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Title: Considerations for the Bioengineering of Advanced Cardiac *in vitro* Models of Myocardial Infarction

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ABSTRACT

Despite the latest advances in cardiovascular biology and medicine, myocardial infarction (MI) remains one of the major causes of deaths worldwide. While reperfusion of the myocardium is critical to limit the ischaemic damage typical of an MI event, it causes detrimental morphological and functional changes known as “reperfusion injury”. This complex scenario is poorly represented in currently available models of ischaemia/reperfusion injury, leading to a poor translation of findings from the bench to the bedside. However, more recent bioengineered *in vitro* models of the human heart represent more clinically relevant tools to prevent and treat MI in patients. These include 3D cultures of cardiac cells, the use of patient-derived stem cells and 3D bioprinting technology. This review aims at highlighting the major features typical of a heart attack while comparing current *in vitro, ex vivo* and *in vivo* models. This information has the potential to further guide in developing novel advanced *in vitro* cardiac models of ischaemia/reperfusion injury. It may pave the way for the generation of advanced pathophysiological cardiac models with the potential to develop personalized therapies.

Keywords

Myocardial infarction, *in vitro* and *in vivo* models, 3D cardiac cultures, advanced cardiac models.

1. INTRODUCTION

1.1. Myocardial Ischaemia/Reperfusion Injury.

Cardiovascular disease (CVD), including myocardial infarction (MI) and stroke, is the leading cause of death worldwide.[1] CVD causes more than 17 million deaths and costs US $863 billion worldwide annually.[2] A heart attack is characterized by prolonged (~20 minutes) myocardial ischaemia following the blockade of coronary blood flow due to rupture of mature atherosclerotic plaque and resulting thrombus (**Figure 1**).[3, 4]

This is followed by reactive oxygen species (ROS) production, cardiomyocyte death and fibroblast infiltration.[3, 4] Cardiac ischaemic events account for 85% of total deaths caused by CVD.[5] Restoration of blood flow to the ischaemic area with reperfusion therapy is critical to limit the ischaemic damage.[6] However, myocardial reperfusion following ischaemia is followed by detrimental morphological and functional changes within the myocardium that is known as "myocardial ischaemia/reperfusion (I/R) injury".[7] Given the complexity of molecular, intracellular and extracellular changes typical of the myocardial I/R injury (**Figure 2**), it is critical to better understand their roles during the ischaemic and reperfusion events, as described below.

1.2. Pathophysiology of Myocardial I/R Injury.

Myocardial I/R injury is characterized by changes in *adenosine triphosphate* (*ATP) levels*. During myocardial ischaemia, oxygen depletion inhibits the mitochondrial oxidative phosphorylation, reduces ATP production and shifts cardiomyocyte metabolism from aerobic to anaerobic respiration.[8] The intracellular calcium overload also decreases ATP production, even during the reperfusion event due to the excessive mitochondrial oxygen and ROS production.[9] Altogether, the constant reduction in ATP levels leads to irreversible cardiac injury/cell death.[9, 10]

During ischaemia also the *intracellular pH* decreases below physiological levels due to the accumulation of lactate and protons causing intracellular acidosis.[11, 12] During reperfusion, the pH increases as lactate is removed from the cell and this activates the Na^+-H^+ exchanger and the Na^+-HCO^{3-} symporter to extrude protons.[13] The rapid restoration of physiological pH leads to mitochondrial permeability transition pore (MPTP) opening together with ROS production. This is followed by hypercontracture due to the indirect effect of ion fluxes resulting in increased intracellular calcium and subsequent increase in contractile activity. Together, ROS and cardiomyocyte hypercontracture lead to cardiac muscle death.[14]

Intracellular acidosis typical of the ischaemic insult is followed by the activation of the sodium-hydrogen (Na^+-H^+) exchanger, with increased intracellular sodium and hydrogen levels (*intracellular ionic imbalance*). Together, the acidic environment and the lack of ATP inhibit the sodium-potassium adenosine triphosphatase (Na^+-K^+ ATPase) exchanger that further promotes intracellular sodium accumulation and cell swelling. The sodium overload activates sarcolemmal sodium-calcium (Na^+-Ca^{2+}) exchanger, followed by intracellular calcium accumulation.[15] Upon reperfusion, rise in energy levels activates Na^+-K^+ ATPase exchanger to balance the sodium overload. However, the rapid pH normalization through activation of Na^+/H^+ exchanger and sodium-bicarbonate (Na^+/HCO^{3-}) cotransporter extrudes hydrogen ions causing a further increase in intracellular sodium. This in turn

reactivates reverse mode of sarcolemmal Na^+-Ca^{2+} exchanger, thereby increasing calcium levels and promoting cardiomyocyte death.[16]

ROS are generated in both ischaemic and reperfusion events.[17] During ischaemia, low pH and mitochondrial dysfunction activate ROS synthesis during ischaemia. Hyperoxygenation, xanthine oxidase and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase are responsible for ROS generation during reperfusion.[18] Increased ROS levels cause cell death through damage to DNA, lipids, proteins and triggers MPTP opening.[19] During reperfusion, increased ROS levels lead to cytotoxic peroxynitrite (a *reactive nitrogen specie*, or RNS) by their reaction with nitric oxide (NO).[20] During the reperfusion event, intracellular calcium opens MPTP, activates mitochondria-localized calpains, damages electron transport chain (ETC), releases cytochrome c and produces ROS, all contributing to *mitochondrial dysfunction* and cell death.[21]

Myofibril contracture is inhibited during ischaemia due to a decreased pH, whereas it is increased during reperfusion due to rapid increased in pH levels (*cardiomyocyte hypercontracture*).[16] Calcium overload and calpain-mediated hydrolysis of sarcolemmal and cytostructural proteins during early reperfusion decrease the ability of cardiomyocytes to handle mechanical stress and leads to cytolysis.[16]

Calcium overload during ischaemia and reperfusion leads to activation of intracellular calcium-dependent cysteine proteases, such as *calpain.*[22] This hydrolyzes structural and functional proteins, including sarcoendoplasmic reticulum (SR) calcium transport ATPase (*SERCA*) and ryanodine receptor (*RyR*), and plays a major role in structural remodelling through the production of mitochondrial proapoptotic factors.[22]

Non-myocytes, such as cardiac fibroblasts and endothelial cells, represent approximatively 70% of the cells in the human heart and play a major role during myocardial I/R injury. The primary function of *fibroblasts* is to control the extracellular matrix (ECM) and to maintain the structural integrity of myocardium.[23] Fibroblasts remain scattered in the ECM around the cardiomyocytes to provide electrical and mechanical stimulation to the cardiomyocytes.[24] Following the ischaemic insult, resident cardiac fibroblasts release inflammatory cytokines and chemokines while becoming activated into motile myofibroblasts.[25, 26] These mediate cardiac fibrosis by the excessive production of collagen type I and eventually heart failure.[25, 26] *Endothelial cells* control cardiomyocyte homeostasis, contraction and ventricular relaxation via paracrine signalling. For instance, physiological NO production by endothelial cells is altered during reperfusion injury, as NO reacts with ROS and produces toxic RNS together with other inflammatory factors.[27, 28] Inactivated *platelets* circulate in the bloodstream and only during injury they promote thrombosis and haemostatis.[29, 30] They are recruited and activated in the area surrounding the infarcted zone following reperfusion.[31] They first infiltrate in the myocardium and then adhere to capillaries and venular endothelium resulting in formation of microthrombi. Activated platelets mainly attach to leucocytes and release vasoconstrictive and pro-inflammatory substances. Altogether, microthrombi, vasoconstrictive and pro-inflammatory agents cause cardiac damage following reperfusion post-ischaemia.[31]

To date, there is no optimal model that can completely recapitulate the complex human heart microenvironment as described above. This has led to several challenges in translating preclinical studies from bench to the bedside for patients suffering from a heart attack. Latest developments in three-dimensional (3D) modelling of human cardiac tissues for *in vitro* testing have demonstrated how the molecular, cellular and extracellular microenvironment is critical for optimal human heart biochemistry, physiology and pharmacology. This review article aims to describe the complex scenario of a heart attack or MI in humans and how this has been modelled in animals *in vivo,* isolated organs/ tissues *ex vivo,* and using cells *in vitro*. A thorough description of currently available models is used to identify the pros and cons of both to better engineer new models to study myocardial infarction, focusing on the complex 3D microenvironment of human heart tissues. The goal of this review is to provide insights for the optimal engineering of novel *in vitro* cardiac models by highlighting current challenges in recapitulating the pathophysiology of the human heart described above.

2. EXPERIMENTAL MODELS OF MYOCARDIAL I/R INJURY

2.1. *In vivo* & *ex vivo* Animal Models of Myocardial I/R Injury

Despite their limitations, animal models have been used for a long time as they recapitulate the majority of the features typical of the complex 3D microenvironment of the heart.[32, 33] These include *in vivo* and *ex vivo* models.

Ex vivo Models.

These models were first established in 1895 by Oscar Langendorff to induce ischaemia/reperfusion injury using isolated hearts. The animal heart is attached to a glass water-jacketed spiral column through which a physiological buffer is delivered *via* retrograde perfusion directly into the aorta **(Figure 3A).**[34, 35] The buffer is perfused with constant pressure and is supplied with a mixture of oxygen (95%) and carbon dioxide (5%), kept at physiological pH and temperature conditions. Ischaemia can be induced in two ways either *i*) through a coronary artery ligation; or *ii*) by stopping (or decreasing) the buffer perfusion.[34, 36, 37]

Benefits. *Ex vivo* isolated Langendorff preparations are simpler and cheaper myocardial I/R injury models compared to *in vivo* animal models. *Ex vivo* hearts allow more reproducible ischaemic event experiments to a region of myocardium or to the entire heart. with direct access to the area of interest. While infrequent, even a human heart can be used in a Langendorff preparation.[38] Overall, *ex vivo* models are used to evaluate biochemical, physiological, and pharmacological features regulating the myocardial I/R injury independently of the neurohumoral control.[39]

Limitations. Animal cells differ from human cardiac cells in several biochemical, morphological, physiological and pharmacological features, such as cytoskeletal protein levels and expressions, contractile activity and biochemical interactions.[40, 41] *Ex vivo* animal models poorly translate to

humans as the continuous coronary blood flow may cause tissue oedema. Another major challenge is represented by the lack of physiological and pathophysiological ROS conditions in *ex vivo* I/R models.[42] Another major limitation of these models is that their contractile and chronotropic functions decrease by approximately 5% per hour, despite the fact the tissue may survive for several hours.[39]

In vivo Models.

Ischaemia/reperfusion injury in animals is induced *via* the temporary ligation of either the left anterior descending (LAD) coronary artery or left circumflex artery (LCx).[42] LAD occlusion causes more damage to global left ventricular function over the occlusion of LCx.[43] Both LAD and LCx are occluded in bigger animals (such as pigs and sheep) to induce a bigger infarct.[42] It is an invasive procedure and requires highly skilled staff. A range of animals, from small rodent to large pigs, are being used for *in vivo* models of myocardial I/R injury.[33, 43-46] Anesthetized mice are intubated through the trachea and a cut in the skin of less than 0.5 cm in the middle of a line between xyphoid and left axilla is made to open the thoracic cavity (**Figure 3C**). Another cut in the fourth intercostal space is made to place the retractors for complete view of the heart and the pericardium is removed. Finally, the LAD/ LCx is ligated using a silk suture for 30-60 minutes (ischaemic event) followed by its removal to allow reperfusion of the myocardium (**Figures 3B** and **3D-F**). At this point, the chest cavity is closed with prolene sutures until the animal fully recovers and presents spontaneous breathing.[47]

Benefits. *In vivo* models are optimal for longitudinal studies for repeated imaging *via* echocardiographic ultrasound to measure cardiac function and deformation. Myocardial anatomy and physiology, perfusion, wall motion and contractility can be analysed with another type of imaging that is cardiac magnetic resonance imaging (MRI). Important clinical parameters that can be obtained from these *in vivo* studies are: *i*) infarct size; *ii*) area at risk; *iii*) haemodynamics; *iv*) inflammatory response; *v*) fibrosis; *vi*) neovascularization; and *vii*) myocardial damage.[42, 48] Animal models are advantageous as they integrate the complexity of the whole organism, allow prolonged studies, genetic manipulation and identify effects of metabolites and therapeutic drugs.[49]

Limitations. The LAD/ LCx ligation may vary depending on how consistently the suture is placed in each animal. Difference in branching and direction of the LAD/ LCx among animals can pose various problems reflected on infarct size variation and direct delivery of drug to the heart at the time of reperfusion. Therefore, a very rigorous and long training is needed for the operator to achieve reproducibility of the model.[50] The use of anaesthetic agents during the procedure may play both a negative and positive role on the procedure outcomes as well.[13]The use of anaesthetic agents during the procedure may play both a negative and positive role on the procedure outcomes as well.[13] Furthermore, the models do not recapitulate fully the clinical setting such as the ischemic time, preconditioning and the duration of reperfusion depend on the species and other factors such as gender, age and temperature.[51, 52] For example, the ischemic time for small animals such as mice, rats and rabbit are around 30-40 min while large animals including dogs, pigs and monkeys have an ischemic duration between 60 to 180 min. In

humans, the duration of index ischemia of humans is around 198-411 min, hence the data demonstrated that human hearts are very robust and less sensitive compared to animals.[51]

Further Considerations. Although large animals represent more physiological models of the human heart compared to rodents, they also present several disadvantages, such as higher costs and difficult handling and housing issues. This makes smaller animals preferred models for early preclinical studies. Nevertheless, due to the intrinsic differences between rodent and human cells, bigger animals are better models for studying the metabolism and function of cardiac cells. Data generated by using big animal models better translate into humans as they include clinically relevant infarct size and cardiac function measurements.

Limitations common to both *in vivo* and *ex vivo* animal models: While both *in vivo* and *ex vivo* models recapitulate some of the features typical of human IR injury, such as 3D microenvironment, infarct size, fibrosis and prolonged studies, they still present intrinsic differences with human hearts, such as morphology and hemodynamic parameters and molecular, cellular and extracellular features typical of the human heart. These are heart rate, blood pressure, ejection fraction, myocardial fibre orientation and function, action potential and electromechanical function.[25, 33, 42] Unlike humans, response to ischaemia and pharmacological agents following anaesthesia is masked in animals.[43] Furthermore, patients with myocardial IR injury have frequently co-morbidities such as diabetes, hypertension and renal failure that are routinely treated with additional drugs; for example, beta-blockers, anti-diabetics medication and aspirin compare to animal models.[48]

2.2. *In vitro* Myocardial I/R Injury Models

In vitro disease modelling of myocardial I/R injury allows the use of isolated cardiac cells from either human or animal origin to mimic changes in oxygen levels (hypoxia/normoxia), typical of the ischaemic and reperfused states of an infarcted heart. To date, cells used in *in vitro* disease models of myocardial I/R injury include: *i*) *cultures of primary cardiomyocytes*;[53-56] *ii*) *induced-pluripotent stem cell (iPSC)-derived cardiac cells*;[57, 58] *iii*) *cardiac cell lines*;[59-64] and *iv*) *cardiac progenitor cells*.[65, 66] These cells have been primarily used in 2D monolayer culture, while in some cases these were employed in 3D cultures.[67] Monolayer cultures poorly translate into clinical studies as they lack the complex 3D microenvironment of the heart *in vivo*, including cell-cell and cell-matrix interactions, a complete and hierarchical vascular network and a physiological contractile activity.[68] *In vitro* studies using human cardiac cells represent more clinically relevant tools as animal cells differ in biochemical, physiological and pathophysiological features from the ones present in the human heart.[68] More recent *in vitro* studies using bioengineered heart tissues aimed at developing 3D cultures that better approximate the human microenvironment to overcome the limitations presented by 2D monolayer cultures (**Table 2**). These include: *i*) engineered heart tissues (EHTs), *ii*) cell sheets, *iii*) microfluidics devices, *iv*) heart-on-a-chip, *v*) organoids/spheroid cultures, *vi*) patient-specific stem cell-derived cardiac cells; and *vii*) 3D bioprinted cardiac tissues.[40, 68-71]

2D Monolayer Cultures.

The majority of 2D monolayer cultures used to study myocardial I/R injury presents cardiac myocytes alone.[42, 72] These include either freshly isolated adult rat ventricular cardiomyocytes (ARVCs) or neonatal rat ventricular cardiomyocytes (NRVCs) in hypoxic conditions.[73-75] Other rodent cell types used include stem cell-derived cardiac cells, myogenic cell lines such as Sprague–Dawley rats cardiac stem cells,[76] embryonic rat heart–derived myogenic cell line H9c2,[73, 77, 78] HL-1 cardiomyocytes.[79] Human cardiac cells freshly isolated from heart biopsies are used to overcome the limitations that are species specific.[49] As access to human heart biopsies is restricted to availability, cardiomyocytes differentiated from human-induced pluripotent cell-derived cardiomyocytes (hiPS-CMs) or human embryonic stem cells (hESCs) have been employed to study myocardial I/R injury *in vitro*.[57, 80, 81]

These cells are generally grown on either i) uncoated tissue plastic or *ii*) laminin-, fibronectin-, gelatin- or PEGylated fibrin- precoated tissue plastic.[82-84] More recently, homotypic 2D cultures that better mimic blood flow and contractile activity of the heart microenvironment have been developed as *iii*) microfluidics devices and *heart-on-a-chip* (discussed below).[84, 85]

Benefits. 2D monolayer cultures of cardiac cells are simple to use and well-established IR models. Cells are exposed to controlled oxygen levels and medium conditions for optimal control of nutrients and growth factors.[86]

Limitations. Unfortunately, these cultures present several limitations for *in vitro* testing of myocardial I/R injury, which are specific to their origin.[87] The lack of a 3D microenvironment, including its vascularization and the other prominent cell types relevant to I/R injury, makes studies from 2D models poorly translatable into clinic.[42] Cardiac myocytes in 2D present a different intrinsic sensibility to oxygen level changes compared to human cardiac cells *in vivo* and only recently they have been cultured the physiological 3-5% oxygen levels, compared to the classic 20% oxygen present in an incubator.[88] Critical parameters, such as changes in infarct sizes and cardiac function, cannot be replicated in monolayer cultures. Cell shape cannot be controlled in 2D myocardial I/R injury cells, which determines biophysical cues affecting cell bioactivities, including sarcomere retention and contractile activity and response.[68] In particular, freshly isolated cardiomyocytes cultured *in vitro* present a reduced viability compared with cells *in vivo*. Regarding the use of animal cardiomyocytes, these cultures present morphological, biochemical and functional features that differ from human heart cells.[49] Neonatal and iPSC-derived cardiomyocytes differ from adult cardiomyocytes for several biochemical and physiological features, including cytoskeletal protein expression pattern, metabolism and contractile activity.[49, 89, 90] This controls their sensibility to low oxygen levels, as they do not die in hypoxic conditions (<1% oxygen).[89, 91] Adult primary cardiomyocytes and cardiac cell lines responds to hypoxic conditions similar to adult human cardiomyocytes *in vivo*. However, these 2D cultures of adult cells fail at recapitulating the complex cardiac tissue morphology, haemodynamics and electrophysiology. Other factors that limit adult cardiomyocyte cultures to fully recapitulate the scenario typical of *in vivo* I/R injury in humans is represented by the lack of: *i*) the role played by endothelial cells, fibroblasts and cells regulating the

inflammatory response; *ii*) ECM proteins; *iii*) a vascular network; and *iv*) paracrine signalling(**Figure 4**).[42, 49, 68, 87, 90]

3D Cultures.

While 2D cultures of cardiac myocytes are widely used, other cell types found in the human heart, such as endothelial cells, fibroblasts and circulating cells, play a major role during I/R injury (**Figure 4**).[42, 87] For this reason, 3D cultures of myocardial I/R injury are generated from either cardiac cells alone (homotypic),[67, 92] or by co-culturing cardiomyocytes with other cell types (heterotypic).[71, 93-95] Generation of 3D cultures include: *i*) organoid/spheroid cultures, *ii*) cultures embedded within a scaffold or grown on/in a prefabricated scaffold, *iii*) cell sheets, i*v*) microfluidics devices; and *v*) 3D bioprinted cardiac tissues **(Table 2)**.[96].

Non-cardiomyocyte cells in 3D cultures. The native microenvironment typical of the human heart includes other cell types than cardiomyocytes alone, such as fibroblasts, endothelial cells, inflammatory/immune cells and platelets.[68] For this reason, more recent *in vitro* studies use 3D cultures that included non-cardiomyocyte cells to better study their role in cardiac pathophysiology (**Figure 5**).[70, 93] These non-cardiomyocyte cell populations play a major pathophysiological role in ECM deposition and vascular network formation in the human heart. In particular, post injury, endothelial cells, fibroblasts and platelets promote injury as discussed earlier. More recently, Sebastiao, Serra *et al.*, have developed an *in vitro* human heterotypic model of myocardial I/R injury by co-culturing human cardiac progenitor cells (hCPCs) and hiPS-CMs in 2D cultures at 3% oxygen (normoxic conditions) followed by their treatment with a serum-deficient culture medium in presence of 0.4% oxygen (control cultures were exposed to), better replicating the *in vivo* I/R conditions.[97] In another study, human adipose-derived stem cells (hADSCs) were also co-cultured with human iPSC-derived cardiomyocytes, cardiac fibroblasts and endothelial cells in cardiac organoid cultures.[95] However, the role played by hADSCs is not clear, and the *in vitro* testing of hypoxic conditions required extensive (10 days) exposure to 10% oxygen, which is known to be above *in vivo* 5% (normoxic) oxygen levels.[88] Therefore, the presence of additional cell types is critical in regulating the hypoxic/normoxic conditions for cardiomyocytes and should be considered for the development of novel *in vitro* models to study myocardial I/R injury.

Cultures embedded within a scaffold. To further promote cell-matrix interactions in 3D, cardiac cells have been embedded in scaffolds made of biocompatible polymers (typically hydrogels).[72, 98] Optimal scaffolds are designed to eventually disintegrate over time for physiological remodelling of the tissue in 3D by the same cells.[99] A first scaffold-based contractile cardiac tissue was generated by embedding neonatal rat ventricular cardiomyocytes in collagen sponges using Matrigel.[100] Following studies used this approach to mimic left ventricular wall thinning, a typical event that follows myocardial infarction in a patient.[101] One of the most commonly used models of cardiac cells embedded in a scaffold is represented by EHTs. They were first developed by Eschenhagen *et al.*, in 1997.[102] In the original model, chicken embryonic cardiomyocytes were embedded in collagen hydrogels using casting molds with metallic spacers. Direct measurements of forces as contractile activity under isometric conditions are possible by growing EHTs between two silicon-coated hook-and-loop fastener tubes.[102] A further modified development of these EHTs includes

the replacement of the silicone tube with Velcro-coated glass tubes and chicken cardiomyocytes with rat neonatal cells.[103] Later on, ring structures have been used instead of the original lattices and hiPSC-CMs instead of rodent primary cells.[104, 105] Thanks to these latest developments in using immature cardiac cells, EHTs have been used to study cellular maturation by applying a range of force (stretching) intensity and electrical pacing. Similarly, they have been used in preclinical drug development and safety toxicology to monitor effects of drugs on contractile function, pace making activity, and relaxation. Another 3D model of cardiac cells is the Biowire, developed to improve electrophysiological and calcium handling properties of cardiac constructs.[106] In this 3D model, cardiac cells are embedded in collagen gels around a template suture in a microfabricated well, while electrical field stimulation can be progressively increased in frequency.[106]

Cell sheets. In this model, multiple 2D cellular sheets of a variety of rodent and human cardiac cells types are stacked on top of each other.[107] Cell sheets are generated by using temperature-responsive polymer surfaces to supply controlled release of cell layers and free-floating sheet of cohesive cells that have been tested *in vivo* as well.[108] While less popular recently, this approach has been successfully used to replicate the pulsatile contractile function typical of cardiomyocytes *in vivo.*[109] Similarly, this technology has been used together with biodegradable 3D scaffolds to regenerate cardiac tissue.[84, 109-124]

Organoids and spheroid cultures. These are scaffold-free 3D cellular aggregates by culturing one or more cell types. In order to generate spheroids with defined cellular and extracellular features, including a uniform size, cardiac cells have been cultured in: *i*) hanging drops, *ii*) non-adhesive micro-molds and multiwells plates and *iii*) microfluidics devices; whereas spheroids presenting a non-uniform shape can be generated by using iv) rotation systems.[72, 90, 98] These cultures provide cells with optimal conditions for growth, migration and differentiation, closer to the human 3D microenvironment.[90] Spheroid size is controlled and limited by the development of hypoxia in the center, typical of any tissue thicker than 100-200μm in diameter.[98] More recently, prevascularization of spheroids allowed the culture and growth of cardiac myocytes together with fibroblasts and endothelial cells. When generated from hiPSC-CMs, they possess spontaneous contractile activity and have been used for disease modeling, such as cardiac fibrosis, and cardiac toxicity studies.[71, 94, 125-127] Heterotypic spheroid cultures generated from cardiomyocytes, fibroblasts and endothelial cells present biophysical and biochemical cues similar to *in vivo* human heart tissue.[71, 94, 126-130] Heterotypic vascularized cardiac spheroids have been employed to study the toxic effects of doxorubicin (a well-known cardiotoxic agent used to treat leukemia, lymphoma and breat cancer) at concentrations found in the bloodstream of treated patients.[71, 94, 126] Cardiac spheroids have been also employed together with other biocompatible polymers for 3D bioprinting applications and microfluidics devices.[69]

Microfluidics devices. In order to mimic blood flow, cells are seeded inside a porous bioreactors made of inert polymers, generally poly(dimethylsiloxane) (PDMS).[131, 132] This model is being studied over the years for cardiac disease investigations for cell behaviour analysis.[131-136] The microfluidic devices generated using cardiomyocytes, whose structure allows them to function as heart as called as heart-on-a-chip.[134, 137] The original heart-on-a-chip was modified to controls injection of drugs, embedded electrodes, a metallic base to maintain physiological temperature, and a transparent

cover to visualize any defects. A study has demonstrated the use of heart-on-a-chip in *in vitro* myocardial I/R injury by introducing hypoxia and reoxygenation.[137]

3D bioprinted cardiac tissues. 3D bioprinting technology has emerged in the past fifteen years as a promising tool to engineer tissues with defined geometry and composition for optimal cell viability and function.[68, 69, 138, 139] In particular, a lot of studies have focused on the development of viable and functional 3D bioprinted cardiac tissues for both *in vitro* and *in vivo* applications.[69, 136, 140] Several 3D bioprinting platform are currently available, with a preference for extrusion-based bioprinters due to their reduced pressure and shear stress applied to cells during the bioprinting process.[69] Extrusion of cardiac cells through the nozzle of a 3D bioprinted allows the layer-by-layer deposition of cells in 3D to better approximate the complex 3D hierarchical tissue-like structures with a morphology resembling the one typical of the human heart.[69, 136, 140-142] Cells are mixed with hydrogels to generate cardiac bioinks, which can be 3D bioprinted in different geometries.[143, 144] Therefore, considerations around polymer biocompatibility and structural support for direct encapsulation of cells in tissues are critical.[136] Despite so many recent advances in this field, to date limited human heart tissues have been successfully 3D bioprinted using either single cells,[145] or pre-formed cardiac microtissues.[146] Heterotypic 3D bioprinted cardiac tissues have been developed by co-culturing cardiac myocytes with non-cardiomyocytes, such as endothelial cells and fibroblasts.[136, 143, 144] In a first study, cardiomyocytes were seeded into an endothelialized bioprinted microfibrous lattice, similar to a heart-on-a-chip model.[136] In another study mouse iPSC-CM and human umbilical vein endothelial cells (HUVECs) were bioprinted with microfluidics printing head for differential spatial distribution and to create vascular network.[143] More recently, a study demonstrated the feasibility of 3D bioprinting of patient-derived cells to form human cardiac patches in a smaller scale.[144] While bioprinted cardiac tissues have been used to model calcific aortic valve disease (CAVD),[145] and for electrical and mechanical strength analysis, there is no study reporting the use of 3D bioprinted heart tissues to study myocardial I/R injury *in vitro*.[136, 146]

Benefits. 3D cell cultures allow the use of cardiomyocytes in optimal culturing conditions for: i) oxygen and nutrient gradients; ii) cell shape; iii) tissue size. When co-cultured in 3D, heterotypic cultures present improved: *i*) cell-cell and cell-matrix interactions; *ii*) spatio-temporal gradients of oxygen, nutrients and cytokines; *iii*) ECM; and *iv*) vascular network formation **(Figure 4)**.[68, 94] Altogether, they enhance cardiomyocyte viability and contractile function *in vitro*, with retained sarcomeric structure, essential for optimal contractile activity.[67, 70, 147, 148] In regards to 3D bioprinting, the use of cardiac-tailored bioinks for 3D bioprinting technology comprising patient-specific cells in combination with permissive hydrogels provide potential tools for the development of personalized medicine to both treat and prevent MI-induced damage in patients.[69] Cardiac tailored hydrogels presenting mechanical and electrical features that promote cardiac cell viability and function are at the forefront in this field.[41] From the engineering point of view, the improved survival rate of bioprinted 3D tissues allows the generation of cardiac tissues with complex geometries and composition thereby creating porous inner structures to facilitate oxygen and nutrients supply.[149, 150]

Limitations. *In vitro* 3D cultures of cardiomyocytes alone lack the paracrine effects played by other cell types and the ECM, reflecting on their poor translation into human pathophysiology.[68, 70] In

particular, the inner core of any tissue >100μm in diameter naturally develops tissue hypoxia in absence of a proper vascular network typical.[68, 88].Except for microfluidics devices, *in vitro* co-cultured models continue to lack the dynamic microenvironment typical of the *in vivo* human heart, including continuous blood stream and mechanical forces (*shear stress* and *pressure*) applied on cardiac blood vessels.[68] The presence of endothelial cells is not sufficient for a proper branched vascular network and is regulated by several factors including the presence of non-cardiomyocyte cells at specific ratios.[70] A standardized protocol for generation of bioengineered heart tissues is missing and may affect the performance of 3D cultures following their exposure to myocardial I/R injury, limiting their use as high throughput assays to model and study myocardial I/R injury *in vitro*.[49, 151]

Despite the latest developments in tissue engineering of cardiac tissues using stem cells, bioengineered 3D cardiac tissues are still far from presenting optimal cell viability and function. For instance, the pressure and shear stress applied on cells through the nozzle of a 3D bioprinter, together with suboptimal electrophysical properties, bioactivity and conductivity of hydrogels, negatively affects the viability and function of these cells in 3D. [69, 152] Ultimately, one of the major challenges to overcome remains the generation of a fully developed vascular network within the 3D cardiac tissue [69, 153]. To date, 3D *in vitro* cardiac tissues still fail at fully providing optimal conditions for cells to survive and contract at pathophysiological conditions, features that are critical for the modeling of myocardial I/R injury *in vitro*.[69]

3. DISCUSSION

In this review we have focused on considerations for the optimal modeling of human cardiac tissues to study myocardial I/R injury, as summarized in **Table 1**. We have briefly discussed the cause of MI, its epidemiology, treatment, factors influencing the pathophysiology of myocardial I/R injury and clinical manifestations. To better engineer novel cardiac models to such a complex pathophysiology, our review of previously published studies provides insights on how conventional *in vivo* and *ex vivo* myocardial I/R injury animal models can still provide useful tools depending on whether we use small or large animals. We have highlighted benefits and limitations of currently available *ex vivo*, *in vivo* and *in vitro* myocardial I/R injury models and mentioned emerging technologies, including scaffold-based 3D cultures, scaffold-free organoid/spheroid cultures, cell sheets, microfluidics devices and 3D bioprinted cardiac tissues. Altogether, the knowledge provided by this review article aims at highlighting how a model may be preferred over the others depending on the parameters to be evaluated for myocardial I/R injury patients.

Animal models have been extensively studied in the past for their ease to use based on previously established protocols and their 3D physiological microenvironment. However, their limited potential to directly translate findings to humans have promoted the development of novel *in vitro* assays including 2D and 3D cultures of human cardiac cells. The use of animals fails at fully recapitulating the human heart biochemistry, physiology and pharmacology besides the fact that it presents ethics considerations that may limit their use. .

Studies using monolayer cultures of cardiac cells are not able to replicate pathophysiological myocardial oxygen and nutrient gradients typical of the *in vivo* environment of an infarcted heart. 3D *in vitro* models of the human heart allow a better functional maturation of human cardiomyocytes *in vitro*, together with an improved level of cell-cell and cell-matrix interactions. Also, 3D models overcome other limitations typical of 2D cultures, such as oxygen, metabolite, and nutrient gradients in the culture conditions.[93] However, considerations around dynamic conditions that promote blood flow and its mechanical changes on the human heart, together with the paracrine effects played by other cells and tissues in our body are critical for future considerations in developing advanced cardiac tissues to study the complex scenario typical of myocardial I/R injury (**Figure 6**). Recent studies demonstrated that *in vitro* 3D cardiac cultures may represent improved physiological and pathological *in vitro* models of cardiotoxicity and cardiac fibrosis compared to previous ones, as they better provide the opportunity to replicate complex features of the human heart, such as the cell-cell and cell-matrix interactions observed *in vivo*.[71, 94] The use of human cells in *in vitro* 3D cultures provides promising alternatives to studying myocardial I/R injury, as intrinsic biochemical and physiological features differ between human and animal cells.[42, 93, 154]

Despite the latest advancements in the field of cardiac 3D culture technologies and engineered tissues over the past 20 years, they are still missing the complex integration of critical features such as paracrine effects due to the inflammatory response, blood flow and pathophysiological contractile activity. While current models still fail at fully recapitulating such a complex scenario, there is a hope for future studies that may benefit by the integration of different technologies to generate hybrid cultures for dynamic and static conditions. This may involve the use of 3D bioprinted tissues using microfluidics devices, patient-derived stem cells and optimal oxygen/nutrient conditions. While this field is still evolving given the continuous development of novel biomaterials (including conductive and photoactivated polymers), future studies may benefit from the use of novel advanced *in vitro* models to study myocardial I/R injury for both personalized medicine and high-throughput assays.

Overall, with the continuous progress in cardiovascular tissue engineering we have access to several 3D *in vitro* models that better recapitulate some features of the native like intracellular and extracellular microenvironment.[2, 72, 73] However, the lack of a fully functional 3D vascular network and optimal contractile function prevents the direct translation of findings from the bench to the bedside.

4. CONCLUSIONS

Currently available *ex vivo*, *in vivo* and *in vitro* models are able to address specific questions regarding cues around the myocardial I/R injury. However, they all fail at fully recapitulating the complex *in vivo* scenario of the human heart microenvironment. The engineering of advanced cardiac models to address the gap in current cardiovascular research is expected to include optimal cell composition, biomaterials, technology development and potential for personalized medicine and high throughput assay generation.

Conflict of Interest

The authors declare that this publication was conducted in the absence of any commercial/financial engagements that could be construed as potential conflicts of interest of this review paper.

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FIGURES and TABLES

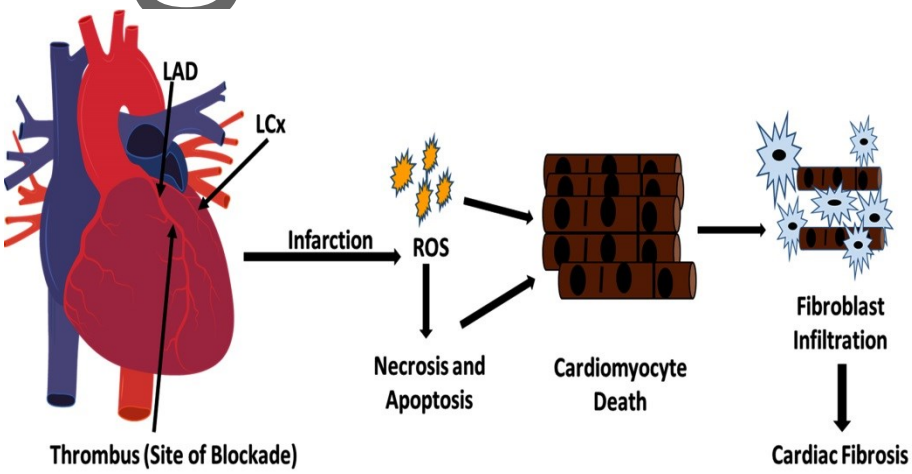

Figure 1

Myocardial Infarction *in vivo*. A schematic summarizing the effects of the ischaemic events following myocardial infarction. These include ROS production followed by cardiomyocyte death and fibroblast infiltration leading to cardiac fibrosis.

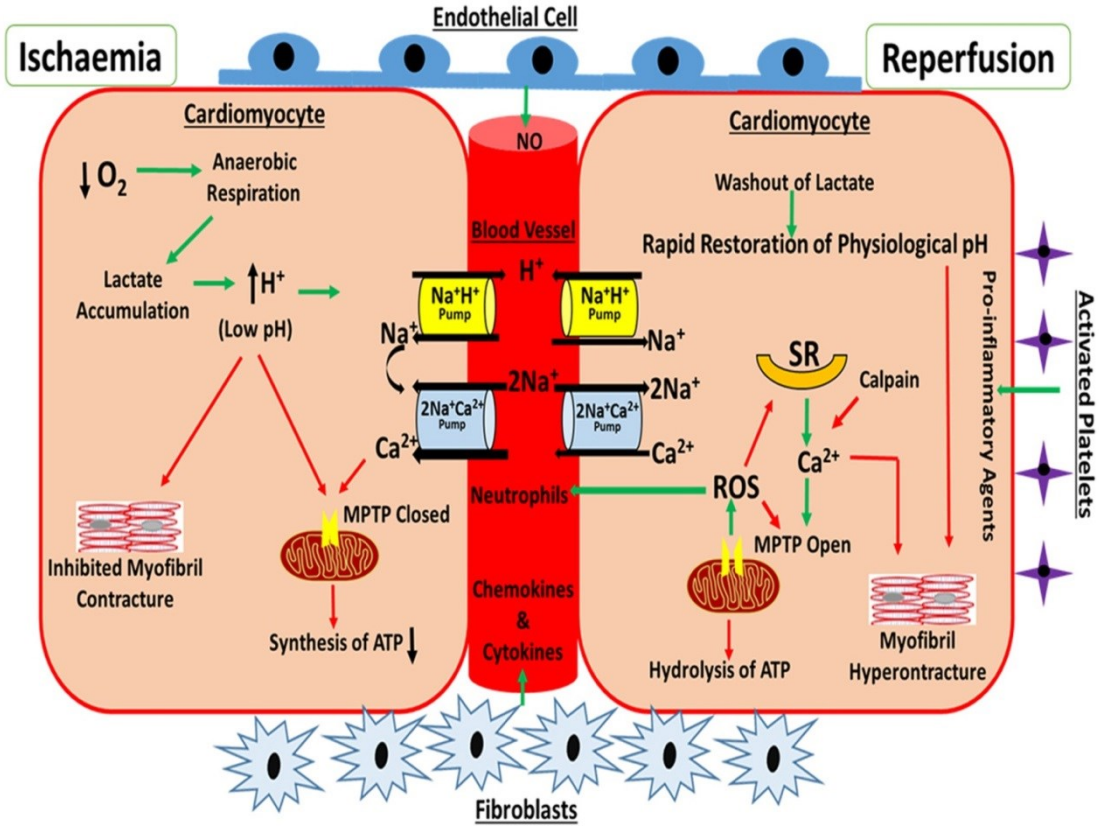

Figure 2

Molecular, Intracellular and Extracellular Mechanisms Regulating Myocardial Ischaemia/Reperfusion Injury. During ischaemia, intracellular pH decreases due to lactate production, activation of the Na^+-H^+ exchanger and the Na^+-Ca^{2+} exchanger. This leads to intracellular Ca^{2+} overload and inhibition of the MPTP opening on the mitochondrial membrane and cardiomyocyte contractility. During reperfusion, there is rapid restoration of physiological intracellular pH, MPTP reopening and cardiomyocyte abnormal contractile activity. Other cells like platelets and fibroblasts exacerbate the inflammatory response, while endothelial cells release toxic levels of NO.

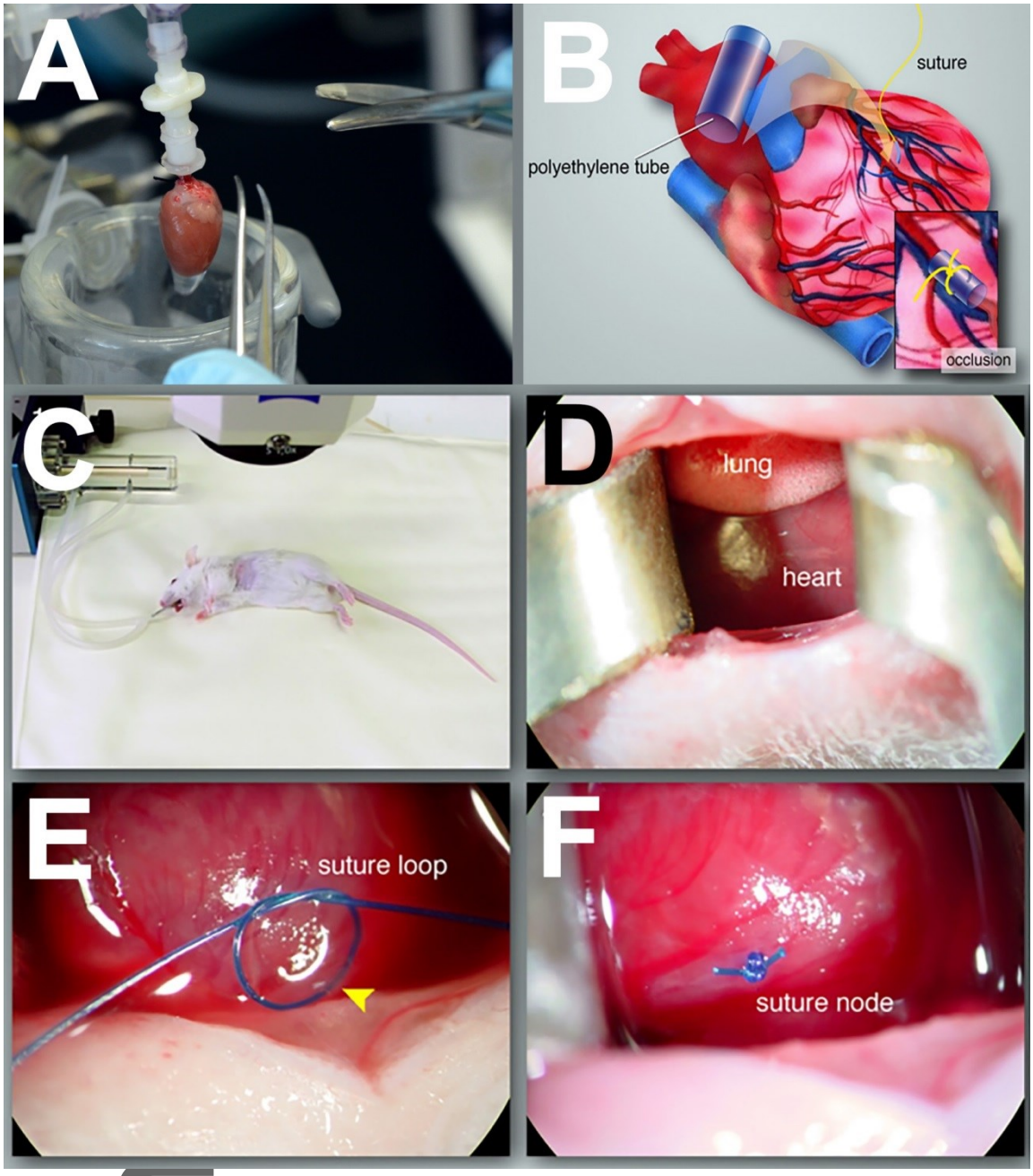

Figure 3

***Ex vivo* and *in vivo* Models of Myocardial I/R injury.** (A) Langendorff heart preparation for *ex vivo* myocardial I/R injury. Reprinted with permission.[155] 2015, Jove (B-F) *In vivo* myocardial I/R injury in mice. To evaluate the acute effects of myocardial I/R injury, the knot is temporarily closed with a polyethylene tube, which is released to reperfuse the heart (B). (C-F) Images showing the position of the suture and the steps of the surgical procedure to model MI *in vivo*. Following the thoracotomy in a ventilated mouse (C), the chest cavity is opened (D), a silk suture is passed beneath the LAD/LCx (E) and a tight knot is made to occlude the artery (F). Reproduced with permission.[156] 2007, Oxford University Press.

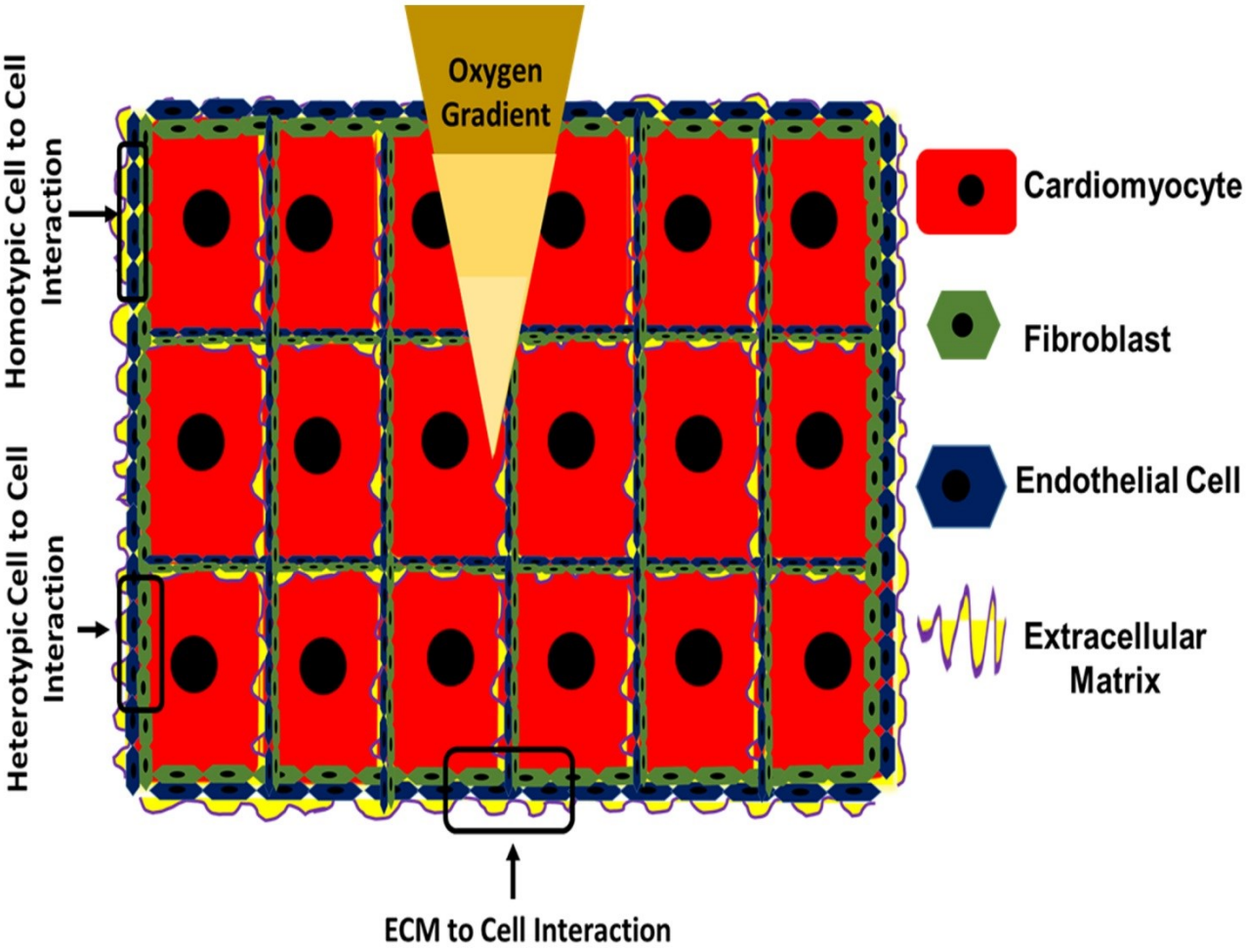

Figure 4

Considerations for the Bioengineering of the Cardiac Microenvironment. This schematic shows cell-cell, cell-matrix interactions and gradients typical of the cardiac tissue microenvironment.

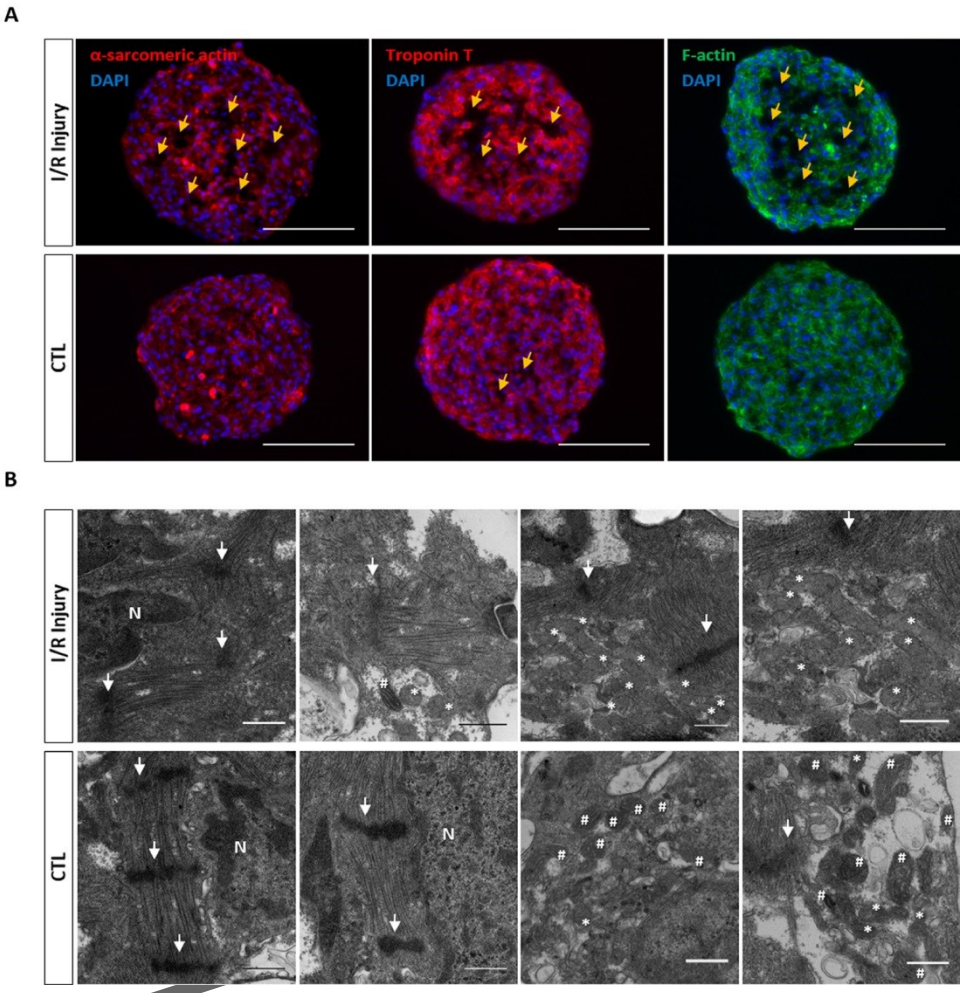

Figure 5
Bioengineered 3D Cell Cultures to study Myocardial Ischaemia/Reperfusion Injury *in vitro*. (A) Fluorescence images of hiPSC-CM spheroid cultures present lacunae (arrows) following I/R injury *in vitro*. (B) TEM analysis showing altered mitochondria (*) following I/R injury. Reproduced with the permission.[93] 2019, Elsevier.

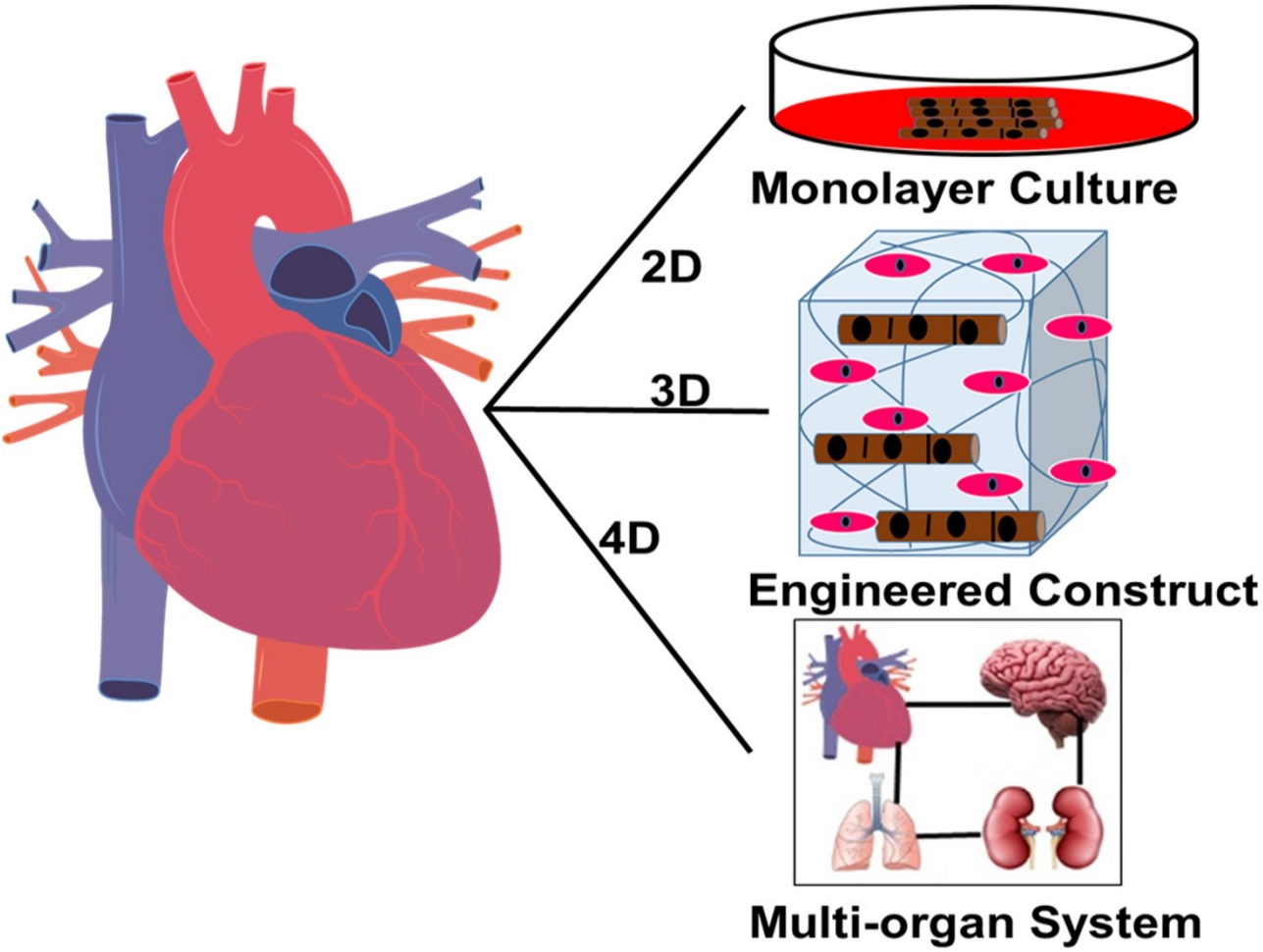

Figure 6

***In vitro* Modeling of Cardiac Tissues.** Complex architectural engineering of advanced *in vitro* models using cardiac cells requires several considerations around static and dynamic conditions, including their interaction with other cellular and extracellular cues from other organs as wells.

Table 1. Features typical of the several in vivo, ex vivo and in vitro myocardial I/R Models.
A comparative summary of the different features typical of the scenario found in MI patients and recapitulated in the different models. ✓ = fully recapitulated; X = not recapitulated; X/✓ = partially recapitulated/requiring additional considerations.

Features Typical of Human Myocardial Tissue for Advanced Model Considerations	*in vivo* Big Animal	*in vivo* Small Animal	*Ex vivo* Animal	*in vitro 2D*		*in vitro 3D*		
				Animal	Human	Animal	Human Homo	Human Hetero
Human Cardiomyocytes	X/✓	X/✓	X/✓	X/✓	✓	X/✓	✓	✓
Human Fibroblasts	X/✓	X/✓	X/✓	X	X	X	X	✓
Human Endothelial Cells	X/✓	X/✓	X/✓	X	X	X	X	✓
Human Platelets	X/✓	X/✓	X/✓	X	X	X	X	✓
Extracellular Matrix	✓	✓	X	X	X	X	X	✓
Vascular Network	✓	✓	X	X	X	X	X	✓
Blood flow	✓	✓	X	X	X	X	X	X
Contractile Function	X	X	X	X	X	X	X	✓
Haemodynamics	X	X	X	X	X	X	X	X
Fibrosis	X/✓	X/✓	X	X	X	X	X	✓
Paracrine Signalling	X/✓	X/✓	X	X	X	X	X	✓
Cardiac function	X/✓	X	X	X	X	X	X	✓
Reduction in ATP Levels	X/✓	X/✓	X/✓	✓	✓	✓	✓	✓
Intracellular pH Changes	✓	✓	✓	X/✓	X/✓	X/✓	X/✓	X/✓
Intracellular Ionic Imbalance	✓	✓	✓	X/✓	X/✓	X/✓	X/✓	✓
Reactive Oxygen Species (ROS)	✓	✓	X/✓	X/✓	X/✓	X/✓	X/✓	X/✓
Mitochondrial Dysfunction	X/✓	X/✓	X/✓	X/✓	✓	X/✓	✓	✓

Cardiomyocyte Hypercontracture	X/✓	X/✓	X/✓	X/✓	✓	X/✓	✓	✓
Calpain System	X/✓	X/✓	X/✓	X	X	X	X	✓
Reactive Nitrogen Species (RNS)	X/✓	X/✓	X/✓	X/✓	X/✓	X/✓	X/✓	✓

Table 2. Pros and Cons of 2D and 3D Engineered Cardiac Tissues

***In vitro* Models**	**Cell Source**	**Biomaterials**	**Pros**	**Cons**
2D Cultures	Primary rodent cells (adult and neonatal)[73-75] Rodent cell line (HL-1 and H9c2)[73, 77-79, 84] Human iPSC-derived cardiomyocytes[80, 81] (Patient-derived cell[83]) Rodent stem cell-derived cardiac cells[76]	Un-coated tissues plastic [81] Fibronectin-Gelatin-precoated surface[82, 83] PEGylated fibrin-precoated tissue plastic[84]	Previously established methods, easy to generate and maintain Suitable for drug testing as high-throughput assays High proliferation rate with low density of cardiomyocytes (*not for adult cells*) Direct access over cardiac muscle and function, useful in testing electromechanical properties.	Cytoskeletal proteins and contractile activity differs between adult and neonatal/iPSC-derived cardiomyocytes Lacks vascularization Limited cell-cell interactions Limited cell-matrix interactions Low ECM production Response to drugs and oxygen changes differ from cells *in vivo*[49] Lacks paracrine signalling and oxygen, and nutrient gradients Lacks *in vivo* morphology

				Static conditions
EHTs	Primary rodent cells (adult and neonatal)[100, 104, 157-162] Chicken embryo[102] Human iPSCs derived cardiomyocytes[105, 106, 158, 163-166]	Collagen sponges[100] Matrigel[100, 104] Fibrogen/matigel[159] Micromolded gelatin[163] Chitosan-enhanced extracellular-matrix (ECM) hydrogels[164] Fibrin-based EHT agarose casting molds with solid silicone racks[105] Polydimethysiloxane (PDMS) racks[158] Velcro-covered rods[102]	Prolonged viability and measurable contractile properties *in vitro* Maturation of neonatal and iPSC-derived cardiomyocytes[167] Can be customized for organotypic functions and diseases Co-cultures with endothelial cells (vascularized constructs) Sensors can be integrated Cardiomyocytes can be stimulated mechanically and electrically Optimal for drug testing as high-throughput assays	Limited diffusion capacity of oxygen and nutrients Limited tissue thickness achievable Cell death in the middle of construct Fragile Lacks paracrine signalling and oxygen, and nutrient gradients Low proliferation rate Static conditions

Cell Sheets	Primary rodent cells (adult and neonatal)[109, 122-124] Primary human cells[118] Rodent cell line (HL-1 and H9c2)[84, 119-121] Human iPSC-derived cardiomyocytes[110-118] Rodent stem cells derived cells[168, 169] [170, 171]	Scaffold-free approach [146, 172] Polycaprolactone (PCL) [120] Polypyrrole[120] Poly(l-lactic acid) (PLA) blending with polyaniline (PANI)[119] Temperature responsive cell culture plates, poly-(N-isopropylacrylamide) (PIPAAm)[173] Fibrin gel[110] Polyglycolic acid, gelatin, alginate, collagen, and fibrin gel[173]	Co-cultures with endothelial cells and fibroblasts (vascularization and ECM production) Mechanical and electrical stimulations, Temperature control	Limited diffusion capacity, Cell death between sheets Limited paracrine signalling and diffusion gradients Lacks the complex hierarchical vascular network found *in vivo* Static conditions
Organoids and Spheroid Cultures	Primary rodent cells (adult and neonatal)[71, 127, 130] Primary human cells[71, 94, 126, 128, 129] Human iPSC-derived cardiomyocytes[94, 126, 129, 174]	Non-adhesive agarose hydrogel molds containing microwells with hemispheric bottoms[129] Scaffold-free (hanging drop culture plates)[126] [71, 94] Gelatin-coated cell culture plastic[174] PEG–fibrinogen hydrogels[130]	Co-cultures with endothelial cells and fibroblasts (vascularization and ECM production) Cell-cell interactions Cell-matrix interactions Paracrine signalling and oxygen, and nutrient gradients Mimics *in vivo* morphology	Hydrogel-embedded spheroids possess limited control on the distribution and shape of cells Spheroid handling requires experienced skills Static conditions Improper nutrient and waste transport

		Gyratory shaking for low attachment well plate[128]		
Microfluidics Devices	Primary rodent cells[132, 136] Primary human cells[136] Rodent cell line (HL-1 and H9c2)[131, 135] Human iPSCs derived cardiomyocytes[132-134]	PDMS[132, 134, 135]	Good control on the dynamic microenvironment Recapitulation of *in vivo* mechanical and chemical factors [175] Fluid flow provides mass transport Ability to integrate with various sensors and actuators Vascularized Cell-cell interactions Cell-matrix interactions Generation of multiple compartments (*body-on-a-chip*) Control of paracrine	Difficult to scale up External pumps, tubing, connectors, and valve are required to operate Cells are grown on stiff substrates (PDMS)

			signalling, pH, oxygen, and nutrient gradients	
3D Bioprinted Tissues	Primary rodent cells (adult and neonatal)[136, 140] Primary human cells[136] Human iPSC-derived cardiomyocytes[69, 143, 144]	GelMA-alginate gel[136] Fibrin-based composite hydrogel[140] Poly(ethylene glycol)-fibrinogen hydrogels[69] Omentum hydrogel[144] Alginate and polyethylene glycol monoacrylate-fibrinogen[143]	Defined location of cells in 3D Hierarchical tissue-like structures Anisotropic growth of cells Co-cultures with endothelial cells and fibroblasts (vascularization and ECM production) Cell-cell interactions Cell-matrix interactions Better mimics the *in vivo* morphology	Forces resulting from hydrogels before, during and after bioprinting process may affect cell viability Fragile constructs Still lacking optimal balance between optimal cell viability and printability of cardiac tissues

Comparison of different *in vitro* cardiac culture based on types constructs they form upon using different cells and biomaterials.

1oo word Gentile

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He is an internationally recognized expert in the field of 3D bioprinting and stem cell technologies for his studies using vascularized cardiac spheroids as novel *in vitro* models of the human heart to study and treat cardiovascular disease.

Major molecular, cellular and extracellular features typical of a heart attack in humans together with a direct comparison of currently available *in vivo*, *ex vivo* and *in vitro* models are summarised in this

review article with the goal to generate advanced bioengineered *in vitro* models better recapitulating the complex *in vivo* microenvironment typical of an infarcted heart.