PROTECTION AND REPAIR OF THE ISCHEMIC HEART

by

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PROTECTION AND REPAIR OF THE ISCHEMIC HEART

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Voor Jolein,
mijn idee van schoonheid

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CHAPTER 1

Introduction

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CLINICAL PERSPECTIVE

Ischemic heart disease (IHD) plagues industrialized nations and increasingly also the developing countries as a disabling disease that can be held responsible for a large proportion of the health-care budget and resources.[1-3] In the European Union (EU) alone, over 1 million cases of acute myocardial infarction (AMI) are treated annually.[2] Current treatment for AMI is aimed at restoration of the blocked coronary blood flow by means of percutaneous coronary intervention (PCI) with stent implantation and pharmacological inhibition of thrombus formation. When applied swiftly, additional loss of contractile cells (cardiomyocytes) can be minimized and, as a result, the short-term mortality of patients enduring an AMI improved considerably to ~ 4-6%, compared to ~ 25-30% in the 1960s.[3] Yet, roughly 1 billion myocytes, which reflect on average 20% of the total cardiomyocyte fraction of the heart is lost after an averaged sized MI. Moreover, our ability to successfully treat the acute stage of the disease came at the expense of a vast increase in patients left behind with a chronic condition that can lead to cardiac adverse remodeling and eventually to congestive heart failure (CHF)(Figure 1A). At present, CHF is suffered by an estimate of 15 million EU citizens with an incidence of ~50.000 new patients each year.[4] Furthermore, as the CHF population rises we are increasingly confronted with the fact that we do not have effective therapies to our disposal that could stop -or even reverse- disease progression with the exception of heart transplantation or assist devices. Unfortunately, these treatment options are only available to a minute fraction of the CHF population in need of treatment due to donor scarcity and is accompanied by costly and continuous need for immunosuppression with its many deleterious side effects. Taken together, these numbers make a strong argument for the development of a new, effective treatment protocol that can minimize initial loss of cardiomyocytes and/or reverse the adverse remodeling processes in the post-MI heart whilst ensuring it is low-cost and readily available to unlimited numbers of patients.

CLINICAL AIMS IN ISCHEMIC HEART DISEASE

As shown in Figure 1B, ischemic heart disease can be viewed as a progressive disorder in which three phases can be distinguished, namely; 1) the acute setting of blocked coronary artery flow leading to myocardial cell death, 2) numerous progressive deleterious changes in left ventricular (LV) architecture and size as well as neurohumoral and adrenergic responses that follows in response to the index event (i.e. acute MI in case of IHD) to ensure adequate cardiac output on short term, recapitulated as 'adverse remodeling', and 3) congestive heart failure in which the heart cannot meet the body's demand on sufficient cardiac output. Over the entire frontier of ischemic heart disease, strategies aimed to abrogate this continuous negative spiral are under intense investigation.[5-7] These strategies can be broadly divided by two different clinical aims, namely; 1) strategies aimed to protect the heart again further cardiomyocyte loss in the acute phase of the MI thereby increasing the initial threshold to enter the vicious circle that can ultimately give rise to heart failure, and 2) strategies aimed to repair and (re)generate lost cardiac myocytes and blood vessels by newly formed counterparts thereby putting a halt to the vicious circle that

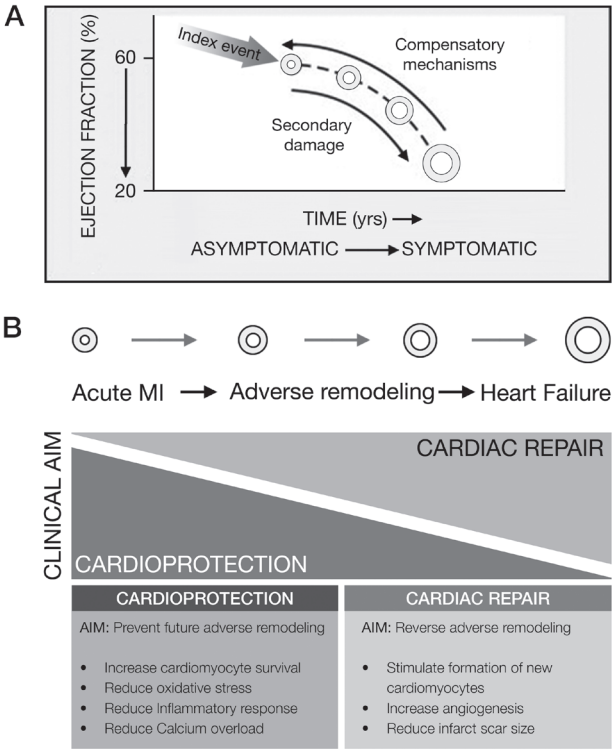

Figure 1. Pathogenesis of congestive heart failure
(A) CHF is the end-stage of a negative cascade that starts after an index event, an acute myocardial infarction in the case of ischemic heart disease, which misbalances the number of force generating cardiomyocytes and the myocardial force that is needed for sufficient cardiac output. **(B)** New clinical therapies for ischemic heart disease can be divided in two different clinical aims, which have their own distinct optimal time span. During or close to the index event, cardioprotection aims to minimize the loss of cardiomyocytes whereby the abovementioned misbalance is improved towards a more favorable ratio. After the onset of adverse remodeling and/or heart failure, cardiac repair compels different cardioregenerative approaches that are aimed to restore the pool of force generating cardiomyocytes thereby reversing the adverse remodeling. *(Figure 1A; Adapted from Mann DL, Mechanisms and Models in HF: A Combinatorial Approach. Circulation 1999:100;999-1008)*

would otherwise have continued and led to CHF. These two clinical aims will now be briefly summarized. A more detailed appraisal of current available evidence on pharmacological regiments for cardioprotection is described in Chapter 2. The concept of heart regeneration and therapeutic use of cardiac stem cells is portrayed in Chapter 4.

CARDIOPROTECTION

The vast majority of MI is based on progressive coronary atherosclerosis. In the event of an acute MI, this is generally superimposed by thrombus formation after the thrombogenic lipid-rich core of the lesion is exposed by rupture of the thin plaque surface.[2] Intuitively, quick restoration of the blood flow ensues rescue of the myocardium that was vascularized by the culprit artery. However, reperfusion of the endangered myocardium paradoxically gives rise to further cell damage and results in the death of cardiomyocytes that were still viable at the moment the vessel was opened.[8-10] Despite the fact that it was still a relatively unknown phenomenon, animal studies brought ischemia/reperfusion (I/R) injury out of limelight as it was estimated to account for a striking ~50% of the total infarct size.[11,12]

Reperfusion injury

Following reperfusion, a cacophony of interconnected mechanisms emerges that collectively culminates in increased cell death.[7,8,13] For simplicity sake, the key mechanisms of cell death following I/R injury can be brought back to four major mechanisms; 1) the occurrence of calcium overload, leading to myocyte hypercontracture, 2) a rapid recovery towards physiological pH, 3) a rapid increase in reactive oxygen species (ROS) and last, 4) neutrophil infiltration of the ischemic area. As a result, a group of proteins called reperfusion injury salvage kinases (RISK)[14-16] and the opening of the mitochondrial permeability transition pore (mPTP)[7,17] have been found as essential targets by which these mechanisms act. The mPTP is a nonselective channel in the inner mitochondrial membrane. Opening of the mPTP channel incapacitates the mitochondria's ability for oxidative phosphorylation that results in ATP depletion and cell death.[18] During ischemia, the mPTP remains closed. However, within the first minutes following reperfusion, mitochondria are overwhelmed by the presence of ROS and the elevated intracellular Ca^{2+} concentration resulting in mPTP opening.[19] Therefore, the mPTP is a crucial determinant of I/R injury and hence has been proven an important new target for cardioprotection. A detailed list of RISK and/or mPTP interference to reduce infarct size is provided in Chapter 2. As means to abrogate necrosis has been investigated in this thesis, this will be briefly introduced.

Necroptosis

Classically, cell death has been viewed as either controlled, cell-induced apoptosis or uncontrolled, cell-independent necrosis. However, this classical view on cell death has failed to withstand the test of time. In 1999, a controlled form of inducible necrosis was reported by means of death receptor stimulation under apoptosis deficient conditions (i.e. caspase inhibition). These conditions could still induce cell death with morphological features of necrosis in certain cell types, supporting the existence of regulated necrosis.[20,21] This new concept of programmed necrosis was termed 'necroptosis' by Degterev and coworkers and they reported an indispensable role for the serine/threonine kinase activity of RIP-1.[22,23] Several reports have shown that RIP-1 modulation yield promising results and small molecule interference with its kinase activity can reduce infarct size up to 30% in rodents.[24,25]

CARDIAC REPAIR

In response to the acute ischemic event, changes in left ventricular (LV) dimension, shape and wall thickness that occur are collectively named adverse remodeling, which by itself further influences cardiac function and prognosis.[26] Besides infarct size as the initial trigger, the two most important factors that influence post-MI remodeling are left ventricular loading conditions and infarct artery patency.[27] As the deficit in cardiomyocytes is aggravated by adverse remodeling rather than improved, the patient finds himself in a vicious circle that terminates in CHF. In essence, opposed to cardioprotection, the overall goal is not primarily to save pre-existing cardiomyocytes, the focus of cardiac repair rather lies within restoration of the cardiomyocyte pool by replacement of lost myocytes with newly generated counterparts. Several biologic and pharmacologic therapies

are currently being tested for this purpose. Given the scope of this thesis, we will focus on one in particular, which is (stem) cell therapy.

Cell based cardiac repair

Formation of new cardiomyocytes based on autologous stem/progenitor cells relies on the premise that these non-committed cells can differentiate into cardiomyocytes, endothelial cells or smooth muscle cells. Among the first generation of cells that were tested for this purpose were skeletal muscle precursor cells, or skeletal myoblasts, as they were relatively resistant to hypoxia and easy to harvest and expand.[28,29] Initial studies showed that there was an improvement in left ventricular systolic function, measured as the ejection fraction (EF) of blood that is pumped out of the LV.[28] However, safety issues were raised when various reports showed that the skeletal myoblasts showed no electromechanical coupling with endogenous preexisting cardiomyocytes and there was an increased event rate of potentially lethal ventricular arrhythmias.[30]

Bone marrow mononuclear cells

Bone marrow mononuclear cells (BMCs) did not seem to have these safety issues and a single report by Orlic[31], published in Nature, showed that large numbers of BMCs could successful be differentiated into functionally contracting cardiomyocytes. Shortly after this break-through in regenerative medicine, BMC therapy was propelled with unprecedented pace towards numerous first-in-man clinical trials. The results of the various initial phase-I safety trials reported BMC therapy to be safe with similar rates of arrhythmias or adverse events between BMC treated patients and controls.[32-34] In line with animal studies, results were highly promising with a LVEF increase of ~15% compared to placebo treated patients.[35,36] Meanwhile, independent research groups failed to reproduce the original work by Orlic and coworkers, thereby questioning the validity of the first report on BMC induced cardiac regeneration.[37] Nevertheless, numerous trials were initiated and, at present, roughly 1500 patients have been treated with BMC therapy word-wide. Recent meta-analyses summarizing the effect of the mononuclear fraction of the bone marrow showed a ~3% increase in LVEF compared to placebo treated patients.[38,39] As many studies that have investigated bone marrow cells were hampered by inappropriate and/or underpowered study design, a large phase III trial is currently being set out to robustly answer the question as to whether bone marrow cell therapy can reduce mortality and improve outcome in AMI patients (BAMI-trial, clinicaltrials.gov: NCT01569178).

Devoid of evidence in support of the principal hypothesis that BMCs have cardiomyogenic potential[37,40], at present, it is widely advocated that the biological black box by which the BMC therapy beneficially influence the heart can be recapitulated as the 'paracrine' effect.[41,42] What this means is that BMC modulate and improve the myocardium by their secretion of a variety of cytokines, various growth factors, and small strands of non-coding RNA (microRNAs) that modulate transcription of messenger RNA. Animal studies have shown that BMC therapy induce neovascularization, protect preexisting cardiomyocytes from apoptotic stimuli, and, interestingly, activate and recruit the endogenous cardiac stem cell compartment to enhance endogenous cardiac repair.[40,43-45]

Besides bone marrow cells, different alternative sources of (stem) cells have been opted to fulfill the unmet need of a cardioregenerative treatment, i.e. the mesenchymal stem cell fraction of the bone marrow[46], adipose derived stem cells[47], tissue-specific (cardiac) stem cells[48] and the cautious use of pluripotent embryonic stem cells (ESCs)[49] or induced *pluripotent* stem cells (iPS cells) under influence of Oct3/4, Sox2, c-Myc and Klf4.[50] Since these are the most commonly used cell types at present to ensue cardiac repair, they will now be briefly outlined.

Mesenchymal stem cells

Mesenchymal stem cells (MSCs), together with hematopoietic stem cells constitute a small subset of the bone marrow mononuclear fraction and have the potential to differentiate towards lineages of mesenchymal origin, i.e. cartilage, bone, fat, connective tissue, smooth muscle and hematopoietic stromal tissue.[51] MSCs were already described in the fifties -called 'stromal cells'- as bone marrow cells that could adhere to plastic.[46] However, during the last decades, several reports showed the multipotent differential capacity of bone marrow MSCs. As of since, they have been isolated from several tissue, including the adipose tissue.[52] One unresolved question pertains to the characterization of MSCs, their immunological status and molecular phenotype. In general, MSCs express cell surface antigens CD73, CD90 and CD105 whilst lacking the expression of surface molecules like major histocompatibility complex II, endothelial (CD31) and hematopoietic markers (CD34, CD45).[53] MSCs can be viewed as immunomodulatory cells that readily interact with a wide range of immune cells as they are a potent inhibitors of T cell activation.[54] Although MSCs cannot differentiate towards a cardiomyocyte phenotype, several combination of cardiopoetic factors could in fact stimulate MSCs to express cardiac genes corresponding to contractile proteins such as alfa sarcomeric actinin and troponin.[55,56] Additionally, their secretome has been found to ameliorate cardiac repair and abrogate cardiac remodeling when tested in animals.[57] When combining the low immunogenic profile with a potent paracrine action, various clones of MSCs are currently developed as an allogeneic 'off-the-shelf' therapy that can be administered for cardioprotective purposes such as in AMI, but also in the post-MI heart.[58,59]

Cardiac stem/progenitor cells

Undoubtedly, one of the most promising cell types for cardiac repair is based the discovery of cardiac stem/progenitor cells, spread throughout the atria and ventricles, of which a small subset have a strong cardiomyogenic potential.[60,61] Similar problems as in the MSC research field exist in underpinning the exact molecular footprint of cardiac stem/progenitor cells (CSCs) and similarities and differences between various types of CSCs are reviewed in more detail in Chapter 4. At present, there is still paucity in our understanding about different CSC types and their relation to each other. Plausibly, as there is considerable overlap between different CSCs and cell surface markers (e.g. c-kit, Sca-1), these cells could represent different phases in stemness or lineage commitment rather that individual CSC populations.

Contrary to bone marrow cells, swift translation of CSC delivery as a new CHF therapy was not facilitated by five decades of research and knowledge on bone marrow transplantation, safety, and handling. Therefore, since their initial discovery in 2003, to date, there have only been two small phase I safety trials published.[62,63] Most importantly, autologous CSC delivery appeared to

be safe and feasible. Regarding efficacy, there was a marked increase in LVEF by 12% compared to control treated patients in the SCIPIO trial.[63] The CADUCEUS trial showed a reduction in infarct size but did not show any change in the LVEF.[62]

Embryonic stem cells and induced pluripotent stem cells

Stemness has been randomly attributed to various 'stem' cells in the research field of cardioregenerative medicine so that it has become almost devoid of meaning. For clarity sake, in this thesis the following definition of stem cells by Potten and Loeffler is used[64]: '*stem cells are defined as cells capable of (a) proliferation, (b) selfmaintenance, (c) the production of a large number of differentiated functional progeny, (d) regenerating the tissue after injury, and (e) a flexibility in the use of these options*'.

ESCs and iPS cells display an unprecedented rate of cellular proliferation and production of functional progeny that is able to differentiate towards any of the somatic cell lineages and germ lines as well as the extra-embryonic tissues that derive from the inner cell mass. Unfortunately, the versatility of ESCs and iPS cells is their strength but on the same page their weakness, as safety issues regarding hyperplasia and teratoma formation still preclude a care-free translation towards a clinically feasible therapy. Recently, a case report emerged in which for the first iPS cells were used in a patient with ischemic heart failure.[65] Time will proof whether the safety hurdles have been overcome and ESCs and/or iPS cells can serve as a potent cell source for cardiac repair.

SCOPE OF THIS THESIS

The overall aim of this thesis is to increase mechanistic understanding on cardiac stem/progenitor cells behavior and function, to advance and translate novel treatment protocols that have shown efficacy and feasibility in rodents to reduce the burden of ischemic heart disease with the use of large animal models, and finally, to optimize a reproducible and reliable large animal models that, as it closely mimics the human situation, can serve as a proxy for human cardiovascular disease.

With the above-mentioned aims in view, **Chapter 2** provides an overview of the concept of cell death in the reperfused heart following an ischemic event. It entails the different mechanisms by which and to what extent cell death occurs and summarizes the results that have been booked in this research field of cardioprotection.

As contemplated in Chapter 2, **Chapter 3** describes the rationale and use of necrostatin-1, a small molecule compound that inhibits the kinase activity of RIP1, when tested in a pig model of ischemia followed by reperfusion.

In **Chapter 4**, we sought to critically appraise the concept of cardiomyocyte renewal in the adult mammalian heart. As is turns out, the heart is not as 'static' and terminally differentiated as previously postulated. Additionally, the role of tissue specific stem/progenitor cells herein is evaluated and evidence on other explanations that may underlie cardiomyocyte renewal are also evaluated.

Chapter 5 and **6** cover the work on the development and validation of a new hydrogel that could be administered to the heart by direct transendocardial injections. Next, this new hydrogel served as a drug delivery scaffold in which a combination of growth factors, IGF-1 and HGF, was tested in a pig model of chronic myocardial infarction.

Chapter 7 entails the rationale and study design of a clinical trial involving cell therapy in patients with refractory angina involving repeated MSC delivery.

Chapter 8 touches on the subject of having a placebo-controlled subjects in clinical trials involving autologous cell therapy for heart disease. In particular, the risks that control subjects are exposed to which include cell harvesting (i.e. bone marrow aspiration, cardiac biopsy, etc.) and delivery to the heart (i.e. the catheterization procedure) is weighed against the benefit of having a double-blinded study design.

Chapter 9 and **10** pertain to the myocardial infarct model in pigs and aims to optimize that protocol. The infarct protocol, as developed in the UMCU, is first presented (Chapter 9). Next, the usability of hyperemic flow reserve was investigated as a functional outcome measure that can provide the investigator information on the microvascular resistance of the infarcted myocardium (Chapter 10).

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CHAPTER **2**

Targeting cell death in the reperfused heart: Pharmacological approaches for cardioprotection

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ABSTRACT

During acute myocardial infarction and in the reperfused heart, loss of cardiomyocytes is mostly caused by apoptosis and necrosis. As apoptosis was considered as the only form of regulated cell death for many years, initial studies investigating cardiomyocyte cell death mainly focused on direct inhibition of apoptosis. However, it has become clear that ischemic conditioning protocols - the application of alternating periods of non-lethal ischemia and reperfusion - can reduce necrotic cell death in the reperfused heart. Research on the signal- transduction pathways responsible for this phenomenon resulted in the discovery of many pharmacological targets to limit cell death after reperfusion, in which the activation of survival kinases and inhibition of mitochondrial permeability transition pore (MPTP) play an important role. Very recently, a regulated form of necrotic cell death (called 'necroptosis') was identified together with potential pharmacological inhibitors, which may also protect the myocardium from lethal reperfusion injury. This review highlights the role of apoptosis and necrosis in the reperfused hearts, including its execution and regulation and the emerging role of programmed necrosis (necroptosis). Furthermore, we will focus on the results of pharmacological interventions in experimental studies as well as relevant proof- of-concept clinical trials trying to limit apoptosis, necrosis and necroptosis in the reperfused heart. Although the list of cardioprotective compounds is promising, large multi-center clinical trials, with enough statistical power, will be necessary to determine whether they can improve clinical outcome and can be applied in patients as adjuvant therapy next to reperfusion.

INTRODUCTION

Acute myocardial infarction (MI) remains one of the leading causes of morbidity and mortality in the western world as this leads to irreversible loss of cardiomyocytes.[1] Myocyte cell death during ischemia-reperfusion (I/R) is mainly caused by apoptosis and necrosis. Currently, the most effective therapy is early reperfusion, as infarct size is a major determinant of cardiac remodeling and prognosis after MI.[2,3] However, within minutes after the restoration of blood flow, reperfusion itself results in additional damage also known as myocardial ischemia-reperfusion (I/R) injury.[4] Based on experimental studies it has become clear that I/R injury contributes to a significant amount of cell death taking place after the onset of reperfusion, also referred to as lethal reperfusion injury.[5,6] Although numerous experimental studies have shown that both pharmacological postconditioning and ischemic postconditioning can lead to infarct size reduction, translation of these cardioprotective approaches has largely failed in the clinical setting.[7-9] Pharmacological approaches that target programmed cell death and especially the role of programmed necrosis, a relatively new level of cell death regulation, may offer novel therapeutic opportunities to limit cell death in the ischemic heart.[10,11]

Cell death in the reperfused heart

Cell death can be classified based on various criteria, but the major types are apoptosis, autophagy and necrosis.[12] Where apoptosis was considered as a regulated form of cell death (i.e. the cell starts its suicide program), necrosis was generally seen as a passive and unregulated process resulting from externally-induced cellular injury. Autophagy on the other hand is mainly considered to be a survival mechanism by which cells recycle their proteins, lipids and organelles under energy and nutrient-deprived conditions, which may become detrimental.[13] This process is induced in the ischemic heart and seems to be cardioprotective rather than being responsible for myocyte death and therefore beyond the scope of this review. The reader is referred to a comprehensive review on the role of autophagy in cardiac disease elsewhere.[14]

Apoptosis was reported to be detectable from approximately 4 hours after coronary artery occlusion and involves cardiomyocytes as well as non-cardiomyocytes.[15-18] Real-time imaging of apoptotic cell death in mice even showed that apoptosis occurs within minutes after ischemia when using Annexin-V labelling[19, 20] Ischemia-reperfusion was suggested to be a stronger stimulus for acute apoptosis than permanent occlusion[21,22], which could even be visualized in patients suffering from myocardial infarction.[23] Necrosis, in contrast, appeared at 2 hours and continued to increase until 24 hours after MI.[15] Therefore, the process of necrosis-induced myocardial cell death mainly takes place within the first 24 hours, after which the inflammatory phase begins.[24,25]

During the last decades, our view and understanding on cell death in the ischemic heart has changed markedly. As apoptosis was seen as the only regulated form of cell death for many years, genetic and pharmacological approaches targeting necrosis are relatively rare.[26] During the last two decades, a growing body of evidence clearly demonstrated that oxidative stress and its effect on mitochondria are very important in lethal reperfusion injury and ischemic cell death.[27,28] It has become clear that at least some part of this necrotic cell death can be regulated, in which opening of the mitochondrial permeability transition pore (MPTP) plays an important role.[29,30]

Moreover, emerging evidence has demonstrated that the serine/threonine kinase activity of receptor-interacting protein 1 (RIP1) and its interaction with RIP3 after death receptor stimulation are necessary for programmed necrosis.[31,32] Necrostatin-1 (Nec-1), a small molecule capable of inhibiting the kinase activity of receptor interacting protein-1 (RIP1), was shown to inhibit programmed necrosis without affecting other RIP1-mediated processes and efficiently prevented necrotic cell death.[33,34] Next to irreversible loss of cardiomyocytes, another important aspect of post-MI recovery is inflammation.[25,35] Apoptosis is characterized by cell shrinkage and the formation of apoptotic bodies in order to avoid inflammation (as plasma integrity is generally maintained). In contrast, necrosis is accompanied by a gain in cell volume, rupture of the plasma membrane and loss of intracellular contents leading to a profound immune response. The possibility to target (programmed) necrosis in the ischemic heart can therefore exert beneficial effects in terms of cardioprotection and may indirectly influence the inflammatory response.[36,37] This is particularly important as inflammation-induced ROS can lead to necrotic cell death as well[38], which could explain why modulation of pro-inflammatory signals that are re-expressed post-MI can attenuate cardiac remodeling accordingly.[39-42]

APOPTOSIS

Apoptosis is a well-defined process by which the cell undergoes cell death following a variety of different stimuli, finally resulting in the activation of a special family of death proteases known as caspases.[43,44] Over 14 different caspases have been identified, of which many are involved in regulating apoptosis. One type of caspases (i.e. initiator caspases) act upstream thereby initiating the apoptotic cascade, including caspase-2, -8, -9 and -10. On the other hand, caspase-3 and -7 were found to be involved in downstream signaling in the apoptosis pathway and are therefore called effector caspases. Like in non-cardiac cells and tissues, caspases are the central players in myocardial apoptosis during pathological conditions such as myocardial infarction and heart failure.[45,46] Caspases are synthesized in an inactive form and remain present in the cytosol as pro-caspases. Once activated, initiator (upstream) caspases cleave and activate the effector caspases, caspase-3 and caspase-7. These downstream caspases then inactivate the enzyme poly ADP-ribose polymerase (PARP), cleave structural nuclear proteins and induce DNA fragmentation via endonucleases including caspase-activated DNAse (CAD).[47] Apoptosis can be separated into two distinct pathways - the extrinsic pathway and the intrinsic pathway -, both leading to the activation of downstream effector caspase-3 and -7 (Figure 1).

The extrinsic pathway

The extrinsic pathway is initiated by binding of extracellular death ligands, tumor necrosis factor-α (TNF-α) and Fas-ligand, to their transmembrane death receptors present on the cell surface.[48] After binding to the death receptor, several death adaptor molecules are recruited originating from the cytoplasmic side, including TNF-receptor-associated death domain (TRADD), TNF-receptor-associated factor (TRAF).[49] This multiprotein complex, known as the death inducing signalling complex (DISC) or Complex I, then starts binding initiator (pro)caspase-8. As more procaspase-8

will be recruited, autoactivation leads to formation of active caspase-8, thereby causing activation of effector caspases such as caspase-3 more downstream.

The intrinsic pathway

The intrinsic (mitochondrial) pathway is induced by a variety of extracellular and intracellular stimuli such as physical stress or oxidative stress and DNA damage. The balance between pro-apoptotic (Bid, Bax, Bak) and anti-apoptotic (Bcl-2, Bcl-xL) members of the Bcl-2 protein family are crucial for the initiation of this pathway.[50] Upon stimulation, Bax translocates to the mitochondria to form a complex with Bak on the outer mitochondrial membrane (OMM). The OMM then becomes permeabilized, leading to the release of cytochrome c and other pro-apoptotic proteins (apoptogens).[51] Once in the cytosol, cytochrome c interacts with apoptotic protease activating factor-1 (APAF-1), forming a complex known as the apoptosome. This complex then activates caspase-9, enabling further activation of effector caspases-3/7 and thus the execution of apoptotic cell death.[52] Other apoptogens that are released upon OMM permeabilization include SMAC (second mitochondria-derived activator of caspase, also known as DIABLO) and apoptosis-inducing factor (AIF).[53] Caspase-8 activation (extrinsic apoptosis pathway) can also activate the mitochondrial pathway by cleavage of Bid (BH3-interacting domain death agonist), also resulting in Bax/Bak translocation and apoptogens release.[54]

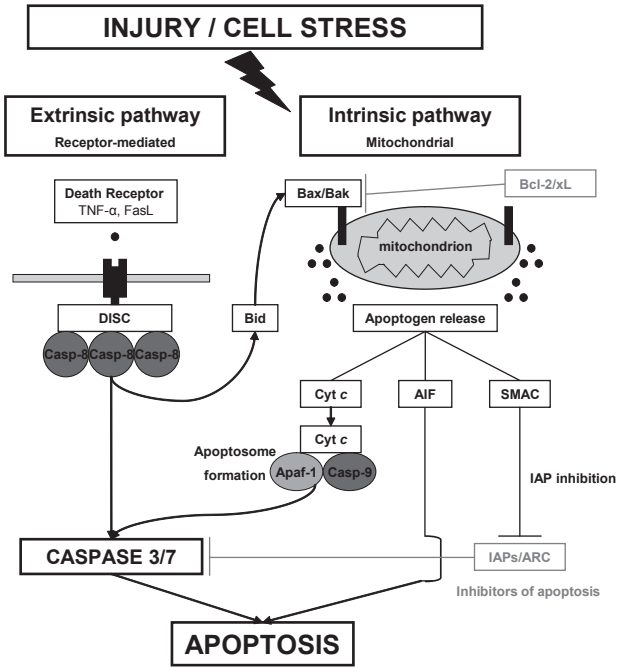

Figure 1. Schematic overview of both the extrinsic (death receptor-mediated) and intrinsic (mitochondrial) pathways leading to apoptosis, as well as the cross-talk between extrinsic apoptosis via Bid cleavage by caspase-8 resulting in Bax/Bak translocation

Regulators of apoptosis

Activation of apoptosis generally occurs upon binding of death ligands to their death receptors (extrinsic pathway) or after specific death-inducing stimuli (intrinsic pathway). Next to the Bcl-2 proteins family, several other proteins are present in the cell that modulate effector caspases, also known as IAPs (inhibitor of apoptosis proteins).[55] The action of IAPs, including the well-studied XIAP (X-chromosome linked IAP), is mainly to inhibit caspase-3 and to prevent the cell from extensive apoptosis. However, upon activation of the intrinsic pathway and their release from the mitochondria, SMAC[56,57] and a protein known as Omi/ HtrA2[58,59], both inhibit IAPs, favouring apoptosis (see Figure 1). ARC (apoptosis repressor with a caspase recruitment domain) is another apoptosis suppressor which interacts with caspase-2 and caspase-8 via its caspase recruitment domain (CARD).[60] Interestingly, ARC can regulate both the extrinsic and intrinsic pathway by inhibiting formation of the DISC complex ("caspase-independent") and inhibition of Bax activation, both via its CARD.[61] Furthermore, nuclear ARC also negatively regulates p53, which normally induces apoptosis.[62,63] More recently it was suggested that ubiquitylation (and consecutive breakdown) of anti-apoptotic proteins and caspases might play an important regulatory role in apoptosis as well64, including the cardiovascular system.[65,66]

NECROSIS

Necrosis is characterized by a gain in cell volume, swelling of organelles, rupture of the plasma membrane and loss of intracellular contents, which is traditionally seen as a passive and unregulated process. Although necrosis plays a major role after myocardial infarction, most studies investigating cell death mainly focused on apoptosis. However, it became clear that a considerable amount of caspase-independent cell death with necrotic characteristics seemed to be occurring during different models of cardiovascular disease.[29,30,67] In this type of necrotic cell death, the opening of the MPTP in the inner mitochondrial membrane (IMM) plays an important role. Furthermore, it was shown that stimulation of the death receptors in the presence of caspase inhibitors resulted in cell death with necrotic characteristics.[68-70] Although current knowledge is still in its infancy, at least two different events can be distinguished which lead to necrosis: mitochondrial MPTP opening and stimulation of the death receptors resulting in necroptosis (Figure 2).

MPTP opening

It has become clear that MPTP opening plays a central role during necrotic cell death and is an interesting target for cardioprotection (reviewed in ref 71). In the normal mitochondrion, electron transport by the respiratory chain results in a transmembrane proton gradient between the IMM and OMM. This mitochondrial transmembrane potential (known as the $\Delta\Psi m$) is essential to generate ATP from ADP and phosphates[72], providing the necessary amount of energy for the cell. Therefore, IMM integrity is crucial and opening of the MPTP during reperfusion has two major consequences: redistribution of solutes and ions accompanied by entry of water and swelling of the mitochondrion (osmotic gradient change) and decline of intracellular ATP due to loss of the

ΔΨm.[73] Mitochondrial swelling can also lead to OMM rupture, causing the release of apoptogens like cytochrome c, followed by caspase activation.[29]

However, ATP depletion and loss of plasma membrane integrity due to activation of proteases are thought to be primarily responsible for necrotic cell death following MPTP opening. MPTP opening itself is mainly triggered by Ca2+, but opening can also occur without a dramatic rise in intracellular calcium levels as many factors (including oxidative stress) influence MPTP sensitivity towards the available amount of Ca2+.[74,75] Although the exact molecular mechanism remains unclear, it was suggested that a number of proteins in some way regulate MPTP structure and function.[71,76] Of special interest is Cyclophilin D (CypD), which interacts with other components of the MPTP and can be inhibited by the drug cyclosporin A.[77,78] Deletion of *ppif*, the gene encoding for CypD, prevented MPTP opening while overexpression strongly induced MPTP opening leading to necrotic cell death.[29,30]

Death receptor-induced necrosis (necroptosis)

Stimulation of the death receptor family members (Fas and TNF receptor) by their ligands (Fas-ligand, TNF-α) normally leads to the activation of the extrinsic apoptotic pathway. Surprisingly, death receptor stimulation under apoptosis deficient conditions (i.e. caspase inhibition) could still induce cell death with morphological features of necrosis in certain cell types, supporting the existence of regulated necrosis.[68,79,80] This type of necrotic cell death was later referred to as "necroptosis" by

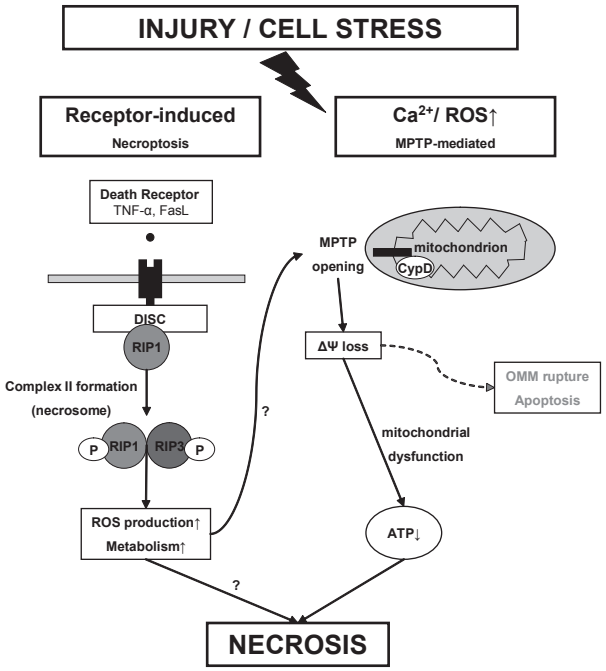

Figure 2. Schematic overview of both necroptosis (receptor-induced) and MPTP- mediated (mitochondrial) necrosis, as well as the crosstalk between MPTP opening and intrinsic apoptosis via rupture of the OMM resulting in release of apoptogens from the mitochondria

Degterev et al[33], in which the serine/ threonine kinase activity of RIP1 plays an essential role.[69,70] Since then, research on necroptosis quickly expanded with the aid of Necrostatin-1 (Nec-1), a small molecule capable of inhibiting the kinase activity of RIP1 without affecting other RIP1-mediated processes[34], and other necrostatin family members. Necroptosis is induced by different extracellular death ligands including TNF-α and Fas-ligand, in which the role of TNF receptor-1 is most extensively studied.[32,49,81]

Upon ligand binding, a multiprotein complex associates with the TNF receptor at the intracellular side (complex I). This complex includes TRADD, TRAF, the inhibitor of apoptosis 1/2 (cIAP1/2) and RIP1.[49] Interestingly, cIAP1/2 are also known to function as RIP1- ubiquitylating enzymes, thereby promoting nuclear factor-γB (NF-γB) survival pathways.[82,83] Removal of K63-ubiquitination of RIP1 by deubiquitinating enzymes A20 and cylindromatosis (CYLD) is necessary to recruit RIP1 to complex II[84,85], providing the first switch between life or death. Like in apoptosis, complex II formation highly depends on ubiquitylation status of RIP1, suggesting that this system might regulate both apoptotic and necrotic cell death.[86] A second switch will then determine if the cell dies from apoptosis or necrosis. RIP1 will assemble with the Fas-associated death domain (FADD), caspase-8, RIP3 and in some cases with TRADD, forming pro-necrotic complex II. Active caspase-8 will cleave RIP1 and induce apoptosis.[87] Furthermore, in the presence of SMAC mimetics (counteracting IAPs), an alternative complex II is formed independently of TRADD, which also leads to caspase-8 activation.[88] When caspase-8 is pharmacologically (or genetically) inhibited, apoptosis cannot be initiated thereby leading to programmed necrosis as an alternative form of cell death.[70,79] Nec-1 inhibits this type of cell death, suggesting that RIP1 kinase activity is required for RIP1/3 complex formation, although RIP3 can also phosphorylate RIP1.[89] Altogether, complex II can promote necroptosis (“necrosome complex” in the presence of RIP1 and RIP3) or apoptosis in the presence of caspase-8.[90]

Current understanding of necroptosis is limited, although several mechanisms are thought to contribute to its execution, including ROS production, metabolic changes, and changes in cellular energetics.[89-92] Interestingly, one of the ideas is that due to cellular stress and in an attempt to survive, the cell increases its energy metabolism turnover thereby facilitating the production of large amounts or ROS, leading to necrotic cell death.[32,93]

PHARMACOLOGICAL APPROACHES TO LIMIT CELL DEATH IN THE REPERFUSED HEART

In the context of myocardial infarction, much effort has been put in the modulation of cell death as infarct size is the major determinant of prognosis after MI.[2] In order to find the most optimal strategy for limiting cell death in the ischemic heart, we must critically evaluate experimental studies targeting cell death after myocardial infarction to avoid future problems in a clinical setting.[9,94] Ranging from genetic to pharmacological approaches, apoptosis inhibition has been studied extensively.[95,96] Most studies, targeting apoptotic and necrotic cell death, used either caspase inhibitors (and other related compounds) or aimed at the prevention of MPTP opening, including pre- and postconditioning protocols.[7] However, influencing (programmed) necrosis directly has

gained considerable scientific attraction over the last few years as this interesting field may lead to new therapeutic approaches.[27,97] An overview of the most promising compounds that influence cell death can be found in Figure 3.

Drugs targeting myocardial apoptosis

Genetic approaches clearly showed that inhibition of myocyte apoptosis reduces myocardial infarct size after cardiac I/R (reviewed in ref. 26). This mainly occurred via interference in the signal transduction of the extrinsic and intrinsic pathway (i.e. death receptor interference) as well as via genetic modification of anti- and pro-apoptotic genes (Bcl-2, Bid, Bax, IAPs). Although these studies provided important mechanistical insights on anti-apoptotic strategies, pharmacological studies have been rather inconclusive in a variety of experimental animal models as discussed below.

Caspase inhibition

Caspases play a pivotal role in the initiation and activation of apoptosis, providing a convincing rationale to use caspase inhibition to limit infarct size post-MI. During the last two decades, several studies investigated the effect of caspase inhibition in various models of myocardial I/R with conflicting results (Table 1).[98-111] Several studies have shown that the application of the pan-caspase inhibitor ZVAD after myocardial I/R limited infarct size (IS) in both rats and rabbits by ~15%.[98,101,102,106,110] However, mice subjected to a similar protocol and treatment, as well as Langendorff-perfused rabbit hearts, did not show any effect.[105,110] YVAD, a selective caspase-1/4

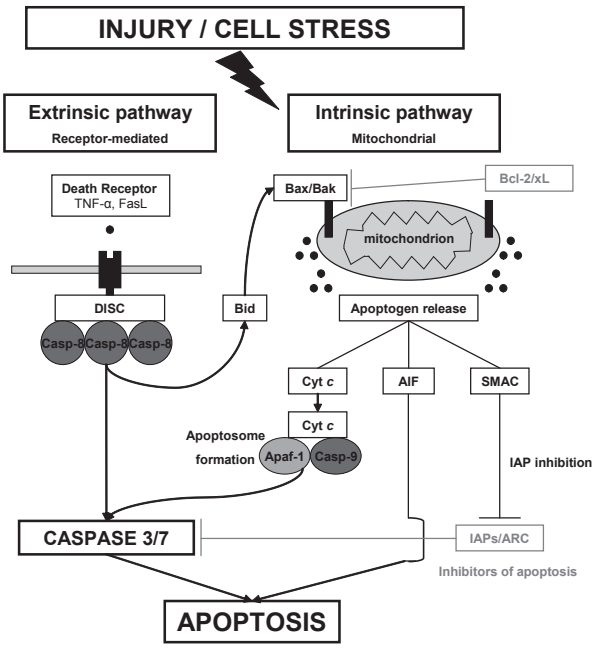

Figure 3. Schematic overview of pharmacological interventions to prevent cell death in the ischemic heart, including the most promising candidates with proven efficacy from both experimental (caspase inhibitors, SfA and Nec-1) and proof-of-concept clinical studies (MCC-135, ANP, adenosine, GLP-1 and CsA).

inhibitor exerted cardioprotection in rabbits and rats[99,103], but failed in two other studies using the same species.[100,106] DEVD, a selective caspase-3/7 inhibitor was even less successful in limiting IS – one positive study[106] compared to three negative studies.[100,101,103] Interestingly, DEVD seemed to improve contractile function independent (or even without) inhibition of myocyte apoptosis, although the exact mechanism is still unclear.[104,109] Caspase-9 inhibition using Z-LEHD in rats resulted in both positive and negative results[101,103], caspase-8 inhibition (Z-IETD) prior to reperfusion did not show any effects.[103] Neither MMPSI (considered as a more potent non-peptide selective caspase-3/7 inhibitor) nor IDN6734 and Z-Asp-DCB (two other pan-caspase inhibitors) were able to reduce IS compared to control animals.[106-108] Although caspase inhibition did not seem to reduce IS in all cases, several studies did observe a reduction of TUNEL-positive cardiomyocytes, reduced caspase activity or increased functional recovery.[100,104,105,107,108,111] This raises the question whether the beneficial effects solely depend on cardiomyocytes, as non-myocyte cell death (i.e. fibroblasts) and caspase-dependent cleavage of intracellular substrates (i.e. contractile proteins) may influence cardiac remodeling as well. Therefore, clinical application is currently too far away as evidence on apoptosis-dependent cell death and other caspase- dependent processes remains controversial. Moreover, only a minority of these studies investigated the use of caspase inhibitors in a clinically relevant animal model of *in vivo* ischemia-reperfusion.

Other compounds targeting apoptosis

Next to the administration of readily available caspase inhibitors, compounds targeting other components of the apoptotic pathway are very limited. One possible option is the mitochondrial serine protease Omi/ HtrA2, a regulator of apoptosis by inducing caspase activation and which is activated during myocardial I/R.[112] UCF-101 is a specific inhibitor of Omi/HtrA2, which was shown to prevent inactivation of IAPs via direct binding and cleavage, thereby inhibiting its proteolytic capacity with very little aspecific activity for other serine proteases.[113] Two independent reports showed that UCF-101 administration reduced IS by limiting XIAP inactivation and caspase-3/9 activity.[114,115] Next to ZVAD, Minatoguchi et al. also investigated whether serine protease inhibition influenced IS.[105] Although less TUNEL- positive cardiomyocytes were present, no IS reduction was observed. A more recent paper failed to show reduced caspase-3 activity upon UCF-101 treatment in diabetic mice.[116] Rather than anti-apoptotic, UCF-101 was suggested to downregulate several enzymes responsible for the degradation of AMP-activated protein kinase (AMPK), thereby preventing cardiac dysfunction during diabetes. Follow-up studies investigating UCF-101 in pre-clinical large-animal models and additional knowledge on its exact mechanism are needed.

Drugs targeting myocardial necrosis

MPTP opening results in myocyte necrosis, characterized by mitochondrial swelling, ATP depletion and cardiomyocyte cell death.[28,117] Although the exact molecular composition of the MPTP remains controversial, this has been a challenging area in research involving myocardial I/R injury.[73,118,119] Via pre- and post-conditioning protocols, delayed MPTP opening seems to be mediated through CypD and activation of the Reperfusion Injury Salvage Kinase (RISK) pathway.[120,121]

Next to CypD, several other proteins have been proposed in regulating MPTP opening, including

voltage- dependent anion channel (VDAC) and adenine nucleotide translocase (ANT). Therefore, most studies aiming for MPTP inhibition targeted these specific proteins (Table 2). Additionally, evidence from both neuronal and myocardial disease models firmly suggested that necroptosis could be an interesting candidate to limit cell death in cardiovascular and many other diseases.[97]

Drugs targeting MPTP opening

As soon as several groups reported that Cyclosporin A (CsA) could inhibit mitochondrial MPTP opening after I/R77,[122-124], Weinbrenner et al. were the first to report IS reduction following CsA treatment. They reached a similar level of inhibition as ischemic preconditioning[125], which was reported by many others afterwards (Table 2, only studies pharmacologically targeting MPTP during I/R are shown).[126-132] Interestingly, hyperglycaemia during ischemia was found to inhibit the protective action of CsA.[133] Furthermore, CsA was shown to interact with cyclophilin A as well, thereby inhibiting calcineurin signalling and providing cardioprotection via different pathways.[134,135] A few years later though, Leshnower et al. showed that calcineurin inhibitor FK506 did not affect infarct size (although administered prior to ischemia), in favour of calcineurin-independent cardioprotection by CsA.[136]

Nevertheless, this controversy led to the development of several CsA analogs including NIM811[137], which was shown to reduce IS significantly *in vivo*.[138] Similarly, sanglifehrin A (SfA), another inhibitor of MPTP opening and independent of calcineurin signalling, was shown to protect against myocardial I/R injury by lowering LDH levels[139] and reducing IS.[140] Interestingly, Javadov et al. reported a reduced LDH release upon CsA and SfA treatment in Langendorff-perfused rat hearts, which was less effective than ischemic preconditioning.[141] Importantly, Lim et al. elegantly demonstrated that the protective effect of both CsA and SfA is at least partly mediated via MPTP opening *in vivo* since the observed IS reduction was absent in CypD knockout mice.[131] Furthermore, Debio-025 (alisporivir), another cyclophilin inhibitor, exerted cardioprotective effects similar to postconditioning protocols.[142]

Piot et al. showed for the first time that CsA administration prior to percutaneous coronary intervention (PCI) reduced infarct size in STEMI patients at 5 days measured by MRI (P=0.04)[143], without any effect on LV function[144], although patient numbers were rather low (n=58), as illustrated by non-significant changes in troponin I levels (P=0.15). Recently, CsA reduced IS in pigs, again demonstrating its cardioprotective properties.[145] Therefore, it remains unclear why three other reports failed to demonstrate a cardioprotective of CsA in pre-clinical models of I/R[146-148], although Lie et al. reported a significantly larger area-at- risk in the CsA-treated animals.[146] Although the reported pig studies are not consistent, CsA could still be considered as a therapeutic approach, considering the available cardiac and non-cardiac experimental and clinical data.[143,144,149]

Table 1. Caspase inhibition in heart disease models

Type of inhibitor	Study	Animal	I/R model	Dose & Timing	Effect
Pan-caspase inhibitor	Yaoita et al. 1998[98]	Rat	30min / 1day *in vivo*	3.3mg/kg ZVAD.fmk; bolus prior to ischemia followed by every 6 hours	IS↓ TUNEL+ CM↓
	Mocanu et al. 2000[101]	Rat	35min / 2hrs Langendorff	0.1μM ZVAD.fmk in perfusate; 5 minutes prior to reperfusion for 15 minutes	IS↓
	Huang et al. 2000[102]	Rat	45min / 3hrs *in vivo*	500μg/rat ZVAD.fmk or BOCD.fmk; prior to ischemia or 10min prior to reperfusion	IS↓; ZVAD.fmk (both protocols) BocD.fmk (protocol 1
	Minatoguchi et al. 2001[105]	Rabbit	30min / 2days *in vivo*	0.8mg/kg ZVAD.fmk; 20 minutes prior to ischemia	IS unaffected TUNEL+ CM ↓
	Chapman et al. 2002[106]	Rabbit	30min / 2hrs *Langendorff*	10μM ZVAD.fmk; 15 min prior to ischemia	IS↓ TUNEL+ CM↓
	Yarbrough et al. 2003[107]	Pig	60min / 7days *in vivo*	2mg/kg IDN6734; bolus at reperfusion, followed by 2mg/kg for 24 hrs	IS unaffected (at 7 days post-MI) LV dimension change↓
	Chandrashekhar et al. 2004[108]	Rat	permanent ligation *in vivo*	2mg/kg Z-Asp-DCB.mk prior to surgery, followed by 2mg/day for 28 days	LV function↑ (28 days) Caspase-3 activity↓
	Mersman et al. 2008[110]	Mouse	20min / 4days *in vivo*	1.5mg/ kg Q-VD-Oph or ZVAD.fmk 1.5mg/kg; prior to ischemia, followed by 1.5mg/kg twice a day	IS unaffected
	Mersman et al. 2008[110]	Rat	25min/ 7days *in vivo*	1.5mg/kg Q-VD-Oph; prior to ischemia, followed by 1.5mg/kg injections twice a day	IS↓ LV function↑
	Yarbrough et al. 2010[111]	Pig	60min / 7days *In vivo*	6mg/kg IDN6734; bolus at reperfusion, followed by 6mg/kg for 24 hrs	IS unaffected LV function↑
Caspase-1/4 inhibitor	Holly et al. 1999[99]	Rabbit	30min / 3hrs *in vivo*	1.5mg/kg YVAD.cmk; prior to ischemia, followed by a bolus at reperfusion	IS↓ TUNEL+ CM↓
	Okamura et al. 2000[100]	Rat	30min / 6hrs *in vivo*	3.5mg/kg YVAD.cho; 5 min prior to ischemia	IS unaffected, but TUNEL+ CM↓ Caspase activity↓
	Kovacs et al. 2001[103]	Rat	30min / 2hrs *Langendorff*	0.5μM YVAD.cmk; at reperfusion	IS↓ TUNEL+ CM↓

follow up table 1

	Chapman et al. 2002[106]	Rabbit	30min / 2hrs *Langendorff*	20μM YVAD.cmk and MMPSI (non-peptide inhibitor); 15 min prior to ischemia	IS unaffected (YVAD) IS↓ (MMPSI)
Caspase-3/7 inhibitor	Okamura et al. 2000[100]	Rat	30min / 6hrs *in vivo*	3.5mg/kg DEVD.cho; 5 min prior to ischemia	IS unaffected, but TUNEL+ CM↓ Caspase activity↓
	Mocanu et al. 2000[101]	Rat	35min / 2hrs *Langendorff*	0.07μM Ac-DEVD.cmk in perfusate; 5 minutes prior to reperfusion for 15 minutes	IS↓
	Kovacs et al. 2001[103]	Rat	30min / 2hrs *Langendorff*	0.2μM Ac-DEVD.cmk; at reperfusion	IS unaffected
	Ruetten et al. 2003[104]	Rat	30min / 30min *Langendorff*	1μM Ac-DEVD.cmk; prior to ischemia or at reperfusion	TUNEL+ CM unaffected Caspase-3 activity↓
	Chapman et al. 2002[106]	Rabbit	30min / 2hrs *Langendorff*	20μM DEVD.fmk; 15 min prior to ischemia	IS↓
	Balsam et al. 2005[109]	Mouse	permanent ligatio	1.6mg/kg DEVD.cho; started prior to surgery, continued twice a day	TUNEL+ CM↓ (1 day) LV function↑ (28 days)
Caspase-8 inhibitor	Mocanu et al. 2000[101]	Rat	35min / 2hrs *Langendorff*	0.07μM Z-IETD.fmk in perfusate; 5 minutes prior to reperfusion for 15 minutes	IS↓
Caspase-9 inhibitor	Mocanu et al. 2000[101]	Rat	35min / 2hrs *Langendorff*	0.07μM Z-LEHD.fmk in perfusate; 5 minutes prior to reperfusion for 15 minutes	IS↓
	Kovacs et al. 2001[103]	Rat	30min / 2hrs *Langendorff*	0.2μM Z-LEHD.fmk; at reperfusion	IS unaffected
Serine-protease inhibitor	Minatoguchi et al. 2001[105]	Rabbit	30min / 2days *in vivo*	2mg/kg DCI; 20 minutes prior to ischemia	IS unaffected TUNEL+ CM ↓

IS = infarct size; TUNEL = Terminal deoxynucleotidyl transferase dUTP nick end labelling; CM = cardiomyocytes; iv. = intravenously; LV = left ventricle/ventricular; MMPSI = (S)-(+)-5-1-(2-methoxymethylpyrrolidinyl)sulfonylisatin (non-peptide selective caspase-3/7 inhibitor);

Table 2. Targeting MPTP opening in heart disease models

Type of inhibitor	Study	Animal/Human	I/R model	Dose & Timing	Effect
Cyclosporin A	Weinbrenner et al. 1998 [125]	Rabbit	30 min / 2 hrs Langendorff	750nM CsA in perfusate; pre-ischemia or 10 minutes post-ischemia	IS↓*
	Squadrito et al. 1999 [126]	Rat	30 min / 48 hrs *in vivo*	0.25, 0.5 or 1mg/kg CsA; 5 min after ischemia	IS↓
	Minners et al. 2000 [127]	Rat	35 min / 2 hrs **Langendorff**	0.2μM CsA in perfusate; 5 min pre-ischemia	IS↓
	Hausenloy et al. 2002 [128]	Rat	35 min / 2 hrs **Langendorff**	200 nM CsA; 5 min prior to reperfusion for 15 minutes	IS↓
	Argaud et al. 2004 [129]	Rabbit	30 min / 4 hrs *in vivo*	10mg/kg CsA; 15 min prior to ischemia	IS↓
	Argaud et al. 2005 [138]	Rabbit	30 min / 4 hrs *in vivo*	10 mg/kg CsA or NIM811 (analogue); at reperfusion	IS↓
	Krolikowski et al. 2005 [130]	Rabbit	30 min / 3 hrs *in vivo*	5 and 10 mg/kg CsA; at reperfusion	IS↓ (only in case of 10mg/kg CsA)
	Lim et al. 2007 [131]	Mouse WT and CypD KO	45 min / 2 hrs *in vivo*	10mg/kg CsA; at reperfusion	IS↓
	Gomez et al. 2008 [132]	Mouse	60 min/24 hrs *in vivo*	10mg/kg CsA; 5 min prior to reperfusion	IS↓†
	Piot et al. 2008 [143]	Human	28 patients with STEMI in need for reperfusion therapy	2.5mg/kg CsA; 5 min prior to reperfusion	IS↓ (measured by MRI)
	Skyschally et al. 2010 [145]	Pig	90 min / 2 hrs *in vivo*	5mg/kg CsA; 5 min prior to reperfusion	IS↓

follow up table 2

	Lie et al. 2010 [146]	Pig	40 min / 3 hrs *in vivo*	10mg/kg CsA; 5 min prior to reperfusion	IS unaffected
	Karlsson et al. 2010 [147]	Pig	45 min / 2 hrs *in vivo*	10mg/kg CsA; 3 min prior to reperfusion	IS unaffected
	Karlsson et al. 2011 [148]	Pig	45 min / 4 hrs *in vivo*	2.5mg/kg CsA; 7 min prior to reperfusion	IS unaffected
Sanglifehrin A	Hausenloy et al. 2003 [140]	Rat	35 min / 2 hrs **Langendorff**	1μM CsA; at reperfusion or after 15 min of reperfusion	IS↓ (only when given at reperfusion)
	Lim et al. 2007 [131]	Mouse WT and CypD KO	45 min / 2 hrs *in vivo*	10mg/kg CsA; at reperfusion	IS↓
Debio-025 (alisporivir)	Gomez et al. 2007 [142]	Mouse	25 min / 1 day *in vivo*	10mg/kg Debio-025; at reperfusion	IS↓

MPTP = mitochondrial permeability transition pore; CsA = cyclosporin A; SfA = sanglifehrin A (does not inhibit calcineurin); *effect was gone when given 20 minutes after onset of ischemia; †effect was independent of GSK3β activity;

A recently published meta-analysis on CsA appeared to be rather negative, but also included (negative) studies with administration of CsA prior to ischemia and studies primarily investigating other mechanisms than CsA-induced cardioprotection.[150] Furthermore, the success of CsA in large-animal models seems to be related to the time of ischemia applied to the animal (Table 2; 90 vs. 45 minutes ischemia followed by reperfusion), which is in line with recent findings.[151] Interestingly, several clinical CsA- studies are currently ongoing, including a study investigating the effect of CsA administration on markers of cardiac injury after bypass surgery (clinicaltrials.gov, NCT01002859). Although promising, both knowledge on the molecular identity of MPTP and follow-up studies will be needed to investigate to what extent CypD-mediated MPTP opening can salvage damaged myocardium in a clinically relevant setting, requiring particular attention to the model (i.e. duration of ischemia), application (i.e. relevant concentration in the early phase of reperfusion) and hard end-points.

Drugs targeting necroptosis

In contrast to the role of MPTP opening in cardioprotection, necroptosis is a relatively new and exciting field with clinical relevance in a variety of diseases.[32,97] After discovery of Nec-1, a small molecule capable of inhibiting the kinase activity of RIP[133,34], the group of Yuan focused on the development of Nec-1 and other chemical variants of necrostatins (i.e. Nec-3, Nec-4, Nec-5 and Nec-7).[152-155] Until now, experimental studies investigating necroptosis mainly used Nec-1. Interestingly, Nec-7 was found to inhibit TNF-α – induced necroptosis in human Jurkat cells without inhibiting RIP1 kinase.[155] Recently, both RIP1-dependent and independent protection from cell death upon Nec-1 treatment were reported[156], pointing towards the existence of additional targets that can regulate this complex pathway. Degterev et al. were the first to show that that Nec-1 administration after 2 hours occlusion of the mid-cerebral artery followed by reperfusion resulted in a significant reduction of infarct size.[33] Furthermore, a follow-up study also showed that Nec-1 decreased brain damage in a model of traumatic brain injury, accompanied by improved cerebral function and less inflammation.[157] Recently, several others showed that Nec-1 exerted neuroprotective effects in models of retinal ischemia[158,159] and after hypoxia in neonatal mice by blocking necrosome formation.[160] Xu et al. were even able to show a synergistic effect when Nec-1 was administered in combination with humanin, an apoptosis inhibitor, upon cerebral I/R.[161] Altogether, these data strongly suggest that necrosis is an important form of cell death after ischemic injury *in vivo* which can be inhibited by Nec-1.

The possibility to inhibit necrotic cell death has important implications for cardiovascular disease, particularly in the context of myocardial ischemia-reperfusion. Until now, both *in vitro* and *in vivo* data on the use of Nec-1 in models of cardiac injury is limited (summarized in Table 3). Smith et al. provided the first indication that Nec-1 protects against myocardial injury.[162] Nec-1 administration resulted in less necrotic cell death under oxidative stress (measured by propidium iodide uptake) *in vitro* and treatment with 100μM (but not 30μM) Nec-1 delayed MPTP opening in isolated rat ventricular myocytes. However, 100μM Nec-li (inactive analogue) also delayed MPTP opening and reduced infarct size to a similar extent as Nec-1 after I/R in a Langendorff model. As a final experiment, the authors performed an open-chest model of 30 minutes ischemia, followed by 2 hours reperfusion in C57BL/6J mice demonstrating an infarct size reduction upon Nec-1 treatment.

Table 3. Targeting necroptosis in heart disease models

Type of inhibitor	Study	Cells/Animal	I/R model	Dose & Timing	Effect
Cells	Smith et al. 2007 [162]	C2C12 myoblasts (mouse)	Oxidative stress for 5.5 hrs; stimulation with peroxide	30μM and 100μM Nec-1; together with stimulus	50% reduction of cell death (30 and 100μM)
	Smith et al. 2007 [162]	H9c2 cardiac cells (rat)	Oxidative stress for 4 hrs; stimulation with peroxide	30μM and 100μM Nec-1; together with stimulus	20-30% reduction of cell death (30 and 100μM)
	Smith et al. 2007 [162]	Isolated rat cardiomyocytes	Laser illumination to stimulate ROS generation	30μM and 100μM Nec-1; 10 minutes pre-treatment before stimulus	Delayed MPTP opening* (only in case of 100μM)
	Liu et al. 2010 [165]	CMPCs (human)	Oxidative stress for 16 hrs; stimulation with peroxide	30μM Nec-1; 30 minutes pre-treatment before stimulus	40% reduction of necrosis measured flow cytometry
Animals	Smith et al. 2007 [162]	Mouse	35 min / 35 min Langendorff	30μM and 100μM Nec-1; at reperfusion	IS↓ (30μM)* IS↑ (100μM)
	Smith et al. 2007 [162]	Mouse	30 min / 2 hrs in vivo	1.65mg/kg Nec-1; ip-injection prior to reperfusion	IS↓ with 40%
	Lim et al. 2007 [163]	Mouse WT vs. CypD -/-	30min/120min, in vivo, open chest model	1.65mg/kg Nec-1; tail vein injection at reperfusion	IS↓ with 70%; no additional IS↓ in CypD -/-

Nec-1 = Necrostatin-1; ROS = reactive oxygen species; MPTP = mitochondrial permeability transition pore; CMPC = cardiomyocyte progenitor cell; ip = intraperitoneal; CypD -/- = Cyclophilin D knockout mice; *similar effect seen with Nec-li (inactive form);

Using the same approach, this laboratory later showed that the cardioprotective effect of Nec-1 was lost in CypD knockout animals[163], which could suggest that the activation of the RISK pathway is involved in the protective mechanism following RIP1 inhibition.[131,164]
On the other hand, the absolute infarct size reduction in wild-type animals was much more than in the CypD knockout mice (70% vs. 40%), suggesting that Nec-1 could also exert cardioprotective effects independent of CypD. While investigating the role of microRNA-155 (which targets RIP1) in cardiomyocyte progenitor cells *in vitro*, our laboratory for the first time showed that Nec-1 exerts cardioprotective effects against oxidative stress in human cells165, suggesting that Nec-1 could also be used to improve cell survival during cell transplantation. This implicates that Nec-1, next to limiting reperfusion injury, could also be used in cell transplantation studies in an attempt to improve cardiac recovery by increasing cell retention after infarction[166].
Especially in combination with the data obtained from models of cerebral injury (as mentioned earlier), Nec-1 seems to be promising a cardioprotective compound. However, these data were obtained from a limited number of animals, the protocol was limited to 2 hours of reperfusion only and no functional follow-up was performed. Many interventions aiming at infarct size reduction, although successful in experimental studies, failed in clinical settings.[9] Furthermore, it is unclear whether Nec-1 directly targets necrotic cell death after cardiac I/R *in vivo*, whether apoptosis is involved and if RIP1 and ROS are affected. More importantly, long-term efficiency needs to be established, including large-animal studies. We therefore believe that additional research is warranted before further steps towards a clinical setting can be made.

Compounds reducing infarct size via the activation of survival kinases

The Reperfusion Injury Salvage Kinase (RISK) pathway plays an important role in cardioprotection, which can be activated mechanically (pre- and postconditioning protocols) and with pharmacological compounds.[4] In experimental models, several compounds were identified that could activate these survival kinases, thereby inhibiting MPTP opening[167], which may be useful in the clinic as adjunctive therapy in addition to reperfusion. Unfortunately, translation of these compounds in clinical trials has proven to be difficult, as infarct size reduction was not observed in all clinical studies (Table 4; randomized clinical studies reporting IS (or enzyme release) and LV function only).
Already about twenty years ago, several groups reported that pre-treatment with adenosine (or a selective adenosine A1-receptor agonist) could reduce infarct size to a similar extent as pre-conditioning, which was not effective when administered prior to reperfusion.[168,169] Many others confirmed this type of cardioprotection in different animal models (extensively reviewed in ref. 170). Interestingly, Norton et al. were the first to report that stimulation of the A2-receptor at the onset of reperfusion exerted cardioprotective effects.[171] Activation of adenosine receptors either during ischemia (A1 and A3 subtype) or at reperfusion (A2 subtype) can be effective, as illustrated by a considerable amount of evidence.[170] Although the adenosine-mediated cardioprotection might be mediated through KATP channels, protein kinase C signalling and Akt (RISK activation), the exact mechanism remains unclear.[170] As shown in Table 4, clinical studies were less consistent. Although the Acute Myocardial Infarction STudy of ADenosine (AMISTAD) trial in patients undergoing thrombolysis reported an infarct size reduction (especially in anterior infarct patients), a positive

Table 4. Experimental versus clinical use of several potential cardioprotective agents

Agent (mechanism)	Experimental use	Effect	Clinical use	N	Effect
Adenosine	Summarized in[170]	IS↓	Mahaffy et al. 1999[172] prior to thrombolysis	236	IS↓*
(RISK-mediated)	Both during ischemia and prior to reperfusion		Marzilli et al. 2000[173] prior to reperfusion	54	CK↓ ACE↓
			Quintana et al. 2003[174] at onset of thrombolysis	608	LV function unaffected
			Ross et al. 2005[175] prior to intervention	2218	IS↓† (high dose only)
			Fokkema et al. 2009[176] 2x bolus after intervention	448	IS (by CK) unaffected
ANP	Takagi et al. 2000[178] *in vivo*, at reperfusion	IS↓	Hayashi et al. 2001[183] after reperfusion	65	LV remodeling↓ vs. NTG
(RISK-mediated)	Sangawa et al. 2004[179] *Langendorff*, at reperfusion	LV function↑	Kuga et al 2003[184] after reperfusion	37	LV function ↑ (angiography)
	Yang et al. 2006[180] *Langendorff*, at reperfusion	IS↓	Kasama et al. 2007[185] upon presentation	50	LV remodeling↓ vs. nitrate
			Kitakaze et al. 2007[186] after reperfusion for 3 days	535	IS↓ (by CK), LV function ↑ ACE↓
GLP-1 (or analogues)	Bose et al. 2005[195] *Langendorff /in vivo*, pre-ischemia	IS↓	Nikolaidis et al. 2004[190] after reperfusion, for 72hrs	21	LV function↑
(RISK-mediated)	Bose et al. 2005[196] *Langendorff*, at reperfusion	IS↓	Lønborg et al.[187] 15 min before reperfusion	105	IS↓# salvage index↑ #
	Bose et al. 2007[197] *Langendorff*, pre-ischemia	IS↓			
	Sonne et al.[198] *in vivo*, at reperfusion	IS↓§			
	Timmers et al.[199] *in vivo*, 5 min prior to reperfusion	IS↓#			
MCC-135	Yarbrough et al. 2003[208] *in vivo*, at reperfusion	LV fuction↑‡	Bär et al. 2006[211] prior to reperfusion	387	no beneficial effects
(Reducing Ca_{2+})	Satoh et al. 2004[209] *Langendorff*, at reperfusion	LV fuction↑	Jang et al. 2008[212] prior to reperfusion	486	no beneficial effects
	Kawasumi et al. 2007[210] *in vivo*, prior to reperfusion	IS↓			

AR-agonist = adenosine receptor agonist; IS = infarct size; CK = creatine kinase; ACE = adverse cardiac events; ANP = atrial natriuretic peptide; vs. = versus; NTG = nitroglycerine; * in case of anterior infarcts; † when treated 70µg/kg per minute adenosine (high dose); ‡ measured by regional contractility; § using exendin-4; # using exenatide (Byetta);

trend towards more adverse events was observed as well.[172] Marzilli et al. reported significantly less adverse events and lower creatine kinase (CK) levels after adenosine treatment prior to reperfusion[173], while another randomized study did not observe functional improvements when adenosine was given at onset of thrombolysis.[174] Although infarct size was comparable between all adenosine-treated patients vs. placebo, the AMISTAD-II trial did show beneficial effects in patients receiving a high dose of adenosine which was correlated with less adverse events.[175] In contrast, a bolus injection after thrombus aspiration followed by another injection after stenting failed to show any beneficial effects.[176] Overall, adenosine might provide myocardial protection, but larger studies with enough statistical power will be necessary to establish the observed beneficial effects.

Atrial Natriuretic Peptide (ANP), a circulating hormone with profound effects on blood pressure and the neuro-hormonal response upon cardiac injury, has also been considered as promising cardioprotective agent.[177] Several small animal studies reported beneficial effects on functional recovery (rat) and infarct size reduction (dog, rabbit) upon ANP administration which might active the RISK pathway[178-180], as well as less ischemia-induced arrhythmias[181], and infarct size reduction in ANP knockout mice after permanent ligation.[182] In AMI patients, ANP infusion after reperfusion reduced LV remodelling compared to nitroglycerine or isosorbide dinitrate and reduced the incidence of arrhythmias.[183-185] Major limitations of these studies are the lack of a proper control population and the way LV function (i.e. angiography) and other clinical parameters were measured. More convincing evidence was provided by a clinical trial comparing ANP with a placebo (J-WIND-ANP) given at onset of reperfusion, which resulted in a significant infarct size reduction (measured by CK), preserved long-term ejection fraction and reduced cardiac death.[186] Clearly, ANP holds a great potential, but larger multi-center trials are necessary to establish its clinical benefit.

Very recently, compelling evidence was reported on the clinical application of exenatide (Byetta) after thrombolysis (n=105), although its exact mechanism still remains to be elucidated.[187] Glucagon-like peptide-1 (GLP-1; exenatide is a synthetic GLP-1 analogue), is a hormone which stimulates insulin secretion and is released into the gastrointestinal tract.[188,189] Unlike GLP-1, exenatide and other analogues are resistant for the enzyme dipeptidyl peptidase-IV (DPP-IV), normally responsible for its rapid breakdown. In an attempt to restore normal glucose uptake, the group of Shannon et al. demonstrated that GLP-1 infusion improved LV function in patients after primary angioplasty (n=21) and with chronic heart failure (n=21), as well as in different dog models of cardiomyopathy, suggestive of both metabolic and inotropic effects.[190-194] Bose and Yellon were the first to use GLP-1 upon reperfusion, showing that the cardioprotective effect of GLP-1 (combined with a DPP-IV inhibitor) on infarct size was blunted when PI3K-Akt signaling (part of the RISK pathway) was blocked in rat.[195-197] Sonne et al. showed similar effects using exendin-4, a peptide constituent of the venom of *H. suspectum* lizard, which also acts as a GLP-1 receptor agonist.[198] Timmers et al. elegantly showed that exenatide administration 5 minutes prior to reperfusion reduced infarct size and preserved systolic function after 3 days of reperfusion in a clinically relevant porcine model, which was associated with increased p-Akt levels and reduction of caspase-3 activity.[199] Finally, with the use of GLP-1 receptor knockout mice, Ban et al. showed that the cardioprotective effects of GLP-1 and its isoform were receptor-dependent, but that of its metabolite GLP-1(9-36) receptor-independent.[200, 201] As administration of exenatide (started

15 minutes prior to reperfusion) resulted in a significantly larger salvage index ([AAR-IS]/AAR) and corresponding decrease in myocardial infarct size (cardiac MRI), this can certainly be considered as a promising cardioprotective compound.[187] Interestingly, a large safety trial using exenatide in patients suffering from myocardial infarction is currently ongoing.[202] Next to these initial studies, large multi-center clinical trials will be necessary to answer the question whether exenatide can improve clinical outcomes as well.

Other compounds capable of limiting infarct size

Many mediators of I/R injury not only cause damage by itself, but may lead to a chain reaction of lethal injury when ROS levels reach a certain threshold.[203] Not surprisingly, several compounds have been identified capable of reducing cardiac reperfusion injury, mainly by limiting ROS levels or, more indirectly, by influencing pH, calcium levels and via clinically available drugs[204,205] (a complete list can be found in ref 8).

One particular compound that has been investigated in the clinical setting is MCC-135 (or Caldaret; Mitsubishi Pharma Corporation, Osaka, Japan).[206,207] MCC-135 is thought to reduce intracellular calcium overload by enhancing uptake by the sarcoplasmic reticulum, which might reduce MPTP opening. Yarbrough et al. were the first to demonstrate that MCC-135 attenuated contractile dysfunction in an in vivo porcine model of ischemia-reperfusion[208], which was confirmed by others.209 MCC-135 decreased both heart rate and CK-MB release, troponin levels were comparable with the control group though. Although experimental data on infarct size was very limited[210], Bär et al. performed the first study in patients receiving MCC-135 prior to revascularization.[211] Both in this safety study as in a later study, MCC-135 failed to demonstrate any beneficial effects[212], except for a sub-study reporting a significant decrease in the incidence of patients with an ejection fraction <30% (P=0.03).[213] Based on two relatively large clinical studies and very limited experimental data on infarct size, it remains to be seen whether MCC-135 truly has cardioprotective properties in the acute setting of MI. More recently, Toll-Like Receptor (TLR) 2 on the circulating cells (but not on cardiomyocyte) was identified as the mediator of TLR2-dependent myocardial I/R injury. Anti-TLR2 therapy using a monoclonal antibody blocking this TLR2 exerts its action by reducing leukocyte influx, cytokine production and pro-apoptotic signaling.[214] In addition, exosomes were identified as the cardioprotective factor in MSC conditioned media.[215] Interestingly, very low concentrations of exosomes resulted in an impressive reduction of infarct size, mainly by delivery of cardioprotective substances (proteins, miRNA) to endangered cardiomyocytes (paracrine effect). As with all experimental compounds, follow-up studies will be necessary to evaluate the protective effects these compounds in large-animal models and in patients in a later stage.

CONCLUDING REMARKS

Cardioprotection has been extensively investigated in the last three decades, but many therapeutic strategies with proven efficacy in experimental studies have failed in the clinical setting. This might be attributed to the use of non-clinically applicable cell- and animal models, wrong timing of

compound administration (i.e. during early ischemia) and clinically relevant end points (infarct size, cardiac function, mortality). However, a number of pharmacological strategies, mainly focusing on the MPTP (Table 2) or mimicking pre/postconditioning by activation of pro-survival kinases (Table 4), have been able to reduce infarct size when given at time of reperfusion (Figure 3). The discovery of a regulated form of necrotic cell death (necroptosis) and its pharmacological inhibitor (Necrostatin-1) may be another opportunity to limit cell death in the ischemic heart, although additional research is warranted before further steps towards a clinical setting can be made (Table 3).

As therapeutic interventions administered at the time myocardial reperfusion have been proven to reduce infarct size in both experimental and clinical models, the existence of lethal reperfusion injury and its contribution to ischemic cell death can no longer be ignored. Patients presenting with an acute ST-segment elevation myocardial infarction (STEMI) will therefore benefit from therapy aiming for 1) timely reperfusion most likely via primary percutaneous coronary intervention, and 2) prevention of lethal reperfusion injury. Only this dual approach will make sure that patients can benefit completely from the myocardial salvage obtained by reperfusion therapy.

In conclusion, the list of cardioprotective agents that can be used as adjuvant therapy next to reperfusion is promising. Large multi-center clinical trials with enough statistical power will be necessary to establish observed beneficial effects and to answer the question whether they can improve clinical outcomes as well. To prevent translational failure, particular attention must be paid to proper selection of patients (who will benefit the most), application (relevant concentration in the early phase of reperfusion) and hard end-points.

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CHAPTER **3**

Necrostatin-1 Alleviates Reperfusion Injury Following Acute Myocardial Infarction in Pigs

Submitted to Cardiovascular Research

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ABSTRACT

Aims

In rodents, it has previously been shown that Necrostatin-1 (Nec-1) inhibits RIP1, a central regulator of programmed necrosis, thereby decreasing cell death and reducing infarct size after ischemia/reperfusion (I/R) injury. To address unanswered questions on feasibility and efficacy of Nec-1 in a large animal model, we assessed the effects of Nec-1 in a porcine I/R model, relevant to human disease.

Methods and Results

In Dalland landrace pigs (n=22, 69±3kg), I/R injury was induced by a 75-min surgical ligation of the left circumflex coronary artery (LCx). Ten minutes prior to reperfusion, pigs were randomly allocated to different Nec-1 doses (1.0mg/kg or 3.3mg/kg) or vehicle treatment (CTRL). Functional endpoints and immunohistological analyses were performed 24 hours after reperfusion. Nec-1 3.3mg/kg significantly reduced infarct size (24.4±15.6%) compared to Nec-1 1.0 mg/kg (54.8±16.9%) or CTRLs (62.1±26.6%; P=0.016). In line, LVEF was significantly higher in Nec-1 3.3mg/kg, compared to Nec-1 1.0mg/kg or CTRL treated animals (50.0±12.0% vs 32.5±12.9% vs 31.9±6.6% respectively, P=0.015). Hemodynamically, a preserved contractility was observed (end systolic volume at 100 mmHg (ESV100)) at 24-hours follow up (87.6±17.3 ml vs. 74.5±41.1 ml vs. 56.8±11.8 ml, respectively; P=0.032), reflecting improved cardiac function.

Conclusions

In the pig model of I/R injury, intravenous administration of Nec-1 prior to reperfusion was an effective and above all practical therapeutic strategy that significantly reduced infarct size and preserved left ventricular function. These data highlight the potential of cardioprotection as a promising adjuvant therapy in the setting of early reperfusion following I/R injury.

INTRODUCTION

Acute myocardial infarction (AMI) leads to cardiomyocyte loss, scar formation and post-MI adverse remodeling thereby remaining one of the leading causes of morbidity and mortality in the Western world.[1] To date, treatment for AMI is aimed at early restoration of myocardial blood flow by means of percutaneous coronary intervention (PCI) and/or use of thrombolytic pharmacologic therapy. However, reperfusion of the ischemic territory comes at the expense of additional injury.[2,3] This additional damage, also known as myocardial reperfusion injury, leads to increased cell death in the endangered myocardium. In fact, ~50% of the total infarct size has been attributed to the detrimental effects of reperfusion injury.[4] Reperfusion injury can be characterized as a cacophony of interrelated mechanisms that culminates in cell death, either via apoptosis or necrosis.[4] Although inferences on true causality are difficult to draw, several key mechanisms via which reperfusion injury occurs are 1) intracellular calcium overload induced hypercontracture, 2) formation of reactive oxygen species, 3) mitochondrial dysfunction and swelling and 4) subsequent intracellular proteolysis and cell death.[4,5]

Fortunately, reperfusion injury is a process that, despite its complexity, can be steered and alleviated at multiple levels (i.e. mitochondrial, cellular, inflammatory mediators or subsequent innate immune response) thereby reducing the final infarct size.[4,6-9] Thus, the search is ongoing for novel compounds that can serve as an adjunctive therapy in the acute phase of the MI that are able to inhibit additional cardiomyocyte loss following reperfusion of the culprit coronary artery. However, the majority of compounds that have shown efficacy in rodents have yet failed to do so in the clinic.[10-12] This might be attributed to the use of non-clinically applicable animal models, wrong timing of compound administration (i.e. during early ischemia) and the lack of clinically relevant end points (infarct size, cardiac function, mortality).[3] Therefore, large animal studies are a necessary step for the translation of promising compounds to limit reperfusion injury.

For many years, apoptosis was considered as the only form of regulated cell death in the ischemic heart.[13,14] However, emerging evidence has demonstrated that at least some part of necrotic cell death is a regulated process, in which the kinase activity of receptor-interacting protein 1 (RIP1) and its interaction with RIP3 are critically involved.[3,15] Receptor interacting protein kinases are serine/threonine kinases and are composed of a seven-member family of key regulatory proteins involved in cell survival and death.[16] The serine/threonine kinase activity RIP1 is required to initiate a cascade that, via phosphorylation of RIP3, ultimately leads to cell death due to programmed necrosis.[17] The concept of programmed necrosis and the possibility to inhibit this in the ischemic heart gives rise to new therapeutic options to prevent myocardial cell death. Recently, several reports have shown that interference with the RIP1 signaling pathway can prevent the initiation of necrosis at the mitochondrial level.[18,19] Necrostatin-1 (Nec-1), a small molecule capable of inhibiting RIP1 kinase activity, was shown to inhibit programmed necrosis without affecting other RIP1-mediated processes and efficiently prevented necrotic cell death after ischemic brain injury.[20,21]

We and others have recently reported on the encouraging cardioprotective effects of Necrostatin-1, previously investigated in rodents.[22,23] When infused intravenously 5 minutes prior to reperfusion, Nec-1 led to a ~30% infarct size reduction following MI.[23] In order to advance this compound towards a first-in-man clinical trial, we addressed the next phase of testing in a large animal model

that permit a better view on the cardioprotective effects of Nec-1 as the pig heart size and function closely resembles that of the human situation. To this end, we performed a dose-escalating study to determine the therapeutic efficacy of Nec-1 on infarct size and cardiac function in the pig model of I/R injury.

METHODS

Animals and Experimental Study Design

All animal experiments were conducted in accordance with the national guidelines on animal care and with prior approval by the Animal Experimentation Committee of the Faculty of Medicine, Utrecht University, the Netherlands.

Twenty-two female Dalland landrace pigs (van Beek SPF pig farm B.V., Lelystad, the Netherlands; weight 68±3kg; age: ~6 months) were subjected to a 75-min temporary ligation of the proximal circumflex coronary artery (LCx) followed by 24 hours of reperfusion, as previously described.[24] Animals were randomly assigned to receive either Necrostatin-1 treatment in two different doses (1.0 mg/kg, or 3.3 mg/kg) or served as controls receiving vehicle treatment of 20% dimethyl sulfoxide (CTRL)(Suppl. Figure 1). A total of 20 ml experimental compound or empty vehicle was administered intravenously 10 minutes prior to reperfusion at a rate of 150 ml/hr. Twenty-four hours after reperfusion, functional endpoints were measured and animals were euthanized by exsanguination under deep general anesthesia. Primary endpoint of this study was infarct size (IS) as determined by 1% triphenyl-tetrazolium chloride (TTC) staining and was expressed as a percentage of the area at risk (AAR), delineated by 1% Evans Blue. Analyses of IS/AAR and functional endpoints were performed by an investigator blinded to the treatment allocation.

Necrostatin-1: Dose rationale and Constituents

Based on previous work in rodents, optimal dose was set at 3.3mg/kg.[23] Commercially available Nec-1 (sc-200142, Santa Cruz) was slowly dissolved in 20 ml phosphate buffered saline (PBS) with 20% dimethyl sulfoxide (DMSO). Two different doses were tested, being the previously defined optimal dose of 3.3 mg/kg and a lower dose of 1.0 mg/kg.

Anesthesia and Medication

All animals were pretreated with amiodarone (400 mg/d, orally) for 10 days until surgery, clopidogrel (75 mg/d, orally) for 3 days until surgery, acetyl salicylic acid (80 mg/d, orally) for 10 days until surgery, and a fentanyl patch (25mcg/hr) 1 day prior to surgery to minimize postsurgical pain. The pigs were sedated with an intramuscular injection of ketamine (10 mg/kg), midazolam (0.5 mg/kg), and atropine (0.04 mg/kg) and subsequently induced with thiopental (4 mg/kg) before they were intubated and connected to positive pressure ventilator with a mixture of oxygen and air (FiO2 of 0.50). The ear vein was canulated and used for continuous admission of saline and anesthesia. Anesthesia was maintained throughout the surgical procedure by continuous intravenous infusion of midazolam (0.7 mg/kg/h), sufentanil citrate (6 µg/kg/h) and pancurorium bromide (0.1 mg/kg/h). Prior to infarction, a bolus of 7500 I.E. of heparin was given intravenously

to minimize the occurrence of no-reflow. In addition, 300 mg of amiodarone was infused intravenously in 45 minutes to minimize the onset of cardiac arrhythmias.

Echocardiography

The echocardiographic examination was performed with the animals under general anesthesia, lying in the right lateral position. Ultrasound data was acquired using a Philips iE33 scanner (Philips Healthcare, Eindhoven, the Netherlands) with a S5-1 phased array transducer (1-5 MHz) for two-dimensional datasets and the X3 transducer for three-dimensional datasets. Standard parasternal long and short axis views were obtained. Left ventricular (LV) dimensions and wall thickness were measured in accordance with the standards of the American Society of Echocardiography.[25] Systolic Wall thickening (SWT) was defined as the change in systolic wall thickness compared to diastolic wall thickness (WT_{syst}-WT_{diast}/WT_{diast} x 100). During the open-chest procedure, apical views (four-, two-, three-chamber) were obtained epicardially. Three-dimensional LV datasets were acquired under breath hold over six consecutive heart beats to assess LV end systolic volume (ESV), end diastolic volume (EDV) and ejection fraction (EF) using Qlab software (Philips, The Netherlands). LV diastolic parameters were also assessed. Pulsed wave Doppler imaging was used to assess transmitral flow patterns. Tissue Doppler Imaging from the four-chamber view was used to determine the mitral annular motion at the basal part of the septum and lateral wall. Analysis included the average of three peak diastolic velocities (E') for both annular sites combined. LV filling index was defined as the ratio of transmitral flow velocity to annular peak diastolic velocity (E/E'). The observer performing the echocardiographic analysing was blinded to the treatment allocation.

Pressure Volume loop analysis

Pressure-volume (PV) loops were measured using a 7-F conductance catheter, as previously described.[26] Briefly, LV pressure and volumes were measured and stored using a Leycom CFL-512 (CD-Leycom, Zoetermeer, the Netherlands). After correct placement in the LV, checked by the individual segmental conductance signals, the conductance signals were calibrated by cardiac output based on transonic flow probe measurement (Transonic Systems Inc., Ithaca, NY, USA). All data were collected while mechanical ventilation was paused. Regarding data recorded under different preload conditions, temporary inferior caval vein ligation was used. Data analysis and calculations were performed offline using custom-made software (CD Leycom, Zoetermeer, the Netherlands), as described previously.[26] Systolic LV function was assessed by various parameters, such as ESV at 100mmHg (ESV_{100}), LV ejection fraction, dP/dT+-peak, stroke work and preload recruitable stroke work. Diastolic function was assessed by dP/dT- analysis. The observer analysing the PV loop data sets was blinded to the treatment allocation.

Immunohistochemical staining

Snap frozen tissue samples from the infarct zone and remote area were embedded in Tissue-Tek (Sakura) and 5 µm cryosections were prepared on a microtome (Leika). Sections were dried for 30 min at room temperature (RT) and fixed in acetone.For the staining of polymorphonuclear neutrophils (PMN), endogenous biotin was blocked with Biotin blocking system (X0590, Dako), followed by

incubation with 10% normal horse serum for 30 minutes and mouse anti-pig neutrophils (1:100, Ls-c58180, LifeSpan BioSciences Inc.). The secondary antibody that was used was biotin labeled horse anti-mouse (1:200, BA-2000. Vector Laboratories), followed by streptavidin-horseradish peroxidase (1:1000, 7100-05, Southern Biotech). The stained neutrophils were visualized using the chromogen 3-amino-9-ethyl carbazole (AEC). Neutrophils were counted in 15 random fields in the infarct area in all included animals. The neutrophils were counted per field (40x).
Oxidative stress was assessed using the fluorogenic probe CELLROX red (5μM, Invitrogen). Cryosections were incubated for 30 minutes at 37°C and fixed in formaldehyde. Quantification was based on signal intensity in 5 random fields in the infarct area at 40x and analyzed using ImageJ (version 1.44g). TUNEL was analyzed using a commercially available apoptosis detection kit (11684795910, Roche) according to the manufacturer's recommendation. All analyses were performed by an investigator blinded to the treatment allocation.

Exclusion criteria

Two criteria were used to exclude animals for all subsequent analyses: 1) failure to have successful reperfusion, defined as a transmural infarct comprising the entire area at risk (>95% IS/AAR), and 2) a small AAR, defined as AAR comprising <20% of the left ventricle.[8] Animals were excluded by the blinded observer analyzing the IS/AAR.

Statistics

Data are presented as mean ± SEM. Functional data, area at risk and infarct size were analyzed by one-way ANOVA. When a significant difference was found, post-hoc analysis was performed with Bonferroni's test. When the assumption of the homogeneity of variance between groups was violated, nonparametric statistical analysis based on Kruskal-Wallis was performed. To test whether the follow-up values differed from baseline values in each group, a paired T-test was used. All statistical tests were two-sided. Differences were regarded significant at the $p<0.05$ level.

RESULTS

Mortality and procedural data

One animal (allocated to Nec-1 1.0 mg/kg) died shortly after the induction of ischemia due to refractory ventricular fibrillation before treatment was given. During 24 hours of follow-up, all of the remaining animals survived. Four animals were excluded. Three animals were excluded based on an area at risk smaller than 20% of the left ventricle (one in each group) and one that did not have successful reperfusion after I/R (allocated to the Nec-1 1.0 mg/kg group). Thus, for end-point analyses the Nec-1 1.0mg/kg group consisted of 5 animals, the Nec-1 3.3mg/kg of 6 animals, and the control group of 6 animals.

Necrostatin-1 delivery prior to reperfusion reduces infarct size

No differences existed with regard to the AAR between the control group (Figure 1B; 33.8 ± 5.6%), the Nec-1 1.0 mg/kg group (Figure 1B; 31.3 ± 12.3%) and Nec-1 3.3 mg/kg group

(Figure 1B; 33.1 ± 9.5%; P=0.902). However, whereas IS was comparable between the Nec-1 1.0 mg/kg group and the control group, administration of Nec-1 3.3 mg/kg resulted in a significant reduction compared to the control group (Figure 1C; 24.4 ± 15.6% vs. 62.1 ± 26.6%; P=0.02). Next, we investigated commonly used serum marker for myocardial damage, i.e. lactate dehydrogenase (LDH), creatinin kinase (CK) and the MB-isoenzyme fraction of CK (CK-MB).

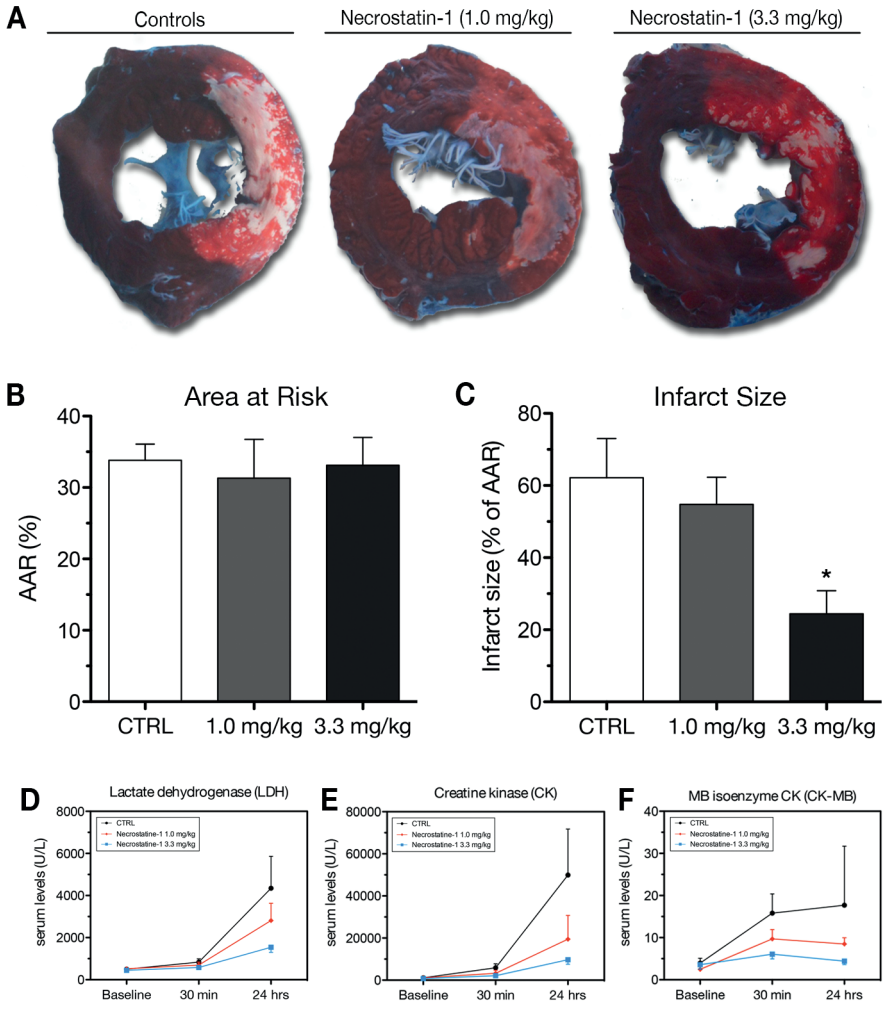

Figure 1. Necrostatin-1 administration reduces infarct size after MI.
(A) Representative photographs of the TTC staining showing the area at risk (bright red) and the infarct area (white). **(B)** The area at risk did not differ between groups. **(C)** Infarct size, expressed as a percentage of the AAR, was significantly reduced between Nec-1 3.3 mg/kg group compared to controls. **(D)** Circulating levels of serum markers for myocardial injury 24 hours following reperfusion of the LCX show extensive tissue damage that can be ameliorated in a dose-dependent manner by Necrostatin-1 treatment. * represents p<0.05 based on Bonferroni post-hoc analysis vs CTRL. All data are mean ± SEM, n=6, 5, 6 for CTRL, Nec-1 1.0 mg/kg and Nec-1 3.3 mg/kg respectively.

In line with the IS data, all serum markers showed a non-significant but consistent trend towards increased levels in the control group and Nec-1 1.0 mg/kg, compared to Nec-1 3.3 mg/kg treated animals (Figure 1D to F; ANOVA analysis of LDH: P=0.387, CK; P=0.285; CK-MB: P=0.525, respectively).

Necrostatin-1 influences cell death by reducing oxidative stress but not apoptosis

As a next step, we investigated the effect of Nec-1 treatment on cell death disaggregating nuclear oxidative stress and apoptosis mediated DNA fragmentation by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). Nuclear ROS levels were significantly decreased upon Nec-1 infusion, in a dose dependent manner (Figure 2 A to C; controls 5.25±0.46 vs Nec-1 1.0mg/kg 2.94±0.79 vs Nec-1 3.3mg/kg 2.01±0.28; P=0.007). However, apoptosis was not influenced by Nec-1 treatment (Figure 2D; Suppl Figure 2), highlighting as well that the diminished infarct size was mainly due to a reduction in necrotic cell death.

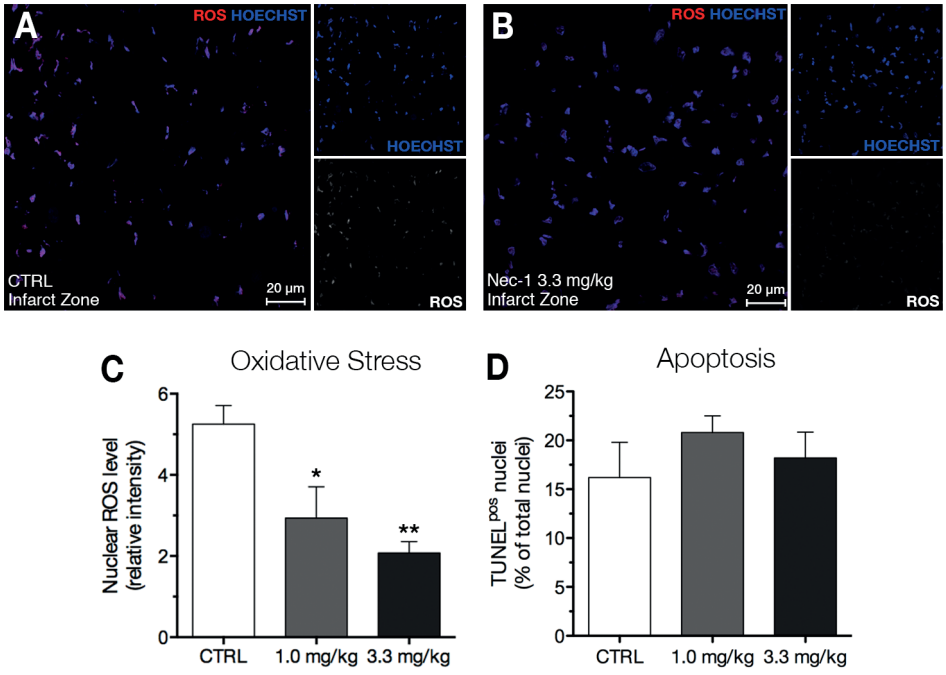

Figure 2. Necrostatin-1 influences cell death by decreasing oxidative stress but not apoptosis

(A,B) Representative microscopic confocal images of nuclear expression levels of oxidative stress (red signal) in the infarct zone 24 hours after reperfusion, quantified in **(C)** as a intensity relative to the remote non-ischemic myocardium. **(D)** Apoptosis was assessed by TUNEL staining, showing similar levels of apoptosis between all groups. * represents p<0.05; ** represents p<0.01, both based on Bonferroni post-hoc analysis vs CTRL. All data are mean ± SEM, n=6, 5, 6 for CTRL, Nec-1 1.0 mg/kg and Nec-1 3.3 mg/kg respectively.

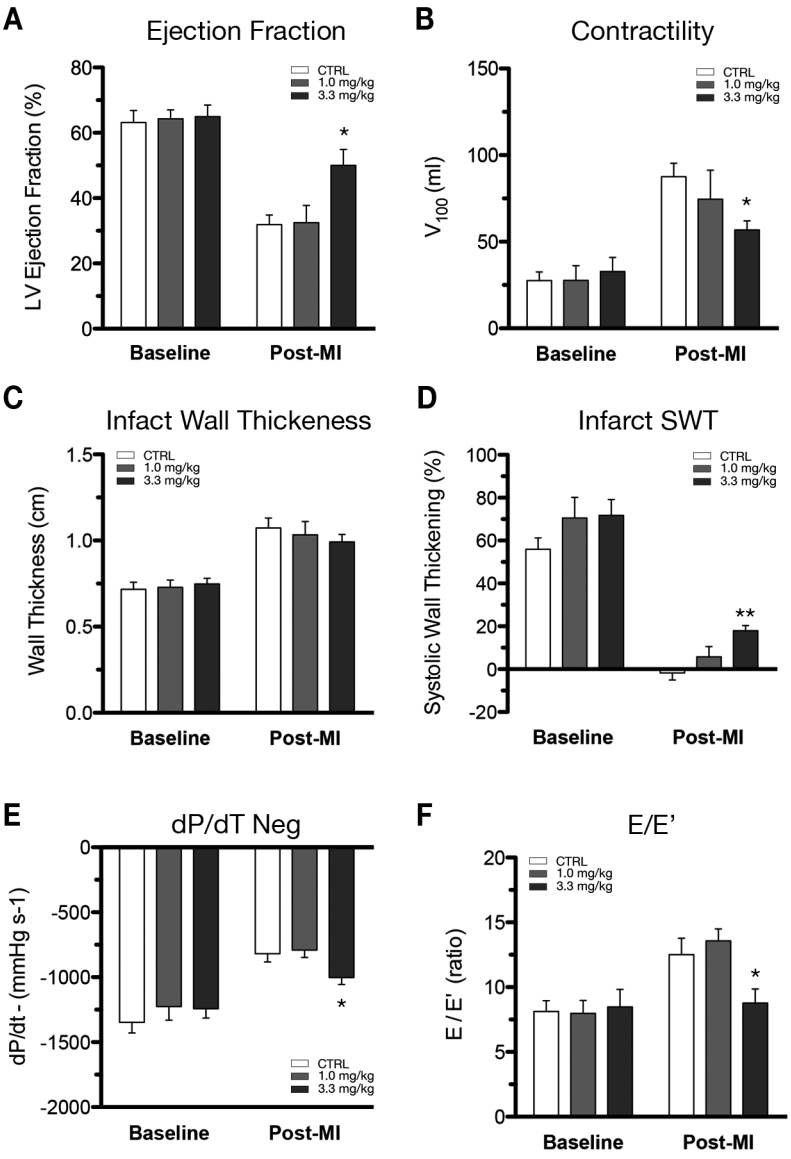

Figure 3. Necrostatin-1 improves cardiac function and hemodynamics.
(A) LV ejection fraction (LVEF) was significantly increased in the Nec-1 3.3 mg.kg animals at follow up. **(B)** Preload-independent measure of contractility, expressed as the ESPVR derived volume at 100 mmHg pressure showed a dose dependent improvement in the Nec-1 treated animals. **(C)** Wall thickness measured by 2D echocardiography showed an overall increase in end-diastolic wall thickness regardless of treatment allocation. However, **(D)** systolic wall thickening was significantly higher in the Nec-1 3.3 mg/kg group compared to CTRL treated animals. **(E)** Intracardiac pressure recordings showed a significant improvement in the diastolic peak dP/dT. (F) Ratio of peak transmitral early inflow and annular peak diastolic velocity. * represents $p<0.05$ based on Bonferroni post-hoc analysis vs CTRL. All data are mean ± SEM, n=6, 5, 6 for CTRL, Nec-1 1.0 mg/kg and Nec-1 3.3 mg/kg respectively.

Necrostatin-1 improves cardiac function after I/R injury

At baseline, no differences existed between the groups with regard to cardiac function (Figure 3A). One day after reperfusion, Nec-1 at the dose of 3.3 mg/kg significantly increased LVEF, compared to the groups receiving Nec-1 1.0mg/kg or vehicle treatment (Figure 3B; 50.0 ± 12.0% vs 32.5 ± 12.9% vs 31.9 ± 6.6%; P=0.040). Likewise, the preload independent measure of contractility V_{100} was also significantly decreased by Nec-1 3.3 mg/kg (Figure 3B; 56.8 ± 11.8 ml vs. 74.5 ± 41.1 ml vs 87.6±17.3 ml; P=0.032). Regional edema in response to the ischemic insult was apparent in all groups, indicated by an increased thickness in the infarcted wall compared to baseline reference values (Figure 3C). However, systolic wall thickening was significantly lower in the controls and low dose group (Figure 3D; -1.7 ± 7.6% and 5.6 ± 9.6% respectively) compared to Nec-1 3.3 mg/kg (Figure 3D; 17.9 ± 5.4%; P=0.005). Regarding diastolic function, intracardiac pressure recordings revealed a significantly preserved negative

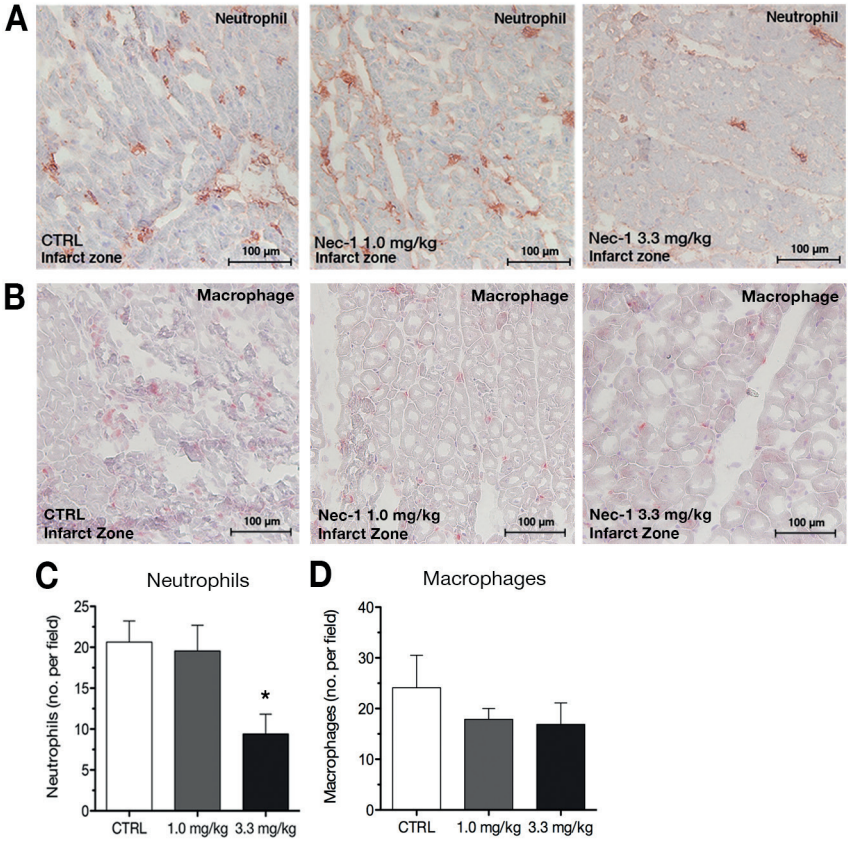

Figure 4. Necrostatin-1 limits neutrophils influx in the infarct area

(A to C) Representative microscopic images of neutrophils (red signal) showing that Necrostatin-1 treatment reduced the influx of PMN 24 hours after the MI. **(D)** Quantification of neutrophils. * represents p<0.05 based on Bonferroni post-hoc analysis vs CTRL. All data are mean ± SEM, n=6, 5, 6 for CTRL, Nec-1 1.0 mg/kg and Nec-1 3.3 mg/kg respectively.

peak pressure drop (dP/dT-) in the Nec-1 3.3 mg/kg treated animals compared to controls (Figure 3E; -1002 ± 133 mmHg/s vs -819 ± 140 mmHg/s; P=0.040). In line with this observation, echocardiographic analysis of diastolic function reflected by the E/E' showed a significantly lower ratio in the Nec-1 3.3 mg/kg group (Figure 3F; 8.7 ± 2.2) compared to Nec-1 1.0 mg/kg (Figure 3F; 13.6 ± 1.8) or the control group (Figure 3F; 12.5 ± 2.8; P=0.038). Thus, both the intracardiac pressure recordings and the mitral inflow patterns point towards a more favorable diastolic function in the Nec-1 3.3 mg/kg treated animals compared to the Nec-1 1.0 mg/kg group and control group.

Necrostatin-1 abrogates inflammatory response following I/R injury

As circulating inflammatory cells also contribute to myocardial reperfusion injury, we studied the influx of neutrophils, which are the first responders following an ischemic cardiac insult.[27] One day after reperfusion, neutrophil influx within the infarct area –delineated by TTC staining as white colored- was significantly reduced in animals receiving the 3.3 mg/kg dose of Nec-1 compared to Nec-1 1.0 mg/kg and control animals (Figure 3 A to D; 9 ± 4/field vs 23 ± 4/field vs 21 ± 3/field respectively; p = 0.005). Next, we studied the early infiltration of macrophages, which follow primary infiltration of polymorphic neutrophilic granulocytes between 24-72 hours after the onset of infarction. At 24 hours following reperfusion, there was already a non-significant decrease in the number of macrophages based on Nec-1 (controls 24±6/field vs Nec-1 1.0mg/kg 18±2/field vs Nec-1 3.3mg/kg 17±4/field; P=0.20).

DISCUSSION

In the present study, the cardioprotective effects of Nec-1 were tested for the first time in a large animal model of acute I/R injury, closely resembling the human situation. We have demonstrated a significant reduction in the infarct size in the Nec-1 treated animals compared to vehicle treatment, in line with previous efficacy data in rodents.[23,28] Secondly, another important finding of this study was the observed improvement in cardiac function upon Nec-1 treatment.

Over the recent years, the research field on reperfusion injury brought cardioprotection out of limelight and reported on many promising biologics and small molecules that were able to reduce infarct size.[3] As of yet, however, the translation towards much needed clinical protocols, is still lacking behind. This is not based on the absence of attempts to strive forwards and test new compounds in large animals or in patients, but rather the results of negative study outcomes that are in contrast with the previous positive results based on small animal studies.[2] Based on many negative trial results, several statement papers on optimizing this translational roadmap have acted as a handhold for future studies on cardioprotection.[12,29,30] In line with these recommendations, this study was designed specifically to investigate the dose-optimizing process and efficacy of Nec-1 in a large animal model. Both the timing of the admission, 10 minutes prior to reperfusion, as well as the intravenous delivery method ensures that this present treatment protocol can be applied in clinical care regarding ST-elevated MI patients in the setting of the catheterization laboratory. Using this treatment protocol, we have shown that the delivery of Nec-1 at a dosage

of 3.3 mg/kg led to a ~40% reduction in IS compared to empty vehicle infusion, in line with previous studies.[31] Interestingly, the dose-escalating study design also revealed that a low dose of 1.0 mg/kg already led to a non-significant but noticeable decrease in IS compared to controls (54.79 ± 7.54% vs 62.15 ± 10.87%; P=0.607). Furthermore, the reduction in myocardial IS was further substantiated by a concomitant decline found in the level of circulating biomarkers for cell damage, such as LDH, CK and CK-MB in the serum one day after reperfusion. Taken together, these findings support the notion that: 1) targeting reperfusion injury in the pig heart can be an effective strategy to reduce the total infarct size, and, 2) this can be effectively achieved in a dose dependent manner by delivery of Nec-1.

Nec-1 improved global and regional cardiac systolic function following I/R injury (Figure 2). One day after reperfusion, EF was significantly improved by Nec-1 treatment, as was the preload-independent measure of contractility $V_{100mmHg}$ (the volume of the left ventricle at 100 mmHg, based on the linear end-systolic pressure volume relationship (ESPVR)) (Figure 2). Moreover, echocardiographic analysis of regional deformation showed that there was some slight residual systolic movement in the Nec-1 treated animals, whereas the 1.0 mg/kg dose or controls resulted in a complete loss of systolic thickening of the infarcted wall. In fact, many of the control animals displayed a dyskinetic outward movement during systole, as shown by a negative value of SWT. Importantly, on a cellular level, these results imply that Nec-1 not only reduced the extent of cardiomyocyte loss (Figure 1), but also preserved the functionality of those salvaged cardiomyocytes (Figure 2).

Finally, Nec-1 reduced the inflammatory influx during the first 24 hours following reperfusion. This reduction in neutrophils acts beneficially for the heart in two ways. First, as the first response mechanisms to an ischemic insult, invading cells such as neutrophils involves secretion of pro-inflammatory cytokines and the production of ROS.[32] Recently, it was shown that neutrophil depletion reduced the myocardial infarct size following ischemia/reperfusion injury.[33] Second, necrotic cell death itself is associated with pro-inflammatory cytokines (i.e. TNF-α) that act as a strong stimulus for neutrophil activation and migration. Hence, lower levels of neutrophils might coincide with the Nec-1 mediated reduction of necrotic cell death limits the neutrophil influx is reduced in the Nec-1 treated animals.

Study Limitations

As concerns of safety, we observed one animal in which reperfusion was not restored, one in the Nec-1 1.0 mg/kg group. The cause for no reflow is most likely based on limitations pertaining to the study model, since compression of the coronary artery by a suture ligation can cause damage to the coronary artery leading to higher changes of local tissue injury triggering vasospasm.[34] Moreover, we did not administer vasodilatory drugs such as nitroglycerin to minimize the change of no-reflow directly after reperfusion.[24] Last, we did not observe this adverse event in our previous work on Nec-1 in rodents or in the 3.3 mg/kg Nec-1 treated animals.[23]

Secondly, the time of ischemia, 75 minutes, was deliberately chosen to closely mimic the clinical daily routine care although it is conceivable that the effect of reperfusion injury will attenuate as longer duration of total coronary occlusion leads to higher initial levels of cardiomyocyte loss prior to reperfusion ('time is muscle').[6] However, as aging impairs the success rate of cardioprotection,

the aged population at risk in clinical care with all known and unknown cardiovascular risk factors differ from that of the 6 month-old pigs used in this study.[35]
Thirdly, Necrostatin-1 pharmacokinetics could not be assessed in the present study. This is a critical step since interference in cell death signaling could potentially accelerate (occult) malignant processes in the aged patient that endures an AMI. We therefore hypothesized that a single doses just prior to reperfusion holds great promise in the search to maximize cardioprotection whilst minimizing potential off-target side effects.

Conclusions

In summary, three major conclusions originate from this study: 1) Intravenous administration of Nec-1 10 minutes prior to reperfusion leads to a significant reduction in infarct size; 2) Administration of Nec-1 improves hemodynamics in the acute phase of myocardial infarction leading to improved systolic and diastolic function; 3) The administration of Nec-1 leads to a decreased inflammatory influx in the infarct zone following MI. Taken together, the present study provides the first evidence for Necrostatin-1 as a cardioprotective compound in a pre-clinical large animal model relevant to human disease, which can be applied in an effective, simple and widely available protocol of cardioprotection in STEMI patients.

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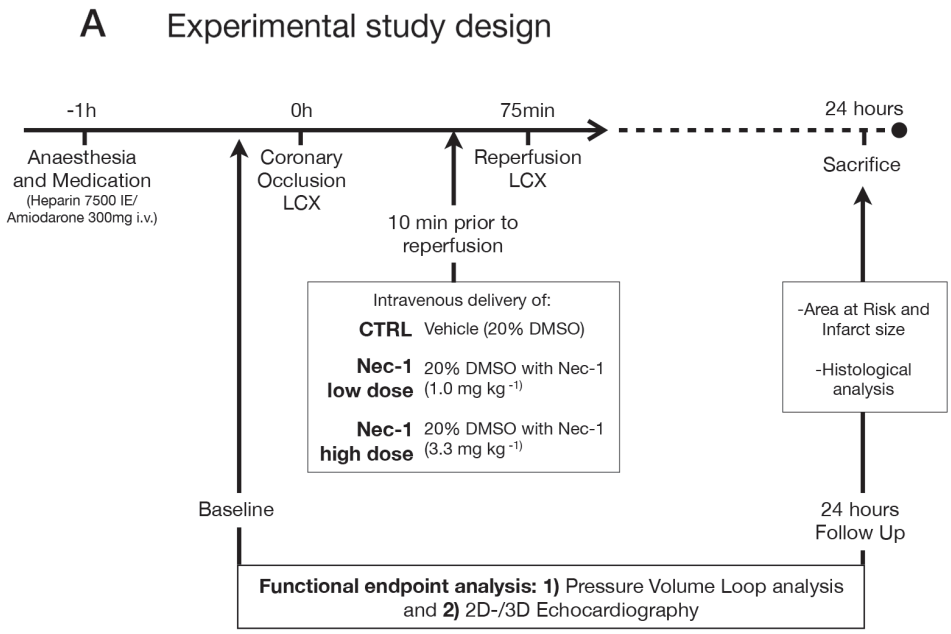
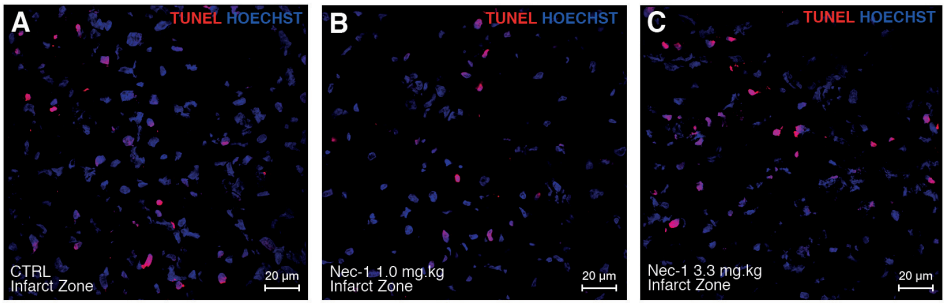
SUPPLEMENTARY INFORMATION



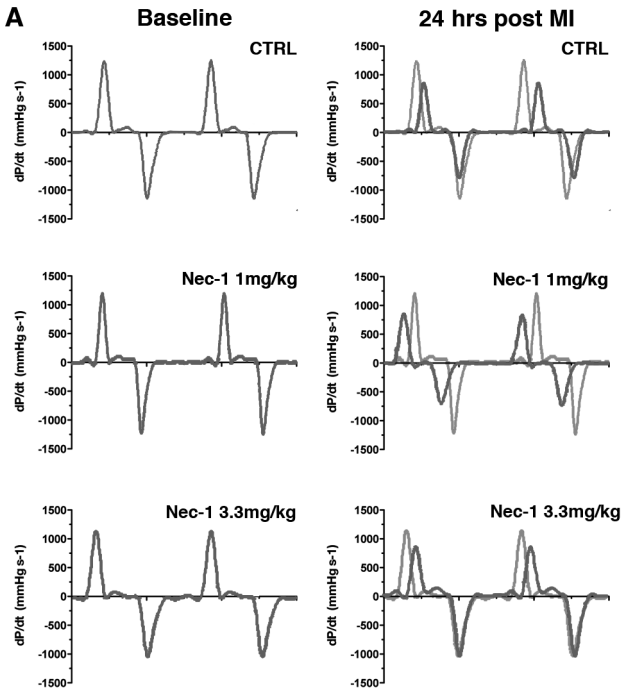
Supplementary Figure 1. Experimental Study Design

(A) Schematic overview of the *in vivo* study design of an acute MI model and different treatment groups (box) administered 10 minutes prior to opening of the occluded coronary artery.



Supplementary Figure 2. TUNEL staining

Representative confocal images of TUNEL staining in the infarct zone 24 hours after reperfusion for **(A)** controls, and Nec-1 treated animals with a dosage of **(B)** 1.0 mg/kg and **(C)** 3.3 mg/kg.



Supplementary Figure 3. Intracardiac pressure recordings after MI

(A) Representative intracardiac recordings of pressure showing a marked decrease after MI compared to baseline (prior to MI) in force generating capacity (positive dP/dT peak) and relaxation (negative dP/dT peak) in the low dose Nec-1 and CTRL treated animals, whereas the high dose Nec-1 treated animals preserved peak dP/dT.

CHAPTER **4**

Heart Regeneration and the Role of Cardiac Stem Cells

Published as

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ABSTRACT

Acute myocardial infarction leads to irreversible loss of cardiac myocytes thereby diminishing the pump function of the heart. As a result, the strenuous workload imposed on the remaining cardiac myocytes often gives rise to subsequent cell loss until the vicious circle ends in chronic heart failure (CHF). Thus, we are in need of a therapy that could ameliorate -or even reverse- the disease progression of CHF. Endogenous regeneration of the mammalian heart has been shown in the neonatal heart, and the discovery that it may still persist in adulthood sparked hope for novel cardioregenerative therapies. As the basis for cardiomyocyte renewal, multipotent cardiac stem/progenitor cells (CSCs) that reside in the heart have been shown to differentiate into cardiac myocytes, smooth muscle cells and vascular endothelial cells. These CSCs do have the potential to actively regenerate the heart but clearly fail to do so after abundant and segmental loss of cells, such as what occurs with MI. Therefore, it is vital to continue research for the most optimal therapy based on the use or *in situ* stimulation of these CSCs. In this review, we discuss the current status of the cardioregenerative field. In particular, we summarize the current knowledge on CSCs as the regenerative substrate in the adult heart and its use in preclinical and clinical studies to repair the injured myocardium.

INTRODUCTION

Since the 20th century, remarkable progress has been made in the treatment of coronary artery disease. Most of the cardiovascular risk factors, which were unravelled by observations in large cohorts like the Framingham study, served as a substrate for pharmacological intervention.[1, 2] Besides these milestones in preventive cardiology, the last three decades were marked by several major breakthroughs in the treatment for acute myocardial infarction (AMI), such as the introduction of the coronary care unit (CCU), pharmacological reperfusion (i.e. thrombolysis), pharmacological interventions (e.g. beta blockade, ACE inhibitors, antiplatelet drugs, statins) and improvements in interventional cardiology (i.e. primary percutaneous coronary intervention, PPCI).[3] As a result, our ability to successfully treat the acute moment of the disease came at the expense of a vast increase in patients left behind with a chronic condition. In particular, the chronic sequels of AMI such as congestive heart failure (CHF) or life-threatening cardiac arrhythmias4 are not only frequent, they also lack effective therapy that could stop -or even reverse- disease progression. This would avoid the necessity of current last resource measures, which are heart transplantation (infrequently performed due to donor shortage)[5], or left ventricular assist devices either as bridge-to-transplant or destination therapy.[6]

As the average life expectancy rises in the developed world combined with its population persistently subjecting itself to traditional risk factors, we are faced with an increase of epidemic magnitude in chronic heart disease that requires vast amounts of human resources and burden on our healthcare budget. In the USA alone, a total of 8 million people have endured an AMI, with an estimate of 785 000 new cases of AMI annually.[7] Of those, roughly 5.7 million patients have CHF accounting for approximately $30 billion annually in health care costs in 2008, with a predicted triplicate in costs rising to $97 billion annually in 2030.[7] Given the initial loss of functional cardiac myocytes as the trigger of a subset of adverse remodelling processes that eventually lead to CHF[8], it is imperative to develop new treatments that either i) further reduce the loss of cardiac myocytes during AMI, or, ideally, ii) can replace lost cardiac myocytes with newly generated counterparts. The latter option can be regarded as the holy grail of cardiac regenerative medicine and has been a controversial subject for centuries.[9] Luckily, it was nature herself, which reassured the validity of this regenerative paradigm, evidenced by various research groups that the adult mammalian heart, by itself, possesses an intrinsic form of cellular homeostasis that permits regeneration and formation of new cardiac myocytes and vasculature, and subsequent replacement of lost cardiac myocytes for physiological turnover.[10-13] These exciting findings were received with initial scepticism since it was in sharp contrast with the previously embraced paradigm that relied on the notions that i) all cardiac myocytes are terminally differentiated, and thus incapable of re-entering the cell cycle ('the heart is a post mitotic organ') and the fact that ii) there are no stem and/or progenitor cells in the heart that can differentiate into functional cardiac myocytes. One of the presumed causal factors accounting for endogenous cardiac regeneration are the tissue-specific stem/progenitor pool in the heart that creates offspring capable of differentiating into mature functional cardiac myocytes and vasculature.[10] This endogenous repair mechanism is clearly not sufficient enough to repair large segmental loss, such as that occurs in an MI. Indeed, a common challenge raised by the skeptics is to question why an MI evolves

into a scar if regenerating CSCs are present in the myocardium? What this question overlooks is that the sudden obstruction of a main parenchymal artery of any organ, no matter how abundant its resident stem cells (e.g. testicle, bone marrow, skin, intestine etc.), always evolves into a scar. This is so because during the evolution of long-lived organisms the presence of adult stem cells is likely to have been selected to not regenerate catastrophic acute segmental cell losses but as a mechanism to repair minor lesions and maintain the normal wear and tear of the tissue.
In this review, we aim to summarize the evidence as to whether the heart has intrinsic regenerative capacity, and if so, to what extent regeneration is based on its own tissue-specific stem/progenitor cells. Secondly, we discuss the current status of the results achieved thus far in preclinical studies and in two pioneering first-in-man clinical trials that used these CSCs as the basis for cardiac regeneration in the injured heart.

EVIDENCE FOR CARDIOMYOCYTE REGENERATION IN THE HEART

Regeneration of the heart in amphibians and fish

The impression that cardiac regeneration does not occur in humans was emphasized by observations in certain species, like the newt or zebrafish, which can easily regenerate large parts of organs or body parts, including the heart. In 1768, the biologist Lazzaro Spallanzani reported complete regeneration of the salamander limb after its removal. The capacity to fully regenerate the excised apex of the left ventricle -corresponding with a loss of ~20% of total cardiac myocytes- without any apparent signs of scar formation was described for the newt[14, 15], and more recently, the zebrafish.[16] The relative ease of genetically altering the zebrafish enabled scientists to show that it were in fact the cardiac myocytes adjacent to the resection wound that responded by a process of dedifferentiation, and break down of their contractile apparatus, before activating a set of early cardiac transcription factors such as GATA4.[17-19] Thus, these studies provide evidence of naturally occurring regeneration of the heart and its underlying mechanism involved. The question remains as to what extent mechanisms behind heart regeneration in these species could also be present in mammals, and if so, which factors lead to an apparent dormant state hereof in the adult mammalian heart.

Regeneration in the neonatal mammalian heart

During embryonic development, the mammalian heart shows remarkable capacity for regeneration. Drenckhahn and colleagues used a cardiomyocyte specific conditional knockout of the X-linked Holocytochrome c synthase (Hccs) gene to create female progeny that, by random X chromosome inactivation, had a mosaic heart.[20] As expected, early female embryos displayed a ratio of 50:50 between normal and *Hccs*-null cardiac myocytes. During embryonic development, proliferating functional cardiac myocytes gradually replaced dysfunctional *Hccs* null cardiac myocytes. As a result, by birth, approximately 90% of all cardiac myocytes were the progeny of cardiac myocytes with one functional *Hccs* allele. This regenerative response was also observed in the first days of the neonatal mouse heart and lost by 7 days of age.[21] With the use of a tamoxifen-inducible Cre recombinase under control of the αMHC promotor,

newly generated cardiac myocytes in the neonatal heart were shown to have originated from pre-existing cardiac myocytes.[21] These findings were reinforced by the observation of a marked decline in telomerase reverse transcriptase (Tert)-GFP expressing cells in the adult heart compared to the neonatal heart. Interestingly, among Tert-GFP+ cells were both Sca-1+ CSCs as well as mature cardiac myocytes.[22]

Regeneration in the adult mammalian heart

With the exception of some tissues such as the liver, skin and intestine, mammals have largely lost its regenerative potential following embryonic and the early post-natal period.[20] After an AMI, massive loss of cardiac myocytes is replaced by fibrosis and subsequent scar formation.[23] Distinguishing between the -albeit very limited- presence or absence of a regenerative potential of the adult mammalian heart is of utter importance since closely mimicking or augmenting a biological process already present in nature is easier than to initiate a new process that does not play a role in normal cellular homeostasis and/or turnover. Until the last decade, two main clinical observations served as the basis for the old paradigm that the heart is a post-mitotic organ[24]; 1) Until then, observations on functionally significant myocardial regeneration in the mammal heart had not been documented; 2) The occurrence of primary tumours arising from the myocardium has been rarely observed in the adult mammalian heart.[24] Since then, there has been a slow but steady reconsideration of this paradigm after a series of reports on the presence of cardiomyocyte renewal in the adult mammalian -including human- heart.[11-13, 25-27] In 2009, the group of Bergman[13] elegantly rendered the vast increase in atmospheric ^{14}Carbon levels--based on post World-War II nuclear bomb testing-- into a pulse-chase experiment of global magnitude to determine the age of cardiac myocytes in relation to the age of the given individual. After the Partial Test Ban in 1962, the increased levels 14C in the atmosphere declined rapidly as it was absorbed in the biosphere. Thus, as DNA was synthesized within this given time period, the levels of ^{14}C incorporated in the DNA corresponded with the registered levels of ^{14}C in the atmosphere, providing the Bergman group the necessary means to accurately establish the date of DNA synthesis. If indeed the post-mitotic heart lacked any regenerative potential, the age of all cardiac myocytes should coincide within the timeframe of the foetal development and early postnatal period. In contrast, it showed that the adult human heart contained cardiac myocytes that were generated throughout the human life-span. Correcting for polyploidization as the basis for newly synthesized DNA in older cardiac myocytes without cell division (cytokinesis), the investigators predicted an approximate annual turnover rate of cardiac myocytes in the order of 1% at the age of 25, declining towards 0.45% by the age of 75.[13]

Recently, the second report based on this approach to estimate the rate of cardiomyocyte turnover in the adult human heart came from the group of Anversa.[28] Strikingly, Kajstura et al calculated a 16-fold higher rate in which the myocyte fraction of the heart is completely replaced approximately 8 times during the human life-span. These findings are in sharp contrast with the 50% replacement of cardiac myocytes during life[13], the preclinical data in rodents by the group of Loren Field, in which DNA synthesis in the adult heart was virtually absent (0.0006%)[29], as well as the group of Richard Lee[30], where low rate (0.76%) of cardiac myocyte turnover was observed during normal aging.

In conclusion, in contrast with previous studies that showed that cardiomyocyte renewal is virtually absent, multiple research groups have independently shown the presence of a regenerative mechanism in the adult mammalian heart. There is accumulating evidence that the adult human heart is characterized by DNA synthesis and formation of cardiac myocytes. The actual turnover rate of cardiac myocytes, however, varies widely in more than one order of magnitude.

CARDIAC STEM/PROGENITOR CELLS

Evidence for Stem/Progenitor Cell involvement in Cardiac Regeneration

Since the concept of cardiac regeneration in the mammalian heart slowly emerged as a reconsidered paradigms on the adult heart cellular homeostasis, one of the next questions that remained to be answered was the cellular source of these newly formed cardiac myocytes. To address this question, the group of Lee designed a genetic lineage tracing experiment that -for the first time- shed light on the cellular homeostasis that governs cardiomyocyte renewal.[27] In a double transgenic MerCreMer/ZEG inducible cardiomyocyte reporter mouse, a tamoxifen-induced pulse caused an irreversible genetic switch -only in the cardiac myocytes- from β-galactosidase to the expression of green fluorescent protein (GFP). Hence, if during the chase any GFP negative stem or progenitor would form new myocytes, these would still express β-galactosidase. Two major findings emerged from this landmark report; 1) Various models of myocardial injury (MI model and chronic pressure overload) resulted in a significant increase in GFP- β-gal+ cardiac myocytes and a corresponding decrease in GFP+ β-gal- cardiac myocytes; 2) During normal aging of the rodent heart, there was no decrease in GFP+ β-gal- cardiac myocytes, suggesting an absence of stem cell based physiologic cardiomyocyte renewal.[27] Recent work from the same group, however, brought to light that, upon induction of MI, there is a high rate of cardiomyocyte turnover in the adult mammalian heart that originates from pre-existing GFP+ cardiac myocytes, rather than non-cardiomyocytes. With regard to physiological aging, using multi-isotope imaging mass spectrometry with their previous transgenic mouse model[27], they calculated that, in young adult hearts, cardiac myocytes are replaced by proliferation of dedifferentiated pre-existing cardiac myocytes at an annual rate of 0.76%.[30] In contrast with these findings, a similar genetic fate mapping approach from the group of Eduardo Marbán showed that new cardiac myocytes not only arise from pre-existing cardiac myocytes, but also from stem/progenitor cells following MI.[31] In line with previous reports, cardiomyocyte turnover predominantly occurs through proliferation of pre-existing cardiac myocytes at an annual rate of 1.3-4% during normal aging.[31] Taken together, it seems that, unlike zebrafish, which solely regenerate based on proliferation of dedifferentiated cardiac myocytes[17, 19], the mammalian heart appears to rely on two mechanisms for endogenous regeneration: 1) a source of stem/progenitor cells that -upon differentiation and maturation- reconstitute the lost cardiac myocytes as occurs in injury[27, 31]; 2) the proliferation of dedifferentiated cardiac myocytes that can re-enter the cell cycle and can give rise to mono-nucleated, newly formed cardiac myocytes.[30, 31] Although a major step towards a better understanding of mammalian regeneration of the heart, these studies could not pin down the exact anatomic location or molecular footprint of these stem and/or progenitor cells.

Types of Cardiac Stem/Progenitor Cells

By lack of consensus, different sources of stem and/or progenitor cells have been proposed as the causal factor for cardiac regeneration. One of these sources is a compartment of endogenous stem or progenitor cells directly from the heart itself as a logical source for maintaining the cardiomyocyte pool by a continuous process by replenishing old dying cardiac myocytes with new ones (Figure 1). Almost ten years ago, the first report was published on an endogenous cardiac stem cell (CSC) from the mammalian heart with regenerative potential based on the tyrosine kinase receptor c-kit.[12] Since then, numerous reports were published on different markers used to identify CSCs in the heart, such as Sca-1[32-36], Isl-1[37-39], Side Population (SP) cells[40-44] or Cardiosphere Derived Cells (CDCs)[45-50]. A schematic overview of resident stem/progenitor cells in the mammalian heart is depicted in Table 1. The abundance in different types of CSCs on the one hand and the clinical observation of such limited regenerative potential in the heart on the other hand led some to the conclusion that most types are in fact one type of CSC in different phases of differentiation and/or maturation.[51] Nevertheless, we will briefly outline the molecular characteristics of the most commonly described CSCs.

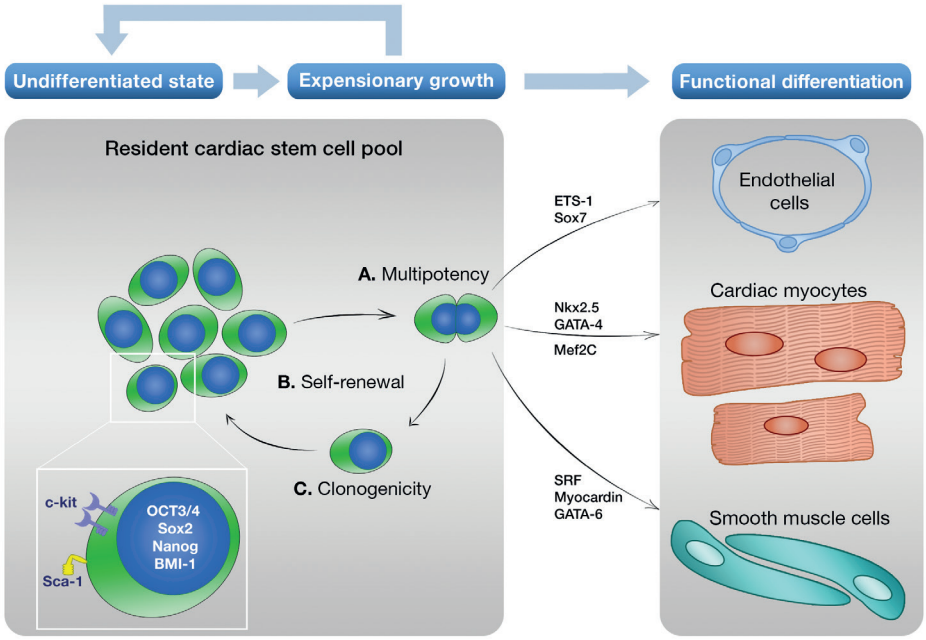

Figure 1. Cardiac stem/progenitor cell-based regeneration in the adult mammalian heart.
The proposed mechanism of cardiac regeneration mediated by endogenous CSCs present in the heart is shown. This mechanism is based on the observation that cardiac stem/progenitor cells (CSCs) are multipotent **(A)**, self-renewing **(B)**, and clonogenic **(C)**. In their undifferentiated state, CSCs express low levels of pluripotency markers such as OCT3/4, Sox2, and Nanog (see inset). The majority of CSCs also express key regulators such as BMI-1 that controls cell cycle inhibitors P19 and P21 to maintain and regulate their ability to proliferate. Once activated, CSCs can re-enter the cell cycle and subsequently can give rise to progeny that both maintain their own pool of undifferentiated stem cells and mature into three different lineages (see functional differentiation) under the influence of various lineage-specific transcription factors.

Table 1. Resident stem/progenitor cells in the mammalian heart

CSC type	Isolation marker	Stem cell molecular markers		Species	Fetal/ Neo-natal	Adult	Stemness criteria[55]			Cardiac myocyte formation in vitro?	Description	Ref
		Positive	Negative				Multi-potent	Self-renewal	Clono-genic			
c-kit	c-kit	c-kit, Sca-1, MDR-1, CD90	CD34, CD45	Mouse, rat, dog, pig, human	Yes	Yes	Yes	Yes	Yes	Yes	Co-cultured with neonatal rat cardiomyocytes	12,52,53,67
CMPC	Sca-1	Sca-1, c-kit, CD105, CD31 CD90	CD14, CD34, CD45	Mouse, human†	Yes	Yes	Yes	Yes	Yes	Yes	Differentiation protocol based on TGF-β/'5-AZA	33,36,54
CDC	None§	c-kit, CD105, Sca-1, CD90, CD34, CD31	MDR-1, CD133, CD45	Mouse, rat, pig, human	Yes	Yes	Yes	Yes	Yes	Yes	Co-cultured with rat cardiomyocytes	46,49,50,68
SP cells	Abcg2‡	Abcg2, Sca-1, MDR-1, CD133	CD31, CD34 CD45, c-kit	Mouse, rat	Yes	Yes	Yes	Yes	No	Yes	Co-cultured with mouse cardio-myocytes	32,40,41,42,43
Isl-1	Islet-1	Islet-1	Sca-1, c-kit, CD31	Mouse, rat, human	Yes	No	Yes	Yes	Yes	Yes	Co-cultured with neonatal mouse cardiomyocytes	37,38,39

† Note: Sca-1 does not exist in humans, the antibody against Sca-1 binds to an unknown Sca-1-like antigen.

§ Isolation based on enzymatically digested single cell suspension of myocardial biopsies. After several days in culture, loosely adherent CSs are formed that can be separated from fibroblast-like cells attached to the fibronectin coated culture dish.[50]

‡ Isolation based on FACS analysis selecting for the G-member protein $Abcg2^{pos}$ cells that are able to efflux Hoechst dye.[32]

Abbreviations: CSC denotes cardiac stem cell; CMPC denotes cardiomyocyte progenitor cell; '5 AZA denotes '5 azacytadine; CDC denotes cardiosphere derived cell; SP denotes side population; Isl-1 denotes transcription factor Islet-1; CM denotes cardiomyocyte; CSs denotes cardiospheres; FACS denotes fluorescence activated cell sorting.

c-kit^{pos} lineage^{neg} cardiac stem cells

The most extensively studied CSC is based on the presence of the c-kit receptor that can be activated by stem cell factor (SCF). In 2003, Beltrami and co-workers[12] described the isolation of c-kit^{pos} lineage-negative CSCs in the adult mammalian rat heart. The criteria for properties of bona fide stem cells were shown for these CSCs; being self-renewing, clonogenic and multipotent. *In vitro* and *in vivo* experiments these c-kit^{pos} CSCs were able to differentiate towards cardiac myocytes, smooth muscle cells and vascular endothelial cells. In 2005, the *in vivo* potential was further demonstrated by Dawn et al, showing that a few GFP^{pos} c-kit^{pos} CSCs had formed GFP^{pos} cardiac myocytes in the infarcted myocardium in rats.[52] Next, Bearzi[53] showed that the adult human heart also contained c-kit^{pos} CSCs. Upon successful isolation and characterisation *in vitro*, these human CSCs were tested in an infarction model based on immunodeficient mice. The observation of a chimeric heart containing human CSC derived cardiac myocytes dispersed in between the rodent myocardium further strengthened the regenerative potential of these CSCs.

Sca-1^{+} cardiomyocyte progenitor cells

In 2003, a supposed different population was documented by Oh and colleagues[33] based on stem-cell antigen 1 (Sca-1). In 6- to 12-week-old mice, these Sca-1^{+} CSCs were able to differentiate towards cardiac myocytes upon induction with the cytosine analogue '5-azacytadine. When tested in an experimental model of myocardial infarction, Sca-1^{+} were infused intravenously and homed towards the infarcted myocardium, where *in vivo* differentiation towards cardiac myocytes was observed. Despite the fact that Sca-1 does not exist in humans, the group of Doevendans and Goumans[36] reported the successful isolation of a cardiac progenitor cell population in the human foetal and adult heart based on a antibody directed against the mouse Sca-1 epitope. Akin to its rodent counterparts, these human Sca-1^{+} cardiomyocyte progenitor cells (CMPCs) showed a capability for self-renewal and multipotency by differentiating towards cardiac myocytes and/or vascular tube-like endothelial cells positive for PECAM-1. When tested in immunodeficient mice for their regenerative capacity, foetal human Sca-1^{+} CMPCs improved cardiac function following infarction and showed in vivo differentiation towards a cardiomyocyte-like phenotype based on the presence of troponin I.[54]

Side Population cells

Contrary to its name, cardiac side population (SP) cells have gained an extensive body of evidence as a distinct entity of cells capable of producing progeny that can renew cardiac myocytes during normal development and disease. SP cells are isolated based on their ability to efflux DNA binding dyes through an ATP-binding cassette (ABC) transporter.

In 2002, Hierlihy and co-workers described the isolation of cells with stem cell-like behaviour which resided in the adult heart that appeared on the 'side' on fluorescence-activated cell sorting (FACS) because these cells were able to efflux Hoechst 33342 using the ABC reporter Abcg2.[40] Since then, the existence of cardiac SP cells have been confirmed by several independent groups.[32, 41-43] Regarding other molecular markers to identify SP cells is based on Sca-1, expressed by 80-90% of SP cells.[44] However, they constitute less than 1% of all Sca-1^{+} cells in the heart.[44] Hence, given the large overlap with the more heterogeneous Sca-1^{+} CMPCs that do not show

signs of clonogenicity, it is conceivable that these SP cells could in fact be the active cell compartment isolated by the groups that investigate Sca-1$^+$ CMPCs.

Cardiosphere-derived cells

In 2004, Messina and co-workers reported the isolation of adult CSCs that grow in self-adherent clusters -designated as cardiospheres (CSs)- and are comprised of a mixture of differentiating progenitor cells, cardiac myocyte-like cells and/or vascular cells.[50] Messina suggested these are the progeny of a small subset of undifferentiated cells within CSs, which are self-renewing, clonogenic, and express different stem cell-markers like c-kit and Sca-1.[50] These cardiosphere-derived cells (CDCs) were isolated from the adult murine and human heart and could be easily expanded *in vitro* based on this cardiosphere forming isolation protocol.

Taken together, the last decade of intense research led to a vast increase in our knowledge on the existence of different types of CSCs that show true characteristics of stem-progenitor cells[55] and their role in cardiomyocyte renewal in the mammalian heart. However, *in vitro* study results obtained with CSCs should be interpreted with the necessary caution as they more likely reflect the presence of a regenerative potential of CSCs, rather than the actual role hereof in tissue cellular homeostasis. As contemplated by Simons and Clevers[56], current methods such as the search for a unique CSC specific molecular marker and quantitative analysis based on immunohistochemistry provide only a small glimpse of CSC behaviour at best. Thus, in order to reliably asses the true regenerative role of CSCs, we need to advance the CSC research field by the introduction of research methods such as lineage tracing based on inducible genetic labelling that can clearly and unambiguously provide insights in CSC dynamics and behaviour *in vivo*.

CARDIAC STEM CELLS AS THE BASIS OF CARDIAC REGENERATIVE THERAPY

Cardiac Stem Cell Therapy: Preclinical Results

Despite the uncertainties pertaining to normal CSC behaviour and dynamics *in vivo*, most reports on CSCs also include experimental data on the use of these cells as the treatment for left ventricular improvement in acute and/or chronic MI. Unlike earlier studied sources for stem cell therapy, like bone marrow mononuclear cells, that do show a modest beneficial effect albeit via other mechanisms than true regeneration[57], most types of CSCs show signs of formation of new cardiac myocytes besides formation of new vasculature. As shown in the Forrest plot (Figure 2), two important findings emanate from the cumulative evidence regarding usage of CSCs as novel cardioregenerative treatment in rodents: 1) Overall, there is an absolute increase in left ventricular ejection fraction (LVEF) based on CSCs by ~12%; 2) The type of CSC, based on different molecular markers (e.g. c-kit, MDR1, Sca-1), does not seem to show superiority of one stem cell marker over the other.

The translational approach as currently practiced requires the use of larger animal models that more closely mimic human disease to establish as to whether the beneficial effects found in rodents actually holds in a (pre)clinical setting.[58] In particular, pigs are often used for this purpose since they fulfill this criterion by their close resemblance in physiology and anatomy to the human cardiovascular system.

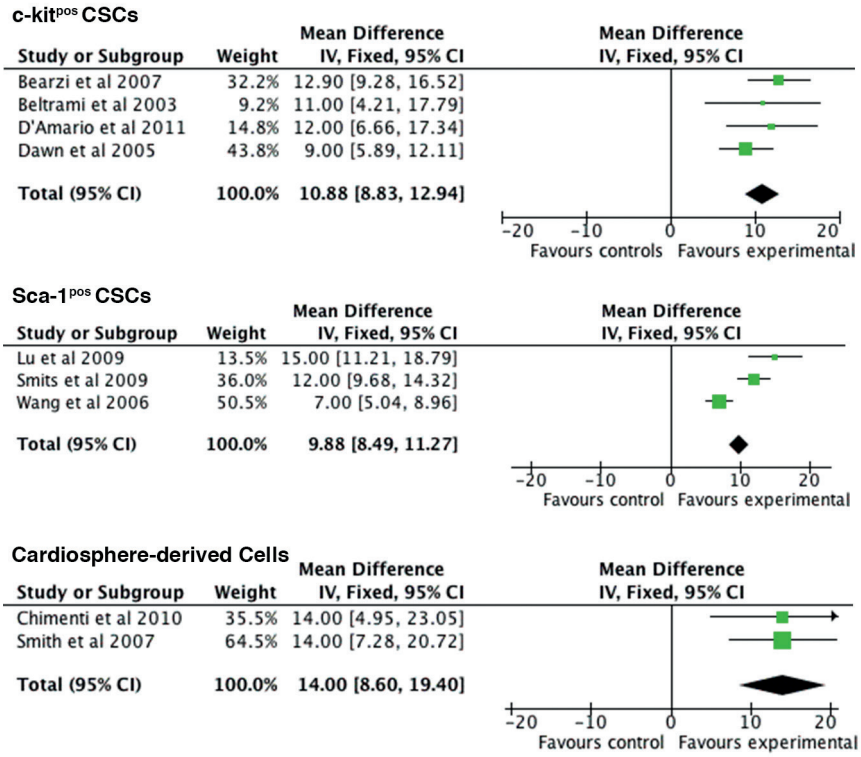

Figure 2. Preclinical studies on the efficacy of exogenous CSC delivery in myocardial infarction in rodents. Shown is a Forrest plot showing the pooled results of the mean difference on left ventricular ejection fraction (LVEF) at follow-up (21–28 days) compared with the baseline of exogenous CSC delivery in rodent models of acute myocardial infarction. Different CSC therapy led to a roughly equal improvement in LVEF for c-kitpos CSCs (+10.88%; [95% CI: 8.83–12.94]), Sca-1pos CSCs (+9.88%; [95% CI: 8.49–11.27]), or CDCs (+14.00%; [95% CI: 8.6–19.40]) at follow-up. Abbreviations: CI, confidence interval; CSC, cardiac stem/progenitor cell; IV, inverse variance; Fixed, fixed effects analysis.

To date, there are only two published reports on the usage of exogenously administered CSCs in a large animal model, both testing the use of CDCs. In 2009, the group of Eduardo Marbán proved the short-term safety of intracoronary delivery of CDCs. Regarding treatment efficacy, they used a porcine model of ischemic cardiomyopathy, in which 300, 000 CDCs/kg was compared to vehicle alone (calcium-free phosphate-buffered saline with 100 U/ml heparin and 250 µg/ml nitroglycerin). Strikingly, CDC treatment led to considerable formation of new cardiac myocytes and a significant reduction of infarct scar tissue.[46] Next, in 2011 the Marbán group extended these data in large animals by a new comparison between cardiosphere-derived cells (CDCs), the CSs themselves or placebo in a mini-pig model of chronic MI. In total 10 x10^6 cells were injected intramyocardially four weeks after an anteroseptal myocardial infarction. After four weeks follow-up, the difference in LVEF between placebo and CDCs or CSs was approximately 7% and 4% respectively (LVEF at follow-up 40±7% vs 47±5 vs 44±5).[59]

Table 2. Update on research development programs of CSC therapy for ischemic heart disease

	Preclinical research in laboratory animals		Clinical research in humans			
CSC type	Proof of efficacy in small animals (e.g. rodents) (year)[Ref]	Safety/efficacy in large animals (e.g. dogs, pigs, sheep) (year)[Ref]	Phase 1 Safety trial (year)[Ref]	Phase 2 a/b Safety / efficacy trial (year)[Ref]	Phase 3 efficacy trial (year)Ref	Routine clinical care / Phase 4 (year)[Ref]
c-kit	Yes (2003)[12]	Yes (2006)*	Yes† (2012)[62]	Yes† (2012)[62]	No	No
CMPC	Yes (2009)[54]	In progress (2013) ‡	No (Anticipated: 2014)¶	No (Anticipated: 2014)¶	No	No
CDC	Yes (2004)[50]	Yes (2009)[46]	Yes§ (2012)[63]	Yes§ (2012)[63]	No	No
SP cells	Yes (2011)[41]	No	No	No	No	No

* This study has never been published and has been presented at the American Heart Association Conference in 2006
†,§ These first-in-man studies both made use of a combined phase 1/2 study design
‡ Started Q1 2013 in the UMC Utrecht, the Netherlands; Principal investigator: Dr. S.A.J. Chamuleau
¶ Expected to start Q3 2014 by the Leiden UMC and the UMC Utrecht, the Netherlands; Principal investigators: Prof. M.J. Goumans and dr. S.A.J. Chamuleau
Abbreviations: CSC denotes cardiac stem cell; CMPC denotes cardiomyocyte progenitor cell; CDC denotes cardiosphere derived cell; SP denotes side population; IHD denotes ischemic heart disease

In 2005, Linke and colleagues described the activation of CSCs in the dog heart in response to hepatocyte growth factor (HGF) and insulin-like growth factor-1 (IGF-1). Upon induction of myocardial infarction, intramyocardial HGF/IGF-1 injections in the borderzone of the infarct led to the formation of new cardiac myocytes and coronary vessels within the infarct.[60] Analogous to this approach, another study in the porcine model by Ellison and colleagues[61] showed activation of the endogenous cardiac stem cell compartment based on these two previously established activators of CSCs.[60] A growth factor cocktail of IGF-1 and HGF was administered intracoronary 30 minutes after AMI. As a result, the growth factor treated animals showed a preserved LVEF compared to placebo, at 2 months follow up. The thymidine analogue bromodeoxyuridine (BrdU) was used to visualize cell generation, which revealed extensive new cardiomyocyte formation that coincided with the activation, proliferation and differentiation of the endogenous CSC compartment.[61]

Cardiac Stem Cell Therapy: Clinical Results

Table 2 provides an overview of the current research programs of the different types of CSC therapy. Despite of the scarcity of experimental data in large animal models for CSCs, clinical trials are already underway.[62, 63] The first trial, SCIPIO (cardiac Stem Cells In Patients with Ischemic

Cardiomyopathy), initiated by the combined effort of Anversa and Bolli, is an open-label phase I trial that randomly allocated sixteen patients with post-infarction LV dysfunction (LVEF ≤ 40%), who had undergone coronary artery bypass grafting, to intracoronary infusion of 0.5-1 x10^6 c-kit positive, lineage-negative cardiac stem cells or standard care as usual.[62] The delivery of c-kit^{pos} CSCs was approximately four months after surgery. LVEF increased by 8% at four months after c-kit^{pos} CSC delivery, whereas the patients receiving standard care as usual did not show any signs of change. Strikingly, a progressive improvement in LVEF was observed in the first eight patients that reached the 1-year follow up with an increase in 12% compared to controls.[62]
The second trial, CADUCEUS (CArdiosphere-Derived aUtologous stem CElls to reverse ventricUlar dySfunction), initiated by Eduardo Marbán, was a prospective, randomised phase I safety trial that investigated the effect of intracoronary admission of CDCs in patients with a recent acute myocardial infarction (with LV ejection fraction of 25-45%) on major cardiac and non-cardiac adverse events and formation of neoplasia.[63] In total, 25 patients were enrolled of which 17 received CDCs 1.5-3 months after the index event. Eight patients served as controls and received guideline-based care as usual. During follow up, four patients (24%) in the CDC group experienced a serious adverse events compared with one control (13%). Regarding functional analysis at 6 months, MRI analysis of patients treated with CDCs showed a significant reduction in scar mass, increased viable heart mass and regional systolic wall thickening. However, changes in end-diastolic volume, end-systolic volume, and LVEF did not differ between groups at 6 months.[63]
However tempting to look at the highly anticipated functional analysis, these phase I trials can merely provide information on what they were designed to investigate, which is the safety of CSC therapy on short-term follow-up. As a reminder from history, only seven years ago, the cardiovascular scientific community received similar initial reports that sparked intense hope that bone marrow derived cell therapy could reduce the burden of ischemic heart disease. However, the initial report[64] of a 15% improvement in LVEF was gradually reduced to an significant increase of 2.87% published very recently in the Cochrane library that reported on the pooled analysis of in total 2.533 patients.[65] We speculate that the evidence as summarized in this review justifies CSCs as one of the currently investigated cell types with the highest potential to ameliorate the repercussion of massive cardiomyocyte loss following AMI.

CELL THERAPY: PROBLEMS & PITFALLS FROM A TRANSLATIONAL PERSPECTIVE

Previously, different versions of autologous cell therapy (e.g. bone marrow derived mononuclear cells) also reached the stage of clinical testing. Most of these therapies have been proven marginally effective but failed to solve the severe healthcare problem that CHF imposes or have a measurable impact in the day-to-day clinical practice of CHF treatment. Even when the preliminary results of the CADUCEUS and SCIPIO trials truly reflect the marked improvement that can be expected based on CSC therapy, this autologous cell approach is still hampered by several pitfalls that need to be resolved in the near future. Firstly, a low engraftment of exogenously administered cells in the heart possibly dilutes the treatment effect of cell therapy. In our center, we compared the delivery efficiency of three commonly used delivery strategies (intracoronary

infusion, intramyocardial injection of surgical injection). Four hours after delivery, we could only detect ~10% of all delivered ^{111}indium labelled mesenchymal stem cells, regardless of delivery method.[69] Secondly, autologous stem/progenitor cell therapy relies on a complex infrastructure of both human expertise and costly facilities that are needed for its isolation, cleaning and culturing of CSCs under GMP conditions to enable successful autologous cell therapy. Thirdly, autologous therapies, at present, still fail to satisfy the cost constrain posed by the need to make the treatment affordable to a very large number of candidate patients. Moreover, handling of the cell product should be devised in such a way that it can be prepared and administered not only in the tertiary cardiovascular centers but also in the majority of hospitals with access to a catheterization laboratory. Therefore, novel strategies activating the endogenous CSC compartment are under current investigation and aimed to 'bypass' the abovementioned shortcomings of exogenous CSC therapy (Figure 3).

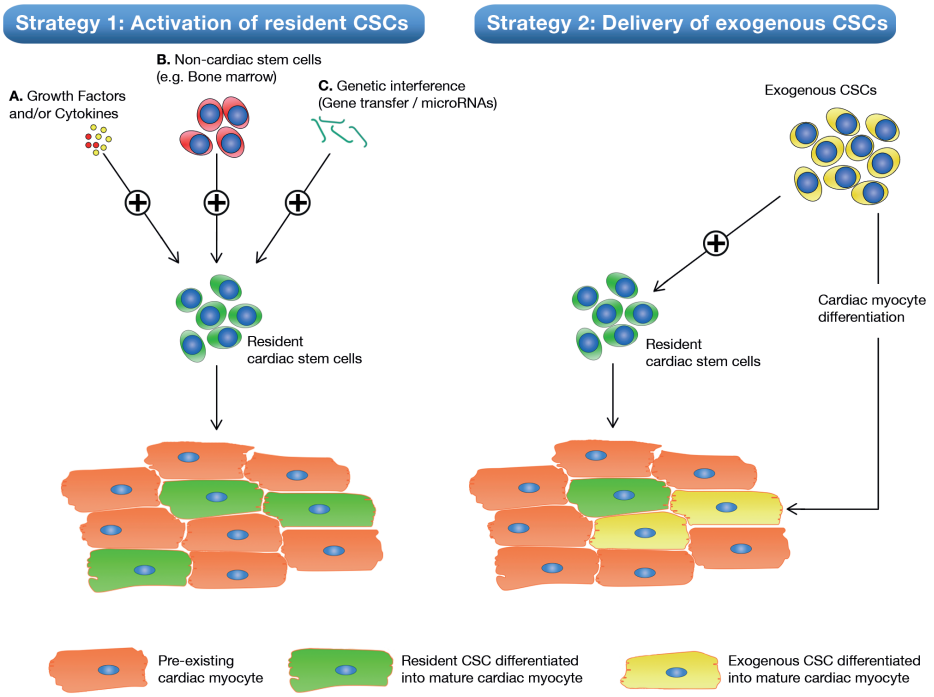

Figure 3. Novel strategies for CSC-based myocardial repair.
Schematic overviews of current strategies used to make use of CSCs for myocar- dial repair are shown. Strategy 1 is based on activation of endogenous CSCs by various means, e.g., growth factors **(A)**, noncardiac stem cells **(B)**, or gene therapy **(C)**. Upon activation, resident endogenous CSCs can proliferate and mature into newly formed cardiac myocytes (green cardiac myocytes). Strategy 2 is based on the delivery of autologous CSCs that have been isolated from small myocardial biopsies and scaled up outside the patient to sufficient numbers. Exogenous CSCs are also shown to be capable of activating the local endogenous CSC compart- ment. In addition, exogenously delivered CSCs are hypothesized to mature and differentiate into functional cardiac myocytes (yellow cardiac myocytes) that are electromechanically coupled with the pre-existing cardiac myocytes (orange cardiac myocytes)31. Abbreviation: CSC, cardiac stem/progenitor cell

FUTURE DIRECTIONS

In this review, we have summarized the current status of the cardiac regenerative research field and highlighted the major breakthroughs that led us to rethink old paradigms with regard to the regenerative capacity of the adult heart that coincides with the presence of an endogenous stem/progenitor cell pool that shows self-renewing and multipotency both i*n vitro* and *in vivo*. Next to these major breakthroughs, we have also shown controversial points in which numerous contradicting reports still preclude a broad consensus. Resolving several issues is paramount in order to advance the field towards development of novel therapies that can augment the regenerative potential of CSCs. We would like to specifically mention two that are in our view of most importance; Firstly, the low level of naturally occurring regeneration in the human heart indicates that CSCs are part of a tightly regulated process that controls the number of progeny that can give rise to new cardiac myocytes. Which factors can activate quiescent CSCs and subsequently let these cells take place in new rounds of cell division? In addition, which factors govern the newly formed progeny towards activation of a set of cardiomyogenic transcription factors that lead to formation of new cardiac myocytes? As shown by Sluijter et al, micro-RNA (miR) interference could successfully govern the differentiation efficiency of Sca-1^{pos} CSCs towards cardiomyocyte-like cells by overexpressing miR 1 and 499.[66]

Secondly, despite extensive preclinical data in young rodents, the effects of aging on behaviour and viability of these CSCs in elderly patients with coronary artery disease (CAD) remains largely unexplored. Cellular aging is governed by the expression of nuclear proteins that regulate cell cycle inhibition and irreversible growth arrest. Therefore, if the regenerative response of CSCs to an ischemic insult is to be further explored as a new treatment for post-MI heart failure, it is imperative to unravel which individual patient characteristics affect CSC viability and, above all, their irreversible state of cellular senescence.

In the foreseeable future, both the highly anticipated results of clinical trials involving the use of CSCs and preclinical clues that can further tailor the high potential of CSCs could pave the way for CSC based myocardial repair on a clinical relevant scale. Given the anticipated increase in socio-economic costs related to heart failure, any reduction in the change to develop heart failure following an acute myocardial infarction could drastically reduce the burden of ischemic heart disease on our healthcare resources and, above all, improve the patient's quality of life.

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CHAPTER **5**

A fast pH-switchable and self-healing supramolecular hydrogel carrier for guided, local catheter-injection in the infarcted myocardium

In press

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ABSTRACT

Minimally invasive intervention strategies after myocardial infarction use state-of-the-art catheter systems that are able to combine mapping of the infarcted area with precise, local injection of drugs. To this end, catheter delivery of drugs that are not immediately pumped out of the heart is still challenging, and requires a carrier matrix that in the solution state can be injected through a long catheter, and instantaneously gelates at the site of injection. To address this unmet need, a pH-switchable supramolecular hydrogel is developed. The supramolecular hydrogel is switched into a liquid at pH > 8.5, with a viscosity low enough to enable passage through a 1-m long catheter while rapidly forming a hydrogel in contact with tissue. The hydrogel has self-healing properties taking care of adjustment to the injection site. Growth factors are delivered from the hydrogel thereby clearly showing a reduction of infarct scar in a pig myocardial infarction model.

INTRODUCTION

Coronary artery disease is a progressive disease that can be held responsible for over seven million deaths worldwide each year, and the increased aging of the population will even yield a further rise in mortality and morbidity.[1] New strategies are aiming at the prevention of the progression of post-myocardial infarction towards heart failure. Catheter-based drug delivery injection approaches[2,3] are substantially less invasive than for example surgical implantation of in-vitro engineered tissues,[4] patches[5,6] or drug delivery carriers.[7] Therefore catheter-injection strategies are the method of choice with regard to clinical applicability. State-of-the-art is the NOGATM catheter which enables precise control over the injection location via a special mapping system.[8] A 3D electromechanical image of the myocardium can be obtained using an ultra-low magnetic-field energy source and a sensor-tipped catheter to locate the catheter position. This mapping allows for the accurate identification of normal and infarcted myocardium, and in this way enables excellent spatial control over the injection of drugs. Generally, the injected drugs are substantially fast removed from the pulsatile heart when not delivered via a solid or gelated carrier material. Therefore, the development of carrier materials that can be injected through the extremely long and narrow lumen of such catheters, and instantaneously gelate in contact with the myocardial tissue, is prerequisite. The dimensions of the catheters, combined with the dynamic beating environment of the heart, require a material with Janus-like material properties; i.e. low viscosity to flow through the long, narrow lumen of the catheter, and high stability to form a local drug delivery reservoir and provide mechanical support in the tissue after injection. This dual character is proposed to be obtained using a stimuli-responsive, switchable hydrogel. Furthermore, ideally this hydrogel shows self-healing properties within the high shear environment of the contracting heart muscle.

Natural hydrogels for myocardial injection therapies that have been investigated include fibrin,[9] collagen,[10] alginate,[11] Matrigel,[12] hyaluronic acid[13] and chitosan.[14] These hydrogels can potentially be delivered in combination with cells or drugs, however, they are derived from a natural source and therefore show batch-to-batch differences. Furthermore, their switching behavior can be poorly controlled. As a consequence, although injection of these hydrogels via a syringe was accomplished relatively easily, the translation to catheter-based delivery has yet remained an unmet challenge. Recently, decellularized porcine extracellular matrix (ECM)-derived hydrogels have been reported as catheter-injectable scaffolds for cardiac regeneration.[15] However, these ECM-derived hydrogels did not show very fast gelation upon injection, and their compatibility with clinical use will be poor because of their animal origin. We propose that synthetic materials fulfill the extensive list of requirements, amongst others that they do not show batch-to-batch differences, they are more easy to switch from sol-gel, and they are not animal, bacteria, or carcinoma-derived.[16] Several synthetic systems have been explored for cardiac injection today, including self-assembling peptides[17] and synthetic hydrogels that are formed after injection via in situ chemical or physical cross-linking,[18] photo-induced polymerization,[19] self-assembly[20] or thermal switching.[21] Again, although easy syringe injection can be obtained with these systems, catheter-compatibility remains difficult and has not been shown.

Differences in pH form interesting parameters to induce switchable behavior in material properties. Since variations of pH naturally occur in the human body, exploiting these for hydrogel formation is appealing. Various pH responsive systems appear throughout literature. Importantly the vast amount of pH responsive systems are used to enable controlled release of hydrogel payload rather than used to persue a switch in material properties thereby controlling catheter-guided injection.[22] Synthetic supramolecular hydrogelators[23-29] that are held together by directed, non-covalent interactions are proposed to allow for full control of their sol-gel switching behavior under mild conditions using the dynamic nature of the supramolecular interactions. When exploited to the fullest, supramolecular switchable hydrogels systems might be the solution for future clinical catheter-delivery therapies. Therefore, our approach to a synthetic, catheter-injectable hydrogel uses the four-fold hydrogen bonding supramolecular ureido-pyrimidinone (UPy) units[30] coupled via alkyl-urea spacers to 10k or 20k poly(ethylene glycol) (PEG) chains (Figure 1a).[31,32] These UPy-modified PEG-hydrogels form fibers in aqueous solution that are able to cross-link forming transient supramolecular networks. Here, we show that this unique UPy-transient network is pH-responsive which enables a sol-to-gel switch in a subtle pH range, and therefore is compatible with the NOGATM catheter system. The natural pH of the tissue instantaneously transforms the injected solution into a drug-loaded hydrogel reservoir. Local in-vivo delivery of MRI contrast agents, and active growth factors (GF) hepatocyte growth factor (HGF) and insulin-like growth factor-1 (IGF-1) is demonstrated in a large animal model of ischemic heart disease.

METHODS

Preparation of UPy-hydrogels: The UPy-hydrogelators with Mn,PEG = 10 kg/mol or Mn,PEG = 20 kg/mol, i.e. UPy-10k and UPy-20k, respectively, were synthesized by SyMO-Chem BV, Eindhoven, The Netherlands.[31] For solution studies, i.e. using concentrations of 1 wt%, the PEG-20k hydrogel was used since the shorter hydrophilic domain of the PEG-10k materials causes precipitation in time. For the gel and biomedical experiments, the higher stability of the PEG-10k is preferred considering the resulting prolonged lifetime of the gel.

For the preparation of the hydrogels, polymer solutions were prepared by dissolving 10 wt% in PBS pH 7.4 by stirring at 70 °C for 2 hours and subsequently cooled to RT. To liquefy the polymer solution, the pH was raised by adding 2 µL aliquots of a 0.1 M NaOH stock solution. Human recombinant IGF-1 (Miltenyi Biotec) and human recombinant HGF (Miltenyi Biotec) were mixed in by slow stirring for 10 minutes yielding a final concentration of 500 ng/mL of each growth factor. For non-invasive assessment of the UPy-hydrogel by cardiac MRI in vivo, SPIOS were mixed with UPy-gel pH 9 by gentle stirring at room temperature for 3 minutes yielding a final concentration of 13.6 µg/mL. Solutions were then UV-sterilized for at least 1 hour prior to use.

Rheology on gel samples: Oscillatory rheology experiments were performed on an Anton Paar Physica MCR 501 rheometer. Mechanical properties of the viscoelastic hydrogel material under neutral or neutralized conditions were assayed using a parallel (PP 25)-plate geometry. The linear viscoelastic regime of the samples was obtained from amplitude sweep experiments that were from 0.1 to 1000% strain at 1 rad/s angular frequency. Frequency sweep measurements were

recorded from 0.1 to 100 rad/s at 1% strain. Measurements were performed at 20 °C unless stated otherwise.

Viscosity measurements on basic solution: Steady-state viscosity measurements were performed using a cone (CP-25-1) -plate geometry with a 1˚ angle. UPy-10k was dissolved in PBS buffer (pH 7.4) at 70 °C, cooled to room temperature and transferred to the injectable fluid state by addition of a small aliquot of NaOH, yielding a 10 wt% hydrogel. The viscosity as a function of shear rate was measured from 0.1 to 100 rad/s at 20 °C. The zero-shear viscosity, η0, was extrapolated from the Newtonian plateau to the y-axis.

Cryogenic transmission electron microscopy (Cryo-TEM): Sample preparation was performed by pipette addition of 1 wt% UPy-20k solutions (3 µL; neutral and basic conditions) onto holey carbon film (hole diameter = 2 µm) supported by a copper TEM grid prior to manual blotting at 100% relative humidity and 21 °C. Immediate vitrification was performed by automated plunging of the grid into liquid ethane. Upon completion of the set time, automated vitrification was performed. Samples were stored under liquid nitrogen prior to imaging. Cryo-TEM was performed on a FEI Tecnai 20 (type Sphera) TEM operating with a 200 kV LaB6 filament and a bottom mounted 1024 x 1024 Gatan msc 794™ CCD camera.

Dynamic Light Scattering (DLS): Dynamic light scattering experiments on a 1 wt% UPy-20k solution under neutral and basic conditions were conducted on an ALV/CGS-3 MD-4 compact goniometer system equipped with a multiple tau digital real time correlator (ALV-7004) (solid state laser: λ = 532 nm; 40 mW). Typical experiments covered a scattering angle from 20 to 150°, averaging over 5 × 30 s runs at a temperature of 20-40 °C in 10 °C intervals. Data was processed using after ALV freeware and scattering artifacts were excluded from the analysis. The hydrodynamic radius (Rh) was extracted from the measured diffusion coefficient $D0=\Gamma(x)/q2$ assuming validity of the Stokes–Einstein relation, $Rh = kT/6\pi\eta D$ (k being the Boltzmann constant and η the solvent viscosity) for spherical objects.

Infrared spectroscopy (IR): UPy-10k samples (neutral and basic conditions) were prepared with a total concentration of 1 wt% in D2O. Solution infrared spectra were recorded on a Perkin-Elmer spectrum One FT-IR spectrometer and measured at a resolution of 4 cm–1, by co-adding 128 scans. Samples were loaded in a fixed path length (50 µm) cell with CaF2 windows, and used immediately for IR measurements.

Cell cultures and biocompatibility testing: Cardiomyocyte progenitor cells (CMPC) derived from human fetal hearts were isolated as described.[43] Briefly, human fetal hearts were collected following elective abortion, after written informed consent was provided. The protocol was approved by the Medical Ethics committee of the University Medical Center Utrecht and is in line with the principles outlined in the Declaration of Helsinki. Briefly, the CMPC were isolated by magnetic bead sorting using Sca-1 coupled beads (Milteny). CMPC were then maintained in growth medium and used for different assays. The toxicity of UPy-10k hydrogel formulations on CMPC was tested in vitro using a lactate dehydrogenase (LDH) assay (Sigma Aldrich). CMPC were cultured in M-199 medium (Sigma Aldrich) with 2% FBS (Invitrogen) and incubated with 100 µL of 10 wt% UPy-hydrogel based on milliQ, NaCl 0.9 or PBS (all buffers were tested both at pH 7.4 and pH 9.0). After three days of incubation, medium was collected and tested for the presence of LDH as a marker for cell damage according to manufacturer's instruction.

Growth factor release and bioactivity in vitro: Human recombinant HGF and human recombinant IGF-1 dissolved in milliQ water were added to liquefied UPy-10K hydrogel in PBS pH 9 yielding a final concentration of 500 ng/mL of each growth factor. 100 µL of UPy-hydrogel with growth factors was incubated in 200 µL of medium in 48-well plates on a rotational shaker (90 RPM) at 37 °C for 7 days. 75 µL of medium was removed daily and replaced with fresh medium. The collected medium was quantified for HGF and IGF-1 using ELISA detection (R&D Systems). The bioactivity of the growth factors released was investigated as follows: CMPC were subjected to serum starvation for 18 hours, followed by supplementation of medium collected from the HGF/IGF-1 release study for 15 minutes. Cells were lysed and western blot analysis was performed for phosphorylated c-Met for HGF (Cell Signaling) and phosphorylated IGF receptor 1 (pIGFR1) for IGF-1 (Cell Signaling). IGF-1 (100 ng/mL) and HGF (100 ng/mL) dissolved in MilliQ water served as a positive control.

Animal experiments and study design: In total thirteen pigs (female Dutch landrace, weighing approximately 70 kg) received humane care in compliance with the national guidelines on animal care and prior approval by the Animal Experimentation Committee of the Faculty of Medicine, Utrecht University, the Netherlands. Myocardial infarction was induced by 75 minutes of intracoronary balloon occlusion of the proximal left circumflex artery. Four weeks later, the animals underwent 3-dimensional electromechanical mapping (EMM) of the left ventricle for infarct and borderzone localization using the NOGA™ catheter system (Biosense Webster, Cordis, Johnson & Johnson, USA). Intramyocardial delivery was performed using the NOGA™ Myostar system (Biosense Webster, Cordis, Johnson & Johnson, USA).

Short term *in vivo* tracking of growth factor distribution: Regarding the in vivo tracking of UPy-hydrogel, four injections of 0.2 cc were placed, two injections with SPIOS-labeled UPy-10k hydrogel in the anteroseptal wall of the left ventricle and two injections with HGF/IGF-1 loaded UPy-hydrogel to the borderzone of the infarction (lateral wall). Six hours after injection, two animals underwent *in vivo* tracking of the hydrogel by cardiac MRI. Tissue samples collected from the septal wall and the injection site were snap frozen for western blot analysis for human IGF-1 (Abcam; dilution 1:1000) and immunofluorescent staining for human anti-IGF-1 (Abcam; dilution 1:100) and for alfa-sarcomeric actin (Sigma, dilution 1:50).

The effect of sustained release on cardiac function: Four weeks after myocardial infarction induction ten animals were injected with pristine UPy-10k hydrogel (n=3), saline dissolved HGF/IGF-1 (both 500 ng/mL) (n=3) and UPy-hydrogel loaded with HGF/IGF-1 (both 500 ng/mL) (n=4). Then the animals were followed for four weeks, and subsequently euthanized by exsanguination under general anesthesia. After excision of the heart, the heart was cut into five slices from base to apex and infarct size was determined by 1% triphenyl-tetrazolium chloride staining. Tissue samples collected of the septal wall (remote) and the infarction zone were snap frozen for histological analysis. Cryosections (7 µm) were prepared on a microtome (Leika) and fixed in 4% formalin for 10 minutes. Next, the sections were incubated in 0.1% Sirius red (BDH) in picric acid (Sigma) for 8 minutes at room temperature. After counterstaining the nuclei with hematoxylin for 1 minute, the sections were dehydrated, cleared and mounted. The quantification was performed on 30 fields per animal of UV polarized light on a microscope (Olympos DP71) at 40x magnification and automatically quantified for the percentage of collagen per field using

ImageJ software for Macintosh (version 1.44g). The analysis was performed by an investigator blinded to the treatment allocation.
Statistical analysis: The data is expressed as mean ± SEM. Differences in data were evaluated with a one-way analysis of variance (ANOVA) followed by Tukey post-hoc analysis. Data analysis was performed on SPSS v19.0.0 software for Macintosh. Probabilities of $P < 0.05$ were considered to be statistically significant; $P < 0.05$ is depicted as *.

RESULTS AND DISCUSSION

Our supramolecular UPy-hydrogel can be made fluid at basic pH, with a threshold at pH 8.5, and reversibly transferred back into a gel state at neutral pH (Figure 1b). This allows the basic polymer solution to be injected in a solution of neutral pH (Figure 1c). Almost instantaneous gelling of the polymer material was observed upon the experienced pH drop. Rheology was performed to investigate the material properties of the hydrogel before and after pH-switching (Figure 1d) and demonstrated that the mechanical properties of the restored hydrogel after pH-switching are identical to the gel before switching. Furthermore, the storage modulus of the 10 wt% UPy-10k gel matches the mechanical stiffness of the natural cardiac tissue, e.g. 24 kPa for the UPy-gel, vs 26 kPa for adult rat heart muscle[33] (Figure 1d). In addition, the UPy-PEG hydrogels show self-healing behavior (Figure 1f). UPy-PEG hydrogels behave liquid-like at larger deformations ($G' < G''$), but recover within minutes when the deformation is removed (Figure 1f). Viscosity measurements on the basic UPy-10k polymer solution show a viscosity of 0.8 Pa (Figure 1e), which is very low for a polymer solution and in the same order of magnitude of a solution of plain PEG (without UPy-moieties).[34] After neutralization of the sample, immediately a gel-like sample is formed, confirming the macroscopic observations (Figure 1c). This makes our UPy-hydrogelator an ideal catheter-injectable material. Since the sol-gel transition is fast, we propose that the pH-switchability is caused by breaking of the cross-links between the fibers that form the transient network instead of complete disassembly of the fibers. This results in the fast switching behavior shown. Cartoons are shown to clarify the assembly (and disassembly) process (Figure 2a-d). 1D UPy-stacks in water are formed by UPy-dimerization and stacking by additional lateral hydrogen bonding provided by the urea-moieties surrounding the alkyl spacers (that form a hydrophobic pocket) (Figure 2a). These stacks cluster together to form a hierarchical fiber (Figure 2a,b) which entangles and cross-links into a transient network, forming a hydrogel above the gelation onset (Figure 2c,d). The UPy-UPy-interactions are inherently dynamic, thus can dynamically associate and dissociate in the assembled fiber forming the cross-links that build up the network (Figure 2c).
As stated above, under basic conditions these cross-links are broken leading to disintegration of the transient network. To investigate this hypothesis in more detail we studied the hydrogelator morphology at nanometer level using cryo-transmission electron microscopy (cryo-TEM), atomic force microscopy (AFM), dynamic light scattering (DLS) and Fourier-transformed infrared (FT-IR) spectroscopy both at neutral and basic conditions. Cryo-TEM shows the presence of rigid and elongated fibers in both neutral and basic solution, with average lengths of 75 nm and 104 nm,

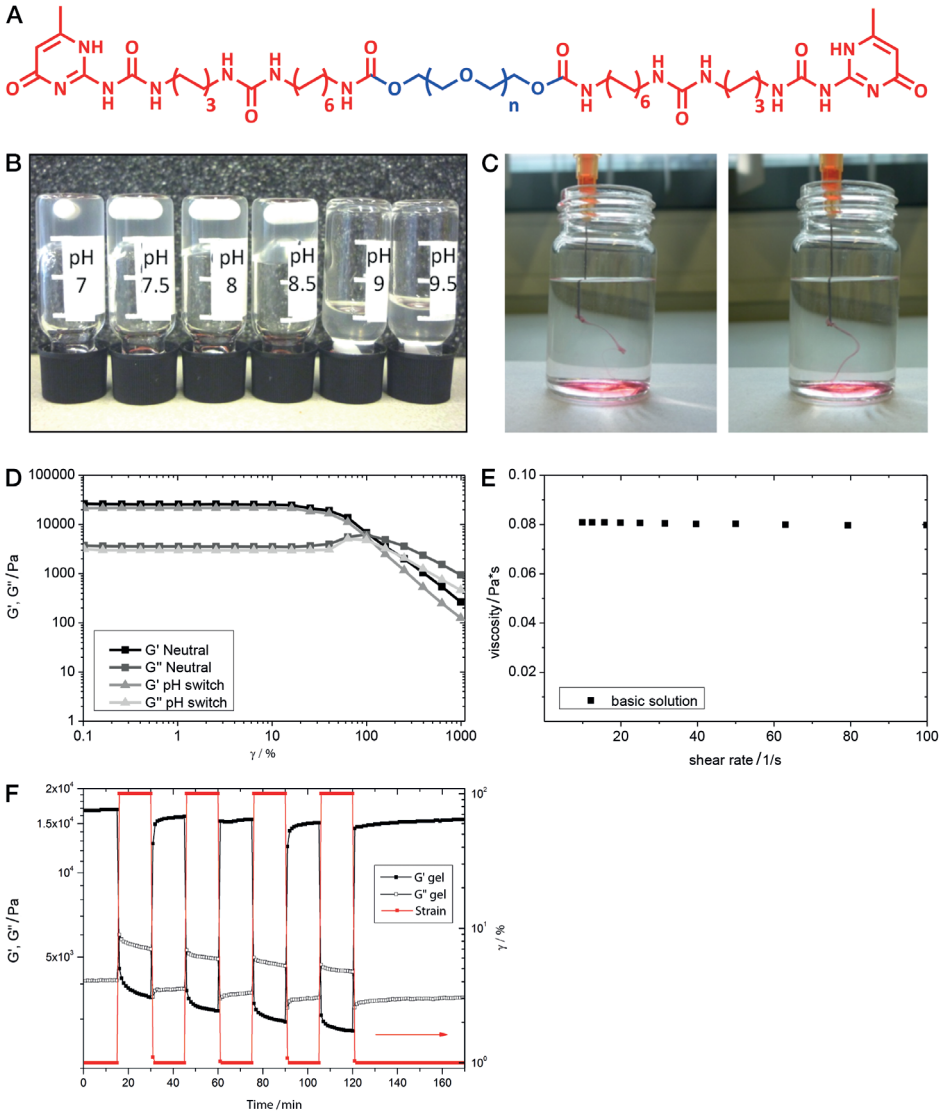

Figure 1 . Structure, pH behavior, and rheological properties of UPy-hydrogelator polymers.
(A) Structure of the UPy-hydrogelators with the hydrophilic PEG block (n = 227/454, M n = 10/20 kDa) in blue and the UPy-alkyl-urea end groups in red. **(B)** Overview of the pH-dependent behavior at a pH range from 7 to 9.5. **(C)** Injection of a liquefi ed 10 wt% UPy-10k sample in PBS (colored in red) into a neutral PBS solution. Immediate gelation occurs when the basic solution comes in contact with the neutral solution. **(D)** Rheology measurements of a 10 wt% UPy-10k gel before and after the pH switch. **(E)** Viscosity measurement of the liquefi ed 10 wt% UPy-10k gel matching the state upon catheter injection. **(F)** Dynamic strain amplitude test of 10 wt% UPy-10k gel at 37 ° C showing the self-healing behavior over four cycles. hydrogelator

respectively (Figure 2e,f, Supp. info). This shows that a high pH does not influence the fiber formation. AFM imaging shows a hierarchical assembly of fibers for the polymers in water (Supp. info). In the basic pH sample, the assemblies seem more rigid and the variation in height is significantly less than in the neutral assembly, suggesting that the fibers are assembled out of fewer individual stacks. This indeed indicates a disrupted lateral self-assembly due to loss of cross-linking in the basic state. Furthermore, DLS confirms the cryo-TEM measurements. DLS demonstrates the presence of two distinct distributions, which are proposed to be fibers and micelles, in both the neutral and basic pH environment (Figure 2g). The size dimensions calculated using the Stokes-Einstein equation to translate diffusion to hydrodynamic radius (with the assumption of spherical and non-interacting particles) match the cryo-TEM measurements. The breaking of the cross-links, and therefore the sol-gel transition, is proposed to be the result of deprotonation of the UPy-moiety when present in the enol-tautomer, yielding the enolate (Figure 2h).

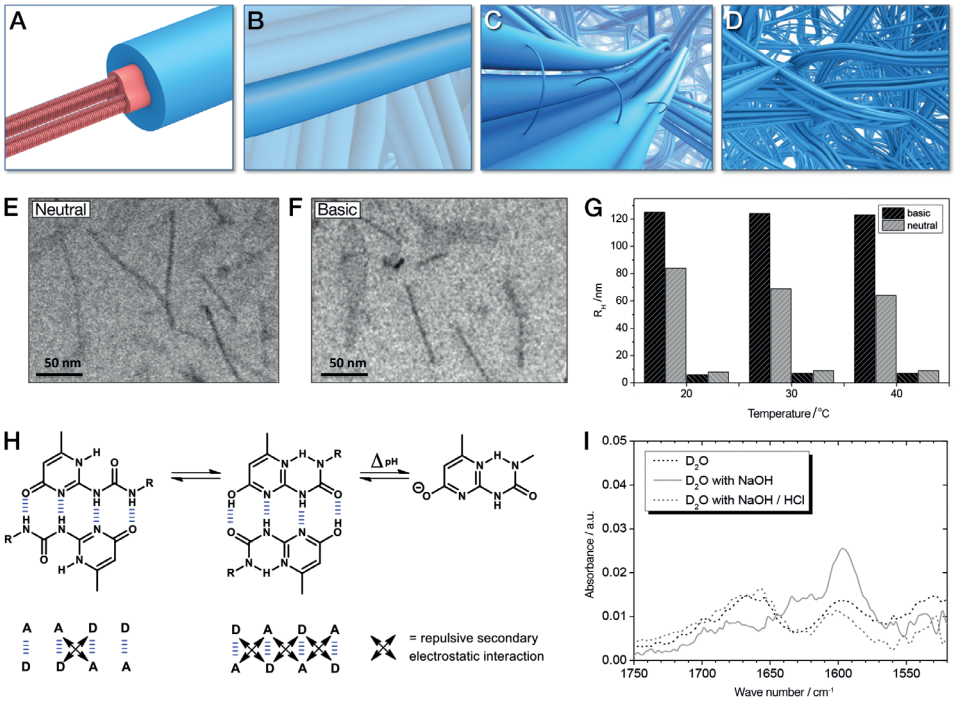

Figure 2 . Structural analysis of UPy hydrogelators in neutral and basic environment.
(A-D) Proposed schematic molecular picture of the hydrogel mate- rial: zoom into the hydrogel fi bers. **(A)** Fibers are composed of multiple UPy-stacks (red), surrounded by a hydrophilic PEG corona (blue). **(B)** Fibers can align and **(C)** cross-link by UPy-polymers from one fi ber into a neighboring fi ber. **(D)** UPy-fi bers assemble into a transient network. **(E** and **F)** Cryo-TEM of 1 wt% solution of UPy-20k demonstrates the existence of elongated fi bers in both **(E)** neutral and **(F)** basic environment. **(G)** DLS on 1 wt% UPy-20k solutions at various temperatures indicate the presence of two distinct populations in solution, micelles, and fi bers, for both pH conditions. **(H)** UPy- tautomeric forms: the keto-tautomer and the enol-tautomer can form homodimers, whereas the enolate formed after deprotonation cannot dimerize. **(I)** FT-IR on UPy-10k solutions of the C ? O stretch vibration indicating the existence of the enolate at basic conditions. self-assembly

FT-IR confirms this enolate-formation (Figure 2i). Modeling of the tautomeric states of the UPy-moiety in various pH environments shows a pKa of the enol-OH of ~ 7.2, and the enolate anion being the dominant species present in a pH range from 8.5 to 12.5.[35] Upon dissociation, the UPy-group experiences a different environment than inside the fiber and a change in tautomeric form occurs, affecting the stability of the assembled system. The charged species cannot get incorporated back in the UPy-fiber to form a cross-link in order to stabilize the UPy-gel. The preservation of molecular ordered fibrillar assemblies forms the fundamental origin of this hydrogel material. As a second hierarchy in order, the switching between gel-like and solution-like material results from the spatio-temporal formation of pH-sensitive inter-fiber cross-links. It is this combination of selective, hierarchical responsiveness that renders our material unique in its kind, and enables catheter-injection to be explored for in vivo application.

Cell compatibility is essential for the system to be applicable *in vivo*. The hydrogelator formulation needs to be biocompatible both at neutral and basic pH. The pH effect of different buffers on cardiomyocyte progenitor cells (CMPC) was screened, being milliQ, 0.9% NaCl, and PBS,

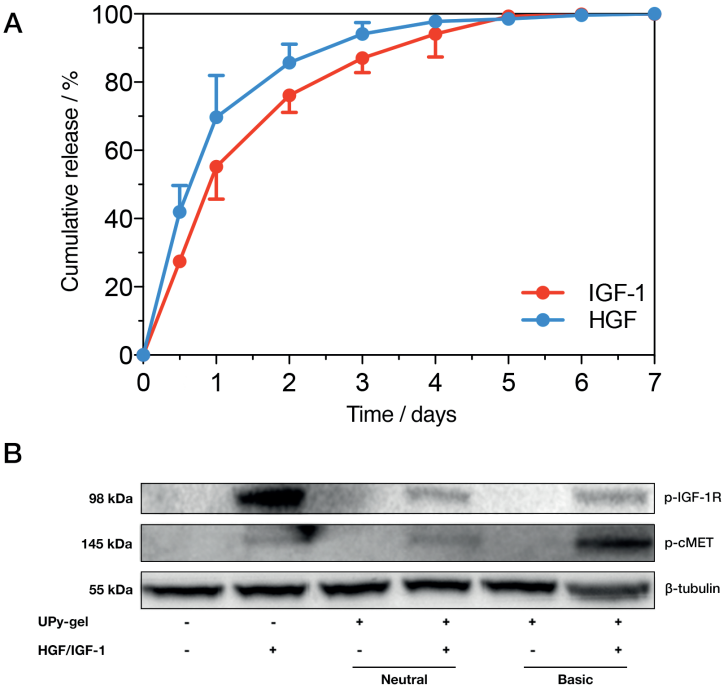

Figure 3. Release profile and bioactivity of HGF/IGF-1 embedded in the UPy-hydrogel.
(A) In-vitro release characteristics of IGF-1 and HGF from the UPy-10k hydrogel by daily collection of medium at 37 °C. Both HGF and IGF-1 display an initial outburst (5 minutes after start of the experiment) followed by a four day release. **(B)** Western blot analysis showing that CMPC express IGF-1R and cMET receptors that can be activated by IGF-1 (100 ng/mL) and HGF (100 ng/mL), respectively. At both pH levels, pristine UPy-hydrogel did not cause phosphorylation of IGF1R and cMET. Medium collected from release studies of UPy-hydrogel loaded with HGF/IGF-1 show that the released HGF and IGF-1 can still activate their receptors, both in UPy-hydrogel with a pH 7.4 (neutral) and the injectable pH 9 (basic).

all buffers at both neutral and basic pH (Supp. info). No toxicity was observed for the UPy-10k hydrogels prepared in different buffers at a physiological pH. For the elevated pH 9 preparations, only the UPy-gel based on PBS did not show any signs of cell toxicity. These in-vitro studies show that the hydrogel system at both neutral (gel-state) and elevated pH (sol-state) in PBS is biocompatible. Additionally, biomaterials used for *in-vitro* or *in-vivo* applications need to be free of contaminating bacteria and endotoxins. To evaluate the presence of lipopolysaccharides present in our materials, an endotoxin test was performed. The concentration of endotoxin for all samples prepared in PBS was below the FDA approved endotoxin value of 0.5 EU/mL (Supp. info).

Stimulation of endogenous cardiac repair through GF injection can benefit significantly from a sustained release over time instead of a single bolus injection.[36,37] Data from different preclinical studies show the ability of selected growth factors, HGF and IGF-1, to enhance endogenous cardiac stem cell response to an ischemic site of injury.[38-40] Here, both HGF and IGF-1 were incorporated in the UPy-10k hydrogel, and their release profile was subsequently studied in vitro. A prolonged HGF and IGF-1 release from the UPy-hydrogel was achieved during 7 days (Figure 3a). The release of HGF was characterized by an initial outburst of 41%, followed by a sustained release until 97% was released by the end of day four. For IGF-1, 27% was initially released whereas subsequent release showed similar release kinetics as HGF, with a 94% release by day four. The difference in burst release between the different growth factor proteins might be attributed to the difference in size, which is 103 kDa for HGF and 17 kDa for IGF-1, respectively. Since PEG is non-fouling, we propose that there are no aspecific non-covalent interactions between the proteins and the hydrogel, suggesting that the difference in release can only be attributed to a size-dependent release profile. Following incubation in the basic UPy-hydrogel both IGF-1 and HGF showed preserved bioactivity shown by their affinity and ability to phosphorylate their corresponding receptors IGF-1R and c-MET in CMPC (Figure 3b). Furthermore, after seven days of in-vitro incubation the UPy-10k hydrogel was visually still present, indicating the stability of the hydrogel.

After the initial studies *in vitro*, we assessed the feasibility of intramyocardial delivery in a porcine model of myocardial infarction. In total four injections of 0.2 cc were placed in one heart: two injections with SPIOS-labeled UPy-10k hydrogel in the anteroseptal wall of the left ventricle and two injections with UPy-hydrogel loaded with human recombinant HGF/IGF-1 in the lateral (infarcted) wall. These injections were guided to the borderzone of the infarction based on electromechanical mapping (EMM) (Figure 4a-b). Six hours after injection, the labeled UPy-hydrogel was successfully visualized by cardiac MRI (Figure 4a), and subsequently by histological analysis (Supp. info). With regard to safety issues, we observed no cardiac arrhythmias or tamponade within 6 hours after the injection. Next, we assessed the growth factor distribution within the pig heart by quantifying the human specific HGF and IGF-1 at the injection site in the infarct borderzone and in the opposite septal wall (i.e. remote area). Due to cross-reactivity with endogenous porcine HGF, only human specific IGF-1 was successfully identified at the site of injection (borderzone) and in the septal wall of the right ventricle (remote area) (Figure 4. C-d). These data suggest an effective intramyocardial gradient of growth factors, increasing towards the borderzone of the infarction. Attraction of endogenous cardiac stem cells towards the infarct borderzone has shown to be beneficial for recovery in terms of improved blood flow to the infarcted

area and formation of new viable cardiomyocytes.[38] These *in vivo* data show feasibility of the UPy-hydrogel system as intramyocardial drug carrier and appears to be safe on short term follow-up, up to 6 hours.

The effect of UPy-hydrogel mediated release on HGF/IGF-1 treatment in a porcine model of chronic ischemia was evaluated. Four weeks after an acute myocardial infarction, ten NOGATM guided injections were intramyocardially placed in the infarct borderzone. Three treatment groups were tested, consisting of the pristine UPy-10k hydrogel, HGF/IGF-1 in saline, and UPy-10k hydrogel loaded with HGF/IGF-1. Four weeks after injection, no adverse events (e.g. fever, cardiac arrhythmias, shortness of breath) were recorded during follow-up, and liver and renal functions were comparable in all groups (data not shown). The myocardial scar tissue was visualized by

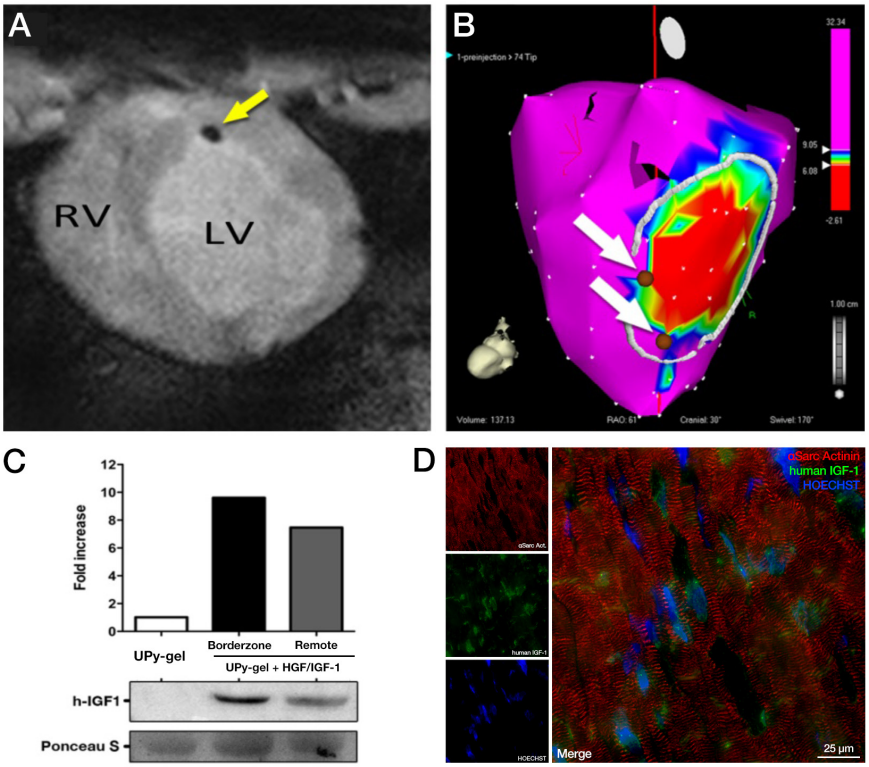

Figure 4 . Transendocardial injection of UPy-hydrogel loaded with HGF/IGF-1.
(A) Cardiac MRI short axis plane showing the UP-gel containing SPIOS, visible as focal depressed T2 ∗ intensity, indicated by the yellow arrow. **(B)** Electromechanical mapping of the left ventricle indicating the UPy-hydrogel + HGF/IGF-1 injections (white arrows) in the borderzone of the infarct (red signal, defi ned as < 6% on the local linear shortening map). **(C)** Western blot analysis of human IGF-1 in the myocardial borderzone injected with pristine UPy-hydrogel, or UPy-hydrogel + HGF/IGF-1 injected into the borderzone (BZ). After transendocardial injection, human IGF-1 was also detected in the opposing septal wall of the right ventricle (Remote). Ponceau S was used as a loading control to correct for differences in protein quantity. **(D)** Immunofl uorescent labeling of human IGF-1 (in green) shows cytoplasmatic and predominately perinuclear uptake by cardio- myocytes. Alfa-sarcomeric actin is shown in red. Nuclei are stained by HOECHST (blue).

1% triphenyl-tetrazolium chloride. All treatment groups macroscopically and histologically displayed roughly similar scar tissue formation in the lateral wall of the left ventricle (Figure 5a-c; 5g-i).

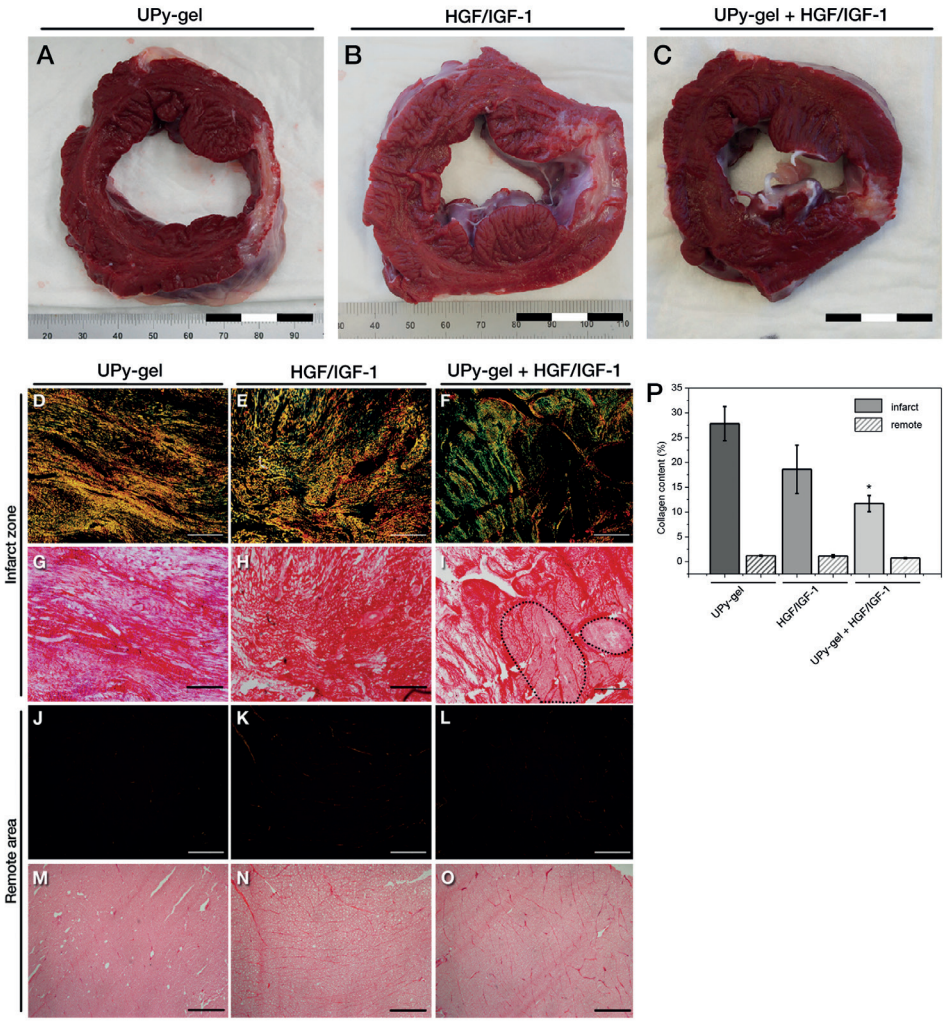

Figure 5. Adverse remodeling and growth factor induced cardiac repair.
(A-C) The infarct scar is visible as a white color stained with 1% triphenyl-tetrazolium chloride in all three treatment groups; **(A)** pristine UPy-hydrogel, **(B)** HGF/IGF-1 saline solution, and **(C)** UPy-hydrogel loaded with HGF/IGF-1. **(A-C)** Scale bars represent 3.0 cm. **(D-F/J-L)** Cryosections were stained with Picrosirius Red to quantify collagen deposition using polarized light on the infarct zone **(D-F)** and the remote area showing viable myocardium with scarcely dispersed collagen **(J-L)**. **(G-I/M-O)** The bright field images of the Picrosirius Red staining. **(I)** Patches of cardiomyocytes were observed within the infarct zone in the UPy-hydrogel with HGF/IGF-1 treated group (demarcated areas). **(P)** Quantification of total collagen content per field demonstrates reduced collagen content in the infarct scar in the UPy-hydrogel with HGF/IGF-1 compared to the pristine UPy-hydrogel (* denotes P = 0.02). The data is expressed as mean ± SEM. Differences in data were evaluated with a one-way analysis of variance (ANOVA) followed by Tukey post-hoc analysis. **(D-O)** Scale bars represent 500 µm.

However, after quantification of the Picrosirius Red stained tissue slices (Figure 5d-f, 5j-l) it was shown that the collagen content of the infarct scar in the UPy-hydrogel loaded with HGF/IGF-1 treated animals was reduced (12.9 ± 3.1%) compared to the pristine UPy-hydrogel (25.2 ± 5.7%; $p = 0.02$) (Figure 5p). Remarkably, patched-like clusters of viable myocardium were present dispersed in between the dense collagen fibers in the UPy-hydrogel loaded HGF/IGF-1 group (Figure 5i). These results suggest a more favorable remodelling process in the UPy-hydrogel loaded with HGF/IGF-1 than in soluble HGF/IGF-1 treated animals.

CONCLUSION

Here we showed the development of a synthetic pH-switchable supramolecular hydrogel that is injectable through the long, narrow lumen of a state-of-the-art catheter mapping system. The catheter-injected hydrogelator solution transforms into a locally controlled drug release reservoir by immediate gelation upon contact with heart tissue and additionally is proposed to self-heal at the site of injection. The preservation of molecular ordered fibrillar assemblies forms the fundamental origin of this hydrogel material. As a second hierarchy in order, the switching between gel-like and solution-like material results from the spatio-temporal formation of pH-sensitive inter-fiber crosslinks. When loaded with the growth factors HGF and IGF-1, we showed that the supramolecular hydrogelator system is able to reduce scar collagen in a chronic myocardial infarction pig model. To our knowledge, this is the first large animal study where NOGATM catheter-injection of a synthetic pH-switchable hydrogel system loaded with growth factors for controlled release is reported. Because the NOGA™ catheter-system is currently used in dedicated clinical centers, it will be together with our unique pH-switchable hydrogel system eminently valuable for the development of a novel therapy for treatment of ischemic heart disease.[41,42]

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SUPPLEMENTARY INFORMATION

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I. DETAILED EXPERIMENTAL PROCEDURES

Preparation of UPy-hydrogel

The UPy-hydrogelators were synthesized as described before.[1] For the preparation of the hydrogels, UPy-polymer solutions were prepared by dissolving 10 wt% in PBS pH 7.4 by stirring for 2 hours at 70 °C. Subsequently, solutions were UV-sterilized for at least 1 hour prior to use. To liquefy the polymer solution, the pH was raised by adding 2 µL aliquots of a 0.1 M NaOH stock solution.

To visualize gel formation after injection of a liquefied UPy-gel into pH 7.4 PBS buffer, the UPy-solution was colored red using a drop of Azorubin (E122). UPy-solution was then injected into a bulk PBS reservoir via a syringe. Gelation was monitored visually and measured by rheology.

For the animal experiments, once the sterilized UPy-hydrogel was liquefied, IGF-1 and HGF were mixed in by slowly stirring for 10 minutes in a final concentration of 500 ng/mL. For non-invasive assessment of the UPy-hydrogel by cardiac MRI *in vivo*, SPIOS were mixed into sterilized UPy-hydrogel by gentle stirring for 3 minutes at a final concentration of 13.6 µg/mL.

Atomic force microscopy

AFM was performed under ambient conditions with asylum research #Olympus AC240TS tips (77 kHz) using an Asylum Research MFP-3D in AC (tapping) mode. AFM samples were prepared from a 1 wt% UPy 20k polymer solution on a mica substrate by applying a 20 µL aliquot on the surface and allowing 10s substrate contact. The liquid was then absorbed by placing filter paper at the corner.

Biocompatibility

The toxicity of UPy-hydrogel on CMPC was tested *in vitro* based on a lactic dehydrogenase essay (Sigma-Aldrich). CMPC were cultured in M-199 medium with 2% FBS and incubated with 100 µL of UPy-hydrogel 10% w/v, based on phosphate-buffered saline (PBS), NaCl 0.9% or milliQ.

To test the possibility of altered toxicity following increased pH, both the unchanged UPy-hydrogel and liquefied UPy-hydrogel were tested. After three days of incubation, medium was collected and tested for presence of LDH as a marker for cell damage according to manufacturer's instruction.

Growth factor release and bioactivity

Human recombinant HGF (Miltenyi Biotec) and human recombinant IGF-1 (Miltenyi Biotec) dissolved in Milli-Q water were incubated simultaneously and gently stirred in liquefied UPy-hydrogel (pH 9.0) at a final concentration of 500 ng/mL. To test the subsequent release from the hydrogel, 100 µL of UPy-hydrogel with growth factors was incubated in 200 µL of medium (M-199, 1% Pen-Strep) in 48-well plates on a rotational shaker (90 RPM) at 37 °C for 7 days. 75 µL of medium was removed daily and replaced with fresh medium. The collected medium was quantified for HGF and IGF-1 using ELISA detection kits (Quantikine, R&D systems).

To test the persistent bioactivity of the released growth factors, CMPC were subjected to serum starvation for 18 hours and followed by supplementation of medium collected from the HGF/IGF-1 release study for 15 minutes. Cells were lyzed (62.5 mM Tris-HCl, 2 w/v % SDS, 10% glycerol and 50 mM DTT) and western blot analysis was performed for pIGF-R1 and phosphor-cMet) by loading 10 µg of protein on PVDF membranes. Membranes were incubated with the following primary antibodies: phos-cMET (1:1000, Cell Signaling technology), phos-IGF1-R (1:1000, Cell Signaling technology), and β-tubulin (Cell Signaling technology, Danvers, USA) according to the manufacturer's recommendations.

Animals and experimental study design

In total thirteen pigs (female Dutch landrace, weighing approximately 70 kg) received humane care in compliance with the national guidelines on animal care and prior approval by the Animal Experimentation Committee of the Faculty of Medicine, Utrecht University, the Netherlands. The animals were sedated with ketamine (10 mg/kg), midazolam (0.5 mg/kg), and atropine (0.04 mg/kg) and subsequently induced with thiopental (4 mg/kg) before they were intubated and connected to positive pressure ventilator. The ear vein was canulated and used for continuous admission of saline and anesthesia. Anesthesia was maintained by continuous infusion of midazolam (0.7 mg/kg/h), whereas analgesia was preserved by continuous infusion of sufentanil citrate (6 µg/kg/h) and finally muscle relaxation via pancurorium bromide (0.1 mg/kg/h). Prior to infarction, 300 mg of amiodarone was infused intravenously in 45 minutes to minimize onset of cardiac arrhythmias. Myocardial infarction was induced by 75 minutes of intracoronary balloon occlusion of the proximal left circumflex artery. For this, the communal carotid artery was canulated with an 8F sheath and a balloon catheter was advanced in the proximal part of the left circumflex artery. Next, the balloon was inflated for 75 minutes, after visual confirmation of successful occlusion by blocked contrast injection. 150 Joule (biphasic) was used to externally defibrillate whenever ventricular fibrillation occurred.

Four weeks later, the animals underwent 3-dimensional electromechanical mapping (EMM) of the left ventricle for infarct and borderzone localization using the NOGA™ catheter system (Biosense Webster, Cordis, Johnson & Johnson). Intramyocardial delivery was performed using the NOGA™

system (Biosense Webster, Cordis, Johnson & Johnson) as described previously.[2] Briefly, the mapping catheter was used to simultaneously measure both conduction and myocardial movement at different points within the left ventricle. These points were interpolated to form a map of the left ventricle. This electromechanical map was used to target the area of interest by identifying the viable infarct borderzone, defined as unipolar voltage signal > 2.5 mV in absence of active movement (hibernating myocardium). The Myostar injection catheter (Biosense Webster, Cordis, Johson & Johnson) was used to navigate towards the demarcated area of interest and inject the UPy-hydrogel in the LV wall.

Short term *in vivo* tracking of growth factor distribution. Regarding the *in vivo* tracking of UPy-hydrogel loaded with HGF/IGF-1, four injections of 0.2 cc were placed, two injections with SPIOS-labeled UPy-hydrogel in the anteroseptal wall of the left ventricle and two injections with HGF/IGF-1 loaded UPy-hydrogel to the borderzone of the infarction (lateral wall). Six hours after injection, two animals underwent *in vivo* tracking of the hydrogel by cardiac MRI. In order to permit visual recognition of the needle puncture in the myocardium, tissue samples were collected one hour after injection in one animal for short-term detection of IGF-1. The following primary antibody was used for western blot analysis on homogenized tissue samples: human recombinant IGF-1 (1:1000, Abcam). For immunofluorescent staining of hIGF-1 a 1:100 dilution was used.

UPy-hydrogel behavior in vivo: MRI

The *in vivo* tracking of UPy-hydrogel loaded with SPIOS after intramyocardial injection was assessed by magnetic resonance imaging (MRI). Cardiac MRI was performed six hours after injection. Animals were scanned in a supine position using a 1.5 Tesla (Philips Medical Systems) scanner with a five-element phased-array cardiac coil for signal reception. Images were acquired along the short axis and longitudinal axis with an inversion recovery segmented k-space 3D gradient echo sequence. The following parameters were used: 2.8 ms echo time, 5.9 ms repetition time, 3 mm slice thickness, 15° flip angle and 160x160x60 field-of-view. Analysis was performed on serial short-axis views by endocardial and epicardial borders using dedicated software (Viewforum, Philips Medical Systems).

The effect of sustained release on cardiac function: follow-up study.

In total ten animals were injected at four weeks after the myocardial infarction with pristine UPy-hydrogel (n=3), saline dissolved HGF/IGF-1 (both 500 ng/mL) (n=3) and UPy-hydrogel loaded with HGF/IGF-1 (both 500 ng/mL) (n=4). The animals were then followed for four weeks and subsequently euthanized by exsanguination under general anesthesia. After excision of the heart, the heart was cut into five slices from base to apex and infarct size was determined by incubation in 1% triphenyl-tetrazolium chloride dissolved in PBS at 37 °C for 15 minutes. Digital photographs were taken (Sony Alfa 55) and automatically quantified using ImageJ for Macintosh (version 1.44g) by an investigator blinded for the treatment allocation. Tissue samples, collected of the septal wall (remote) and the infarction zone, were snap frozen in liquid nitrogen and stored at -80 °C until used for histological analysis. Cryosections (7 µm) were prepared on a microtome (Leika) and fixed in 4% formalin for 10 minutes. Next, the sections were incubated in 0.1% Sirius red (BDH) in picric acid (Sigma) for 8 minutes at room temperature. After counterstaining the nuclei with

hematoxylin for 1 minute, the sections were dehydrated, cleared and mounted. The quantification was performed on 30 fields per animal of UV polarized light on a microscope (Olympos DP71) at 40x magnification and automatically quantified for the percentage of collagen per field using ImageJ software for Macintosh (version 1.44g). The analysis was performed by an investigator blinded to the treatment allocation.

II. RESULTS

Macroscopic self-healing

The non-covalent interactions that form supramolecular materials are inherently dynamic, which yields the materials to become stimuli-responsive to heat, light, pH or even contact pressure. Therefore, self-healing properties are intrinsic to many supramolecular materials.[3]
The UPy-hydrogels show a self-healing behaviour in time, occurring at room temperature without any external interference. Annealed samples of 10 wt% UPy-10k or UPy-20k were manually ground and the self-healing was kinetically monitored. After complete destruction of the gel, at room temperature, UPy-10k healed in approximately one week while the faster dynamics of UPy-20k caused healing already within 2-3 days (Figure SI-1). The faster kinetics for UPy-20k is expected due to the inherent higher water solubility of the polymer caused by the larger hydrophilic block.

Cryo-TEM analysis

Cryo-TEM was performed on 1 wt% UPy-20k samples in both water and basic (0.1 M TRIS, pH 9) buffer. The images show that rigid and elongated fibers are present in the dilute solution regime for both basic and neutral solutions. Image analysis on the fiber-length distribution yielded fiber-averages of 75 nm and 104 nm for the neutral and basic samples, respectively (Figure SI-3). Although the mean of the fibers in the basic solution appears larger, it must be noted that the overall number of observed fibers was significantly lower (e.g. n = 89 vs 17 for neutral vs basic, respectively) and dominated by the presence of 2 large fibers. Quantitative conclusions are therefore difficult to draw. However, a quantitative proof of the existence of fibers in both neutral and basic aqueous environment is given.

Gelation onset at higher wt%

It was found that the UPy-system displays a pH-dependent sol-gel behavior with a threshold at pH 8.5. Above this pH, a 10 wt% solution appears as a (viscous) liquid, while below this pH, a solid network is obtained. We evaluated if a higher polymer concentration would result in gel formation at acidic and basic pH. Figure SI-4 shows that for the basic state (0.1 M NaOH, pH 12), even increasing the polymer content to 25 wt% does not result in gel formation.

Existence of UPy-fibers in basic environments by AFM

The UPy-polymers supramolecularly assemble into elongated fibers due to the interaction of H-bonds and separation of the hydrophilic and hydrophobic domains. To investigate the effect of

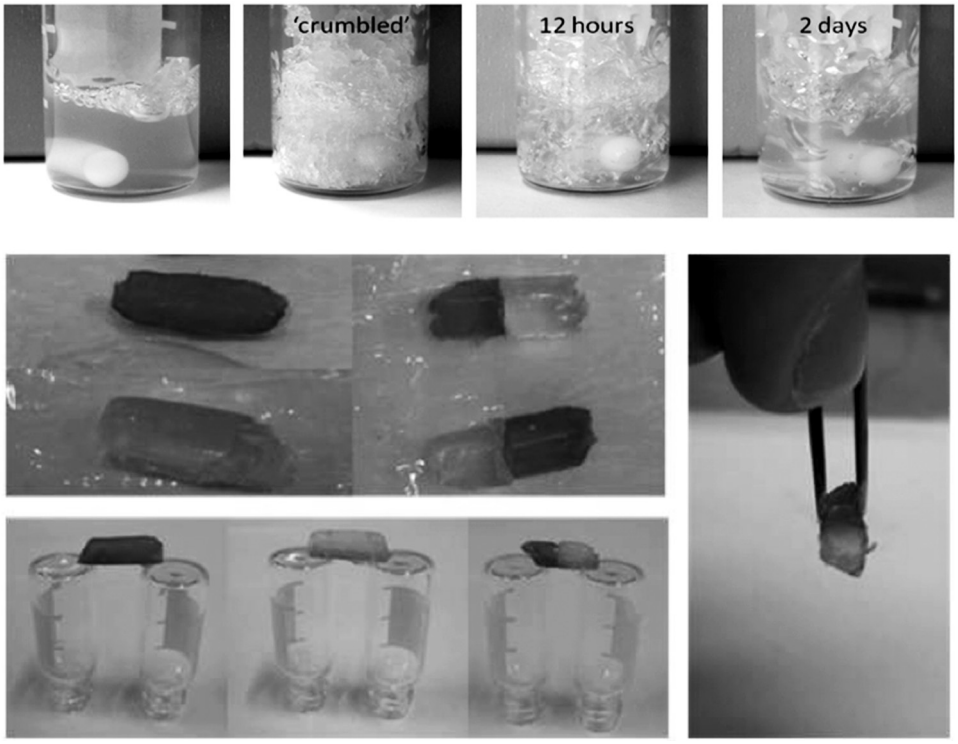

Figure SI-1: Pictorial demonstration of the self-healing character of the UPy-hydrogel system.
After preparation of a 10 wt% gel, the system was manually "crumbled" and self-healing over a time frame of days was observed. Upper panel: a hydrogel was formed in a vial after stirring (stir bar visible), and subsequently crumbled using a spatula. The hydrogel autonomously "reformed" in time, referred to as "self-healed". Lower panel: two hydrogels blocks were made with blue and red food-colorant. These blocks were cut and the red and blue halves were put together and allowed to form one block in time (resulting in two blocks composed of a red and a blue part).

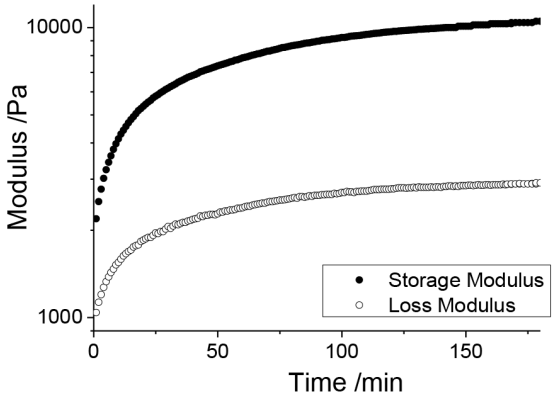

Figure SI-2: Build up of storage and loss modulus over time after adding acid at 37°C showing the immediate formation of a gel (G' > G") and consequent strengthing. The measurement was started within 1 minute after neutralizing.

pH changes on this supramolecular order, we visualized the self-assembled UPy-aggregates by AFM (Figure SI-5). UPy-20k hydrogelators were used because of solubility issues with the UPy-10k polymer at 1 wt% concentrations. However, both systems show comparable characteristics and can be interchanged in these analysis.

The polymers were set to assemble in neutral MQ solution as well as in a pH 9 TRIS buffered solution. For AFM, 10 µL of the 1 wt% solution was drop-cast on mica and residual fluid was drained with tissue paper. Samples were dried overnight and measured at room temperature. AFM shows a hierarchical assembly of fibers resembling collagen fibers for the polymers in water. In the basic pH sample, the assemblies seem more rigid and existing of fewer individual fibers, indicating a disrupted lateral self-assembly. However, the presence of salts hinders the AFM imaging and analysis but still a clear formation of fibers is visible.

Gelation and buffer compatibility

The toxicity of the UPy-moiety and different UPy-polymers has been extensively studied before.[i, iv] We have shown that the UPy-moiety is not toxic *in vitro*.[iv-a] Furthermore, many *in-vivo* studies on UPy-PCL polymers[iv-a,b,c] and UPy-PEG hydrogels[i, iv-d] have shown that depending on the composition, these materials trigger hardly any or a mild tissue response. PEG is a commonly used component in current biomedical applications; therefore, the possible toxicological effects *in vivo* of PEGs and PEG-protein/drug conjugates have been studied by others showing that there is a large therapeutic window for the use of PEG as a biological reagent.[v]

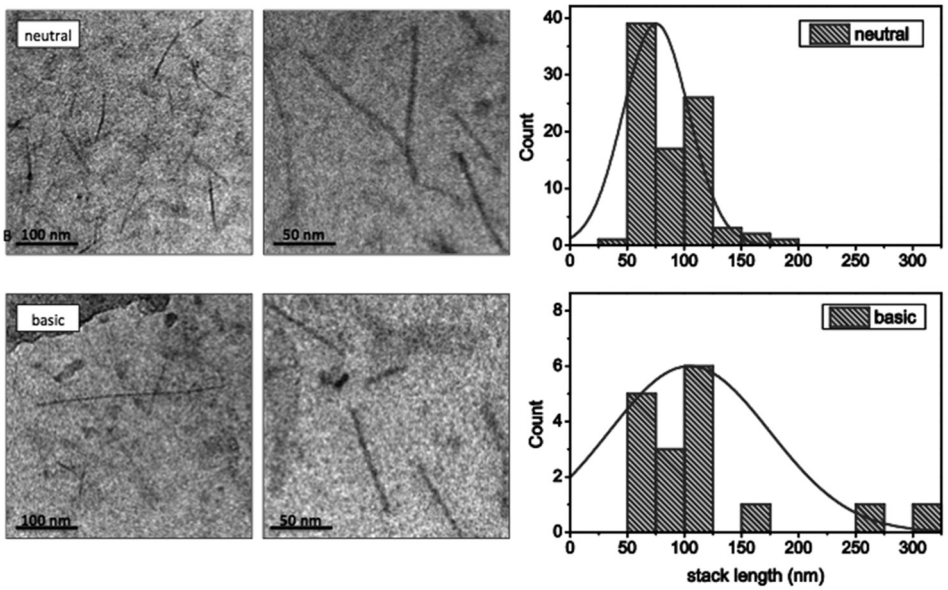

Figure SI-3: Cryo-TEM images of a 1 wt% UPy20k solution in water **(A)** and basic buffer **(B)**, together with a histogram of the statistical length variation of the fibers. The line represents a normal distribution fit, with mean length found to be 75±29 nm and 104±70 nm for neutral and basic samples, respectively. For both neutral and basic solution, the existence of high aspect ratio fibers in the dilute regime is demonstrated.

For a successful treatment, biocompatibility of the gel at neutral and basic pH is required. Additionally, the growth factors should remain active in both sol and gel state. To find the optimal compatibility between cells and material, we screened various (buffered) systems, being milliQ,

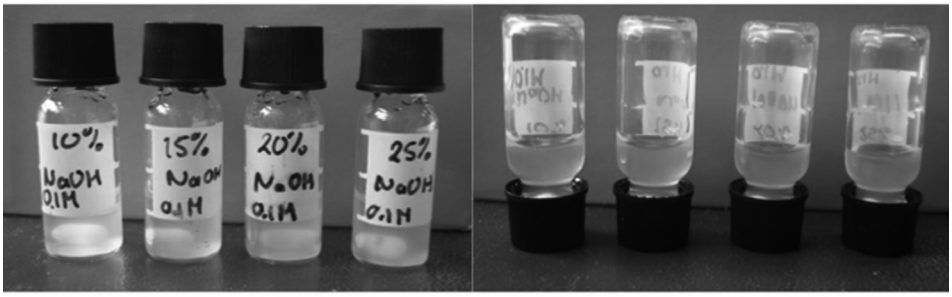

Figure SI-4: gel inversion tests of UPy samples in 0.1 M NaOH, ranging from 10 wt% - 25 wt%, indicating that an increase in wt% does not result in gel formation when dissolved in basic solution.

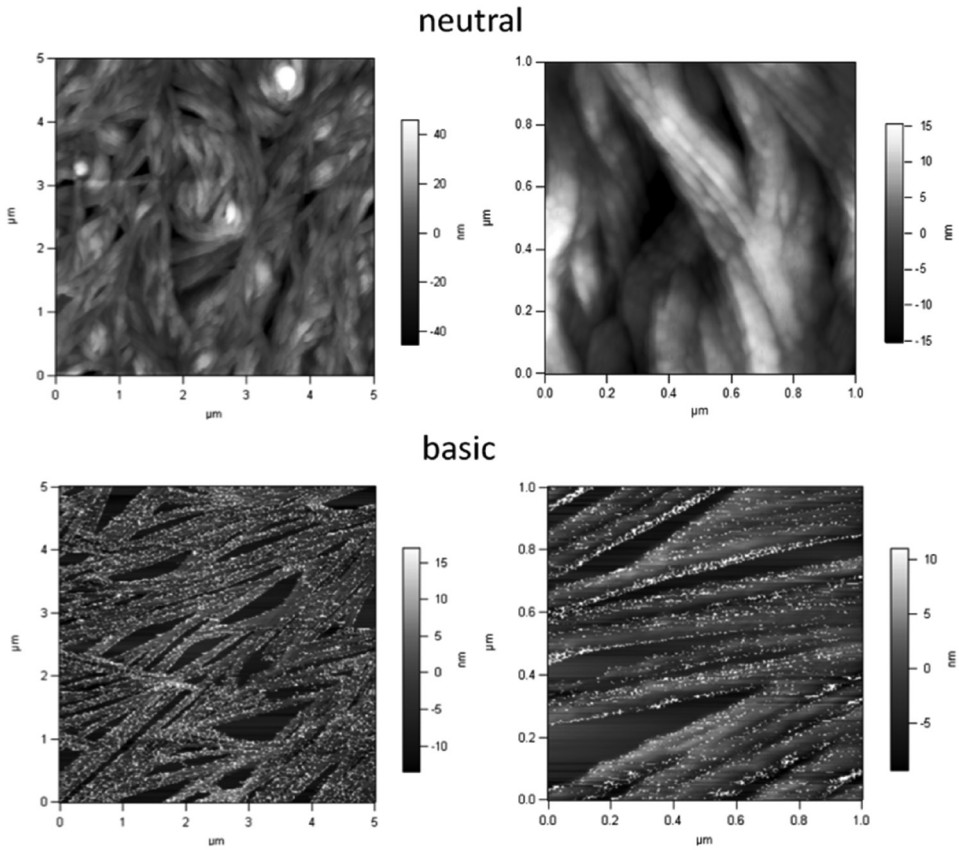

Figure SI-5: AFM height images of a drop-cast 1 wt% UPy-20k solution prepared in MQ water or pH 9 buffer and drop-cast on MICA substrates. In both environments, elongated stacked objects are visualized.

0.9% NaCl, PBS and TRIS at pH 9 (Figure SI-6). Upon a pH increase, the UPy-hydrogel prepared in milliQ induced more cell damage with subsequent LDH release. On the contrary, when the UPy-hydrogel was prepared in PBS the LDH level was similar to the reference LDH level at pH 7.4. These *in-vitro* studies show that the hydrogel system at elevated pH, in the sol-state is compatible with the CMPC. Best results were obtained for the PBS samples, making this the preparation method of choice for further study.

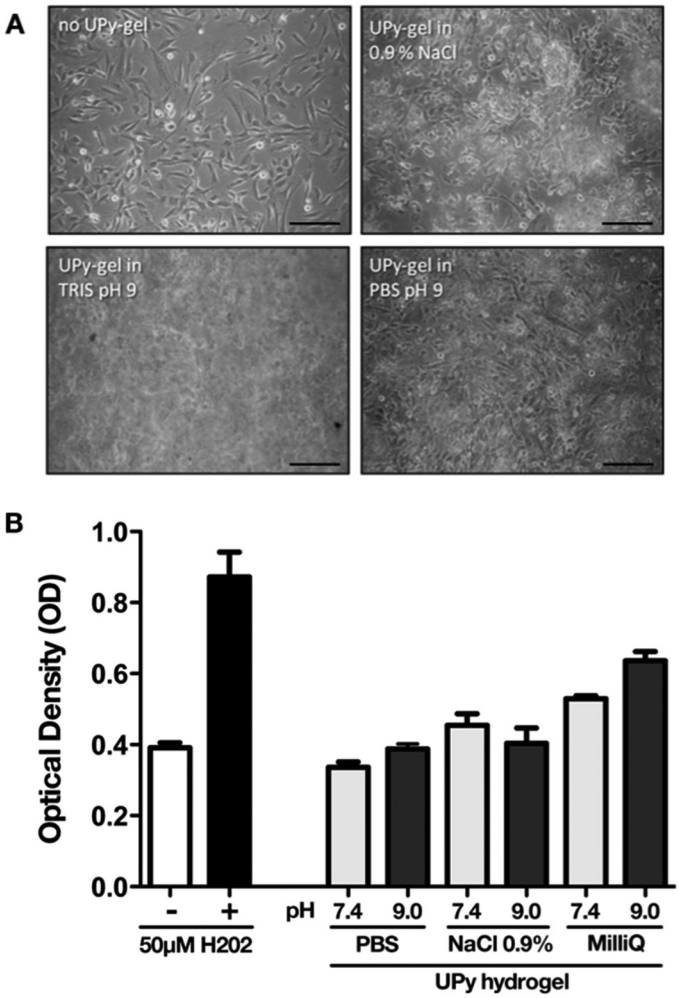

Figure SI-6: (a) Screening of the direct gelation of the UPy-hydrogelators after injection into culture medium. First, the CMPC were cultured in 0.1% gelatin coated dishes, after which the UPy-hydrogelators were injected on top of the cells. The gelled structures were directly formed upon injection (white objects on the images). UPy-gel was prepared in different media, milliQ, physiological salt solution, TRIS pH 9 and PBS pH 9, to screen for cell compatibility. The micrographs of the cells are taken when the gels were still on top of the cells; the cells did not change in morphology. (b) Cell compatibility screening for different UPy-hydrogels prepared in different media using the LDH leakage test. Optical density was assessed at 490 nm and corrected for background absorbance at 690 nm.

LAL assay

Biomaterials used for *in-vitro* or *in-vivo* applications need to be free of contaminating bacteria. To evaluate the amount of bacteria present in our materials, an endotoxin test was used. Samples were prepared in PBS at pH 9 and pH 7, UV-sterilized for one hour comparable to the sterilization protocol used for *in vivo* injection and incubated for 24 hours at 37 ℃. The concentration of endotoxin for all samples was below the FDA approved endotoxin value of 0.5 EU/mL (Figure SI-7).

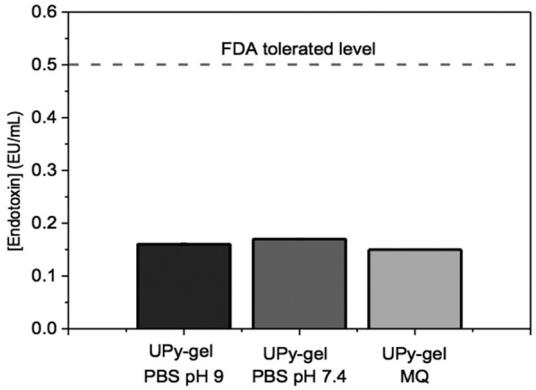

Figure SI-7: LAL assay results on UPy-gel samples prepared in PBS pH 7, PBS pH 9 and water all reside well below the FDA tolerated amount (0.5 EU/mL).

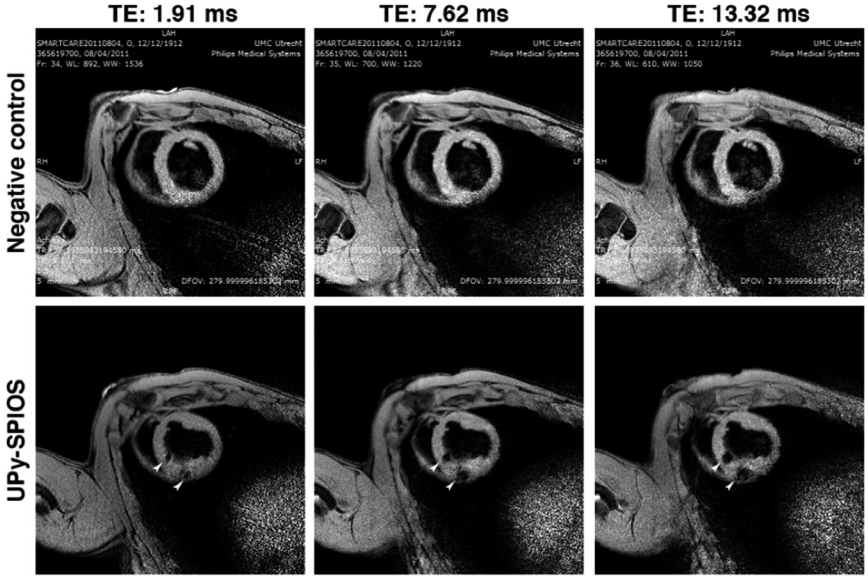

Figure SI-8: Visualization of SPIOS by T2* cardiac MRI. Representative T2* MRI images of a healthy heart (negative control) and a heart in which two NOGA injections of UPy-hydrogel labeled by SPIOS (depicted as UPy-SPIOS) have been placed. These UPy-SPIOS injections are visible as black dots of increasing intensity as echo time increases (white arrows). TE denotes Time to Echo.

Visualization of UPy-hydrogel with MRI

To evaluate the specificity of T2* gated cMRI imaging for SPIO detection, we evaluated different sequential increments in time to echo (TE). Optimal TE protocol enabled highly accurate anatomic location of SPIOS based in increasing distortion in the T2* gated images, whereas no differences was observed in the untreated porcine heart (negative control) (Figure SI-8). Histological data are shown in Figure SI-9.

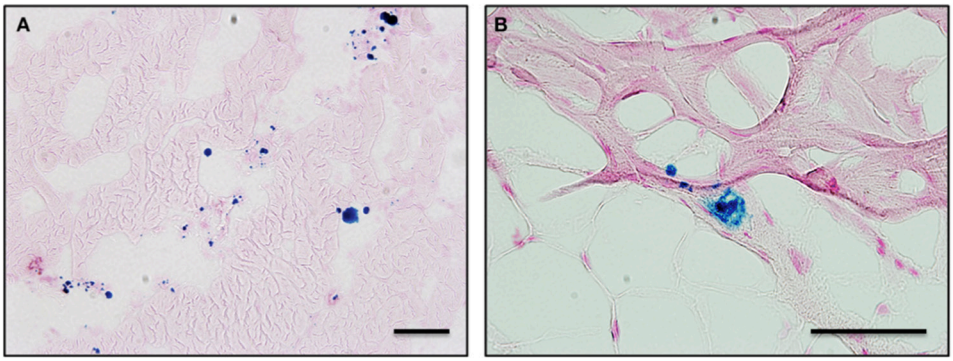

Figure SI-9: Representative microscopic images at 200x (a) and 400x (b) magnifications for Prussian Blue stained peri-infarct/borderzone sections showing small clusters of SPIOS (blue) dispersed in between the cardiac myocytes after intramyocardial injection of UPy-SPIOS . Scale bars indicate 125 μm.

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CHAPTER **6**

Sustained Delivery of Insulin-Like Growth Factor-1/Hepatocyte Growth Factor Stimulates Endogenous Cardiac Repair in the Infarcted Pig Heart

Koudstaal S, Bastings MM, Feyen DAM, Waring CD, Dankers PYW, van Slochteren FJ, Torella D, Sluijter JPG, Nadal-Ginard B, Doevendans PA, Ellison GM, Chamuleau SAJ. Sustained Delivery of Insulin-Like Growth Factor-1/Hepatocyte Growth Factor Stimulates Endogenous Cardiac Repair in the Infarcted Pig Heart.
Journal of Cardiovascular Translational Research 2013

ABSTRACT

Activation of endogenous cardiac stem/progenitor cells (eCSCs) can improve cardiac repair after acute myocardial infarction (AMI). We studied whether the in-situ activation of eCSCs by insulin-like growth factor 1(IGF-1) and hepatocyte growth factor (HGF) could be increased using a newly developed hydrogel in chronic MI. One-month post-MI pigs underwent NOGA-guided intramyocardial injections of IGF-1/HGF (both 0.5µg/ml; n=5, GF), or IGF-1/HGF incorporated in UPy-hydrogel (both 0.5µg/ml, n=5, UPy-GF). UPy-hydrogel without added growth factors was administered to 4 control (CTRL) pigs. LV ejection fraction was increased in the UPy-GF and GF animals compared to CTRLs. UPy-GF delivery reduced pathological hypertrophy, led to the formation of new, small cardiomyocytes and increased capillarisation. The eCSC population was increased almost fourfold in the borderzone of the UPy-GF treated hearts, compared to CTRL hearts. These results show that IGF-1/HGF therapy led to an improved cardiac function in chronic MI and that effect size could be further increased by using UPy-hydrogel.

INTRODUCTION

Despite early revascularization, acute myocardial infarction leads to irreversible loss of cardiomyocytes. As a consequence, the increased workload on the survived cardiomyocytes often initiates a cascade of additional cardiomyocyte loss, myocardial remodeling, until the vicious circle ends in chronic heart failure (CHF).[1] In the USA alone, approximately 5.7 million patients have CHF accounting for roughly $30 billion annually in health care costs in 2008, which are predicted to triplicate by 2030.[2] Given the initial loss of functional cardiomyocytes as the trigger of adverse remodeling processes that eventually lead to CHF[1], it is imperative to develop new low-cost, widely available treatments that are able to ameliorate the natural disease progression following an MI to reduce the occurrence of post-MI heart failure. One of the emerging therapeutic approaches relies on the notion that the adult mammalian heart fosters an innate capacity for cardiomyocyte regeneration and different approaches to upscale this phenomenon to a clinically relevant level of myocardial regeneration are under intense investigation.[3,4]

The presence of tissue specific, endogenous cardiac stem/progenitor cells (eCSCs) that reside in the heart and, upon activation, can create progeny that mature into functional cardiomyocytes and vasculature, have been put forward as the causal agent for the regenerative capacity of the heart.[5-7] Recently, accumulating evidence supported the notion that the regenerative response of eCSCs towards the ischemic myocardium can be stimulated by means of in situ administration of various growth factors, such as IGF-1 and HGF.[8-10] We have previously shown that the co-administration of IGF-1 and HGF led to activation of eCSCs, increased cardiomyogenesis and significantly improved cardiac function.[9] Yet, like previous studies, these results were reported on animal models addressing the acute phase of the MI, which, by itself, is a complex and powerful initiator of numerous molecular signaling processes in response to the ischemic insult.[11] Given the unmet clinical need for development of new therapeutics to treat post-MI heart failure, we investigated whether the effect of IGF-1/HGF therapy is also effective in the post-MI heart, in which cardiac adverse remodeling is already an active process. To this end, we used the pig model of chronic MI, as the pig heart closely resembles the human size and hemodynamics.

Besides the validation and identification of growth factors and signaling pathways that can stimulate cardiac repair, novel drug delivery systems such as biomaterials are extensively being studied to increase effect size. Previous reports showed that by combining growth factors with an injectable biomaterial, the biomaterial could serve as a controlled drug release platform thereby improving functional outcome.[10,12] Therefore, we investigated the added value of incorporating the growth factors within a smart hydrogel that can serve as a release scaffold upon catheter-based delivery in the infarcted heart to generate sustained GF levels at the site of dysfunction over time. Recently, we have reported on a new, pH-switchable and self-healing hydrogel carrier that could be injected in the heart by transendocardial delivery using the NOGA™ catheter system (Biosense Webster, Johnson & Johnson Co.). We have previously shown that release kinetics *in vitro* showed a four-day time span for both IGF-1 and HGF in absence of protein degradation based on the increased pH of the hydrogel.[13] Furthermore, IGF-1/HGF loaded hydrogel injections in the borderzone of the infarct created an effective spatial gradient of growth factors within the heart in which growth factor concentrations increased towards the site of injection.[13]

Here, we present the first results on efficacy of this new hydrogel system in combination with growth factors IGF-1/HGF on cardiac function and the progression of post-MI adverse remodeling in the pig chronic MI model.

METHODS

A detailed method section can be found in the supplement data. Briefly, MI was induced by 75-min intracoronary balloon occlusion of the LCx followed by reperfusion, in 6-month old female Dalland landrace pigs (~70kg). Four weeks later, 10 intramyocardial injections of 0.2mL each were placed in the infarct borderzone with either IGF-1/HGF in 0.9% saline (both 0.5µg/ml, GF), IGF-1/HGF in UPy hydrogel (both 0.5µg/ml, UPy-GF), or UPy hydrogel alone as control (CTRL) were administered. Four weeks after treatment, cardiac function was assessed by 2D- and 3D echocardiography, and pressure volume loop analysis. Regional microvascular resistance was quantified by simultaneous assessment of intracoronary pressure-/and flow velocity parameters. Cardiomyocyte hypertrophy, cell proliferation, new cardiomyocyte and capillary formation, c-kit^{pos} CD45^{neg} eCSC number and their committed progeny were characterized by immunohistochemistry and confocal microscopy.

RESULTS

Mortality and Procedural data

A schematic of the study design is depicted in Figure 1A. Three animals died during the induction of ischemia by LCx occlusion as a consequence of refractory ventricular fibrillation. One animal died four weeks later, prior to the intervention. Of the survived 14 animals, 5 animals were randomly allocated to UPy-GF, 5 animals to GF and the remaining 4 animals to UPy hydrogel alone, serving as controls.

IGF-1/HGF administration improves cardiac function in chronic MI

First, the controls, UPy hydrogel without growth factors, were compared against a historical cohort of identical MI procedure and NOGA injections with phosphate buffered saline (PBS) one month after MI. There were no differences in any echocardiographic or PV-loop derived parameters (Suppl. Figure 1). Thus, with no indication that the UPy hydrogel by itself influenced post-MI remodeling, we considered the empty UPy hydrogel as negative controls. As a reference value, prior to MI, the LV end diastolic volume (EDV) was on average 81.2 ± 6 ml. Two months after MI, there was a slight increase in LVEDV by ~15% in all groups but LVEDV did not differ between treatment groups (Figure 2A; CTRL vs GF vs UPy-GF; 94.9 ± 10.8ml vs 94.0 ± 8.9ml vs 92.4 ± 6.6 ml respectively, p = 0.915).

On the other hand, the mean change relative to baseline in LV end systolic volumes (ESV) was significantly improved in the UPy-GF group compared to GF treated animals and controls (Figure 2B; -1.1 ± 2.3ml vs -10.3 ± 9.9ml vs -9.5 ± 4.6ml; p=0.03). Likewise, progressive deterioration

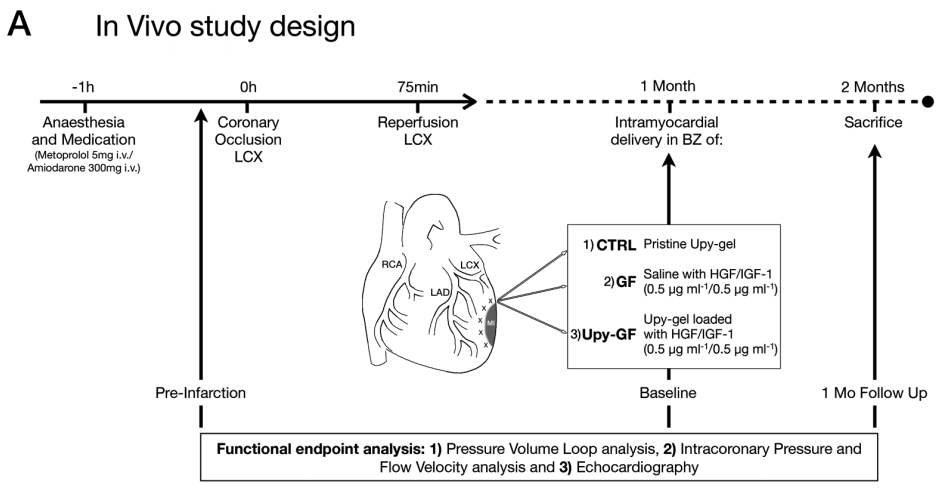

Figure 1. Study design

(A) Schematic study design, showing the targeted intramyocardial delivery in the MI borderzone of 1) empty UPy-hydrogel as control (CTRL), 2) IGF-1/HGF dissolved in saline, denoted as GF, or 3) UPy-hydrogel with IGF-1/HGF, denoted as UPy-GF.

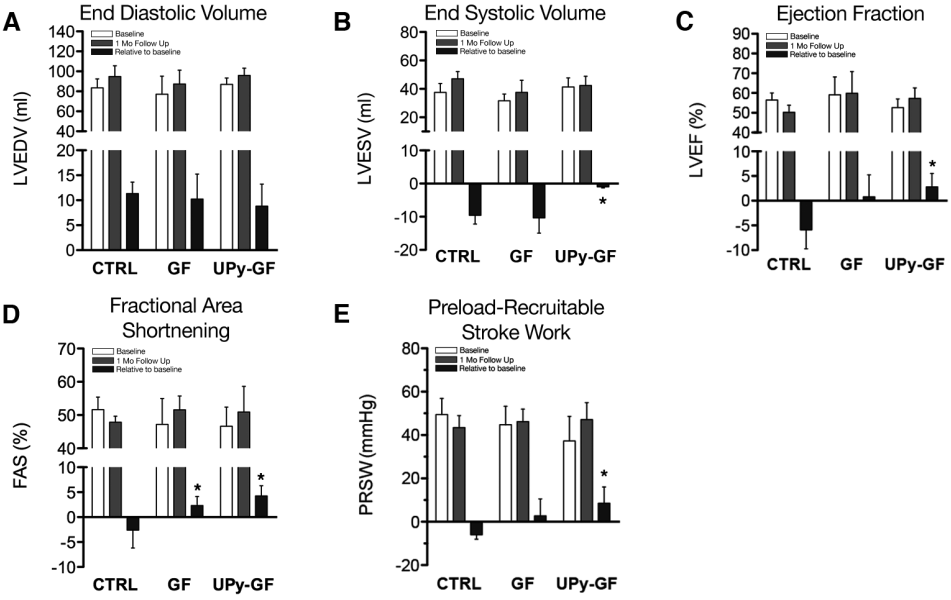

Figure 2. UPy-IGF-1/HGF therapy improves cardiac function in chronic MI.

(A to B) LV end-diastolic and end-systolic volumes at baseline, 1 month after injection and the relative change between both timepoints and **(C)** LV ejection fraction. **(D)** FAS measured by 2DE at the level of the papillary muscles. **(E)** preload recruitable stroke work (PRSW) measured by intracardiac pressure-volume loop recordings. * denotes $p<0.05$ vs CTRL. All data are mean ± SD, n = 3, 5, 5 for CTRL, GF and UPy-GF respectively.

in left ventricular ejection fraction was also significantly reversed in the UPy-GF group (Figure 2C; mean change +2.8 ± 2.7%), compared to CTRL animals (Figure 2D; -5.9 ± 3.8%, p = 0.02), but did not significantly differ from the GF group (Figure 2C; 0.8 ± 2.0%; p = 0.410). Fractional area shortening (Suppl. Figure 2) was significantly improved in both the GF and UPy-GF groups compared to the CTRL animals (Figure 2D; +2.3±1.8% vs +4.2±2.0% vs -2.6±3.6%; p = 0.008). With regard to diastolic function of the heart, the ratio of transmitral flow velocity to annular peak diastolic velocity (E/E') was preserved in the IGF-1/HGF treated animals (GF 7.7 ± 0.3; UPy-GF 7.4 ± 1.1), compared to CTRLs (9.3 ± 0.6; p = 0.04).

Targeted intramyocardial IGF-1/HGF delivery attenuates cardiomyocyte hypertrophy and fibrosis in chronic MI

Histological analyses have been summarized in Table 1. As a reference, average cardiomyocyte diameter in the healthy pig heart was 18±3 µm. Four weeks after the NOGA-guided injections, histological analysis revealed significant cardiomyocyte hypertrophy in the borderzone of the CTRL hearts (Figure 3A, Table 1). In contrast, both GF and UPy-GF treatment attenuated cardiomyocyte hypertrophy as well as increased the number of relatively small (<18µm) cardiomyocytes, compared to CTRL (Figure 3B,I; GF 18.47 ± 2.56µm vs UPy-GF 16.04 ± 1.85µm vs CTRL 21.20 ± 2.81µm respectively; p = 0.04). In line with previous pilot data[13], both the GF and the UPy-GF treated hearts showed a trend towards reduction in fibrosis, shown by picric Sirius red staining (Figure 3C to H, Table 1), compared to the CTRL group (p=0.27).

Intramyocardial IGF-1/HGF administration leads to formation of new cardiomyocytes

Immunohistological analyses have been summarized in Table 1. Different myocardial cell types express growth factor receptors for IGF-1 and/or HGF. Thus, we sought to investigate the level of cell proliferation in the borderzone of the chronic MI after GF treatment. Even 30 days after the injection procedure, an increased proliferation rate assessed by Ki67 expression was present within the GF treated hearts, which was greater in the UPy-GF treated hearts (Figure 4A,B, Table 1). In particular, the borderzone of the GF and UPy-GF treated animals harbored newly formed, small, immature $Ki67^{pos}$ cardiomyocytes, which amounted to ~1 every 1000 cardiomyocytes (Figure 4C). These small $Ki67^{pos}$ cardiomyocytes accounted for >10% of the total proliferating $Ki67^{pos}$ cells, in the GF treated hearts, making their existence physiologically significant. Although $Ki67^{pos}$ cardiomyocytes were also observed in the CTRL hearts, these were only witnessed in ~1 every 3000 cardiomyocytes (p = 0.016). To verify that these $Ki67^{pos}$ cardiomyocytes were newly formed, we measured their size and compared this with $Ki67^{neg}$ cardiomyocytes. Indeed, the $Ki67^{pos}$ cardiomyocytes were on average smaller (Figure 4E; 12.52 ± 3.97µm) compared to their $Ki67^{neg}$ counterparts (Figure 4E; 17.48 ± 3.85; p=0.0006), suggestive of a newly formed and immature cardiomyocyte subpopulation.[6,9]

IGF-1/HGF delivery leads to the formation of new capillaries in the infarct borderzone

The IGF-1/HGF treatment led to an increased number of capillaries in the infarct borderzone, favoring the UPy-GF group (Figure 5 A to B, Table 1; UPy-GF 8.6 ± 0.9/0.2mm^2 vs GF 7.8 ±

0.9/0.2mm^2 vs CTRL 6.3 ± 0.8/0.2mm^2 respectively; p=0.022). Consistent with the increased capillarisation, the hyperemic microvascular resistance index (HMR) (a simultaneously measured intracoronary pressure-/ and flow velocity derived parameter) was decreased in the infarct related artery in the UPy-GF group compared to the HMR value measured just prior to the intramyocardial treatment delivery (Figure 5C; p = 0.053).

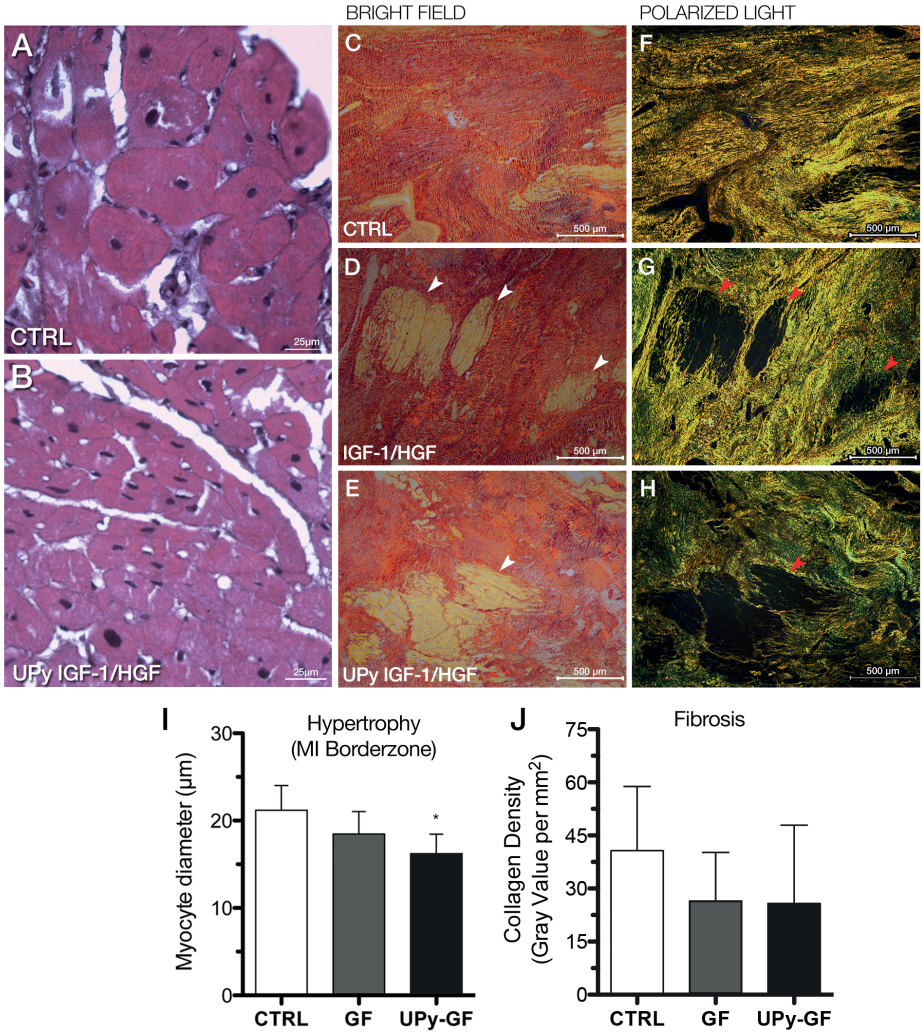

Figure 3. IGF-1/HGF treatment reduced pathological hypertrophy in the MI borderzone.
(A,B) Representative MI borderzone sections (hematoxylin and eosin (H&E) staining) showing adverse cardiac hypertrophy in the control treated animals **(A)**, which was not observed in the UPy-GF treated animals **(B)**. (C to H) Picric Sirius red staining in bright field images **(C to E)** and under polarized light **(F to H)** showing extensive scar tissue in all groups depicted as red staining in bright field microscopy. Under polarized light, color depended on the collagen fiber density (yellow for higher intensity, green for lower intensity). In both growth factor treated groups, small myocardial islands were visible in the infarct area (see arrowheads). Quantification of **(I)** cardiomyocyte diameter in the MI borderzone and **(J)** fibrosis. * denotes p<0.05 vs CTRL. All data are mean ± SD, n = 3, 4, 5 for CTRL, GF and UPy-GF respectively. MI denotes myocardial infarction.

IGF-1/HGF administration leads to expansionary growth of the epCSC compartment and induces cardiogenic precursors

To elucidate potential mechanisms governed by IGF-1/HGF stimulation that are responsible for the observed new cardiomyocyte and capillary formation, we determined the number and precursor state of the previously described c-kit^{pos} CD45^{neg} epCSCs.[9] We found increased c-kit^{pos} cells in the infarct and borderzone with GF treatment, however ~73% of all c-kit^{pos} cells also co-expressed CD45, identifying cardiac mast cells (Figure 6Aiii; Suppl. Figure 3).[9] Furthermore, there was an infiltration of CD45^{pos} c-kit^{neg} cells into the infarct and borderzone (Figure 6Aii). c-kit^{pos} CD45^{neg} epCSCs (Figure 6Ai;B) had a relatively small cytoplasm to nuclei ratio and in the infarct zone, the total number of epCSCs was increased four-fold by IGF-1/HGF delivery, compared to CTRL hearts (Figure 6C, Table 1; 0.37 ± 0.09% vs 0.43 ± 0.14 vs 0.12 ± 0.07% respectively, p = 0.004). With regard to the borderzone, the highest increase in c-kit^{pos} epCSC number was observed in the UPy-GF group (Figure 6C, Table 1; 0.24 ± 0.06%) compared to GF or CTRL hearts (Figure 6C; 0.14 ± 0.06% vs 0.12 ± 0.01%, p = 0.03). Of those epCSCs, sustained IGF-1/HGF release induced a modest increase in the number of progenitor epCSCs (~40%) that co-expressed the early cardiac transcription factor Nkx2.5, indicative of their commitment towards

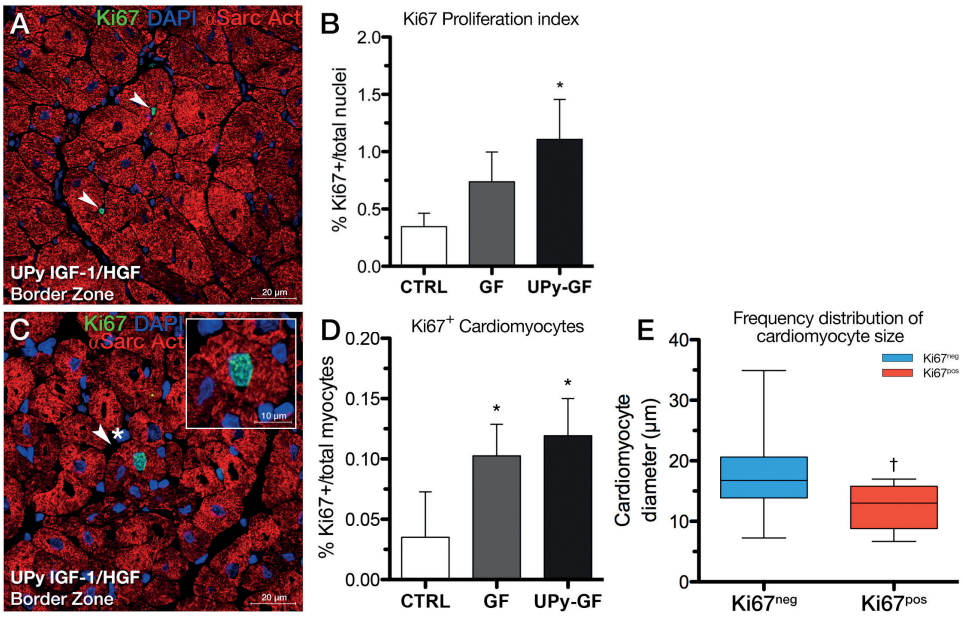

Figure 4. IGF-1/HGF administration leads to formation of new cardiac myocytes.
(A to B) Expression of cellular proliferation marker Ki67 (green) showed increased proliferation index of cells (arrowheads) in the UPy-GF treated animals, compared to CTRL. **(C to D)** Increased newly formed Ki67pos (green) cardiomyocytes (arrowheads, asterix, see inset) after GF treatment, compared to CTRL in the peri-infarct/borderzone. **(E)** Ki67pos cardiac myocytes were smaller than the quiescent Ki67neg cardiomyocyte fraction, indicative of their immature, newly formed nature. * denotes p<0.05 vs CTRL. † denotes p<0.05 vs Ki67neg cardiac myocytes. All data are mean ± SD, n = 3, 4, 5 for CTRL, GF and UPy-GF respectively.

the cardiomyogenic lineage (Figure 6D to E, Table 1). Furthermore, another subset of epCSCs expressed the transcription factor Ets-1, indicative of their commitment to the endothelial lineage, and the generation of capillaries (Figure 6F, Table 1).[9]

Table 1. Histological and Immunohistological Analysis at 1 Month Follow-Up

	CTRL	IGF-1/HGF	UPy-IGF-1/HGF
Cardiac Adverse Remodeling			
CM Hypertrophy (μm)	21.2 ± 2.8	18.4 ± 2.6	16.0 ± 1.9*
Fibrosis (Gray Value per mm^2)	40.7 ± 18.1	26.5 ± 13.7	25.8 ± 22.1
Proliferation			
Proliferation index (% $Ki67^{pos}$nuclei/total nuclei) (%)	0.3 ± 0.1	0.7 ± 0.3	1.1 ± 0.3*
$Ki67^{pos}$ CM (Border Zone) (%)	0.03 ± 0.03	0.10 ± 0.03*	0.12 ± 0.03*
Cardiac Stem Cells			
c-kit^{pos} CSCs (Border Zone) (%)	0.12 ± 0.1	0.14 ± 0.1	0.24 ± 0.1*
c-kit^{pos} CSCs (Infarct Zone) (%)	0.13 ± 0.1	0.37 ± 0.1*	0.43 ± 0.1*
c-kit^{pos} $Nkx2.5^{pos}$ CSCs (Border Zone) (%)	25.2 ± 5.2	37.5 ± 5.9	45.5 ± 8.5*
c-kit^{pos} $Nkx2.5^{pos}$ CSCs (Infarct Zone) (%)	29.2 ±10.0	33.7 ± 5.2	52.4 ± 14.8
c-kit^{pos} Ets-1^{pos} CSCs (Border Zone) (%)	16.9 ± 3.6	20.2 ± 2.9	23.0 ± 4.0
c-kit^{pos} Ets-1^{pos} CSCs (Infarct Zone) (%)	19.1 ± 5.1	24.2 ± 6.7	24.8 ± 5.3
Angiogenesis			
vWF^{pos} capillaries (Border zone) (No per 0.2 mm2)	6.3 ± 0.8	7.8 ± 0.9	8.7 ± 0.9*

Data are represented as mean±SD. * indicates $p < 0.05$ vs CTRL; † indicates $p < 0.05$ vs IGF-1/HGF
Abbreviations: CTRL denotes controls; IGF-1 denotes insulin-like growth factor-1; HGF denotes hepatocyte growth factor; UPy denotes ureido-pyrimidinone; CM denotes cardiomyocyte; CSC denotes cardiac stem/progenitor cell

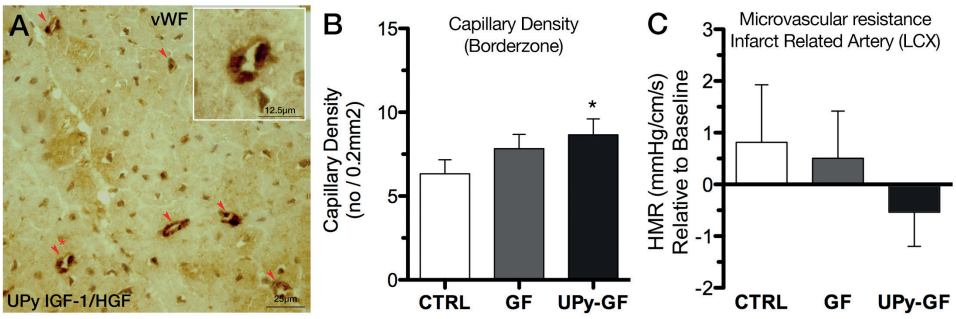

Figure 5. IGF-1/HGF leads to increased capillerisation and reduces microvascular resistance.
(A) Staining for Von Willebrand factor (vWF) show small capillary structures (red arrowheads, asterix, see inset) in the borderzone of the UPy-GF treated heart. **(B)** Number of capillaries in the peri-infarct/borderzone area. **(C)** Relative change, compared to baseline, in simultaneously measured intracoronary pressure and flow derived hyperemic microvascular resistance (HMR). * denotes p<0.05 vs CTRL. All data are mean ± SD, n = 3, 4, 5 for CTRL, GF and UPy-GF respectively.

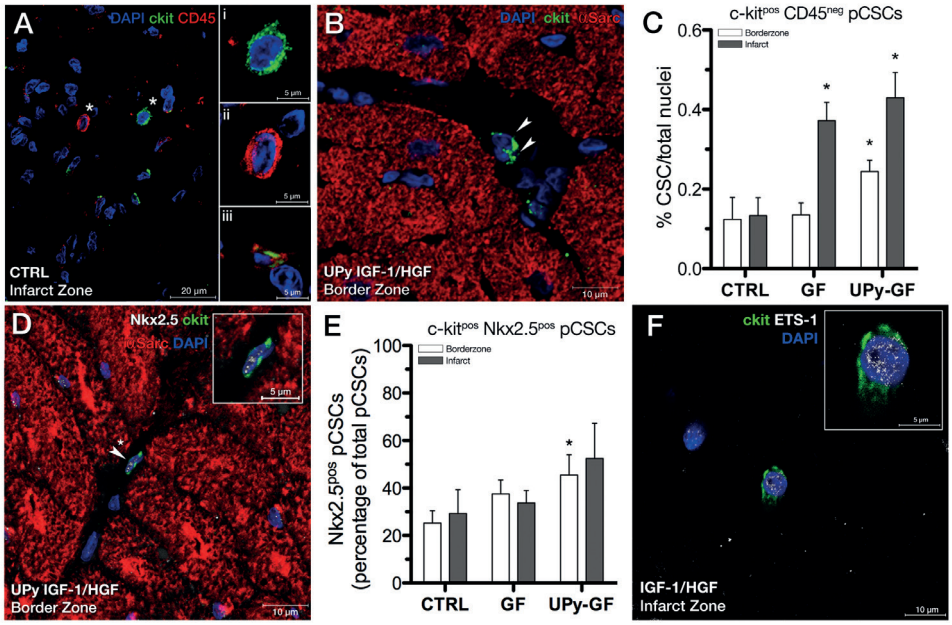

Figure 6. IGF-1/HGF treatment increases the epCSC compartment and drives their cardiac commitment in Chronic MI.
(A) The infarct area harbors various cell types, such as i) c-kitpos CD45neg epCSCs, ii) c-kitneg CD45pos cells or iii) c-kitpos CD45pos cells (including mast cells). **(B)** Endogenous epCSCs were a morphologically distinct subset of small cells showing perinuclear expression of c-kit (green)(arrowheads) and negative for CD45. **(C)** Quantification of epCSCs in the peri-infarct/border and infarct zone. **(D)** A c-kitpos (green) myogenic progenitor (arrowhead, asterix, see inset), expressing the early cardiac transcription factor, Nkx2.5 (white). **(E)** Quantification of Nkx2.5pos epCSCs in the peri-infarct/border and infarct zone. * denotes p<0.05 vs CTRL. All data are mean ± SD, n = 3, 4, 5 for CTRL, GF and UPy-GF respectively. **(F)** Some c-kitpos epCSCs also expressed the transcription factor ETS-1 (arrowhead, asterix, see inset).

DISCUSSION

In the present study, we sought to investigate the functional and histological/cellular effects of intramyocardial administration of IGF-1/HGF in chronic MI in the pig. We show that improved delivery of IGF-1/HGF by a newly developed UPy-hydrogel carrier holds potential as a novel treatment for chronic MI. Four weeks after delivery, UPy-IGF-1/HGF treatment led to a reduction in pathological cardiac remodeling, activated and increased the number of epCSCs, and led to the formation of new cardiomyocytes and capillaries. Importantly, the repair and regeneration of the damaged myocardial tissue was associated with a significant improved in cardiac function.

Heart regeneration and eCSCs

To date, the presence of endogenous mechanisms for cardiomyocyte renewal in the post-natal heart remains a subject of intense debate.[14] Our findings presented here challenge the prevalent view that the adult mammalian heart, at best, can only increase its myocyte volume by means of a hypertrophic response of existing cardiac myocytes in the absence of new myocyte formation. Here, we show that the adult infarcted pig heart contains immature cardiac myocytes that are substantially smaller than normal, non-hypertrophied, myocytes and do not reside in the quiescent G0 phase of the cell cycle, as would be expected given the hypothesis that the heart is a post-mitotic organ. Importantly, this regenerative potential of the adult heart could be effectively boosted by sustained release of the growth factors IGF-1 and HGF. These findings further ascertain the definitive presence of cardiomyocyte renewal in the adult mammalian heart as deducted from elaborate pulse-chase experiments published by various independent research groups.[3,15-17]
Secondly, our present findings document that following IGF-1/HGF administration, the number of resident c-kit^{pos} epCSCs in the peri-infarcted area increased (Figure 6) analogously to the increase in the presence of newly formed, immature, Ki67^{pos} cardiomyocytes (Figure 4). Indeed, the majority of eCSCs in the peri-infarct region also co-expressed the nuclear transcription factors Nkx2.5 and Ets-1, indicative of their commitment towards the myogenic and vasculature lineage, respectively. However, as to what extent these newly formed cardiomyocyte reflect the differentiated progeny of eCSCs[7,15], or, are the result of an endogenous regeneration mechanism that was indirectly mediated by paracrine actions[18,19] could not be answered in this translational large animal model.

Growth factors to stimulate endogenous cardiac repair

Recently, essential growth factor/signaling pathways for cardiomyogenesis during the embryonic period have been summarized.[20] Various growth factors have been identified as potential candidates to guide post-natal stem-progenitor cells towards a cardiomyogenic fate.[8,9,21-23] In a recent report by Chimenti and co-workers, the possibility was raised that eCSCs are not just mere consumers of growth factors, but actively secrete a wide range of growth factors themselves, providing intricate networks of auto-/ and paracrine feedback loops.[19] We have previously documented that the effects of a single administration of IGF-1/HGF is still measurable 2 months after its application suggesting the existence of a feed-back loop triggered by the external stimuli that activates the production of growth and survival factors by the targeted cells, which explains the persistence and long duration of the regenerative myocardial response.[9] Since here we have

observed effects on cell proliferation detectable one month after delivery of a single dose of IGF-1/HGF, we speculate a similar auto/paracrine feedback loop that leads to sustained epCSC activation and proliferation and resultant cardiomyocyte formation, long after the primary stimulus has disappeared.[9]

Sustained release of GF using a bioscaffold

Previous proof of concept experiments validating the UPy hydrogel showed that the hydrogel created a successful gradient of growth factors towards the infarcted area.[13] As a next step, the present study was undertaken to determine the therapeutic value provided by the sustained release of IGF-1/HGF using the UPy hydrogel carrier. This subsequent report advances initial findings by showing that IGF-1/HGF incorporated in the UPy-hydrogel increased the effect of IGF-1/HGF therapy but did not show statistical significance compared to equal concentrations of IGF-1/HGF dissolved in saline in both functional and histological endpoints (Figure 2; Table 1). However, when comparing the different growth factor treated groups to the control treated animals, only UPy-GF treated animals showed improvement with statistical significance as opposed to the GF group in which significance was not reached for ESV and EF as well several histological outcomes (i.e. CM hypertrophy, eCSC numbers in the MI borderzone). Altogether, there is a highly consistent trend visible showing that the UPy-GF treated animals outperformed the GF treated animals on all levels of outcome measures (i.e. cardiomyocyte formation, number of c-kit^{pos} eCSCs, cardiac function).

Clinical perspective

By avoiding myocardial biopsies to extract eCSCs that need *ex vivo* up scaling to acquire clinically relevant numbers for subsequent delivery, one escapes from several drawbacks of cellular products as a novel treatment for ischemic heart disease.[24,25] First and foremost, cellular therapy requires dedicated clinical centers that have both the expertise and high-cost resources for isolating, culturing and handling of the stem cell products to pursue cardiac repair. Secondly, it relies on an available time-span necessary for culturing stem/progenitor cells that is not present as in the case of acute myocardial infarction. Therefore, *in situ* activation of the eCSC compartment could bypass the aforementioned limitations of exogenous stem cell therapy. This holds true in particular for the chronic MI patients, in which aging and co-morbidities also reduce the potency of the eCSC compartment. One particular aspect is the dramatic increase in cellular senescence of eCSCs to ~70% of all eCSCs in aged mice.[26] Work by Torella and colleagues further showed that growth factors such as IGF-1 are capable to reverse this process in aged mice and restore function of aged-senescent eCSCs.[26]

Previous work on the therapeutic efficacy of IGF-1/HGF relied on transepicardial injections during open-chest surgery as the route of delivery.[8,10,27] In contrast, we used a percutaneous approach with the NOGA catheter system to acquire information on the infarct location and used the MYOSTAR catheter for targeted intramyocardial delivery in the peri-infarct/borderzone of the chronic MI. As a consequence, the entire study protocol employed in this present work is clinically feasible and can be performed at a conventional catheterization laboratory. Work to address use of UPy hydrogel synthesized under GMP conditions for human use is currently in progress.

Limitations

Given the dynamicity in temporal expression pattern of Ki67 in cycling cells, our histology, at best, provides a 'snapshot' of cellular homeostasis in the post-MI heart at one month follow up.[28] Therefore, we cannot draw inferences on the absolute number of newly formed cardiomyocytes in any of the treatment groups. Although we specifically characterized the contribution of tissue specific c-kit^{pos} CD54^{neg} eCSCs, we cannot exclude that other stem/progenitor cell populations or other mechanisms of cardiomyogenesis contributed to new cardiac cell formation, and, if so, to what extent. Furthermore, given the immature nature and low numbers of small, newly formed cardiomyocytes, the increase in cardiac function is most likely also caused by numerous other unknown factors, commonly designated as 'paracrine effects'.[29,30] The identification of these biological processes can provide further clues to improve growth factor mediated cardiac repair and regeneration. Unraveling hereof is warranted in order to advance the cardioregenerative field to clinically relevant levels of myocardial regeneration.

Last, although experimental *in vitro* work on release by UPy hydrogel showed a ~3 day sustained release of both IGF-1 and HGF, extrapolation towards the *in vivo* situation warrants certain caution. Since we did not choose to sacrifice additional animals shortly after the GF injections, we cannot conclude whether the highest improvement in LV function seen in the UPy-GF group was actually caused by sustained release of growth factors, or, that the hydrogel was capable of retaining higher initial levels of growth factors compared to the saline solution. Despite careful placement of the intramyocardial injections, there is considerable back-flow into the left ventricular cavity and/or venous drainage that could be potentially be minimized by the UPy hydrogel.

CONCLUSION

In summary, four major conclusions can be deducted from this study: (1) targeted intramyocardial IGF-1/HGF injections attenuated pathologic cardiac remodeling and increased the formation of small newly formed cardiomyocytes in the borderzone of the infarct scar, in the post-MI adult pig heart; (2) IGF-1/HGF admission gave rise to a robust increase of the c-kit^{pos} epCSC compartment of the heart and increased their commitment towards the cardiomyogenic and vasculature lineage; (3) intramyocardial IGF-1/HGF injections in the borderzone of the infarct scar led to an improvement in cardiac systolic and diastolic function when compared to control treated hearts; (4) the use of a smart hydrogel carrier that acts as a sustained release platform increased the effectiveness of growth factor therapy as a treatment for chronic MI. Taken together, these results provide rationale to further develop experimental work on growth factor therapy for myocardial repair and regeneration. Moreover, these findings identify the UPy hydrogel carrier system as a practical, affordable and widely applicable therapeutic strategy designated to counteract the adverse remodeling and natural disease progression in the post-MI heart that would otherwise lead to congestive heart failure.

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SUPPLEMENTARY INFORMATION

METHODS

UPy Hydrogel and Growth Factors

The UPy-hydrogelators were synthesized by SyMO-Chem BV, Eindhoven, the Netherlands.[1] For the preparation of the hydrogel, polymer solutions were dissolved at 10 wt% in phosphate buffered saline (PBS) by stirring at 70 °C for 2 hours and subsequently cooled to room temperature (RT). To liquefy the polymer solution, the pH was raised by adding 2 µL aliquots of a 0.1 M NaOH stock solution. Solutions were then UV-sterilized for at least 1-hour prior to use. Next, human recombinant HGF (Miltenyi Biotec) and human recombinant IGF-1 (Miltenyi Biotec) were mixed in by slow stirring for 10 minutes yielding a final concentration of 0.5 µg/mL of each growth factor.

Animals and Study Design

All *in vivo* experiments were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources. Experiments were approved by the Animal Experimentation Committee of the Medicine Faculty of the Utrecht University, the Netherlands. Eighteen female Dalland Landrace pigs (age ~6 months, weighing 68 ± 4 kg) were pretreated with clopidogrel 75 mg for 3 days and 500 mg acetylsalicylic acid one day before the operation. Prior to ischemia, the animals received 300 mg amiodarone and 5 mg metoprolol intravenously in 45 minutes to minimize the onset of cardiac arrhythmias. Myocardial infarction (MI) was induced by intracoronary balloon occlusion, under general anesthesia[2], of the proximal left circumflex artery for 75 minutes. Heparin was administered at 100 IU/kg body weight intravenously. Four weeks later, the animals underwent 3-dimensional electromechanical mapping (EMM) of the left ventricle for infarct and borderzone localization using the NOGA™ catheter system (Biosense Webster, Cordis, Johnson & Johnson, USA). Intramyocardial delivery was performed using the NOGA™ Myostar system (Biosense Webster, Cordis, Johnson & Johnson, USA), as previously described.[3] After randomized treatment allocation, ~10 injections of 0.2 mL were placed in the borderzone of the infarct with: 1) empty UPy-hydrogel (CTRL); 2) HGF/IGF-1 (0.5 µg ml^{-1}/0.5 µg ml^{-1}) (GF); 3) UPy-hydrogel loaded with HGF/IGF-1 (0.5 µg ml^{-1}/0.5 µg ml^{-1}) (UPy-GF). One month after the intramyocardial delivery, the animals underwent functional endpoint analysis and were sacrificed by exsanguination under general anesthesia. After excision of the heart, the left ventricle was cut into five slices from base to apex and incubated in 1% triphenyl-tetrazolium chloride dissolved in phosphate buffered saline (PBS) at 37°C for 15 minutes. Next, the slices were washed in PBS and photographed digitally (Sony Alfa 55). Infarct size was determined using ImageJ software (version 1.44g). The treatment allocation was kept blind to the investigator analyzing the infarct size.

Intracoronary pressure and flow velocity assessment

Intracoronary pressure and flow velocity were measured simultaneously by using the Combowire® (Volcano Corporation, San Diego, USA) as previously described.[4,5] Pressure and flow velocity signals,

combined with aortic pressure and ECG signals were recorded using the ComboMap® system (Volcano Corporation, San Diego, USA). Intracoronary pressure and flow velocity were assessed prior to the infarction and four weeks after MI in the infarct related artery (LCX) and the reference artery (LAD). Nitroglycerin (200 mcg) was injected intracoronarily to prevent coronary spasms. Next, the Combowire was placed in the proximal section of the LCX and the LAD. Velocity and pressure signals were recorded during rest and maximal hyperemia. Hyperemia was induced by intracoronary bolus of 60 mcg adenosine. At least 3 representative measurements were performed per vessel.
Datasets were stored digitally and analyzed offline using AMC Study manager, a custom software package (written in Delphi vs. 6.0, Borland Software Corporation and Delphi vs. 2010, Embarcadero, CA, USA). CFVR was calculated as CFVR = pAPV / bAPV, where APV is average peak flow velocity in cm/s. The bAPV and pAPV were calculated as the mean of four beats at rest and the mean of three successive beats with the highest flow velocity respectively. HMR was calculated as HMR = P_d / pAPV, where both P_d and pAVP were derived from the mean of three beats at hyperemia. (6) The treatment allocation was kept blind to the investigator who performed the analysis.

Echocardiography

The echocardiographic examination was performed with the animals under general anesthesia, lying in the right lateral position. Ultrasound data was acquired using a Philips iE33 scanner (Philips Healthcare, Eindhoven, the Netherlands) with a S5-1 phased array transducer (1-5 MHz) for two-dimensional datasets and the X3 transducer for three-dimensional datasets. Standard parasternal long and short axis views were obtained. Left ventricular dimensions and wall thickness were measured in accordance with the standards of the American Society of Echocardiography.[7] Fractional area shortening was determined on the parasternal short axis view at papillary muscle level and expressed as a percentage of the LV internal area shortening in systole compared to the diastolic LV internal area. During the open-chest procedure at follow up, apical views (four-, two-, three-chamber) were obtained epicardially. Three-dimensional LV datasets were acquired under breath hold over six consecutive heartbeats to assess LV volumes and ejection fraction. Pulsed wave Doppler imaging was used to assess transmitral flow patterns. Tissue Doppler Imaging from the four-chamber view was used to determine the mitral annular motion at the basal part of the septum and lateral wall. Analysis included the average of three peak diastolic velocities (E') for both annular sites combined. LV filling index was defined as the ratio of transmitral flow velocity to annular peak diastolic velocity (E/E'). The treatment allocation was kept blind to the investigator who performed the analysis.

Pressure Volume loop analysis

Pressure-volume (PV) loops were assessed using a 7-F conductance catheter. Briefly, LV pressure and volumes were measured and stored using a Leycom CFL-512 (CD-Leycom, Zoetermeer, the Netherlands). After correct placement in the LV, checked by the individual segmental conductance signals, the conductance signals were calibrated by cardiac output estimated by thermodilution of 5mL NaCl 0.9% at room temperature infused by Swan Ganz catheter. All data were collected while mechanical ventilation was paused. Data analysis and calculations were performed off-line using custom-made software (CD Leycom, Zoetermeer, the Netherlands), as

described previously.[2] Systolic LV function was measured as LV ejection fraction, dP/dT^{+}-peak, stroke work and preload recruitable stroke work. Diastolic function was assessed by dP/dT^{-} analysis. The treatment allocation was kept blind to the investigator performing the analysis.

Immunohistochemistry

Tissue sections were sampled from the infarct zone (visible as white area by TTC staining), border zone (viable myocardium directly adjacent to infarct zone) or the septal wall that served as remote area. Following fixation in 4% formalin, tissue samples were embedded in paraffin. Next, 7 µm sections were prepared on a TP1020 microtome (Leica). Hematoxylin and eosin staining was used for measurement of cardiac hypertrophy. The cardiomyocyte diameter of across the nucleus was measured in the borderzone of the infarct area on a light microscope (Nikon E1000M) using Lucia G software. A total of 100 cardiomyocytes/animal was measured. The mean value per animal was used in the subsequent analysis. Next, sections were incubated in 0.1% Fast Blue RR in magnesium borate buffer at pH 9 (Sigma) for 30 minutes. Following wash steps with distilled water, the slides were incubated in 0.1% Sirius red (BDH) in picric acid (Sigma) for 8 minutes at room temperature. After washing, the sections were dehydrated, cleared and mounted. The quantification was performed on 10 fields per animal under normal light and UV polarized light on a microscope (Olympos DP71) at 10x magnification and automatically quantified for the percentage of collagen per field using ImageJ software for Macintosh (version 1.44g). To quantify newly formed cardiomyocytes, tissue sections were stained for Ki67 (Vector), cardiomyocytes were identified by α-sarcomeric actin (Sigma) and nuclei stained by DAPI to identify cycling cells and cycling cardiomyocytes. In total 20 random fields of the peri-infarct borderzone were counted at 40x for cycling cells and cycling cardiomyocytes. To identify c-kitpos CSCs, numbers of positive for c-kit (DAKO) and CD45 (Santa Cruz) was counted. DAPI was used to identify nuclei. Tissue sections were also stained with c-kit and Nkx2.5 (R&D) and ETS-1 (Santa Cruz) to identify lineage commitment. For the borderzone and infarct area, 5 random fields/area at 40x was counted. Capillary density was assessed by von Willebrand factor (vWF, Millipore) and counterstained with hematoxylin to identify nuclei. Vessels with a circumference spanning 1-3 endothelial cells were counted as capillaries.[8] In total, 10 fields/section at 40x were counted per animal in the borderzone. The amount of capillaries was expressed per $0.2mm^2$. Fluorescence images were visualized and acquired with confocal microscopy (Zeiss LSM710, LSM software). All analysis was performed by investigators who were blind to the group assignment.

Statistics

Continuous variables were presented as mean±SD unless stated otherwise and compared by one-way ANOVA. When a significant difference was found, post-hoc analysis was performed with Bonferroni's test. When the assumption of the homogeneity of variance between groups was violated, nonparametric statistical analysis based on Kruskal-Wallis was performed. The assumption of a normal distribution was checked by QQ plots and the Kolmogorov-Smirnov test. To test whether the follow-up values differed from baseline values in each group, a paired T-test was used. All tests were performed using SPSS Statistics 17.0. Probability values of $p < 0.05$ were considered statistically significant.

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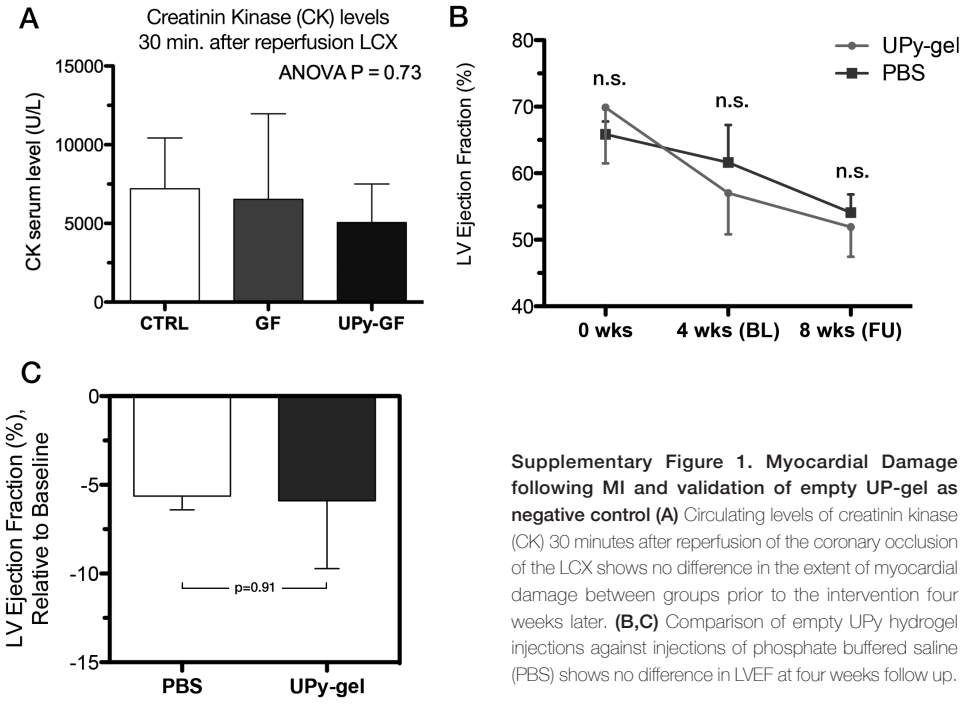
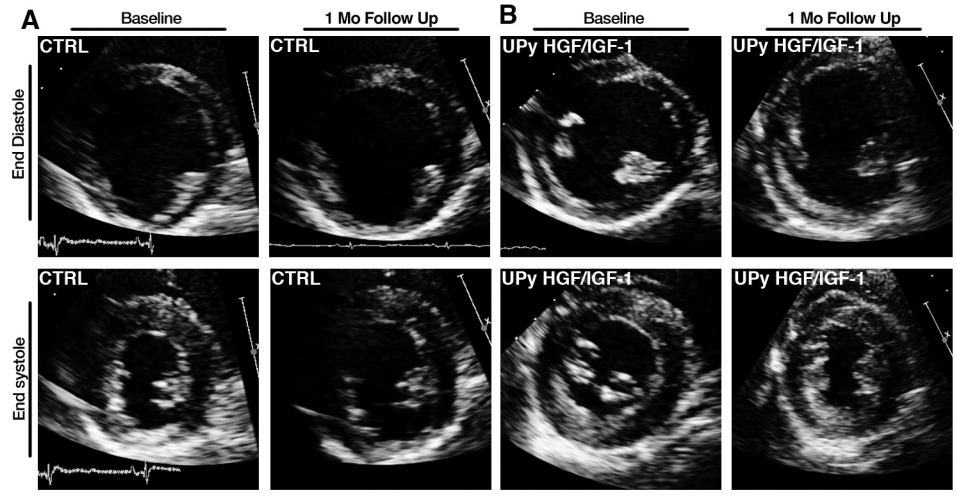
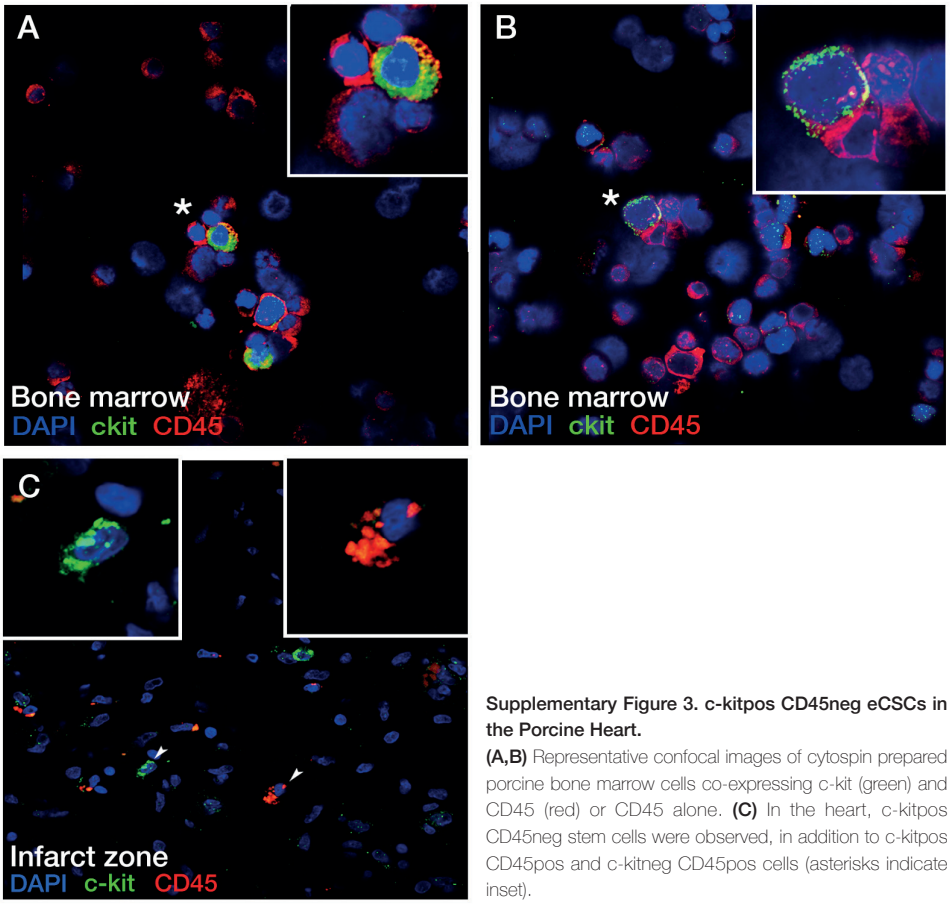


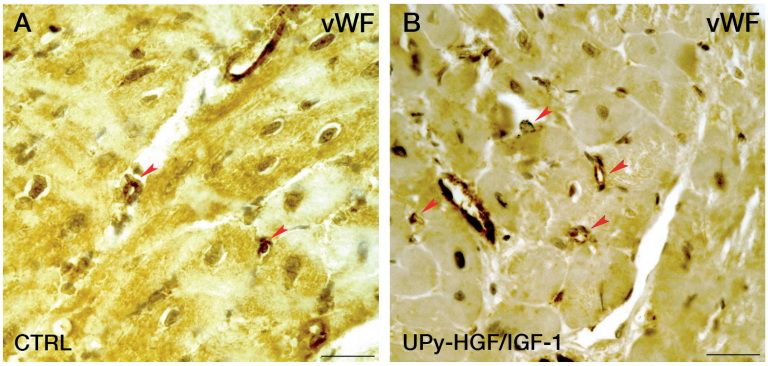
Supplementary Figure 1. Myocardial Damage following MI and validation of empty UP-gel as negative control (A) Circulating levels of creatinin kinase (CK) 30 minutes after reperfusion of the coronary occlusion of the LCX shows no difference in the extent of myocardial damage between groups prior to the intervention four weeks later. **(B,C)** Comparison of empty UPy hydrogel injections against injections of phosphate buffered saline (PBS) shows no difference in LVEF at four weeks follow up.



Supplementary Figure 2. Echocardiographic data.
Representative 2D b-mode echocardiographic images of the short-axis view at the papillary muscle level showing the fractional area shortening for **(A)** CTRL and **(B)** UP-GF treated animals, at baseline and 1 month after intramyocardial delivery



Supplementary Figure 3. c-kitpos CD45neg eCSCs in the Porcine Heart.
(A,B) Representative confocal images of cytospin prepared porcine bone marrow cells co-expressing c-kit (green) and CD45 (red) or CD45 alone. **(C)** In the heart, c-kitpos CD45neg stem cells were observed, in addition to c-kitpos CD45pos and c-kitneg CD45pos cells (asterisks indicate inset).



Supplementary Figure 4. HGF/IGF-1 Mediated Angiogenesis.
(A,B) vWF staining of the borderzone in CTRL **(A)** and UPy-HGF/IGF-1**(B)** treated animals show capillaries (red arrowheads) as defined by 1 or 2 endothelial cells spanning the vWF positive vessel circumference (shown by DAB staining in brown). Scale bar represents 25 μm

CHAPTER 7

Rationale and Design of the Repetitive Intramyocardial cell delivery in no-option Patients with refractory coronary Artery disease and Stable Anginal complaints (RIPASSA) trial:
A randomized, double-blind, placebo-controlled study of safety and efficacy

In preparation

ABSTRACT

Background

Refractory angina pectoris (RAP) is a debilitating condition that plagues patients with severe coronary artery disease who are deemed ineligible for conventional revascularization strategies and experience anginal complaints in spite of optimal medical care. Preclinical work has put forward mesenchymal stem cells (MSCs) as a promising new strategy to improve symptoms.

Study Design

The RIPASSA is a Dutch, multi-centre, double-blind, placebo-controlled clinical trial evaluating the safety of repetitive intramyocardial injection of autologous mesenchymal stem cells derived from bone marrow (10^7) and its effect on cardiopulmonary exercise testing in patients with refractory angina. A total of one hundred fifty patients with anginal complaints CCS class II or higher that have no option for conventional revascularisation will be randomized in a 1:2:2 fashion to placebo, autologous MSC delivery, or repetitive MSC delivery (i.e. 6 months after initial dose). The primary endpoint is difference in VO_2 max as assessed by cardiopulmonary exercise testing 12 months after initial therapy, as compared to baseline. The second endpoints will include change in CCS class, left ventricular ejection fraction, perfusion scintigraphy and change in the Seattle Questionnaire for Anginal complaints.

Conclusion

The RIPASSA trial will evaluate the safety and efficacy of repetitive intramyocardial delivery of autologous bone marrow derived MSCs in patients with coronary artery disease and refractory anginal complaints.

INTRODUCTION

Refractory angina (RAP) is a debilitating disease tormenting coronary artery disease (CAD) patients with severe, incessant cardiac pain, resistant to all conventional treatments for CAD.[1-3] Despite a relatively low mortality rate of approximately 4% per year, RAP patients are challenged on a daily basis by recurrent and disabling pain that give rise to anxiety, depression, impaired physical activity and thus societal participation, and reduced health-related quality of life.[4-9] Prevalence of RAP is not well established and estimates range from 600,000 to 1.8 million people in the United States alone with 50,000 new cases annually.[3] Several registries suggested that ~10% of patients with symptomatic stable CAD referred for angiography cannot be controlled by a combination of medical therapy, percutaneous revascularization and/or coronary bypass surgery and are thereby deemed 'no-option' patients.[1,10]

For these patients, the recently published ESC guideline on management of stable CAD [11] recommends 3 additional treatment options that might be considered in these 'no-option' RAP patients to reduce symptoms, i.e. enhanced external counterpulsation (EECP), spinal cord stimulation (SCS) and TENS[12-16]. In contrast, transmyocardial revascularization (TMR) has been abandoned as it is deemed ineffective in RAP patients (class III, level of evidence A).[17-21]

Unfortunately, these new therapies have thus far only been proven marginally effective. In addition, even if their efficacy were to be markedly increased, these techniques would impose a high demand in procedural time, human and economic resources, rendering these techniques only eligible to a minute fraction of the candidate patients.

One of the emerging therapeutic approaches relies on stem cells to repair and ameliorate the damaged ischemic heart.[22,23] Initial enthusiasm fuelled high expectations for bone marrow mononuclear cells as a new treatment for ischemic heart disease.[24,25] At present, meta-analyses have shown that bone marrow derived cell therapy is safe and modestly effective in increasing left ventricular ejection fraction (LVEF).[26-28] However, what these meta-analyses overlook is the fact that anginal pain is the culprit in RAP patients, not LV systolic function. Studies on the effect of cell therapy for RAP have shown significant improvements in angina frequency and exercise tolerance while little change was seen on systolic LV function.

One of the key questions that remain unanswered is: which cell type supports the strongest regenerative boost following delivery to the heart? One of these high-potential 'second-generation' cell types is the mesenchymal stem cells (MSC), a small subset of the bone marrow mononuclear cell compartment. Based on preclinical evidence, these MSCs have a greater potential than bone marrow mononuclear cells to repair the ischemic myocardium by activating the endogenous regenerative potential of the heart.[29-34] Moreover, MSC have been found to release cytoprotective and proangiogenic soluble factors that exert powerful myocardial protection (e.g. inhibited apoptosis, angiogenesis, immunomodulatory effects), leading to functional improvement in animal models.[35] These results led to a phase I-II clinical trial in patients with severe CAD and RAP. Even at the three-year follow-up, there was a sustained improvement in angina frequency, nitroglycerine use, hospital admissions and exercise tolerance.[36]

After intracoronary infusion, it has been shown that both delivery efficiency and survival of transplanted cells is low within the hostile environment of damaged myocardium (ischemic and

scar tissue), potentially caused by reduced perfusion of the area of interest and insufficient supply of oxygen and nutrients.[37,38] In addition, intracoronary delivery is dependent on the distribution based on the infarct related vessel and hence still delivers only a minute fraction of the cells to the ischemic target area. Direct intramyocardial injection of the cells avoids the potential adverse effects related to cell infusion in the diseased and narrowed coronary vasculature seen in RAP patients and has been used in the majority of cell therapy trials in RAP patients. In addition, in order to advance the accuracy of targeting, there is a need for high resolution imaging that provides information on the extent and location of the infarcted territory. To this end, we have developed a real-time image-fusion of late gadolinium enhanced (LGE)-cMRI with electromechanical mapping whereby the operator has information on infarct scar location by LGE-cMRI directly projected onto the electromechanical map. As a result, for the first time, the cells can be injected with a precision of ~2-3mm to the target ischemic area.

Finally, there is still controversy regarding the long-term effects of cell therapy. The 5-year follow up of the BOOST trial showed complete resolution of the previously observed improvement based on bone marrow-derived cells.[39] On the contrary, Mathiasen and coworkers reported on the observation that a single delivery of MSCs can provide sustained effects, even 3 years following injection.(36) Therefore, the RIPASSA trial will use repetitive MSC delivery in order to determine as to whether there is an incremental treatment effect based on the second MSC delivery and to what extent single or repetitive MSC treatment differ in long-term effect. , The concept of repetitive cell delivery has been reported safe and feasible using surgical injections in animals(40) and intracoronary infusion in patients.[41-43]

Study Rationale

The purpose of the RIPASSA trial is to advance cell based therapy for refractory angina by comparing for the first time repetitive or single MSC delivery to standard care and using a novel double imaging approach to accurately locate and deliver cells to the target area.

PROTOCOL & METHODS

Study Objectives

The primary objective of the RIPASSA study is to assess the safety and feasibility of autologous MSCs repetitively administered by intramyocardial injection in no-option patients with refractory angina, and the second objective is to demonstrate the efficacy.

Study Design

The RIPASSA study will be a national, multicentre, double blind, placebo controlled trial, held in the Netherlands.

Patient population and enrolment

One hundred fifty patients with RAP which are classified Canadian Cardiovascular Society (CCS) class 2 or higher despite maximal guideline-based clinical care (i.e. medication) that have no

options for revascularisation approaches such as 1) percutaneous coronary intervention or 2) coronary artery bypass surgery, will be selected for inclusion. All in-/exclusion criteria are shown in table 1. Patients are considered eligible if the left ventricular ejection fraction (LVEF) ≥35% and there are at least 2 segments of reversible/irreversible ischemia on SPECT or MRI.
Patients will be excluded if presenting with any of the following conditions: 1) liver and/or kidney dysfunction, 2) hematologic disorder, including coagulopathies, 3) history of non curatively treated malignancy within 5 years (See full exclusion list in Table 1).

Table 1. RIPASSA in-/exclusion criteria

Inclusion Criteria	
1.	Age >18 yrs and <90 yrs
2.	Must be able to provide written informed consent
3.	Maximal medical therapy, including:
	a) Beta-blockers unless contraindicated or causing unacceptable side effects.
	b) Calcium channel blockers or long acting nitrates when beta-blockers cannot be prescribed.
	c) Calcium channel blockers or long acting nitrates in combination with beta-blockers.
	d) Sublingual nitroglycerin or nitroglycerin spray.
4.	No candidate for revascularization via:
	a) Percutaneous coronary intervention
	b) Coronary artery bypass surgery
5.	Refractory Angina
	a) Canadian Cardiovascular Scale ≥ 2
6.	Left ventricular ejection fraction ≥ 35%, measured by CMR, echocardiogram, gated blood pool scan or SPECT scan within the prior 6 months in not in the setting of an ischemic event.
7.	Screening SPECT scan shows reversible and/or irreversible perfusion defects in at least 2 segments within the prior 6 months.
Exclusion Criteria	
1.	Have a noncardiac disease with a life expectancy <1yr
2.	Have a known serious allergy against radiographic contrast agents
3.	Have a baseline glomerular filtration rate <30mL/min per 1.73 m^2
4.	Currently participating –or have participated in the last 3 months- in an investigational new therapy trial of device trial
5.	Have a hematologic disorder as characterized by hematocrit <0.30, leucocytes <4.0; >10.0 x10^9/l or platelet value <150 x10**9**/l
6.	Have a coagulopathy (international normalized ratio > 1.2) not caused by medical therapy (e.g. vitamin K antagonists)
7.	Be serum positive for HIV, hepatitis B, hepatitis C or Treponema Pallidum.
8.	Be a woman who is pregnant, nursing, or has childbearing potential in absence of contraceptive methods. Female patients with childbearing potential must undergo a pregnancy test within 14 days before injection.
9.	Have a history non curatively treated malignancy within 5 yrs (except low grade and fully resolved non-melanoma skin malignancy)
10.	Have claustrophobia or other contra-indication for CMR scan

After screening, eligible patients that are included will be randomized in a 2:2:1 fashion to the following groups: Group A (60 patients) that will receive $100x10^6$ MSCs twice by intramyocardial injection with a 6 months interval in between; Group B (60 patients) that will receive $100x10^6$ MSCs by intramyocardial injection once and a placebo solution 6 months later, and: Group C (30 patients) that will receive a placebo solution twice with a 6 months interval in between. The RIPASSA study design is shown in Figure 1. MCSs and/or placebo will be injected via the NOGA injection catheter (Biosense Webster, Johnson & Johnson, USA) aided by the image-fusion software CARTCare providing detailed infarct location based on a pre-acquired LGE-cMRI.
It is estimated that the 150 patients will be enrolled in a 3-year period, and the study will be finished 1 year after the enrolment of the last patient. This trial will be listed at clinicaltrials.gov.

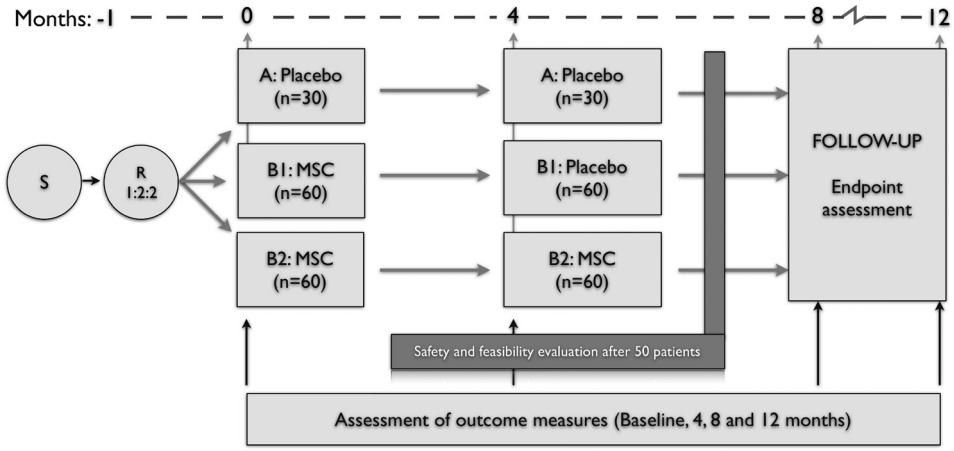


Figure 1. Schematic overview of the RIPASSA trial design.

Outcome measures for safety

The safety of the patients will be investigated both on short terms with specific focus on the serious adverse events related to the cell delivery procedure as well as long-term safety with regard to the effect of the MSCs itself on various safety measures.
Short-term safety of the heart catheterization and cell infusion procedure will be assessed during the two-day admission on the Coronary Care Unit following cell injection. During these two days, vital signs, heart rhythm, biochemical cardiac markers and spontaneous complaints will be recorded. The primary endpoint of safety consists of a 30-day incidence of a composite of serious adverse events, i.e. death, non-fatal MI, stroke, cardiac related hospitalization, coronary dissection, cardiac perforation/tamponade, sustained ventricular arrhythmias –defined as ventricular arrhythmias >30 seconds or giving rise to hemodynamic instability-, ventricular fibrillation or atrial fibrillation.
Next, patients will be closely monitored for long-term safety at the outpatient clinic at six and twelve months after the cell delivery. The main focus of the secondary safety outcome at twelve

months is on serious adverse events potentially caused by the cells itself: ventricular and/or atrial arrhythmias, neoplasia and MACE such as death, MI, stroke, hospitalization.

Outcome measures for efficacy

Efficacy end points will consist of the change in peak oxygen consumption, as assessed via cardiopulmonary exercise testing, CMR, 2-/3D echocardiography and SPECT scan, all evaluated at baseline, six (i.e. before second injection, and twelve months after the first MSC delivery. Based on these investigations, measures such as left ventricular function, end diastolic and systolic volumes, regional deformation and number and location of irreversible/reversible perfusion defects will be used to test treatment efficacy.

Since symptoms of angina are a pathognomonic sign of RAP, the RIPASSA trial will emphasize on subjective measures of association as well. Therefore, during the visits to the outpatient clinic, patients will be asked by their consulted physician about their frequency of angina, nitroglycerine usage and other complaints. In addition, the consulted physician will determine the CCS functional class. The patient will also be asked to fill in the Seattle Angina Questionnaire to determine the burden of coronary artery disease in 5 dimensions: 1) Physical limitations, 2) anginal stability, 3) anginal frequency, 4) treatment satisfaction and 5) disease perception.

Randomization and blinding

Randomisation will be accomplished by means of permuted block randomisation. Treatment allocation will be distributed by email to the cell therapy core facility in the UMCU. The time of randomization will be after the harvesting of the bone marrow from the patient. In the laboratory, the laboratory assistant will prepare the treatment allocation corresponding with the randomization number. If the patient is allocated to infusion with mesenchymal stem cells, the bone marrow will be isolated for MSCs, which will be expanded to 10×10^7 cells under GMP conditions. If the patient is allocated to placebo, the bone marrow will be stored for research and placebo solution will be prepared. The cultured MSCs or placebo solution will be aspirated and divided into a darkened 2.5 cc syringe and transported to the catheterization laboratory.

The randomization procedure takes place in the laboratory, therefore the patient's treatment allocation is not known to the interventional cardiologist, performing the infusion procedure nor by the patient himself.

Cell harvesting and processing

Approximately 80-100 ml of bone marrow will be aspirated in all patients from the posterior iliac crest under conscious sedation and local analgesia by a hematologist under aseptic conditions. The mononuclear cell fraction will be separated using density gradient centrifugation in the cell therapy facility of the UMCU as core center for cell handling. The mononuclear cells will be washed with PBS and the number of viable cells will be counted. In the patients allocated to treatment group A and B, MSCs will be generated under GMP conditions by seeding the BMNCs into 225 cm^2 tissue culture flasks with M-199 (Lonza, Belgium) supplemented with 10% fetal bovine serum and penicillin/streptomycin. After 21-28 days in culture, 20×10^7 MSCs will be harvested by trypsin treatment and, after resuspension in 5% dimethyl sulfoxide, frozen in liquid nitrogen.

Once the MSCs are requested for intramyocardial injection, MSCs will be thawed in a 37°C water bath, and centrifuged at low speed. The cell pellet will be resuspended in phosphate-buffered saline. Viability will be assessed and the MSCs will be delivered to the catheterization laboratory.

Dose rationale

10×10^7 MSCs, or roughly 0.13 million cells/kg, has been carefully set as the dose. The dosage is based on current available evidence, which varies widely (intracoronary admission of MSCs in patients ranging from 2×10^6 to 10×10^9).[44-46]

Given the absence of: 1) dose-response relation between 1.3 million cells/kg and 5 million cells/kg[47], and 2) the fact that no adverse events were witnessed with the admission of 10×10^9 cells[44], we estimate 10×10^7 to be the optimal dosage.

Injection Procedure

First, a three-dimensional model of the left ventricle will be constructed based on ~80-100 electromechanical mapping points taken by the NOGA catheter (Biosense Webster, Cordis, USA) (Figure 2). Infarct area and borderzone were identified by combining information on absence of local wall movement and a decreased unipolar conduction signal, defined as <6mV (Figure 3A). Next, infarct area localization was further substantiated by real-time image fusion of late gadolinium enhanced cMRI images and the NOGA EMM map (Figure 3B). In the case of mismatch between both imaging modalities, the localization of infarct scar by cMRI outweighed the EMM map. After careful selection of the target area, the MYOSTAR catheter (Biosense Webster, Cordis, USA) was used to navigate towards the infarct and 10 injections of 0.2 ml in a concentration of 5×10^6 cells/ml were placed in the demarcated area. The volume per injection was injected in ~5-10 seconds and ~5 seconds after injection.

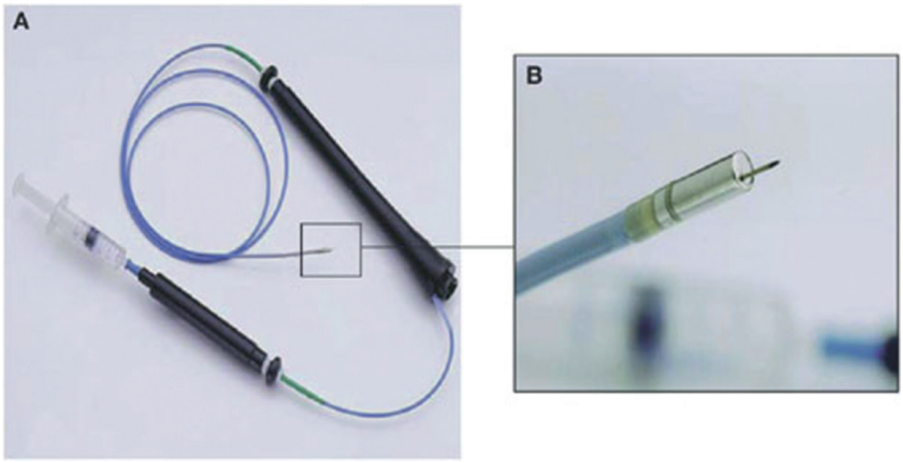


Figure 2. The NOGA catheter system.
(A) The NOGA catheter system will be used for targeted cell delivery by means of a 27-Gauge needle (**(B)** see inset) that can be deployed and retracted at the base of the catheter. Illustrations kindly provided by BDS Biosense Webster, a Johnson and Johnson company

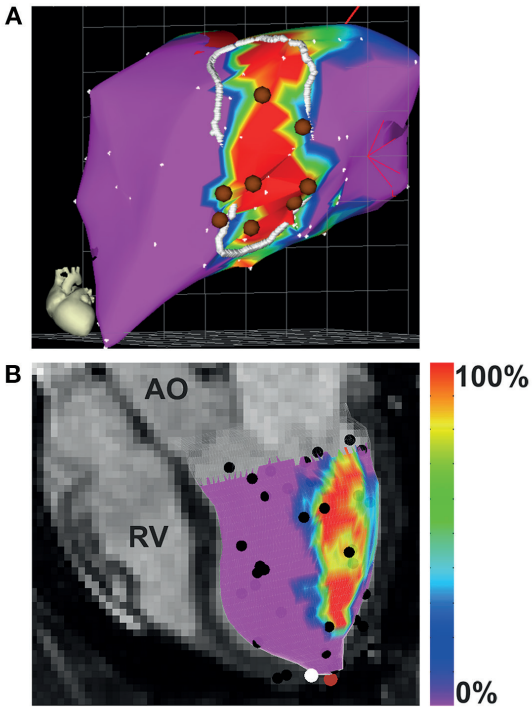


Figure 3. Double image-fusion based delivery
(A) a standard NOGA electromechanical (EMM) map is acquired based ~70-90 individual points registered by the NOGA mapping catheter equally spread throughout the left ventricle. **(B)** Next, a pre-acquired LGE-cMRI will be fused with the EMM map to provide real-time information on the location of the infarcted area.

Sample size calculation

The RIPASSA trial is powered at 85% (α .025) to demonstrate a minimum of 5% difference in peak oxygen consumption between subjects randomized to the autologous MSC-treated group (54 for each group) and control subjects (n=27) at 12 months, To account for a 10% drop out in each group, 60 patients per group will be included in the autologous MSC treated groups and 30 patients in the control group. The evaluation between single and repetitive MSC delivery is powered at 85% (α .025) to detect a minimal difference of an additional 6% in peak oxygen consumption (n=54 for each group).

Statistical analysis

Continuous variables will be presented as mean (±SD) or median (IQR). Categorical data will be presented by frequencies and percentages. For the analysis of normally distributed CPET, MRI, SPECT and echocardiographic variables, we will conduct an repeated measure ANOVA to assess treatment effect at both months 4, 8 and 12, adjusted for the baseline reference. In variables where normality is not presumed, Kruskal Wallis one-way analysis of variance will be performed to test treatment effect among the three study groups. All tests will be two-sided and assessed at the 5% significance level.

The RIPASSA trial is not adequately powered to accurately examine subgroups. However, explorative subgroup analysis will be performed to determine the occurrence relation between outcome and treatment effect in different subgroups divided by 1) severity of anginal complaints

(CCS class), 2) baseline cardiac function (LVEF <45%; ≥45%) and 3) baseline cardiopulmonary exercise capacity. These analyses should be regarded as hypothesis generating rather that providing firm evidence, given the presence of confounding of non pre-specified subgroup analysis in small randomized clinical trials.[48]

After the first 50 patients, treatment allocation will be unblinded and provided to the Safety Monitoring Board. Univariate analysis of adverse events rates (e.g. troponin serum levels as a marker of myocardial damage) will be performed to gain insights in safety of intramyocardial injection of autologous mesenchymal stem cells.

Safety and monitoring

We will establish an independent Safety Monitoring Committee, which will closely monitor and evaluate potential adverse events in the treated patients. If the committee beliefs the adverse events are related to the treatment, than it is in power to take all necessary steps in order to prevent the recurrence of such adverse events. Besides the evaluation of adverse events, a fixed unblinded analysis of the first 50 treated patients is planned which will specifically focuses on safety and feasibility.

DISCUSSION

To this date, cell therapy in IHD patients leads to a modest 3-4% improvement in LVEF compared to placebo. The investigated cells mainly concerned bone marrow mononuclear cells and the majority of studies used an intracoronary delivery route. In order to advance cell therapy, the ESC has commissioned a task force on stem cells and repair of the heart, that identified several unresolved issues in a recent statement paper: 1) What is the best cell type? 2) What is the optimal delivery route for cell based therapies? 3) What is the underlying mechanism of action? The RIPASSA trial presented here is designed to advance cell therapy by addressing two of those key unresolved issues: 1) the efficacy of mesenchymal stem cell of the bone marrow mononuclear fraction (~0.5-1.5%), and 2) the added value of novel image-fusion guided intramyocardial injection method.

Mesenchymal Stem Cells

Numerous cell types have been proposed to trigger a cardiac repair mechanism after they have been transplanted into the heart. However, it is not clear how these cell types differ in their ability to stimulate cardiac repair. We have recently shown that MSCs outperformed BMNCs in a head-to-head comparison, evidenced by a greater increase in cardiac systolic function in the post-MI pig heart. (unpublished data) This finding is in line with our previous meta-analysis in which a meta-regression between MSCs and BMNCs already hinted at a greater effect size of MSCs. One of the underlying mechanisms that can explain this difference is the protective secretome of MSCs and their unique immunomodulatory function. MSCs secrete various cytokines and growth factors such as VEGF, FGF-2, HGF, IGF-1 and TB4 -in particular under hypoxic conditions- that culminate in vasculogenesis, reduction in fibrosis and cardiomyogenesis. In addition, MSCs have been shown

to stimulate the recruitment of endogenous c-kitpos cardiac stem/progenitor cells (eCSCs), which in turn can differentiate into contractile myocytes, smooth and endothelial vascular cells. Taken together, the relative ease of harvesting and culturing MSCs in combination with their powerful paracrine actions makes a strong argument to explore this cell type to ensue cardiac repair.

Repetitive delivery

Little is known about the effects of repetitive delivery in cell therapy. The RIPASSA trials also investigates the added value of repeated delivery of cells in terms of absolute effect size (i.e. does the repeated delivery lead to bigger improvement?) as well as preservation of previously observed effects that otherwise is often blunted after 12 months of follow up (i.e. is the observed improvement sustained over a longer time period?). As expected, in negative studies on cell therapy, repeated delivery did not made a difference, that is, both the first admission of cells did not led to a substantial improvement in cardiac systolic function nor did the second one. Unfortunately, the assessment of additional value of repeated delivery is often coalesced with the cardioregenerative effects of the particular cells used in these studies. Therefore, inferences on effect of repetitive delivery can only be drawn in studies in which there is a measurable effect of the single dose.

For example, Yao and co-workers showed that a single delivery of BMNCs 3-7 days after an acute MI increased LVEF by 7% in patients at 12 months follow-up. When they repeated the first BMNCs infusion by a second one three months later they observed an almost doubled increase on LVEF of 12% at 12 months follow up compared to the single dose. In conclusion, these preliminary results from a small phase II study show that repeated delivery could indeed increase effect size of cell therapy and warrants further investigation.

Image-fusion based delivery method

Currently, intracoronary infusion of cells is often performed due to its relative ease, short procedure time and straightforward approach. However, this technique is heavily dependent on a patent coronary vasculature that leads to the designated ischemic area. Hence, advanced coronary artery disease such as exist in the patient population of the RIPASSA trial often precludes this delivery approach. Alternatively, direct transendocardial injection into the infarcted tissue has been proposed as a delivery method that bypass this problem. Accurate targeting is mandatory to maximize the therapeutic potential of the cell product whilst minimizing the changes of myocardial perforation. We have previously shown that this method leads to an accurate identification of the target site in the post-MI pig heart. As the next step, the RIPASSA trial will investigate if higher accuracy of delivering cells to the target area indeeds corresponds with greater therapeutic potential.

CONCLUSION

In conclusion, the RIPASSA trial will evaluate for the first time the safety and efficacy of repetitive intramyocardial injection of MSCs in RAP patients in improving anginal complaints and reducing persistent ischemia.

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CHAPTER 8

Placebo in Autologous Cell Therapy Trials: Hard Pill to Swallow?

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To the Editor

Cell-based strategies are under intense investigating in the pursuit to develop new effective treatment protocols for ischemic heart disease (IHD). These strategies have been mainly based on the use of tissue-specific autologous stem/progenitor cells such as cells from bone marrow, adipose tissue, or the heart itself.[1] Several of these cell types have reached the phase of clinical testing.[2,3] Clinical trials entail inherent scientific and ethical challenges, but especially cell-based clinical trials involve scientific and ethical issues. Due to the combination of the relative lack of experience with cell-based interventions, the complexity, the variability (especially when autologous cells are used), and the invasive character, traditional ethical issues get a new perspective. One of these issues is the choice for the comparator.[4]

Treatment effect is generally assessed in clinical trials by means of superiority of the novel intervention over standard clinical care or placebo. Placebo is used to conceal intervention allocation both for the patient and investigator-physician.[5] In pharmaceutical clinical trials, the placebo is often a capsule or tablet indistinguishable from the investigational new drug. However, in clinical trials assessing autologous cell interventions for heart disease, proper blinding can only be achieved when participants allocated to the placebo group undergo the identical harvesting procedure (e.g. bone marrow aspiration in the case of bone marrow mononuclear cells) as well as a sham delivery procedure to the heart undistinguishable from the cell delivery procedure. If the interventional cardiologist is not involved in the follow-up and/or outcome analysis, the sham procedure might not necessitate an actual injection with a placebo solution identical to the cell suspension. Either way, in contrast to placebo tablets, autologous cell interventions expose the control group to risk and harm, raising ethical concerns.

To date, little is known about the extent to which sham interventions in cell-based trials are performed. Therefore, we conducted a systematic review on published reports of randomized clinical trials (RCTs) investigating efficacy of autologous cell interventions in patients with IHD.

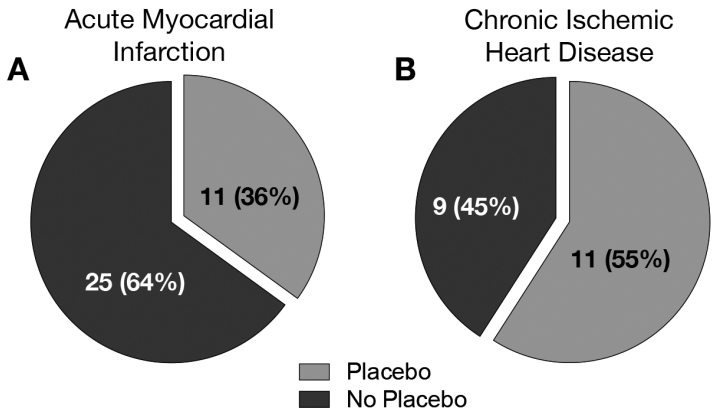

Figure 1. Use of Placebo in Autologous Cell Therapy Trials.

Number of studies that used a double-blinded study design based on placebo for **(A)** acute myocardial infarction and **(B)** chronic ischemic heart disease (i.e. refractory angina and/or post-MI congestive heart failure).

We assessed how the RCTs were designed regarding cell harvesting and/or cardiac sham delivery, whether this depended on the type and stage of cardiac disease, and the adverse events rate in sham procedure patients. A search syntax was developed based on relevant synonyms for domain, which is patients with IHD, and determinant, which is cell intervention delivered to the heart (i.e. intracoronary delivery, intramyocardial injection or epicardial delivery). The outcome – efficacy of cell intervention- was deliberately withheld from our search syntax to avoid potential reporting and retrieval bias. A systematic literature search was conducted in MEDLINE and EMBASE on the 13th of May 2013. Two reviewers (SK, JW) independently screened title and abstract of studies in accordance of in-/exclusion criteria (see SI). The selected articles were crosschecked to identify relevant studies missed by the initial search using ISI Web of Science®.

A total of 56 RCTs were identified that were published between 2001 and 2013. In total 3610 patients were included in these studies, of which 2189 (61%) received autologous cells, compared to 1421 (39%) controls. In 75% of studies, bone marrow mononuclear cells were the investigational cell type. Diagnosis was acute MI in 2463 patients (982 controls (40%)), compared to 1147 patients (439 controls (38%)) with chronic IHD (i.e. refractory angina or post-MI heart failure).

Combined cell harvesting and sham cardiac delivery, thereby ensuring patient and investigator blinding, was performed in 22 of the 56 studies (39%). Analysis divided for IHD type revealed that 11 of 36 studies (31%) used sham delivery in acute MI and 11 out of 20 studies (55%) in chronic IHD (Figure 1 A to B; chi-square P=0.09). Apparently, use of placebo is generally not preferred in the acute setting of MI in contrast with an elective cell intervention procedure for chronic IHD. Furthermore, in one study, participants allocated to the control group underwent cell harvesting but no sham cardiac injections, while the rest of the studies used usual care as a comparator.

Out of 22 studies that used placebo, adverse events in controls were reported in five studies (23%), all of which investigating bone marrow cells. A combined endpoint analysis of major cardiovascular events revealed that intracoronary infusion of a placebo solution did not lead to heightened mortality or serious morbidity in these five studies. With regard to the cell harvesting procedure no adverse events were reported.

Three major conclusions emanate from our study: 1) 39% of RCTs investigating efficacy of autologous cell intervention used a double-blind trial design based on a sham procedure; 2) trials using sham delivery were less frequently observed in AMI compared to CHD; and 3) 23% of these trials reported data on (minor) adverse events. This report shows that the choice for the control group for cell-based interventions in cardiology differs among research groups, which will be related to various views on scientific necessity and ethical acceptability of sham. This empirical report supports the need to clarify when and under what conditions sham is scientific necessary and ethically acceptable, and when another comparator is more appropriate in clinical trials investigating cell-based interventions for cardiology and other medical fields. This will, among others, probably correlate with the study population, and the risk profile of the sham procedure. Hence, this report can function as a starting point to formulate guidelines for researchers that aim to set up a cell-based clinical trial.

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SUPPLEMENTARY INFORMATION

SEARCH SYNTAX MEDLINE

#1: heart[Title/Abstract] OR cardia*[Title/Abstract] OR myocardia*[Title/Abstract] OR coronary*[Title/Abstract] OR cardiomyopath*[Title/Abstract]

#2: failure[Title/Abstract] OR decompensation[Title/Abstract] OR infarction[Title/Abstract] OR ischemi*[Title/Abstract] OR ischaemi*[Title/Abstract] OR disease[Title/Abstract] OR dysfunction[Title/Abstract] OR disfunction[Title/Abstract] OR angina[Title/Abstract]

#3: stem*[Title/Abstract] OR progenitor*[Title/Abstract] OR (bone[Title/Abstract] AND marrow*[Title/Abstract]) OR precursor*[Title/Abstract]

#4: cell*[Title/Abstract]

#5: myoblast*[Title/Abstract]

#6: transcoronar*[Title/Abstract] OR intracoronar*[Title/Abstract] OR transendocardial*[Title/Abstract] OR intramyocardial*[Title/Abstract] OR intravenous*[Title/Abstract] OR transvenous[Title/Abstract]

Search: #1 AND #2 AND ((#3 AND #4) OR (#5)) AND #6

SEARCH SYNTAX EMBASE

#1: heart:ti,ab OR cardia*:ti,ab OR myocardia*:ti,ab OR coronary*:ti,ab OR cardiomyopath*:ti,ab

#2: failure:ti,ab OR decompensation:ti,ab OR infarction:ti,ab OR ischemi*:ti,ab OR ischaemi*:ti,ab OR disease:ti,ab OR dysfunction:ti,ab OR angina:ti,ab

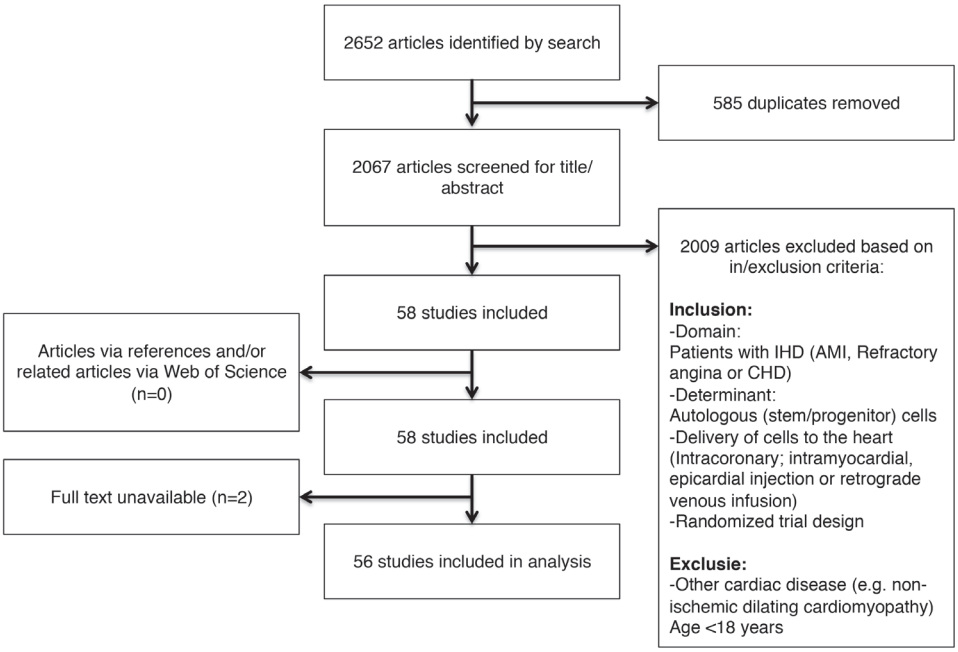
#3: stem*:ti,ab OR progenitor*:ti,ab OR (bone:ti,ab AND marrow*:ti,ab) OR cardia*:ti,ab OR precursor*:ti,ab

#4: cell*:ti,ab

#5: myoblast*:ti,ab

#6: transcoronar*:ti,ab OR intracoronar*:ti,ab OR transendocardial*:ti,ab OR intramyocardial*:ti,ab OR intravenous*:ti,ab OR transvenous:ti,ab

Search: #1 AND #2 AND ((#3 AND #4) OR (#5)) AND #6



Supplementary Figure 1. Flowchart

Flowchart of the systematic search conducted on 13th of May, 2013 on Medline and Embase. IHD denotes ischemic heart disease, AMI; acute myocardial infarction, CHD; chronic ischemic heart disease.

Table 1. Characteristics of studies included in the analysis

Source	Year	Sample size	Type of IHD	Cell type	Route of Delivery	Cell harvesting in controls	Sham delivery procedure
Ang et al[1]	2008	63	CHD	BMNC	IC	BM	No
Assmus et al[2]	2006	92	CHD	BMNC	IC	None	No
Assmus et al[3]	2013	103	CHD	BMNC	IC	BM	Yes
Bolli et al[4]	2011	23	CHD	CSC	IC	None	No
Cao et al[5]	2009	86	AMI	BMNC	IC	BM	Yes
Chen et al[6]	2004	69	AMI	MSC	IC	BM	Yes
Colombo et al[7]	2011	10	AMI	CD133+ cells	IC	None	No
Dohmann et al[8]	2005	21	CHD	BMCN	IM	None	No
Erbs et al[9]	2005	26	CHD	BCPC	IC	Venous blood	Yes
Ge et al[10]	2006	20	AMI	BMNC	IC	BM	Yes
Grajek et al[11]	2006	45	AMI	BMNC	IC	None	No
He et al[12]	2010	41	CHD	BMNC	IC	None	No
Hendrikx et al[13]	2006	20	CHD	BMNC	EI	BM	Yes
Herbots et al[14]	2009	67	AMI	BMNC	IC	BM	Yes
Hirsch et al[15]	2010	200	AMI	BMNC/BCPC	IC	None	No
Huikuri et al[16]	2008	80	AMI	BMNC	IC	BM	Yes
Janssens et al[17]	2006	67	AMI	BMNC	IC	BM	Yes
Kang et al[18]	2004	27	AMI	BCPC	IC	None	No
Karpov et al[19]	2005	44	AMI	BMNC	IC	None	No
Lipiec et al[20]	2009	39	AMI	BMNC	IC	None	No
Lunde et al[21]	2005	49	AMI	BMNC	IC	None	No
Lunde et al[22]	2006	100	AMI	BMNC	IC	None	No
Makkar et al[23]	2012	25	AMI	CSC	IC	None	No
Malagoli et al[24]	2010	41	AMI	BMNC	IC	None	No
Meluzin et al[25]	2006	66	AMI	BMNC	IC	None	No
Menasche et al[26]	2008	97	CHD	SM	EI	Surgical biopsy	Yes
Miettinen et al[27]	2010	78	AMI	BMNC	IC	BM	Yes
Nasseri et al[28]	2013	77	AMI	CD133+ cells	IM	None	No
Noguiera et al[29]	2009	30	AMI	BMNC	IC	None	No
Penicka et al[30]	2007	27	AMI	BMNC	IC	None	No
Perin et al(31)	2011	30	CHD	BMNC	IM	BM	Yes

Table 1. continued

Perin et al(32)	2012	20	CHD	BMNC	IM	BM	Yes
Perin et al(33)	2012	92	CHD	BMNC	IM	BM	Yes
Perin et al(34)	2003	21	CHD	BMNC	IM	None	No
Piepoli et al[35]	2010	38	AMI	BMNC	IC	None	No
Plewka et al[36]	2009	60	AMI	BMNC	IC	None	No
Pokushalov et al[37]	2009	99	CHD	BMNC	IM	None	No
Pokushalov et al[38]	2010	109	CHD	BMNC	IM	None	No
Povsic et al[39]	2011	20	CHD	SM	IM	Surgical biopsy	Yes
Quyyumi et al[40]	2009	31	AMI	CD34+ cells	IC	None	No
van Ramshorst et al[41]	2009	50	CHD	BMNC	IM	BM	Yes
Sanchez Fernandez et al[42]	2012	120	AMI	BMNC	IC	None	No
Schächinger et al[43]	2006	204	AMI	BMNC	IC	BM	Yes
Shihong et al[44]	2012	112	CHD	CD34+ cells	IC	BM	Yes
Silva et al[45]	2009	30	AMI	BMNC	IC	None	No
Tendera et al[46]	2009	200	AMI	BMNC	IC	None	No
Terrovitis et al[47]	2011	22	AMI	CD34+cells	IC	None	No
Traverse et al[48]	2011	87	AMI	BMNC	IC	BM	Yes
Traverse et al[49]	2012	120	AMI	BMNC	IC	BM	Yes
Trzos et al[50]	2009	62	AMI	BMNC	IC	None	No
Tse et al[51]	2007	28	CHD	BMNC	IM	BM	Yes
Wohrle et al[52]	2010	42	AMI	BMNC	IC	BM	Yes
Wollert et al[53]	2004	60	AMI	BMNC	IC	None	No
Xu et al[54]	2009	80	CHD	BMNC	IC	None	No
Zhan-quan et al[55]	2007	70	AMI	BCDC	IC	None	No
Zhang et al[56]	2007	70	AMI	CD34+ cells	IC	None	No

CHD denotes chronic ischemic heart disease; AMI denotes acute myocardial infarction; BMNC denotes bone marrow mononuclear cell; BCPC denotes blood-derived circulating progenitor cell; SM denotes skeletal myoblast; BM denotes bone marrow aspiration

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CHAPTER 9

Myocardial infarction and Functional Outcome Assessment in Pigs

In print

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ABSTRACT

Introduction of newly discovered cardiovascular therapeutics into first-in-man trials depends on a strictly regulated ethical and legal roadmap. One important prerequisite is a good understanding of all safety and efficacy aspects obtained in a large animal model that validly reflect the human scenario of myocardial infarction (MI). Pigs are widely used in this regard since their cardiac size, hemodynamics and coronary anatomy are close to that of humans. Here, we present an effective protocol for using the porcine MI model using a closed-chest coronary balloon occlusion of the left anterior descending artery (LAD), followed by reperfusion. This approach is based on 90 minutes of myocardial ischemia, inducing large left ventricle infarction of the anterior, septal and inferoseptal walls. Furthermore, we present protocols for various measures of outcome that provide a wide range of information on the heart, such as cardiac systolic and diastolic function, hemodynamics, coronary flow velocity, microvascular resistance and infarct size. This protocol can be easily tailored to meet study specific requirements for the validation of novel cardioregenerative biologics at different stages (i.e. directly after the acute ischemic insult, in the sub-acute setting or even in the chronic MI once scar formation has been completed). This model therefore provides a useful translational tool to study MI, subsequent adverse remodeling and the potential of novel cardioregenerative agents.

INTRODUCTION

Acute myocardial infarction (AMI) and its long-term sequelae such as chronic heart failure (CHF) profoundly impact patient prognosis and quality of life, let alone the high cost restraints imposed on our available healthcare resources.[1] The prevalence of CHF in the western world is estimated at 1-2%, of which ~60% of cases are the consequence of AMI as primary cause.[2] In the USA alone, about 5.7 million patients suffer from CHF accounting for approximately $30 billion in annual healthcare costs in 2008, with a predicted triplicate in costs rising to $97 billion annually in 20301. Taken together, these numbers make a strong argument for the development of new cardioregenerative treatments that, for swift translation, rely on a reproducible and reliable large animal myocardial infarction model that accurately mimics the human scenario.

Pigs (Sus scrofa) are increasingly being used in cardiovascular research for pharmacological and toxicological testing.[3] One of the traits responsible for this success as a translational research tool is their similarity in cardiac function and anatomy with the human heart.[4,5] For instance, pig heart-to-body weight ratio, cardiac size and coronary artery anatomy distribution have all shown to be remarkably similar to man.[4] Moreover, cardiomyocyte metabolism, electrophysiological properties and response to an ischemic insult such as AMI have been reported to show high levels of agreement with the human situation.[6,7] Ultimately, to fulfill the above described criteria, a standardized MI-protocol that produces robust and sustainable MI for testing of investigational new drugs (IND) is needed. Here, we present such a standardized model that uses a 90-minutes closed-chest coronary balloon occlusion of the left anterior descending artery (LAD) followed by reperfusion, thereby creating reproducible myocardial infarction covering the anteroapical, septal and inferoseptal walls of the left ventricle.

PROTOCOL

All *in vivo* experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources. Experiments were approved by the local Animal Experimentation Committee.

Part 1. Medication, anesthesia, venous access and intubation

1. Medication and Anesthesia

1.1. Pre-medication

1.1.1. Start amiodarone 150 mg/kg 10 days prior to surgery to prevent arrhythmias. Continue amiodarone in a dose of 100 mg/kg from the day of the procedure until day 28. Reduce the dosage to 50 mg/kg on day 29 and continue until the end of the study.

1.1.2. Start anti-platelet therapy, 75 mg clopidogrel from day 3 prior to surgery and 320 mg acetylsalicylic acid 1 day before surgery. Continue clopidogrel 75 mg per day and acetylsalicylic acid 80 mg per day.

1.1.3. Start pain medication 1 day prior to surgery by Fentanyl patch, 25 microgram/hour.

1.1.4. Fast the animal for 24 hrs, maintain unlimited access to water.

1.2. Anesthesia

1.2.1. To prevent unnecessary stress and discomfort, sedate the pig in its stable by intramuscular injection of a mixture of midazolam 0.4 mg/kg, ketamine10 mg/kg and atropine 1mg.

1.2.2. Obtain venous access by canulating the ear vein with an 18G i.v. cannula . Induce anesthesia by intravenous administration of 5 mg/kg sodiumthiopental, and give 1000/100mg amoxicillin/clavulanic acid to prevent infections. The pig will receive 1000/100mg amoxicillin/clavulanic acid the day after surgery as antibiotic treatment.

1.2.3. Intubate the pig by using an endotracheal tube (size 8.5 for pigs weighing ~70 kg). If necessary perform balloon-ventilation with a frequency of 12/minute and transport the pig to the operating theater.

1.2.4. At arrival on the operation theater, immediately start mechanical positive pressure ventilation with FiO2 0.50, 10ml/kg tidal volume and a frequency of 12/min under continues capnography.

1.2.5. Start balanced anesthesia by continues intravenous infusion of a combination of midazolam 0,5 mg/kg/h, sufentanil 2,5 microg/kg/h and pancuronium bromide 0.1 mg/kg/h.

1.2.6. Infuse 300 mg of amiodarone in 500 ml venofundin 6% intravenously.

1.2.7. Monitor heart rhythm by a 5-leads ECG.

1.2.8. Shave and clean the neck and hind limb area.

1.2.9. Insert a Foley catheter.

Part 2. Transthoracic echocardiography

1.1. Place the animal in the right lateral position. In landrace pigs such as Dalland pigs, only parasternal views (long and short axis) can be obtained. Due to the shape of the thorax, apical view acquisition is not feasible.

1.2. Orient and obtain parasternal long axis view in 2D (B-mode). Determine the LV dimensions at end diastole and end systole in M-mode.

1.3. Rotate the echo probe 90° clockwise whilst maintaining its parasternal position to acquire the LV short axis views at the levels of the mitral valve, papillary muscle and apex. The short axis view of the papillary muscle and apex may require placement of the echo probe one or two intercostal spaces lower relative to the position for the mitral valve short axis view.

Part 3. Surgical preparation and vascular access

1.1. Disinfect the surgical areas with iodine 2% and use sterile surgical drapes to cover the non-sterile parts of the pig.

1.2. Make a medial incision in the neck. Pass the linea alba to minimize muscle damage and bluntly approach the carotid artery and internal jugular vein next to the trachea.

1.3. Carefully isolate the carotid artery and internal jugular vein. Make sure the vagal nerve is undamaged. Place vicryl 2-0 sutures around both vessels to gain vessel control. Achieve arterial access by canulating the internal carotid artery with an 8F sheath using the Seldinger technique. Fix the sheath to the artery, make sure the artery is not fully occluded by the suture. Venous access can be acquired by canulating the jugular vein

with a 9F sheath also using the Seldinger technique. Before securing the sheath make sure the vein is ligated.

1.4. Administer 100 IE/kg heparin immediately after inserting the sheaths to inhibit thrombus formation.

1.5. For a stable and constant arterial pressure measurement, canulate one of the smaller arteries in one of the hind limbs by making a small incision above the artery. The artery is found just under the skin, pulsations can be felt through the skin. Isolate the artery from its surrounding tissue. Place 2 vicryl 2-0 sutures around the vessel, 1 proximal and 1 distal. Ligate the distal side and insert an 18G i.v. cannula and secure tightly connect the pressure.

Part 4. Invasive Pressure Volume loop analysis

1.1. Insert a Swann-Ganz catheter (SG) via the previously placed sheath in the internal jugular vein.

1.2. Connect a cardiac output device to the part of the SG that culminates in the proximal lumen.

1.3. Inject 5 mL of 0.9% saline into the proximal lumen of the SG and measure cardiac output; repeat this three times and average the indices.

1.4. Calibrate the PV system by using the previously determined cardiac output.

1.5. Insert the 7F conductance catheter via the carotid artery into the left ventricle under fluoroscopic guidance.

1.6. Select the largest segment present in the LV for volume measurements and perform a baseline scan under apnea.

1.7. After volume calibration is completed, record 10-15 beats under apnea.

1.8. Remove the SG and place a balloon catheter in the inferior caval vein at the level of the diaphragm under fluoroscopic guidance.

1.9. Perform preload reduction by inflating the balloon under apnea and record the corresponding PV-Loops.

Part 5. Intracoronary pressure and flow measurement

1.1. Dilute nitroglycerin in a concentration of 100 microgram/ml and dilute adenosine in a concentration of 30 microgram/ml.

1.2. Position an 8F-guiding catheter in the ostium of the left coronary artery.

1.3. Place the combined pressure/flow wire in the proximal part of the left coronary artery.

1.4. Administer 200 micrograms of nitroglycerin intracoronary and normalize the distal pressure (Pd), measured by the wire, with the arterial pressure.

1.5. Place the wire in the mid part of the left anterior descending artery (LAD).

1.6. Start measuring baseline pressure and flow. Induce hyperemia by administering 60 micrograms of adenosine intracoronary, flush with 2 mL of saline and measure hyperemic pressure and flow. Wait for the flow to restore to baseline values. Repeat the measurement twice.

1.7. Infuse another 200 mcg nitroglycerin intracoronary and repeat step 1.6 and 1.7 for the left circumflex coronary artery.

Part 6. Induction of MI

1.1. Place the intracardiac defibrillation catheter in the right ventricle using the venous sheath. The distal electrodes should be in the apex of the ventricle, the proximal electrodes in the atrium and/or superior caval vein. Connect the catheter to the defibrillator and set it to 50J.

1.2. Measure the diameter of the LAD distal from the second diagonal (D2) in AP and LAO 30° view.

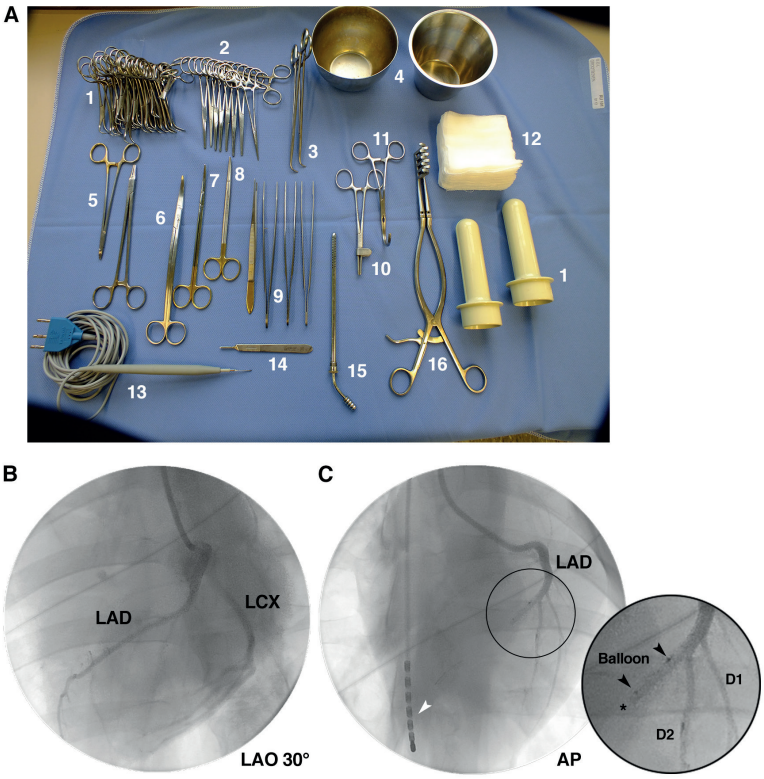

Figure 1. MI model based on LAD balloon occlusion.

(A) Standard surgical equipment with: 1) towel clamps; 2) mosquitos; 3) dissecting forceps; 4) round container; 5) needle holders (fine and rough); 6) Klinkenberg scissor; 7) dissecting scissors (straight and curved); 9) forceps (De-Bakey, fine and rough); 10) hose clamp; 11) anastomosis clamp; 12) gauzes; 13) electrosurgical pencil; 14) scalpel holder; 15) Dreesman (suction); 16) retractor; 17) lamp holders. **(B)** Left anterior oblique fluoroscopic view of the LAD and the LCX. **(C)** After visualizing the second diagonal branch, position the two radiopaque markers (see inset, black arrowheads) of the balloon just distally of the D2. Inflate and ensure that coronary blood flow is successfully blocked by contrast injection (see asterisk). Intracardiac defibrillator lead can be seen in the right ventricle (see white arrowhead). LAD denotes Left anterior descending artery; LCX denotes left circumflex artery; LAO denotes left anterior oblique view; AP denotes anterior posterior view; D1 denotes first diagonal branch; D2 denotes second diagonal branch.

1.3. Choose an angioplasty balloon with a diameter according the diameter of the LAD distal from the D2 (Figure 1).
1.4. Position a guidewire through the guiding catheter distally in the LAD.
1.5. Advance the balloon catheter over the guidewire. Place the balloon distal from the D2.
1.6. Administer 30 IE/kg heparin.
1.7. Inflate the balloon until the pressure matches the right diameter of the LAD.
1.8. Check total occlusion of the LAD by angiography (Figure 1).
1.9. Cover the sterile working field and the wound in the neck with sterile drapes cloths. Free the chest from any coverage to make it available for chest compressions or transthoracic defibrillation.
1.10. Check the pressure in the balloon during the next 90 minutes and restore the pressure if necessary.
1.11. In case of ventricular fibrillation:
1.11.1. Immediately start chest compressions with a frequency of 100/minute.
1.11.2. Administer 300 milligram amiodarone intravenously as a fast bolus (~1 minute).
1.11.3. Start intracardiac defibrillation, give shocks of 50J.
1.11.4. After 5 unsuccessful shocks, restart chest compressions. Change intracardiac defibrillation to transthoracic defibrillation and shock with 150J. In case of unsuccessful shock, change to 200J.
1.11.5. If necessary, administer another dose of 150 milligrams amiodaron and/or 1 milligram adrenalin. Repeat adrenalin twice with intervals of 3-5 minutes, when necessary.
1.11.6. Continue chest compressions, interspersed with transthoracic defibrillation

Finishing the surgical procedure (for long term follow up)

1.1. After 90 minutes check by angiography if the LAD is still fully occluded.
1.2. Administer another 30 IE/kg heparin and deflate the balloon. Check for reperfusion. Remove the deflated balloon with the guiding catheter from the carotid sheath.
1.3. Carefully remove the arterial sheath and clamp the carotid artery immediately with an anastomosis clamp (Figure 1). Use continues stitches (6-0 prolene) to close the carotid artery. Remove the clamp and check for leakages.
1.4. Remove the internal defibrillation catheter and remove the sheath from the internal jugular vein. Ligate proximal of the sheath entry.
1.5. Close the subcutis and skin of the neck in two layers using 2-0 vicryl.

Part 7. Cardiac Magnetic Resonance Imaging

1.1. Place the animal on the MRI table head first in the supine position under continuous anesthesia.
1.2. Place a dedicated phased-array cardiac coil over the chest of the animal.
1.3. For image planning obtain scout images in short axis and two-chamber long axis views.
1.4. Acquire ECG-gated steady-state free precession (SSFP) cine of short axis (from apex to base of LV) and two chamber long-axis views.

1.5. Late gadolinium enhancement (LGE) can be acquired using an inversion recovery 3D-turbo-gradient-echo-technique 15 minutes after double-dose i.v. bolus injection of a gadolinium based contrast agent.
1.6. Perform offline analysis with validated software of functional parameters. Assess left-ventricular ejection fraction (LVEF), LV mass, end diastolic volume, end systolic volume, stroke volume, cardiac output and scar mass.

Part 8. End of study and Infarct size

1.1. At the end of the study, follow part 1-5 and 7 to acquire follow up measurements.
1.2. Make a median 30-40 cm incision from just below the suprasternal notch to a point just below the xiphoid process. Advance through the linea alba down to the sternum. Split the xiphoid and use Klinkenberg scissors to separate the posterior sternum from the pericardium with caution. After using the scissors bluntly continue further separatation. Perform a sternotomy by e.g. using a hammer and Lebsch knife. Bone marrow bleeding is minimized by rubbing bone wax on the marrow. Open the thorax with a sternum retractor.
1.3. Enter the 3rd pleural space and locate the inferior caval vein in the mediastinum.
1.4. Humanely euthanize the animal by cutting the inferior caval vein under deep anesthesia. Remove blood with a suction device. Place a 9V battery on the apex to induce ventricular fibrillation.
1.5. After excision of the heart, cut the right and left ventricle into five slices from base to apex and incubated in 1% triphenyl-tetrazolium chloride dissolved in 0.9% saline at 37°C for 15 minutes. Next, wash the slices in 0.9% saline and photograph the slices from both sides.

REPRESENTATIVE RESULTS

Mortality and Infarct size

In our center, out of 32 pigs (Female Dalland Landrace, 6 months old, ~70 kg) that were subjected to this MI protocol, five (15.6%) died due to refractory ventricular fibrillation during ischemia. This protocol creates an infarct covering approximately 10-15% of the left ventricle, located in the anteroseptal, septal and inferoseptal walls (Figure 2A). If serial non-invasive assessment of infarct size is warranted, late gadolinium enhancement (LGE) on CMR can be used to follow the non-viable infarct area over time (Figure 2B).

Cardiac Function and remodeling

Four weeks after MI, global and regional parameters reflecting cardiac function should be decreased compared to healthy baseline values. Specifically, LV ejection fraction (LVEF) should decrease to approximately ~35-45% four weeks post-MI. Besides global systolic function, several parameters reflecting post-MI adverse remodeling can also be measured, such as LV morphology and diameters using CMR and echocardiography (Figure 3A,B). Four weeks after MI, an increase in end diastolic volume (EDV) as a sign of adverse remodeling can be expected (Figure 3A,B).

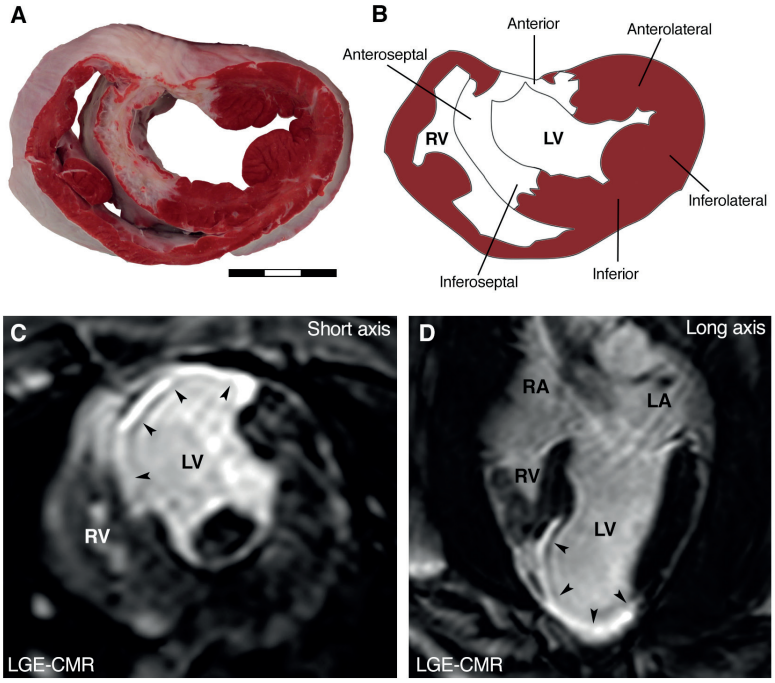

Figure 2. Infarct size after MI.
(A) The 90-minute balloon occlusion of the LAD leads to extensive myocardial damage and scar formation (white color), visualized by TTC staining at 1 month follow up. **(B)** Schematic infarct distribution shows that the infarction is located in the anterior, anteroseptal and inferoseptal segments of the heart. **(C,D)** Short and long axis late gadolinium enhanced CMR images show the extensive infarct scar (white signal, see black arrowheads) localized in the anterior, anteroseptal and inferoseptal segments of the heart. LGE-CMR denotes late gadolinium enhanced cardiac magnetic resonance. Scale bar denotes 3 centimeters.

Coronary flow and pressure parameters

Angiogenesis and formation of new capillaries are often regarded as important treatment goals in ischemic heart disease.[8] Assessment of microvascular resistance can be indirectly based on the combined measurement of intracoronary pressure and flow velocity. Representative pressure and flow velocity measurement under normal conditions and maximal hyperemia is shown in Figure 4. Four weeks after MI, the hyperemic microvascular resistance should be increased in the infarct related coronary artery (LAD) compared to the baseline situation.[8]

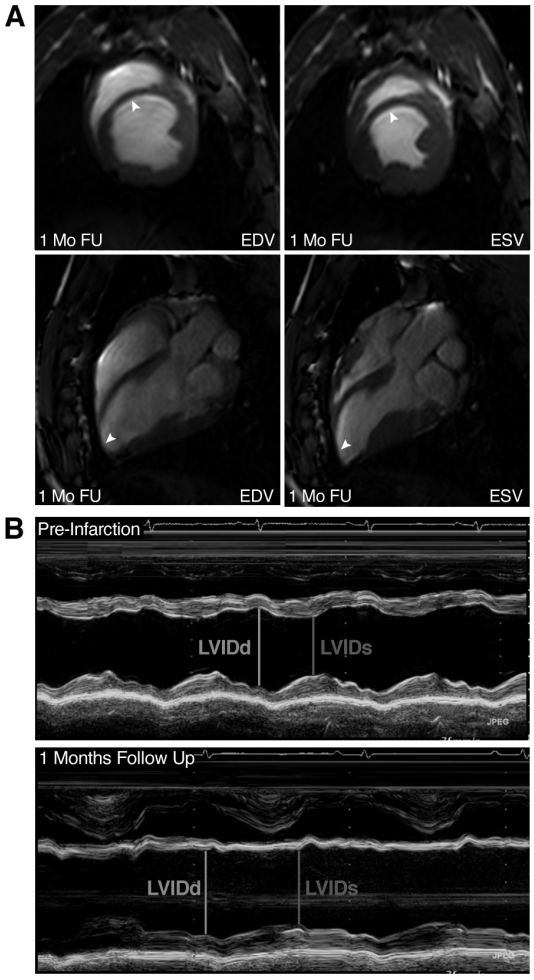

Figure 3. Assessment of cardiac function in ischemic MI models.
(A) Representative CMR cine-loop images at end diastole and end systole showing functional impairment of the infarct scar segments. **(B)** M-Mode image of 2D parasternal long axis by echocardiography, showing LV dilatation (increase in LVIDd) 1 month after MI, as well as functional impairment (absence of septal thickening). EDV denotes end diastolic volume; ESV denotes end systolic volume; LVIDd denotes left ventricular internal diameter at diastole and LVIDs denotes left ventricular internal diameter at systole.

DISCUSSION

Intracoronary balloon occlusion of the LAD provides a reproducible and consistent preclinical MI model in pigs that can be used to investigate safety and the efficacy of new cardiovascular therapies that closely mimics the human situation. As shown in Figure 5, the presented ischemia/reperfusion infarction model provides the platform that can be further tailored to investigate different phases of MI and post-MI remodeling whilst the initial ischemia/reperfusion injury is identical for both.

The success of the described protocol outlined here is dependent on the myocardial ischemia as the most critical phase of the protocol. Correct placement of the balloon distal to the second diagonal branch of the LAD is crucial for reaching adequate infarct size whilst ensuring a high

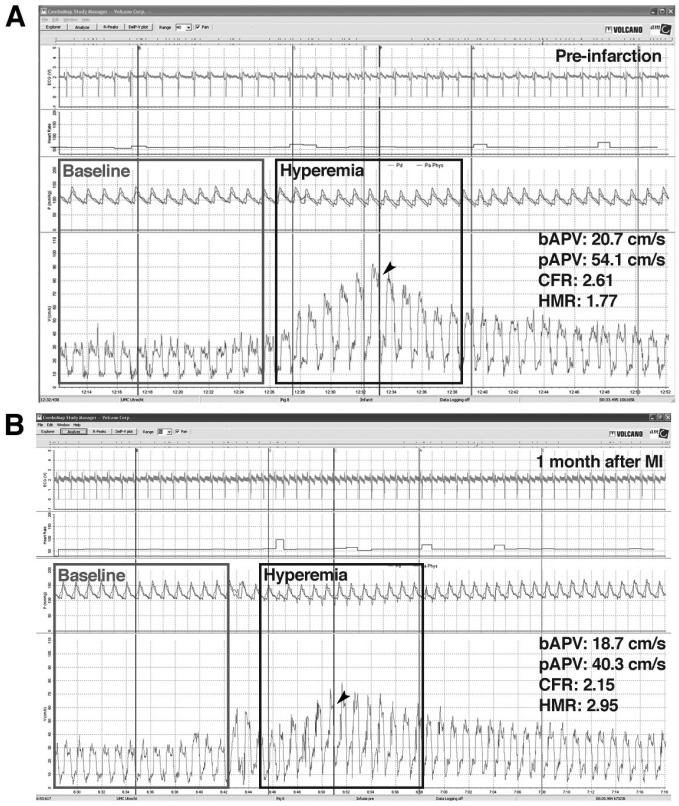

Figure 4. Intracoronary pressure and flow velocity derived parameters.
Intracoronary pressure and flow velocity recordings using the Combowire (Volcano Corp., USA), showing **(A)** reference values prior to MI with high response to hyperemia (black arrowhead). **(B)** 1 month after MI, the infarct related artery (LAD) has a decreased hyperemic response in coronary flow velocity (black arrowheads). As a result, pressure and flow velocity derived parameters (HMR) or flow velocity reserve (CFR) are decreased compared to the baseline. bAPV denotes basal average peak velocity; pAPV denotes peak average peak velocity; CFR denotes coronary flow reserve; HMR denotes hyperemic microvascular resistance.

survival rate. Based on this MI model, a ~15 percent mortality rate was observed, while extensive mid and apical segments of the anterior, septal and inferior walls were infarcted as seen on CMR and TTC staining (Figure 2A,B). The duration of ischemia can be tailored according to the desired infarct size. Although we have used landrace pigs in this protocol, minipigs (i.e. Göttingen minipigs) usually require longer durations of myocardial ischemia (e.g. 150 min occlusion).

Outcome analysis in preclinical and clinical MI studies is often based on LVEF. Although lower LVEF has been firmly associated with increased risk for cardiovascular mortality, it remains dependent on hemodynamical parameters such as preload.[9] Arguably, given that on average only 10-15% of the LV is infarcted, several conceptual and practical limitations are related to LVEF being a global measure of LV systolic function rather than reflecting local improvement.[10] Therefore, the proposed measures of outcome used in this model shed light on different aspects

of MI and post-MI remodeling thereby providing the investigators the means to accurately assess the efficacy of new therapies on multiple levels.

To optimize translation from pre-clinical models to clinical practice, we choose using large pigs instead of minipigs. Hemodynamic measurements, medication dosages and surgical devices can easily be exchanged with clinical practice. Compared to minipigs, large pigs gain relatively much weight. This may cause a problem in long-term follow up, with regard to comparability of serial results. Female Dalland Landrace pigs weigh approximately 70 kg at an age of 6 months. To prevent abundant weight gain during the follow up period, animals are kept on a restricted diet. Pigs receive 750 gram of custom made low calorie food (containing: proteins 15.6%, fat 2.0%, fibers 14.8%, ashes 8.8%, calcium 0.9%, phosphor 0.57%, magnesium 0.29% and potassium 0.18%) twice a day and gain about 10 kg of weight in 4 weeks.

McCall and coworkers have previously published a similar protocol for myocardial infarction in pigs.[11] Considerable overlap exists between this protocol and theirs, emphasizing the preference for the LAD rather than the left circumflex artery (LCX) or the right coronary artery (RCA). In our experience, there is a lesser extent of infarct size of the total left ventricle using the LCX while the RCA infarction is accompanied with higher chance of unwanted conduction disturbances (i.e. sinus node dysfunction, AV-node dysfunction). One difference between the two protocols pertains to the use of increased pharmacological platelet inhibition in this protocol, as we have observed higher rates of no-reflow based on thrombus formation as the result of 90 minutes of hemostasis in the occluded coronary artery. This observation is in line with known hypercoagulability observed in pigs.[12] Although McCall proposed using a single, high-dose, bolus of heparin, this protocol

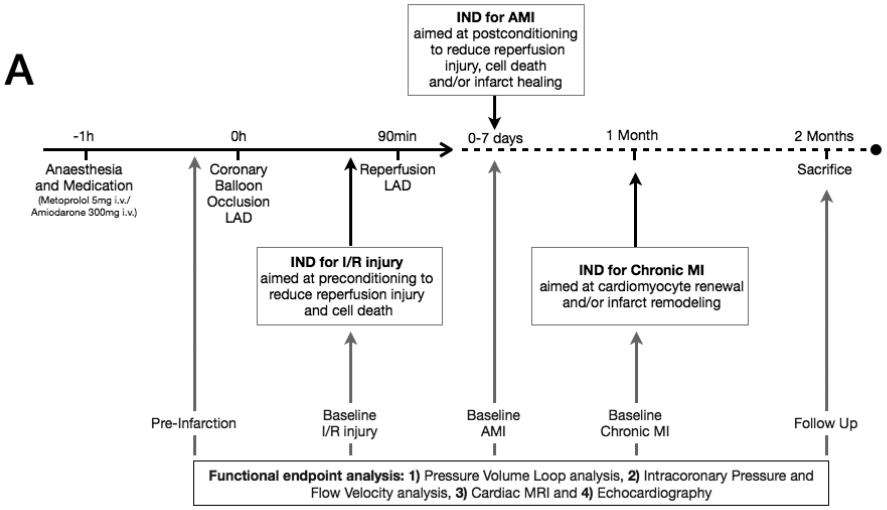

Figure 5. Overview of different study designs. (A) Schematic of multiple possible study designs to validate investigational new drugs (INDs) in various stages of MI using this LAD MI pig model. Dependent on the chosen phase of MI that is under investigation, functional analysis can be performed just prior to the treatment allocation as the baseline value and assessment of the area at risk.

relies on the use of heparin in multiple lower doses spread throughout the surgery to minimize thrombotic complications.

In summary, we present a porcine MI model that enables researchers to make use of an effective, reproducible and above all practical large animal model of human disease to study new therapeutics as an essential step towards a first-in-man clinical trial.

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CHAPTER **10**

10

Assessment of Coronary Microvascular Resistance in the Chronic Infarcted Pig Heart

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ABSTRACT

Background

Preclinical studies aimed at treating ischemic heart disease (i.e. stem cell- and growth factor therapy) often consider restoration of the impaired microvascular circulation an important treatment goal. However, serial in vivo measurement hereof is often lacking.

Objectives

The purpose of this study was to evaluate the applicability of intracoronary pressure and flow velocity as a measure of microvascular resistance in a large animal model of chronic myocardial infarction (MI).

Methods

MI was induced in Dalland Landrace pigs (n=13;68.9±4.1kg) by a 75-minute balloon occlusion of the left circumflex artery(LCX). Intracoronary pressure and flow velocity parameters were measured simultaneously at rest and during adenosine induced hyperemia, using the Combowire (Volcano) before and four weeks after MI. Various pressure and/or flow derived indices were evaluated.

Results

Hyperemic microvascular resistance (HMR) was significantly increased by 28% in the infarct related artery, based on a significantly decreased peak average peak flow velocity (pAPV) by 20% at four weeks post-MI (p=0.03). Capillary density in the infarct zone was decreased compared to the remote area (658±207/mm^2 vs 1650±304/mm^2, p=0.017). In addition, arterioles in the infarct zone showed excessive thickening of the alpha smooth muscle actin (αSMA) positive cell layer compared to the remote area (33.55±4.25 μm vs 14.64±1.39 μm, p=0.002).

Conclusions

Intracoronary measurement of HMR successfully detected increased microvascular resistance that might be caused by loss of capillaries and arteriolar remodeling in the chronic infarcted pig heart. Thus, HMR may serve as a novel outcome measure in preclinical studies for serial assessment of microvascular circulation.

INTRODUCTION

Coronary artery disease is a major cause of mortality and morbidity worldwide that can be held responsible for seven million deaths annually.[1] Myocardial ischemia is associated with a poor prognosis and could give rise to disabling complaints of refractory angina pectoris.[2] The concept of restoration of impaired blood flow by formation of new capillaries (angiogenesis) to treat ischemia in tissue has a high scientific appeal.[3] Therefore, numerous broadly ranging strategies to promote angiogenesis (e.g. stem cell therapy, growth factor delivery, microRNA interference) are currently being explored in the preclinical setting.[4-7] Serial in vivo assessment of the status of the myocardial microcirculation remains cumbersome.[8] Thus, angiogenesis is often reported based on ex vivo histologic analysis of the area of interest.

Several intracoronary pressure- and flow velocity-derived indices have been studied for the ability to draw inferences on the healthy or diseased status of the coronary circulation. Among these indices, there is the clinically widely used fractional flow reserve (FFR), based on intracoronary pressure, to steer clinical decision-making in epicardial stenoses.[9,10] Coronary flow velocity reserve (CFVR), derived from intracoronary flow velocities, represents the ability to increase coronary flow under hyperemic conditions. Unfortunately, CFVR varies between and within patients as it depends on several parameters such as metabolic demand, the diastolic time fraction, blood pressure and microvascular disease.[11,12] Relative flow velocity reserve, the ratio of CFR in the stenosed and healthy coronary artery, has been proposed as an alternative but did not lead to clinical application.[13] An alternative method to assess the functioning of myocardial vasculature is by pressure-/ and flow velocity derived microvascular resistance.[14] This has become possible by the combination of simultaneously measured pressure and flow velocity, to yield an index referred to as hyperemic microvascular resistance (HMR).[15,16]

We hypothesized that elevated microvascular resistance could serve as a novel outcome measure for preclinical studies that investigate novel treatment strategies to restore ischemia in myocardial tissue by means of arteriogenesis and/or angiogenesis. The aim of this study was to investigate the effect of chronic myocardial infarction in a large animal model on microvascular resistance and to study the potential underlying mechanisms reflecting this parameter.

METHODS

Animals and Study Design

Thirteen 6-month old female Dalland Landrace pigs (weighing 69 ± 4 kg) received care in accordance with the Guide for the Care and Use of Laboratory Pigs prepared by the Institute of Laboratory Animal Resources. Experiments were approved by the Animal Experimentation Committee of the Medicine Faculty of the Utrecht University, the Netherlands. First, intracoronary pressure and flow velocity and pressure volume (PV) loop analysis was measured in healthy animals. Next, these animals were subjected to myocardial infarction (MI), induced by a 75-minute balloon occlusion of the LCX. Four weeks after the MI, functional endpoint analysis was repeated. The schematic study design is shown in supplementary Figure 2.

Myocardial infarction

The MI was induced as previously described.[17] Briefly, animals were sedated and general anesthesia was maintained by continuous infusion of midazolam (0.7 mg/kg/h), sufentanil citrate (6 µg/kg/h) and pancurorium bromide (0.1 mg/kg/h) via the canulated ear vein. The animals were mechanically ventilated with a positive pressure ventilator (FiO2 0.50) under continuous capnography.

Arterial access was achieved by canulating the internal carotid artery and MI was induced by a 75-minute balloon occlusion of the proximal left circumflex artery. Prior to the infarction, a bolus of amiodarone (300mg) and metoprolol (5 mg) was infused intravenously in 45 minutes to minimize onset of cardiac arrhythmias.

Intracoronary pressure and flow velocity assessment

Intracoronary pressure and flow velocity were measured simultaneously by using the Combowire® (Volcano Corporation, San Diego, USA) as previously described[15,18]. Pressure and flow velocity signals, combined with aortic pressure and ECG signals were recorded using the ComboMap® system (Volcano Corporation, San Diego, USA). Intracoronary pressure and flow velocity were assessed prior to the infarction and four weeks after MI in the infarct related artery (LCX) and the reference artery (LAD). Nitroglycerin (200 mcg) was injected intracoronarily to prevent coronary spasms. Next, the Combowire was placed in the proximal section of the LCX and the LAD. Velocity and pressure signals were recorded during rest and peak hyperemia. Hyperemia was induced by intracoronary bolus of 60 mcg adenosine. At least 3 representative measurements were performed per vessel.

Analysis of pressure-/flow velocity derived indices

Datasets were stored digitally and analyzed offline using AMC Studymanager, a custom software package (written in Delphi vs. 6.0, Borland Software Corporation and Delphi vs. 2010, Embarcadero, CA, USA). CFVR was calculated as CFVR = pAPV / bAPV, where APV is average peak flow velocity in cm/s. The bAPV and pAPV were calculated as the mean of four beats at rest and the mean of three successive beats with the highest flow velocity respectively. HMR was calculated as HMR = Pd / pAPV, where both Pd and pAVP were derived from the mean of three beats at hyperemia.[19]

Pressure-Volume loop protocol

PV loops were acquired using a 7-F conductance catheter that was placed in the left ventricle. The catheter was connected with a signal processor (Leycom CFL, Zoetermeer, the Netherlands). Data were collected during steady-state conditions with the respirator system turned of at end-expiration. Data analysis and calculation was performed on custom-made software (CD Leycom, Zoetermeer, the Netherlands).

Histology

Four animals, that served as control treated animals in a larger study20, were euthanized 8 weeks after MI by exsanguination under general anesthesia (see SI Figure 2). After excision of the heart,

the left ventricle was cut into five slices from base to apex and incubated in 1% triphenyl-tetrazolium chloride dissolved in phosphatase buffered saline (PBS) at 37°C for 15 minutes. Next, the slices were washed in PBS and photographed digitally (Sony Alfa 55). Snap frozen tissue samples from the infarct zone and remote area (septal wall) were embedded in Tissue-Tek (Sakura) and 7 µm cryosections were prepared on a microtome (Leica). Sections were dried for 30 min at room temperature (RT) and fixed in acetone. Subsequently, slides were incubated with 0.1% Triton X-100 (Sigma) in PBS with 1% bovine serum albumin (BSA), blocked in 10% goat serum, incubated overnight at 4°C in 1% goat serum with primary antibodies against α-smooth muscle actin (αSMA) (1:1500, Mouse monoclonal, Clone 1A4, Sigma) and CD31 (1:100, rabbit polyclonal, Abcam) and then incubated for 1 hour at RT with secondary antibodies (Invitrogen). Slides were mounted in Fluoromount (Southern Biotech) and fluorescence images were acquired on an Olympus DP71 microscope. For image analysis, the number of arterioles (defined as aSMA-positive vessels > 20 µm and < 300 µm) and wall thickness (defined as the wall thickness of the aSMA-positive cell layer) was measured in 10 different fields/section in the infarct zone and the border zone at x40 magnification using ImageJ (1.44g). The number of arterioles was expressed per 1.0mm^2.

The density of capillaries in the infarct region and remote area was assessed by staining with an antibody against CD31 (1:100, mouse monoclonal, AbD Serotec). Endogenous peroxidase in the cryosections was blocked with 0.15% hydrogen peroxide in acetone for 15 min at RT. The 2° antibody used was horse anti-mouse, biotinylated (1:200)(Invitrogen) for 60 min at RT followed by a streptavidin-HRPO in PBS (1:1000) for 60 min at RT. The chromogen 3-amino-9-ethylcarbazole (AEC) (Sigma) was used to visualize capillaries. The slides were counterstained with hematoxylin for identification of nuclei. The number of capillaries (defined as 1 or 2 cells spanning the CD31-positive vessel circumference) was determined by counting 10 fields/section in the infarct zone and the peri-infarct border zone at x40 magnification. The number of capillaries was expressed per 1.0 mm^2.

Statistics

Continuous variables are presented as mean±SD. Analysis was performed by paired or two sample t-test. The assumption that the variable must be normally distributed was checked by QQ plots and the Kolmogorov-Smirnov test. All tests were performed using SPSS Statistics 17.0. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Effect of chronic MI on coronary pressure and flow velocity

The mean coronary diameter at baseline was 3.3 ± 0.7 mm and 3.1 ± 0.8 mm in the LCX and LAD, respectively, and did not change over time. Coronary pressure- and flow-velocity derived indices at baseline and follow-up are shown in Table 1. HMR in the infarct related artery (LCX) was significantly higher at follow up (Figure 1A; 2.4±1.1 mmHg/cm/s) compared to baseline (Figure 1A; 1.9±0.6 mmHg/cm/s; p=0.03), indicating an increase in regional microvascular

resistance in the area of interest. The increased HMR in the LCX was based on an impaired peak coronary flow response to distal vasodilation at four weeks compared to baseline (Figure 1B; 42.5±11.4 cm/s versus 53.0±17.3 cm/s, respectively; p=0.05). The distal intracoronary pressure did not show any change between four weeks after MI and baseline (Figure 1C; 92.4±21.2 mmHg versus 92.4±19.1 mmHg; p=0.50). Coronary flow velocity at rest was also impaired four weeks after MI compared to baseline (SI Figure 1). As a result, the CFVR remained unchanged following MI compared to baseline values (SI Figure 1). The reference artery (LAD) did not show any change in coronary pressure and velocity derived indices (Figure 1A-C), indicating a stable and unchanged ability to regulate flow velocities to myocardial demand.

The effect of decreased vascular density of the scar tissue on microvascular resistance

To examine the role of myocardial vascular density in microvascular resistance, we quantified the CD31+ capillaries (Figure 2A,B) and the αSMA+ positive arterioles (Figure 2 C,D) in both the infarct area and the remote area. The number of CD31+ capillaries was reduced in the scar tissue

Table 1. Parameters before and 4 weeks after MI.

		Baseline	4 weeks	Difference		Sign.
		Mean ± SD	Mean ± SD	Mean	%	
	MAP (mmHg)	95.7 ± 20.1	96.4 ± 20.6	0.67	0.7%	0.91a
	HR (b/min)	64.4 ± 10.1	57.0 ± 12.9	-7.36	-11.4%	0.16a
	EF (%) (n=12)	65.7 ± 6.7	55.3 ± 8.5	-10.13	-15.4%	0.00c
	Weight (kg)	68.9 ± 4.1	72.3 ± 4.0	3.42	5.0%	0.01a
Infarct related artery (LCX) n=13	FFR	1.0 ± 0.0	1.0 ± 0.0	0.01	0.9%	0.83b
	CFVR	2.9 ± 0.4	3.0 ± 0.7	0.02	0.7%	0.46c
	bAPV (cm/s)	18.0 ± 4.2	14.9 ± 4.0	-3.17	-17.6%	0.03c
	pAPV (cm/s)	53.0 ± 17.3	42.5 ± 11.4	-10.46	-19.7%	0.05c
	Pd (mmHg)	92.4 ± 19.1	92.4 ± 21.2	0.00	0.0%	0.50c
	HMR (mmHg/cm/s)	1.9 ± 0.6	2.4 ± 1.1	0.53	28.4%	0.03c
Reference artery (LAD) n=12	FFR	1.0 ± 0.0	1.0 ± 0.0	0.00	0.5%	0.53b
	CFVR	2.8 ± 0.5	3.0 ± 0.5	0.13	4.8%	0.21c
	bAPV (cm/s)	17.3 ± 3.0	16.5 ± 2.9	-0.79	-4.5%	0.25c
	pAPV (cm/s)	50.0 ± 13.3	47.7 ± 5.2	-2.25	-4.5%	0.25c
	Pd (mmHg)	88.9 ± 19.8	87.9 ± 20.7	-1.00	-1.1%	0.42c
	HMR (mmHg/cm/s)	1.9 ± 0.7	1.9 ± 0.5	-0.04	-1.9%	0.42c

T=0, before MI; T=4wk, 4 weeks after MI; MAP, Mean aortic pressure; HR, heart rate; EF, Left ventricular ejection fraction; CK-MB ratio, ratio of CK-MB before ischemia and 30 minutes after reperfusion; FFR = Pd/aortic pressure; CFVR=pAPV/bAPV; Pd = intracoronary pressure; HMR = Pd/pAPV.
a: Two tailed paired T-test. b: Wilcoxon Signed Ranks test. c: One tailed paired T-test.

(Figure 2E; 658±207 per mm^2) compared to the remote area (Figure 2E; 1650±304 per mm^2, p=0.0009). Regarding the number of arterioles, there was no change in the infarct area (Figure 2F; 199±30 per mm^2) compared to the remote area (Figure 2F; 222±52 per mm^2, p=0.369). Thus, these results indicate that the balance between capillaries and larger arterioles is shifted in the infarct scar on the basis of a decreased number of capillaries per number of arterioles.

Adverse remodeling of αSMA+ arterioles and changes in extracellular matrix composition

Next, we examined the morphology of the αSMA+ arterioles to elucidate the role of adverse remodeling as a mechanism for impaired coronary response to hyperemia. Strikingly, the αSMA+ arterioles within the infarct zone were characterized by a pronounced thickening of the αSMA+ cells forming a dense layer surrounding the CD31+ endothelial cells compared to the remote area where αSMA+ cells appeared as a small rim surrounding the arterioles (Figure 3A-B). The average thickness of the αSMA+ cells per arteriole was increased in the infarct area (Figure 3E; 36±14 μm) compared to the remote area (Figure 3E; 15±4 μm; p=0.002).

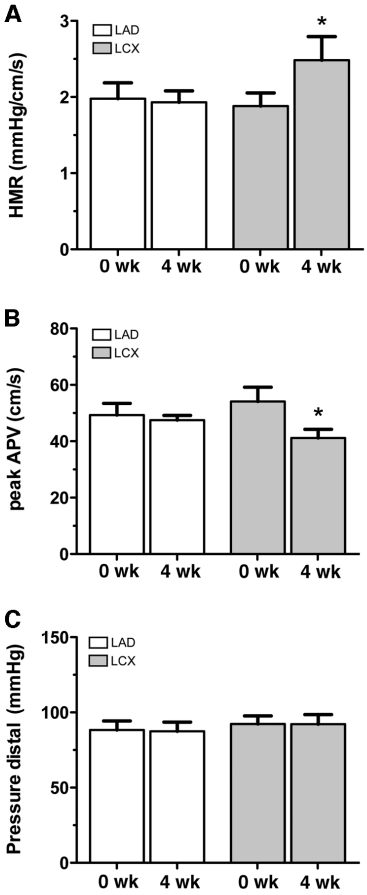

Figure 1. Coronary pressure-/flow derived assessment of microvascular circulation.

(A) Combined pressure and peak hyperemic flow were used to calculate HMR in both the reference artery (white bars) and the infarct related artery (grey bars). Four weeks after MI, HMR in the LCX was increased (* denotes p = 0.03). **(B)** The peak APV was decreased in the LCX at four weeks after MI compared to baseline (* denotes p = 0.05). **(C)** Intracoronary pressure measured by the Combowire did not change throughout the study. Error bars represent SEM.

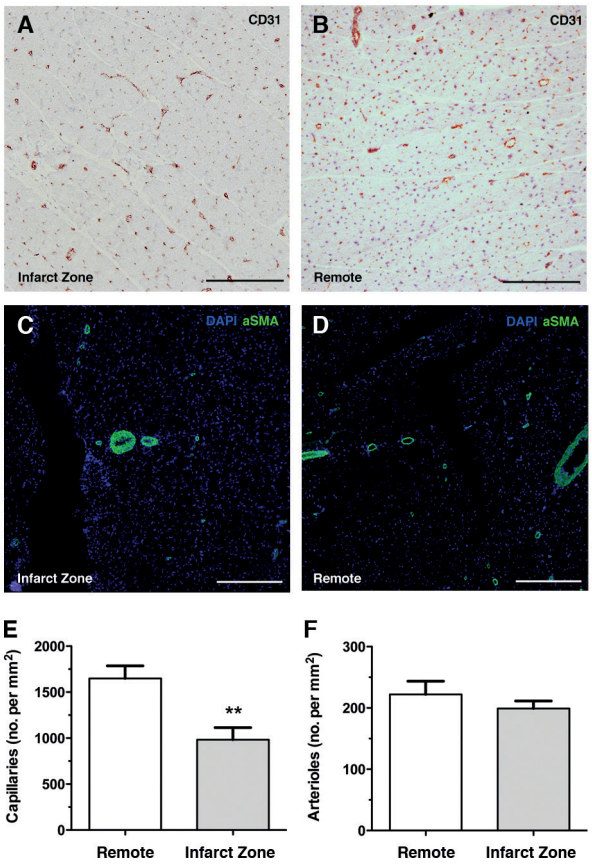

Figure 2. Altered vascular density of the scar tissue impairs microvascular resistance.
Representative photographs showing microscopic fields of transversally oriented cardiomyocytes with CD-31 positive capillaries **(A,B)** or immunofluorescent-labeled αSMA **(C,D)**. **(A)** Peri-infarct zone in the LCX vascularized area shows a decreased number of CD-31 positive capillaries compared to **(B)** the remote area of the LAD **(C,D)**. The number of αSMA positive arterioles is not different between infarct zone and remote area. Quantification for **(E)** CD31 positive capillaries (** denotes p = 0.0009) and **(F)** αSMA+ arterioles (remote vs infarct zone; p = 0.366). All scale bars represent 500μm. Error bars represent SEM.

Furthermore, when combining the αSMA+ fluorescent images with the polarized light microscopy of picrosirius stained collagen, arterioles appeared entrapped in between the dense collagen fibers (Figure 3 C) in the infarct zone. In contrast, the remote area revealed a small layer of collagen surrounding the arterioles dispersed in between the viable myocardium (Figure 3 D). Altogether, these findings indicate that both adverse remodeling of the αSMA+ vessels and altered extracellular matrix composition could be potential underlying mechanisms leading to impaired coronary flow response and increased hyperemic microvascular resistance.

DISCUSSION

Since the 1990s, intracoronary pressure and flow velocity measured with sensor-tip guidewires have been introduced as a novel approach for assessment of coronary hemodynamics.[21] The use of guidewire-based assessment of coronary hemodynamics in the cardiac catheterization laboratory is well established to guide clinical decision-making.[15] Yet, this powerful tool has thus

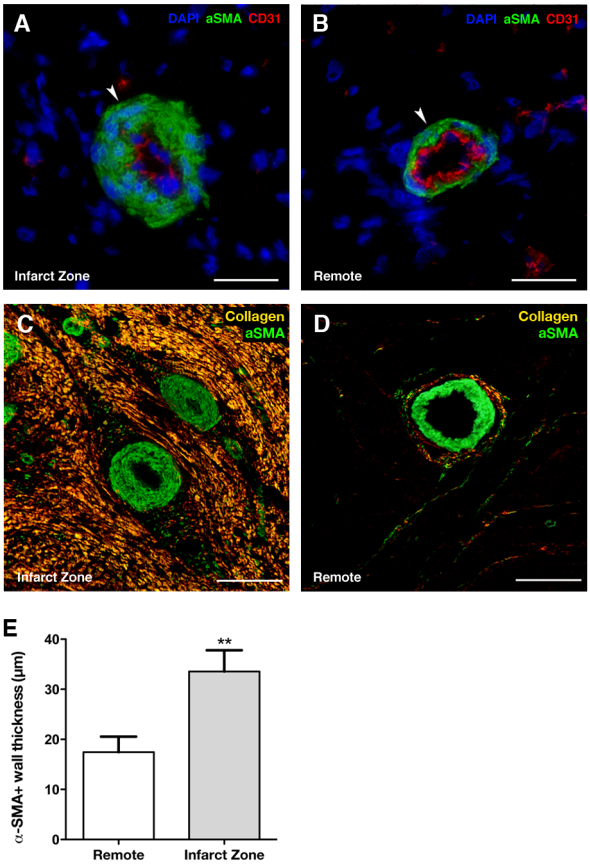

Figure 3. Structural changes in arterioles and extracellular matrix increase microvascular resistance. Representative photographs showing microscopic fields of co-staining with immunofluorescent-labeled αSMA (green signal), CD-31 (red signal) and nuclei counterstained with 4',6-diamino-2-phenylindole (DAPI). **(A)** In the infarct zone, there is pronounced thickening of the αSMA + cells of the arterioles. **(B)** This phenomenon did not occur in arterioles from samples of the LV remote area. **(C)** In the infarct zone αSMA+ arterioles are embedded within dense collagen fibers (yellow signal). In contrast, **(D)** the remote area displayed small amounts of collagen dispersed in between patches of viable cardiomyocytes and constituted a small rim surrounding the arterioles. **(E)** Quantification of αSMA+ wall thickness shows a twofold increase (** denotes p = 0.002). All scale bars represent 50µm. Error bars represent SEM.

far not been implemented as a functional endpoint in large animal models of ischemic heart disease and in particular those designed to validate new angiogenic therapies such as growth factors/cytokines, stem cell therapy or gene therapy.

Here, we have shown that intracoronary pressure-/flow velocity could successfully detect an increased hyperemic microcirculatory resistance in a porcine model of chronic MI. Potential mechanisms pertaining to this increased microvascular resistance are three-fold. First, capillary density in the infarct scar tissue was reduced two-fold while larger arterioles were present in similar numbers as in the remote area. Second, arterioles within the infarct zone were characterized by a marked thickening of the αSMA+ cell layer. Third, infarct zone arterioles were dispersed in between dense collagen fibers that could hamper the dilatory capacity of these vessels to hyperemic stimuli. Collectively, these data indicate a dual origin for the increase in hyperemic microvascular resistance, both on the level of arterioles as well as on the level of capillaries.

The role of capillaries in microvascular dysfunction

From previous data in dogs, Jayaweera and coworkers showed that, in absence of a stenosis or

hyperemia, vasoconstriction of the arterioles constitute the largest resistance (61 ± 5 %) of total myocardial vascular resistance on coronary flow, compared to the capillary (25 ± 5%) or venous (14 ± 4) compartments.[22] Work by Friedman et al. confirmed the modest role of capillaries at rest by showing that increased flow recruited to opening of additional capillaries thereby facilitating the additional flow.[23] Due to the redistribution of coronary resistance, the capillary compartment had the highest vascular resistance at maximal vasodilation, accounting for 75% of the total myocardial vascular resistance.[22] Thus, the observed reduction in capillary density in the infarct zone and its effect on the increase in hyperemic microvascular resistance support the notion that the capillary compartment plays a crucial role during a hyperemic response of the coronary flow. The mean capillary density of 1650 ± 304 /mm^2 in the healthy porcine myocardium is in reasonable agreement with the previously reported 1956 ± 231/mm^2 in the human heart.[24] More importantly, we observed a capillary density in the porcine infarct scar area of 658±207/mm^2, which mimicked reported values of human ischemic cardiomyopathy of 1124 ± 226/mm2 by Karch24 and 771 ± 68/mm2 by Mehrabi25 in explanted hearts from patients with ischemic cardiomyopathy undergoing heart transplantation.

Microvascular dysfunction and arteriolar remodeling in the infarct zone

Another finding of the present study is the contribution of increased arteriolar wall thickness on the curtailed coronary flow. This phenomenon has been observed previously in patients with arterial hypertension.[26] Furthermore, although in a distinct patient population, increased arteriolar wall thickness in heart allografts was linearly associated with an increase in hyperemic microvascular resistance.[27] Coronary arterioles tend to structurally adapt to a wide variety of pathophysiological situations. In pigs that underwent a gradual coronary stenosis, a range of structural changes and impaired response to bradykinin-1 in arterioles distant to a stenosis was observed.[28] Additionally, type-2 diabetes induced similar narrowing of coronary arteriolar lumen by increase in wall thickness.[29] Given the role of arteriolar dilation during hyperemic peak flow, it is conceivable that this arteriolar remodeling negatively affects their ability to relax and hence impair their vasodilatory capacity and thereby increases vascular resistance.

The effect of the extracellular matrix on the microvascular resistance

Besides the altered vasculature, change in extracellular matrix has also been shown to moderate the vascular ability to respond to different flow conditions.[26] In addition, Berry et al. showed that myocardial tissue after infarction is more rigid than healthy myocardium.[30] In line with these findings, we report an increased layer of dense collagen fibers in the close proximity of arterioles. Although the effect of fibrosis on arteriolar vasodilatation was not quantified in this study, we speculate that the increased collagen content further reduced the normal hyperemic flow response.

Taken together, these results provide support for the notion that HMR measured by the Combowire can play an important role in preclinical large animal models in which serial assessment of microvascular circulation is warranted.

Study Limitations

When extrapolating the current findings to an equivalent patient population of chronic MI, it should

be kept in mind that several determinants of increased HMR have not been incorporated in this animal model, such as age, diabetes or reduced pressure due to atherosclerosis and/or subsequent formation of thrombus and/or thrombotic emboli- in the epicardial vessels.[31]
Secondly, it should borne in mind that the use of adenosine has a few practical limitations that precludes an unbiased estimate of the maximal coronary flow. We cannot rule out that dysfunction of vessels and thereby improper reaction on adenosine might also contribute to the increased HMR as well. In coronary artery disease and after myocardial infarction, α-adrenergic vasoconstriction occurs and adenosine does not dissolve this α-adrenergic vasoconstriction.[32]
Thirdly, regarding mechanisms of increased microvascular resistance, undoubtedly, numerous processes in the chronic infarct can account for an increase in microvascular resistance, such as mechanical stresses and strain of the microvessels during myocardial contraction and relaxation or neurologic and/or metabolic dysregulation on vascular alpha-tones.
Finally, morphometric analysis of arterioles may be influenced by fixation of the myocardial tissue. However, we did not observe any signs of arteriolar remodeling in the healthy myocardium, suggesting a dominant role for the chronic infarct as a causal factor.

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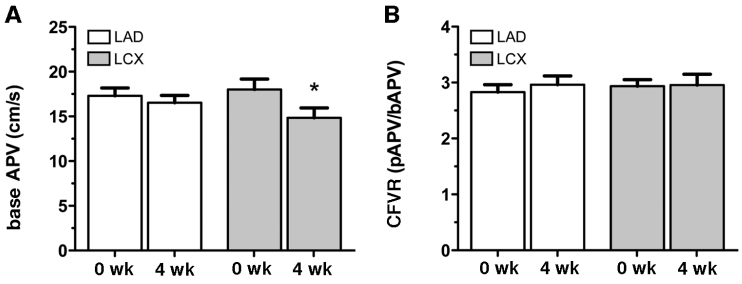
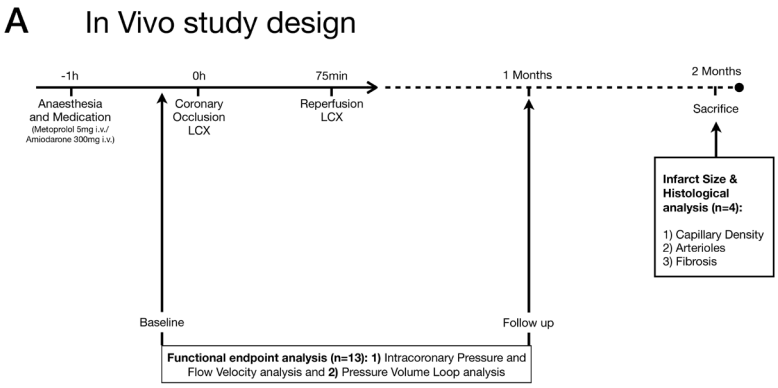
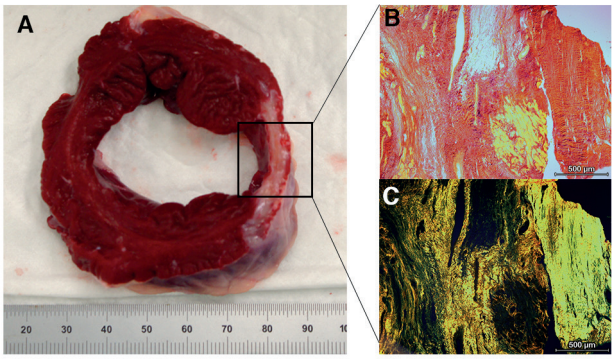
SUPPLEMENTARY INFORMATION



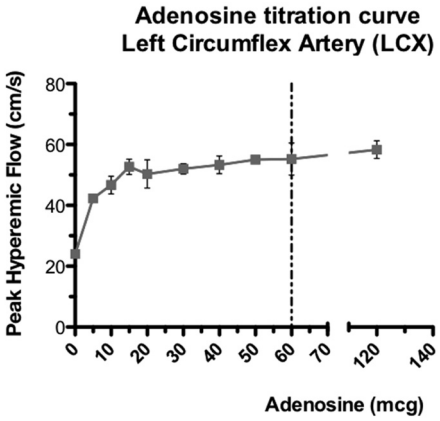
Supplementary figure 1. Assessment of Coronary Flow Velocity Reserve.
(A) At four weeks follow up, coronary flow velocity under base condition was decreased. **(B)** As a result, the ratio between peak and base APV, reflected by the CFVR, remained similar throughout the follow up duration.



Supplementary figure 2. Experimental Study Design.
(A) Schematic overview of the *in vivo* study design of a chronic MI model and different time points of intracoronary pressure and flow velocity analysis, namely before induction of MI (baseline) and at 1 month follow up. Histology is obtained at two months after MI in a subset of animals (n=4) which have been used as a negative control group described elsewhere.[1]



Supplementary figure 3. Infarct size.
(A) Representative photograph of triphenyltetrazolium chloride (TTC) staining of the left ventricle at the level of the papillary muscle show varying forms of transmural infarct size (white color) in the viable myocardium (red color). **(B)** Picric red staining shows the infarct collagen (red signal) and viable myocardium (yellow signal). **(C)** Under polarized light, picric red staining for collagen is visible as a bright yellow signal.



Supplementary figure 4. Dose-finding of intracoronary adenosine and peak hyperemia.
Average of three consecutive measurements of peak hyperemic flow in response to increasing doses of adenosine administered intracoronary. For further experiments, intracoronary admission of 60 mcg was set as the adenosine dose.

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PART FIVE | DISCUSSION AND SUMMARY

CHAPTER **11**

General Discussion

Over the time course of this 3 year-PhD program, roughly 22 million people have died worldwide due to coronary artery disease.[1] Myocardial infarction leads to the irreversible loss of functional, contractile myocytes and none of the existing clinical therapies can restore this deficit in cardiomyocytes.[2] After the index event, post-MI remodelling further aggravates cardiomyocyte death. Although substantially lower than the massive loss during MI, post-MI remodelling can still increase myocyte loss by 100-fold compared to non-failing hearts.[3] Hence, two different approaches are equally promising to reduce the loss of myocytes in ischemic heart disease: 1) strategies to protect cardiomyocytes against the ischemic insult so that more cardiomyocytes survive this critical moment, and, 2) cardiac regenerative medicine aimed to replace the lost myocyte fraction by newly generated counterparts. In this thesis both were studied, i.e. cardioprotection against reperfusion injury and cardiac regeneration in the post-MI heart. The experimental work presented in this thesis was designed with the clinical applicability in mind and conducted in a large animal model of MI. This ensures an accurate proxy of human cardiovascular disease is used thereby bridging the translational gap between bench and bedside.

PART ONE
CARDIOPROTECTION

Myocardial infarction is in the majority of cases the functional consequence of progressive coronary atherosclerosis, superimposed by thrombus formation due to rupture of the plaque surface. As a result, blocked coronary blood flow culminates in the loss of on average one billion cardiomyocytes, accounting for ~20% of the total myocyte fraction. Current treatment options includes a combination of swift restoration of blood flow and pharmacological platelet inhibition.[4] However, as extensively reviewed in **chapter 2**, reperfusion of the ischemic territory paradoxically accelerates myocyte injury and death, termed ischemia/reperfusion (I/R) injury. Different forms of cell death (i.e. apoptosis, necrosis or necroptosis), the concept of I/R injury in the heart and current results of pharmacological strategies to alleviate I/R injury have been critically and systematically reviewed. From **chapter 2**, four important new insights emanate: 1) the presence of I/R injury in the heart is substantiated by a large body of evidence and is estimated to account for ~50% of the final infarct size; 2) the concept of programmed necrosis, or necroptosis, is a new and important form of cell death in the reperfused heart; 3) pharmacological strategies for cardioprotection have shown that the effect of I/R injury can indeed be abrogated and final infarct size can be reduced; 4) the clinical translatability of preclinical evidence is disappointingly low as numerous therapies that were successful in animal studies failed to show efficacy in clinical trials.

Necrostatin-1 exerts cardioprotective properties against I/R injury

In an experiment as elegant as simple, Degterev and colleagues showed that TNF-α stimulation, normally activating apoptosis, could still induce cell death even when the apoptosis pathway was silenced.[5] Termed necroptosis, this new form of programmed cell death involved a family of seven members of receptor interacting protein (RIP) kinases.[6] Experimental data showed that the RIP1/RIP3 complex leads to a detrimental increase in metabolism by glycolysis, glutaminolysis and

oxidative phosphorylation.[7,8] As a result, reactive oxygen species (ROS) are formed leading to accumulating cellular damage and necrosis. We and others have shown that Necrostatin-1 (Nec-1) is a potent small molecule that blocks the kinase activity of RIP-1 thereby inhibiting programmed necrosis.[9] In **chapter 3**, this direct inhibitor of RIP-1 was tested for its ability to reduce infarct size in a pig model of I/R injury. Intravenous administration of Nec-1, ten minutes prior to reperfusion decreased final infarct size in a dose-dependent manner and improved systolic and diastolic function in our pig model. Akin to previous work on Nec-1 in different models of myocardial injury[9], there was a profound decrease in circulatory necrotic markers (i.e. lactate dehydrogenase, creatinin kinase and creatinin kinase MB isoenzyme) whereas apoptosis was not influenced. As a next step, we showed that the inflammatory mechanism in response to necrotic myocardial damage was also reduced. A shifted balance between apoptosis and necrosis may be responsible for this observation as the latter is the strongest stimulator of inflammatory responses. Given the additional increase of cell death due to the inflammatory response itself, the reduction in final infarct size of ~50% in the Nec-1 treated animals could be viewed as the composite of two distinct processes, namely: 1) direct inhibition of necrotic cell death and 2) less cell death based on the subsequent inflammatory response to the ischemic insult. Taken together, experimental data from **chapter 3** collectively support the notion that I/R injury exists in the pig heart and that rodent data on the efficacy of Nec-1 in reducing infarct size could be reproduced in a large animal model. Therefore, we believe that Nec-1 is a promising candidate to serve as an adjunctive treatment besides primary percutaneous coronary intervention (PPCI) for the treatment of acute MI.

Clinical translation of cardioprotection

Despite its undisputed necessity, 30 years of research did not lead to the incorporation of cardioprotection against reperfusion injury into clinical guidelines for treatment of acute MI.[10] Although promising results were observed in preclinical studies, what is the reason so many therapies against I/R injury got lost in translation? Some have argued that pharmacological cardioprotection using one single, fairly upstream, signaling pathway has less change to succeed because of the complex and often interrelated mechanisms that are involved (i.e. different forms of cell death pathways, intracellular calcium overload, reactive oxygen species formation etc.) (**chapter 2**). However, this view is challenged by the observation that cyclosporin A, an inhibitor of the more downstream mPTP pore, has shown efficacy in smaller patient studies and a larger randomized and blinded trial is currently underway (CIRCUS trial, NCT01502774). Interestingly, the group of Yellon observed that isolated mitochondria treated with Nec-1 showed a significant delay in opening of the mPTP.11 In conclusion, the observations of successful cardioprotection by delay in the downstream target of the mPTP opening and the first results of Nec-1 in a large animal model presented in **chapter 3** collectively justifies future work on its pharmacodynamics, safety, toxicity as a last step before advancing towards a first-in-man clinical trial.

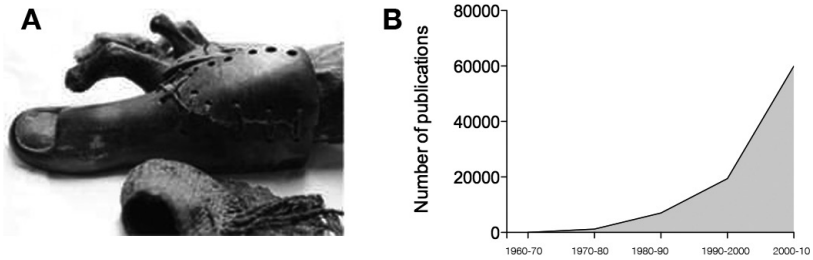

Figure 1. Biomaterials and medicine.
(A) Wooden toe, dating from ~1065–740 B.C., used as a prosthetic to replace an amputated toe (Image courtesy of J. Finch, KNH Centre for Biomedical Egyptology, University of Manchester, UK, and The Egyptian Museum, Cairo.) **(B)** Number of publications related to biomaterial science indexed in MEDLINE.

PART TWO
STRATEGIES FOR CARDIAC REPAIR: PRECLINICAL RESULTS

Dynamic supramolecular biomaterials for in situ tissue engineering

In the ancient world, biomaterials already played an important role, when natural materials such as wood were used to replace the tissue lost by trauma or disease (Figure 1A). Historically, materials serving as biomaterials were selected based on their natural availability. However, during the early part of the twentieth century, the introduction of synthetic polymers propelled the use of biomaterials. Nowadays, it has become an increasingly studied research field (Figure 1B). For instance, controlled drug delivery by polymer-coated devices such as drug eluting stents are frequently used in cardiology to treat patients with coronary artery disease.[12,13]

Supramolecular biomaterials have unique advantages in terms of self-assembled *in situ* tissue engineering, processability, tunable biodegradability and convenient incorporation of biologics.[14] The ureido-pyrimidinone (UPy)-hydrogel system described in this thesis is a supramolecular polymer,based on quadruple hydrogen bonding UPy moieties.[15,16] Based on the reversible nature of hydrogen bonding, as opposed to covalent bonding, the UPy-polymer can be easily processed into different forms of scaffolds, such as hydrogel, films, fibers, electrospun scaffold or meshes.[16,17] A major part of this thesis is focused on the development and validation of a new cardiac regenerative strategy that involves a catheter-based delivery of a smart biomaterial for local growth factor release in the heart. To this end, we developed a new technique to deliver growth factors in the heart by making use of this UPy-hydrogel system. In **chapter 5**, we report on the first *in vitro* and *in vivo* experiments aimed to validate UPy-hydrogel as a new drug delivery system to the heart. After the discovery that the enolate form of the UPy-hydrogelators cannot dimerize, we showed that, under the influence of pH, UPy-hydrogel gel could be injected by catheter in the heart. Upon delivery, the physiological pH of the surrounding myocardium switched the deprotonated form back into keto-tautomer thereby rapidly forming homodimers. As a result, growth factors IGF-1 and HGF could be loaded in the UPy-hydrogel that gelates in situ and their protein activity could be confirmed by western blot six hours after injection.

As a next step, we tested this new approach *in vivo* using a pig model of myocardial infarction. The delivery of IGF-1 and HGF by itself already showed an improvement in cardiac function and structural remodeling (**chapter 6**). The release by UPy-hydrogel showed a trend towards increased effect of growth factor therapy compared to sole delivery of the growth factors. This finding is in contrast with various reports which show that biologics in the absence of a biomaterial were only marginally effective.[18-20] Apparently, in our experiments, the animals treated with IGF-1/HGF already showed a considerable increase in cardiac function and histology compared to control treated animals. This might explain why we failed to show a significant difference in the comparison between IGF-1/HGF and UPy-IGF/HGF. Based on this marginal increase, extending group size was not considered as it would require 190 additional animals to detect a ~2% increase in LV ejection fraction between both groups with a power of 0.90. Interestingly, there was not one single parameter in which the UPy-IGF-1/HGF group did not outperform saline dissolved IGF-1/HGF. From a clinical point of view, it is unlikely that this increased effect of UPy-hydrogel system observed in **chapter 6** will yield a clinically relevant improvement for patients with ischemic heart disease. However, from a translational point of view, these results highly encourage further work on the use of the UPy-hydrogel system as a novel strategy to improve growth factor efficacy. Partly based on the results of this thesis, a start-up company Suprapolix aims to further develop and improve the patented UPy-hydrogel system and its pH tunability.

Intramyocardial delivery of IGF-1 and HGF and cardiac repair

Life is regenerative, by definition. But humans lack, and by large, the regenerative capacity as evidenced in lower amphibians and teleost fish.[21,22] However, there are several organs and tissues such as the liver, intestine and skin that readily regenerate following aging and/or injury. Unfortunately, accumulating insults of injury, aging or diseases wreak havoc on those that do not. This is why heart disease, an organ infamous for its inability to substantially regenerate, leads the chart as one of the leading causes of morbidity and mortality in the world.[1] In **chapter 4**, we asked ourselves the question if and to what extent cardiac regeneration occurs in the adult mammalian heart. As most tissues in the human body are regenerated based on the differentiation of stem/progenitor cells, a second objective was to shed light on the role that cardiac stem/progenitor cells (CSCs) play in this process. By careful review of the available literature, we show that there is a substantial body of evidence favoring the presence of cardiomyocyte renewal in the adult mammalian heart as confirmed by various independent research groups. However, at this point consensus is replaced by two diametrically opposed views on the cause of cardiomyocyte renewal. On the one hand, data has been presented showing that new myocytes are derived from pre-existing cardiomyocytes.[23,24] Other groups have presented evidence in support of a polar view, which hypothesizes that newly formed cardiomyocytes are the direct progeny of stem/progenitor cells.[25-28] In favor of the latter view, our own research group has reported on the observation that transplanted (human) cardiomyocyte progenitor cells in the infarcted heart of immunodeficient mice can indeed lead to the formation of (human) cardiomyocytes.[29] Recently, Ellison and coworkers showed that clinically relevant regeneration was present in isoprotenerol-induced myocyte loss.[26] Furthermore, by genetically tagging the resident c-kit^{pos} CSCs with a yellow fluorescent protein (YFP), for the first time, they showed that

the regenerated myocytes also expressed YFP thereby confirming for the first time that indeed these are the progeny of resident CSCs.
In **chapter 6**, we showed the presence of Ki67, a marker for proliferation, in cardiomyocytes adjacent to the infarct area. Analogous this the increase in small, cycling cardiomyocytes, increased numbers of CSCs were present following growth factor treatment. Given our choice for a large animal model using outbred pigs, we could not unambiguously address causality between increased numbers of eCSCs in relation to $Ki67^{pos}$ cardiomyocytes. However, from a biological point of view, these results do provide support for the notion that stem cell based tissue-/cellular homeostasis in the heart does not seem to differ from other organs previously regarded incapable of self-renewal, such as the brain[30] or the skeletal muscle.[31]

PART THREE
CELL BASED CARDIAC REPAIR: CLINICAL PERSPECTIVE

A recent Forbes analysis valued the total costs of creating a single new drug at $350 million. In the US alone, a ten-fold $3.7 billion dollar has been invested by the NIH for stem cell research. Since then, cell-based therapy is a promising option for IHD patients but numerous small clinical trials have produced mixed results.[32-34] Despite many cell types used, the quest is still on to identify the 'perfect', or, even a 'reasonably good' cell type as the basis for cardiac regenerative therapy. Amidst the high potentials, MSCs are a very promising cell type for cardiac repair, both in preclinical studies as well as clinical trials.[35-37] Combined with the fact that MSCs are harvested with relative ease, can be easily expanded, we addressed the efficacy of MSCs compared it to BMNCs in a head-to-head comparison regarding cardiac function. Interestingly, MSCs secrete a variety of growth factors –including IGF-1 and HGF- that, in concurrence with the results of **chapter 6**, can activate endogenous CSCs thereby leading to endogenous regeneration.[36] In post-MI pigs, MSCs outperformed BMNCs in terms of LV systolic function (unpublished data). As a next step, pigs that already received BMNCs were injected with MSCs. Perplexingly, similar levels of improved cardiac function observed in the MSC cohort was replicated in the initial BMNC-injected group following secondary MSC delivery. Therefore, in **chapter 7**, we report and discuss the rationale and study design of a double-blinded, placebo controlled, randomized clinical trial to investigate the effect of repetitive MSC delivery in patients with refractory angina pectoris. The RIPASSA trial is designed to advance cell therapy by: 1) testing the repetitive use of these 'next generation' MSC and 2) testing a newly developed multimodality imaging approach based on cMRI and NOGA to improve targeted delivery in the peri-infarcted area.
Placebo -Latin for '*I shall please*'- is defined as 'an inactive substance or preparation used as a control in an experiment or test to determine the effectiveness of an investigational new drug'. It is commonly implemented in clinical trials to untangle the 'true' effect of the new medicinal drug. However, in the case of autologous cell therapy delivered to the heart by a percutaneous approach, this would imply that all patients that have been allocated placebo treatment would still receive a harvesting procedure (e.g. bone marrow aspiration) as well as an intracardiac delivery of placebo by coronary infusion or intramyocardial injection. No literature exists on the subject,

but common sense would led one to argue that complications related to swallowing a placebo pill is negligible. On the contrary, placebo treatment in patients participating in autologous cell therapy trials are confronted with a very low change to experience a serious adverse event related to harvesting (i.e. hematoma, infection) or cardiac delivery (i.e. stroke, recurrent myocardial infarction, tamponade, coronary dissection). To this date, there is no published report on the ethical considerations that this imposes on the clinical scientist that designs and coordinates cell-therapy trials. Hence, if the harvesting and delivery are not performed, the patient is by definition not blinded and study outcome could be biased accordingly. In **chapter 7**, we have taken a first step towards a statement paper by first carefully analysing how previous trials involving autologous cells delivered to the heart were designed and conducted. We have shown, to our surprise, that approximately 57% of all trials were designed to investigate efficacy of cell therapy without placebo-controlled subjects as comparison. Of the remainder 43%, both harvesting as well as placebo infusion was performed to ensure patient blindness. Hence, based on the observations described in **chapter 7**, the current heterogeneity in trial design underscores the necessity of consensus with regard to placebo controlled cell-therapy studies, as risk for patients must be weighed against potentially biased study results. Ethical considerations cannot be expressed as a probability value with a <0.05 significance cut-off. Nevertheless, we propose to tailor the clinical trial design of autologous cell therapy trials so that two prerequisites are met: 1) study aimed to investigate efficacy must use a placebo controlled double blinded trial design to ensure the validity of results, meanwhile, 2) to minimize the risk for the patients that participate in such trials, a 1:1 treatment versus placebo ratio must be avoided and replaced by smart powering of the study so that a bare minimum of patients will receive placebo.

PART FOUR
OPTIMIZATION OF THE PORCINE MYOCARDIAL INFARCTION MODEL

Approximately 95% of animal experiments are based on rodents, in particular mice (*Mus musculus*), yet the outcome of which are intended to predict human response. For the heart, this imposes severe limitations on the usability of rodent data. For instance, large differences exist between both in terms of heart rate, intracardiac pressure volume relationships and metabolic rate. Hence, before testing promising new biologics in men, large animals such as the pig function as a necessary bridge to close the gap between basic science and proof-of-concept rodent data and a first-in-man study. In **chapter 9**, we described our protocol for the pig model of myocardial infarction based on intracoronary balloon occlusion. We show that 90 minutes of LAD occlusion suffices in an infarct size of ~25% of the left ventricle. The protocol has been developed partly based on work published in this thesis with the LCX occlusion. Although our LCX model led to a declined EF, increased EDV and increased restrictive diastolic function (**chapter 3 and 6**), this small range in functional decline could be improved to detect effect of a given new treatment modality. Hence, the LAD-model we developed indeed resulted in a larger ischemic insult and subsequent post-MI remodelling whilst only increasing mortality by 5% to 16%. By producing an easy-to-follow protocol and accompanying video, researchers

that aim to validate new therapies for ischemic heart disease could use this protocol as the basis for their own experiments.
As a next step, we sought to validate a novel way to quantify angiogenesis and capillary density in the infarcted area. To this end, we tested whether minimally invasive measurement of intracoronary pressure-and flow derived parameters could reflect microvascular resistance in our pig model of myocardial infarction. **Chapter 10** described the increase in hyperemic microvascular resistance in the infarcted coronary artery following MI. Furthermore, this was associated with a decrease in capillary density, thereby highlighting that, under hyperemic conditions, capillaries account for ~ 75% of the resistance. Since this study was conducted before the development of the LAD infarction model, it is worthwhile to reproduce these results for the LAD-MI model as well.

CLINICAL IMPLICATIONS AND FUTURE PERSPECTIVES

This thesis aimed to elucidate and advance strategies for cardioprotection and cardiac repair, translate preclinical findings towards future clinical studies and optimize the translational research process for IHD.
So far, many cardioprotective strategies against reperfusion injury could not be successfully replicated in clinical studies, despite promising results in experimental studies. Failure to translate these promising findings could be the consequence of several factors, such as lack of testing in a large animal model, administration of the drug at a clinically irrelevant timepoint (i.e. prior to induction of ischemia) or fundamental differences in reperfusion injury in humans compared to laboratory animals. Fortunately, evidence in favor of reperfusion injury in humans is present rendering it a promising target to reduce cardiomyocyte loss.[38] We designed a dose-optimizing study in pigs to investigate a clinically applicable treatment protocol. The outcome is presented in **Chapter 3**, showing for the first time that necrostatin-1 is also effective at reducing infarct size in a large animal model of I/R injury. Hence, these findings support the rationale to continue work on necrostatin-1. In particular, it is imperative that future work focuses on pharmacokinetics and safety profile as a prerequisite to advance towards a first-in-man clinical trial.
The presence and growth factor mediated recruitment and activation of eCSCs in **chapter 6** identify IGF-1 and HGF as one of many possible combinations of cardiotrophic factors to stimulate endogenous cardiac repair. Future work should investigate and identify key mediators to activate and bring about a robust regenerative response in the heart. In particular, cellular senescence in eldery patients that could impair this regenerative response should be studied to ensure that rejuvenation of eCSCs would lead to more effective regenerative therapy. As shown in **chapter 6**, biomaterials can facilitate local delivery of biologics and most importantly, can increase the effectiveness of new cardioregenerative therapies. It is imperative that ongoing development of biomaterials such as the UPy-gel system is aimed at increasing the tunability of its growth factor release and its biodegradation *in vivo*.

In summary, cardioprotection and cell based cardiac repair both represent unique and exciting research fields which should not be viewed as direct competitors but rather can act as adjuvant therapies that collectively provide new treatment options for patients that endure the burden of IHD. As the first phase III trials are currently underway in both research areas, cardioprotection and cell based therapy could provide new options for IHD patients in the foreseeable future.

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PART FIVE | DISCUSSION AND SUMMARY

CHAPTER **12**

Samenvatting in het Nederlands

Gedurende dit 3-jarige promotietraject, zijn 22 miljoen mensen overleden wereldwijd aan de gevolgen van ischemisch hartlijden.[1] Het acute myocardinfarct (MI) leidt tot een onomkeerbaar verlies van contractiele hartspiercellen (cardiomyocyten), en geen van de huidige behandelingen is in staat om dit ontstane tekort in cardiomyocyten te herstellen.[2] Na het acute myocardinfarct, leidt remodellering van het hart tot verder verlies van functionele cardiomyocyten. Hoewel het verlies van cardiomyocyten beduidend lager is in vergelijking met het massale verlies tijdens een hartinfarct, kan het verlies aan cardiomyocyten nog steeds 100-voudig verhoogd zijn ten opzichte van myocyten sterfte in het gezonde hart.[3] Met bovenstaande inachtneming zijn er daarom twee verschillende invalshoeken denkbaar die beiden even beloftevol lijken om de negatieve spiraal van het verlies van cardiomyocyten in verschillende fasen van ischemische hartziekten te doorbreken: 1) strategieen die tijdens het acute moment van een myocardinfarct de cardiomyocyten beter bestendig maken tegen het zuurstofgebrek zodat meer cardiomyocyten dit kritieke moment overleven, of, 2) cardiale regeneratieve geneeskunde dat gericht is op het vervangen van de verloren cardiomyocyten door nieuwgevormde cardiomyocten.
In dit proefschrift zijn beiden strategieen onderzocht, dat wil zeggen cardioprotectie tegen reperfusieschade en cardiale regeneratie in het reeds beschadigde na een myocardinfarct. Het experimentele onderzoek wat beschreven staat in dit proefschrift was ontworpen met de klinische toepasbaarheid in ogenschouw nemend. Hierdoor zijn veel experimenten uitgevoerd in het groot proefdier model van het myocardinfarct, teneinde de generaliseerbaarheid naar de humane situatie van hart- en vaatziekten te kunnen vergroten zodat er een brug wordt geslagen tussen basaal onderzoek enerzijds en de klinische patientenzorg anderzijds.

PART ONE
CARDIOPROTECTIE

Het acute myocardinfarct is in het leeuwendeel gevolg van voortschrijdende verkalking (artherosclerose) van de coronairarteriën, waar bij zich een bloedstolsel vormt op de plek waar de kwetsbare vaatwand is gescheurd. Dit heeft als gevolg dat de bloedvoorziening volledig wordt geblokkeerd, hetgeen leidt tot massale sterfte van cardiomyocyten. Huidige behandeling bestaat uit het zo snel mogelijk openen van de afgeloten coronairarterie en medicamenteus ingrijpen op de vorming van het bloedpropje door remming van de geactiveerde bloedplaatjes.[4]
In **hoofdstuk 2** geven wij een overzicht van de huidige stand van zaken in het onderzoeksgebied dat zich richt op de behandeling van reperfusieschade, ofwel de paradoxale schade die ontstaat in het ischemische gebied nadat de bloedvoorziening is hersteld. Verschillende vormen van celdood (bv apoptose, necrose en necroptose), het concept van reperfusieschade en de huidige resultaten met pharmacologische therapieen om reperfusieschade te beperken worden beschreven. Uit **hoofdstuk 2** komt een aantal conclusies naar voren, namelijk: 1) het vóórkomen van reperfusieschade in het hart wordt ondersteund door een groot aantal studies in proefdieren en in mensen, en geschat wordt dat ~50% van het uiteindelijke infarctlitteken; 2) het concept van geprogrammeerde necrose, ofwel necroptose, is een nieuw en belangrijke vorm van celdood in het hart na reperfusie; 3) Reperfusieschade is een succesvol doelwit voor medicamenteuze

experimentele behandeling gebleken waarbij cardioprotectie tijdens het acute myocardinfarct de overleving van cardiomyocyten kan bevorderen, en tot slot, 4) de vertaling van succesvolle behandeling van reperfusieschade in kleine proefdieren naar grote proefdieren of de mens is teleurstellend laag.
Om die reden, hebben wij onderzocht in hoofdstuk 3 of de succesvolle resultaten van een nieuw middel tegen necroptose, genaamd necrostatine-1, behalve in muizen5, ook kon worden gereproduceerd in varkens. De hartgrootte, hartfunctie en hemodynamiek van varkens lijken nauwgezet op die van de mens.6 Uit de resultaten van de studie blijkt dat necrostatine-1 in staat is in om de infarctgrootte te reduceren om ~50% vergeleken met infusie van alleen de oplossingsvloeistof zonder necrostatine-1. Ook was de hemodynamiek en hartfunctie van de nec-1 behandelde varkens minder sterk verslechterd ten opzichte van de controle groep. Tevens bleek uit histologisch onderzoek van het infarctgebied blijkt er een afname was opgetreden van restschade in de celkern die veroorzaakt is door zuurstofradicalen in nec-1 behandelde dieren. Bovendien was er een afname van de influx van reactieve afweercellen, genaamd neutrofiele granulocyten, hetgeen de suggestie wekt dat er mogelijk ook minder ontsteking aanwezig is in het geinfarceerde gebied na nec-1 behandeling.

PART TWO
STRATEGIEN VOOR CARDIAAL HERSTEL: PREKLINISCHE RESULTATEN

In het tweede deel van dit proefschrift wordt het onderzoek beschreven dat gericht is op het herstel van reeds opgetreden schade aan het hart en diens negatieve hermodellering. In **hoofdstuk 4** vroegen wij ons af of cardiale regeneratie optreedt in het volwassen hart van zoogdieren en zo ja, in welke mate. Aangezien de meeste weefsels in het lichaam geregenereerd worden op basis van differentiatie van stam-/voorlopercellen, namen wij de rol van hartstamcellen in het proces van cardiale regeneratie onder de loep. Uit de resultaten van hoofdstuk 4 blijkt dat er aanzienlijk wetenschappenlijk bewijs bestaat dat het vóórkomen van regeneratie van cardiomyocyten ondersteunt, ook in het volwassen hart van zoogdieren, hetzij in een zeer laag tempo optreedt. Daarna blijken de meningen sterk verdeeld over de daadwerkelijk oorzaak wat ten grondslag ligt aan de vorming van nieuwe cardiomyocyten. Enerzijds wordt bewijs aangeleverd dat gedifferentieerde cardiomyocyten zichzelf kunnen dedifferentieren, en in de nieuwe, meer primitieve status, via celdeling nieuwe dochtercellen vormen die wederom uitgroeien tot cardiomyocten. Anderzijds laten andere onderzoeksgroepen zien dat nieuwe cardiomyocyten gevormd worden als gevolg van deling van stam-/voorlopercellen, wiens dochtercellen uiteindelijk differentieren tot cardiomyocyten. In tegenstelling tot bijvoorbeeld het onderzoeksveld gericht op neuronale regeneratie, bestaat er in het cardiale regeneratie onderzoeksveld grote verdeeldheid met sterk uiteenlopende onderzoeksresultaten.
Hoofdstuk 5 beschrijft het werk dat verricht voor de ontwikkeling van een nieuwe hydrogel, in samenwerking met de Technische Universiteit Eindhoven. De voorwaarden waaraan deze nieuwe gel moest voldoen waren meervoudig. Ten eerste moest de nieuwe hydrogel minimaal invasief te injecteren zijn in het hart via een catheter zodat er geen open hart chirurgie nodig is voor de

lokale toediening. Ten tweede moest de hydrogel in het hart geleren zodat er een lokaal gelreservoir ontstaat van waaruit groeifactoren uit de hydrogel in het hart werkzaam kunnen zijn. Tot slot moest de gel biologisch afbreekbaar zijn en dezelfde rheologische eigenschappen hebben als het hart. De supramoleculaire ureido-pyrimidione moieiteiten is als basis gebruikt waarbij deze hydrogel onder invloed van pH modificatie aan alle bovenstaande eigenschappen bleek te voldoen.[7] Uit de experimenten van hoofdstuk 5 blijkt dat de pH modificatie van de UPy-gel reversibel is. Ook zijn de groeifactoren IGF-1 en HGF na contact met de basische UPy-gel (pH 9) nog steeds biologisch actief. Tot slot is in varkens de nieuwe hydrogel voor het eerste met succes geïnjecteerd. De volgende stap is beschreven in **hoofdstuk 6** waar we onderzochten wat de effectiviteit was van groeifactoren therapie met of zonder de UPy-gel als lokale afgiftereservoir. In een chronisch infarctmodel in het varken bleek ook de combinatie van groeifactoren HGF en IGF-1 direct geïnjecteerd in het randgebied van het infarct een gunstig effect te hebben op de hartfunctie en structurele remodellering van het hart. De afgifte van dezelfde concentratie groeifactoren door de UPy-gel toonde een niet-significante trend richting verbetering van de groeifactortherapie. Immunohistologische analyse van het infarctgebied toonde een toename van het aantal c-kit^{pos} CD45^{neg} cardiale stamcellen (CSC) in de groeifactorengroepen vergeleken met de controle groep, waarbij de hoogste aantallen werden gevonden in het UPy-gel-groeifactoren groep. Tot slot, wat betreft het aantal bloedvaatjes, bleek groeifactoren injectie het aantal capillairen in het geinfarceerde hartspierweefsel te bevorderen.

PART THREE
STRATEGIEN VOOR CARDIAAL HERSTEL: KLINISCHE PERSPECTIEF

In **hoofdstuk 7** beschrijven wij een studieopzet voor een nieuwe klinische trial met als doel om wetenschappelijk bewijs aan te voeren om de veelbelovende preklinische resultaten van mesenchymale stamcel (MSC) therapie te valideren. MSCs blijken met relatief gemak geoogst te kunnen worden uit het lichaam.[8] Tegelijkertijd leent deze cel zich goed voor het opkweken in het laboratorium zodat genoeg cellen kunnen worden verkregen om vervolgens terug te plaatsen. De RIPASSA trial is onderscheidend ten opzichte van eerdere studies in drie aspecten. Ten eerste zijn de meeste studies naar het gebruik van (stam)cellen tot op heden uitgevoerd met beenmerg mononucleaire cellen.[9] Uit een recente meta-analyse van onze groep bleek dat MSCs beter lijken te presteren dan beenmerg mononucleaire cellen.[10] Dit vermoeden wordt bekrachtigd door een studie in varkens waar wij deze twee cel types direct met elkaar vergeleken hebben (niet gepubliceerde data). Hieruit viel op te maken dat MSCs aanmerkelijk beter in staat zijn de hartfunctie te verbeteren in het chronisch hartinfarctmodel. Ten tweede wordt er een nieuwe strategie gebruikt teneinde de injectieprecisie te verbeteren. Dat wordt bereikt door middel van fusie van MRI beelden met de electromechanische data van het NOGA injectie systeem (niet gepubliceerde data). Tot slot is zullen wij de MSCs repetitief toedienen in vergelijking met een eenmalige toediening. Deze multicenter studie zal patiënten met refractaire angina pectoris randomiseren tussen drie groepen: placebo behandeling, eenmalig MSC toediening of tweemalige MSC toediening. De primaire uitkomst is het verschil in VO2 max 12 maanden na toediening van

de eerste therapie, vergeleken met de placebo groep.
Vervolgens vragen wij in **hoofdstuk 8** aandacht voor een lastige ethische afweging ingeval van het gebruik van placebo injecties in het hart in stamcel studies. In tegenstelling tot een nieuw te testen geneesmiddel, waarbij de placebo vaak als vergelijkbare tablet/capsule wordt aangeboden, is bij stamcelstudies zowel het nieuw te testen geneesmiddel –d.w.z. de stamcellen van de patiënt zelf- als de toediening via een katheter in het hart niet zonder risico's. Wij onderzochten de afweging van onderzoekers om al dan niet een placebo groep op te nemen in het ontwerp van de klinische studies naar het gebruik van stamceltherapie. Uit 56 gerandomiseerde klinische trials bleek in 22 studies (39%) gebruik te worden gemaakt van een placebogroep. Dat houdt in dat deze controle patienten wel een beenmergpunctie ondergingen en vervolgens een niet werkzame zout-oplossing toegediend kregen in het hart. Uit dit empirisch onderzoek blijkt dat er geen consensus bestaat over de (on)zin van placebo gebruik in klinische stamcelstudies. Er bestaat een noodzaak om tot een breed gedragen consensus te komen zodat patientveiligheid en validiteit van de onderzoeksresultaten elk worden gewogen in een aanbeveling voor toekomstige stamcelstudies.

PART FOUR
OPTIMALISATIE VAN HET VARKENSMODEL VAN HARTINFARCT

Ongeveer 95% van alle dierproeven wordt uitgevoerd in kleine proefdieren, met name de muis (*Mus Musculus*). Alhoewel deze studies lastig te generaliseren zijn richting de mens, blijken ze vaak gebruikt te worden om het effect te voorspellen van nieuwe behandelingen in de mens. Wat betreft het hart, brengt dat enkele beperkingen met zich mee. Zo zijn er bijvoorbeeld grote verschillen wat betreft de hartfrequentie, drukken in het hart en metabolisme van het hart. Omdat het varkenshart veel meer op het mensenhart lijkt, beschrijven we in hoofdstuk 9 de noodzakelijke onderzoeksmethodologie om nieuwe experimentele behandelingen te testen in een varkensmodel van het hartinfarct. We tonen dat 90 minuten occlusie van een deel van de linker anterior descenderende coronairarterie (LAD) een infarct creert van ongeveer 25% van de linkerkamer. Het protocol van **hoofdstuk 9** is verfilmd en staat open-access op de site van the Journal of Visualized Experiments, teneinde onderzoek wereldwijd met gebruik van grote proefdieren te bevorderen. Ons einddoel hierbij is dat meer veelbelovende basale ontdekkingen gevalideerd zullen worden in grote proefdieren, hetgeen moet leiden tot betere behandelingmogelijkheden van tot nu toe uitbehandeld ischemisch hartlijden.
Tot onderzoeken we in **hoofdstuk 10** een nieuwe manier om angiogenese en dichtheid van capillairen te bepalen in het geinfarceerde hart. Dit is belangrijk omdat de tot nu toe gebruikte methode, namelijk histologie van weefsel, zich per definitie niet leent voor een serieele meting. In de varkensstudie van hoofdstuk 6 hebben wij intracoronaire druk-/flow afgeleide parameters gebruikt om het effect van het infarct op capillaire dichtheid te meten. De hyperemische microvasculaire weerstand bleek toe te nemen vier weken na het induceren van het hartinfarct. Op histologisch niveau werd een afname van de capillaire dichtheir gezien en een verdikking van de laag gladde spiercellen van de arteriolen, mogelijk als gevolg van veranderde flow na het infarct.

KLINISCHE BETEKENIS EN TOEKOMSTVISIE

Dit proefschrift heeft tot doel om nieuwe behandelingen voor cardioprotectie en cardiaal herstel te onderzoeken, om preklinische basale resultaten te vertalen naar onderzoek in grote proefdieren en om dit translationeel proces te bevorderen. Tot dusver zijn er veel cardioprotectieve middelen met succes getest in experimentele dierstudies; het grootste deel van deze resultaten konden niet in klinische studies gereproduceerd worden. Hieraan kunnen meerdere redenen ten grondslag liggen, zoals het ontbreken van grote proefdierstudies, veranderingen in de tijd en/of toedieningswijze van het nieuwe geneesmiddel of fundamentele verschillen in het optreden van reperfusieschade tussen mensen en proefdieren. Gelukkig is er toenemend bewijs dat reperfusieschade wel degelijk optreedt in de mens, hetgeen dus als substraat kan worden gebruikt om de gevolgen van een hartinfarct te beperken. Met bovenstaande inachtneming hebben wij een studie opgezet en uitgevoerd in varkens met als doel om de eerder behaalde resultaten te valideren in grote proefdieren. De resultaten uit hoofdstuk 3 tonen aan dat het middel necrostatine-1 in elk geval in staat zijn in een hart vergelijkbaar met dat van de mens de gevolgen van het acuut hartinfarct drastisch te beperken. De volgende stap is gedegen onderzoek naar pharmacokinetiek en het veiligheidsprofiel alvorens een eerste studie in mensen op te zetten.

De aanwezigheid van cardiale stamcellen en de mogelijkheid om deze te activeren door groeifactoren zoals beschreven in hoofdstuk 6 tonen aan de IGF-1 en HGF een van de vele mogelijkheden zijn van groeifactoren die endogeen cardiaal herstel kunnen bevorderen. Toekomstig onderzoek zou naar mijn idee zich moeten richten op het ontrafelen en identificeren van essentiele factoren die deze regeneratieve respons gunstig kunnen beinvloeden. Een van de hordes die genomen moet worden is de constatering dat cellulaire senescentie –ofwel het onvermogen van cellen om de cellcyclus te activeren- frequent optreed in de oudere patientenpopulatie, een fenomeen dat deze regeneratieve respons negatief kan beinvloeden. Tevens laten de resulaten in hoofdstuk 6 zien dat biomaterialen de lokale afgifte van bioactieve stoffen kan faciliteren. Verder onderzoek binnen dit opkomende onderzoeksgebied moet op termijn de mogelijkheid bieden om de biomaterialen dusdanig te beinvloeden dat de afbraak van het biomateriaal en de afgifte volledig gereguleerd kan worden.

In conclusie, cardioprotectie en cardiaal herstel zijn beide unieke en veelbelovende onderzoeksgebieden die niet moeten worden beschouwd als directe tegenhangers; mogelijkerwijs kunnen zij bij toekomstige patienten beiden als adjuvante therapieen worden gebruikt om de last van ischemische hartziekten drastisch te verminderen. Gezien het feit dat beide onderzoeksvelden recent de eerste phase III trials zijn gestart, zou kunnen betekenen dat er nieuwe behandelopties zijn voor ischemische hartzieken in de nabije toekomst.

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PART SIX | APPENDIX

CHAPTER **13**

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Na 3 jaren keihard werken is het dan zover: mijn proefschrift is af!! Alhoewel, mijn? Inderdaad; onderzoek is teamwerk. En velen die in dit proefschrift hun eigen inbreng herkennen ben ik dan ook zeer dankbaar. Zonder iemand tekort te doen, wil ik graag een aantal in het bijzonder noemen.

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Aan veel artikelen in dit proefschrift hebben verschillende studenten hard meegewerkt. Aafke, Jasmijn, Justin, Moira, Patricia en Patrick, bedankt hiervoor en heel veel succes met jullie eigen (onderzoeks)pad!

En dan nu een illuster tweetal in het bijzonder, mijn bunckerbuddies Frebus van Slochteren en Tycho van der Spoel. Allereerst harde werker Tycho…kuch…man...! Het punt is dat ik je heel erg ben gaan waarderen en een voorbeeld neem aan de tomeloze werklust waarmee jij je hebt ingezet voor Champie's stamcelclub. Alhoewel ik nog steeds niet goed snap hoe je moet leven als een hart, weet ik door al die jaren heen dat jouw hart absoluut op de goede plek zit. Bedankt voor al je adviezen en dat we maar veel mogen blijven tennissen ondanks alle drukte in de kliniek! Dan Slootje, met je aanstekelijke vrolijkheid kennelijk resistent voor de barre omstandigheden van de Buncker. In het holst van het UMCU, ver verscholen van daglicht, hebben we mooie projecten gelanceerd en heb ik veel aan je gehad. Mooi dat je mijn paranimf wilt zijn en je bent altijd welkom voor de befaamde Ko&Slo Mc-Sunny-Side-Up burger, die is ontwikkeld na de Sherman meeting in het ijskoude NY.

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LIST OF ABBREVIATIONS

AAR	Area at Risk
AMI	Acute Myocardial Infarction
APV	Average Peak Velocity
BMCs	Bone marrow mononuclear cells
BrDU	Bromodeoxyuridine
c-kitpos	c-kit positive
CAD	Caspase-activated DNAse
CAD	Coronary Artery Disease
CCS scale	Canadian Cardiovascular Society scale
CCU	Coronary Care Unit
CD45neg	CD45 negative
CDC	Cardiac Derived Cell
CFVR	Coronary Flow Velocity Reserve
CHF	Congestive Heart Failure
CK	Creatinin kinase
CK-MB	MB-isoenzyme fraction of CK
CM	Cardiomyocyte
CMPC	Cardiac Myocyte Progenitor Cell
cMRI	Cardiac Magnetic Resonance Imaging
CPET	Cardio Pulmonary Exercise Test
CsA	Cyclosporin A
CSC	cardiac stem/progenitor cell
CTRL	control
DMSO	Dimethyl sulfoxide
E/E'	Ratio of transmitral flow velocity to annular peak diastolic velocity
ECM	Extracellular Matrix
eCSC	endogenous cardiac stem/progenitor cell
EDV	end diastolic volume
EF	ejection fraction
EMM	Electro Mechanical Map
ESC	European Society Cardiology
ESCs	Embryonic Stem Cells
ESPVR	End-systolic Pressure Volume Relationship
ESV	end systolic volume
ESV100	End Systolic Volume at 100mmHg
FAS	fractional area shortening
GF	growth factors IGF-1/HGF
GFP	Green Fluorescent Protein
GMP	Good Manufacturing Practices
Hccs	Holocytochrome c synthase
HGF	hepatocyte growth factor

HMR	Hyperemic Microvascular Resistance
HR	Heart Rate
I/R injury	Ischemia/Reperfusion Injury
IGF-1	insulin-like growth factor-1
IHD	Ischemic Heart Disease
iPSs	Induced Pluripotent Cells
IS	Infarct Size
LAD	left anterior descending artery
LCx	left circumflex artery
LDH	Lactate Dehydrogenase
LV	left ventricle
MACE	Major Adverse Cardiac Event
MAP	Mean arterial pressure
MI	myocardial infarction
mPTP	Mitochondrial permeability transition pore
MSCs	Mesenchymal Stem Cells
Nec-1	Necrostatin 1
PARP	Poly ADP-Ribose Polymerase
PBS	Phosphate buffered saline
PCI	Percutaneous Coronary Intervention
PEG	Polyethylene glycol
PV	Pressure-Volume
RAP	Refractory Angina Pectoris
RIP-1	Receptor-Interacting Protein 1
RISK	Reperfusion Injury Salvage Kinase
ROS	Reactive Oxygen Species
RT3DE	real-time 3-dimensional echocardiography
Sca-1	Stem-cell antigen-1
SP	Side Population
SWT	Systolic Wall Thickening
TNF-α	Tumor Necrosis Factor- alpha
TTZ	Triphenyl-tetrazolium chloride
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labelling
UPy	ureido-pyrimidinone moieties
UPy-GF	growth factors IGF-1/HGF embedded in UPy hydrogel
WT	Wall Thickness
αSMA	Alpha Smooth Muscle Actin

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CURRICULUM VITAE

Stefan Koudstaal werd op 16 januari 1985 bij een gevoelstemperatuur van -11.4 °C geboren in de forenzenstad Hellevoetsluis. Na 8 jaar verhuisde het jonge gezin Koudstaal naar Rotterdam, waar hij in 2003 zijn gymnasium diploma behaalde aan het Marnix Gymnasium te Rotterdam. In datzelfde jaar begon hij de studie geneeskunde aan de Universiteit Utrecht. Hij heeft in 2005 zijn studie voor een jaar onderbroken om zitting te nemen in het bestuur van de faculteitsvereniging MSFU "Sams". Na aanvang van de co-schappen in 2006 werd zijn interesse gewekt voor de cardiologie, waar hij in 2008 een keuze co-schap liep bij de cardiologie in het Meander MC in Amersfoort onder supervisie van dr. P.J. Senden. Een wetenschappelijke basis werd in datzelfde jaar gelegd tijdens een onderzoeksstage onder leiding van dr. M.J. Cramer, bij dr. B. de Boeck en drs. G.E. Leenders. Na het behalen van de artsenbul in 2010 vervolgde hij zijn onderzoek, onder leiding van dr. S.A.J. Chamuleau en prof dr. P.A.F.M. Doevendans, naar nieuwe behandelingen van chronisch ischemisch hartlijden. Dit heeft geleid tot een 3-jarig promotietraject. Tijdens dit traject heeft hij tegelijkertijd met goed gevolg de post-graduate master epidemiologie doorlopen en is sinds voorjaar 2014 geregistreerd als klinisch epidemioloog. Na zijn promotietraject is hij sinds 1 november 2013 klinisch werkzaam als cardioloog in opleiding bij de interne geneeskunde van het Diakonessenhuis Utrecht (opleider: dr. A.F. Muller). In 2016 zal hij in het UMCU zijn cardiologie opleiding vervolgen onder supervisie van zijn hoofdopleider, Dr. J.H. Kirkels. Hij is gelukkig getrouwd met Jolein en zij wonen in Utrecht.