study the response in patient-specific backgrounds. The human tissue engineered model of IRI presented in this study offers much potential in gaining insights into the pathophysiology of IRI, its treatments, and how we can translate therapies into the clinical setting.

Issues with Current Preclinical Ischemia-Reperfusion Injury Models

In vitro models are useful tools to capture the direct effect of treatments or drugs on cardiomyocytes, while isolating out the confounding factors that are encountered in animal models. *In vivo* animal models are important in their physiological simulation of ischemia-reperfusion and their ability to capture the interactions between various cell types. However, these preclinical models still fail to fully simulate the clinical setting. Future models will need to address these shortcomings, and a tissue engineered cardiac construct may be most suitable to account for them in preclinical studies of IRI (**Figure 19**).

Accounting for Comorbidities

A common criticism levied on preclinical IRI studies is that therapies are tested on a homogenous, young, and healthy set of small animals. In contrast, myocardial infarction primarily affects a diverse and older population with many comorbidities, such as diabetes and cardiac hypertrophy. These factors alter the cardiomyocyte response to IRI in different ways, usually be increasing their susceptibility to reperfusion

injury and decreasing the effectiveness of treatments (e.g. ischemic preconditioning) against it [207, 257, 258].

In recent years, the researchers have begun demonstrating that hiPS-CMs can recapitulate the key features of chronic diseases. Drawnel et al. established a diabetic hiPS-CMs screening platform using cells exposed to a prodiabetic environment and hiPS cell lines derived from diabetic patients [141]. The diabetic patient-specific hIPS-CMs were not exposed to any diabetes-specific environmental signals, and yet, they demonstrated a diabetic phenotype and the corresponding responses to drugs. These findings indicate that the hIPS-CMs recapitulating the disease phenotype should be a part of patient-specific diabetic models. A diabetic phenotype was also demonstrated in an engineered heart tissue model, utilizing neonatal rat cardiomyocytes [259]. In addition, increased diabetic susceptibility to IRI has been demonstrated in an animal model [260], but not *in vitro* using human cells or tissues.

Tissue engineering could also be instrumental for modeling comorbidities *in vitro*. In some models, engineered heart tissue was suspended between two attachment sites to allow for mechanical stress and auxotonic contractions [261]. Hirt et al. was able to expand upon this concept by inserting metal braces to increase the afterload sensed by the cardiac tissue. Consequently, the cardiomyocytes developed a pathologic cardiac hypertrophic phenotype. The results were validated against a hypertrophy-promoting pharmacologic conditioning protocol [262]. In summary, there is increasing evidence that tissue engineering enables various approaches to model comorbidities of interest, and could thus provide a more biomimetic model of IRI.

Concomitant Drugs

It has been found that many of the medications taken to treat the comorbid conditions found in patients can alter the effects of IRI and could be cardioprotective themselves. For example, statins are

cardioprotective, but also interfere with the cardioprotective effects of ischemic preconditioning [263]. Some of the anesthetics used during PCI to reperfuse the artery are also cardioprotective, while others are not [264, 265]. Further complicating the matter, it has been found that comorbid conditions, such as diabetes, can interfere with the cardioprotective effect of anesthetics and statins [266, 267]. These interactions are not being systematically tested and identified in preclinical animal models, and may play a role in the mixed results from clinical trials of promising drugs. In fact, the concomitant drug may be providing cardioprotection through the same pathway as the therapeutic of interest, or it may be actively hindering the therapy.

The cardioprotective features of anesthetics have been demonstrated on isolated human atrial trabeculae *ex vivo* [268]. *In vitro* models would be ideal for performing these studies in a high-throughput manner. In particular, tissue engineered cardiac constructs derived from patient-specific hIPS-CMs will be of value in identifying the drug-drug interactions. As the patient population grows older and requires more drugs for treating multiple comorbidities, such screenings will become even more important. Reductionist models could be very helpful for understanding how different drugs and conditions interact, in order to avoid false negative results.

Modeling Microvascular Obstruction

In addition to the direct effects of reperfusion on the cardiomyocytes, a long-lasting source of damage is microvascular obstruction [18, 269, 270]. In the heart, microvascular obstruction is the failure of coronary artery reperfusion to fully reperfuse the underlying myocardial microvasculature. This is an independent risk factor that correlates with worsened clinical outcomes [271]. Reperfusion causes the influx of leukocytes and platelets, endothelial damage, and vasoconstriction, which all contribute to microvascular obstruction [272–274]. These phenomena could be studied *in vitro* in a heart muscle engineered to contain microvasculature. There have been many advances in engineering vascularized cardiac tissue for

implantation [82, 84, 275], and the existing platforms could potentially be further developed to examine microvascular obstruction in a tissue model of IRI. With a functional microvasculature and surrounding pericytes, it would be possible to study *in vitro* the endothelial damage and vasoconstriction associated with microvascular obstruction. Tissue engineered constructs with a functional microvasculature can help better recapitulate the clinical setting of IRI on a highly manipulatable platform.

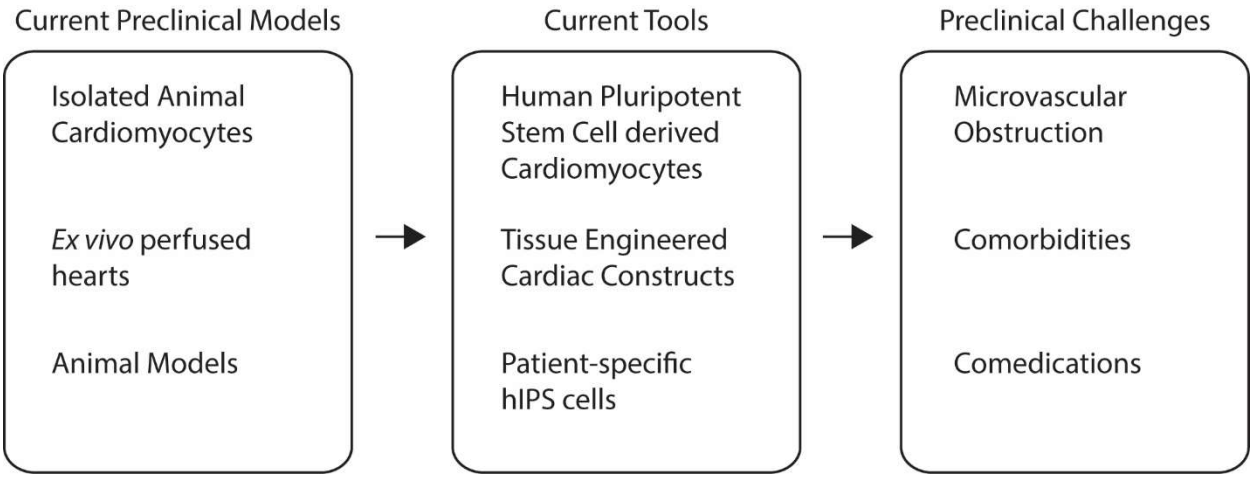


Figure 19: **Improving preclinical studies of ischemia-reperfusion injury**. Current studies primarily rely on animal models, but also include the use of isolated animal cardiomyocytes and perfused hearts. Current tools at our disposal include hiPS-CMs and tissue engineering techniques to mature them, and these tools were the focus of this thesis. Patient-specific hIPS cells have also been developed to more accurately model a particular patient phenotype. Future studies will need to incorporate comorbidities and medications that patients have to better assess clinical efficacy. Microvascular obstruction is also an important source of reperfusion injury that also needs to be accounted for.

Future Directions

Improvements to the model

Part of the next steps in this research will involve some alterations to the platform design to improve cardiomyocyte development and platform usability for future studies. A current issue with the design is that the construct thickness made it difficult to utilize assays that relied on optical visualization of the cardiomyocytes within and made for an inhomogeneous distribution of cardiomyocytes throughout due to diffusion limitations. We want to focus on forming a thinner construct by decreasing the height of the construct formation well and diameter of the flexible pillars. This would also allow us to use fewer cardiomyocytes per construct. A prototype version of the thinner construct platform mold has already been constructed, but it was not used due to time constraints. It involved very few changes to the manufacturing process for the mold besides utilizing a very small diameter end mill for the flexible pillars. We think that these changes will improve the use of the platform as a model for IRI.

The oxygen and nutrient diffusion limit can also be overcome by culturing the constructs on a rocker. This method is superior to perfusion systems for simultaneous analysis of multiple platforms because it does not require setting up pumps and tubing. Furthermore, this method eliminates potential sources of contamination due to tubing connections. There is a concern that the shear stress from media movement may negatively impact cardiomyocyte development. However, Jackman et al. demonstrated that culture on a rocker, termed “dynamic culture”, improved the distribution and maturation of cardiomyocytes in the engineered cardiac construct through improved nutrient delivery compared to static culture [107]. Our platform can accommodate culture on a rocker with its two large spaces for media on each side of the construct formation well, and we briefly explored its use in initial experiments. However, we discontinued its use to troubleshoot some other aspects of construct formation. Dynamic culture can be reintroduced back to the construct culture process if the thinner construct platform does not achieve a

homogenous cardiomyocyte distribution. Furthermore, we can utilize the change from dynamic culture under normoxia to static culture under hypoxia to further simulate loss of blood flow and nutrient availability during ischemia.

Electrical stimulation of engineered cardiac constructs has also demonstrated that it can help mature hiPS-CMs [73, 75], and it should be utilized in future studies. The platform incorporates carbon rods that can be utilized for electrical stimulation of the constructs. However, we had to remove electrical stimulation from our platform after initial studies indicated that it caused cell death. Multiple cardiac tissue engineering platforms have successfully utilized electrical stimulation in their culture protocols [73, 75], and it will require some troubleshooting to understand what design changes led to issues in our platform. Reincorporation of electrical stimulation will also allow for better simulation of ischemic conditions by forcing the cardiomyocytes to contract during ischemia. This will prevent cardiomyocytes from going into hibernation to reduce energy requirements, and this will better simulate the *in vivo* environment where the heart still needs to meet contractile demands.

Preconditioning Threshold for Cardioprotection

There is a great variance in preconditioning regimens in the field, and no standard has been developed as of yet. There have been a few studies that have compared ischemic conditioning regimens in animals by varying the number of cycles and duration of ischemia [208, 209, 276, 277]. They suggest that conditioning regimens have to cross a threshold of duration or number of cycles to provide a stimulus for cardioprotection, but additional duration or cycles after that do not provide additional cardioprotection. Furthermore, extending the ischemic duration too much may lead to a loss of protection.

We want to compare preconditioning regimens in our platform to determine the signaling transduction changes that are required to meet the minimum threshold for cardioprotection. To accomplish, we will examine the number of ischemic preconditioning cycles required to achieve cardioprotection. It will be technically simpler to compare number of cycles compared to ischemia duration due to our method of inducing hypoxia by flushing anoxic gas in a hypoxic chamber. In our experiments, we utilized one cycle of 30 minutes of ischemia and 15 minutes of reperfusion for preconditioning. This indicates that we will need to significantly reduce the ischemia duration for each cycle in order to obtain a conditioning regimen that does not demonstrate cardioprotection. We will measure cell death to determine when the threshold for ischemic preconditioning is met.

We want to assess changes in Akt activation on Western blot to determine if the threshold is met due to activation of cell survival proteins. That is, we do not expect a gradual increase in Akt activation with increasing preconditioning stimulus, but rather, a significant increase when the threshold is met. Furthermore, we want to assess changes in the other proteins of the RISK pathway, such as ERK1/2 and GSK3β, to confirm our results. By characterizing the differences when the threshold is met, we can begin to assess when the ischemic preconditioning stimulus should be cardioprotective. Similarly, if we are able to characterize these changes in protein activation, we will want to utilize serial dilutions of PI3K-Akt pharmacologic activators to demonstrate that we can recapitulate the presence of a threshold for preconditioning. We will then want to assess how changes to the construct baseline, such as maturity, chronic medications, comorbidities, change the threshold for activation to better characterize cardiomyocyte response.

More clinically relevant model

Our goal is to create a human tissue platform that is predictive of the clinical setting. As part of this goal, we need to identify the influence of the patient background on IRI in the cardiac constructs. Patient comorbidities and comedications have been found to have an effect on myocardial susceptibility to IRI and therapeutic cardioprotection [257]. The impact of the patient background is not routinely assessed in preclinical studies, but it is believed to heavily contribute to neutral results of cardioprotective therapeutics in clinical studies [3, 17, 233]. We aim to start by testing the influence of medications and disease backgrounds on cardioprotection by ischemic preconditioning. Ischemic preconditioning demonstrates the strongest and most robust cardioprotection [190], and identification of the setting and changes that cause it to fail will be important to clinical translation of therapies.

Chronic Statin Use

Statins are commonly taken by patients with cardiovascular disease for hyperlipidemia, and are commonly cited as a confounding factor in clinical studies [3, 257, 278]. Statin therapy is thought to be inherently cardioprotective [279], and a retrospective analysis indicated that patients on chronic statin treatment have reduced injury after an acute MI [280]. Thus, there is a concern that cardioprotective therapeutics cannot provide any additional reduction in IRI. However, there is also a concern that statin therapy interferes with ischemic conditioning protocols and contributes to neutral results [205, 281]. We wish to clarify the interaction between chronic statin use and ischemic preconditioning to better understand the clinical setting.

A study using chronic lovastatin in rat hearts confirmed a reduction in infarct size [263]. Moreover, they found that acute and chronic lovastatin treatment prevented cardioprotection by ischemic preconditioning and postconditioning respectively. Both acute and chronic lovastatin treatment

decreased baseline Akt phosphorylation but maintained ERK1/2 activity. Another study that examined acute and chronic atorvastatin treatment in ischemic postconditioning found that only ischemic postconditioning with chronic atorvastatin use did not demonstrate a reduced infarct size [282]. Chronic atorvastatin abolished ischemic-postconditioning induced Akt activation. These studies indicate that chronic statin use negatively inhibits the ability of cardiomyocytes to adapt to ischemic conditioning protocols, and further suggests that their combined use increases injury over chronic statin use alone. Chronic lovastatin use is associated with an increase in PTEN expression and levels, where PTEN is a negative regulator of Akt activity [283]. This suggests that chronic statin use directly inhibits Akt-dependent ischemic conditioning cardioprotection, but it separately maintains cardioprotection through other mechanisms.

Our experiments will focus on chronic statin treatment because it is the most representative of the clinical setting. Our first step is to characterize cell death after simulated ischemia-reperfusion in the setting of chronic atorvastatin use, ischemic preconditioning, and their combination. Atorvastatin is a first line statin [284]. We need to first establish under which conditions is there a reduction in IRI. We expect that chronic atorvastatin and ischemic preconditioning will both demonstrate reduced cell death compared to non-treated reperfused constructs, but their combination will not. We will then assess Akt, ERK1/2, and GSK3β phosphorylation and PTEN levels between the groups and in baseline non-ischemic chronic atorvastatin use. This will allow us to assess the parallel activators of the RISK pathway (Akt, ERK1/2), their downstream target (GSK3β), and the PI3K-Akt inhibitor (PTEN). Critically, we need to correlate phosphorylation of GSK3β and its inhibition with reduction of IRI. Any group that demonstrates a reduction in cell death without increased GSK3β phosphorylation suggests cardioprotection through a RISK-independent pathway. In that case, we would need to explore other reperfusion survival pathways, such as the SAFE pathway and STAT activation.

Our next step is to assess if we can reestablish cardioprotection by ischemic preconditioning in the setting of chronic statin treatment. We would expect low GSK3β phosphorylation in the combined treatment group due to inhibition of PI3K-Akt through PTEN activation in chronic statin treatment. However, chronic statin treatment should not have an effect on ERK1/2 signaling, and this discrepancy needs to be assessed by examining GSK3β and ERK1/2 phosphorylation in the chronic atorvastatin treatment group. If GSK3β phosphorylation is low, we would first aim to reestablish cardioprotection through pharmacological inhibition of GSK3β and then target activation of upstream PI3K-Akt. If GSK3β phosphorylation is high despite no reduction in cell death, we would then aim to inhibit PTEN or directly activate Akt to identify if reactivation of the PI3K-Akt pathway can restore cardioprotection independently of GSK3β inhibition.

Identification that the combination of ischemic preconditioning and chronic statin treatment inhibits cardioprotection by each independent therapy will be important in establishing the utility of the platform. Chronic statin treatment is utilized by many patients and could help explain neutral results in clinical trials. Furthermore, by assessing how we can reestablish cardioprotection, we can identify what additional therapeutics and targets we must incorporate to reduce IRI in patients.

Diabetes

We would like to further demonstrate the utility of the platform by studying IRI utilizing hiPS-CMs that recapitulate the comorbidities that patients have. One study found that they were able to guide hiPS-CMs into a diabetic phenotype by culturing then under pro-diabetic conditions or by deriving them from patients with type 2 diabetes [141]. We want to expand upon these initial studies by assessing the response of diabetic hIPS-CMs to ischemia-reperfusion and preconditioning. Studies on diabetic animal models and atrial appendage slices from patients with diabetes have demonstrated that the protective effects of ischemic preconditioning are not present in diabetes [285–287]. Another study on isolated atrial

trabecula from diabetic patients demonstrated that the normal ischemic preconditioning protocol did not provide cardioprotection, but a longer preconditioning protocol could [207]. They also found that the samples from diabetic patients had decreased Akt phosphorylation, which could explain the need for a greater preconditioning stimulus. One confounding aspect of human studies is that patients are being managed on therapeutics. The commonly prescribed drug, metformin, is an activator of AMPK, which initiates autophagy, and acute treatment with metformin has demonstrated a decrease in IRI [288]. However, it is unclear how chronic metformin treatment influences the cardiomyocytes. We want to separate the medications and first examine only the influence of diabetes on IRI to better understand its role.

We first want to subject diabetic constructs to ischemia-reperfusion and compare cell death to normal reperfused constructs. Diabetic patients have demonstrated increased infarct size after an acute MI [289], and we expect to see increased cell death in the diabetic constructs. Furthermore, we want to compare ischemic preconditioning cardioprotection in diabetic and normal constructs. We expect a decrease in cardioprotection in the diabetic constructs due to impairment of PI3K-Akt signaling [290]. We want to identify if we can overcome this decrease through addition of pharmacologic agents or a stronger ischemic preconditioning stimulus. We want to test if a longer ischemic duration or additional cycles in preconditioning can lead to increased Akt activation to demonstrate increased cardioprotection in diabetic cardiac constructs. Furthermore, activators of PI3K or Akt should provide a strong stimulus to overcome baseline decrease to allow for cardioprotection by ischemic preconditioning. We also want to assess phosphorylation of GSK3β to ensure inhibition of the downstream target.

Cyclosporine A

We also want to further investigate cyclosporine A in a more clinically-representative setting. The phase III clinical trial for cyclosporine A demonstrated no benefit [1]. Some of the criticisms of the trial and reasoning for lack of response were centered around the different formulation of cyclosporine A and the higher use of $P2Y_{12}$ platelet inhibitors [233]. Intralipid, the newer formulation of cyclosporine A used, has demonstrated increased cardioprotection over cyclosporine A [291]. Intralipid use further demonstrated phosphorylation of Akt and GSK3β that was not seen with cyclosporine A use. The $P2Y_{12}$ platelet inhibitor, clopidogrel, also demonstrated a reduction in infarct size that was blocked by wortmannin, which suggested activation of PI3K-Akt for cardioprotection [292]. Thus, these results suggest that PI3K-Akt activation with GSK3β inhibition demonstrates a stronger cardioprotective effect compared to direct inhibition of the MPTP.

We want to compare the cardioprotective effects of cyclosporine A, intralipid, and clopidogrel to identify if cyclosporine A is able to demonstrate any additional reduction of IRI. Furthermore, we want to assess if intralipid and clopidogrel interfere with each other and cyclosporine A to cause neutral results. Because intralipid and clopidogrel have both indicated cardioprotection by PI3K-Akt signaling, we also want to use inhibitors of the pathway and test if cyclosporine A can still demonstrate cardioprotection in this setting. This will further let us assess if cyclosporine A can still decrease cell death in the a patient background of impaired PI3K-Akt signaling [290].

Many clinical studies of cardioprotective therapeutics have demonstrated no benefit. It is thought that this failure is due to the patient background, where patient comorbidities and comedications can interfere with or mask any potential cardioprotective benefit. We want to test our cardioprotective therapeutics on a modified background to indicate that we can recapitulate these potential confounding factors.

Furthermore, we want to identify the changes at baseline in these constructs to understand what we need to target to overcome impaired activation of pro-survival pathways. Testing these therapies in a setting that is more representative of the patient background will allow us to better predict the clinical response.

Preconditioning Proteomics

In this thesis, we identified that preconditioned constructs demonstrated increased activation and phosphorylation of Akt that could be contributing to the decrease in cell death. Animal studies have also identified activation of Akt through preconditioning, but preconditioning involves changes across multiple signaling pathways [210]. The full identity of these pathways still needs to be sorted out, while the scope is still expanding. Furthermore, it has been recognized that there may be species differences in the proteins that are relevant. For example, STAT5 activation, but not STAT3, was found to be cardioprotective in humans, while the opposite was found in rodents [200, 201]. Therefore, we want to utilize proteomics and phosphoproteomics in future studies to identify the proteins that drive cardioprotection by ischemic preconditioning in an unbiased and wide-ranging manner. Many of the signaling changes in ischemic conditioning are driven by phosphorylation due to the time scale of these changes [293], which makes phosphoproteomics important in the identification of the activated and relevant proteins.

There was a proteomics study that compared LV biopsies taken from patients undergoing CABG surgeries with and without remote ischemic preconditioning [294]. However, they did not find a statistically significant difference between the two groups. They attributed the lack of difference to the high baseline variation seen in the patients. The patients had many differences in their comorbidities and comedications, and furthermore, these comorbidities and comedications have been found to affect the myocardial response to ischemic conditioning [257]. Our platform is advantageous in providing a homogenous baseline sample and will allow us to better detect differences.

We want to continue studying ischemic preconditioning because this cardioprotective strategy has shown the strongest and most robust reduction in IRI. The initial groups we want to examine are normoxic controls, preconditioned constructs prior to the ischemic insult, reperfused constructs, and reperfused constructs with prior ischemic preconditioning. These groups will allow us to assess changes in the initial and end state due to preconditioning that are leading to and causing the differences in outcome. We would like to also further test constructs immediately after the ischemic insult to establish a time course of changes.

Comparing constructs subjected to ischemia-reperfusion with and without ischemic preconditioning will allow us to identify the proteins that are relevant to cardioprotection. In particular, we would like to identify which proteins are phosphorylated and activated as part of changes in signal transduction pathways in order to target therapeutic development. Furthermore, we want to compare the initial and end states to assess how protein and phosphorylation levels change over time.

Finally, differences between the normoxic controls and preconditioned constructs before the ischemic insult may give us an indication into how patient comorbidities and comedications change the response to IRI and preconditioning. Proteomics allows us to identify the changes in the initial state caused by preconditioning that are needed to protect the cardiomyocytes during ischemia and reperfusion. This gives us an indication of how comorbidities or medications fail to allow the heart to adapt to these changes, and thus, prevent cardioprotection by ischemic conditioning.

There have been some proteomic studies done in animal models, but we want to perform these studies in our human tissue model to establish if there are any species differences. A review of proteomic studies done in animals for ischemic preconditioning and pharmacologic therapy to simulate ischemic preconditioning found that changes in signal transduction only demonstrated the fourth most number of changes [295]. Of course, we expect that signal transduction changes should have an outsized impact

compared to the relative percentage of changes. However, these results indicate that we need to expand our scope of analysis. The top 3 categories of changes were energy production and conversion; post-translational modification, protein turnover, and chaperones; and cytoskeleton. Our discussion has so far focused on mitochondria and changes in PI3K-Akt signaling that affect the mitochondria because mitochondria are at the center of energy production in cardiomyocytes. But, we need to also assess the role of autophagy and ER stress in IRI and cardioprotection by ischemic preconditioning.

Autophagy

Autophagy is an important aspect of IRI, and it should be characterized in future experiments on the human tissue platform. Currently, the role of autophagy in IRI remains to be fully defined. Autophagy is activated during ischemia due to nutrient deprivation, and at low levels, autophagy promotes cell survival by degrading damaged cellular components to generate amino acids and ATP [183, 184]. However, overactivation of autophagy leads to excessive degradation and cell death [187]. Thus, autophagy can be cardioprotective or contribute to IRI depending on the circumstances, and these conditions need to be identified. Furthermore, reperfusion leads to a change in autophagy. It has been demonstrated that reperfusion leads to increased formation of autophagosomes through a mechanism distinct from that of ischemia [183], but this increase may be due to decreased clearance autophagosomes in the lysosomes [186]. Both inhibition [296, 297] and stimulation [32, 129, 185] of autophagy has been found to be cardioprotective in ischemia-reperfusion. Ischemic preconditioning has also been found to increase autophagy for cardioprotection [298, 299]. Furthermore, different cell sources have different activation of autophagy, which was seen in increased autophagosome formation in neonatal rat cardiomyocytes over H9c2 cells [60]. Study on the human tissue model will be important to better assess and clarify the importance of autophagy in human cardiomyocytes.

The first assessment needs to characterize autophagy during simulated ischemia. Ischemic conditions involve depletion of nutrients. The resulting decrease in ATP and increase in AMP activates AMP-activated protein kinase (AMPK). AMPK activates autophagy directly by recruiting proteins to form the autophagosome and indirectly by inhibiting mammalian target of rapamycin (mTOR) inhibition of autophagy [183]. Beclin1 is another protein that is involved in the formation of the autophagosome, and increased levels correspond to an increase in initiation of autophagosome formation. Formation of the autophagosome is associated with an increase in LC3-II levels. We want to identify when autophagy switches from being cardioprotective to causing cell death by comparing cell death in constructs exposed to short and long ischemic times, with and without moderators of autophagy. We believe that stimulation of autophagy will decrease cell death in short ischemic times, but it will increase cell death in long ischemic times due to autophagy-related cell death.

There are many targets that we can modify to inhibit or activate autophagy to characterize it in regards to ischemic time. Class I phosphatidylinositol 3-kinase (PI3K) inhibits autophagy through activation of Akt and mTOR, while class III PI3K activates autophagy directly by helping to form the autophagosome [300]. Wortmannin is generally considered to be an inhibitor of autophagy, but it affects all PI3K classes. Instead, we would like to use 3-methyladenine (3-MA) as a specific inhibitor of class III PI3K. We can also use inhibitors of AMPK to control initiation of autophagy during ischemia. Beclin 1 knockout can also be utilized to inhibit initiation of autophagy. To stimulate autophagy, we can utilize rapamycin to inhibit mTOR inhibition of autophagosome formation or utilize AMPK activators. There are many indirect and direct activators of AMPK available [301]. We aim to inhibit or stimulate autophagy and characterize changes in the downstream protein levels and cell death. An ischemic time that induces mild activation of autophagy with cardioprotection should see an increase in cell death with inhibition of autophagy. Conversely, an ischemic time that results in excessive autophagy should see either a decrease or no change in cell death with inhibition of autophagy. No change might be seen because the large amounts of

necrotic cell death may overwhelm any signal from autophagy. Many studies have identified stimulation of autophagy to be a cardioprotective strategy [32, 129, 185], and we must establish if this is dependent on the ischemic time.

The next step is to characterize autophagy during reperfusion. We aim to clarify whether the increase in autophagosomes seen during reperfusion is due to increased initiation of autophagy or decreased clearance of autophagosomes [183, 186]. As previously discussed, autophagic flux can be measured by either comparing LC3 levels with and without inhibition of lysosomal clearance or transfecting the cells with RFP-GFP-LC3 lentiviral vectors and measuring red fluorescence to assess quenching of the GFP signal in the acidic lysosome. We want to assess if inhibition of autophagic flux using bafilomycin to inhibit autophagosome fusion with the lysosome or chloroquine to inhibit lysosomal degradation leads to an increase in reperfusion injury [186, 302]. We also want to identify if reperfusion autophagy is different depending on the nature of autophagy during ischemia. It has been found in studies that autophagy stimulation was only cardioprotective in the border zone of the infarct [32]. The ischemic insult to the border zone is presumably lower due to collateral circulation, and it would have lower levels of autophagy that would be cardioprotective compared to the center of the infarct.

We believe that the beneficial effects of autophagy stimulation are reliant on increasing autophagic flux, and we want to test this by inhibiting autophagic flux on reperfusion to determine if this leads to a reduction in the beneficial effects of autophagy stimulation. We expect that the use of AMPK activators or rapamycin for increased activation of autophagy with bafilomycin or chloroquine inhibition of lysosome fusion or degradation will lead to an increase in cell death. We think that stimulation of autophagy for cardioprotection is dependent on increasing autophagic flux to overcome the impaired autophagic flux that is seen during reperfusion, and stimulation of autophagy initiation is not cardioprotective in itself.

Furthermore, we also want to clarify the role of ischemic preconditioning in promoting autophagy. Ischemic preconditioning leads to increased formation of autophagosomes, and it has been shown that 3-MA and bafilomycin inhibition of autophagy both prevented neuroprotection by ischemic preconditioning [299, 303]. We believe that cardioprotection by ischemic preconditioning is also dependent on increasing autophagic flux. To test this, we first want to characterize the formation of autophagosomes and identify increased clearance of them due to ischemic preconditioning.

We want to identify if preconditioning leads to increased activation of AMPK and clarify preconditioning-associated activation of PI3K-Akt. Activation of Akt leads to activation of mTOR, where mTOR is an inhibitor of autophagy. However, AMPK acts to inhibit mTOR inhibition. It is clear that ischemic preconditioning acts to increase autophagy activation [299, 303]; therefore, we want to determine if addition of rapamycin to block any increase in mTOR activation through PI3K-Akt can lead to further cardioprotection in ischemic preconditioning.

We then want to identify if treatment with bafilomycin or chloroquine to inhibit autophagosome clearance will abolish the protective effects of ischemic preconditioning. Furthermore, we want to identify if inhibition of autophagic flux causes a change in RISK pathway signaling by assessing Akt, ERK1/2, and GSK3β. The RISK pathway is thought to provide cardioprotection by targeting the mitochondria and inhibiting the MPTP. If the signaling of this pathway remains while cardioprotection is abolished, that could indicate that cardioprotection is not only reliant on inhibition of the MPTP, but also clearance of mitochondria. Thus, we will need to continue our assessment by examining mitophagy in IRI.

Mitophagy

Further characterization of ischemia-reperfusion needs to assess mitophagy. Mitophagy is clearance of damaged mitochondria to help maintain cellular homeostasis. Mitophagy is also critical for reducing IRI because it has been recognized that injured mitochondria cause further damage to neighboring healthy mitochondria in a viscous cycle [304]. Thus, removal of these damaged mitochondria before they can inflict widespread damage is important to limiting cell injury. Mitophagy is primarily mediated by the interactions of the proteins PINK1 and Parkin. PINK1 is normally maintained at low levels through transport and cleavage in the mitochondria [305, 306]. However, loss of mitochondrial membrane potential and increase in permeability prevents the cleavage of PINK1, and this results in accumulation of PINK1 on the mitochondrial surface [307]. PINK1 accumulation recruits and activates Parkin, which is normally in the cytosol, to the damaged mitochondria to direct mitochondria clearance [308, 309]. Thus, mitophagy can be assessed through western blot by increased PINK1 levels overall and in the mitochondrial fraction, and by translocation of Parkin from the cytosol to mitochondrial fraction. Furthermore, p62 has been recognized to bind to the mitochondria and assist in recruitment to the autophagosome [298].

We can also assess mitophagy by examining mitochondrial mass. However, we are cautious of the suggested methods of mitochondrial mass quantification using cardiolipin content, citrate synthase activity, and complex I activity because they are inherently affected by IRI and ischemic preconditioning [310]. It has also been demonstrated that fluorescent dyes that target the mitochondria, such as Mitotracker, are also inherently influenced by the mitochondrial membrane potential and are not appropriate for mitophagy analysis in the setting of IRI [117]. It may be more appropriate to monitor mitochondrial proteins, such as TOMM20 or TIMM23, and compare ratios to cytosolic housekeeping proteins to determine mitochondrial mass [118].

We want to assess whether cardioprotection by autophagy stimulation is dependent on mitophagy, and if impairments in mitophagy lead to an increase in IRI. Mitochondria are recognized as critical effectors of cell injury overall and especially in IRI [225], and we want to better understand the importance of mitochondria as targets for cardioprotective strategies. To accomplish this, we need to selectively target and inhibit mitophagy while maintaining autophagy intact. Mitophagy can be targeted through either genetic editing (e.g. CRISPR) or introduction of siRNA against Parkin. In this manner, we can monitor an increase in PINK1 levels to confirm that mitophagy is activated, but a decrease in p62 translocation to mitochondria to confirm that the mitochondria are not being directed to the autophagosomes. Furthermore, we want to confirm that autophagic flux is maintained through previously described methods. We next want to stimulate autophagy with drugs when it is cardioprotective (i.e. short ischemic times). We expect that autophagy stimulation with mitophagy inhibition will still reduce cell death during the ischemic period due to the autophagy degrading cellular contents for nutrients. However, we expect that autophagy stimulation with mitophagy inhibition will result in no change to increased cell death compared to no stimulation during the reperfusion period due to impaired clearance of damaged mitochondria. Opening of the MPTP and depolarization of the mitochondrial membrane is a critical step in reperfusion injury, and damaged mitochondria negatively impact neighboring healthy mitochondria [253]. We suspect that cardioprotection by autophagy stimulation is dependent on clearance and isolation of damaged mitochondria from the otherwise viable cardiomyocyte.

We also want to assess if cardioprotection by ischemic preconditioning is reliant on increases in mitophagy. Ischemic preconditioning mediates cardioprotection partially through autophagy and mitophagy. Knockout of Parkin has also been demonstrated to negate the cardioprotective effects of ischemic preconditioning [298]. We wish to demonstrate this in our human tissue model. It is suggested that ischemic preconditioning promotion of mitophagy helps to eliminate the mitochondria with the lowest threshold for opening of the MPTP and depolarization. It has also been previously established that

preconditioning reduces opening of the MPTP [202]. Thus, the decrease in preconditioned mitochondrial membrane permeability that we demonstrated in preconditioned constructs can be due to both elimination of mitochondria with low thresholds and increased resilience of healthy mitochondria. We want to distinguish these two mechanisms by assessing GSK3β phosphorylation and mitochondrial membrane potential in the setting of mitophagy inhibition. If GSK3β phosphorylation is maintained but mitochondrial membrane potential and cell death continue to increase due to mitophagy inhibition, this indicates that preconditioning is reliant on increased mitophagy. MPTP inhibition could just be a bridge to eliminating the damaged mitochondria. It has also been demonstrated that Akt signaling leads to mitophagy [311], and we want to identify if inhibition of PI3K-Akt signaling by ischemic preconditioning abolishes its increase in mitophagy.

We expect that ischemic preconditioning not only leads to increased mitophagy after the preconditioning regimen, but also it increases autophagic flux to clear the damaged mitochondria during reperfusion. Furthermore, mitochondrial dysregulation has been identified in chronic cardiovascular diseases such as heart failure and diabetes [312, 313]. Disruptions in mitophagy may explain why patient comorbidities lead to increased susceptibility to IRI and decreased cardioprotection by ischemic preconditioning [257]. We think that mitophagy and isolation and clearance of damaged mitochondria is critical to cardioprotective strategies.

ER stress

Ischemia disrupts endoplasmic reticulum (ER) function and results in ER stress, which is the accumulation of misfolded and unfolded proteins in the ER. To alleviate ER stress, the unfolded protein response (UPR) is initiated to decrease protein translation to prevent further accumulation of unfolded proteins, upregulate chaperones to assist in protein folding, and increase ER-associated protein degradation (ERAD)

components to help clear the proteins [314]. Activation of the UPR can help restore cell homeostasis, but overactivation or prolonged activation of the UPR leads to apoptosis [315]. ER stress and the UPR have been recognized as having important roles in cardiovascular disease and ischemic injury [316].

We want to first characterize ER stress and the UPR in the cardiac constructs. Direct measurements of ER stress and the misfolded and unfolded proteins are difficult. Instead, we will assess ER stress and the UPR through the UPR master regulators: inositol-requiring enzyme 1 (IRE1); protein kinase RNA-like ER kinase (PERK); and activating transcription factor 6 (ATF6). Normally, BiP (immunoglobulin binding protein) is a chaperone protein that binds to the master regulators to keep them inactivate. ER stress causes BiP to dissociate from the regulator proteins, and activation of the UPR can be measured through phosphorylation of IRE1 and PERK and cleavage of ATF6 in western blots. We will also measure downstream signals of UPR activation and ER stress. We will measure CHOP (CCAAT/enhancer-binding protein homologous protein, downstream of PERK) and caspase 12 levels. These two proteins are important to the activation of ER stress related apoptosis [317–319]. Furthermore, we can also monitor PERK-mediated phosphorylation of eIF2α that leads to the decrease in protein translation seen in the UPR [320]. We can also use the drug MG132 to block ERAD to inhibit the UPR [321].

We want to understand the role of ER stress and the UPR in IRI and as a target for therapeutic strategies. Stimulation and inhibition of ER stress through drugs has demonstrated a respective increase and decrease in infarct size [31]. We want to first establish that direct stimulation and inhibition of ER stress can respectively increase and decrease cell death after IRI in our constructs. In the study on rat hearts, dithiothreitol (DTT) was utilized to quickly stimulate ER stress by inhibiting disulfide-bond formation [31]. The chemical chaperone, sodium 4-phenylbutyrate (PBA), was used to directly inhibit ER stress [322]. We wish to test the effects of these compounds in our model and assess changes in the UPR.

Studies suggest that ischemic preconditioning is dependent on activation of the UPR rather than any direct reduction in ER stress. It has been seen that ischemic preconditioning protocol itself without ischemic injury leads to activation of the UPR, which suggests that the preconditioning regimen causes mild ER stress [323]. It has also been identified that inhibition of the UPR prevents cardioprotection by ischemic preconditioning [323, 324]. Furthermore, another study demonstrated that postconditioning demonstrated an increase in UPR activation, but remote ischemic conditioning did not [325]. They found that addition of the chemical chaperone (PBA), which directly reduces ER stress, abolished cardioprotection by ischemic postconditioning only and prevented UPR activation. This suggests that by preventing ER stress directly with PBA, the cells could not activate the UPR and decrease injury through the UPR. However, this result conflicts with the previous study that demonstrated that PBA reduced infarct size [31]. We need to reconcile these results in our platform to by testing both direct inhibition of ER stress with PBA and ischemic preconditioning. We also want to identify if we can inhibit cardioprotection by ischemic preconditioning through UPR inhibitors in IRE1 or PERK inhibition [314]. This would allow any changes in ER stress to still occur but block the construct response to ER stress.

Furthermore, we wish to assess if activation of the UPR can be cardioprotective in itself, regardless of ER stress. The UPR is controlled by three master regulators, IRE1, PERK, and ATF6, where IRE1 and PERK are activated by phosphorylation and ATF6 by cleavage. Unfortunately, it is unclear if there are any direct pharmacologic activators of these proteins available. There is a PERK activator that has been tested [326], and we can also validate its activity in our platform by examining downstream eIF2α phosphorylation and CHOP levels. Any potential IRE1 activators can be validated by demonstrating splicing of the intron in its downstream target, XBP1 mRNA [327]. We may need to introduce transgenic constitutively activated IRE1, PERK, or ATF6 instead. We can also utilize salubrinal as an inhibitor of eIF2α dephosphorylation to potentiate eIF2α activity and UPR signaling [328]. We want to assess if activation of the UPR in the setting of ER stress reduction by PBA demonstrates cardioprotection. This would allow us to determine if the UPR

has other beneficial effects outside of ER stress reduction. Furthermore, we wish to determine if CHOP inhibition can provide any further therapeutic benefit on top of induction of the UPR.

Conclusion

In this thesis, we established a human tissue engineered model of ischemia-reperfusion injury. A cardiac construct was formed by seeding a hiPS-CM and hydrogel mixture around flexible pillars that provided mechanical conditioning for cell maturation. The cardiac construct demonstrated aligned and striated cardiomyocytes along the edge of the construct, and functional maturity with both a chronotropic and ionotropic response to β-adrenergic stimulation. The constructs subjected to simulated ischemia and reperfusion demonstrated an increase in cell death after reperfusion compared to ischemia only.

We also demonstrated a decrease in IRI through ischemic preconditioning of the cardiac constructs prior to simulated ischemia-reperfusion. Ischemic preconditioning is difficult to study in patients, and this model has the potential to further our understanding of the cardioprotective mechanisms in ischemic conditioning regimens. We further demonstrated a decrease in IRI by modifying the reperfusion conditions. Reperfusion with acidic media helped decrease cellular injury by attenuating rapid normalization of intracellular pH. We also targeted opening of the MPTP and oxidative stress with cyclosporine A and N-acetyl-L-cysteine respectively, but only cyclosporine A demonstrated a decrease in IRI.

This model of IRI demonstrated comparable results to animal studies, but it has greater potential by providing a platform to study human responses to ischemia and reperfusion. Animals and humans have critical differences in their physiology that make animal models less suitable for preclinical use. Furthermore, we anticipate that the growing use of patient-specific hIPSCs will allow for a better model that recapitulates patient comorbidities and more accurately simulates the clinical setting.

Our future studies will focus on better defining the factors that lead to differences in cell death that we saw in our experiments. We need to identify the molecular changes that underly different cell behavior in ischemia, reperfusion, and ischemic conditioning. This will allow us to understand how we need to modify

construct response to reduce IRI and help in developing clinical predictive utility of the platform by understanding how baseline changes may lead to a lack of response. The human tissue engineered model developed in this thesis has demonstrated key aspects of IRI and will help in developing therapies to reduce IRI and improve clinical outcomes.

Research Design and Methods

Bioreactor Fabrication

A custom mold for the bioreactor was designed in SOLIDWORKS and manufactured using a computer numerical control (CNC) milling machine (Haas OM 2) to mill a sheet of Delrin Acetal Resin. Sylgard 184 Polydimethylsiloxane (PDMS) was mixed in a 10:1 ratio with Sylgard 184 curing agent, and poured into the custom mold. Carbon electrode rods (CST T319Lm, 1.5 mm diameter) were also inserted into the molds. The molds were placed under vacuum to remove bubbles, and left to cure in the oven at 60°C overnight. The subsequently formed reactors were washed and plasma bonded to a glass slide (75 x 50 mm). The bioreactors were sterilized by autoclaving prior to construct seeding and culture. The pillars of the bioreactor are 4.5 mm apart, and the tissue well is 6 x 8 mm. Each bioreactor supported the formation of four constructs.

Cell Culture

The human induced pluripotent stem (hIPS) cell line WTC11 was obtained from Dr. Bruce R. Conklin (Gladstone Institute of Cardiovascular Disease, UCSF). The hIPS cells were cultured in mTeSR 1 (Stemcell Technologies) supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C in a humidified incubator with room air and 5% CO_2. The hIPS cells were continuously passaged at 60-70% confluency into 6 well plates. 2 µM of Thiazovivin (Tocris 3845) was added to the mTeSR media immediately after passaging, but was then switched out for normal mTeSR media on subsequent days. The plates were coated with 1 mL of Growth Factor Reduced Matrigel (Corning 356231) diluted 1:80 in DMEM/F12 media prior to use.

Cardiomyocyte Differentiation

hIPS cultures were differentiated at 90-95% confluency. Differentiation was started by washing the wells with PBS, and adding CDM3 (RPMI 1640 (ThermoFisher 11875093) supplemented with 213 µg/mL ascorbic acid (Wako Chemicals 321- 44823), 500 µg/mL albumin (ScienCell OsrHSA), and 100 U/mL penicillin and 100 µg/mL streptomycin) supplemented with 3 µM CHIR 99021 (Tocris 4423). After two days, the media was changed to CDM3 supplemented with 2 µM Wnt-C59 (Tocris 5148). Media was continuously changed with normal CDM3 every two days afterwards.

Cardiac Construct Formation

Cardiomyocytes differentiated from hIPS cells (hiPS-CMs) began spontaneously beating after 8-10 days in culture. They were harvested between 12-17 days after the start of differentiation to be formed into tissue engineered constructs. hIPS-CM monolayers were dissociated using 0.2% collagenase II (Worthington LS004177) in HBSS. Prior to construct seeding, the construct formation wells were coated with 4% Pluronic F127 (Sigma P2443) for 30 minutes, washed, and then allowed to air dry for 30 minutes. This pretreatment helped to prevent hydrogel attachment to the sides and bottom of the well during condensation of the construct structure. Approximately 3 x 10^6 hiPS-CMs were mixed into a collagen-fibrinogen hydrogel (final concentration of 1.5 mg/mL rat tail collagen type I (Corning CB354249), 4 mg/mL bovine fibrinogen (Sigma F8630), 10 units/mL thrombin (Sigma T7513)) and seeded into a well of the bioreactor to form a cardiac construct.

Construct Culture

The constructs were cultured in RPMI 1640 (Thermofisher 11875) supplemented with 2% B27 supplement (Thermofisher 17504044), 213 µg/mL ascorbic acid, 100 U/mL penicillin and 100 µg/mL streptomycin.

Aprotinin (33 µg/mL, Sigma A3428) was added to the media for the first 7 days of culture, and the media was changed every two days. Ischemia-reperfusion experiments were started two weeks after tissue formation.

Isoproterenol Dose Response

To assess the response of constructs to β-adrenergic stimulation, serial dilutions of isoproterenol (Sigma I6504) were made in normal culture media. The constructs were allowed to incubate at 37°C for 15 minutes before being moved to the microscope to take twenty second videos. The videos were then analyzed in MATLAB using custom code to analyze pixel displacement to determine beat frequency and threshold masked to determine fractional area change (FAC). Dose-response curves were then analyzed in GraphPad Prism 6 software using built-in nonlinear regression tools to determine EC50 for beat frequency and significance compared to a horizontal line for FAC.

Ischemia

The constructs were washed in PBS and then 120 µL of ischemic solution (in mM, NaCl 119, KCl 12, NaH_2PO_4 1.2, $MgSO_4$ 1.3, $MgCl_2$ 0.5, $CaCl_2$ 0.9, Sodium Lactate 20, HEPES 5, pH 6.4) was added to each tissue well. The reactors were then placed into a hypoxic chamber, and anoxic gas (95% N_2, 5% CO_2) was flushed in. The chambers were placed into the incubator at 37°C for 6 hours. Constructs subjected to ischemia-only (referred to as "*Isch*") were analyzed immediately after 6 hours of simulated ischemia.

Reperfusion

Reperfusion was achieved by returning the constructs (referred to as "*Rep*" or "*Rep (pH 7.4)*") to room air and adding RPMI 1640 supplemented with 1.4 mM calcium chloride (for a final calcium concentration of

1.8 mM) and 2% B27 supplement, minus antioxidants (Thermofisher 10889038) to achieve a physiological calcium concentration. Normoxic constructs were also cultured in this media for the duration of the experiment. Constructs were placed in the incubator at 37°C for 3 hours before endpoint analysis was performed.

Treatments to Reduce IRI

Ischemic preconditioning (referred to as "*PreC*") was simulated by subjecting the constructs to simulated ischemia for 30 minutes, followed by simulated reperfusion for 15 minutes. The constructs then proceeded as normal through simulated ischemia for 6 hours followed by reperfusion for 3 hours.

The other treatment groups were all subjected to simulated ischemia the same as before, but simulated reperfusion was modified in an attempt to decrease IRI. Reperfusion with acidic media (referred to as "*Rep pH (6.4)*") was tested by titrating the simulated reperfusion media down to a pH of 6.4. For testing the effects of drugs, cyclosporine A (CsA, 1 µM, Sigma C1832, group referred to as "*CsA*") and N-acetylcysteine (NAC, 5 mM, Sigma A9165, group referred to as "*NAC*") were added to the reperfusion to solely examine their effects on reperfusion. The comparison groups are summarized in **Figure 20**.

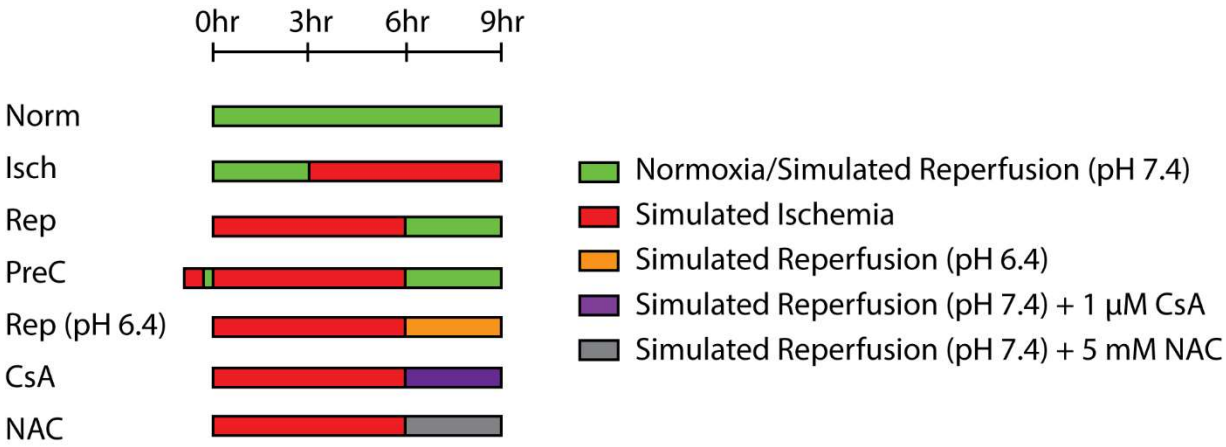


Figure 20: Graphical summary of various comparison and treatment groups to assess the validity of the engineered cardiac construct as a model of IRI.

Assays

Cell death was assessed using LDH release (Thermofisher 88954) and adenylate kinase release (Toxilight, Lonza LT07) into the media. Cell viability was determined using RealTime-Glo (Promega G9712). Construct reactive oxygen species levels were measured using ROS-Glo (Promega G8820). Construct intracellular pH was measured using pHrodo Green dye (ThermoFisher P35373). Mitochondria membrane permeability was assessed using JC-1 dye and measuring excitation at 485 nm, and comparing emission at 528 and 590 nm. All kits were executed according to manufacturer's instructions.

Imaging

For histology, constructs were fixed in 4% paraformaldehyde in PBS, transferred to 30% sucrose in PBS, and then frozen in OCT embedding compound. 10 µm sections were stained for using cardiac troponin T antibody (1:100, Thermofisher MS-295-P1), and nuclei were counterstained using DAPI. Secondary antibody used was Goat anti-mouse Alexa Fluor 488 conjugate (Thermofisher A-11001). Coverslips were

mounted using VECSTASHIELD antifade mounting medium (Vector H1200), and images were taken under epifluorescence and confocal microscope.

Western Blot

Constructs were flash-frozen in liquid nitrogen and then transferred to a -80°C freezer until they were ready for protein isolation. Three-four constructs were pooled together, and were homogenized using TissueRuptor II (Qiagen) in Tissue Protein Extraction Reagent (Thermofisher 78510) supplemented with 1X Protease/Phosphatase Inhibitor Cocktail (Cell Signaling Technology 5872S). The homogenized constructs were then placed on a vortex mixer at 4°C for 2 hours. Protein concentrations were then determined using a Pierce BCA protein assay kit (Thermofisher 23225).

For the western blot, 30 µg of total protein were added to each lane. Blots were transferred onto nitrocellulose membranes (0.2 µm) (Bio-Rad 1620112) and blocked using 5% non-fat milk in TBS-T. Membranes were probed using antibodies and developed under chemiluminescence. Antibodies used were: cleaved caspase-3 (Cell Signaling Technology 9664), total caspase-3 (Cell Signaling Technology 9668), phospho-Akt (Ser473) (Cell Signaling Technology 4060), Akt (pan) (Cell Signaling Technology 2920), and β-tubulin (Cell Signaling Technology 86298). Quantification was done in Licor Image Studio software.

Statistics

Statistical tests were performed using GraphPad Prism 6 software. ANOVA was performed with post-hoc Tukey's HSD to determine significant differences between groups. $P < 0.05$ was used to assess for statistical significance.

References

1. Cung T-T, Morel O, Cayla G, et al (2015) Cyclosporine before PCI in Patients with Acute Myocardial Infarction. N Engl J Med 373:1021–31 . doi: 10.1056/NEJMoa1505489

2. Dominguez-Rodriguez A, Abreu-Gonzalez P, de la Torre-Hernandez JM, et al (2017) Effect of intravenous and intracoronary melatonin as an adjunct to primary percutaneous coronary intervention for acute ST-elevation myocardial infarction: Results of the Melatonin Adjunct in the acute myocaRdial Infarction treated with Angioplasty trial. J Pineal Res 62:e12374 . doi: 10.1111/jpi.12374

3. Hausenloy DJ, Garcia-Dorado D, Bøtker HE, et al (2017) Novel targets and future strategies for acute cardioprotection: Position Paper of the European Society of Cardiology Working Group on Cellular Biology of the Heart. Cardiovasc Res 113:564–585 . doi: 10.1093/cvr/cvx049

4. Hausenloy DJ, Yellon DM (2015) Targeting Myocardial Reperfusion Injury--The Search Continues. N Engl J Med 373:1073–5 . doi: 10.1056/NEJMe1509718

5. Rossello X, Yellon DM (2016) A critical review on the translational journey of cardioprotective therapies! Int J Cardiol 220:176–184 . doi: 10.1016/j.ijcard.2016.06.131

6. Lecour S, Botker HE, Condorelli G, et al (2014) ESC Working Group Cellular Biology of the Heart: Position Paper: improving the preclinical assessment of novel cardioprotective therapies. Cardiovasc Res 104:399–411 . doi: 10.1093/cvr/cvu225

7. Lu HR, Mariën R, Saels A, De Clerck F (2001) Species plays an important role in drug-induced prolongation of action potential duration and early afterdepolarizations in isolated Purkinje fibers. J Cardiovasc Electrophysiol 12:93–102 . doi: lu01

8. Holmes A, Bonner F, Jones D (2015) Assessing drug safety in human tissues - what are the barriers? Nat Rev Drug Discov 14:585–7 . doi: 10.1038/nrd4662

9. Cook D, Brown D, Alexander R, et al (2014) Lessons learned from the fate of AstraZeneca's drug pipeline: a five-dimensional framework. Nat Rev Drug Discov 13:419–31 . doi: 10.1038/nrd4309

10. Sanchis-Gomar F, Perez-Quilis C, Leischik R, Lucia A (2016) Epidemiology of coronary heart disease and acute coronary syndrome. Ann Transl Med 4:256–256 . doi: 10.21037/atm.2016.06.33

11. McNamara RL, Wang Y, Herrin J, et al (2006) Effect of door-to-balloon time on mortality in patients with ST-segment elevation myocardial infarction. J Am Coll Cardiol 47:2180–6 . doi: 10.1016/j.jacc.2005.12.072

12. Hausenloy DJ, Yellon DM (2013) Myocardial ischemia-reperfusion injury: a neglected therapeutic target. J Clin Invest 123:92–100 . doi: 10.1172/JCI62874

13. Murry CE, Jennings RB, Reimer KA (1986) Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation 74:1124–1136 . doi: 10.1161/01.cir.74.5.1124

14. Argaud L, Gateau-Roesch O, Raisky O, et al (2005) Postconditioning inhibits mitochondrial permeability transition. Circulation 111:194–197 . doi: 10.1161/01.CIR.0000151290.04952.3B

15. Hahn J-Y, Song Y Bin, Kim EK, et al (2013) Ischemic postconditioning during primary percutaneous

coronary intervention: the effects of postconditioning on myocardial reperfusion in patients with ST-segment elevation myocardial infarction (POST) randomized trial. Circulation 128:1889–96 . doi: 10.1161/CIRCULATIONAHA.113.001690

16. Le Page S, Bejan-Angoulvant T, Angoulvant D, Prunier F (2015) Remote ischemic conditioning and cardioprotection: a systematic review and meta-analysis of randomized clinical trials. Basic Res Cardiol 110:11 . doi: 10.1007/s00395-015-0467-8

17. Rossello X, Yellon DM (2016) Cardioprotection: The Disconnect Between Bench and Bedside. Circulation 134:574–5 . doi: 10.1161/CIRCULATIONAHA.116.022829

18. Kloner RA, Ganote CE, Jennings RB (1974) The “no-reflow” phenomenon after temporary coronary occlusion in the dog. J Clin Invest 54:1496–508 . doi: 10.1172/JCI107898

19. Vághy PL (1979) Role of mitochondrial oxidative phosphorylation in the maintenance of intracellular pH. J Mol Cell Cardiol 11:933–40 . doi: 10.1016/0022-2828(79)90385-7

20. Robergs RA (2004) Biochemistry of exercise-induced metabolic acidosis. AJP Regul Integr Comp Physiol 287:R502–R516 . doi: 10.1152/ajpregu.00114.2004

21. Halestrap AP, Wang X, Poole RC, et al (1997) Lactate transport in heart in relation to myocardial ischemia. Am J Cardiol 80:17A–25A . doi: 10.1016/S0002-9149(97)00454-2

22. Theroux P, Chaitman BRR, Danchin N, et al (2000) Inhibition of the sodium-hydrogen exchanger with cariporide to prevent myocardial infarction in high-risk ischemic situations. Main results of the GUARDIAN trial. Guard during ischemia against necrosis (GUARDIAN) Investigators. Circulation 102:3032–8 . doi: 10.1161/01.CIR.102.25.3032

23. Marban E, Kitakaze M, Kusuoka H, et al (1987) Intracellular free calcium concentration measured with 19F NMR spectroscopy in intact ferret hearts. Proc Natl Acad Sci U S A 84:6005–9

24. Qian T, Nieminen AL, Herman B, Lemasters JJ (1997) Mitochondrial permeability transition in pH-dependent reperfusion injury to rat hepatocytes. Am J Physiol 273:C1783-92

25. Hernando V, Inserte J, Sartório CL, et al (2010) Calpain translocation and activation as pharmacological targets during myocardial ischemia/reperfusion. J Mol Cell Cardiol 49:271–279 . doi: 10.1016/j.yjmcc.2010.02.024

26. Raedschelders K, Ansley DM, Chen DDY (2012) The cellular and molecular origin of reactive oxygen species generation during myocardial ischemia and reperfusion. Pharmacol Ther 133:230–255 . doi: 10.1016/j.pharmthera.2011.11.004

27. Baines CP (2009) The mitochondrial permeability transition pore and ischemia-reperfusion injury. Basic Res Cardiol 104:181–8 . doi: 10.1007/s00395-009-0004-8

28. Griffiths EJ, Halestrap AP (1995) Mitochondrial non-specific pores remain closed during cardiac ischaemia, but open upon reperfusion. Biochem J 307 (Pt 1):93–8

29. Baines CP, Kaiser RA, Purcell NH, et al (2005) Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. Nature 434:658–62 . doi: 10.1038/nature03434

30. Ottani F, Latini R, Staszewsky L, et al (2016) Cyclosporine A in Reperfused Myocardial Infarction the Multicenter, Controlled, Open-Label CYCLE Trial. J Am Coll Cardiol 67:365–374 . doi:

10.1016/j.jacc.2015.10.081

31. Zhang C, Tang Y, Li Y, et al (2017) Unfolded protein response plays a critical role in heart damage after myocardial ischemia/reperfusion in rats. PLoS One 12:e0179042 . doi: 10.1371/journal.pone.0179042

32. Xie M, Kong Y, Tan W, et al (2014) Histone Deacetylase Inhibition Blunts Ischemia/Reperfusion Injury by Inducing Cardiomyocyte Autophagy. Circulation 129:1139–1151 . doi: 10.1161/CIRCULATIONAHA.113.002416

33. He X, Li S, Liu B, et al (2017) Major contribution of the 3/6/7 class of TRPC channels to myocardial ischemia/reperfusion and cellular hypoxia/reoxygenation injuries. Proc Natl Acad Sci U S A 114:E4582–E4591 . doi: 10.1073/pnas.1621384114

34. Pabla R, Buda AJ, Flynn DM, et al (1996) Nitric oxide attenuates neutrophil-mediated myocardial contractile dysfunction after ischemia and reperfusion. Circ Res 78:65–72 . doi: 10.1161/01.RES.78.1.65

35. Thielmann M, Dörge H, Martin C, et al (2002) Myocardial dysfunction with coronary microembolization: signal transduction through a sequence of nitric oxide, tumor necrosis factor-alpha, and sphingosine. Circ Res 90:807–13 . doi: 10.1161/01.RES.0000014451.75415.36

36. Heusch G, Skyschally A, Schulz R (2011) The in-situ pig heart with regional ischemia/reperfusion - Ready for translation. J Mol Cell Cardiol 50:951–963 . doi: 10.1016/j.yjmcc.2011.02.016

37. Shen YT, Fallon JT, Iwase M, Vatner SF (1996) Innate protection of baboon myocardium: effects of coronary artery occlusion and reperfusion. Am J Physiol 270:H1812-8

38. Hamlin RL, Altschuld RA (2011) Extrapolation from mouse to man. Circ Cardiovasc Imaging 4:2–4 . doi: 10.1161/CIRCIMAGING.110.961979

39. Gibbs CL (2003) Cardiac energetics: Sense and nonsense. Clin Exp Pharmacol Physiol 30:598–603 . doi: 10.1046/j.1440-1681.2003.03878.x

40. O'Hara T, Rudy Y (2012) Quantitative comparison of cardiac ventricular myocyte electrophysiology and response to drugs in human and nonhuman species. AJP Hear Circ Physiol 302:H1023–H1030 . doi: 10.1152/ajpheart.00785.2011

41. Tsang A, Hausenloy DJ, Mocanu MM, Yellon DM (2004) Postconditioning: A form of "modified reperfusion" protects the myocardium by activating the phosphatidylinositol 3-kinase-Akt pathway. Circ Res 95:230–232 . doi: 10.1161/01.RES.0000138303.76488.fe

42. Kalogeris T, Baines CP, Krenz M, Korthuis RJ (2012) Cell biology of ischemia/reperfusion injury. Int Rev Cell Mol Biol 298:229–317 . doi: 10.1016/B978-0-12-394309-5.00006-7

43. DIAZ R, WILSON G (2006) Studying ischemic preconditioning in isolated cardiomyocyte models. Cardiovasc Res 70:286–296 . doi: 10.1016/j.cardiores.2005.12.003

44. Diaz RJ, Harvey K, Boloorchi A, et al (2014) Enhanced cell volume regulation: a key mechanism in local and remote ischemic preconditioning. Am J Physiol Cell Physiol 306:C1191-9 . doi: 10.1152/ajpcell.00259.2013

45. Si J, Wang N, Wang H, et al (2014) HIF-1α signaling activation by post-ischemia treatment with astragaloside iv attenuates myocardial ischemia-reperfusion injury. PLoS One 9:1–10 . doi:

10.1371/journal.pone.0107832

46. Strijdom H, Genade S, Lochner A (2004) Nitric Oxide synthase (NOS) does not contribute to simulated ischaemic preconditioning in an isolated rat cardiomyocyte model. Cardiovasc drugs Ther 18:99–112 . doi: 10.1023/B:CARD.0000029027.50796.84

47. Cavalheiro RA, Marin RM, Rocco SA, et al (2010) Potent cardioprotective effect of the 4-anilinoquinazoline derivative PD153035: involvement of mitochondrial K(ATP) channel activation. PLoS One 5:e10666 . doi: 10.1371/journal.pone.0010666

48. Song M, Huang L, Zhao G, Song Y (2013) Beneficial effects of a polysaccharide from Salvia miltiorrhiza on myocardial ischemia-reperfusion injury in rats. Carbohydr Polym 98:1631–6 . doi: 10.1016/j.carbpol.2013.08.020

49. Sedmera D, Kucera P, Raddatz E (2002) Developmental changes in cardiac recovery from anoxia-reoxygenation. Am J Physiol Regul Integr Comp Physiol 283:R379–R388 . doi: 10.1152/ajpregu.00534.2001

50. Portal L, Martin V, Assaly R, et al (2013) A Model of Hypoxia-Reoxygenation on Isolated Adult Mouse Cardiomyocytes: Characterization, Comparison With Ischemia-Reperfusion, and Application to the Cardioprotective Effect of Regular Treadmill Exercise. J Cardiovasc Pharmacol Ther 18:367–375 . doi: 10.1177/1074248412475158

51. Chang G, Zhang D, Liu J, et al (2014) Exenatide protects against hypoxia/reoxygenation-induced apoptosis by improving mitochondrial function in H9c2 cells. Exp Biol Med (Maywood) 239:414–22 . doi: 10.1177/1535370214522177

52. Pálóczi J, Varga Z V, Apáti Á, et al (2016) Exogenous Nitric Oxide Protects Human Embryonic Stem Cell-Derived Cardiomyocytes against Ischemia/Reperfusion Injury. Oxid Med Cell Longev 2016:1–9 . doi: 10.1155/2016/4298945

53. Lopaschuk GD, Ussher JR, Folmes CDL, et al (2010) Myocardial fatty acid metabolism in health and disease. Physiol Rev 90:207–258 . doi: 10.1152/physrev.00015.2009.

54. Burridge PW, Matsa E, Shukla P, et al (2014) Chemically defined generation of human cardiomyocytes. Nat Methods 11:855–60 . doi: 10.1038/nmeth.2999

55. Larsson L, Ohman S (1978) Serum ionized calcium and corrected total calcium in borderline hyperparathyroidism. Clin Chem 24:1962–5

56. Tiburcy M, Hudson JE, Balfanz P, et al (2017) Defined Engineered Human Myocardium With Advanced Maturation for Applications in Heart Failure Modeling and Repair. Circulation 135:1832–1847 . doi: 10.1161/CIRCULATIONAHA.116.024145

57. Ostadalova I, Ostadal B, Kolár F, et al (1998) Tolerance to ischaemia and ischaemic preconditioning in neonatal rat heart. J Mol Cell Cardiol 30:857–65 . doi: 10.1006/jmcc.1998.0653

58. Milerova M, Charvatova Z, Skarka L, et al (2010) Neonatal cardiac mitochondria and ischemia/reperfusion injury. Mol Cell Biochem 335:147–153 . doi: 10.1007/s11010-009-0251-x

59. Teixeira G, Abrial M, Portier K, et al (2013) Synergistic protective effect of cyclosporin A and rotenone against hypoxia-reoxygenation in cardiomyocytes. J Mol Cell Cardiol 56:55–62 . doi:

10.1016/j.yjmcc.2012.11.023

60. Cao X, Wang X, Ling Y, et al (2014) Comparison of the degree of autophagy in neonatal rat cardiomyocytes and H9c2 cells exposed to hypoxia/reoxygenation. Clin Lab 60:809–814 . doi: 10.7754/Clin.Lab.2013.130521

61. Kuznetsov A V., Javadov S, Sickinger S, et al (2015) H9c2 and HL-1 cells demonstrate distinct features of energy metabolism, mitochondrial function and sensitivity to hypoxia-reoxygenation. Biochim Biophys Acta - Mol Cell Res 1853:276–284 . doi: 10.1016/j.bbamcr.2014.11.015

62. Rundell VLM, Manaves V, Martin AF, de Tombe PP (2005) Impact of beta-myosin heavy chain isoform expression on cross-bridge cycling kinetics. Am J Physiol Hear Circ Physiol 288:H896--903 . doi: 10.1152/ajpheart.00407.2004

63. Davidson MM, Nesti C, Palenzuela L, et al (2005) Novel cell lines derived from adult human ventricular cardiomyocytes. J Mol Cell Cardiol 39:133–147 . doi: 10.1016/j.yjmcc.2005.03.003

64. Wu K, Hu M, Chen Z, et al (2017) Asiatic acid enhances survival of human AC16 cardiomyocytes under hypoxia by upregulating miR-1290. IUBMB Life 660–667 . doi: 10.1002/iub.1648

65. Huo L, Shi W, Chong L, et al (2016) Asiatic acid inhibits left ventricular remodeling and improves cardiac function in a rat model of myocardial infarction. Exp Ther Med 11:57–64 . doi: 10.3892/etm.2015.2871

66. Huang X, Zuo L, Lv Y, et al (2016) Asiatic acid attenuates myocardial ischemia/reperfusion injury via Akt/GSK-3β/HIF-1α signaling in rat H9c2 cardiomyocytes. Molecules 21:1–14 . doi: 10.3390/molecules21091248

67. Hsieh A, Feric NT, Radisic M (2015) Combined hypoxia and sodium nitrite pretreatment for cardiomyocyte protection in vitro. Biotechnol Prog 31:482–92 . doi: 10.1002/btpr.2039

68. Sun N, Yazawa M, Liu J, et al (2012) Patient-Specific Induced Pluripotent Stem Cells as a Model for Familial Dilated Cardiomyopathy. Sci Transl Med 4:130ra47-130ra47 . doi: 10.1126/scitranslmed.3003552

69. Khan JM, Lyon AR, Harding SE (2013) The case for induced pluripotent stem cell-derived cardiomyocytes in pharmacological screening. Br J Pharmacol 169:304–317 . doi: 10.1111/j.1476-5381.2012.02118.x

70. Paşca SP, Portmann T, Voineagu I, et al (2011) Using iPSC-derived neurons to uncover cellular phenotypes associated with Timothy syndrome. Nat Med 17:1657–1662 . doi: 10.1038/nm.2576

71. Wei W, Liu Y, Zhang Q, et al (2016) Danshen-Enhanced Cardioprotective Effect of Cardioplegia on Ischemia Reperfusion Injury in a Human-Induced Pluripotent Stem Cell-Derived Cardiomyocytes Model. Artif Organs. doi: 10.1111/aor.12801

72. Guo L, Abrams RMC, Babiarz JE, et al (2011) Estimating the risk of drug-induced proarrhythmia using human induced pluripotent stem cell-derived cardiomyocytes. Toxicol Sci 123:281–9 . doi: 10.1093/toxsci/kfr158

73. Nunes SS, Miklas JW, Liu J, et al (2013) Biowire: a platform for maturation of human pluripotent stem cell-derived cardiomyocytes. Nat Methods 10:781–7 . doi: 10.1038/nmeth.2524

74. Shadrin IY, Allen BW, Qian Y, et al (2017) Cardiopatch platform enables maturation and scale-up

of human pluripotent stem cell-derived engineered heart tissues. Nat Commun 8:1825 . doi: 10.1038/s41467-017-01946-x

75. Ronaldson-Bouchard K, Ma SP, Yeager K, et al (2018) Advanced maturation of human cardiac tissue grown from pluripotent stem cells. Nature 556:239–243 . doi: 10.1038/s41586-018-0016-3

76. Li RA, Keung W, Cashman TJ, et al (2018) Bioengineering an electro-mechanically functional miniature ventricular heart chamber from human pluripotent stem cells. Biomaterials 163:116–127 . doi: 10.1016/j.biomaterials.2018.02.024

77. Agarwal A, Goss JA, Cho A, et al (2013) Microfluidic heart on a chip for higher throughput pharmacological studies. Lab Chip 13:3599–608 . doi: 10.1039/c3lc50350j

78. Katare RG, Ando M, Kakinuma Y, Sato T (2010) Engineered Heart Tissue: A Novel Tool to Study the Ischemic Changes of the Heart In Vitro. PLoS One 5:e9275 . doi: 10.1371/journal.pone.0009275

79. Argaud L, Gateau-Roesch O, Muntean D, et al (2005) Specific inhibition of the mitochondrial permeability transition prevents lethal reperfusion injury. J Mol Cell Cardiol 38:367–374 . doi: 10.1016/j.yjmcc.2004.12.001

80. Dow J, Simkhovich BZ, Kedes L, Kloner RA (2005) Washout of transplanted cells from the heart: a potential new hurdle for cell transplantation therapy. Cardiovasc Res 67:301–7 . doi: 10.1016/j.cardiores.2005.04.011

81. Radisic M, Deen W, Langer R, Vunjak-Novakovic G (2005) Mathematical model of oxygen distribution in engineered cardiac tissue with parallel channel array perfused with culture medium containing oxygen carriers. Am J Physiol Heart Circ Physiol 288:H1278-89 . doi: 10.1152/ajpheart.00787.2004

82. Sekine H, Shimizu T, Sakaguchi K, et al (2013) In vitro fabrication of functional three-dimensional tissues with perfusable blood vessels. Nat Commun 4:1399 . doi: 10.1038/ncomms2406

83. Zhang YS, Arneri A, Bersini S, et al (2016) Bioprinting 3D microfibrous scaffolds for engineering endothelialized myocardium and heart-on-a-chip. Biomaterials 110:45–59 . doi: 10.1016/j.biomaterials.2016.09.003

84. Zhang B, Montgomery M, Chamberlain MD, et al (2016) Biodegradable scaffold with built-in vasculature for organ-on-a-chip engineering and direct surgical anastomosis. Nat Mater. doi: 10.1038/nmat4570

85. Zhang D, Shadrin IY, Lam J, et al (2013) Tissue-engineered cardiac patch for advanced functional maturation of human ESC-derived cardiomyocytes. Biomaterials 34:5813–20 . doi: 10.1016/j.biomaterials.2013.04.026

86. Wang G, McCain ML, Yang L, et al (2014) Modeling the mitochondrial cardiomyopathy of Barth syndrome with induced pluripotent stem cell and heart-on-chip technologies. Nat Med 20:616–23 . doi: 10.1038/nm.3545

87. Mathur A, Loskill P, Shao K, et al (2015) Human iPSC-based cardiac microphysiological system for drug screening applications. Sci Rep 5:8883 . doi: 10.1038/srep08883

88. Ulmer BM, Stoehr A, Schulze ML, et al (2018) Contractile Work Contributes to Maturation of

Energy Metabolism in hiPSC-Derived Cardiomyocytes. Stem Cell Reports 10:1–14 . doi: 10.1016/j.stemcr.2018.01.039

89. Tulloch NL, Muskheli V, Razumova M V, et al (2011) Growth of engineered human myocardium with mechanical loading and vascular coculture. Circ Res 109:47–59 . doi: 10.1161/CIRCRESAHA.110.237206

90. Mannhardt I, Breckwoldt K, Letuffe-Brenière D, et al (2016) Human Engineered Heart Tissue: Analysis of Contractile Force. Stem cell reports 7:29–42 . doi: 10.1016/j.stemcr.2016.04.011

91. Boudou T, Legant WR, Mu A, et al (2012) A microfabricated platform to measure and manipulate the mechanics of engineered cardiac microtissues. Tissue Eng Part A 18:910–9 . doi: 10.1089/ten.tea.2011.0341

92. Schaaf S, Shibamiya A, Mewe M, et al (2011) Human engineered heart tissue as a versatile tool in basic research and preclinical toxicology. PLoS One 6:e26397 . doi: 10.1371/journal.pone.0026397

93. Turnbull IC, Karakikes I, Serrao GW, et al (2014) Advancing functional engineered cardiac tissues toward a preclinical model of human myocardium. FASEB J 28:644–54 . doi: 10.1096/fj.13-228007

94. Mannhardt I, Breckwoldt K, Letuffe-Brenière D, et al (2016) Human Engineered Heart Tissue: Analysis of Contractile Force. Stem cell reports 7:29–42 . doi: 10.1016/j.stemcr.2016.04.011

95. Hirt MN, Boeddinghaus J, Mitchell A, et al (2014) Functional improvement and maturation of rat and human engineered heart tissue by chronic electrical stimulation. J Mol Cell Cardiol 74:151–161 . doi: 10.1016/j.yjmcc.2014.05.009

96. Tandon N, Cannizzaro C, Figallo E, et al (2006) Characterization of electrical stimulation electrodes for cardiac tissue engineering. Conf Proc . Annu Int Conf IEEE Eng Med Biol Soc IEEE Eng Med Biol Soc Annu Conf 1:845–8 . doi: 10.1109/IEMBS.2006.259747

97. Soong PL, Tiburcy M, Zimmermann W-H (2012) Cardiac Differentiation of Human Embryonic Stem Cells and their Assembly into Engineered Heart Muscle. Curr Protoc Cell Biol 1–21 . doi: 10.1002/0471143030.cb2308s55

98. Hinson JT, Chopra A, Nafissi N, et al (2015) HEART DISEASE. Titin mutations in iPS cells define sarcomere insufficiency as a cause of dilated cardiomyopathy. Science 349:982–6 . doi: 10.1126/science.aaa5458

99. Ruan JL, Tulloch NL, Razumova M V., et al (2016) Mechanical Stress Conditioning and Electrical Stimulation Promote Contractility and Force Maturation of Induced Pluripotent Stem Cell-Derived Human Cardiac Tissue. Circulation 134:1557–1567 . doi: 10.1161/CIRCULATIONAHA.114.014998

100. Leychenko A, Konorev E, Jijiwa M, Matter ML (2011) Stretch-Induced hypertrophy activates NFkB-Mediated VEGF secretion in adult cardiomyocytes. PLoS One 6:1–9 . doi: 10.1371/journal.pone.0029055

101. van Meer BJ, de Vries H, Firth KSA, et al (2017) Small molecule absorption by PDMS in the context of drug response bioassays. Biochem Biophys Res Commun 482:323–328 . doi: 10.1016/j.bbrc.2016.11.062

102. Wang JD, Douville NJ, Takayama S, Elsayed M (2012) Quantitative analysis of molecular absorption into PDMS microfluidic channels. Ann Biomed Eng 40:1862–1873 . doi: 10.1007/s10439-012-0562-z

103. Parsa H, Wang BZ, Vunjak-Novakovic G (2017) A microfluidic platform for the high-throughput study of pathological cardiac hypertrophy. Lab Chip. doi: 10.1039/c7lc00415j

104. Huebsch N, Loskill P, Deveshwar N, et al (2016) Miniaturized iPS-Cell-Derived Cardiac Muscles for Physiologically Relevant Drug Response Analyses. Sci Rep 6:1–12 . doi: 10.1038/srep24726

105. Leonard A, Bertero A, Powers JD, et al (2018) Afterload promotes maturation of human induced pluripotent stem cell derived cardiomyocytes in engineered heart tissues. J Mol Cell Cardiol 118:147–158 . doi: 10.1016/j.yjmcc.2018.03.016

106. Ricchiuti V, Voss EM, Ney A, et al (1998) Cardiac troponin T isoforms expressed in renal diseased skeletal muscle will not cause false-positive results by the second generation cardiac troponin T assay by Boehringer Mannheim. Clin Chem 44:1919–24

107. Jackman CP, Carlson AL, Bursac N (2016) Dynamic culture yields engineered myocardium with near-adult functional output. Biomaterials 111:66–79 . doi: 10.1016/j.biomaterials.2016.09.024

108. Brandenburger M, Wenzel J, Bogdan R, et al (2012) Organotypic slice culture from human adult ventricular myocardium. Cardiovasc Res 93:50–59 . doi: 10.1093/cvr/cvr259

109. Pillekamp F, Haustein M, Khalil M, et al (2012) Contractile Properties of Early Human Embryonic Stem Cell-Derived Cardiomyocytes: Beta-Adrenergic Stimulation Induces Positive Chronotropy and Lusitropy but Not Inotropy. Stem Cells Dev 21:2111–2121 . doi: 10.1089/scd.2011.0312

110. van der Velden J, Klein L., van der Bijl M, et al (1998) Force production in mechanically isolated cardiac myocytes from human ventricular muscle tissue. Cardiovasc Res 38:414–423 . doi: 10.1016/S0008-6363(98)00019-4

111. Bers DM (2002) Cardiac excitation-contraction coupling. Nature 415:198–205 . doi: 10.1038/415198a

112. Binah O, Dolnikov K, Sadan O, et al (2007) Functional and developmental properties of human embryonic stem cells-derived cardiomyocytes. J Electrocardiol 40:S192-6 . doi: 10.1016/j.jelectrocard.2007.05.035

113. Doss MX, Diego JM Di, Goodrow RJ, et al (2012) Maximum Diastolic Potential of Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes Depends Critically on IKr. PLoS One 7:e40288 . doi: 10.1371/JOURNAL.PONE.0040288

114. Feric NT, Radisic M (2016) Maturing human pluripotent stem cell-derived cardiomyocytes in human engineered cardiac tissues. Adv Drug Deliv Rev 96:110–34 . doi: 10.1016/j.addr.2015.04.019

115. Lopaschuk GD, Spafford MA, Marsh DR (1991) Glycolysis is predominant source of myocardial ATP production immediately after birth. Am J Physiol Circ Physiol 261:H1698–H1705 . doi: 10.1152/ajpheart.1991.261.6.H1698

116. Lopaschuk GD, Belke DD, Gamble J, et al (1994) Regulation of fatty acid oxidation in the mammalian heart in health and disease. Biochim Biophys Acta 1213:263–76

117. Xiao B, Deng X, Zhou W, Tan E-K (2016) Flow Cytometry-Based Assessment of Mitophagy Using MitoTracker. Front Cell Neurosci 10:76 . doi: 10.3389/fncel.2016.00076

118. Chen L, Ma K, Han J, et al (2017) Monitoring Mitophagy in Mammalian Cells. Methods Enzymol 588:187–208 . doi: 10.1016/bs.mie.2016.10.038

119. Seidlmayer LK, Juettner V V., Kettlewell S, et al (2015) Distinct mPTP activation mechanisms in ischaemia-reperfusion: contributions of Ca2+, ROS, pH, and inorganic polyphosphate. Cardiovasc Res 106:237–48 . doi: 10.1093/cvr/cvv097

120. Davidson SM, Foote K, Kunuthur S, et al (2015) Inhibition of NAADP signalling on reperfusion protects the heart by preventing lethal calcium oscillations via two-pore channel 1 and opening of the mitochondrial permeability transition pore. Cardiovasc Res 108:357–366 . doi: 10.1093/cvr/cvv226

121. Sun L, Zhao M, Yu X-J, et al (2013) Cardioprotection by acetylcholine: a novel mechanism via mitochondrial biogenesis and function involving the PGC-1α pathway. J Cell Physiol 228:1238–48 . doi: 10.1002/jcp.24277

122. Görbe A, Varga Z V., Pálóczi J, et al (2014) Cytoprotection by the NO-donor SNAP against ischemia/reoxygenation injury in mouse embryonic stem cell-derived cardiomyocytes. Mol Biotechnol 56:258–264 . doi: 10.1007/s12033-013-9704-2

123. Inserte J, Garcia-Dorado D, Ruiz-Meana M, et al (2002) Effect of inhibition of Na(+)/Ca(2+) exchanger at the time of myocardial reperfusion on hypercontracture and cell death. Cardiovasc Res 55:739–48

124. Gertz EW, Wisneski JA, Stanley WC, Neese RA (1988) Myocardial substrate utilization during exercise in humans. Dual carbon-labeled carbohydrate isotope experiments. J Clin Invest 82:2017–2025 . doi: 10.1172/JCI113822

125. Patel KP, O'Brien TW, Subramony SH, et al (2012) The spectrum of pyruvate dehydrogenase complex deficiency: clinical, biochemical and genetic features in 371 patients. Mol Genet Metab 105:34–43 . doi: 10.1016/j.ymgme.2011.09.032

126. Jitrapakdee S, St Maurice M, Rayment I, et al (2008) Structure, mechanism and regulation of pyruvate carboxylase. Biochem J 413:369–387 . doi: 10.1042/BJ20080709

127. Brooks GA (2018) The Science and Translation of Lactate Shuttle Theory. Cell Metab 27:757–785 . doi: 10.1016/j.cmet.2018.03.008

128. Yánez AJ, Nualart F, Droppelmann C, et al (2003) Broad expression of fructose-1,6-bisphosphatase and phosphoenolpyruvate carboxykinase provide evidence for gluconeogenesis in human tissues other than liver and kidney. J Cell Physiol 197:189–97 . doi: 10.1002/jcp.10337

129. Wang L qiao, Cheng X shu, Huang C hua, et al (2015) Rapamycin protects cardiomyocytes against anoxia/reoxygenation injury by inducing autophagy through the PI3k/Akt pathway. J Huazhong Univ Sci Technol - Med Sci 35:10–15 . doi: 10.1007/s11596-015-1381-x

130. Mathupala SP, Kiousis S, Szerlip NJ (2016) A lab assembled microcontroller-based sensor module for continuous oxygen measurement in portable hypoxia chambers. PLoS One 11:1–14 . doi: 10.1371/journal.pone.0148923

131. McDougal AD, Dewey CF (2017) Modeling oxygen requirements in ischemic cardiomyocytes. J Biol Chem 292:11760–11776 . doi: 10.1074/jbc.M116.751826

132. Gatien M, Stiell I, Wielgosz A, et al (2005) Diagnostic performance of venous lactate on arrival at the emergency department for myocardial infarction. Acad Emerg Med 12:106–113 . doi: 10.1197/j.aem.2004.10.012

133. Vermeulen RP, Hoekstra M, Nijsten MW, et al (2010) Clinical correlates of arterial lactate levels in patients with ST-segment elevation myocardial infarction at admission: a descriptive study. Crit Care 14:R164 . doi: 10.1186/cc9253

134. Goodwin ML, Harris JE, Hernández A, Gladden LB (2007) Blood lactate measurements and analysis during exercise: A guide for clinicians. J Diabetes Sci Technol 1:558–569 . doi: 10.1177/193229680700100414

135. Rodríguez B, Trayanova N, Noble D (2006) Modeling cardiac ischemia. Ann N Y Acad Sci 1080:395–414 . doi: 10.1196/annals.1380.029

136. Hill JL, Gettes LS (1980) Effect of acute coronary artery occlusion on local myocardial extracellular K+ activity in swine. Circulation 61:768–78 . doi: 10.1161/01.CIR.61.4.768

137. Weiss J, Shine KI (1982) [K+]o accumulation and electrophysiological alterations during early myocardial ischemia. Am J Physiol 243:H318-27 . doi: 10.1152/ajpheart.1982.243.2.H318

138. Cheng C-J, Kuo E, Huang C-L (2013) Extracellular potassium homeostasis: insights from hypokalemic periodic paralysis. Semin Nephrol 33:237–47 . doi: 10.1016/j.semnephrol.2013.04.004

139. Garlick PB, Radda GK, Seeley PJ (1979) Studies of acidosis in the ischaemic heart by phosphorus nuclear magnetic resonance. Biochem J 184:547–54 . doi: 10.1042/bj1840547

140. Inserte J, Barba I, Hernando V, et al (2008) Effect of acidic reperfusion on prolongation of intracellular acidosis and myocardial salvage. Cardiovasc Res 77:782–790 . doi: 10.1093/cvr/cvm082

141. Drawnel FM, Boccardo S, Prummer M, et al (2014) Disease modeling and phenotypic drug screening for diabetic cardiomyopathy using human induced pluripotent stem cells. Cell Rep 9:810–820 . doi: 10.1016/j.celrep.2014.09.055

142. Imahashi K, Pott C, Goldhaber JI, et al (2005) Cardiac-specific ablation of the Na+-Ca2+ exchanger confers protection against ischemia/reperfusion injury. Circ Res 97:916–21 . doi: 10.1161/01.RES.0000187456.06162.cb

143. Yamamoto T, Swietach P, Rossini A, et al (2005) Functional diversity of electrogenic Na+-HCO3- cotransport in ventricular myocytes from rat, rabbit and guinea pig. J Physiol 562:455–75 . doi: 10.1113/jphysiol.2004.071068

144. Lagadic-Gossmann D, Buckler KJ, Vaughan-Jones RD (1992) Role of bicarbonate in pH recovery from intracellular acidosis in the guinea-pig ventricular myocyte. J Physiol 458:361–84 . doi: 10.1113/jphysiol.1992.sp019422

145. Burtis CA, Ashwood ER, Bruns DE, Tietz NW (2008) Tietz fundamentals of clinical chemistry. Saunders Elsevier

146. Andersen A-D, Poulsen KA, Lambert IH, Pedersen SF (2009) HL-1 mouse cardiomyocyte injury and death after simulated ischemia and reperfusion: roles of pH, Ca2+-independent phospholipase A2, and Na+/H+ exchange. Am J Physiol Cell Physiol 296:C1227–C1242 . doi: 10.1152/ajpcell.00370.2008

147. Casey TM, Arthur PG (2000) Hibernation in noncontracting mammalian cardiomyocytes. Circulation 102:3124–9 . doi: 10.1161/01.CIR.102.25.3124

148. Seiler C (2010) The human coronary collateral circulation. Eur J Clin Invest 40:465–476 . doi: 10.1111/j.1365-2362.2010.02282.x

149. Ren L, Liu W, Wang Y, et al (2013) Investigation of hypoxia-induced myocardial injury dynamics in a tissue interface mimicking microfluidic device. Anal Chem 85:235–44 . doi: 10.1021/ac3025812

150. Nguyen D-HT, Stapleton SC, Yang MT, et al (2013) Biomimetic model to reconstitute angiogenic sprouting morphogenesis in vitro. Proc Natl Acad Sci U S A 110:6712–7 . doi: 10.1073/pnas.1221526110

151. Leucker TM, Ge Z-D, Procknow J, et al (2013) Impairment of endothelial-myocardial interaction increases the susceptibility of cardiomyocytes to ischemia/reperfusion injury. PLoS One 8:e70088 . doi: 10.1371/journal.pone.0070088

152. Tousoulis D, Kampoli A-M, Tentolouris C, et al (2012) The role of nitric oxide on endothelial function. Curr Vasc Pharmacol 10:4–18

153. Depré C, Fiérain L, Hue L (1997) Activation of nitric oxide synthase by ischaemia in the perfused heart. Cardiovasc Res 33:82–87 . doi: 10.1016/S0008-6363(96)00176-9

154. Pabla R, Curtis MJ (1996) Endogenous Protection Against Reperfusion-induced Ventricular Fibrillation: Role of Neuronal versus Non-neuronal Sources of Nitric Oxide and Species Dependence in the Rat versus Rabbit Isolated Heart. J Mol Cell Cardiol 28:2097–2110 . doi: 10.1006/jmcc.1996.0202

155. Kim J-S, Jin Y, Lemasters JJ (2006) Reactive oxygen species, but not Ca2+ overloading, trigger pH- and mitochondrial permeability transition-dependent death of adult rat myocytes after ischemia-reperfusion. Am J Physiol Heart Circ Physiol 290:H2024-34 . doi: 10.1152/ajpheart.00683.2005

156. Chen M, Won DJ, Krajewski S, Gottlieb RA (2002) Calpain and mitochondria in ischemia/reperfusion injury. J Biol Chem 277:29181–29186 . doi: 10.1074/jbc.M204951200

157. Gao E, Boucher M, Chuprun JK, et al (2007) Darbepoetin alfa, a long-acting erythropoietin analog, offers novel and delayed cardioprotection for the ischemic heart. Am J Physiol Heart Circ Physiol 293:H60-8 . doi: 10.1152/ajpheart.00227.2007

158. Gottlieb RA, Burleson KO, Kloner RA, et al (1994) Reperfusion injury induces apoptosis in rabbit cardiomyocytes. J Clin Invest 94:1621–8 . doi: 10.1172/JCI117504

159. Zhao ZQ, Nakamura M, Wang NP, et al (2000) Reperfusion induces myocardial apoptotic cell death. Cardiovasc Res 45:651–60

160. Dumont EAWJ, Hofstra L, van Heerde WL, et al (2000) Cardiomyocyte Death Induced by Myocardial Ischemia and Reperfusion : Measurement With Recombinant Human Annexin-V in a Mouse Model. Circulation 102:1564–1568 . doi: 10.1161/01.CIR.102.13.1564

161. Mocanu MM, Baxter GF, Yellon DM (2000) Caspase inhibition and limitation of myocardial infarct size: Protection against lethal reperfusion injury. Br J Pharmacol 130:197–200 . doi: 10.1038/sj.bjp.0703336

162. McCully JD, Wakiyama H, Hsieh Y-J, et al (2004) Differential contribution of necrosis and apoptosis in myocardial ischemia-reperfusion injury. Am J Physiol Heart Circ Physiol 286:H1923–H1935 . doi: 10.1152/ajpheart.00935.2003

163. Galluzzi L, Aaronson SA, Abrams J, et al (2009) Guidelines for the use and interpretation of assays for monitoring cell death in higher eukaryotes. Cell Death Differ 16:1093–107 . doi: 10.1038/cdd.2009.44

164. Kroemer G, Galluzzi L, Brenner C (2007) Mitochondrial membrane permeabilization in cell death. Physiol Rev 87:99–163 . doi: 10.1152/physrev.00013.2006

165. Griendling KK, Touyz RM, Zweier JL, et al (2016) Measurement of Reactive Oxygen Species, Reactive Nitrogen Species, and Redox-Dependent Signaling in the Cardiovascular System: A Scientific Statement from the American Heart Association

166. Chouchani ET, Methner C, Nadtochiy SM, et al (2013) Cardioprotection by S-nitrosation of a cysteine switch on mitochondrial complex I. Nat Med 19:753–9 . doi: 10.1038/nm.3212

167. Schafer FQ, Buettner GR (2001) Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. Free Radic Biol Med 30:1191–212

168. Poole LB (2015) The basics of thiols and cysteines in redox biology and chemistry. Free Radic Biol Med 80:148–57 . doi: 10.1016/j.freeradbiomed.2014.11.013

169. Julien O, Wells JA (2017) Caspases and their substrates. Cell Death Differ 24:1380–1389 . doi: 10.1038/cdd.2017.44

170. Yi CH, Yuan J (2009) The Jekyll and Hyde Functions of Caspases. Dev Cell 16:21–34 . doi: 10.1016/j.devcel.2008.12.012

171. Prabhakaran K, Li L, Borowitz JL, Isom GE (2004) Caspase inhibition switches the mode of cell death induced by cyanide by enhancing reactive oxygen species generation and PARP-1 activation. Toxicol Appl Pharmacol 195:194–202 . doi: 10.1016/j.taap.2003.11.012

172. Schellenberg B, Wang P, Keeble JA, et al (2013) Bax Exists in a Dynamic Equilibrium between the Cytosol and Mitochondria to Control Apoptotic Priming. Mol Cell 49:959–971 . doi: 10.1016/j.molcel.2012.12.022

173. Li P, Nijhawan D, Budihardjo I, et al (1997) Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 91:479–89

174. Gorczyca W, Bruno S, Darzynkiewicz R, et al (1992) DNA strand breaks occurring during apoptosis - their early insitu detection by the terminal deoxynucleotidyl transferase and nick translation assays and prevention by serine protease inhibitors. Int J Oncol 1:639–48

175. Galluzzi L, Vitale I, Aaronson SA, et al (2018) Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death 2018. Cell Death Differ 25:486–541 . doi: 10.1038/s41418-017-0012-4

176. Talukder MAH, Zweier JL, Periasamy M (2009) Targeting calcium transport in ischaemic heart

disease. Cardiovasc Res 84:345–352 . doi: 10.1093/cvr/cvp264

177. Huebsch N, Loskill P, Mandegar MA, et al (2015) Automated Video-Based Analysis of Contractility and Calcium Flux in Human-Induced Pluripotent Stem Cell-Derived Cardiomyocytes Cultured over Different Spatial Scales. Tissue Eng Part C Methods 21:467–479 . doi: 10.1089/ten.tec.2014.0283

178. Toda T, Kadono T, Hoshiai M, et al (2007) Na+/H+ exchanger inhibitor cariporide attenuates the mitochondrial Ca2+ overload and PTP opening. Am J Physiol Heart Circ Physiol 293:H3517-23 . doi: 10.1152/ajpheart.00483.2006

179. Yu S, Zheng S, Leng J, et al (2016) Inhibition of mitochondrial calcium uniporter protects neurocytes from ischemia/reperfusion injury via the inhibition of excessive mitophagy. Neurosci Lett 628:24–29 . doi: 10.1016/j.neulet.2016.06.012

180. Yoshikawa Y, Hagihara H, Ohga Y, et al (2005) Calpain inhibitor-1 protects the rat heart from ischemia-reperfusion injury: analysis by mechanical work and energetics. Am J Physiol Heart Circ Physiol 288:H1690-8 . doi: 10.1152/ajpheart.00666.2004

181. Inserte J, Garcia-Dorado D, Ruiz-Meana M, et al (2004) Ischemic preconditioning attenuates calpain-mediated degradation of structural proteins through a protein kinase A-dependent mechanism. Cardiovasc Res 64:105–14 . doi: 10.1016/j.cardiores.2004.06.001

182. Hanna RA, Campbell RL, Davies PL (2008) Calcium-bound structure of calpain and its mechanism of inhibition by calpastatin. Nature 456:409–12 . doi: 10.1038/nature07451

183. Matsui Y, Takagi H, Qu X, et al (2007) Distinct roles of autophagy in the heart during ischemia and reperfusion: Roles of AMP-activated protein kinase and beclin 1 in mediating autophagy. Circ Res 100:914–922 . doi: 10.1161/01.RES.0000261924.76669.36

184. Lum JJ, DeBerardinis RJ, Thompson CB (2005) Autophagy in metazoans: cell survival in the land of plenty. Nat Rev Mol Cell Biol 6:439–448 . doi: 10.1038/nrm1660

185. Huang C, Liu W, Perry CN, et al (2010) Autophagy and protein kinase C are required for cardioprotection by sulfaphenazole. Am J Physiol Heart Circ Physiol 298:H570–H579 . doi: 10.1152/ajpheart.00716.2009

186. Ma X, Liu H, Foyil SR, et al (2012) Impaired autophagosome clearance contributes to cardiomyocyte death in ischemia/reperfusion injury. Circulation 125:3170–3181 . doi: 10.1161/CIRCULATIONAHA.111.041814

187. Cao DJ, Gillette TG, Hill JA (2009) Cardiomyocyte autophagy: remodeling, repairing, and reconstructing the heart. Curr Hypertens Rep 11:406–11 . doi: 10.1007/s11906-009-0070-1

188. Biederbick A, Kern HF, Elsässer HP (1995) Monodansylcadaverine (MDC) is a specific in vivo marker for autophagic vacuoles. Eur J Cell Biol 66:3–14

189. Mizushima N, Yoshimori T How to interpret LC3 immunoblotting. Autophagy 3:542–5

190. Jones SP, Tang XL, Guo Y, et al (2015) The NHLBI-Sponsored Consortium for preclinicAl assESsment of cARdioprotective Therapies (CAESAR): A new paradigm for rigorous, accurate, and reproducible evaluation of putative infarct-sparing interventions in mice, rabbits, and pigs. Circ Res 116:572–586 . doi: 10.1161/CIRCRESAHA.116.305462

191. Yellon DM, Alkhulaifi AM, Pugsley WB (1993) Preconditioning the human myocardium. Lancet

342:276–277 . doi: 10.1016/0140-6736(93)91819-8

192. Walsh SR, Tang TY, Kullar P, et al (2008) Ischaemic preconditioning during cardiac surgery: systematic review and meta-analysis of perioperative outcomes in randomised clinical trials. Eur J Cardio-thoracic Surg 34:985–994 . doi: 10.1016/j.ejcts.2008.07.062

193. Muller DW, Topol EJ, Califf RM, et al (1990) Relationship between antecedent angina pectoris and short-term prognosis after thrombolytic therapy for acute myocardial infarction. Thrombolysis and Angioplasty in Myocardial Infarction (TAMI) Study Group. Am Heart J 119:224–31 . doi: 10.1016/S0002-8703(05)80008-0

194. Masci PG, Andreini D, Francone M, et al (2013) Prodromal angina is associated with myocardial salvage in acute ST-segment elevation myocardial infarction. Eur Heart J Cardiovasc Imaging 14:1041–1048 . doi: 10.1093/ehjci/jet063

195. Ikonomidis JS, Tumiati LC, Weisel RD, et al (1994) Preconditioning human ventricular cardiomyocytes with brief periods of simulated ischaemia. Cardiovasc Res 28:1285–91

196. Walker DM, Walker JM, Pugsley WB, et al (1995) Preconditioning in isolated superfused human muscle. J Mol Cell Cardiol 27:1349–57

197. Schulman D, Latchman DS, Yellon DM (2002) Urocortin protects the heart from reperfusion injury via upregulation of p42 / p44 MAPK signaling pathway. Am J Physiol - Hear Circ Physiol 283:1481–1488

198. Uchiyama T, Engelman RM, Maulik N, Das DK (2004) Role of Akt signaling in mitochondrial survival pathway triggered by hypoxic preconditioning. Circulation 109:3042–9 . doi: 10.1161/01.CIR.0000130647.29030.90

199. Hausenloy DJ, Tsang A, Mocanu MM, Yellon DM (2005) Ischemic preconditioning protects by activating prosurvival kinases at reperfusion. Am J Physiol Heart Circ Physiol 288:H971-6 . doi: 10.1152/ajpheart.00374.2004

200. Aleshin A, Ananthakrishnan R, Li Q, et al (2008) RAGE modulates myocardial injury consequent to LAD infarction via impact on JNK and STAT signaling in a murine model. AJP Hear Circ Physiol 294:H1823–H1832 . doi: 10.1152/ajpheart.01210.2007

201. Heusch G, Musiolik J, Kottenberg E, et al (2012) STAT5 activation and cardioprotection by remote ischemic preconditioning in humans: short communication. Circ Res 110:111–5 . doi: 10.1161/CIRCRESAHA.111.259556

202. Clarke SJ, Khaliulin I, Das M, et al (2008) Inhibition of mitochondrial permeability transition pore opening by ischemic preconditioning is probably mediated by reduction of oxidative stress rather than mitochondrial protein phosphorylation. Circ Res 102:1082–1090 . doi: 10.1161/CIRCRESAHA.107.167072

203. Schmidt MR, Redington A, Bøtker HE (2015) Remote conditioning the heart overview: Translatability and mechanism. Br J Pharmacol 172:1947–1960 . doi: 10.1111/bph.12933

204. Hausenloy DJ, Tsang A, Yellon DM (2005) The Reperfusion Injury Salvage Kinase Pathway: A Common Target for Both Ischemic Preconditioning and Postconditioning. Trends Cardiovasc Med 15:69–75 . doi: 10.1016/j.tcm.2005.03.001

205. Verouhis D, Sörensson P, Gourine A, et al (2016) Effect of remote ischemic conditioning on infarct size in patients with anterior ST-elevation myocardial infarction. Am Heart J 181:66–73 . doi: 10.1016/j.ahj.2016.08.004

206. McLeod SL, Iansavichene A, Cheskes S (2017) Remote ischemic perconditioning to reduce reperfusion injury during acute ST-segment-elevation myocardial infarction: A systematic review and meta-analysis. J Am Heart Assoc 6: . doi: 10.1161/JAHA.117.005522

207. Sivaraman V, Hausenloy DJ, Wynne AM, Yellon DM (2010) Preconditioning the diabetic human myocardium. J Cell Mol Med 14:1740–1746 . doi: 10.1111/j.1582-4934.2009.00796.x

208. Schulz R, Post H, Vahlhaus C, Keusch G (1998) Ischemic preconditioning in pigs: A graded phenomenon: Its relation to adenosine and bradykinin. Circulation 98:1022–1029 . doi: 10.1161/01.CIR.98.10.1022

209. Yamasaki K, Fujiwara H, Tanaka M, et al (1997) Preconditioning with 15-minute ischemia extends myocardial infarct size after subsequent 30-minute ischemia in rabbits. Jpn Circ J 61:344–52

210. Heusch G (2015) Molecular basis of cardioprotection signal transduction in ischemic pre-, post-, and remote conditioning. Circ Res 116:674–699 . doi: 10.1161/CIRCRESAHA.116.305348

211. Juhaszova M, Zorov DB, Kim S-H, et al (2004) Glycogen synthase kinase-3β mediates convergence of protection signaling to inhibit the mitochondrial permeability transition pore. J Clin Invest 113:1535–1549 . doi: 10.1172/JCI19906

212. Gomez L, Paillard M, Thibault H, et al (2008) Inhibition of GSK3beta by postconditioning is required to prevent opening of the mitochondrial permeability transition pore during reperfusion. Circulation 117:2761–8 . doi: 10.1161/CIRCULATIONAHA.107.755066

213. Nishino Y, Webb IG, Davidson SM, et al (2008) Glycogen synthase kinase-3 inactivation is not required for ischemic preconditioning or postconditioning in the mouse. Circ Res 103:307–14 . doi: 10.1161/CIRCRESAHA.107.169953

214. Skyschally A, van Caster P, Boengler K, et al (2009) Ischemic postconditioning in pigs: no causal role for RISK activation. Circ Res 104:15–8 . doi: 10.1161/CIRCRESAHA.108.186429

215. Lecour S (2009) Activation of the protective Survivor Activating Factor Enhancement (SAFE) pathway against reperfusion injury: Does it go beyond the RISK pathway? J Mol Cell Cardiol 47:32–40 . doi: 10.1016/j.yjmcc.2009.03.019

216. Taegtmeyer H, Sen S, Vela D (2010) Return to the fetal gene program: a suggested metabolic link to gene expression in the heart. Ann N Y Acad Sci 1188:191–8 . doi: 10.1111/j.1749-6632.2009.05100.x

217. Dirkx E, da Costa Martins PA, De Windt LJ (2013) Regulation of fetal gene expression in heart failure. Biochim Biophys Acta - Mol Basis Dis 1832:2414–2424 . doi: 10.1016/j.bbadis.2013.07.023

218. Hinkle PC (2005) P/O ratios of mitochondrial oxidative phosphorylation. Biochim Biophys Acta 1706:1–11 . doi: 10.1016/j.bbabio.2004.09.004

219. Gumina RJ, Mizumura T, Beier N, et al (1998) A new sodium/hydrogen exchange inhibitor, EMD 85131, limits infarct size in dogs when administered before or after coronary artery occlusion. J

Pharmacol Exp Ther 286:175–83

220. Javadov S, Choi A, Rajapurohitam V, et al (2008) NHE-1 inhibition-induced cardioprotection against ischaemia/reperfusion is associated with attenuation of the mitochondrial permeability transition. Cardiovasc Res 77:416–24 . doi: 10.1093/cvr/cvm039

221. Zeymer U, Suryapranata H, Monassier JP, et al (2001) The Na(+)/H(+) exchange inhibitor eniporide as an adjunct to early reperfusion therapy for acute myocardial infarction. Results of the evaluation of the safety and cardioprotective effects of eniporide in acute myocardial infarction (ESCAMI) trial. J Am Coll Cardiol 38:1644–1650 . doi: S0735109701016084 [pii]

222. Bond JM, Herman B, Lemasters JJ (1991) Protection by acidotic pH against anoxia/reoxygenation injury to rat neonatal cardiac myocytes. Biochem Biophys Res Commun 179:798–803 . doi: 10.1016/0006-291X(91)91887-I

223. Vaughan-Jones RD, Spitzer KW, Swietach P (2009) Intracellular pH regulation in heart. J Mol Cell Cardiol 46:318–331 . doi: 10.1016/j.yjmcc.2008.10.024

224. Leem CH, Lagadic-Gossmann D, Vaughan-Jones RD (1999) Characterization of intracellular pH regulation in the guinea-pig ventricular myocyte. J Physiol 517 (Pt 1:159–80

225. Halestrap AP, Richardson AP (2015) The mitochondrial permeability transition: a current perspective on its identity and role in ischaemia/reperfusion injury. J Mol Cell Cardiol 78:129–41 . doi: 10.1016/j.yjmcc.2014.08.018

226. Skyschally A, Schulz R, Heusch G (2010) Cyclosporine A at reperfusion reduces infarct size in pigs. Cardiovasc drugs Ther 24:85–7 . doi: 10.1007/s10557-010-6219-y

227. De Paulis D, Chiari P, Teixeira G, et al (2013) Cyclosporine A at reperfusion fails to reduce infarct size in the in vivo rat heart. Basic Res Cardiol 108:379 . doi: 10.1007/s00395-013-0379-4

228. Karlsson LO, Zhou A-X, Larsson E, et al (2010) Cyclosporine does not reduce myocardial infarct size in a porcine ischemia-reperfusion model. J Cardiovasc Pharmacol Ther 15:182–9 . doi: 10.1177/1074248410362074

229. Piot C, Croisille P, Staat P, et al (2008) Effect of cyclosporine on reperfusion injury in acute myocardial infarction. N Engl J Med 359:473–81 . doi: 10.1056/NEJMoa071142

230. Mewton N, Croisille P, Gahide G, et al (2010) Effect of Cyclosporine on Left Ventricular Remodeling After Reperfused Myocardial Infarction. J Am Coll Cardiol 55:1200–1205 . doi: 10.1016/j.jacc.2009.10.052

231. Hausenloy D, Kunst G, Boston-Griffiths E, et al (2014) The effect of cyclosporin-A on peri-operative myocardial injury in adult patients undergoing coronary artery bypass graft surgery: a randomised controlled clinical trial. Heart 100:544–549 . doi: 10.1136/heartjnl-2013-304845

232. Chiari P, Angoulvant D, Mewton N, et al (2014) Cyclosporine Protects the Heart during Aortic Valve Surgery. Anesthesiology 121:232–238 . doi: 10.1097/ALN.0000000000000331

233. Heusch G (2015) CIRCUS: A kiss of death for cardioprotection? Cardiovasc Res 108:215–216 . doi: 10.1093/cvr/cvv225

234. Leshnower BG, Kanemoto S, Matsubara M, et al (2008) Cyclosporine preserves mitochondrial morphology after myocardial ischemia/reperfusion independent of calcineurin inhibition. Ann

Thorac Surg 86:1286–92 . doi: 10.1016/j.athoracsur.2008.06.033

235. Rigobello MP, Scutari G, Boscolo R, Bindoli A (2002) Induction of mitochondrial permeability transition by auranofin, a gold(I)-phosphine derivative. Br J Pharmacol 136:1162–8 . doi: 10.1038/sj.bjp.0704823

236. Gill RS, Bigam DL, Cheung P-Y (2012) The Role of Cyclosporine in the Treatment of Myocardial Reperfusion Injury. Shock 37:341–347 . doi: 10.1097/SHK.0b013e31824bc9ab

237. Zhong Z, Theruvath TP, Currin RT, et al (2007) NIM811, a Mitochondrial Permeability Transition Inhibitor, Prevents Mitochondrial Depolarization in Small-for-Size Rat Liver Grafts. Am J Transplant 7:1103–1111 . doi: 10.1111/j.1600-6143.2007.01770.x

238. Zweier JL, Flaherty JT, Weisfeldt ML (1987) Direct measurement of free radical generation following reperfusion of ischemic myocardium. Proc Natl Acad Sci U S A 84:1404–7

239. Toyokuni S (1999) Reactive oxygen species-induced molecular damage and its application in pathology. Pathol Int 49:91–102 . doi: 10.1046/j.1440-1827.1999.00829.x

240. de Lorgeril M, Basmadjian A, Lavallée M, et al (1989) Influence of leukopenia on collateral flow, reperfusion flow, reflow ventricular fibrillation, and infarct size in dogs. Am Heart J 117:523–32

241. Sochman J, Kolc J, Vrána M, Fabián J (1990) Cardioprotective effects of N-acetylcysteine: the reduction in the extent of infarction and occurrence of reperfusion arrhythmias in the dog. Int J Cardiol 28:191–6

242. Forman MB, Puett DW, Cates CU, et al (1988) Glutathione redox pathway and reperfusion injury. Effect of N-acetylcysteine on infarct size and ventricular function. Circulation 78:202–213

243. Sochman J, Vrbská J, Musilová B, Rocek M (1996) Infarct Size Limitation: acute N-acetylcysteine defense (ISLAND trial): preliminary analysis and report after the first 30 patients. Clin Cardiol 19:94–100

244. (2000) Effect of 48-h intravenous trimetazidine on short- and long-term outcomes of patients with acute myocardial infarction, with and without thrombolytic therapy; A double-blind, placebo-controlled, randomized trial. The EMIP-FR Group. European Myocardial Inf. Eur Heart J 21:1537–46 . doi: 10.1053/euhj.1999.2439

245. Flaherty JT, Pitt B, Gruber JW, et al (1994) Recombinant human superoxide dismutase (h-SOD) fails to improve recovery of ventricular function in patients undergoing coronary angioplasty for acute myocardial infarction. Circulation 89:1982–91

246. Molnár Z, Shearer E, Lowe D (1999) N-Acetylcysteine treatment to prevent the progression of multisystem organ failure: a prospective, randomized, placebo-controlled study. Crit Care Med 27:1100–4

247. Chouchani ET, Pell VR, Gaude E, et al (2014) Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS. Nature 515:431–435 . doi: 10.1038/nature13909

248. Valls-Lacalle L, Barba I, Miró-Casas E, et al (2016) Succinate dehydrogenase inhibition with malonate during reperfusion reduces infarct size by preventing mitochondrial permeability transition. Cardiovasc Res 109:374–84 . doi: 10.1093/cvr/cvv279

249. Schieber M, Chandel NS (2014) ROS function in redox signaling and oxidative stress. Curr Biol

24:R453-62 . doi: 10.1016/j.cub.2014.03.034

250. Madamanchi NR, Runge MS (2007) Mitochondrial dysfunction in atherosclerosis. Circ Res 100:460–73 . doi: 10.1161/01.RES.0000258450.44413.96

251. Chen Q, Moghaddas S, Hoppel CL, Lesnefsky EJ (2008) Ischemic defects in the electron transport chain increase the production of reactive oxygen species from isolated rat heart mitochondria. Am J Physiol - Cell Physiol 294:C460–C466 . doi: 10.1152/ajpcell.00211.2007.

252. Zorov DB, Juhaszova M, Sollott SJ (2014) Mitochondrial Reactive Oxygen Species (ROS) and ROS-Induced ROS Release. Physiol Rev 94:909–950 . doi: 10.1152/physrev.00026.2013

253. Zorov DB, Juhaszova M, Sollott SJ (2006) Mitochondrial ROS-induced ROS release: An update and review. Biochim Biophys Acta - Bioenerg 1757:509–517 . doi: 10.1016/J.BBABIO.2006.04.029

254. Smith RA, Porteous CM, Coulter C V, Murphy MP (1999) Selective targeting of an antioxidant to mitochondria. Eur J Biochem 263:709–16

255. Murphy MP, Smith RAJ (2007) Targeting antioxidants to mitochondria by conjugation to lipophilic cations. Annu Rev Pharmacol Toxicol 47:629–56 . doi: 10.1146/annurev.pharmtox.47.120505.105110

256. Monassier L, Ayme-Dietrich E, Aubertin-Kirch G, Pathak A (2016) Targeting myocardial reperfusion injuries with cyclosporine in the CIRCUS Trial - pharmacological reasons for failure. Fundam Clin Pharmacol 30:191–193 . doi: 10.1111/fcp.12177

257. Ferdinandy P, Hausenloy DJ, Heusch G, et al (2014) Interaction of risk factors, comorbidities, and comedications with ischemia/reperfusion injury and cardioprotection by preconditioning, postconditioning, and remote conditioning. Pharmacol Rev 66:1142–74 . doi: 10.1124/pr.113.008300

258. Szilvassy Z, Ferdinandy P, Szilvassy J, et al (1995) The loss of pacing-induced preconditioning in atherosclerotic rabbits: role of hypercholesterolaemia. J Mol Cell Cardiol 27:2559–69 . doi: 10.1006/jmcc.1995.0043

259. Song H, Zandstra PW, Radisic M (2011) Engineered heart tissue model of diabetic myocardium. Tissue Eng Part A 17:1869–78 . doi: 10.1089/ten.TEA.2010.0617

260. Li H, Liu Z, Wang J, et al (2013) Susceptibility to myocardial ischemia reperfusion injury at early stage of type 1 diabetes in rats. Cardiovasc Diabetol 12:133 . doi: 10.1186/1475-2840-12-133

261. Hansen A, Eder A, Bönstrup M, et al (2010) Development of a drug screening platform based on engineered heart tissue. Circ Res 107:35–44 . doi: 10.1161/CIRCRESAHA.109.211458

262. Hirt MN, Sörensen N a, Bartholdt LM, et al (2012) Increased afterload induces pathological cardiac hypertrophy: a new in vitro model. Basic Res Cardiol 107:307 . doi: 10.1007/s00395-012-0307-z

263. Kocsis GF, Pipis J, Fekete V, et al (2008) Lovastatin interferes with the infarct size-limiting effect of ischemic preconditioning and postconditioning in rat hearts. Am J Physiol Heart Circ Physiol 294:H2406–H2409 . doi: 10.1152/ajpheart.00862.2007

264. Cope DK, Impastato WK, Cohen M V, Downey JM (1997) Volatile anesthetics protect the ischemic rabbit myocardium from infarction. Anesthesiology 86:699–709 . doi: 10.1167/8.5.1.

265. Kottenberg E, Thielmann M, Bergmann L, et al (2012) Protection by remote ischemic preconditioning during coronary artery bypass graft surgery with isoflurane but not propofol - a clinical trial. Acta Anaesthesiol Scand 56:30–8 . doi: 10.1111/j.1399-6576.2011.02585.x

266. Tai W, Shi E, Yan L, et al (2012) Diabetes abolishes the cardioprotection induced by sevoflurane postconditioning in the rat heart in vivo: Roles of glycogen synthase kinase-3β and its upstream pathways. J Surg Res 178:96–104 . doi: 10.1016/j.jss.2012.02.021

267. Fan Y, Yang S, Zhang X, et al (2012) Comparison of cardioprotective efficacy resulting from a combination of atorvastatin and ischaemic post-conditioning in diabetic and non-diabetic rats. Clin Exp Pharmacol Physiol 39:938–943 . doi: 10.1111/1440-1681.12014

268. Lemoine S, Durand C, Zhu L, et al (2010) Desflurane-induced postconditioning of diabetic human right atrial myocardium in vitro. Diabetes Metab 36:21–28 . doi: 10.1016/j.diabet.2009.06.006

269. Skyschally A, Walter B, Heusch G (2013) Coronary microembolization during early reperfusion: Infarct extension, but protection by ischaemic postconditioning. Eur Heart J 34:3314–3321 . doi: 10.1093/eurheartj/ehs434

270. Hamirani YS, Wong A, Kramer CM, Salerno M (2014) Effect of microvascular obstruction and intramyocardial hemorrhage by CMR on LV remodeling and outcomes after myocardial infarction: A systematic review and meta-analysis. JACC Cardiovasc Imaging 7:940–952 . doi: 10.1016/j.jcmg.2014.06.012

271. Brosh D, Assali AR, Mager A, et al (2007) Effect of no-reflow during primary percutaneous coronary intervention for acute myocardial infarction on six-month mortality. Am J Cardiol 99:442–5 . doi: 10.1016/j.amjcard.2006.08.054

272. Engler RL, Schmid-Schönbein GW, Pavelec RS (1983) Leukocyte capillary plugging in myocardial ischemia and reperfusion in the dog. Am J Pathol 111:98–111

273. Ito BR, Schmid-Schönbein G, Engler RL (1990) Effects of leukocyte activation on myocardial vascular resistance. Blood Cells 16:145-63; discussion 163–6

274. Galaup A, Gomez E, Souktani R, et al (2012) Protection against myocardial infarction and no-reflow through preservation of vascular integrity by angiopoietin-like 4. Circulation 125:140–149 . doi: 10.1161/CIRCULATIONAHA.111.049072

275. Chiu LLY, Montgomery M, Liang Y, et al (2012) Perfusable branching microvessel bed for vascularization of engineered tissues. Proc Natl Acad Sci U S A 109:E3414-23 . doi: 10.1073/pnas.1210580109

276. Iliodromitis EK, Kremastinos DT, Katritsis DG, et al (1997) Multiple Cycles of Preconditioning Cause Loss of Protection in Open-chest Rabbits. J Mol Cell Cardiol 29:915–920 . doi: 10.1006/jmcc.1996.0328

277. Johnsen J, Pryds K, Salman R, et al (2016) The remote ischemic preconditioning algorithm: effect of number of cycles, cycle duration and effector organ mass on efficacy of protection. Basic Res Cardiol 111:1–10 . doi: 10.1007/s00395-016-0529-6

278. Przyklenk K (2015) Ischaemic conditioning: pitfalls on the path to clinical translation. Br J Pharmacol 172:1961–73 . doi: 10.1111/bph.13064

279. Ludman A, Venugopal V, Yellon DM, Hausenloy DJ (2009) Statins and cardioprotection - More than just lipid lowering? Pharmacol Ther 122:30–43 . doi: 10.1016/j.pharmthera.2009.01.002

280. Ishii H, Ichimiya S, Kanashiro M, et al (2006) Effects of receipt of chronic statin therapy before the onset of acute myocardial infarction: A retrospective study in patients undergoing primary percutaneous coronary intervention. Clin Ther 28:1812–1819 . doi: 10.1016/J.CLINTHERA.2006.11.003

281. Engstrøm T, Kelbæk H, Helqvist S, et al (2017) Effect of Ischemic Postconditioning During Primary Percutaneous Coronary Intervention for Patients With ST-Segment Elevation Myocardial Infarction: A Randomized Clinical Trial. JAMA Cardiol 2:490–497 . doi: 10.1001/jamacardio.2017.0022

282. Fan Y, Yang S, Cao Y, Huang Y (2013) Effects of acute and chronic atorvastatin on cardioprotection of ischemic postconditioning in isolated rat hearts. Cardiovasc Ther 31:187–192 . doi: 10.1111/j.1755-5922.2012.00318.x

283. Teresi RE, Shaiu CW, Chen CS, et al (2006) Increased PTEN expression due to transcriptional activation of PPARγ by Lovastatin and Rosiglitazone. Int J Cancer 118:2390–2398 . doi: 10.1002/ijc.21799

284. Malhotra HS, Goa KL (2001) Atorvastatin: an updated review of its pharmacological properties and use in dyslipidaemia. Drugs 61:1835–81

285. Kersten JR, Toller WG, Gross ER, et al (2000) Diabetes abolishes ischemic preconditioning: role of glucose, insulin, and osmolality. Am J Physiol Circ Physiol 278:H1218–H1224 . doi: 10.1152/ajpheart.2000.278.4.H1218

286. Kristiansen SB, Løfgren B, Støttrup NB, et al (2004) Ischaemic preconditioning does not protect the heart in obese and lean animal models of type 2 diabetes. Diabetologia 47:1716–21 . doi: 10.1007/s00125-004-1514-4

287. Hassouna A, Loubani M, Matata BM, et al (2006) Mitochondrial dysfunction as the cause of the failure to precondition the diabetic human myocardium. Cardiovasc Res 69:450–458 . doi: 10.1016/j.cardiores.2005.11.004

288. Wang X, Yang L, Kang L, et al (2017) Metformin attenuates myocardial Ischemia-reperfusion injury via Up-regulation of antioxidant enzymes. PLoS One 12:1–13 . doi: 10.1371/journal.pone.0182777

289. Alegria JR, Miller TD, Gibbons RJ, et al (2007) Infarct size, ejection fraction, and mortality in diabetic patients with acute myocardial infarction treated with thrombolytic therapy. Am Heart J 154:743–750 . doi: 10.1016/j.ahj.2007.06.020

290. Hotta H, Miura T, Miki T, et al (2010) Angiotensin II type 1 receptor-mediated upregulation of calcineurin activity underlies impairment of cardioprotective signaling in diabetic hearts. Circ Res 106:129–32 . doi: 10.1161/CIRCRESAHA.109.205385

291. Li J, Iorga A, Sharma S, et al (2012) Intralipid, a clinically safe compound, protects the heart against ischemia-reperfusion injury more efficiently than cyclosporine-A. Anesthesiology 117:836–46 . doi: 10.1097/ALN.0b013e3182655e73

292. Yang XM, Liu Y, Cui L, et al (2013) Platelet P2Y12blockers confer direct postconditioning-like

protection in reperfused rabbit hearts. J Cardiovasc Pharmacol Ther 18:251–262 . doi: 10.1177/1074248412467692

293. Porter K, Medford HM, McIntosh CM, Marsh SA (2012) Cardioprotection requires flipping the "posttranslational modification" switch. Life Sci 90:89–98 . doi: 10.1016/j.lfs.2011.10.026

294. Gedik N, Krüger M, Thielmann M, et al (2017) Proteomics/phosphoproteomics of left ventricular biopsies from patients with surgical coronary revascularization and pigs with coronary occlusion/reperfusion: Remote ischemic preconditioning. Sci Rep 7:1–22 . doi: 10.1038/s41598-017-07883-5

295. Kim HK, Thu VT, Heo H-J, et al (2011) Cardiac proteomic responses to ischemia-reperfusion injury and ischemic preconditioning. Expert Rev Proteomics 8:241–61 . doi: 10.1586/epr.11.8

296. Wang Y, Wang Q, Zhang L, et al (2017) Coptisine protects cardiomyocyte against hypoxia/reoxygenation-induced damage via inhibition of autophagy. Biochem Biophys Res Commun 1–8 . doi: 10.1016/j.bbrc.2017.06.027

297. Valentim L, Laurence KM, Townsend PA, et al (2006) Urocortin inhibits Beclin1-mediated autophagic cell death in cardiac myocytes exposed to ischaemia/reperfusion injury. J Mol Cell Cardiol 40:846–52 . doi: 10.1016/j.yjmcc.2006.03.428

298. Huang C, Andres AM, Ratliff EP, et al (2011) Preconditioning involves selective mitophagy mediated by parkin and p62/SQSTM1. PLoS One 6: . doi: 10.1371/journal.pone.0020975

299. Sheng R, Zhang L-S, Han R, et al (2010) Autophagy activation is associated with neuroprotection in a rat model of focal cerebral ischemic preconditioning. Autophagy 6:482–494 . doi: 10.4161/auto.6.4.11737

300. O'Farrell F, Rusten TE, Stenmark H (2013) Phosphoinositide 3-kinases as accelerators and brakes of autophagy. FEBS J 280:6322–6337 . doi: 10.1111/febs.12486

301. Kim J, Yang G, Kim Y, et al (2016) AMPK activators: mechanisms of action and physiological activities. Exp Mol Med 48:e224 . doi: 10.1038/emm.2016.16

302. Przyklenk K, Dong Y, Undyala V V., Whittaker P (2012) Autophagy as a therapeutic target for ischaemia /reperfusion injury? Concepts, controversies, and challenges. Cardiovasc Res 94:197–205 . doi: 10.1093/cvr/cvr358

303. Park HK, Chu K, Jung KH, et al (2009) Autophagy is involved in the ischemic preconditioning. Neurosci Lett 451:16–19 . doi: 10.1016/j.neulet.2008.12.019

304. Zorov DB, Filburn CR, Klotz LO, et al (2000) Reactive oxygen species (ROS)-induced ROS release: A new phenomenon accompanying induction of the mitochondrial permeability transition in cardiac myocytes. J Exp Med 192:1001–1014 . doi: 10.1084/jem.192.7.1001

305. Yamano K, Youle RJ (2013) PINK1 is degraded through the N-end rule pathway. Autophagy 9:1758–69 . doi: 10.4161/auto.24633

306. Deas E, Plun-Favreau H, Gandhi S, et al (2011) PINK1 cleavage at position A103 by the mitochondrial protease PARL. Hum Mol Genet 20:867–79 . doi: 10.1093/hmg/ddq526

307. Narendra DP, Jin SM, Tanaka A, et al (2010) PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. PLoS Biol 8: . doi: 10.1371/journal.pbio.1000298

308. Grenier K, Kontogiannea M, Fon EA (2014) Short mitochondrial ARF triggers Parkin/PINK1-dependent mitophagy. J Biol Chem 289:29519–30 . doi: 10.1074/jbc.M114.607150

309. Narendra D, Tanaka A, Suen D-F, Youle RJ (2008) Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. J Cell Biol 183:795–803 . doi: 10.1083/jcb.200809125

310. Larsen S, Nielsen J, Hansen CN, et al (2012) Biomarkers of mitochondrial content in skeletal muscle of healthy young human subjects. J Physiol 590:3349–60 . doi: 10.1113/jphysiol.2012.230185

311. Larson-Casey JL, Deshane JS, Ryan AJ, et al (2016) Macrophage Akt1 Kinase-Mediated Mitophagy Modulates Apoptosis Resistance and Pulmonary Fibrosis. Immunity 44:582–596 . doi: 10.1016/j.immuni.2016.01.001

312. Yu T, Robotham JL, Yoon Y (2006) Increased production of reactive oxygen species in hyperglycemic conditions requires dynamic change of mitochondrial morphology. Proc Natl Acad Sci U S A 103:2653–8 . doi: 10.1073/pnas.0511154103

313. Chen L, Gong Q, Stice JP, Knowlton AA (2009) Mitochondrial OPA1, apoptosis, and heart failure. Cardiovasc Res 84:91–99 . doi: 10.1093/cvr/cvp181

314. Hetz C, Chevet E, Harding HP (2013) Targeting the unfolded protein response in disease. Nat Rev Drug Discov 12:703–19 . doi: 10.1038/nrd3976

315. Tabas I, Ron D (2011) Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress. Nat Cell Biol 13:184–190 . doi: 10.1038/ncb0311-184

316. Minamino T, Komuro I, Kitakaze M (2010) Endoplasmic reticulum stress as a therapeutic target in cardiovascular disease. Circ Res 107:1071–1082 . doi: 10.1161/CIRCRESAHA.110.227819

317. Tajiri S, Oyadomari S, Yano S, et al (2004) Ischemia-induced neuronal cell death is mediated by the endoplasmic reticulum stress pathway involving CHOP. Cell Death Differ 11:403–15 . doi: 10.1038/sj.cdd.4401365

318. Nakagawa T, Zhu H, Morishima N, et al (2000) Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-β. Nature 403:98–103 . doi: 10.1038/47513

319. Zinszner H, Kuroda M, Wang X, et al (1998) CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. Genes Dev 12:982–95

320. Harding HP, Zhang Y, Ron D (1999) Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. Nature 397:271–274 . doi: 10.1038/16729

321. Fan XM, Wong BC, Wang WP, et al (2001) Inhibition of proteasome function induced apoptosis in gastric cancer. Int J cancer 93:481–8

322. Vilatoba M, Eckstein C, Bilbao G, et al (2005) Sodium 4-phenylbutyrate protects against liver ischemia reperfusion injury by inhibition of endoplasmic reticulum-stress mediated apoptosis. Surgery 138:342–351 . doi: 10.1016/j.surg.2005.04.019

323. Brooks AC, Guo Y, Singh M, et al (2014) Endoplasmic reticulum stress-dependent activation of ATF3 mediates the late phase of ischemic preconditioning. J Mol Cell Cardiol 76:138–147 . doi: 10.1016/j.yjmcc.2014.08.011

324. Shintani-Ishida K, Nakajima M, Uemura K, Yoshida K ichi (2006) Ischemic preconditioning protects cardiomyocytes against ischemic injury by inducing GRP78. Biochem Biophys Res Commun 345:1600–1605 . doi: 10.1016/j.bbrc.2006.05.077

325. Grall S, Prunier-Mirebeau D, Tamareille S, et al (2013) Endoplasmic Reticulum Stress Pathway Involvement in Local and Remote Myocardial Ischemic Conditioning. Shock 39:433–439 . doi: 10.1097/SHK.0b013e31828e4f80

326. Bruch J, Xu H, Rösler TW, et al (2017) PERK activation mitigates tau pathology in vitro and in vivo. EMBO Mol Med 9:371–384 . doi: 10.15252/emmm.201606664

327. Papandreou I, Denko NC, Olson M, et al (2011) Identification of an Ire1alpha endonuclease specific inhibitor with cytotoxic activity against human multiple myeloma. Blood 117:1311–4 . doi: 10.1182/blood-2010-08-303099

328. Boyce M, Bryant KF, Jousse C, et al (2005) A Selective Inhibitor of eIF2 Dephosphorylation Protects Cells from ER Stress. Science (80-) 307:935–939 . doi: 10.1126/science.1101902