ADVANCED CELL-BASED CARDIAC REPAIR

HOW TO MEND A BROKEN HEART

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PART 1

Background and Introduction

CHAPTER 1

General Introduction and outline of this thesis

GENERAL INTRODUCTION

Cardiovascular disease is still the leading cause of death worldwide, accounting for approximately 13 million deaths a year.[1] It is estimated that this number will double in the upcoming 15 years due to aging of the population in combination with an increase of risk factors as hypertension, diabetes mellitus, hypercholesterolemia and obesity. Approximately half of the cardiovascular deaths are related to coronary artery diseases.[1] Ischemic heart failure (HF) is usually caused by an acute myocardial infarction (AMI). An AMI develops when a coronary artery is occluded, whereupon the myocardium is deprived of nutrients and oxygen, which results in apoptosis of the cardiomyocytes. In the initial phase following an AMI, an inflammatory process occurs to clear the wound from death cell debris and activate reparative pathways.[2] This process is followed by the formation of scar tissue. The remaining viable myocardium has to take over the function of the damaged area and compensate for the loss to maintain cardiac output. Eventually the viable myocardium will be unable to do so, whereupon the ventricle dilates, pump function is reduced and the heart starts to fail (figure 1).[3,4]

The current treatment strategy for an AMI, which consists of immediate revascularization in combination with optimal pharmacological treatment, has resulted in improved survival, even of severe cases. This increased survival comes at the cost of an increased prevalence of heart failure. Therefore the search for new therapies to limit the damage of an AMI or reverse HF is ongoing. New therapeutic strategies are continuously developed and investigated in preclinical and clinical settings. Stem cell therapy is one the therapeutic strategies that has emerged in the field of clinical cardiology in the past decade.

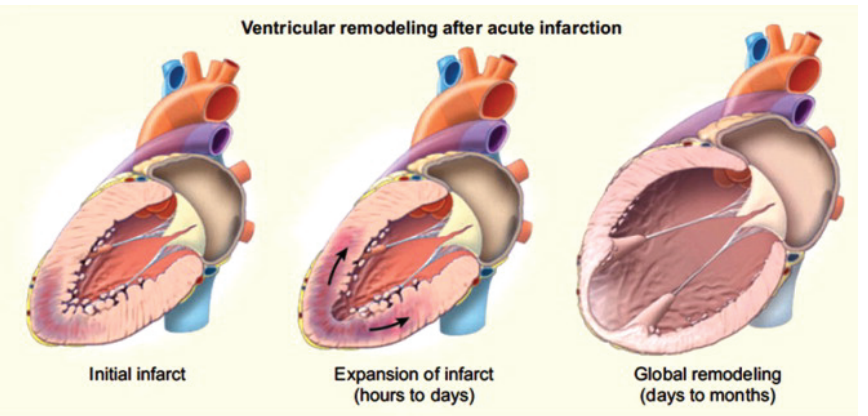

Figure 1. Development of heart failure following an acute myocardial infarction

During and after an acute myocardial infarction (an apical infarction in this case) no clinically significant changes are observed in overall ventricular geometry (left panel). Within hours to days, the area of myocardium affected by the infarction begins to expand and becomes thinner (mid panel). Without treatment, during weeks to months following AMI, global remodeling can occur, resulting in dilatation of the ventricle and decreased systolic function. In some cases, this leads to mitral-valve dysfunction and the formation of an aneurysm (right panel)(Adapted from Jessup *et al*, NEMJ, 2003).[4]

First generation stem cells

A stem cell is an undifferentiated cell that has a multi-lineage potential. The interest in stem cell therapy began more than a decade ago when researchers discovered that some types of stem cells could transdifferentiate into cardiomyocytes in vitro and in vivo.[5] Thereby high hopes were set that these cells could replace the damaged myocardium.

The first candidates for cell-based cardiac repair were skeletal myoblast (SkM), progenitor cells that will become striated skeletal muscle. The initial results in a rodent model of HF were promising and first-in-man clinical trials followed.[6–8] After the first clinical trials, the enthusiasm faded.[8–10] The trials revealed that cardiac function did not improve following intramyocardial SkM injections. Moreover, SkM transplantation resulted in an higher incidence of ventricular arrhythmias (this thesis).[8,11] Therefore, skeletal myoblasts were omitted in clinical trials for heart repair.

In parallel with SkM, bone marrow mononuclear cells (BMMNCs) emerged for the treatment of AMI and post-AMI HF. BMMNC is the whole fraction of bone marrow derived stem cells that is obtained following bone marrow puncture, making this an easy and fast source of stem cells. The bone marrow mononuclear subset consists of hematopoietic progenitor cells, that give rise to red and white blood cells and several other cell types, mesenchymal stem cells (MSC) and endothelial progenitor cells.[12] The first clinical application was preceded by a pivotal mouse study that showed that BMMNC cells could regenerate infarcted hearts by transdifferentiation into cardiomyocytes.[5] The first clinical trials that investigated BMMNCs for the treatment of AMI have proven that intracoronary injection is safe and also suggested therapeutic efficacy, whereupon many trials followed.[13,14] To date, BMMNC therapy improves left ventricular ejection fraction (LVEF) overall by only +2-3% (this thesis).[15] Some trials showed an improvement in cardiac function, while others failed to show any benefit what could be related to the design differences between the clinical trials. Many questions regarding cell-based AMI repair were unanswered when the first clinical trials were initiated, for example: the correct cell number; the optimal time point for injection; the optimal method for stem cell isolation etcetera. To date, many issues yet remain unanswered. The modest effects of BMMNC therapy raised the question whether this cell type is optimal for heart repair. Moreover, preclinical trials and first-in-man clinical trials were emerging with new cell types, like the autologous enriched cells (CD34+/CD133+), autologous mesenchymal stem cells and cardiac derived cells that seem to have more potential.

How does stem cell therapy work?

The working mechanism of stem cell therapy depends on the underlying disease. Stem cell therapy for the treatment of AMI is based on reducing the initial infarct damage and limit infarct size, whereas treatment of HF is based on regenerating scar tissue, blood supply improvement and inhibition of ventricular remodeling. This part only focuses on AMI patients since this is the main topic of this thesis. In the initial phase of stem cell therapy, it was suggested that the first generation stem cells, the BMMNCs, could transdifferentiate into cardiomyocytes, but this was found to be controversial.[16] Preclinical research followed that aimed to investigate the true mechanism of action of BMMNC and other stem cells. Currently, it is believed that the effects of stem cell therapy in general are based on

the release of paracrine factors by the cells. The release of paracrine factors is known to 1) reduce apoptosis thereby reducing infarct scar size, 2) stimulate angiogenesis, 3) be immunomodulatory, 4) improve wound healing, 5) stimulate homing of endogenous stem cells (including cardiac stem cells) to the site of injury.[3,17] It was found that BMMNCs released several paracrine factors, and newer generations of stem cells emerged with a better expression profile for cardiac repair: the autologous enriched cells (second generation). Although BMMNCs are still investigated in clinical trials to date, newer, more potent stem cells are gradually taking over.

Mesenchymal (-like) stem cells

One of the cell types that emerged as potential candidate for heart repair is the mesenchymal stem cell (MSC), that can be derived from a variety of tissues, including bone marrow, liver tissue and adipose tissue.[18,19] MSC are able to differentiate into cardiomyocytes in vitro and also in vivo, but their primary mechanism of action is based on secretion of paracrine factors that are beneficial for cardiac repair following AMI.[20] Data from multiple preclinical studies suggest that the effect of BMMNC is related to the MSC fraction.[21,22] MSC are only a small fraction within the bone marrow (0.001%-0.01%). This indicates that transplantation of 10^9 BMMNC results in only 10^4-10^5cells MSC entering the coronary system. MSC can be easily isolated following bone marrow puncture via plastic adherence, whereupon they can be culture-expanded.[18] Culturing of MSC is time consuming and can take up to 1-2 months. The effects of cell therapy for AMI are based on the release of paracrine factors that enhance wound remodeling, angiogenesis and reduce apoptosis in the acute phase, directly following AMI. Autologous MSC derived from the bone marrow are therefore not suitable for application in AMI, but they might be convenient for application in HF.

An advantage of MSC is their immunological status. MSC lack HLA-II and co-stimulatory factors, they suppress innate immune cells and a T-cell mediated immune response.[20,23] This makes them suitable for allogeneic transplantation without the need for immune suppressive drugs. Allogeneic transplantation has several advantages over autologous transplantation: 1) no painful, time-consuming harvesting procedures are needed; 2) cells are directly available as an off-the-shelf product that can be directly administered following an event; 3) cells are derived from a healthy donor. Clinical research using allogeneic MSC is currently performed in larger preclinical studies and in clinical trials.

As stated above, MSC can be isolated from a variety of tissues. Adipose tissue contains mesenchymal-like stem cells; the adipose tissue derived regenerative cells (ADRC), that, just like MSC, stimulate neo-angiogenesis and cardiomyocyte survival both *in vitro* and *in vivo* by release of various angiotrophic, anti-apoptotic and immunomodulatory factors.[24,25] Sufficient numbers of ADRCs for transplantation can be isolated out of 200 ml adipose tissue that can be obtained by liposuction that is performed directly following AMI. No cell culturing processes are needed and the ADRCs are returned to the patient within 24 hours after the event. ADRCs are currently investigated in clinical trials.

Biomaterials

Besides the discussion on cell type, many other issues remain in the field of cell therapy. One major issue of stem cell therapy is the retention of stem cells in the myocardium. As described above, the effects of stem cells are related to the release of paracrine factors. When many cells are washed out of the myocardium within hours to days, less paracrine factors are released into the myocardium. This results in a decreased efficacy of the new therapy. Biomaterials are developed to improve cell retention. The first biomaterials that were developed in cell therapy were cell sheets that consist of a (soluble) biomaterial and a population of stem cells. Cell sheets were transplanted directly on the epicardial site of the heart during bypass surgery.[26] They are still used to date containing newer generations of stem cells. Their application method makes them however unsuitable for the treatment of an AMI. To improve cell retention of cells in the AMI population, but also in HF, MSC were encapsulated in a highly biocompatible alginate biopolymer (CellBeads™). The idea of encapsulating cells derived from the oncology field, where drug-eluting beads are used.[27,28] The hypothesis of the mechanism of action of encapsulated MSC for cardiac repair is that following intracoronary infusion, the encapsulated MSC will get stuck in the pre-capillary bed due to their size. Here they will locally release paracrine factors that are produced by the MSC for a longer period of time. Moreover, the MSC inside the bead are protected from a host immune response (figure 2). [29]

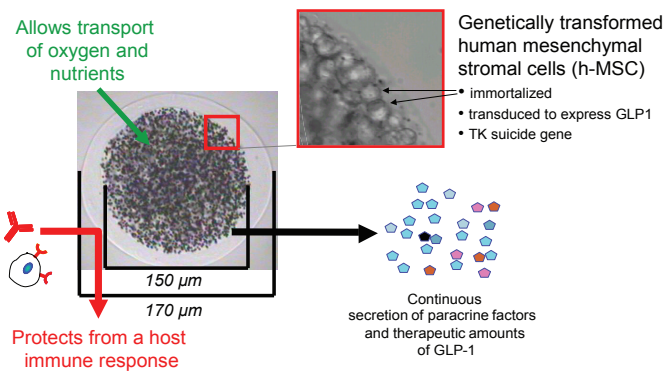

Figure 2. Encapsulated Mesenchymal stem cell

The MSC (grey dots) are genetically modified to produce glucagon-like peptide-1 (GLP-1). The alginate shell surrounding the MSC allows influx of oxygen and nutrients into the cells while protecting them from a host immune response (adapted from Wallrapp *et al*, 2013)[29]

The encapsulated MSC are programmed to produce glucagon-like peptide-1 (GLP-1) that has known cardioprotective capacities. In a preclinical trial and two clinical trials it has been shown that GLP-1 reduces infarct size, thereby improving cardiac function.[30–32] To investigate the safety and feasibility of GLP-1 eluting MSC a pilot study was conducted in the naïve and infarcted porcine myocardium (this thesis). The promising results of the pilot study have resulted in the design of a large dose finding study investigating the long-term safety and feasibility of intracoronary infusion of GLP-1 eluting MSC in a moderate and severe porcine AMI model (this thesis).

Animal models

Before a new therapy can be tested in clinical trials, it has to be extensively explored in models that are representative for the human situation. The infarct process, remodeling of the ventricle and thereby the development of HF is a multifactorial complex process that cannot be mimicked by computer models. Most first stage research that is implemented for safety and efficacy is performed in rodents, mostly mice. Rodent studies can be performed quickly and without large variability (ea. same genetic strain), but translational from results to the human situation is limited. The anatomy of the mouse heart differs from the human heart, not only in size but also for example in heart rate and heart-to body ratio which could influence study results. Another problem is application of new therapies, for example stem cells, to the heart. Minimally invasive methods, like intracoronary infusion or endo-ventricular injection are preferable in the clinical setting, but are not applicable in small-sized hearts.[15] Moreover, translation from a large animal model to human is more convenient.

Therefore, animal models that are representative, relevant and reproducible for human AMI and HF are crucial and need to be employed. The optimal animal model for cardiac diseases is the porcine heart, because 1) heart-to-body ratio is comparable[33]; 2) they have a similar conduction system[34]; 3) the same coronary anatomy[35,36] and 4) a comparable metabolism.[37]

There are several different ways to induce HF in a pig, knowing: 1) pressure overload via aortic banding; 2) volume overload by mitral regurgitation or arterial-venous shunt formation; 3) tachycardia-induced cardiomyopathy; 4) myocardial ischemia.[33,38–42]

Myocardial ischemia can be divided into chronic myocardial ischemia, which is induced by permanent ligation, a bottleneck stent model, infusion of ethanol or coronary artery coiling[31,39,43,44], or ischemia-reperfusion, in which the target coronary artery is revascularized after a period of ischemia. HF that develops following an AMI is best mimicked by an animal model of ischemia reperfusion, as the cornerstone in AMI treatment is revascularization. Therefore, this model was applied and further explored in several chapters in this thesis.

AIM AND OUTLINE OF THIS THESIS

As stated above, many questions and issues remain regarding cell-based cardiac repair. The aim of this thesis was to solve some of these issues and investigate new types of cell therapy for the treatment of AMI. This was executed via research in preclinical animal studies and clinical trials that were developed based on the results of preclinical studies.

Part 1 of this thesis consists of a general introduction on cell-based cardiac repair. **Chapter 2** gives an overview of the different generations of stem cells that are investigated to date. This chapter is followed by two meta-analyses in which the effects of stem cell therapy in general and BMMNC therapy in particular for the treatment of an AMI and HF was investigated (**Chapter 3 and 4)**.

Part 2 of the thesis is devoted to a new generation of stem cells, the ADRCs, that include mesenchymal-like stem cells and endothelial progenitor cells. ADRCs are isolated from the patient's adipose tissue in the first couple of hours following AMI using specialized enzymes and devices. This isolation protocol is described in **chapter 5**. The ADRCs were directly intracoronary infused into the target coronary artery

after isolation. **Chapter 6** describes the results of the APOLLO trial that consisted of 14 patients of whom 10 patients were treated with ADRCs.

As described before, obtaining stem cells for the treatment of cardiovascular disease is a burden for the patient who is already ill. Moreover, stem cell quality of an older patient with comorbidities is less compared to that of a healthy 18 year old donor. Therefore, stem cells that could be given in an allogeneic setting are currently investigated. MSC and their precursor cells (MPC) lack HLA-DR which prevents a T-cell mediated immune response. This, in combination with their paracrine expression profile, makes them suitable candidates for an off-the-shelf therapy. The safety and efficacy of intracoronary infusion of allogeneic MPC was investigated in a sheep model of anterior wall infarction as described in **chapter 7.** The sheep were subjected to an AMI where after they were randomized to 3 dosages of MPC or placebo solution. This was infused 30 minutes after reperfusion into the target vessel. The animals were sacrificed at 8 week follow-up and cardiac function was determined by 2D-echocardiography and pressure-volume loop analysis. Histological analysis was performed to investigate the mechanism of MPC-based repair. The promising results of this study resulted in the design of the AMICI trial, a first-in-man randomized controlled trial in which 225 patients will be subjected to intracoronary infusion of allogeneic MPC directly following revascularization (**Chapter 8**).

Part 3 of this thesis is dedicated to the encapsulated mesenchymal stem cells (CellBeads). Retention of stem cells remains a hurdle in cell-based cardiac repair as described above. MSC were encapsulated to overcome this issue. The safety and feasibility of encapsulated MSC in a porcine AMI model is described in **chapter 9.** The efficacy of this novel product was investigated in large preclinical study. One-hundred female pigs were subjected to an AMI followed by intracoronary infusion of encapsulated MSC or a placebo solution. The pigs were sacrificed eight weeks after infarct induction and cardiac function was assessed by echocardiography and PV-loop analysis (**chapter 10**)

Translation from bench to bedside is an important in preclinical research. Therefore relevant animal models have to be developed, alongside accurate methods to assess outcome. Revascularization is a cornerstone in patients with an AMI. The optimal animal model for the treatment of an AMI or ischemic HF is an ischemia-reperfusion model. In **Chapter 11** ischemia-reperfusion of the left descending coronary artery (LAD) is compared to ischemia-reperfusion of the left circumflex artery (LCx) to investigate the influence of infarct location on infarct size and cardiac function.

Several modalities can be used to assess cardiac function. Echocardiography is a widely used method to assess cardiac function. Pressure-volume loop analysis is an invasive hemodynamic measurement in which specialized catheters are placed into the left ventricle where they locally assess volumes, ejection fraction and additional cardiac parameters. **Chapter 12** describes a study in a porcine AMI-model that compares 3D-echocardiography with 2 methods of pressure volume loop analysis, admittance and conductance, to investigate superiority of one of these systems in assessing left ventricular ejection fraction and volumes opposed to 3D echocardiography.

In **chapter 13**, all results described in this thesis are discussed as well a future prospectives regarding cell-based cardiac repair. Finally, in **chapter 14** this thesis will be summarized.

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CHAPTER 2

A concise review on cell-based therapies for cardiovascular repair: What the clinician needs to know

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INTRODUCTION

Stem cell therapy to heal scarred myocardium or abrogate adverse remodeling following acute myocardial infarction (AMI) has raised high hopes over the past decades. As stem cells are by definition multipotent cells, in theory they can differentiate into cardiomyocytes and replace scar tissue. Moreover, many stem cell types were found to secrete high levels of cardioprotective proteins and pro-angiogenic factors, thereby representing transplantable micro factories of anti-remodeling and angiotrophic agents. To date, numerous preclinical and clinical efforts have attempted to meet the high expectations with variable outcomes.

More than a decade ago, the first patients with ischemic heart failure were treated with stem cell therapy, using intramyocardial injection of skeletal myoblasts (SkM).[1,2] Briefly afterwards the first pilot study was published, in which AMI patients were treated with intracoronary infusion of bone marrow-derived mononuclear cells (BMMNC).[3,4]

Initial optimism concerning the use of SkM in heart failure patients was toned down by issues of possible pro-arrhythmogenicity of the cells, and disappointing results on efficacy in randomized studies.[5–7] Also, the regenerative capacity of bone marrow (BM)-derived cells in AMI patients has been under debate, since the numerous trials that were performed to date show conflicting results. More specifically, a recent meta-analysis evaluating >2,000 AMI patients, who received BM-derived cellular therapy, showed a modest effect of only +2.14% on left ventricular ejection fraction (LVEF) with no effect on clinical end points (see chapter 3 of this thesis).

Importantly, pre-clinical investigations and phase I clinical studies revealed that newer generations of stem cells might have more regenerative capacities than these first generation stem cells[8–10], whereas the future role of totipotent, embryonic stem cells is still undetermined. Moreover, the age-old dogma that the heart only comprises terminally differentiated and post-mitotic myocytes has recently been

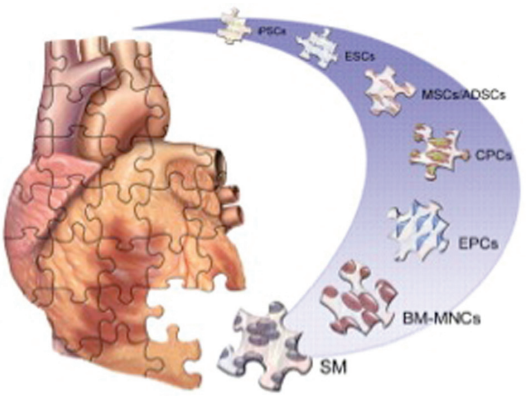

Figure 1. Candidate cell types for cardiovascular regenerative therapy.

A variety of cell sources with differing cardiomyogenic potential and developmental origins are under active investigation for cardiac cell therapy after myocardial infarction. BM-MNCs - Bone marrow mononuclear cells. CSCs -Cardiac stem cells. EPCs - Endothelial progenitor cells. ESC - Embryonic stem cells. iPSC - Induced pluripotent stem cells. MSCs - Mesenchymal stem cells. SM - Skeletal myoblasts. *Courtesy of Deutsch et al. Circ Res. 2013;112:884-890*

Table Ia. Skeletal Myoblasts Trials (First Generation)

	Phase	Cell type	Status	Design	No.	Delivery Method	Condition	Transfer day after PCI	Cell number (mill)	Primary Clinical Outcome	Reference
MAGIC	II	Skel MB	terminated	RDBPC	97	IM (Epi)	ICHF	–	400/800	Safety/Efficacy: MACE/ LVEF (Echo) at 6M,	NCT00102128
CAUSMIC II	II	Skel MB	unknown	RDBPC	23	IM (Endo)	ICHF	–	300	Safety/Efficacy: MLHFQ at 1 year	NCT00626314
SEISMIC	II	Skel MB	complete	ROPC	40	IM (Endo)	ICHF	–	150-800	Safety/Efficacy: LVEF (MUGA) at 6M	NCT00375817
MARVEL	II/III	Skel MB	unknown	RDBPC	170	IM (Endo)	ICHF	–	400/800	Safety/Efficacy: QOLQ, 6min walk test at 1 year	NCT00526253

RDBPC: randomized double-blind placebo controlled, ROPC: randomized open-label placebo controlled, SKelMB: skeletal myoblast, IM: intramyocardial, ICHF: ischemic congestive heart failure, LVEF: LV ejection fraction, MACE: major adverse cardiovascular event, MLHFQ:Minnesota living with heart failure questionnaire, QOLQ: quality of life questionnaire, MUGA: multi gated acquisition scan

abandoned. Studies have shown that the heart contains a resident cardiac stem cell niche, and that cardiomyocytes are replaced several times in a life time.[11,12] This new finding implicates that myocardial tissue has endogenous regenerative capacities, and initiated a new era in cardiovascular regenerative medicine.[13–15]

We therefore pose that there are two potential working mechanisms for cell-based repair of cardiac dysfunction: 1) the enhancement of endogenous regenerative potential, or 2) the delivery of exogenous cells to stimulate or repopulate the heart. This review aims to summarize our current understanding of cardiovascular regenerative medicine by discussing cell types that already have been evaluated in the clinical setting, but also cell types that are currently being evaluated in pre-clinical research. We thereby divide stem cells into first to fifth generation, and will elaborate on the proposed working mechanisms and cardiac disease type they aim to heal.

First generation stem cells for cardiovascular repair – Autologous stem cells

Autologous skeletal myoblasts (table Ia)

Skeletal myoblasts (SkM) are progenitor cells residing in striated skeletal muscle, and are responsible for regeneration of skeletal muscle upon damage. They do not meet the stem cell criteria, as they do not have multi-lineage differentiation potential, but the injection of SkM into scarred myocardium of ischemic heart failure (HF) patients, was considered stem cell therapy in the early days. SkM are easily expandable in cell culture, and because they are destined to become contracting cells, they were a logical candidate for cell replacement therapy. Moreover, in pre-clinical investigations, SkM were able to form functional skeletal myotubes, repopulate the damaged heart, and integrate into host myocardium with formation of electromechanical junctions between host cardiomyocytes and injected skeletal myotubes.[16–18] These data resulted in the first HF patients treated with epicardial SkM injections during bypass surgery, or endomyocardial injections using specialized injection catheters.[1,2] Initial and short-term follow up results were promising with positive effects on global cardiac function, when assessed by stress echocardiography, MRI and/or pressure-volume loop analysis.[2,19,20] However, early enthusiasm subsided, when SkM injection was associated with an increased incidence of sustained ventricular arrhythmias.[21] These ventricular arrhythmias were presumed to be caused by the lack of electromechanical coupling between injected SkM and host myocardium, which is due to the lack of connexin-43 expression. Collections of injected SkM thereby formed electrically isolated islands that functioned as re-entry circuits for ventricular arrhythmias.[22]

The only double-blind, and randomized study performed to date (MAGIC trial; NCT00102128; table Ia), in which patients with depressed cardiac function and scheduled for CABG were randomized between two doses of SkM or placebo, was negative.[6] Also, most other studies that assessed endomyocardial, catheter-based injections failed to show long-term improvement of global cardiac function[5,7], whereas all initiatives for large randomized studies (CAUSMIC: NCT00626314; MARVEL[23]: NCT00526253; table Ia) were halted. Due to the association with ventricular arrhythmias, the lack of obvious long-term efficacy, and more attractive alternatives, SkM therapy currently seems to be abandoned as cellular therapy for cardiac repair.

Bone marrow-derived mononuclear cells (table Ib)

By far the most clinical experience in cardiac cell therapy has been obtained with BM-derived mononuclear cells (BMMNC). The first clinical application was preceded by a pivotal mouse study that showed that BM-derived cells could regenerate infarcted hearts by transdifferentiation into cardiomyocytes.[24] It should be noted however, that only a small percentage of the mononuclear cell fraction of BM consists of stem cells, and that the majority of these stem cells are committed to hematopoietic lineages.[25] It is currently believed that actual transdifferentiation of BMMNC into functioning cardiomyocytes is practically non existing, and that the beneficial effect is primarily evoked by paracrine pro-angiogenic actions, and possibly by incorporation of BMMNC into neo-capillaries.[26]

Although the results of the initial mouse study in 2001 turned out to be controversial[27], reports on the treatment of the first AMI patients followed shortly thereafter.[3,4] Since then, more than 1,300 AMI patients have been treated with BM-derived cells. Even so, results of the various trials have been conflicting. In 2006, the landmark REPAIR-AMI trial was published, which was a double-blind and randomized study to assess the safety and efficacy of BMMNC in over 200 AMI patients (NCT00279175).[28] REPAIR-AMI showed improved LVEF, as assessed by LV angiography at 4 months, and, more importantly, long-term follow up revealed a reduction in clinical end points.[29] Other early studies included the BOOST and ASTAMI randomized trials that showed promising early results, but no sustained benefits after 3 and 5 years of follow up.[30–32] Moreover, recent trials with similar patient numbers, that used cardiac MRI as imaging modality, contradict the beneficial findings on global LV function that were found in the early trials.[33–36] A recent meta-analysis that compiled all clinical evidence to date, revealed a small, but significant, improvement of 2.10% on LVEF (see chapter 3 of this thesis). Intriguingly, this modest beneficial effect disappeared when sub-group analyses were performed for trial design (excluding non-controlled and cohort studies) or imaging modality (only including studies that used MRI-derived measures), thereby reinforcing conclusions from recent negative trial results.[33–36]

The fact remains that long-term follow up of the REPAIR-AMI trial showed sustained effects on clinical end points as recurrent AMI[29], although this effect was not confirmed in the long-term follow up of the BOOST trial (NCT00224536).[32,37] It is hoped that the forthcoming, phase III, BAMI study (NCT01569178), initiated by professor Zeiher and funded by the European Union, which aims to include 3,000 AMI patients, will render definite answers to this unrequited issue.

Although most trials using BMMNC were performed in AMI patients 3-30 days after the index event, some other studies evaluated the effect of BMMNC in ischemic or non-ischemic HF patients. A recent meta analysis analyzed 13 randomized, controlled trials and concluded that BMMNC therapy resulted in a modest increase in global LV function (see chapter 4 of this manuscript).[38] Moreover, intramyocardial delivery was found to be superior to intracoronary delivery. It should be noted however, that also in the treatment of HF patients, the benefit of BMMNC is controversial[39], and several well-designed studies with proper end points rendered only modest or negative results.[40–42]

Also in the field of refractory myocardial ischemia, several exploratory studies have been performed using BMMNC. In most studies, a beneficial effect was found on either cardiac function or angina rates.[43–45] Patient numbers in these studies were low though, and it seems plausible that enriched cell populations with more pro-angiogenic potential will soon replace BMMNC therapy in this specific patient population.

Second generation stem cells for cardiovascular repair – Autologous enriched stem cells

Endothelial progenitor cells (i.e. CD34+ and CD133+ cells; table Ib)

Endothelial progenitor cells (EPC) were first described in 1997 by Asahara *et al.* as circulating CD34+ cells that can differentiate into endothelial cells.[46] They originate from the BM and were shown to repair and repopulate damaged endothelium, and incorporate into foci of pathological or physiological neovascularization. The ability to promote new blood vessel formation renders these cells ideal candidates for cellular therapy in ischemic diseases. However, fifteen years after their discovery, there is still debate on the exact definition of EPC, and a distinctive cell surface marker is lacking.[47] The majority of EPC is derived from BM, and share a progenitor cell with hematopoietic stem cells (HSC), called the hemangioblast. Both EPC and HSC are positive for CD34, CD133, and KDR, which makes distinction based on cell sorting difficult. Once plated in a culture dish, EPC differentiate into so-called early outgrowth cells (EOC) and late outgrowth cells (LOC). The latter appear only after 2-3 weeks in culture, and are able to form vessel-like structures *in vitro*. EOC are positive for the hematopoietic marker CD45, whereas LOC are not. It is therefore believed that LOC functionally represent true EPC, whereas EOC are more related to the white blood cell line.[48]

Nonetheless, these discriminating features did not enter the clinical arena yet. Thus far, studies in cardiac disease pragmatically used the surface markers CD34 or CD133 to define a pro-angiogenic precursor cell type.

In a few pilot studies in AMI patients, BM-derived cell fractions enriched for CD34+ or CD133+ cells resulted in improved perfusion only, but trends towards increased cardiac function.[49–51] Peri-operative intramyocardial injection during bypass surgery of BM-derived CD133+ cells showed favorable safety results, but failed to improve cardiac function.[52] Nevertheless, a phase III trial was designed to definitively evaluate the efficacy of surgical injection adjunct to bypass surgery in HF patients.[53] EPC have also been successfully applied in patient populations with chronic myocardial ischemia.[47] For instance, Losordo *et al.* performed intramyocardial injections in 167 patients with refractory angina pectoris with a positive response on angina rates[54], and the same group also reported favorable results in patients with critical limb ischemia.[55]

Table Ib. Bone Marrow-derived Cells Trials (First Generation)

	Phase	Cell type	Status	Design	No.	Delivery Method	Condition	Transfer day after PCI	Cell number (mill)	Primary Clinical Outcome	Reference
BOOST	I	MNC	complete	RDBPC	60	IC	AMI	4.8	2460	Safety/Efficacy: Regional systolic wall thickening (MRI)	NCT00224536
LEUVEN-AMI	II	MNC	complete	RDBPC	67	IC	AMI	1	304	Efficacy: LVEF (MRI) at 4M	NCT00264316
REPAIR-AMI	III	MNC	complete	RDBPC	204	IC	AMI	4	236	Efficacy: LVEF (LVG) at 4M	NCT00279175
ASTAMI	II	MNC	complete	RSBPC	100	IC	AMI	6	68	Safety/Efficacy: LVEF, EDV, Infarct size	NCT00199823
TIME	II	MNC	not yet recruiting	RDBPC	120	IC	AMI	3 or 7	150	Efficacy: LVEF (MRI) at 6M	NCT00684021
LateTIME	II	MNC	complete	RDBPC	87	IC	AMI	17.4	150	Efficacy: LVEF (MRI) at 6M	NCT00684060
SWISS-AMI	II	MNC	complete	ROPC	200	IC	AMI	5-7 vs. 21-28	156	Efficacy: LVEF (MRI) at 4M	NCT00355186
HEBE	II	MNC	complete	ROPC	200	IC	AMI	6	296	Efficacy: Regional LVEF (MRI) at 4M	ISRCTN 95796863
BAMI	III	MNC	not yet recruiting	ROPC	3000	IC	AMI	3 to 6	–	Safety/Efficacy: All cause death at 3Y	NCT01569178
REGENT	II	CD34/ CXCL4	complete	ROPC	200	IC	AMI	7	1.9	Safety/Efficacy: LVEF (Echo, LVG) at 6M	NCT00316381
Rostock	I	MNC/CD133	complete	N/A	32	IM (Epi)	ICHF	–	N/A		N/A
PERFECT	III	MNC/CD133	recruiting	RDBPC	142	IM (Epi)	ICHF	–	0.5-5	Efficacy: LVEF (MRI) at 6M	NCT00950274
ESCAPE	III	MNC	complete	RDBPC	250	IM (Epi)	ICHF	–	150	Safety/Efficacy: Survival at 1Y	NCT00841958
FOCUS	II	MNC	recruiting	RDBPC	92	IM (Endo-NOGA®)	ICHF	–	100	Safety/Efficacy: MVO2, LVESV, Reversible defect size	NCT00824005
STAR heart	–	MNC/ CD133/34	complete	Cohort	391	IC	ICHF	–	66	(LVEF (LVG), ETT at 1 and 5Y)	–
RENEW	III	CD34/G-CSF	recruiting	ROPC	444	IM (Endo-NOGA®)	CMI	–	0.1	Safety/Efficacy: ETT at 1Y	NCT01508910
Cell-Wave	I/II	MNC/ shock wave	complete	RDBPC	100	IC	CMI	–	N/A	Safety/Efficacy: LVEF (LVG) at 4M	NCT00326989
Chagas	III	MNC	terminated	RDBPC	183	IC	Chagas CM	–	250	Safety/Efficacy: LVEF at 1Y	NCT00349271

RDBPC: randomized double-blind placebo controlled, RSBPC: randomized single-blind placebo controlled, ROPC: randomized open-label placebo controlled, MNC: bone marrow-derived mononuclear cell, G-CSF: granulocyte-colony stimulating factor, IC: intracoronary, IM: intramyocardial, AMI: acute myocardial infarction, ICHF: ischemic congestive heart failure, CMI: chronic myocardial ischemia, CM: cardiomyopathy, LVEF: LV ejection fraction, EDV: LV end diastolic volume, ESD: LV end systolic volume LVG: left ventriculogram, ETT: exercise tolerance test, MVO2: maximal oxygen consumption

Autologous bone marrow-derived mesenchymal stem cells (table II)

The adult BM harbors another stem cell type, which is probably the most investigated adult stem cell type for cardiac repair in the current preclinical arena. They have shown significant beneficial effect in almost all experimental models of heart disease, and the first clinical applications exerted promising safety results.[56] The mesenchymal stem cell (MSC) was discovered 40 years ago as a rare population of plastic adherent cells, comprising 0.01-0.001% of BMMNC cells.[57] They represent a heterogeneous cell population, are self-renewing, can be culture expanded innumerably, and are defined as: 1) plastic adherent; 2) expressing CD90, CD73 and CD105, and not expressing CD34, CD45, HLA-DR, CD14 or CD11b, CD79a, or CD19 by FACS analysis; and 3) able to transdifferentiate into adipocytes, osteoblasts and chondrocytes *in vitro*.[58] Aside from this consensus, the exact phenotype is still under debate, and MSC identification prior to culture remains undetermined.

Over the past few years, it has become apparent that MSC can be found in most post-natal organs, including the heart[59], liver, spleen, thymus, tendon, periodontal ligament, lungs, menstrual blood, and adipose tissue.[60] The physiological role for MSC in the BM is to support the hematopoietic microenvironment. In other organs, it is believed that MSC reside in perivascular tissue, might have pericyte features, and have a role in maintaining tissue homeostasis.[60,61] Adipose tissue-derived MSC are discussed separately below.

MSC have several characteristics that make them very suitable for cardiac repair, excellently reviewed by Choi *et al.* and Williams *et al.*[56,62] First, they are able to transdifferentiate into cardiomyocytes *in vitro* and *in vivo*.[63] It is important to note, however, that *in vitro* differentiation of MSC requires epigenetic modulation, including DNA demethylization and/or histone acetylation.[64,65] The effect of chemically induced DNA demethylization or histone acetylation on genetic stability and possible chromosome aberrations is yet unknown, and may ultimately interfere with cell product safety.

Second, and more importantly, MSC are known to secrete a vast amount of paracrine factors, which can affect angiogenesis, cardiomyocyte survival, extracellular matrix remodeling, cardiac stem cell recruitment, and the cardiac immunologic milieu (table A).[56] It is believed that these paracrine factors are packed in nano particles, called exosomes.[66] Infusion of MSC conditioned medium containing these exosomes improves cardiac function, without the actual presence of MSC.[67,68] However, administration of these factors alone is believed to be less effective than infusion or injection of MSC, which is probably caused by the rapid wash out of the medium and its exosomes. Also, MSC are immune privileged, thereby enabling allogeneic cell transfer. The use and clinical application of allogeneic MSC is discussed separately below.

The biggest limitation of MSC is the very low frequency in which they are found in regular BM aspirations. Before sufficient cell numbers can be obtained for clinical applications, MSC need extensive culture expansion in clean room facilities. This implicates extra costs, but also at least 2-3 weeks between BM harvest and initiation of cell therapy. This excludes the application of autologous MSC in, for instance, the acute phase of an AMI. Importantly, in a meta-analysis of all large animal studies performed to date, MSC were found to be superior to BMMNC.[8]

Table II. Clinical Autologous Mesenchymal Stem Cell Trials (Second Generation)

	Phase	Cell type	Status	Design	No.	Cell source	Delivery Method	Condition	Primary Clinical Outcome	Reference
STEMMI	II	Autologous	complete	RDBPC	78	BM (mobilized G-CSF)	IC	AMI	Safety/Efficacy : regional systolic wall thickening MRI	NCT00135928
PROMETHEUS	I/II	Autologous	complete	RDBPC	45	BM	IM (Epi)	ICHF	Safety/Efficacy: Serious adverse events, Infarct size (MRI)	NCT00587990
C-CURE	II/III	Autologous	complete	RSBPC	240	Guided BM	IM (Endo) (C-Cath®)	ICHF	Safety/Efficacy: LVEF	NCT00810238
APOLLO	I	Autologous	complete	RDBPC	13	Adipose-tissue	IC	AMI	Safety	NCT00442806
ADVANCE	II/III	Autologous	not yet recruiting	RDBPC	360	Adipose-tissue	IC	AMI	Safety and Efficacy: infarct size (MRI)	NCT01216995
TAC-HFT	I/II	Autologous	complete	RDBPC	60	BM	IM (Endo) (Helix®)	CMI	Safety/Efficacy: MRI	NCT00768066
PRECISE	I	Autologous	recruiting	RDBPC	36	Adipose-tissue	IM (Endo) (NOGA®)	CMI	Safety: MACCE at 3Y	NCT00426868
MyStromalCell	II	Autologous	recruiting	RDBPC	60	VEGF-ADRC	IM (Endo) (NOGA®)	CMI	Safety/Efficacy: EET at 6M	NCT01449032

RDBPC: randomized double-blind placebo controlled, RSBPC: randomized single-blind placebo controlled, BM: bone marrow, G-CSF: granulocyte-colony stimulating factor, IC: intracoronary, IM: intramyocardial, AMI: acute myocardial infarction, ICHF: ischemic congestive heart failure, CMI: chronic myocardial ischemia, LVEF: LV ejection fraction, MACCE: major adverse cardiovascular and cerebrovascular event, EET: exercise tolerance test

Autologous MSC have been investigated in clinical pilot studies of AMI, as well as ischemic and non-ischemic HF. Chen and colleagues investigated the effect of intracoronary infusion of autologous MSC in 34 patients with sub-acute AMI, showing improved perfusion and LV function in treated patients when compared to placebo controls.[69] In a recent pilot study, autologous MSC were injected intramyocardially into the border zone of ischemic HF patients, resulting in reversed remodeling on MRI in 4 patients.[70] These results, together with the conflicting results with BMMNC in HF patients, formed the basis for the TAC-HFT trial (NCT00768066).[71] In this trial, a total of 65 ischemic HF patients were randomized to receive either BMMNC (n=22), MSC (n=22) or placebo (n=21). Intramyocardial injection using the Helix catheter was safe. Injection of MSC, but not BMMNC, resulted in a significant reduction of infarct size and regional cardiac function, when compared to placebo controls.[72] This study suggests that MSC are superior to BMMNC, although these data need to be judged with caution due to the small sample size of the TAC-HFT trial. Also, Mathiasen and co-workers will assess the safety and efficacy of autologous MSC versus placebo treatment in ischemic HF patients.[73]

Cardiogenic-oriented mesenchymal stem cells (table II)

Very recently, clinical trial results were published from a specific sub category of autologous MSC. The group of Terzic *et al.* found ways to direct MSC towards a cardiogenic phenotype using a mix of growth factors, but without genetic engineering or modification.[74] Autologous, patient-derived MSC are culture expanded following BM harvest, after which these MSC are exposed to a cardiogenic cocktail of growth factors and chemokines to acquire myocyte-like features. When transplanted in an animal model of ischemic HF, these cells were shown to improve cardiac function,[75] which resulted in the design of the phase I C-CURE trial (NCT00810238). In this randomized study, a total of 48 patients with ischemic HF were included, who received NOGA-guided intramyocardial injection of cardiogenically-oriented MSC or placebo. Two-year follow up results showed safety and feasibility of this approach, whereas global LV function was significantly enhanced after 6 months of follow up, when compared to standard of care.[76]

Autologous adipose tissue-derived regenerative cells (table II)

Adipose tissue was first identified as an alternative source of abundant numbers of multipotent mesenchymal-like stem cells in 2002.[77] Like BM-derived MSC, these cells stimulate neo-angiogenesis and cardiomyocyte survival both *in vitro* and *in vivo* by release of various angiotrophic, anti-apoptotic and immunomodulatory factors.[78,79] The frequency of ADRCs in freshly isolated adipose tissue digestates is ~ 2,500 fold greater than that of freshly aspirated BM, which implies that culture expansion is not required to generate sufficient numbers of therapeutic cells.[80] On average, 20-40 million cells can be isolated within two hours after a liposuction from as little as 200 grams of lipo-aspirate. In a large animal model of AMI, administration of freshly isolated adipose tissue-derived regenerative cells (ADRCs) improved LV function and myocardial perfusion by cardiomyocyte salvage and stimulated neo-angiogenesis in the infarct border zone, resulting in reduced infarct scar formation.[81]

The cardioprotective and pro-angiogenic effect in large animal models were the basis for the phase I

Table A. Cardioprotective paracrine factors secreted by MSC

Secreted factor		Function
Pro-survival		
Insuline-like growth factor-1	IGF-1	Inhibits apoptosis
Secreted frizzled-related protein-2	SFRP-2	Inhibits apoptosis
Homing/recruitment of stem cells		
Thymosin β-4	Tβ-4	Promotes cell migration
Stromal-derived factor	SDF	Promotos cell homing
Cell proliferation		
Basic fibroblast growth factor	bFGF	Proliferation of smooth muscle cells and endothelial cells
Fibroblast growth factor -2	FGF-2	Proliferation of smooth muscle cells and endothelial cells
Fibroblast growth factor -7	FGF-7	Proliferation of endothelial cells
vascular endothelial growth factor	VEGF	Proliferation of endothelial cells en migration
Platelet-derived growth factor	PDGF	Proliferation of smooth muscle cells
Tumor necrosis factor-α	TNF-α	Cell proliferation
Granulocyte colony stimulating factor	G-CSF	Neutrophil proliferation and differentiation
Insuline-like growth factor-1	IGF-1	Regulates cell growth and proliferation
Macrophage colony stimualting factor	M-CSF	Monocyte proliferation and differentiation
Secreted-frizzled-related protein-1	SFRP-1	Enhances cell development
Secreted frizzled-related protein-2	SFRP-2	Enhances cell development
Vessel fomation		
Vascular endothelial growth factor	VEGF	Tube formation
Placental growth factor	PIGF	Promotes angiogenesis
Transforming growth factor-β	TGF-β	Promotes vessel maturation
Metalloproteinase-1	MMP-1	Tubule formation
Metalloproteinase-2	MMP-2	Tubule formation
Remodeling of extracellular matrix		
Metalloproteinase-1	MMP-1	Loosens extracellular matrix
Metalloproteinase-2	MMP-2	Loosens extracellular matrix
Metalloproteinase-9	MMP-9	Loosens extracellular matrix
Plasminogen activator	PA	Degradation of matrix molecules
Tumor necrosis factor-α	TNF-α	Degradation of matrix molecules
Immunomodulatory		
Heme oxygenase-1	HO1	CD4+ T-cell proliferation inhibitor
Hepatocyte growth factor	HGF	T-cell proliferation inhibitor
Indoleamine 2,3-dioxygenase	IDO	Inhibits innate and adaptive immune cell proliferation
Inflammation		
Interleukin-6	IL-6	Inflammation regulator, VEGF induction
Prostaglandin E2	PGE-2	Decreases inflammation
Inducible nitric oxide synthase	iNOS	Decreases inflammation

APOLLO (NCT00442806) and PRECISE (NCT00426868) trials. APOLLO was the first-in-man experience with ADRCs in the treatment of patients with ST-elevation AMI. It showed in 14 patients that performing a liposuction in the acute phase of the AMI, as well as intracoronary infusion of ADRC within 24 hours following the primary PCI, is safe and feasible.[9] Moreover, significant effects were obtained on reduction of infarct size and the perfusion defect, which is concordant to the proposed working mechanism of ADRC therapy. Also, a trend towards improved global cardiac function and decreased LV volumes was found. The currently recruiting phase III ADVANCE trial (NCT01216995), which aims to include a total of 216 AMI patients, will evaluate the true value of ADRC therapy in AMI patients.

In the PRECISE trial, ADRC were injected intramyocardially in patients with depressed cardiac function and proof of refractory ischemia with no other treatment options, using NOGA-XP cardiac mapping. The PRECISE trial demonstrated a statistically significant improvement in VO_2max in patients treated with ADRC, when compared to those treated with placebo, although LVEF did not change (unpublished data). Two other clinical phase I/IIa studies that are aimed to assess safety and feasibility of ADRC in patients with chronic myocardial ischemia, are currently enrolling.[82]

Third generation stem cells for cardiovascular repair – Allogeneic stem cells

Allogeneic mesenchymal stem cells (table III)

As stated above, MSC are immune-privileged cells. This is achieved by several immunological features of MSC: 1) lack of expression of MHC class II antigen, and low levels of MHC class I; 2) lack of co-stimulatory molecules as CD40, CD80, and CD86; 3) secretion of immuno-modulatory factors including nitric oxide, heme-oxygenase I, and interleukin-6; 4) suppress innate immune cells via direct cell-cell contact, but also 5) suppress T-cell proliferation and alter naïve T-cells into an anti-inflammatory state.[56,83] These immunologic properties extend the applicability of MSC as a therapeutic for ischemic heart disease, as the immune system plays a pivotal role in infarct remodeling.[84,85] Moreover, its immuno-modulatory effects enable allogeneic cell transfer without the need for immunosuppressive therapies, which has several important advantages. It avoids a laborious, time-consuming, and potentially dangerous BM puncture, as well as the subsequent culturing steps in clean room facilities. Moreover, it enables the production of “off-the-shelf”, and even commercially available, cell preparations derived from young and healthy donors. Such stable stem cell banks ensure adequate quality control with inherent batch-to-batch consistency. Also, a negative correlation was found between the number and functionality of progenitor cells, and age and cardiovascular risk factors.[86,87] This would make the use of allogeneic MSC, derived from young and healthy donors, in the typically elderly, cardiovascular patient population preferable over autologous cells. More importantly, cell therapy can be initiated directly after the revascularization of an AMI, thereby maximally utilizing the anti-apoptotic and immuno-modulatory capacities of the cells. However, the ideal timing of cell therapy following AMI is still a matter of debate (see below).[8]

Table IV. Clinical Allogeneic MSC/Cardiac Progenitor Cell Trials (Third/Fourth Generation)

	Phase	Cell type	Status	Design	No.	Cell source	Delivery Method	Condition	Primary Clinical Outcome	Reference
PROCHYMAL	II	Allogeneic	recruiting	RDBPC	220	BM	IV	AMI	Safety: (LVESV)	NCT00877903
Mesoblast AMI	I/II	Allogeneic	recruiting	RSBPC	25	BM	IM(Endo) (NOGA®)	AMI	Feasibility/Safety	NCT00555828
MultiStem	I	Allogeneic	complete	ONPC	25	BM	IM(adventitia of CA) (Cricket®)	AMI	Safety: Adverse Event at 1M	NCT00677222
AMICI	II	Allogeneic	not yet recruiting	RDBPC	225	BM	IC	AMI	Safety/Efficacy: Infarct size (MRI) at 6M	EUCTR2010-020497-41-NL
POSEIDON	I/II	Auto/Allo	complete	RONPC	30	BM	IM(Endo) (Helix®)	ICHF CHF	Safety/Efficacy: TE-SAE at 1M	NCT01087996
Mesoblast CHF	II	Allogeneic	unknown	RSBPC	60	BM	IM(Endo) (NOGA®)	(ischemic/idiopathic)	Feasibility and Safety	NCT00721045
SCIPIO	I	Autologous	recruiting	RONPC	40	CPC (c-kit)	IC	ICHF	Short term Safety	NCT00474461
CADUCEUS	I	Autologous	complete	RONPC	31	CPC (Cardiospheres)	IC	ICHF	Safety	NCT00893360

RDBPC: randomized double-blind placebo controlled, RSBPC: randomized single-blind placebo controlled, ONPC: open label, non-placebo-controlled, RONPC: randomized, open label, non-placebo-controlled, BM: bone marrow, CPC: cardiac progenitor cells, IV: intravenous, IC: intracoronary, IM: intramyocardial, CA: coronary artery, AMI: acute myocardial infarction, CHF: congestive heart failure, ICHF: ischemic congestive heart failure, LVESV: LV end systolic volume

Most pre-clinical, but also clinical experience with MSC thus far was obtained using allogeneic MSC.[8] Interestingly, there is some pre-clinical evidence in large animals that small percentages of injected MSC have the capacity to engraft in cardiac tissue, and to transdifferentiate into cardiomyocytes, endothelial cells, and smooth muscle cells.[63,88,89] These results were obtained in pigs, but could not be confirmed in other species. In dogs, MSC seemed to transdifferentiate into vascular cells only[90], whereas in a sheep model of HF no engraftment or transdifferentiation could be detected at all.[91] The fact that there seem to be inter-species differences, as well as the low rate of actual transdifferentiation, make the clinical relevance of this phenomenon questionable. Moreover, the robust functional improvement following MSC transplantation in both AMI and chronic HF models[8], is disproportionate to this low rate of engraftment and transdifferentiation. Hence, other mechanisms must be at play. Most of these mechanisms were already mentioned above, summarized in table A, and are primarily based on the paracrine properties of the cells. More specifically: MSC are known to have anti-apoptotic and pro-survival capacities, secrete pro-angiogenic proteins, and influence the local immune system and extracellular matrix composition. Moreover, recent studies have shown that also the postnatal heart contains resident stem cells.[13] Delivery of MSC to infarcted or hibernating myocardium may regenerate myocardium and improve cardiac function by stimulating these resident cardiac stem cells and cardiomyocytes to (re-)enter the cell cycle, thereby initiating cardiomyocyte generation or proliferation.[13,92–95]

Numerous pre-clinical investigations in small and large animal models preceded the few clinical studies that have been performed in AMI and HF patients using allogeneic MSC to date (see both reviews and the meta-analysis by Van der Spoel *et al.*[8,56,96]). In AMI patients, one clinical study investigated the intravenous administration of allogeneic MSC in 39 AMI patients versus 21 placebo controls, briefly following an AMI.[10] It was found that infusion of a considerable number of allogeneic cells was safe, and did not result in adverse reactions or an immunologic response. Moreover, patients exhibited a reduction of ventricular arrhythmias, increased pulmonary function, and improved LVEF after 3 months. Other clinical experience with AMI patients is lacking, because MSC delivery to recently infarcted hearts has been troublesome. More specifically, endomyocardial injection is prone to perforation so briefly following AMI, and intracoronary delivery resulted in vascular plugging with no-reflow phenomena in several pre-clinical studies.[97–100] This issue was recently overcome in a large pre-clinical AMI study using 88 sheep. In this study, the safety, feasibility and efficacy was assessed of intracoronary infusion of a specific Stro3+, immune-selected, immature sub type of MSC directly following an AMI. It was found that intracoronary infusion of these so-called mesenchymal precursor cells (MPC) is safe, does not hamper coronary flow, and has marked beneficial effects on global and regional cardiac function.[95,101] These effects are evoked by myocardial salvage, neo-vascularization, and stimulation of endogenous cardiac regeneration. The observations in this study resulted in the design of a multi-center, phase IIa/b, double blind, randomized and placebo-controlled clinical trial. The Allogeneic-Mesenchymal-precursor-cell-Infusion-in-myoCardial-Infarction (AMICI) trial (NCT01781390), in which European, Australian and US sites will participate, is aimed to prove safety, feasibility and efficacy of MPC therapy in a minimum of 225 patients with ST-elevation AMI, and recently the first patient was treated successfully.

These Stro3+ MSC are an interesting cell type for cardiac repair, as they were shown to exert extensive cardioprotective effects that exceed the cardioprotective effects of regular MSC.[102,103] It is believed that this difference is evoked by more potent paracrine activity, as well as more extensive multilineage differentiation potential.[102,104] In several pre-clinical investigations, intramyocardial injection of these cells resulted in marked improvement of cardiac function and LV remodeling in models of AMI, ischemic, and non-ischemic heart failure.[91,102,105,106] Recently, the results from a clinical, phase I/II study, assessing the effect of percutaneous endomyocardial injections of allogeneic MPC in 60 HF patients, were presented (Mesoblast-CHF; NCT00721045). Allogeneic MPC injections up to a dose of 150 million cells were shown to be safe and feasible without a clinically significant anti-allogeneic immune response. More importantly, MACCE rate, cardiac mortality and composite end points for heart failure were markedly decreased at 12 month clinical follow up (unpublished data). This study resulted in the preparations of a phase III study analyzing the therapeutic effect of MPC therapy via intramyocardial injections in 120 congestive HF patients.

In the POSEIDON trial (NCT01087996) the difference between autologous and allogeneic MSC in the treatment of ischemic HF was evaluated in 30 patients.[107] It confirmed the safety data of the Mesoblast-CHF study, as no significant anti-allogeneic immune response was found in patients treated with allogeneic MSC. Moreover, cardiac function improved equally in both autologous and allogeneic groups. The future POSEIDON-DCM (NCT01392625) will evaluate the effect of MSC in patients with non-ischemic dilated cardiomyopathy, whereas larger phase III initiatives are much anticipated.

CellBeads

One of the biggest challenges in the cell therapy field today is the poor retention rate of therapeutic cells upon local delivery in the heart, with retention rates as low as 1% after intracoronary delivery.[108,109] Even though permanent engraftment of stem cells is not required to elicit the cardio-protective effect, it seems logical that the greater the number of cells that are retained in the injured myocardium and the longer they reside there, the more pronounced the potential beneficial effect will be. A new concept of stem cell delivery has recently become available owing to advances in the field of biotechnology, as it is currently possible to encapsulate MSC in a biocompatible alginate shell.[110] Alginate encapsulation of varying numbers of MSC results in so-called CellBeads™. These MSCs have been genetically modified to secrete a proprietary recombinant GLP-1 fusion protein, which consists of two GLP-1 molecules bound by an intervening peptide. This form of recombinant GLP-1 is more stable than endogenous GLP-1, rendering a longer half-life and thus prolonged therapeutic potential. The alginate coating of the CellBeads is permeable to the GLP-1 fusion protein and MSC-derived paracrine factors, allowing for continuous delivery, while protecting the MSC from the patient's immune system. Also, oxygen and nutrients can freely pass through the alginate shell, which renders the MSC viable for a long period of time. Thus, Cellbeads are potentially a unique, biological, long-term, local drug delivery platform that is capable of delivering GLP-1, or other therapeutic proteins, in addition to MSC-derived factors (VEGF, MCP-1, IL-6, IL-8, GDNF and NT-3) to any target tissue. These CellBeads can be delivered safely to infarcted myocardium by intracoronary infusion, resulting in engraftment and production of the

recombinant protein for at least 7 days post infusion.[111] The potential efficacy of CellBeads in a large animal model of AMI is currently being analyzed, after which the clinical potential of this promising new therapy will become evident.

Fourth generation stem cells for cardiovascular repair – Cardiac-derived stem cells

Cardiac stem cells (table III)

For decades, the heart has been considered a post-mitotic organ, without the capacity to self-renew or regenerate upon inflicted damage. This dogma has recently been abandoned by the discovery by several groups that considerable cardiomyocyte turnover occurs throughout life in healthy, aged, and damaged hearts.[12,112,113] This self-renewing capacity was found to be based on both the intrinsic capacity of senescent cardiomyocytes to re-enter the cell cycle, as well as the presence of endogenous cardiac stem cells (CSC).[13,114–116] Although the rate of myocyte turnover varied between 40% in a lifetime[112] to over 40% per year[12] depending on the way it was measured, there is now concensus that the cardiomyocyte compartment is substituted approximately 8 times during the adult life time of a healthy indiviual.[113,117] The fact that the heart contains endogenous regenerative potential caused a paradigm shift with regard to cellular therapies to mend broken hearts. It initiated a quest to find ways to direct resident cardiomyocytes to re-enter the cell cycle and start proliferation. Moreover, the isolation of CSC was deemed a potential holy grail for regenerative therapies for CV disease, as these cells might be very effective in regenerating damaged myocardium.

To date, several types of CSC have been identified.[13] The most extensively studied stem cell type is the cKit+ CSC, first described in 2003.[118] They reside in niches in the post-natal heart, which are primarily localized in the atria and in the apex of the heart.[13] This CSC is multipotent, and has the ability to transdifferentiate into endothelial cells, smooth muscle cells and cardiomyocytes.[115] They were found to ameliorate cardiac function when transplanted in pre-clinical models of HF[118,119], which laid the basis for the SCIPIO trial (NCT00474461). In this trial, 16 ischemic HF patients were treated with intracoronary infusion of 1 million autologous CSC. The main finding of SCIPIO was that intracoronary infusion of these cells is feasible and safe, whereas it also resulted in improved cardiac function and decreased infarct size at 1 year follow up.[15] These results warrant further investigation of these cells in larger phase II studies.

Cardiosphere derived cells

Cardiospheres were first described in 2004, and are defined as spherical clusters of undifferentiated cells that evolve when adult cardiac tissue specimens are placed in suspension culture.[120] These cardiospheres contain a heterogeneous cell population with proliferating cKit+ cells in its core, which are surrounded by differentiating cells that express endothelial and cardiac markers. When cardiospheres are plated and culture expanded, cardiosphere-derived cells (CDC) can be obtained reproducibly.[121] These CDC express stem cell markers, as well as markers vital for contractile and electrical function. It is believed that they possess greater regenerative potential than CSC, as they

mimic the stem cell niches that are also found *in vivo*.[122] Also, they have more paracrine activity than pure cKit+ CSC populations.[123] When transplanted in both small and large animal models of ischemic HF, these cells form new cardiac tissue, improve cardiac function, and attenuate adverse remodeling.[121,124] These promising pre-clinical findings initiated the CADUCEUS trial (NCT00893360), in which a total of 17 patients with ischemic HF with baseline LVEF of 25-40% were treated with intracoronary infusion of CDC, as opposed to 8 patients who received standard of care. After 6 months, CDC infusion proved to be safe, whereas MRI analysis showed reduction of infarct size, and improved regional cardiac function. However, LV dimensions and LVEF were not significantly enhanced.[14] It seems that the high hopes that were raised in pre-clinical studies could not be confirmed, although only larger studies as the forthcoming ALLSTAR trial (NCT01458405) can provide definitive answers.

It should be noted that, to obtain cells for both therapies, a cardiac muscle biopsy, but also clean-room facilities, are required. Cardiac biopsy is an invasive procedure with considerable risk of perforation of the right ventricle, and poses a big disadvantage of both CSC and CDC therapy, whereas resident CSC can also be stimulated by MSC transplantation (table A).[92,94,95]

Fifth generation stem cells for cardiovascular repair – Pluripotent stem cells

Embryonic stem cells

Embryonic stem cells (ESC) are derived from the inner cell mass of the developing embryo, and are able to transdifferentiate in all cell and tissue types. This is why the ESC is the prototypical stem cell, capable of unlimited expansion and self-renewal. Theoretically, given their versatility and the possibility of generating beating cardiomyocytes, ESC are the ultimate candidate for cell-based regenerative therapies for cardiovascular disease.[125,126] Moreover, when transplanted into rodent and large animal models of HF, ESC-derived cardiomyocytes engraft into host tissue and improve cardiac function.[127,128] However, cell therapy using ESC raises several concerns that have not been addressed sufficiently yet to proceed to a clinical application: 1) as ESC are derived from embryos, there are ample ethical and societal issues; 2) ESC are prone to teratoma formation, when cells remain in their undifferentiated state; 3) ESC are by definition allogeneic and not immune-privileged, which may lead to immune rejection; 4) the yield of cardiomyocytes from ESC cultures is still too low; 5) competency of ESC-derived cardiomyocytes to electrically and/or mechanically integrate into host myocardium. Extensive research is currently ongoing in order to solve these issues.

Induced pluripotent cells

Recently, a newer cell type was discovered that might circumvent several of the concerns that were raised in the previous paragraph. Induced pluripotent stem cells (iPS) are pluripotent cells that are derived from mature, differentiated cells, such as skin fibroblasts. By overexpressing some reprogramming factors (*i.e.* Sox-2, c-Myc, Oct 3/4, and Kfl4) that are also expressed by ESC, such specialized somatic cells can be reprogrammed to reverse to an embryonic state.[129] iPS have almost identical pluripotent and proliferative potentials as ESC, and can be differentiated into any desired cell

type, including cells from the cardiovascular lineage.[130,131] Thus far, human iPS have been successfully transplanted into the murine heart, resulting in regeneration of the myocardium, and improving cardiac function.[132] Because iPS can be patient-derived cells, the ethical issues that were raised around ESC are circumvented, whereas immune rejection is likely to be absent. However, also with iPS, several issues remain to be solved before the field can advance to a clinical application in cardiovascular patients. Most importantly, the disruption of the genome by inserting genes can cause gene mutations ranging from mild aberrations to tumorigenesis.[133,134] Although several groups have reported the possibility to use vectors that do not permanently change the host's DNA, these new techniques are highly inefficient and need extensive fine tuning. The issues that need to be addressed can be summarized as: 1) effectuate more efficient induction of cardiomyocyte lineages; 2) selective expansion of cells of the cardiomyocyte lineage; 3) purification of differentiated cardiomyocytes, as undifferentiated iPS might develop teratomas; 4) address issues with possible acquired immunogenicity; 5) ensure electromechanical coupling of implanted iPS-derived cardiomyocytes.[133]

Induced cardiomyocytes

The fact that somatic cells can be re-programmed into pluripotent cells raises the question if these somatic cells can not be re-programmed towards a cardiomyogenic fate, without first becoming a progenitor cell. This would circumvent the issue of teratogenesis. Indeed, recently Ieda and coworkers were able to reprogramme murine dermal fibroblasts into functional cardiomyocytes by inserting three developmental transcription factors (Gata4, Mef2c, and Tbx5).[135] Although such reprogrammed fibroblasts might be a source of cardiomyocytes for regenerative purposes, the *in vitro* reprogramming efficiency should be improved significantly. Nonetheless, other reports suggest that cardiac-derived fibroblasts can undergo the same reprogramming into functional cardiomyocytes, even in *in vivo* situations. Resident cardiac fibroblasts, reprogrammed into cardiomyocytes, were shown to improve cardiac function and reduce ventricular remodeling.[136,137] This new approach might signify the next paradigm shift in cellular/gene therapy, as it would enable direct reprogramming of scar tissue into functional myocardium without the need for actual cell transplantation.

What cell for what cardiovascular disease type?

This review describes the use of cells for the treatment of cardiovascular diseases. Although it describes many cell types, we do not claim completeness, and several cells have not been mentioned. Moreover, the cardiovascular patient as such does not exist, and there are many disease types with all different pathogeneses. We will only briefly discuss AMI and HF below, and elaborate on the most logical candidate cell for these two specific disease types, whereas many other cardiovascular diseases will not be addressed.

Most clinical studies to date were performed in AMI patients, and used BMMNC. To determine the optimal cell for AMI patients, however, one should ask what we aim to treat by using cell therapy. Obviously, we should strive to minimize damage inflicted by ischemia and reperfusion, thereby reducing infarct size and thus minimizing LV remodeling.

We believe that the ideal cell for AMI patients has the following charateristics: 1) pronounced paracrine anti-apoptotic, pro-angiogenic, and immuno-modulatory capacities; 2) mobilize or stimulate resident CSC and/or cardiomyocytes to proliferate; 3) available during the (hyper)acute phase of the AMI; 4) non-embryonic; 5) multipotent; 6) autologous or non-immunogenic. BMMNC contain some of those characteristics, but MSC harbor all, whereas its paracrine capacities exceed those of BMMNC.[56] Moreover, the immune-privileged state of MSC renders the possibility of an "off-the-shelf" allogeneic cell product, which enables delivery directly following reperfusion of the AMI. It should be noted that also autologous adipose tissue-derived MSC can be available in the acute phase of the AMI, given their high frequency in, and easy accessibility of, adipose tissue.[9] All other cell types necessitate cell culture expansion, which makes the application within hours following the AMI impossible. Thus, to date, MSC seem the most logical candidate for cellular therapy in AMI patients. The AMICI trial, as well as several other forthcoming phase II studies, will render more insight in the safety and efficacy of allogeneic MSC in AMI patients.

In contrast to AMI, HF is a chronic condition with a much broader time window of possible stem cell transplantation, thereby enabling the use of other autologous cell types. Ideally, in patients with heart failure due to systolic dysfunction, cells should be applied that can 1) contribute to the contractile apparatus; 2) influence the remodeling process; and/or 3) enhance blood and nutrients supply. It is still far from feasible to transplant pluripotent cells that have differentiated into therapeutic amounts of cardiomyocyte-like cells, and that engraft and electromechanically couple with the host myocardium. However, in recent years, several cell types that are already committed towards a cardiomyogenic lineage have entered the (pre-)clinical arena, whereas the role for BMMNC seems to subside.[38,40] Interestingly, the proof of principle of cardiac stem cells and cardiosphere-derived cells in HF has been established, and the near future will probably prove whether these cells have a definite place in this disease type.[14,15,124] Currently the most feasible cell type in HF, however, is the MSC. They are readily available, and both autologous and allogeneic MSC have been shown to reverse cardiac remodeling following intramyocardial injection, which might be associated with their stimulatory effect on resident cardiac stem cells and cardiomyocytes, as well as their pro-survival and pro-angiogenic potential.[8,63,92,106,138] Moreover, MSC driven towards a cardiogenic phenotype were recently shown to have beneficial effects in HF patients.[76] In conclusion, in our opinion, MSC are still the cornerstone of contemporary cardiac cellular therapy until potential new players take over.

Timing of stem cell delivery

One of the unsolved issues in cardiac cell therapy is the ideal timing of cell transplantation following the AMI. Almost all clinical studies that assessed the effect of BMMNC infused the between 2-30 days following the AMI. This was primarily based on results in pilot studies, the landmark REPAIR-AMI trial, as well as logistical considerations.[139–141] The recent SWISS-AMI, TIME and late-TIME trials, but also several meta-analyses showed no difference in benefit, if there was any benefit at all, between the early and late time points within these limits.[34,36,142,143] It should be noted, however, that in AMI patients one of the predominant working mechanism of cell therapy is believed to be

through cardiomyocyte salvage, which is evoked by the anti-apoptotic and pro-survival properties of the cells. This suggests that cell therapy should be initiated soon after reperfusion, as in that period most cardiomyocytes are at risk for necrosis or apoptosis.[144,145] We believe that the anti-apoptotic and pro-survival characteristics of, for instance, MSC are best utilized when these cells are infused briefly following the primary PCI. This implicates that the cells are infused in a hostile environment, and that many transplanted cells may not survive. However, it is this hostile environment that the cells need to ameliorate, not only by their anti-apoptotic and pro-survival capacities, but also by influencing the local immunologic milieu[83] and reducing oxidative stress.[68] It was recently posed that the ideal time window for cell transplantation is within 6 hours after the primary PCI, or 5 days later.[84] Indeed, our group found that intracoronary infusion of Stro3+ MSC directly during reperfusion in an ovine model of AMI, resulted in extremely high cell retentions, a marked reduction of infarct size, and improved global and regional cardiac function.[95] This seems to confirm that, although many MSC may subside directly following AMI, the surviving MSC can exert pronounced beneficial effects.

However, injection of stem cells into sub-acute AMIs of 2-4 weeks old can still preserve myocardium and reverse cardiac remodeling.[8,14] Moreover, as discussed above, stem cell therapy in remodeled and already failing ventricles can still result in reduction of LV volumes.[6,70,71,107] The most beneficial effect in post-AMI patients though, is likely to be achieved in the yet non-dilated ventricle.

Delivery methods

Although the field of cell therapy has advanced considerably, and many cell types are currently in phase II clinical testing, several issues still remain. Finding the most appropriate cell delivery method is one of those issues, and still a matter of debate. Several techniques have been described, including intravenous, intracoronary, percutaneous endomyocardial, surgical epicardial, and retrograde transvenous into the coronary sinus, but percutaneous intracoronary and endomyocardial delivery are the most widely used techniques.[146,147] The best delivery technique largely depends on the disease type. Intracoronary delivery necessitates homing to the site of injury, which includes passage through the endothelial barrier. We currently know that homing signals (*i.e.* SDF-1 expression) are highest within the first few days following an AMI, whereas they subside to sub-clinical levels in chronic HF.[148] This implicates that briefly following an AMI, intracoronary infusion can be applied, and stem cells are attracted to the site of injury.[149–151] This was confirmed in several pre-clinical studies that revealed that intracoronary infusion efficiently targets post-AMI myocardium.[95,99,108] In most clinical studies thus far, a stop-flow technique was adopted by using an over-the-wire balloon that is briefly inflated during stem cell delivery. However, recent studies suggest that continuous infusion without balloon occlusion might result in comparable efficacy results, and even better stem cell homing.[9,95,152]

Because homing signals are mostly absent in failing hearts, intracoronary infusion results in sub-optimal stem cell homing in HF patients.[153] Hence, in this patient population, direct intramyocardial injection has been the preferred mode of stem cell delivery since the beginning of cellular therapy[1,2], and was applied in numerous clinical studies thus far. Intramyocardial injection ensures delivery of the stem cells directly into the interstitial space of the myocardium. However, defining and reaching the target

area remains a challenge. Endomyocardial injection using an endovascular approach, combined with electromechanical mapping (NOGA-XP in combination with MyoStar[154] injection catheter), is currently most widely used[2,40,70,107,155–157], and ensures accurate localization of ischemic, hibernating, or scarred areas.[158] Also, several other injection catheters were developed, all with their specific advantages or disadvantages (Helix[159], MyoCath[160], Stiletto[161], SilverPoint). Direct epicardial injection during (bypass) surgery is yet another option for intramyocardial stem cell delivery, and enables direct visualization of the scar and border zone.[1,6,52,162] However, this approach necessitates sternotomy or thoracotomy, which might be unwanted in this frail patient population.

Considerations and future directions

Cellular therapy for both AMI and HF patients has progressed substantially, since the first patients were treated more than a decade ago. Safety and feasibility has been shown for numerous cell types in phase I/IIa studies, and the field is slowly progressing towards phase IIb and III clinical trials. However, many questions still remain unanswered. It is yet unknown what cell type will prevail, what delivery method is safest and most efficient, and the optimal timing of cell delivery is still controversial.

We believe that the forthcoming, EU-sponsored, phase III BAMI trial will definitively show if there is a place for BMMNC in AMI patients, as it is supposed to be powered to find differences on hard clinical endpoints. Moreover, phase II studies using intracoronary or intravenous delivery of allogeneic MSC will assess their presumed superiority to BMMNC, whereas the role for CDC in AMI patients is yet to be determined. Also the field of HF treatment is progressing, recent promising results of the TAC-HFT trial favoring MSC over BMMNC. Moreover, a phase III study was initiated to assess the efficacy of Stro3+ MSC in 225 ischemic HF patients, and the sequel of the C-CURE trial is much anticipated.

The pre-clinical field is currently progressing rapidly in the ongoing search for new cells, or optimizing existing therapies. Of note are the numerous investigations that are ongoing to enhance stem cell homing and engraftment by gene therapy, or the application of micro-RNA.[163–165] Moreover, numerous biomaterials are currently assessed to improve cell retention, including hydrogels, alginate, and extra cellular matrix surrogates.[166,167] Genetic engineering or preconditioning of stem cells with pro-survival or anti-apoptotic factors were shown to have beneficial effects on stem cell survival, whereas preconditioning of the receiving environment were also beneficial.[168–173]

In conclusion, our knowledge about cellular therapy for cardiovascular repair has progressed significantly over the past two decades. Although it seems that the role for first generation cells is subsiding, cells from the third and fourth generation show pronounced effects in preclinical investigations and favorable results in phase I clinical studies. It seems obvious that cell therapy for cardiovascular disease is here to stay, although several questions remain to be answered in future investigations.

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CHAPTER 3

Intracoronary Stem Cell Infusion Following Acute Myocardial Infarction: A Meta-analysis and Update on Clinical Trials

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ABSTRACT

Background Several cell-based therapies for the adjunctive treatment of acute myocardial infarction (AMI) have been investigated in multiple clinical trials, but the benefits still remain controversial. This meta-analysis aims to evaluate the efficacy of BMMNC therapy in AMI patients, but also explores the effect of newer generations of stem cells.

Methods and Results A random-effects meta-analysis was performed on randomized controlled trials (RCT) investigating the effects of stem cell therapy in patients with AMI that were published between January 2002 and September 2013. The defined endpoints were left ventricular ejection fraction (LVEF), left ventricular end-systolic and end-diastolic volumes (LVESV/LVEDV), infarct size and major adverse cardiac and cerebral event (MACCE) rates. Also several subgroup analyses were performed on BMMNC trials. Overall, combining results of 22 RCTs, LVEF increased by +2.10% (95% CI, 0.68- 3.52 , P=0.004) in the BMMNC group as compared to controls, evoked by a preservation of LVESV (-4.05 ml ; 95% CI, -6.91- -1.18, P=0.006), and a reduction in infarct size (IS; -2.69%; 95% CI, -4.83- -0.56, P=0.01). However, there is no effect on cardiac function, volumes, nor infarct size, when only randomized controlled trials (n=9) were analyzed that used MRI-derived endpoints. Moreover, no beneficial effect could be detected on MACCE rates following BMMNC infusion after median follow-up duration of 6 months.

Conclusions Intracoronary infusion of BMMNC is safe, but does not enhance cardiac function on MRI-derived parameters, nor does it improve clinical outcome. New and possibly more potent stem cells are emerging in the field, but their clinical efficacy still needs to be defined in future trials.

INTRODUCTION

Despite advancements in treatment options, ischemic heart failure (IHF) remains the leading cause of morbidity and mortality in the Western world.[1] Therefore, the search for new therapeutic strategies to prevent adverse ventricular remodeling following acute myocardial infarction (AMI) and subsequent development of IHF is ongoing. More than a decade after the first patient with an AMI was treated with intracoronary infusion of unfractioned bone marrow-derived mononuclear cells (BMMNC)[2], numerous clinical studies have investigated cell-based therapy as an adjuvant treatment in AMI patients. These studies have repeatedly shown that stem cell therapy is safe and feasible. However, although initial results were promising with significant improvement in left ventricular (LV) function and volumes [3–5], other studies showed ambiguous or even negative results.[6–9] This controversy resulted in an continued search for new cell types, and methods to improve outcomes, but still many questions remain.

Thus far, it has been difficult to make solid statements on efficacy and long-term effects on clinical outcomes of cellular therapy due to the limited number of treated patients, and the relatively short follow up (FU) period. However, recently, some larger studies reported their primary results, whereas other studies presented long-term FU data.[5,10–12]

Several meta-analysis regarding BMMNC for the treatment of AMI and ischemic heart failure have been published to date.[13–15] These analyses showed an improvement of only 2-3% on left ventricular ejection fraction (LVEF), and a significant reduction of major adverse cardiac and cerebrovascular events (MACCE; for example all-cause mortality; OR 0.39; 95% CI 0.27-0.55).[14] However, these manuscripts only evaluated the use of autologous BMMNC on cardiac repair, whereas several other cell types have now been investigated to date. Moreover, in the most cited meta-analysis of Jeevanantham, data of AMI and IHF patients were pooled for the evaluation of clinical outcome parameters and subgroup analyses, which might have clouded the outcome in AMI patients.[14]

The current meta-analysis focuses solely on AMI patients, who have been treated with an infusion of BMMNC, but also autologous or allogeneic mesenchymal stem cells (MSC), adipose tissue-derived regenerative cells (ADRC) or cardiosphere-derived cells (CDC). It thereby provides a side-by-side comparison of BMMNC and other cell populations in AMI patients.

METHODS

This meta-analysis was executed according to the Quorum statements.[16] Briefly, a random effect meta-analysis was performed that included all randomized controlled trials regarding stem cell therapy for the treatment of AMI, published on Medline (Pubmed) between July 2002 and September 2013. BMMNC were the main focus in this study, as the majority of studies to date assessed this specific cell type. Moreover, the effects of BMMNC therapy were compared with newer generations of stem cells, including MSC, bone marrow progenitors (CD133+/CD34+ cells), ADRC and CDC. The following search strategy was applied: “stem cells”, “progenitor cells”, “mononuclear cells”, “adipose tissue-derived regenerative cells”, “mesenchymal stem cells’, “cardiac-derived stem cells”, “bone marrow”, “vascular stromal fraction”, “adipose stem cells”, “mesenchymal-like stem cells”, “coronary artery disease”,

"myocardial infarction", "cardiac repair", and "myocardial regeneration". Only articles published in English were included (Supplement Table I). Studies were included that met the following criteria: (1) randomized controlled trials with an appropriate control group who received standard therapy, (2) conducted in patients with an AMI that occurred less than 3 months before, (3) using stem cells that were administered by intracoronary or intravenous injection, (4) total of number of patients enrolled should exceed 10, (5) stem cells were derived from adipose tissue, bone marrow or heart, (6) given in an allogeneic or autologous setting.

Data abstraction and analysis was performed by three different researchers (RdJ, JH, SS) and reported on standardized forms. LVEF, left ventricular end-systolic volume (LVESV), left ventricular end-diastolic volume (LVEDV) and infarct size were assessed as outcome measures as well as clinical outcome. Additional subgroup analyses were performed within the RCTs that investigated BMMNC therapy, in an attempt to gain more insight into possible discriminating parameters or conditions that might improve outcome in future trials.

Subgroup analyses that were conducted are: (1) follow up (FU) duration of 6 months, 6-18 months, and 18-60 months; (2) the different imaging modalities that were used to assess LVEF, LV volumes and infarct size; (3) LVEF at baseline (<40%, <45%, <50%, >50%); (4) the amount of infused cells (<50 million, <100 million, >100 million), (4) timing of delivery (< 2 days, 2-7 days (7 days was the median in this analysis), > 8 days after MI); (5) delivery method (intracoronary 'stop-flow' technique, continuous intracoronary infusion); (6) location of AMI (anterior wall versus all other AMI locations); (7) the used cell preparation method and the use of heparin in the final cell suspension; and (8) Lymphoprep versus Ficoll-based isolation.

Data analysis

Left ventricular function was the primary endpoint of our analysis. In particular, we studied the difference in mean LV ejection fraction change (LVEF, from baseline to follow-up) between patients receiving stem cells and control treatment. We have applied inverse-variance weighting to combine the results from independent studies. Most studies reported mean LVEF ± one standard deviation (SD) at baseline and follow-up. The mean $LVEF_{change}$ was then determined as $LVEF_{follow\text{-}up}$ - $LVEF_{baseline}$, whereas the SD_{change} was estimated according to the method that is described by Hristov *et al*.[17] For studies that report standard errors of the mean (SEM), SDs were determined as SEM*√(sample size). In case interquartile ranges are reported, SDs are estimated as range/4. We applied a random effects model to obtain an overall estimate of the treatment effect, which we report as point estimate and 95% confidence interval (CI). Heterogeneity was analyzed with the I^2 statistic, and was defined as low (25%-50%), intermediate (50%-75%) or high (>75%).

We have applied similar methodology to study several secondary endpoints, including (mean changes in) left ventricular end systolic (LVESV) and end diastolic (LVEDV) volumes, infarct size as measured by cardiac MRI, and perfusion defect as measured by SPECT. We applied the Mantel-Haenszel odds ratio to obtain an overall estimate of the odds ratio for MACCE, again assuming random effects.

All analyses were performed using Review Manager 5.2 analysis software (Rev Man, Version 5.2, Copenhagen, The Nordic Cochrane Centre, The Cochrane collaboration, 2012). We considered p-values <0.05 (two-sided) as statistical significant. Funnel plots were constructed to explore publication bias. A detailed description of the methods can be found in the material and methods section in the data supplement.

RESULTS

Search results

The final search on September 1st 2013 resulted in a total of 386 articles. The majority of articles were excluded, due to study subject (chronic heart failure or G-CSF treatment), duplicate reports or reviews, resulting in a total of 47 studies. When cohort studies were omitted, 42 articles were used in final analysis.[3–7,10,11,18–52]we aimed to assess whether intracoronary transfer of autologous bone-marrow cells could improve global left-ventricular ejection fraction (LVEF Finally, a total of 30 RCTs were used in this meta-analysis, comprising a total of 2037 patients, 1218 of whom were treated with cells (Supplemental Figure A). Twenty-two RCTs investigated BMMNC for cardiac repair, whereas 3 trials investigated MSC or MSC-like cells, 4 trials subjected bone marrow progenitors and 1 trial investigated the effects of CDC.

Study Quality

The quality of the RCT was assessed by the Jüni criteria (Supplemental Table II).[53] In 60% of the RCTs, patients and or investigators were not blinded for the cell intervention. Control patients did not undergo a sham biopsy and infusion of cells in most of these studies. Patient follow-up was completed in all studies.

Study Characteristics

The average of participating patients per study was 68 ± 51 patients, whereas the median was 45 patients (range 14-200). Most studies used a 1:1 randomization scheme. The median follow-up duration in all studies was 6 months (range 3-60 Months). The median amount of infused viable cells was 100 million (range from 5 x 10^6 to 60 x 10^9) and the cells were infused after a median of 7 days (range <24 hours to 3 months). MRI was the imaging modality of choice for FU of LV function in 40% of the RCTs (Supplemental Table III).

BMMNC: Cardiac parameters

Overall, BMMNC infusion increased LVEF by +2.10% (95% CI, 0.68-3.52, P=0.004; Figure 1A; 21 trials). LVEDV decreased by -2.80 ml (95% CI, -6.03-0.44, P=0.09; Figure 1C), whereas LVESV decreased by -4.05 mL (95% CI, -6.91- -1.18, P=0.006; Figure 1D) in the cell therapy group. Infarct size was reduced by -2.69% (95% CI, -4.83- -0.56, P=0.01; Figure 1B).

BMMNC: Subgroup analyses

1. Effects of BMMNC transplantation over time

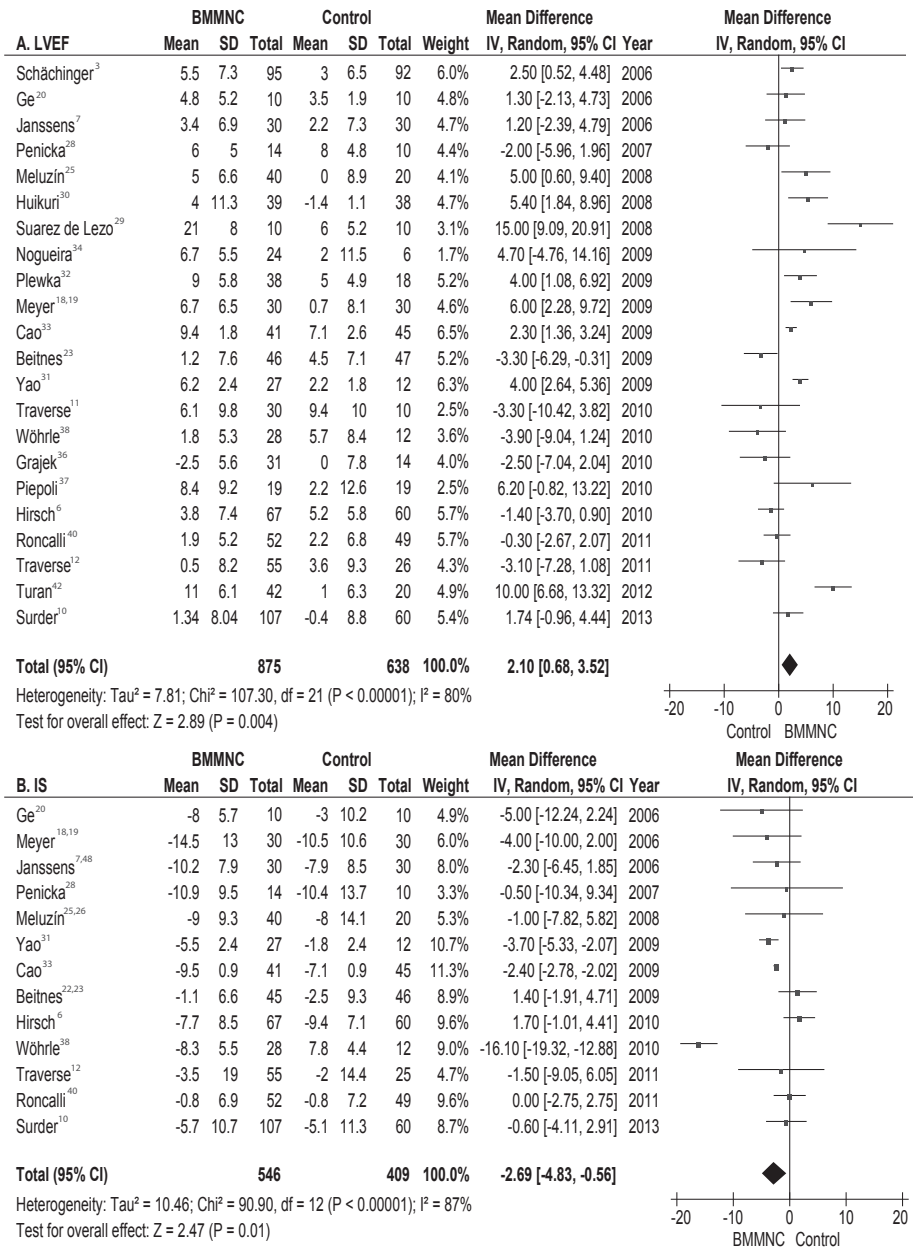
Subgroup analysis revealed that at 6 months FU (n= 21 RCT) the LVEF increased by +2.08% (95% CI, 0.55- 3.60, P=0.008; Table 1). At 12 months (n= 8) this beneficial effect was sustained and increased to more pronounced effect of +3.04% (95% CI, 1.27-4.81, P= 0.0008) when compared to control. At 36-60 months FU (n=3), this treatment effect disappeared to +1.19% (95% CI, -2.74- 5.12, P=0.55) (Table 1). This increase in LVEF up to 18 months FU, was mainly due to a preservation of LVESV in the BMMNC group (as opposed to the control group). In the treatment group, LVESV progressively decreased by -4.84 mL (95% CI, -7.69- -2.00, P=0.0008) at 6 months FU and -3.56 ml at 18 month FU (95% CI, -6.87- -0.25, P=0.03). Infarct size was significantly reduced at 6 months FU (-2.69%, 95% CI,-4.83 - -0.56, P=0.01) and 18 month FU (-3.71%, 95% CI, -6.99- -0.43, P=0.03). This significant effect on infarct size diminished at long term FU (>18 month FU; -0.82%, 95% CI, -3.78-2.15, P=0.59).

Table 1. Effects of BMMNC transplantation over time

LVEF	Difference in mean, (95% CI)	P-value
≤ 6months	2.08 [0.55, 3.60]	0.009
6-18 months	3.04 [1.27, 4.81]	0.0008
> 18-60 months	1.19 [-2.74, 5.12]	0.55
LVEDV		
≤ 6months	-3.18 [-6.59, 0.24]	0.07
6-18 months	-1.75 [-6.28, 2.79]	0.45
> 18-60 months	-1.75 [-6.57, 3.07]	0.48
LVESV		
≤ 6months	-4.84 [-7.69, -2.00]	0.0008
6-18 months	-3.56 [-6.87, -0.25]	0.03
> 18-60 months	-0.44 [-9.94, 9.07]	0.06
Infarct size		
≤ 6months	-2.69 [-4.83, -0.56]	0.01
6-18 months	-3.71 [-6.99, -0.43]	0.03
> 18-60 months	-0.82 [-3.78, 2.15]	0.59

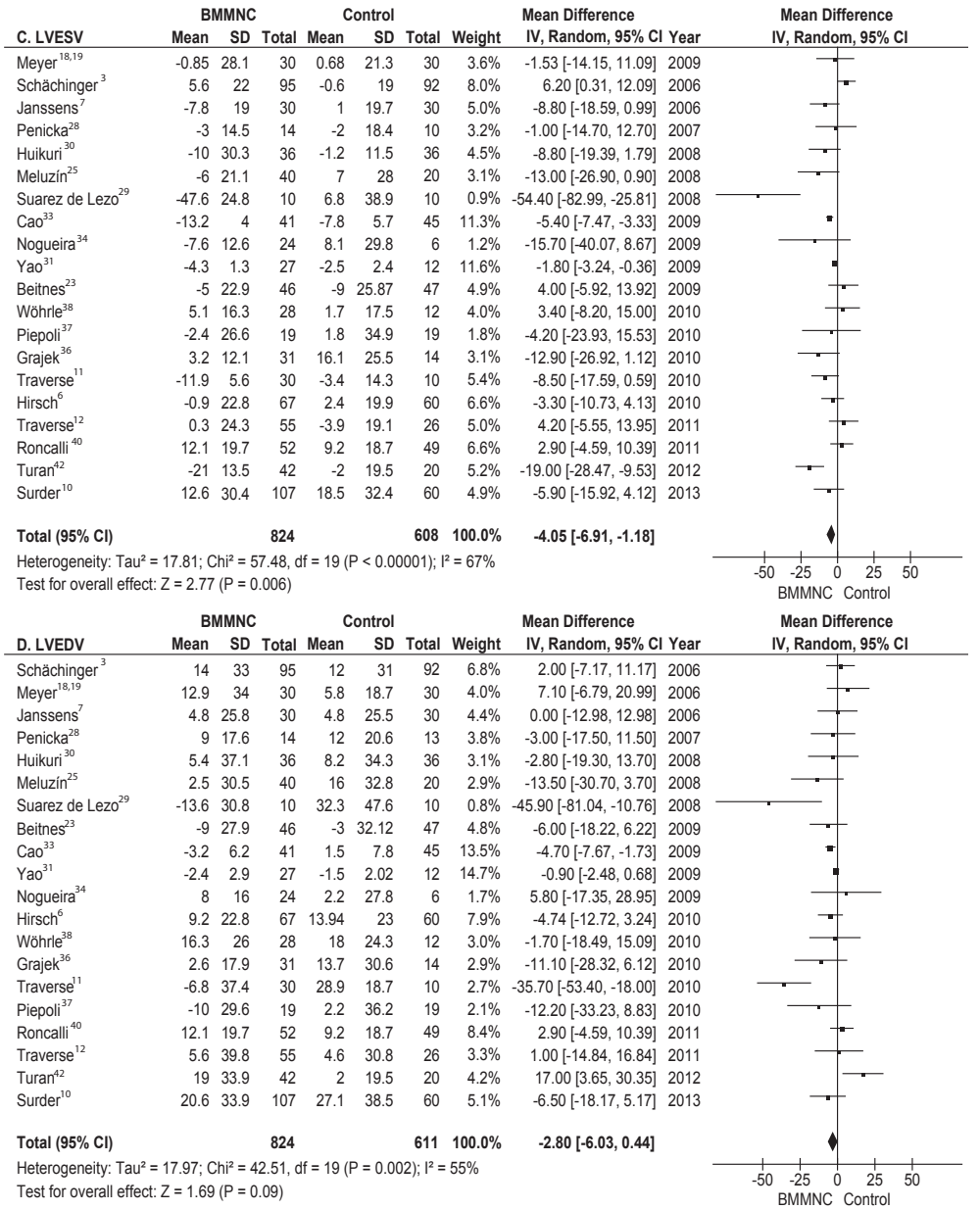
The beneficial effect of BMMNC therapy on left ventricular ejection fraction (LVEF) was maintained during 18 year follow-up. This effect on LVEF was mainly due to a preservation of left ventricular end-systolic volume (LVESV). The effects diminished after the 18 months FU. LVEDV: left ventricular end-diastolic volume.

Figure 1. Forest plot BMMNC therapy



1A: Forest plot of change in left ventricular ejection fraction of BMMNC transplantation (unadjusted difference in mean, 95% CI). Overall LVEF is increased by +2.10 % (95% CI, 0.68-3.52, P=0.004). **1B:** Forest plot of unadjusted difference in mean change in infarct size. Infarct size was reduced by -2.69 (95% CI, -4.83- -0.56 P=0.01).

Figure 1. Continued



C. LVESV	BMMNC Mean	BMMNC SD	BMMNC Total	Control Mean	Control SD	Control Total	Weight	Mean Difference IV, Random, 95% CI	Year
Meyer[18,19]	-0.85	28.1	30	0.68	21.3	30	3.6%	-1.53 [-14.15, 11.09]	2009
Schächinger[3]	5.6	22	95	-0.6	19	92	8.0%	6.20 [0.31, 12.09]	2006
Janssens[7]	-7.8	19	30	1	19.7	30	5.0%	-8.80 [-18.59, 0.99]	2006
Penicka[28]	-3	14.5	14	-2	18.4	10	3.2%	-1.00 [-14.70, 12.70]	2007
Huikuri[30]	-10	30.3	36	-1.2	11.5	36	4.5%	-8.80 [-19.39, 1.79]	2008
Meluzín[25]	-6	21.1	40	7	28	20	3.1%	-13.00 [-26.90, 0.90]	2008
Suarez de Lezo[29]	-47.6	24.8	10	6.8	38.9	10	0.9%	-54.40 [-82.99, -25.81]	2008
Cao[33]	-13.2	4	41	-7.8	5.7	45	11.3%	-5.40 [-7.47, -3.33]	2009
Nogueira[34]	-7.6	12.6	24	8.1	29.8	6	1.2%	-15.70 [-40.07, 8.67]	2009
Yao[31]	-4.3	1.3	27	-2.5	2.4	12	11.6%	-1.80 [-3.24, -0.36]	2009
Beitnes[23]	-5	22.9	46	-9	25.87	47	4.9%	4.00 [-5.92, 13.92]	2009
Wöhrle[38]	5.1	16.3	28	1.7	17.5	12	4.0%	3.40 [-8.20, 15.00]	2010
Piepoli[37]	-2.4	26.6	19	1.8	34.9	19	1.8%	-4.20 [-23.93, 15.53]	2010
Grajek[36]	3.2	12.1	31	16.1	25.5	14	3.1%	-12.90 [-26.92, 1.12]	2010
Traverse[11]	-11.9	5.6	30	-3.4	14.3	10	5.4%	-8.50 [-17.59, 0.59]	2010
Hirsch[6]	-0.9	22.8	67	2.4	19.9	60	6.6%	-3.30 [-10.73, 4.13]	2010
Traverse[12]	0.3	24.3	55	-3.9	19.1	26	5.0%	4.20 [-5.55, 13.95]	2011
Roncalli[40]	12.1	19.7	52	9.2	18.7	49	6.6%	2.90 [-4.59, 10.39]	2011
Turan[42]	-21	13.5	42	-2	19.5	20	5.2%	-19.00 [-28.47, -9.53]	2012
Surder[10]	12.6	30.4	107	18.5	32.4	60	4.9%	-5.90 [-15.92, 4.12]	2013
Total (95% CI)			**824**			**608**	**100.0%**	**-4.05 [-6.91, -1.18]**	

Heterogeneity: Tau² = 17.81; Chi² = 57.48, df = 19 (P < 0.00001); I² = 67%
Test for overall effect: Z = 2.77 (P = 0.006)

Mean Difference IV, Random, 95% CI
-50 -25 0 25 50
BMMNC Control

D. LVEDV	BMMNC Mean	BMMNC SD	BMMNC Total	Control Mean	Control SD	Control Total	Weight	Mean Difference IV, Random, 95% CI	Year
Schächinger[3]	14	33	95	12	31	92	6.8%	2.00 [-7.17, 11.17]	2006
Meyer[18,19]	12.9	34	30	5.8	18.7	30	4.0%	7.10 [-6.79, 20.99]	2006
Janssens[7]	4.8	25.8	30	4.8	25.5	30	4.4%	0.00 [-12.98, 12.98]	2006
Penicka[28]	9	17.6	14	12	20.6	13	3.8%	-3.00 [-17.50, 11.50]	2007
Huikuri[30]	5.4	37.1	36	8.2	34.3	36	3.1%	-2.80 [-19.30, 13.70]	2008
Meluzín[25]	2.5	30.5	40	16	32.8	20	2.9%	-13.50 [-30.70, 3.70]	2008
Suarez de Lezo[29]	-13.6	30.8	10	32.3	47.6	10	0.8%	-45.90 [-81.04, -10.76]	2008
Beitnes[23]	-9	27.9	46	-3	32.12	47	4.8%	-6.00 [-18.22, 6.22]	2009
Cao[33]	-3.2	6.2	41	1.5	7.8	45	13.5%	-4.70 [-7.67, -1.73]	2009
Yao[31]	-2.4	2.9	27	-1.5	2.02	12	14.7%	-0.90 [-2.48, 0.68]	2009
Nogueira[34]	8	16	24	2.2	27.8	6	1.7%	5.80 [-17.35, 28.95]	2009
Hirsch[6]	9.2	22.8	67	13.94	23	60	7.9%	-4.74 [-12.72, 3.24]	2010
Wöhrle[38]	16.3	26	28	18	24.3	12	3.0%	-1.70 [-18.49, 15.09]	2010
Grajek[36]	2.6	17.9	31	13.7	30.6	14	2.9%	-11.10 [-28.32, 6.12]	2010
Traverse[11]	-6.8	37.4	30	28.9	18.7	10	2.7%	-35.70 [-53.40, -18.00]	2010
Piepoli[37]	-10	29.6	19	2.2	36.2	19	2.1%	-12.20 [-33.23, 8.83]	2010
Roncalli[40]	12.1	19.7	52	9.2	18.7	49	8.4%	2.90 [-4.59, 10.39]	2011
Traverse[12]	5.6	39.8	55	4.6	30.8	26	3.3%	1.00 [-14.84, 16.84]	2011
Turan[42]	19	33.9	42	2	19.5	20	4.2%	17.00 [3.65, 30.35]	2012
Surder[10]	20.6	33.9	107	27.1	38.5	60	5.1%	-6.50 [-18.17, 5.17]	2013
Total (95% CI)			**824**			**611**	**100.0%**	**-2.80 [-6.03, 0.44]**	

Heterogeneity: Tau² = 17.97; Chi² = 42.51, df = 19 (P = 0.002); I² = 55%
Test for overall effect: Z = 1.69 (P = 0.09)

Mean Difference IV, Random, 95% CI
-50 -25 0 25 50
BMMNC Control

1C: Forest plot of unadjusted difference in mean change in LVESV. LVESV decreased by -4.05 ml (95% CI, -6.91--1.18, P= 0.006) in the treatment group. **1D:** Forest plot of unadjusted difference in mean LVEDV(with 95% CI). LVEDV decreased by -2.80 ml (95% CI, -6.03-0.44, P=0.09).

2. Imaging modality

Interestingly, when subgroup analysis is performed based on MRI (n=9), which is currently considered as the golden standard to assess cardiac function and volumes, the significant effect of BMMNC therapy on LVEF diminished (0.13%, 95% CI -2.67 -2.93, P=0.93). Also, the beneficial effect on LV volumes and infarct size disappeared (Table 2). This finding could indicate that BMMNC therapy is not beneficial in AMI patients.

Table 2. BMMNC therapy per imaging modality

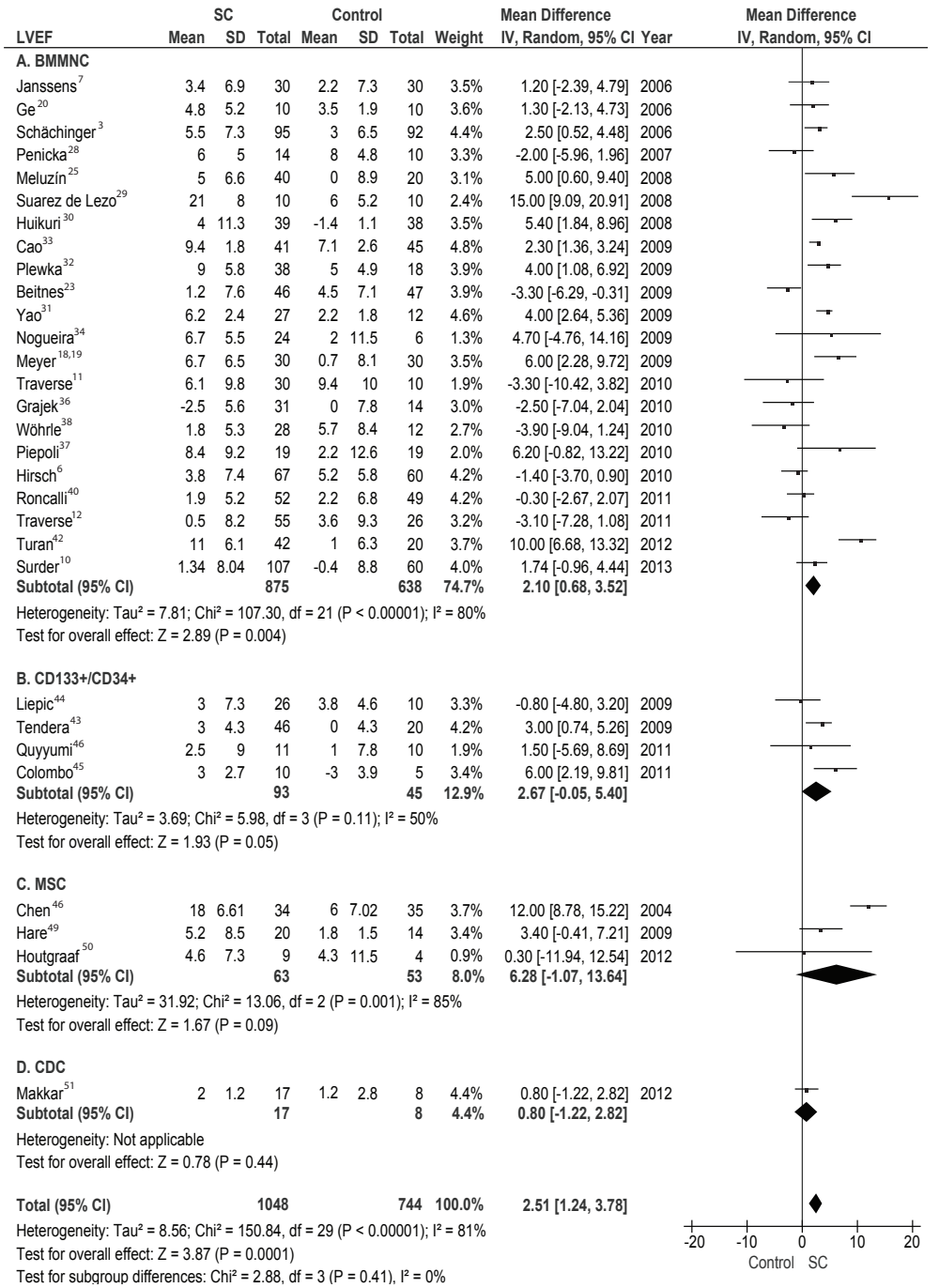
Imaging modality	Difference in mean (95% CI)	P for Z	P for subgroup differences
LVEF			
MRI	0.13 [-2.67, 2.93]	0.93	0.03
Echo	3.05 [1.45, 4.64]	0.0002	
SPECT	5.63 [1.81, 9.44]	0.004	
LVG	7.69 [2.43, 12.95]	0.004	
LVEDV			
MRI	-0.86 [-4.66, 2.94]	0.66	0.13
Echo	-3.21 [-5.99, -0.43]	0.02	
SPECT	-15.24 [-30.88, 0.40]	0.06	
LVG	1.85 [-4.44, 8.13]	0.56	
LVESV			
MRI	-2.65 [-5.28, 0.02]	0.06	0.01
Echo	-6.17 [-8.31, -4.03]	<0.00001	
SPECT	-12.71 [-24.41, -1.01]	0.03	
LVG	-14.88 [-26.85, -2.90]	0.01	
Infarct size			
MRI	-1.11 [-3.74, -1.53]	0.82	0.44
SPECT	-2.40 [-2.85, -1.95]	0.00001	

Parameters are represented as unadjusted difference in mean, with 95% CI. Subgroup analysis of MRI revealed that BMMNC therapy is not beneficial for improvement of left ventricular ejection fraction (LVEF). LVEDV: left ventricular end-diastolic volume; LVESV: left ventricular end-systolic volume; LVG: left ventricular angiography.

3.BMMNC versus other cell types

IC infusion of bone marrow progenitor cells (*i.e.* CD34/CD133+ cells) resulted in a significant increase in LVEF of +2.67% (95% CI: -0.05- 5.40, P=0.05; Figure 2), whereas MSC transplantation resulted in an increase of +6.28% (95% CI: -1.07 - 13.64, P=0.09). LVEF increased with +0.80% after transplantation of CDC (95% CI: -1.21-2.82, P=ns). When data of all stem cells are combined, LVEF increases by +2.51% (95% CI 1.24 - 3.78, P=0.0001; Figure 2). Remarkably, we found a significant increase of LVEDV in the CD133/CD34+ patient group by +14.06 mL (95% CI 9.63-18.48) as opposed to a decrease by -2.80 ml in the BMMNC patient group (-6.03 -0.44, P for subgroup differences<0.00001). Table 3 summarizes functional parameters of other cell types opposed to BMMNC and the other subgroup analysis. CDC were omitted from further comparison, because only one trial investigated this new cell type.

Figure 2. Forest plot of LVEF per stem cell type



Forest plot of unadjusted difference in mean LVEF (with 95% CI) of all trials and stem cells to date. The overall effect on LVEF on all stem cells combined is +2.51% (95% CI: 1.24-3.78; P=0.0001). BMMNC: Bone marrow Mononuclear cell, MSC: mesenchymal stem cell: CDC: cardiosphere derived cells.

Table 3. Subgroup analysis of BMMNC and comparison between BMMNC and other stem cells

		Ejection Fraction		Infarct size	
Subanalyses	**No of RCT**	**Difference in mean**	**P-value subgroup difference**	**Difference in mean**	**P-value subgroup difference**
EF prior to infusion					
EF < 40%	8	3.46 [1.12, 5.81]	0.53	-2.46 [-4.72, -0.21]	0.06
EF ≥ 40%	15	2.52 [0.69, 4.34]		0.08 [-1.38, 1.55]	
EF < 45%	10	3.19 [1.07, 5.30]	0.46	-2.39 [-2.75, -2.02]	0.05
EF ≥ 45%	12	1.95 [-0.54, 4.45]		-0.50 [-2.36, 1.36]	
EF < 50%	17	2.80 [1.07, 4.53]	0.67	-1.81 [-3.64, 0.02]	0.34
EF ≥ 50%	5	1.85 [-2.10, 5.80]		-1.32 [-2.90, 0.27]	
Target Vessel					
LAD/LCx/RCA	13	1.42 [-0.06, 2.79]	0.25	-0.26 [-1.57, 1.04]	0.19
LAD infarct	9	3.11 [0.61, 5.61]		-2.47 [-5.51, 0.56]	
Timing of infusion					
Infusion <8 d	19	3.10 [1.49, 4.70]	0.009	-1.41 [-3.13, 0.32]	0.46
Infusion ≥ 8 d	3	-0.37 [-2.40, 1.66]		-0.40 [-2.46, 1.66]	
Amount of infused cells					
< 50 M cells	3	4.06 [2.81, 5.31]	0.10	-2.40 [-2.78, -2.02]	0.19
≥ 50 M cells	19	2.18 [0.32, 4.04]		-0.97 [-3.07, 1.13]	
< 100 M Cells	6	3.80 [0.66, 6.94]	0.39	-2.05 [-3.71, 0.38]	0.57
≥ 100 M cells	16	2.07 [0.15, 3.99]		-3.16 [-6.64, 0.32]	
Heparine use					
Heparine	15	3.56 [1.60, 5.51]	0.07	-1.57 [-3.62, 0.48]	0.55
No Heparine	5	0.91 [-1.16, 2.99]		-0.72 [-2.93, 0.10]	
BMMNC vs other SC			**versus BMMNC**		
BMMNC	22	2.10 [0.68, 3.52]		-2.69 [-4.83, -0.56]	
CD133+/CD34+	4	2.67 [-0.05, 5.40]	0.74	-2.84 [-6.38, 0.70]	0.96
MSC	3	6.28 [-1.07, 13.64]	0.27	-14.02 [-17.06,10.79]	<0.00001
EF prior to infusion					
EF < 40%	8	-0.69 [-3.49, 2.12]	0.84	-4.51 [-8.51, -0.52]	0.85
EF ≥ 40%	15	-1.41 [-7.58, 4.77]		-4.31 [-9.36, 0.73]	
EF < 45%	10	-1.51 [-4.41, 1.39]	0.70	-4.76 [-8.23, -1.29]	0.78
EF ≥ 45%	12	0.17 [-7.85, 8.19]		-3.80 [-9.72, 2.31]	
EF < 50%	17	-1.20 [-4.82, 2.42]	0.97	-5.62 [-8.84, -2.41]	0.57
EF ≥ 50%	5	-1.01 [-9.69, 7.67]		-3.09 [-11.33, 5.14]	
Target Vessel					
LAD/LCx/RCA	13	-0.73 [-4.38, 2.92]	0.36	-2.04 [-7.03, 2.94]	0.28
LAD infarct	9	-3.78 [-9.14, 1.57]		-5.50 [-9.40, -1.60]	
Timing of infusion					
Infusion <8 d	19	-1.27 [-4.74, 2.20]	0.03	-5.16 [-8.32, -2.00]	0.30
Infusion ≥ 8 d	3	8.38 [0.36, 16.39]		-0.44 [-8.79, 7.91]	

Table 3. Continued

		Ejection Fraction		Infarct size	
Subanalyses	**No of RCT**	**Difference in mean**	**P-value subgroup difference**	**Difference in mean**	**P-value subgroup difference**
Amount of infused cells					
< 50 M cells	3	-4.70 [-12.27, 2.88]	0.38	-6.49 [-8.84, -4.14]	0.26
≥ 50 M cells	19	-0.94 [-4.72, 2.83]		-4.00 [-7.60, -0.39]	
< 100 M Cells	6	4.01 [-8.40, 16.42]	0.35	-7.05 [-11.89, -2.20]	0.64
≥ 100 M cells	16	-2.27 [-6.45, 1.90]		-3.88 [-7.63, -0.13]	
Heparine use					
Heparine	15	-2.81 [-6.76, 1.13]	0.34	-3.69 [-4.82, -2.56]	0.28
No Heparine	5	0.49 [-5.08, 6.07]		-1.12 [-5.65, 3.41]	
BMMNC vs other SC					
BMMNC	22	-2.80 [-6.03, 0.44]		-4.05 [-6.91, -1.18]	
CD133+/CD34	4	14.06 [9.63, 18.48]	<0.00001	-3.00 [-8.07, 2.07]	0.72
MSC	3	-9.76 [-33.27, 13.74]	0.07	-6.68 [-23.86, 10.50]	0.77

RCT indicates randomized controlled trials; LVEF: left ventricular ejection fraction; LVEDV: left ventricular end-diastolic volume: LVESV; left ventricular end-systolic volume; LAD: left descending artery; LCx: left Circumflex artery; RCA: right coronary artery; BMMNC: bone marrow mononuclear cells; MSC: mesenchymal stem cells.

4. Infarct location and LV function at baseline

Patients with an anterior wall AMI due to occlusion of the LAD did not benefit more from stem cell therapy than patients with an AMI located elsewhere (Table 3). According to our data, patients with a lower LVEF (*LVEF* <40% or *LVEF* <45%) at baseline did not benefit more from cell therapy, than patients with a higher LVEF. Only, the beneficial effect of BMMNC therapy on infarct size was significantly greater in patients with a LVEF below 45% (-2.39%; 95% CI, -2.75- -2.02), as opposed to almost no reduction (-0.50%; 95% CI, -2.36- 0.27) in patients with a LVEF above 45% (P for subgroup differences 0.05).

5. Parameters related to cell infusion

The median cell number infused was 100 million cells. Intriguingly, total cell number did not predict outcome. More specifically, patients treated with infusion of <100 million cells did not benefit more or less from cell infusion than patients with higher cell doses. BMMNC transplantation before day 8 resulted in an improved LVEF (+3.10%, 95% CI: 1.49 – 4.70, P=0.009) as opposed to late infusion (-0.37%, 95% CI -2.40 – 1.66).

6. Intracoronary delivery technique

We found that performing a subgroup analysis on intracoronary delivery technique was not feasible, as in all, except 2 studies, the 'stop-flow' technique was adopted.

7. Cell preparation

Subgroup analysis based on the methods of BMMNC isolation revealed that all trials, except for 2[6,33], used Ficoll-based isolation, therefore subgroup analysis was not useful. Final suspension of the cell preparation in heparin-containing saline or in non-heparinized solutions did not have a significant effect on treatment outcome.

Safety of IC cell infusion and MACCE-rates

In the majority of trials, IC infusion of stem cells did not result in procedure-related adverse events, or adverse events were not reported in the manuscript. Only 1 study described a case of thrombosis in the infarct related artery after AMI and 3 cases of intima dissection following balloon inflation during transplantation.[26]

The median follow-up duration for MACCE rates was 6 months. IC infusion of BMMNC did not result in reduction of any MACCE event. More specifically, and in contrast to previous reported meta-analysis, no differences on all-cause mortality, cardiac mortality, hospitalizations for heart failure, restenosis rate, thrombosis, target vessel revascularization (TVR), stroke, recurrent AMI and ICD implantations were detected between BMMNC patients and controls (Table 4). Bone marrow progenitor cell infusion resulted in a reduction in re-hospitalizations for heart failure (OR 0.14, 95% CI: 0.04-0.52; P=0.003) and MSC transplantation resulted in a reduction in VF/VT (OR 0.08, 95% CI: 0.01-0.79; P=0.03) and ICD implantations (OR 0.08; 95% CI: 0.01-0.79, P=0.03)

Table 4. MACCE events represented as Mantel-Haenszel odds ratio (OR)

	BMMNC			BM progenitor cells			MSC		
Outcome	**OR**	**95% CI**	**P**	**OR**	**95% CI**	**P**	**OR**	**95% CI**	**P**
All- cause mortality	0.68	0.36-1.31	0.25	0.50	0.09-2.67	0.41	3.18	0.13-81.01	0.48
Cardiac mortality	0.73	0.32-1.65	0.45		no events			no events	
Recurrent MI	0.5	0.24-1.06	0.07	2.25	0.04-1.42	0.70		no events	
TVR	0.86	0.58-1.27	0.44	0.96	0.1-2.29	0.93	1.59	0.05-7.52	0.79
Stent restenosis	0.95	0.51-1.79	0.88		no events			no events	
Stent thrombosis	0.75	0.08-7.45	0.81	0.61	0.10-3.84	0.60		no events	
Heart failure	0.84	0.44-1.60	0.60	0.14	0.04-0.52	0.003		no events	
CVA	0.62	0.13-2.84	0.53	0.29	0.01-7.76	0.46		no events	
VT/VF	0.60	0.30-1.21	0.16		no events		0.08	0.01-0.79	0.03
ICD	0.98	0.37-2.64	0.97	0.93	0.05-16.39	0.96	0.08	0.01-0.79	0.03

MACCE rates were not reduced in BMMNC treated patients. In patients treated with Bone marrow progenitor cells, the rehospitalizations for heart failure were lower opposed to the control group. Intracoronary infusion of mesenchymal stem cells (MSC) resulted in lower number of VT/VF and ICD implantations. It has to be kept in mind that the trials to date were not sufficiently powered to detect differences in clinical outcome. OR; odds Ratio; CI; confidence interval; MI: myocardial infarction; TVR; target vessel revascularization; VT; ventricular tachycardia; VF: ventricular fibrillation; ICD: Implantable Cardioverter Defibrillator.

Publication Bias

A funnel plot for LVEF showed that studies were equally distributed around the overall estimate, suggesting that there was no sign for publication bias (Supplemental Figure B).

DISCUSSION

In this meta-analysis, which comprises a total of 2037 AMI patients, cell therapy proved to be safe. BMMNC therapy modestly improves LVEF at short and long term FU when all imaging modalities are combined for analysis. The modest improvement in LVEF was mainly due to a sustained LVESV, accompanied by a reduction in infarct size. Interestingly, when only studies are analyzed that used cardiac MRI for measuring volumes and LVEF, this beneficial effect of BMMNC therapy on cardiac function disappeared. Furthermore, the occurrence MACCE events is not reduced in patients treated with BMMNC when compared to controls.

MACCE rates

One of the most salient findings of the current meta-analysis comprises the fact that, despite over 1,500 patients analyzed to date, BMMNC therapy did not affect clinical outcome measures in AMI patients. Our findings seem contradictory to findings in recent meta-analyses by Jeevanantham and coworkers who described a reduction in all-cause mortality, cardiac mortality, recurrent AMI, hospitalizations for heart failure and in-stent thrombosis after BMMNC transplantation. However, in this meta-analysis, both AMI and IHF patients were combined for the assessment of clinical outcome. We hypothesize that ischemic heart failure patients may benefit more from cell therapy, which was corroborated in a recent meta-analysis that solely focused on IHF patients.[15] The current analysis includes, for the first time, recent negative publications as the SWISS-AMI trial and LATE-TIME trial which has modified the results. It should be noted that the median follow-up duration for the assessment of MACCE rates is only 6 months, which might be too short to draw conclusions regarding clinical outcome. We performed a power analysis to calculate the number of patients needed to discriminate a possible beneficial effect of cell therapy in AMI patients. Based on our data, we found that a study of 2,994 patients would be needed to demonstrate a possible effect on MACCE when the incidence of an event is 20% (Supplemental Table IV), whereas >30,000 patients would be needed to demonstrate an effect if the incidence of an event was 2%. In our meta-analysis, the incidence of all-cause mortality was only 2% at a median follow-up duration of 6 months. The forthcoming phase III BAMI trial (NCT01569178) is designed shed more light on the value of BMMNC therapy in improving clinical outcome. It is designed to compare BMMNC transplantation in AMI patients with baseline LVEF <45% to a control group that receives optimal medical care. The primary endpoint in this study is time from randomization to all-cause mortality during 3 year FU. The secondary outcome measures consist of the occurrence of other MACCE events from randomization up until 3 years FU. LV function, volumes and infarct size, are no outcome measures in this trial.

Our power analysis is based on multiple small studies. All these individual studies were primarily designed as safety and feasibility studies, and thus inadequately powered to detect an effect on clinical

end points, whereas the BAMI trial was powered based on long-term FU data of the landmark REPAIR-AMI trial.[24] More importantly, the median FU in our meta-analysis is only 6 months, which is rather short to notice effects on clinical outcome measures in this era of aggressive primary interventions and pharmacotherapy. However, it remains questionable if the BAMI trial, is sufficiently powered to establish definitive answers. Nonetheless, we believe that the BAMI trial will shed more light on several questions concerning BMMNC therapy as adjunctive treatment for AMI patients, and the final results are much anticipated. Supplemental Table V summarizes all upcoming clinical trials on stem cell therapy for AMI. Currently, newer generations of more potent stem cells are emerging in the field of cardiology. Our meta-analysis revealed a reduction in rehospitalizations for heart failure or reduction in ventricular arrhythmias and ICD implantations in patients treated with these newer generation cells. However, the number of clinical trials to date is limited, which is why no statement could be made about the superiority of these cells yet.

Cell therapy-related parameters possibly influencing efficacy outcomes

Timing of cell delivery

The optimal timing of cell therapy with respect to AMI remains unclear to date. Thus far, it was believed that cell therapy should be initiated 3-10 days after the AMI, based on findings in phase I studies, logistical issues, and the assumption that in the first 72 hours, the infarct territory encompasses a too hostile environment for the infused cells. Others argued that stem cells should be infused as soon as possible to prevent cardiomyocyte loss by secreted anti-inflammatory, pro-survival and anti-apoptotic paracrine factors.[54] This hypothesis was recently supported by preclinical and clinical evidence.[50,55] Nevertheless, to date almost all (>90%) other clinical studies infused stem cells >72 hours after the AMI. In this meta-analysis, we found that timing of BMMNC infusion later than 8 days did not appear to be effective. This was confirmed by recent trials aimed to address the question of cell therapy timing.[10–12,39] The TIME and LATE-TIME and SWISS-AMI failed to show any beneficial effect of late infusion opposed to early infusion. Of note, the forthcoming phase II AMICI (NCT01781390) and phase III ADVANCE (NCT01216995) trials will render important information on early infusion of MSC-like cells, whereas the BAMI (BMMNC; NCT01569178), REVITALIZE (BMMNC; NCT00874354), REGEN-AMI (BMC; NCT00765453), AMIRST (BMC; NCT01536106) and the phase II study with Prochymal (allogeneic MSC; NCT00877903) will possibly provide new insights in timing of stem cell administration between 2 and 7 days.

Cell type

It is currently hypothesized that culture-expanded sub populations of BMMNC or other specialized cell types, might exhibit more cardio-protective effects than BMMNC.[56] Indeed, a recent meta-analysis that compared all pre-clinical, large animal studies that were performed to date concluded that MSC appear to have more pronounced beneficial effects on LV function than BMMNC [57], whereas newer generation cells might be even more effective. In this meta-analysis we found a trend towards an improvement in

cardiac function in patients treated with MSC. However, only limited numbers of patients are treated with this stem cell type to date, rendering a high heterogeneity between trials. A power calculation revealed that a study of 106 patients per group is needed to detect a possible significant benefit of MSC over BMC. Forthcoming AMICI (NCT01781390) and ADVANCE (NCT01216995) clinical trials are both phase IIa/IIb trials designed to investigate the effects of mesenchymal-like cells on cardiac repair in over 200 patients. Therefore, they might provide evidence of superiority of mesenchymal stem cells. It should be noted, however, that both studies do not perform head-to-head comparisons of MSC and BMMNC.

Cell preparation and infusion

Recently, it was suggested that the use of heparin in the final stem cell suspension might interfere with the SDF-1/CXCR4 axis , thereby resulting in decreased homing of BMC.[58] However, we found that the use of heparin during cell preparation did not appear to influence therapy outcome. Contrarily, there seems to be a trend towards a beneficial effect on LVEF in heparin-treated cells, which was also found by Jeevanantham et al.[14] We also confirm their finding that the BMMNC isolation protocol did not appear to influence therapy outcome. Noteworthy, only 2 trials prepared BMMNC via a different method than Ficoll isolation.

Cardiac MRI and study design as effect modifier?

Although the effect that we found on LVEF in this meta-analysis is limited, it can have significant clinical implications. For instance, in the studies that assessed the effect of primary PCI following AMI, a similar modest 4% improvement in LVEF was found that eventually mounted up to pronounced effects on mortality.[59,60] However, despite early enthusiasm and several previous positive meta-analyses on cellular therapy [9,14], it seems that some consideration is justified.

First, and most importantly, in most of the earlier cell therapy trials that drove initial enthusiasm, LVEF and volumes were assessed by LV-angiography or echocardiography, whereas cardiac MRI is currently considered as the golden standard.[61] Most recent stem cell trials however, used MRI-based analysis for primary endpoint measures of efficacy and volumes, and 40% of all trials in the current meta-analysis used MRI.[6,7,11,12,23,31,38,49]200 patients with large first AMI treated with primary percutaneous coronary intervention were randomly assigned to either intracoronary infusion of mononuclear BM cells (n = 69 Intriguingly, when our data are corrected for the use of MRI as imaging tool, the positive effect of cell therapy on LVEF, volumes, and infarct size diminishes. This finding corroborates the exploratory findings of Traverse et al[62], and puts the initial enthusiasm concerning BMMNC-based therapies for AMI patients in a different perspective.

Noteworthy, about 50% of the RCTs in this meta-analysis was not executed according to the Jüni criteria (Supplemental Table II), as they do not perform a bone marrow biopsy and sham injection procedure in placebo patients. However, in most studies an unbiased outcome was ascertained by blinded core lab analysis.

Considerations

We believe that the current meta-analysis shows strong indications that BMMNC therapy in AMI patients is not effective in improving clinical outcome. Although the number of patients treated with next generation cell therapies is still too low, and studies performed to date were primarily designed to prove safety and feasibility, these new therapies might prove to be more effective. It is believed that mesenchymal cell populations, or cardiac derived stem cells, exhibit more cardio-protective and regenerative potential. Importantly, preclinical and preliminary clinical evidence indeed show promising benefits of these cell types.[49–51,55,57] Moreover, MSC are immune-privileged cells, and can be administered in an allogeneic setting. This renders the possibility of an allogeneic "off-the-shelf" cell product, which is readily available directly following the primary PCI. This has several logistical advantages, but might also enhance outcome, as it was shown that stem cells derived from young and healthy donors perform better, and have more regenerative potential, than autologous stem cells from the typically elderly cardiovascular patient.[63]

Novelty / Significance

The current meta-analysis, consisting of 30 published, randomized controlled trials, and comprising a total of 2037 patients, is the largest meta-analysis on stem cell therapy for the treatment of AMI patients to date. It includes recently published, relatively large RCTs that used MRI-derived parameters as surrogate end point, and were not included in any meta-analysis yet.[6,10] Also, for the first time, studies that investigated other cell types than BMMNC were included in a subgroup analysis.[50,51] In contrast to a recently published meta-analysis that combined AMI and heart failure patients in most of its subgroup analyses, the current manuscript focuses solely on AMI patients, rendering different and sometimes opposing conclusions.[14]

CONCLUSION

Intracoronary infusion of BMMNC improves LVEF by +2.10%, mostly by reduction of LVESV and infarct size. However, there is no beneficial effect on global LVEF when restricted to cardiac MRI analysis. The improvement in LVEF did not lead to a reduction in clinical outcome. Newer generations of stem cells with a better profile for cardiac repair are emerging, but their future role still needs to be defined in phase II and III studies.

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SUPPLEMENTAL DATA

MATERIALS AND METHODS

Search strategy

Medline (July 2002-September 2013) and the Cochrane Central Register of Controlled trials (CENTRAL) and the website of US Food and drug administration www.fda.gov were searched for relevant articles. The search included all studies reported up to September 1st 2013. We also searched for relevant abstracts and presentations on this topic reported in major cardiology meetings. References in other articles were also investigated and included in the analysis whenever deemed appropriate. Websites, including www.clinicaltrials.gov and www.clinicaltrialregister.eu, were searched for ongoing trials and future trials. The following search strategy was applied: "stem cells", "progenitor cells", "mononuclear cells", "adipose tissue-derived regenerative cells", "mesenchymal stem cells', "cardiac-derived stem cells", "bone marrow", "vascular stromal fraction", "adipose stem cells", "mesenchymal-like stem cells", "coronary artery disease", "myocardial infarction", "cardiac repair", and "myocardial regeneration". Only articles published in English were included. Limitations used in the search were the publication of the study within the last 10 years, limited to clinical trials and randomized controlled clinical trials (Supplemental Table I).

Inclusion and exclusion of studies

Studies were included that met the following criteria: (1) randomized controlled trials with an appropriate control group who received standard therapy, (2) conducted in patients with an AMI that occurred less than 3 months before, (3) using stem cells that were administered by intracoronary or intravenous injection, (4) total of number of patients enrolled should exceed 10, (5) stem cells were derived from adipose tissue, bone marrow or heart, (6) given in an allogeneic or autologous setting.
Only studies with a complete dataset and specified data on the amount of infused cells were included in this meta-analysis. Studies that described the combination of circulating progenitor cells (CPC) or CPC with granulocyte-colony stimulating factor (G-CSF) were excluded from this analysis to circumvent the potential confounding effect of G-CSF therapy on LV function and dimensions, although G-CSF was previously proven ineffective as a mono-therapy for cardiac repair in AMI. When studies compared C-GSF and stem cells, only the patients in the control and stem cell arm were used in this analysis. Cohort studies were excluded from further analysis due to a limited number of studies.

Data abstraction

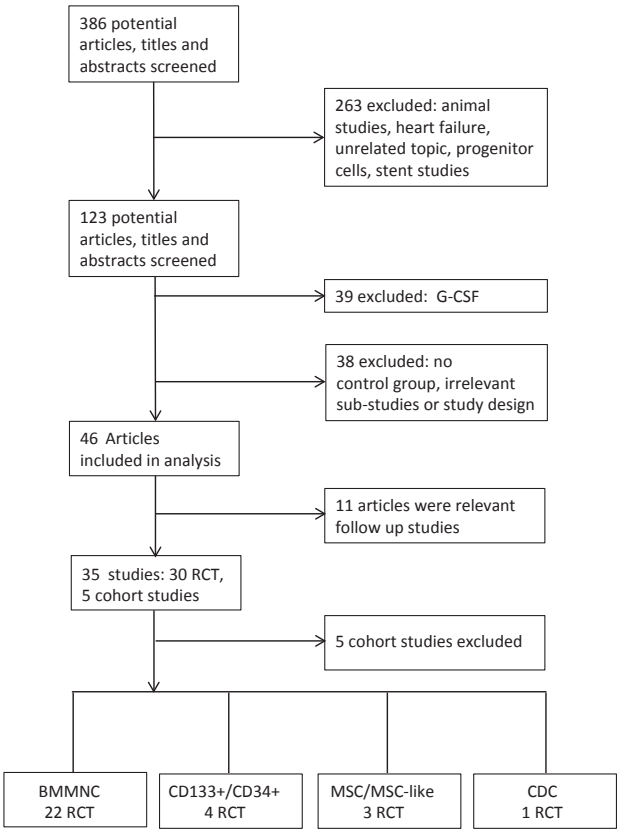
Three reviewers (RdJ, JH, SS) independently screened abstracts and reported their results in a standardized form. Data extracted from the articles were categorized in trial characteristics, functional outcome, scar size and safety. The following parameters were extracted from the articles: Left ventricular ejection fraction (LVEF), LV end-systolic volume (LVESV), LV end-diastolic volume (LVEDV), infarct size (MRI), perfusion defect (SPECT) and major adverse cardiac and cerebral events (MACCE)

rates. MACCE was specified as: all-cause mortality, cardiac mortality, hospitalization for heart failure, in-stent thrombosis and restenosis, target vessel revascularization, ventricular arrhythmia, ICD implantation and stroke. Infarct size was expressed as the percentage of left ventricle infarcted (in %volume or mass). In the various studies, different imaging modalities have been used to determine left ventricular ejection fraction. Cardiac magnetic resonance imaging (MRI) was considered the golden standard. If more than one imaging modality was included, all data was extracted for subgroup analysis. For studies with more than 1 intervention arm (e.g. multiple doses) the weighted mean was calculated and applied for the main analysis.[1] In trials with multiple follow-up time points, the last published follow-up was used in the main analysis.

Quality

The methodological quality of randomized controlled trials was tested by the Jüni criteria.[2]

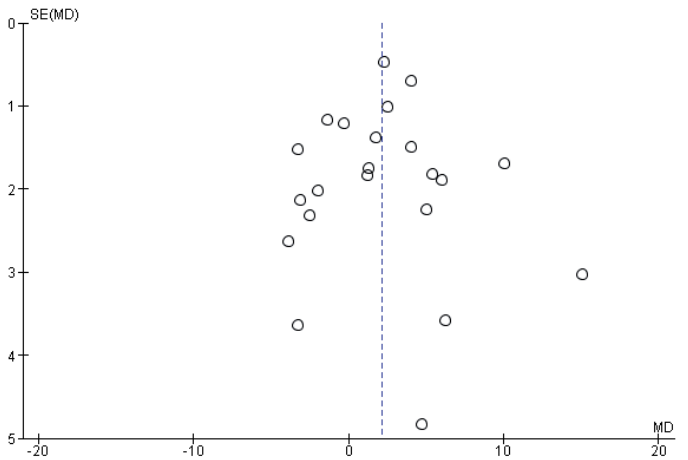
Supplemental Figure A. Flowchart



Flowchart of search: "stem cell therapy for acute myocardial infarction" G-CSF: Granulocyte-Colony Forming Units; RCT: randomized controlled trial; BMMNC: Bone marrow derived mononuclear cells; MSC: mesenchymal stem cells; CDC: cardiosphere derived cells.

Supplemental Figure B. Funnel plot

Funnel plot of left ventricular ejection fraction of BMMNC trials. The dotted line indicates the treatment effect of stem cell therapy. SE (MD): standard error of mean difference; RCT indicates randomized controlled trials; MD: mean difference.



Supplemental Table I. Search strategy

Database:	
PubMed	
User query:	
((((((acute myocardial infarction)) OR (coronary artery disease)) OR (myocardial regeneration)) OR (myocardial infarction))) AND ((((((((stem cells)) OR (bone marrow stem cells)) OR (progenitor cells)) OR (mononuclear cells)) OR ((mesenchymal stem cells) OR mesenchymal-like stem cells)) OR (adipose tissue derived regenerative cells)) OR (vascular stromal cells)) OR (cardiac derived stem cells)) AND ((Clinical Trial[ptyp] OR Letter[ptyp] OR Controlled Clinical Trial[ptyp] OR Randomized Controlled Trial[ptyp]) AND "last 10 years"[PDat] AND Humans[Mesh])	
Translations:	
myocardial infarction	"myocardial infarction"[MeSH Terms] OR ("myocardial"[All Fields] AND "infarction"[All Fields]) OR "myocardial infarction"[All Fields]
coronary artery disease	"coronary disease"[MeSH Terms] OR ("coronary"[All Fields] AND "disease"[All Fields]) OR "coronary disease"[All Fields] OR ("coronary"[All Fields] AND "artery"[All Fields] AND "disease"[All Fields]) OR "coronary artery disease"[All Fields] OR "coronary artery disease"[MeSH Terms] OR ("coronary"[All Fields] AND "artery"[All Fields] AND "disease"[All Fields])
myocardial	"myocardium"[MeSH Terms] OR "myocardium"[All Fields] OR "myocardial"[All Fields]
regeneration	"regeneration"[MeSH Terms] OR "regeneration"[All Fields]
stem cells	"stem cells"[MeSH Terms] OR ("stem"[All Fields] AND "cells"[All Fields]) OR "stem cells"[All Fields]
bone marrow	"bone marrow"[MeSH Terms] OR ("bone"[All Fields] AND "marrow"[All Fields]) OR "bone marrow"[All Fields]
progenitor cells	"stem cells"[MeSH Terms] OR ("stem"[All Fields] AND "cells"[All Fields]) OR "stem cells"[All Fields] OR ("progenitor"[All Fields] AND "cells"[All Fields]) OR "progenitor cells"[All Fields]
cells	"cells"[MeSH Terms] OR "cells"[All Fields]
mesenchymal stem cells	"mesenchymal stromal cells"[MeSH Terms] OR ("mesenchymal"[All Fields] AND "stromal"[All Fields] AND "cells"[All Fields]) OR "mesenchymal stromal cells"[All Fields] OR ("mesenchymal"[All Fields] AND "stem"[All Fields] AND "cells"[All Fields]) OR "mesenchymal stem cells"[All Fields]
adipose tissue	"adipose tissue"[MeSH Terms] OR ("adipose"[All Fields] AND "tissue"[All Fields]) OR "adipose tissue"[All Fields]
stromal cells	"stromal cells"[MeSH Terms] OR ("stromal"[All Fields] AND "cells"[All Fields]) OR "stromal cells"[All Fields]
vascular	"blood vessels"[MeSH Terms] OR ("blood"[All Fields] AND "vessels"[All Fields]) OR "blood vessels"[All Fields] OR "vascular"[All Fields]
cardiac	"heart"[MeSH Terms] OR "heart"[All Fields] OR "cardiac"[All Fields]
Humans[Mesh]	"humans"[MeSH Terms]
Result:386	

Supplemental Table II. Quality assesment score for RCT included in the meta analysis According to Juni Criteria[2]

Author	Year	Was allocation adequate?	Adequate method of randomization described?	Were groups similar at the start of the study?	Were patients/ caregivers blinded for intervention?	Was outcome ascertained blinded?	What percentage was lost in follow up?	Were all patients analyzed in the group to which they were assigned?
BMMNC								
Meyer[3–6]	2004	Y	Y	Y	Y	Y	0	Y
Ge[7]	2006	Y	Y	Y	Y	Y	0	Y
Janssens[40,41]	2006	Y	Y	Y	Y	Y	0	Y
Lunde/ Beitnes[8–10]	2006	Y	Y	Y	N	Y	0	Y
Schachinger[11–13]	2006	Y	Y	Y	Y	Y	0	Y
Meluzin/Panovsky[14–16]	2006	Y	N	Y	N	Y	10	Y
Penicka[17]	2007	Y	N	Y	NR	NR	0	Y
Suarez de Lezo[18]	2007	Y	N	Y	NR	Y	0	Y
Huikuri[19]	2008	Y	Y	Y	Y	Y	0	Y
Yao[20]	2009	Y	N	Y	N	Y	0	Y
Plewka[21]	2009	Y	N	Y	N	Y	0	Y
Cao[22]	2009	Y	Y	Y	NR	Y	0	Y
Nogueira (Silva)[23,24]	2009	Y	Y	Y	N	Y	0	Y
Grajek[25]	2010	Y	Y	Y	N	Y	0	Y
Piepoli[26]	2010	Y	Y	Y	N	Y	0	Y
Wohrle[27]	2010	Y	Y	Y	Y	Y	0	Y
Traverse TIME[28,29]	2010	Y	Y	Y	Y	Y	0	Y
Hirsch[30]	2010	Y	N	Y	Y	Y	0	Y
Roncalli[31]	2011	Y	Y	Y	N	Y	0	Y
Travers LATE-TIME[32]	2011	Y	N	Y	N	Y	1.1	Y
Turan[33]	2012	Y	N	Y	N	Y	0	Y
Surder[34]	2012	Y	Y	Y	N	Y	?	Y

Supplemental Table II. Continued

Author	Year	Was allocation adequate?	Adequate method of randomization described?	Were groups similar at the start of the study?	Were patients/ caregivers blinded for intervention?	Was outcome ascertained blinded?	What percentage was lost in follow up?	Were all patients analyzed in the group to which they were assigned?
CD133+/CD34+								
Tendera[35]	2009	Y	N	Y	N	Y	0	Y
Liepic[36]	2009	Y	N	Y	N	Y	0	Y
Colombo[37]	2011	Y	Y	Y	Y	Y	0	Y
Quyumi[38]	2011	Y	N	Y	N	Y	0	Y
MSC/MSC-like								
Chen[39]	2004	Y	N	Y	Y	Y	0	Y
Hare[42]	2009	Y	Y	Y	Y	Y	0	Y
Houtgraaf[43]	2012	Y	Y	Y	Y	Y	0	Y
CDC								
Makkar[44]	2012	Y	Y	Y	N	Y	0	Y

Y indicate yes; N: no; NR: Not reported

Supplemental Table III. Study characteristics of all RCT included in this analysis

Author	Year	Total no	CT pt	Randomization	Cell type	Number of cells	Time to application	Infusion method	Location of MI	FU (mo)	Imaging modality
BMMNC											
Meyer[3–6]	2004	60	30	1:1	BMC	24.6 ± 9.4 x 10^8	4.8 ± 1.3 d	stop flow	all	60 (6,18)	MRI
Ge[7]	2006	20	10	1:1	BMMNC	40 x 10^6	1 d	Stopflow	all	6	Echo, SPECT
Janssens[40,41]	2006	67	33	1:1	BMMNC	172 ± 72 x 10^6	1 d	stopflow	all	4	MRI, echo
Lunde[8] and Beitnes[9,10]	2006	100	47	1:1	BMMNC	87 ± 47.7 x 10^6	5 -8 d	stopflow	anterior	36 (6,12)	MRI, SPECT, echo
Schachinger[11–13]	2006	204	101	1:1	BMMNC	236 ± 174 x 10^6	3-7 d	stopflow	all	12	LVG
Meluzin[14,15] and Panovsky[16]	2006	66	44	1:1:1	BMMNC	Low 1 x 10^7 High 1x 10^8	7 ± 0.3 d	stopflow	all	12 (3,6)	SPECT
Penicka[17]	2007	27	17	2:1	BMMNC	26.4 x 10^8	4 ± 11 d	stopflow	anterior	4	Echo, SPECT
S. de Lezo[18]	2007	20	10	1:1	BMMNC	9 x 10^8	7 ± 2 d	stopflow	anterior	3	LVG
Huikuri[19]	2008	80	40	1:1	BMMNC	402 ± 196 x 10^6	2-6 d	stopflow	all	6	LVG, Echo
Yao[20]	2009	39	12 15	1:1:1	BMC BMC	1,9 ± 1,3 *10^8 2,1 ± 1,7 *10^8	3-7 d 3-7 d and 3 mo	stopflow	anterior	12 (6)	MRI
Plewka[21]	2009	56	38	2:1	BMC	1.44 ± 0.49 x 10^8	7 ± 2 d	stopflow	anterior	6	Echo
Cao[22]	2009	86	41	1:1	BMMNC	5 ± 1.2 x 10^7	7 d	stopflow	anterior	48 (6,12)	Echo
Nogueira[23,24]	2009	30	14	2:1	BMMNC	100 x 10^6	5.5 ± 1.2 d	stopflow ICA stopflow ICV	all	6	Echo, SPECT
Grajek[25]	2010	45	31	2:1	BMMNC	2.34 ± 1.2 x 10^9	5-6 d	Stopflow	anterior	12 (3,6)	Echo
Piepoli[26]	2010	38	19	1:1	BMMNC	418.8 x 10^6	4-7 d	stopflow	anterior	12 (6)	SPECT/echo
Wohrle[27]	2010	42	29	2:1	BMMNC	381 ± 130 x 10^6	5-7 d	stopflow	all	6	MRI
Traverse TIME[28,29]	2010	40	30	3:1	BMMNC	100 x 10^6	3-10 d	infusion catheter 1ml/min	anterior	6	MRI
Hirsch[30]	2010	134	65	1:1	BMC	BMC 296 ± 164 x 10^6	3-8 d	stopflow	all	4	MRI
Roncalli[31]	2011	101	52	1:1	BMC	98.3 ± 8.7 x 10^6	7-10 d	stopflow	all	3	SPECT
Traverse LATE-TIME[32]	2011	87	58	2:1	BMC	150 x 10^6	14-21 d	stopflow	all	6	MRI
Turan[33]	2012	62	42	2:2	BMC	9.6 ± 3,2 x 10^7	7 d	stopflow	all	6	LVG
Sürder[34]	2013	192	64 64	1:1:1	BMMNC BMMNC	160 x 10^6 140 x 10^6	5-7 d 21-28 d	stopflow	all	12(4)	MRI

Supplemental Table III. Continued

Author	Year	Total no	CT pt	Randomization	Cell type	Number of cells	Time to application	Infusion method	Location of MI	FU (mo)	Imaging modality
Bone marrow progenitors											
Tendera[35]	2009	200	80	2:2:1	CD34+CXCR4+ BMC	1.90 x 10^6	3-12 d	stopflow	anterior	6	MRI
			80		BMMNC	1,78 x 10^6					
Lipiec[36]	2009	39	26	2:1	CD 133 + BMC	0.33 ± 0.17 x 10^6	4-11 d	stopflow	anterior	6	SPECT, echo
					CD34+	3.36 ± 1.87 x 10^6					
Colombo[37]	2011	10	5	1:1	CD 133 + BMC	5.9 x 10^6	10-14 d	stopflow	anterior	12(6)	Echo, PET
Quyumi[38]	2011	31 280	16 207	1:1:1:1	CD 34+ BMC	5-15 x 10^6	8.3 d	stopflow	all	6	MRI, SPECT
MSC											
Chen[39]	2004	69	34	1:1	bone marrow MSC	48-60 x 10^9	18.4 ± 0.5 d	stopflow	all	6	LVG, PET
Hare[42]	2009	53	34	2:1	allogneic MSC	dose escalating 0.5, 1.6 or 5.0 x10^6 MSC/body weight	1-10 d	intravenous	all	12 (3,6)	MRI
Houtgraaf[43]	2012	14	10	3:1	ADRC	17.4 ± 4.1 x 10^6	<24 hours	infusion-catheter	anterior	6	MRI, SPECT
CDC											
Makkar[44]	2012	25	17	2:1	cardiosphere derived cells	low 12.5 x 10^6 High: 25 x 10^6 intermediate 17,3 x 10^6	1,5 - 3 mo	stopflow	all	12 (6)	MRI

Overview of RCT characteristics. No: number; pt: patients; d: days; m: months; CT: cell therapy; BM-MSC: Bone Marrow Mesenchymal stem cells; BMMNC: Bone-marrow mononuclear cells, MSC; mesenchymal stem cells; ICA: intracoronary arterial; ICV: intracoronary venous; ADRC: adipose tissue derived regenerative cells; CDC: Cardiosphere-derived cells; LVG: left ventricular-angiography; all: LCx, LAD and RCA; Mo: months

Supplemental Table IV. Sample size calculations MACCE rates

% of MACCE in control	effect	% of MACCE in treatment group	N per group	Total number of patients	effect	% of MACCE in treatment group	N per group	effect	% of MACCE in treatment group	N per group	total number of patients
2	0,60	1,2	4107	8214	0,70	1,4	7615	0,80	1,6	17837	35674
3	0,60	1,8	2716	5432	0,70	2,1	5034	0,80	2,4	11785	23570
4	0,60	2,4	2021	4042	0,70	2,8	3744	0,80	3,2	8759	17518
5	0,60	3,0	1604	3208	0,70	3,5	2970	0,80	4	6943	13886
6	0,60	3,6	1326	2652	0,70	4,2	2454	0,80	4,8	5733	11466
7	0,60	4,2	1128	2256	0,70	4,9	2085	0,80	5,6	4868	9736
8	0,60	4,8	979	1958	0,70	5,6	1808	0,80	6,4	4220	8440
9	0,60	5,4	863	1726	0,70	6,3	1593	0,80	7,2	3716	7432
10	0,60	6,0	770	1540	0,70	7,0	1421	0,80	8	3312	6624
15	0,60	9,0	492	984	0,70	10,5	905	0,80	12	2102	4204
20	0,60	12,0	353	706	0,70	14,0	647	0,80	16	1497	2994

When the occurrence of a MACCE event in the control group is 2% and the odd ratio (OR) of this event is 0.60, 1.2% of the treated patients will have an event during the study. To show significance between the groups 4107 patients are needed per group. If the occurrence of events rise to 10% only 492 patients are needed in the study. If the odds ratio is 0.80, the differences between the treatment and control group are smaller and more patients will be needed to reach significance. When the occurrence of an MACCE event is 2% in the control group, expected 35.674 patients in total are needed to show a beneficial effect on that event. The longer the follow-up time in the study, the higher the incidence of a MACCE event. Less patients are needed to show a beneficial effect. In our meta-analysis, the all-cause mortality in the control group was 3.4% opposed to 2.1% in the treatment group. The OR of all-cause mortality is therefore 0,60. This would mean that based on this meta-analysis and our calculations, 35.674 patients are needed to show a beneficial effect of cell therapy on all-cause mortality. These calculations are based on a median follow-up duration of 6 months.

Supplemental table V. Upcoming clinical trials on stem cell therapy for the treatment of an AMI

Study name	Trial number	Phase	Route of administration	No. of patients	Cell type	Primary endpoint
BAMI	NCT01569178	III	IC	3000	BMMNC	all-cause mortality during 3 year FU
AMICI	NCT01781390	I/II	IC	225	allogeneic MSCs	reduction of infarcts size between baseline and 6 months FU
ADVANCE	NCT01216995	II/III	IC	216	ADRC	reduction of infarcts size between baseline and 6 months FU
AMIRST	NCT01536106	I/II	IC	30	BMC	adverse events
RELIEF	NCT01652209	III	IC	135	autologous MSC	change in LVEF on MRI
Prochymal-2	NCT 00877903	II	IV	220	allogeneic MSCs	change in ESV on cardiac MRI
REVITALIZE	NCT00874354	I	IC	30	BMMNC	safety and change in LVEF on echo and MRI
ALLSTAR	NCT01458405	II	IC	274	allogeneic CDC	safety and change in infarct size on MRI
REGEN-AMI	NCT00765453	II/III	IC	102	BMMNC	change in LVEF on MRI at 1 year FU

Trial number as found on www.clinicaltrials.gov. IC indicates intracoronary; IV: intravenous;
BMMNC: bone marrow derived mononuclear cells; MSC: mesenchymal stem cell;
ADRC: adipose tissue derived regenerative cells; BMC: Bone marrow stem cells;
CDC: cardiosphere derived cells; LVEF: left ventricular ejection fraction; LVESV: left ventricular end-systolic volume.

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CHAPTER 4

First generation stem cell therapy for ischemic heart disease: a review, a meta-analysis and future prospectives

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ABSTRACT

Background Several cell-based therapies for the adjunctive treatment of acute myocardial infarction (AMI) and heart failure (HF) have been investigated in multiple clinical trials, but the benefits still remain controversial. The first generation of cells, defined as bone marrow-derived mononuclear cells (BMMNC) and skeletal myoblasts (SkM), have been investigated in such extent to merit a meta-analysis. This meta-analysis aims to evaluate the efficacy of this first generation stem in AMI (BMMNC only) and HF (BMMNC and SkM) patients, and gives an overview of newer generations stem cells for cardiovascular repair.

Methods and Results A random-effects meta-analysis was performed on randomized controlled trials (RCT) investigating the effects of stem cell therapy in patients with AMI or HF that were published between January 2002 and December 2013. The defined endpoints were left ventricular ejection fraction (LVEF), left ventricular end-systolic and end-diastolic volumes (LVESV/LVEDV), infarct size and major adverse cardiac and cerebral event (MACCE) rates. Overall, in AMI, LVEF increased by +2.10% (95% CI, 0.68- 3.52 , P=0.004) in the BMMNC group and +3.97% (95% CI: 2.33 – 5.62; P<0.00001), both related to an effect on LVESV. However, there is no effect on cardiac function, volumes, nor infarct size, when only randomized controlled trials were included that used MRI-derived endpoints in AMI. No significant effect on MACCE rates was detected in AMI patients following BMMNC therapy. Interestingly, in HF patients, the significant improvement on LVEF remained, whereas also an effect on all-cause mortality was shown.

Conclusion BMMNC therapy does not improve cardiac function in AMI patients, but mildly improves cardiac function in HF. New generations of stem cells are emerging in the field that might be more potent to mend broken hearts, but they still have to prove their superiority in larger clinical trials.

INTRODUCTION

Cardiovascular disease accounts for the highest mortality worldwide, despite improvements in treatment options.[1] Approximately half of the cardiovascular deaths is related to acute myocardial infarction (AMI), whereas subsequent heart failure (HF) and stable coronary artery disease account for the majority of morbidity. To reduce the burden of cardiovascular disease and health care costs, new therapeutic strategies are continuously developed and investigated. In the past decade, stem cell therapy emerged as a potent candidate for cardiac repair following an AMI and in ischemic HF.

More than a decade ago, the first patients were treated with the first generation of stem cell therapy, using intramyocardial injection of skeletal myoblasts (SkM) in patients with HF,[2,3] and intracoronary infusion of bone marrow-derived mononuclear cells (BMMNC) in AMI patients.[4,5] Initial optimism concerning the use of SkM in HF patients were toned down by issues of possible pro-arrhythmogenicity of the cells, and disappointing results on efficacy in randomized studies.[6–8] Also, the regenerative capacity of bone marrow (BM)-derived cells in AMI and HF patients has been under debate, since the numerous trials that were performed to date, show conflicting results. Several meta-analyses suggested beneficial effects of BMMNC. However, in most of these meta-analyses, both non-randomized and randomized studies were included, whereas AMI and HF patients were pooled for analysis of clinical end points,[9,10] or clinical end points were included in the analysis.[11]

In the current paper, we aim to give an overview of the safety and efficacy of the first generation of stem cell therapy for the treatment of ischemic heart disease by including some relevant sub group analyses. Also, we aim to give a concise overview regarding newer generations of stem cells for cell-based cardiac repair.

METHODS

A detailed description of the search for the meta-analysis can be found in the online supplement. Briefly, the meta-analysis was executed according to the Quorum statements.[12] A random effect meta-analysis was performed that included all clinical trials regarding stem cell therapy for the treatment of AMI, chronic heart failure or ischemic cardiomyopathy, published on Medline between July 2002 and December 2013. BMMNC and SkM (first generation of stem cells) were the main topic in this analysis. Other stem cells included in this analysis were cardiac-derived stem cells (CDC), mesenchymal stem cells (MSC) and adipose tissue-derived regenerative cells (ADRC), although a meta-analysis on these cell types is not feasible due to the small number of clinical trials that were executed to date. However, these clinical trials are briefly described in the review section below.. The following search strategy was applied: “stem cells”, “progenitor cells”, “mononuclear cells”, “adipose tissue-derived regenerative cells”, “mesenchymal stem cells’, “cardiac-derived stem cells”, “bone marrow”, “vascular stromal fraction”, “adipose stem cells”, “mesenchymal-like stem cells”, “skeletal myoblasts”, “coronary artery disease”, “myocardial infarction”, “heart failure” “cardiac repair”, and “myocardial regeneration”.

Studies were included that met the following inclusion criteria: (1) randomized controlled trials with an appropriate control group that received standard therapy; (2) conducted in patients with an

Table 1a. Skeletal Myoblasts Trials

	Phase	Cell type	Status	Design	No.	Delivery Method	Condition	Transfer day after PCI	Cell number (mill)	Primary Clinical Outcome	Reference
MAGIC	II	SkM	terminated	RDBPC	97	IM (Epi)	ICHF	–	400/800	Safety/Efficacy: MACE/ LVEF (Echo) at 6M,	NCT00102128
CAUSMIC II	II	SkM	unknown	RDBPC	23	IM (Endo)	ICHF	–	300	Safety/Efficacy: MLHFQ at 1 year	NCT00626314
SEISMIC	II	SkM	complete	ROPC	40	IM (Endo)	ICHF	–	150-800	Safety/Efficacy: LVEF (MUGA) at 6M	NCT00375817
MARVEL	II/III	SkM	unknown	RDBPC	170	IM (Endo)	ICHF	–	400/800	Safety/Efficacy: QOLQ, 6min walk test at 1 year	NCT00526253

RDBPC: randomized double-blind placebo controlled, ROPC: randomized open-label placebo controlled, SkM: skeletal myoblast, IM: intramyocardial, ICHF: ischemic congestive heart failure, LVEF: LV ejection fraction, MACE: major adverse cardiovascular event, MLHFQ: Minnesota living with heart failure questionnaire, QOLQ: quality of life questionnaire, MUGA: multi gated acquisition scan

Table 1b. Bone Marrow-derived Mononuclear Cell Trials

	Phase	Cell type	Status	Design	No.	Delivery Method	Condition	Transfer day after PCI	Cell number (mill)	Primary Clinical Outcome	Reference
BOOST	I	MNC	complete	RDBPC	60	IC	AMI	4.8	2460	Safety/Efficacy: Regional systolic wall thickening (MRI)	NCT00224536
TCT-STAMI	I	MNC	complete	RDBPC	20	IC	AMI	1	40	Efficacy: LVEF, volumes(echo) at 6M	-
REPAIR-AMI	III	MNC	complete	RDBPC	204	IC	AMI	4	236	Efficacy: LVEF (LVG) at 4M	NCT00279175
Janssen	II	MNC	Complete	RDBPC	67	IC	AMI	1	172	Efficacy: LVEF (MRI) at 4M	NCT00264316
Meluzin	II	MNC	Complete	RSBPC	66	IC	AMI	7	LD 10	Efficacy; perfusion defect at 3,6,12M	
									HD 100	LVEF (SPECT)	

AMI that occurred less than 3 months before or patients diagnosed with heart failure or ischemic cardiomyopathy; (3) using stem cells that were administered by intracoronary, intravenous or intramyocardial injection; (4) total of number of patients enrolled should exceed 10; (5) stem cells were derived from bone marrow, skeletal muscle, adipose tissue, or the heart; (6) given in an autologous or allogeneic setting.

A pre-specified sub-group analysis was performed on the use of the current golden standard to measure cardiac volumes and function: cardiac MRI.

Data abstraction and analysis was performed by two different researchers (RdJ, JH) and reported on standardized forms. Left ventricular ejection fraction (LVEF), left ventricular end-systolic volume (LVESV), left ventricular end-diastolic volume (LVEDV) and infarct size were assessed as outcome measures, as well as the occurrence of major adverse cardiac or cerebral events (MACCE rates). The data of the primary endpoint was used in this analysis.

RESULTS

Autologous skeletal myoblasts in ischemic heart failure patients

Skeletal myoblasts (SkM) are progenitor cells residing in striated skeletal muscle, and are responsible for regeneration of skeletal muscle upon damage. SkM can be easily expanded in cell culture. Because they are destined to become contracting cells, they were a logical candidate for cell-based heart repair. In pre-clinical investigations, SkM were able to form functional skeletal myotubes, repopulate the damaged heart, and integrate into host myocardium with formation of electromechanical junctions between host cardiomyocytes and injected skeletal myotubes.[13]

These promising data resulted in the first HF patients treated with epicardial SkM injections during bypass surgery, or endomyocardial injections using specialized injection catheters.[2,3] Our search revealed 4 clinical trials using SkM,[8,14–16] of which only 2 trials were performed in a double-blind and randomized fashion (MAGIC; NCT00102128; table 1a, and MARVEL; NCT00526253; table 1a[15,16]). Only the MAGIC trial was completed, whereas MARVEL was terminated early with inclusion of only limited patient numbers.[16] In both the CAUSMIC and SEISMIC trials, patients and caregivers were not blinded for the treatment, resulting in exclusion of these studies.[8,14] Because only 1 RCT reached the pre-specified end point, a meta-analysis on cardiac function and volumes was not deemed useful. It should be noted, that in this trial, intramyocardial injection of SkM during bypass surgery failed to improve cardiac function after 6 months of follow up.[15]

Moreover, SkM injection has been associated with increased incidence of ventricular tachyarrhythmias. Indeed, according to our analysis, SkM injection is associated with an increased incidence of VT/VF (OR 2.52; 95% CI 0.99-6.40; P=0.05; Figure 1). These ventricular arrhythmias are hypothesized to be caused by the lack of electromechanical coupling between injected SkM and host myocardium, due to the lack of connexin-43 expression. Collections of injected SkM thereby form electrically isolated islands that can function as re-entry circuits for ventricular arrhythmias.[17] This finding, in combination with the lack of obvious long-term efficacy, and the development of new and better alternatives, SkM therapy currently is abandoned as cellular therapy for cardiac repair.

Table 1b. Contnued

	Phase	Cell type	Status	Design	No.	Delivery Method	Condition	Transfer day after PCI	Cell number (mill)	Primary Clinical Outcome	Reference
Penicka	II	MNC	Early terminated	RSBPC	27	IC	AMI	4	2640	Efficacy: LVEF and volumes(echo), Infarct size (SPECT) at 4M	NCT00389545
S. de Lezo	II	MNC	Complete	RSBPC	20	IC	AMI	7	900	Safety/efficacy LVEF (LVG) 3M	-
FINCELL	II	MNC	Complete	RDBPC	80	IC	AMI	2-6	402	Safety : IVUS/arrhythmias, Efficacy: LVEF(LVG/echo) at 6M	-
ASTAMI	II	MNC	complete	RSBPC	100	IC	AMI	6	68	Safety/Efficacy: LVEF, EDV, Infarct size	NCT00199823
Yao	II	MNC	Complete	RSBPC	39	IC	AMI	3-7 d or 3-7d and 3 M	190 210	Efficacy: LVEF and volumes (MRI), perfusion defect (SPECT) at 6 and 12M	-
Plewka	II	MNC	Complete	RSBPC	60	IC	AMI	7	144	Efficacy: LV diastolic and systolic function (echo at 6M)	-
Cao	II	MNC	Complete	RSBPC	86	IC	AMI	7	50	Efficacy: LVEF and volumes (echo), perfusion defect (SPECT) at 6,12 and 48M	-
Nogueira	I/II	MNC	Complete	RSBPC	30	IC	AMI	5.5	100	Safety, feasibility	
Grajek	II	MNC	Complete	RSBPC	45	IC	AMI	5-6	2340	Efficacy: perfusion defect (SPECT) and LVEF radionucleotide ventriculography at 3,6, 12M	-
Piepoli	I/II	MNC	Complete	RSBPC	38	IC	AMI	4-7	418	Mortality, efficacy: LVEF (Echo) at 6 and 12M	NCT00437710
SCAMI	II	MNC	Complete	RSBPC	42	IC	AMI	5-7	381	Efficacy: LVEF and volumes (MRI) 1,3,12M MACCE 1,3,6,12	NCT00669227
TIME	II	MNC	complete	RDBPC	120	IC	AMI	3 or 7	150	Efficacy: LVEF (MRI) at 6M	NCT00684021
HEBE	II	MNC	complete	ROPC	200	IC	AMI	6	296	Efficacy: Regional LVEF (MRI) at 4M	ISRCTN 95796863
BONAMI	II	MNC	Complete	ROPC	101	IC	AMI	3-8	98.3	Efficacy:LVEF and myocardial viability Thalium scintigraphy 3 and 12M, LVEF echo 1,3,6,12M	NCT00200707

Table 1b. Contnued

	Phase	Cell type	Status	Design	No.	Delivery Method	Condition	Transfer day after PCI	Cell number (mill)	Primary Clinical Outcome	Reference
LateTIME	II	MNC	complete	RDBPC	87	IC	AMI	17.4	150	Efficacy: LVEF (MRI) at 6M	NCT00684060
Turan	II	MNC	Complete	RSBPC	62	IC	Ami	96	7	Efficacy: LVEF, infarctsize(LVG) 6M	-
SWISS-AMI	II	MNC	complete	ROPC	200	IC	AMI	5-7 vs. 21-28	156	Efficacy: LVEF (MRI) at 4M	NCT00355186
Assmus	II	MNC	Complete	ROPC	51	IC	Healed AMI	3 months	205	Efficacy: LVEF (LVG) at 3M	NCT00289822
Hendrikx	II	MNC	Complete	RSBPC	23	IM(Epi)	Post AMI HF	214	60.3	Efficacy: LVEF (MRI), perfusion defect (SPECT) at 4M	-
PROTECT-CAD	II	MNC	Complete	RDBPC	28	IM (Endo-NOGA®)	ICHF	-	16.7	Exercise capacity, CCS. NYHA, LVEF(MRI), perfusion defect 6M	-
Ang	II	MNC	Complete	RSBPC	62	IC or IM (Epi)	ICHF	-	IC 115 IM 84	Efficacy: LVEF and volumes (MRI and echo),regional wall motion 6M	-
Yao	II	MNC	Complete	RDBPC	47	IC	ICHF	-	12	Efficacy: LVEF and infarct size (MRI), LV volumes Echo at 6M	-
Zhao	II	MNC	Complete	RDBPC	36	IM(Epi)	ICHF	-	659	MACE, Efficacy: LVEF(echo), perfusion defect SPECT at 6M	-
Hu	II	MNC	Complete	RSBPC	60	IM(Epi)	ICHF	-	132	Efficacy: LVEF and volumes (MRI), NYHA, exercise test	-
ESCAPE	III	MNC	complete	RDBPC	250	IM (Epi)	ICHF	–	150	Safety/Efficacy: Survival at 1Y	NCT00841958
Turan	II	MNC	Complete	RSBPC	56	IC	ICHF	-	1	Efficacy: LVEF and volumes(LVG)	-
FOCUS-HF	I	MNC	Complete	RSBPC	30	IM (Endo-NOGA®)	ICHF	-	30	Safety/efficacy: MVO2, perfusion defect, LVEF and volumes (echo) at 3 and 6M. electromechanical mapping 6M	NCT00203203
FOCUS-CCTRN	II	MNC	complete	RDBPC	92	IM (Endo-NOGA®)	ICHF	–	100	Safety/Efficacy: MVO2, LVESV, Reversible defect size	NCT00824005
TAC-HFT*	I/II	MNC	Complete	RDBPC	29	IM (helix)	ICHF	-	?	Safety 30d, SAE 1Y, Infarct size and regional myocardial function (MRI) 1Y	NCT00768066

RDBPC: randomized double-blind placebo controlled, RSBPC: randomized single-blind placebo controlled, ROPC: randomized open-label placebo controlled, MNC: bone marrow-derived mononuclear cell, IC: intracoronary, IM: intramyocardial, AMI: acute myocardial infarction, ICHF: ischemic congestive heart failure, CMI: chronic myocardial ischemia, CM: cardiomyopathy, LVEF: LV ejection fraction, LVEDV: LV end diastolic volume, LVESD: LV end systolic volume LVG: left ventriculogram, ETT: exercise tolerance test, MVO2: maximal oxygen consumption, SAE: serious adverse event. *TAC-HFT compares both MSC and BMNNC to a control group.

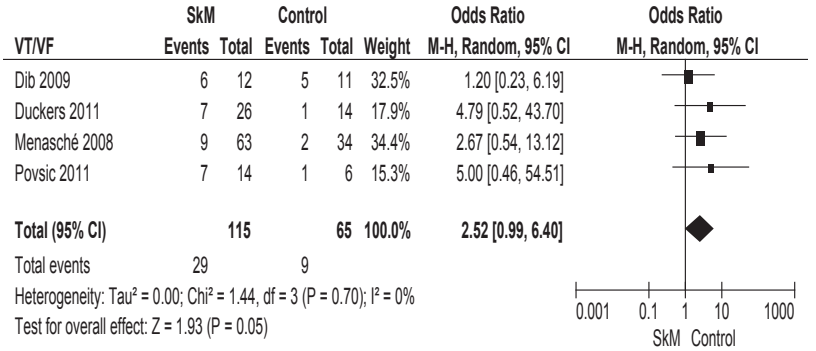

Figure 1. Forest plot of ventricular tachycardia (VT) and ventricular fibrillation (VF) in patients treated with Skeletal myoblasts. Intramyocardial injection of SkM was associated with a significant increase in VF/VT

Bone marrow-derived mononuclear cells in AMI patients

The search resulted in 21 trials that evaluated the effect of BMMNC transplantation in AMI patients (table 1b, supplemental table 3a). A total of 845 patients were treated with BMMNC opposed to 608 control patients. The average of participating patients per study was 68 ± 51 patients, whereas the median was 45 patients (range 14-200). Most studies used a 1:1 randomization scheme. The median follow-up duration in all studies was 6 months (range 3-60 Months). The median amount of infused viable cells was 100 million (range from 5×10^6 to 60×10^9) and the cells were infused after a median of 7 days (range <24 hours to 3 months). MRI was the imaging modality of choice for follow up of LV function in 40% of the RCTs.

Intracoronary infusion of BMMNC resulted in a minimal increase in LVEF by +2.10% (95% CI: 0.68-3.52; P=0.004; figure 2a; table 2), mostly by a preservation in LVESV which decreased by -4.05 mL

Table 2. Treatment effect and imaging modality

	all imaging modalities		**MRI only**	
AMI	**difference in mean (95% CI)**	**p**	**difference in mean (95% CI)**	**p**
LVEF	2.10 [0.68, 3.22]	0.004	-0.13 [-2.67, 2.93]	0.93
LVEDV	-2.69 [-4.83, -0.56]	0.09	-0.86 [-4.66, 2.94]	0.66
LVESV	-4.05 [-6.91, -1.18]	0.006	-2.65 [-5.28, 0.02]	0.06
Infarct Size	-2.80 [-6.03, -0.44]	0.09	-1.11 [-3.74, 1.53]	0.82
HF				
LVEF	3.97 [2.33, 5.62]	<0.00001	3.06 [1.12, 5.01]	0.002
LVEDV	-9.96 [-23.05, 3.13]	0.14	-14.57 [-33.48, 4.34]	0.13
LVESV	-16.80 [-29.62, -2.47]	0.02	-14.41 [-35.03, -6.20]	0.17
Infarct Size	-2.57 [-5.48, 0.35]	0.08	-0.73 [-1.72, 0.27]	0.15

The treatment effect of BMMNC therapy diminished in AMI patients when data is pooled for studies that used MRI derived endpoints. However, in HF patients, the effect on LVEF remains. AMI: acute myocardial infarction; HF: heart Failure; LVEF: left ventricular ejection fraction; LVEDV: left ventricular end-diastolic volume; LVESV: left ventricular end-systolic volume:

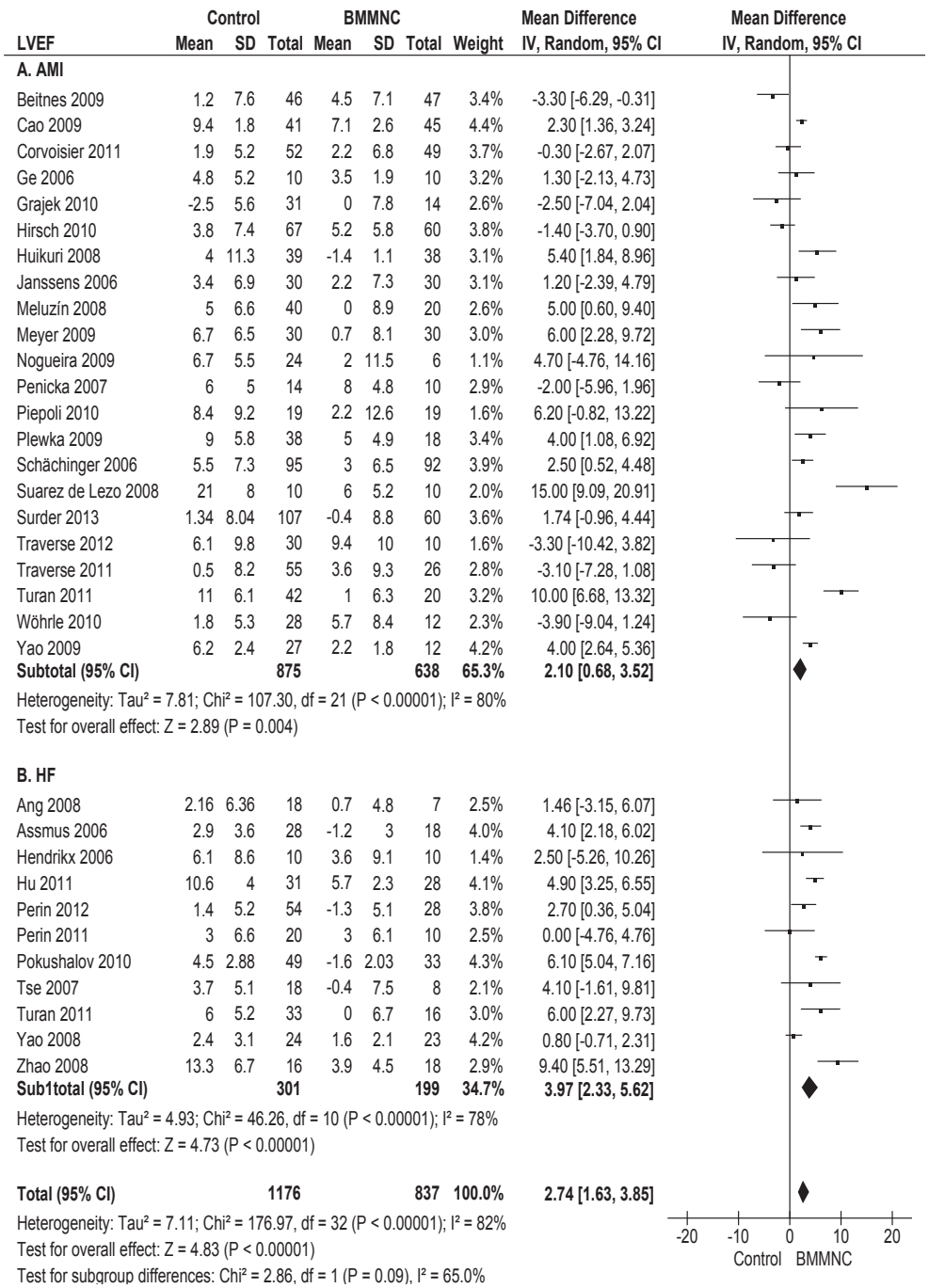

Figure 2. Forest plot of LVEF in patients treated with BMMNC. Figure A represents intracoronary infusion of Bone marrow mononuclear cells (BMMNC) in patients with an acute myocardial infarction (AMI). Figure B represents intramyocardial injection of BMMNC in patients with HF.

(95% CI: -6.91- -1.18 mL; P=0.006). Intriguingly, this modest beneficial effect disappeared (0.13, 95% CI -2.67-2.93; p=0.93) when a sub-group analysis was performed based on imaging modality, and only including studies that used MRI-derived measures (figure 3a; table 2). LVEDV and infarct size did not improve in patients treated with BMMNC, irrespective of imaging modality.

Intracoronary infusion of stem cells did not result in procedure-related adverse events, or adverse events were not reported in the manuscript. No effect on the incidence of MACCE events was detected in this meta-analysis (table 3).

Bone marrow-derived mononuclear cells in ischemic heart failure patients

Although most trials using BMMNC were performed 3-30 days following the AMI, some other studies evaluated the effect of BMMNC in ischemic or non-ischemic HF patients (table 1b, supplemental table 3b). The search resulted in 12 trials, including a total of 500 patients (301 were treated with BMMNC) [s1-11] The average number of BMMNC injected was 128.6 ± 186.6 x 10^6. The median follow-up duration was 6 months and most trials assessed cardiac function by 2D-echocardiography or LV angiography. Endpoints were mainly specified as the effect on LVEF and LV volumes, or exercise capacity in combination with improvement in NYHA classification. Three studies used intracoronary infusion as delivery route; nine studies investigated the effects of intramyocardial injection.

Overall, LVEF improved by +3.97% (95% CI: 2.33 – 5.62; P<0.00001; figure 2b; table 2) mainly evoked by a

Table 3. MACCE rates Bone Marrow-derived Cells in AMI

		BMMNC	
Outcome AMI	**OR**	**95% CI**	**P**
All cause mortality	0.68	0.36-1.31	0.25
Cardiac mortality	0.73	0.32-1.65	0.45
Recurrent MI	0.50	0.24-1.06	0.07
TVR	0.86	0.58-1.27	0.44
In-stent restenosis	0.95	0.51-1.79	0.88
In-stent thrombosis	0.75	0.08-7.45	0.81
Heart failure	0.84	0.44-1.60	0.60
CVA	0.62	0.13-2.84	0.53
VT/VF	0.66	0.32-1.35	0.25
ICD	0.98	0.37-2.64	0.97
Outcome HF*			
All-cause mortality	0.34	0.15-0.75	0.008
Cardiac mortality	0.70	0.14-3.58	0.66
VT/VF	1.64	0.03-83.48	0.8

MACCE rates were not reduced in BMMNC treated patients. It has to be kept in mind that the trials to date were not sufficiently powered to detect differences in clinical outcome. OR; odds Ratio; CI; confidence interval; MI: myocardial infarction; TVR; target vessel revascularization; VT; ventricular tachycardia; VF: ventricular fibrillation; ICD: Implantable Cardioverter Defibrillator; HF: heart failure; AMI: acute myocardial infarction

*In HF patients, only all-cause mortality, cardiac mortality and VT/VF were included in the analyses. Other MACCE parameters were omitted due to a limited number (2-3) trials that presented these results in their paper.

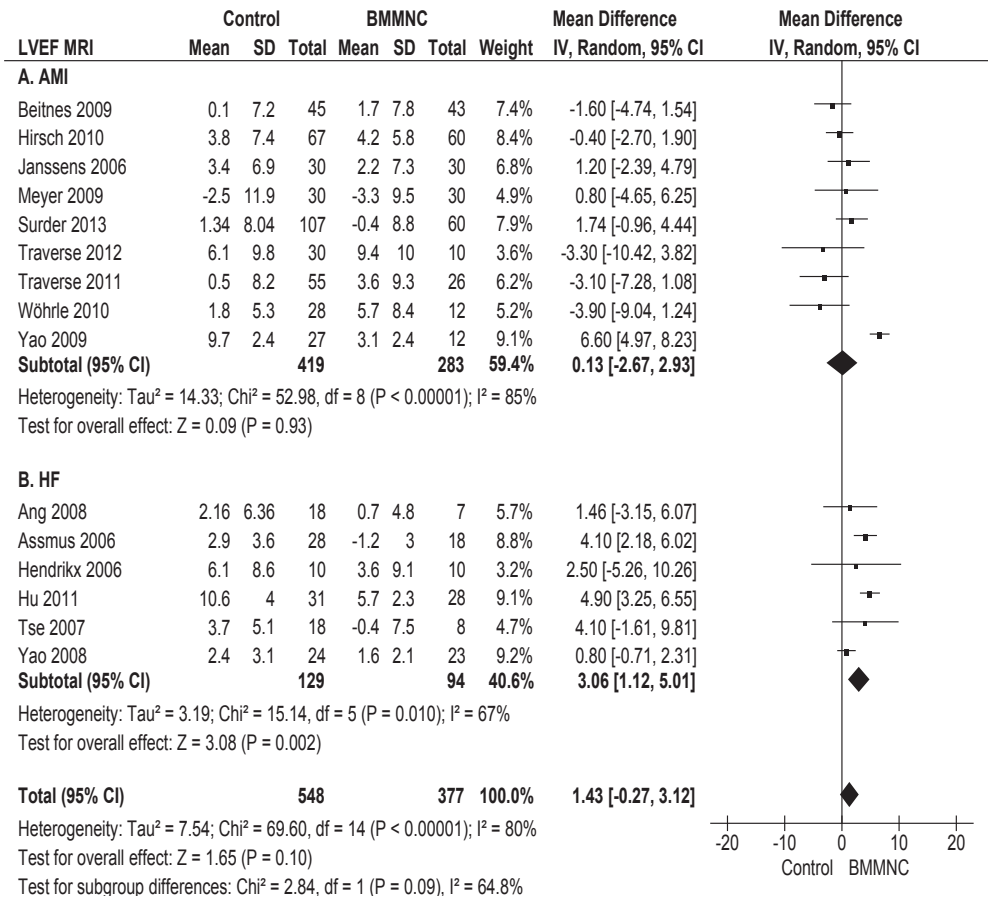

Figure 3. Forest plot of MRI-derived LVEF in patients treated with BMMNC. Figure A represents intracoronary infusion of Bone marrow mononuclear cells (BMMNC) in patients with an acute myocardial infarction (AMI). Figure B represents intramyocardial injection of BMMNC in patients with HF.

reduction in LVESV of -16.05 mL (95% CI: -29.62 - -2.47, p=0.02). LVEDV remained unchanged. Moreover, most trials revealed an improvement in NYHA classification. Unfortunately, the recent study by Heldman *et al.*, which compared BMMNC with autologous mesenchymal stem cells (MSC), did not report the absolute effects on LVEF and volumes.[18] Therefore, this study is not used in our analysis of functional data.

When corrected for studies that used MRI derived parameters, LVEF remains significantly enhanced in patients treated with BMMNC (+3.06%, 95% CI 1.12- 5.01; P=0.002; Figure 3b, Table 2). However, the significant effect on LVESV disappeared (-14.45 ml, 95% CI -35.03 - 6.20; p=0.17).

No serious adverse events were reported in the trials regarding BMMNC transplantation, despite the vulnerable patient population. BMMNC treatment resulted in a reduction of all-cause mortality in ischemic HF patients (OR 0.34, 95% CI 0.15 – 0.75, p=0.008; table 3) but not in other MACCE events.

DISCUSSION

The current meta-analysis comprises 1453 AMI patients and 501 HF treated with BMMNC, as well as 107 HF patients treated with SkM. We defined these cells as first generation of cardiac stem cell therapy, because initial and most experience has been obtained with these cells. We found that BMMNC therapy was safe in both patient groups. However, SkM injection resulted in a significant increase in ventricular arrhythmias in treated HF patients. Our meta-analysis revealed that there is a modest increase of 2% in LVEF in AMI patients treated with BMMNC, but this effect diminishes when only trials are included that use MRI as imaging modality. In HF patients, we found a significant increase in LVEF, irrespective of the imaging modality that was used in the trials. This beneficial effect resulted in a decrease in all-cause mortality in treated patients with HF, which was absent in AMI patients.

Clinical outcome

Several previous meta-analyses have reported beneficial effects of BMMNC therapy on clinical end points in AMI patients as all-cause mortality, cardiac mortality, and the incidence of recurrent myocardial infarction and stent thrombosis.[9,10,19] These results do not correspond with our data. Indeed, we found some discrepancies between these meta-analyses and the current manuscript. First, and most importantly, in these analyses, data of both AMI and HF patients were pooled to increase power. Also, some important negative studies as the HEBE trial were not included in their analysis, whereas other studies were mentioned twice. Moreover, in the current study the odds ratio was calculated to assess the effect on clinical end points, whereas another meta-analysis displayed clinical outcome measures using the relative risk.[11]

The current paper for the first time separates both patient groups, and describes functional outcome, as well as clinical outcome, in one manuscript. We found no clear effect on any pre-specified clinical end point in AMI patients treated with BMMNC, whereas in HF patients the beneficial effect was reduced to all-cause mortality alone. We believe that combining the results of these two distinct patient populations in earlier meta-analyses has clouded outcome, and overestimated the effect of BMMNC therapy. We should also keep in mind that all studies to date regarding BMMNC therapy were designed as safety and feasibility studies. Hence, they were not powered to detect differences on clinical endpoints. We hope that the forthcoming BAMI study (NCT01569178), with all-cause mortality during 3-year follow up as primary end point, will render a definitive answer regarding the role of BMMNC therapy in AMI patients. In this trial, 3000 AMI patients with EF below 45% will be included and randomized to BMMNC therapy or optimal medical care. However, although this study has been announced several years ago, thus far no patient has been included.

Cardiac MRI as reference imaging modality

The studies that drove initial enthusiasm of BMMNC-based heart repair following AMI used either left ventriculography or echocardiography as imaging tool to assess cardiac function.[20,21] Both imaging modalities are known to be less accurate in determining cardiac volumes than the current golden

standard, which is cardiac MRI.[22] We believe that this, in combination with relatively low patient numbers, could have overestimated the effects of BMMNC in AMI patients. In the current meta-analysis, 40% (8 out 21) of all trials used cardiac MRI as imaging modality, and correction for this parameter resulted in a reduction of the treatment effect to non-significant values. This finding corroborates the exploratory findings of Traverse et al.[23], and should put the initial enthusiasm concerning BMMNC-based therapies for AMI patients in a different perspective.

In patients with HF, however, improvement of LVEF remained within significant values, despite correction for MRI as imaging modality. Although this effect of only ~3% seems modest, it could have significant clinical implications. For instance, in the studies that assessed the effect of primary PCI following AMI, a similar modest improvement in LVEF was found that eventually resulted in pronounced effects on mortality.[22,24] The community of HF specialists has embraced these positive findings by incorporating BMMNC therapy as a possible adjunctive treatment of HF patients in the guidelines of the European Society of Cardiology for the treatment of HF.[25]

Are BMMNC the optimal cells for ischemic heart disease?

The mononuclear cell fraction of BM consists of a heterogeneous population of cells, predominantly comprising white blood cells and its precursors. Less than 1% of all BMMNC are actual stem cells, the majority of which are hematopoietic stem cells, but this fraction also includes endothelial progenitor cells and mesenchymal stem cells (~0.01%).[26,27] Following BM harvest, BMMNC are separated from red blood cells and plasma, for instance using a Ficoll gradient, after which this whole heterogeneous fraction is infused or injected.

This meta-analysis shows that even *if* there is an effect of BMMNC therapy in ischemic heart disease patients, it is only modest. In the meanwhile, the field has progressed, and several newer generations of stem cells have emerged. Some of these cells have already been tested is phase I and II clinical studies, whereas others are still in the preclinical testing phase. The next paragraphs summarize our knowledge about some of these next generations of stem cells for cell-based cardiac repair.

What cell for what disease type?

As AMI and HF are two distinct conditions within different stages of the disease process, it seems logical that the cell that fits one condition might not be the ideal cell for the other. Therefore, the purpose of cell therapy should be defined for both diseases separately.

In AMI patients, we should strive to minimize damage inflicted by ischemia and reperfusion, thereby reducing infarct size and thus delaying or abrogating LV remodeling. We believe that the ideal cell for AMI patients has the following characteristics: 1) availability during the (hyper)acute phase of the AMI, and; 2) have pronounced paracrine anti-apoptotic, pro-angiogenic, immuno-modulatory, and anti-remodeling capacities, and/or; 3) mobilize or stimulate resident cardiac stem cell niches or cardiomyocytes, and; 4) be autologous or non-immunogenic.

In contrast to AMI, HF is a chronic condition with a much broader time window for possible stem cell transplantation. Ideally, in patients with heart failure due to systolic dysfunction, cells should be

Table 4. Clinical Autologous and Allogeneic Mesenchymal Stem Cell Trials

	Phase	Cell type	Status	Design	No.	Cell source	Delivery Method	Condition	Primary Clinical Outcome	Reference
STEMMI	II	Autologous	complete	RDBPC	78	BM (mobilized G-CSF)	IC	AMI	Safety/Efficacy : regional systolic wall thickening MRI	NCT00135928
APOLLO	I	Autologous	complete	RDBPC	13	Adipose-tissue	IC	AMI	Safety	NCT00442806
ADVANCE	II/III	Autologous	not yet recruiting	RDBPC	360	Adipose-tissue	IC	AMI	Safety and Efficacy: infarct size (MRI)	NCT01216995
PROMETHEUS	I/II	Autologous	complete	RDBPC	45	BM	IM (Epi)	IHF	Safety/Efficacy: Serious adverse events, Infarct size (MRI)	NCT00587990
C-CURE	II/III	Autologous	complete	RSBPC	240	Guided BM	IM (Endo) (C-Cath®)	IHF	Safety/Efficacy: LVEF	NCT00810238
TAC-HFT	I/II	Autologous	complete	RDBPC	30	BM	IM (Endo) (Helix®)	IHF	Safety/Efficacy: MRI	NCT00768066
PRECISE	I	Autologous	recruiting	RDBPC	36	Adipose-tissue	IM (Endo) (NOGA®)	IHF	Safety: MACCE at 3Y	NCT00426868
PROCHYMAL	I	Allogeneic	complete	RDPBC	53	BM	IV	AMI	safety	-
PROCHYMAL	II	Allogeneic	recruiting	RDBPC	220	BM	IV	AMI	Safety: (LVESV)	NCT00877903
Mesoblast AMI	I/II	Allogeneic	recruiting	RSBPC	25	BM	IM(Endo) (NOGA®)	AMI	Feasibility/Safety	NCT00555828
MultiStem	I	Allogeneic	complete	ONPC	25	BM	IM(adventitia of CA) (Cricket®)	AMI	Safety: Adverse Event at 1M	NCT00677222
AMICI	II	Allogeneic	not yet recruiting	RDBPC	225	BM	IC	AMI	Safety/Efficacy: Infarct size (MRI) at 6M	EUCTR2010-020497-41-NL

Table 4. Continued

	Phase	Cell type	Status	Design	No.	Cell source	Delivery Method	Condition	Primary Clinical Outcome	Reference
POSEIDON	I/II	Auto/Allo	complete	RONPC	30	BM	IM(Endo) (Helix®)	ICHF	Safety/Efficacy: TE-SAE at 1M	NCT01087996
Mesoblast CHF	II	Allogeneic	unknown	RSBPC	60	BM	IM(Endo) (NOGA®)	CHF (ischemic/ idiopathic)	Feasibility and Safety	NCT00721045

RDBPC: randomized double-blind placebo controlled, RSBPC: randomized single-blind placebo controlled, BM: bone marrow, IC: intracoronary, IM: intramyocardial, AMI: acute myocardial infarction, ICHF: ischemic congestive heart failure, CMI: chronic myocardial ischemia, LVEF: LV ejection fraction, MACCE: major adverse cardiovascular and cerebrovascular event, EET: exercise tolerance test

Table 5. Cardiac Progenitor Cell Trials

	Phase	Cell type	Status	Design	No.	Cell source	Delivery Method	Condition	Primary Clinical Outcome	Reference
SCIPIO	I	Autologous	complete	RONPC	40	CSC (c-kit)	IC	ICHF	Short term Safety	NCT00474461
CADUCEUS	I	Autologous	complete	RONPC	31	CDC (Cardiospheres)	IC	ICHF	Safety	NCT00893360
ALLSTAR	I-II	Allogeneic	recruiting	RDBPC	274	Allogeneic CDC	IC	ICHF	Safety/ Infarct size	NCT01458405

RDBPC: randomized double-blind placebo controlled, RSBPC: randomized single-blind placebo controlled, ONPC: open label, non-placebo-controlled, RONPC: randomized, open label, non-placebo-controlled, BM: bone marrow, CSC: cardiac stem cells, CDC: cardiosphere derived cells; IC: intracoronary, ICHF: ischemic congestive heart failure, LVESV: LV end systolic volume

applied that can 1) influence the remodeling process, and have anti-apoptotic capacities and/or; 2) mobilize or stimulate resident cardiac stem cell niches or cardiomyocytes, and/or 3) enhance blood and nutrients supply by inducing angiogenesis; and/or 4) be multipotent, transdifferentiate into functional cardiomyocytes, and contribute to the contractile apparatus.
Although BMMNC contain some of the characteristics mentioned above, we believe that there are more potent candidates. For a complete overview of all cell types we refer to chapter 2 of this thesis.

CONCLUSION

BMMNC are not effective for the treatment of AMI-patients, whereas their benefit in HF patients is modest at most. We believe that, to date, MSC are the promising candidate for cardiac repair in both AMI and HF patients. Autologous and allogeneic MSC have been shown to reverse cardiac remodeling following intracoronary and intramyocardial injection, in AMI and HF patients. This is probably associated with their potent paracrine capacities, resulting in a stimulatory effect on resident cardiac stem cells and cardiomyocytes, as well as reduction of apoptosis and extracellular matrix remodeling, and a stimulation of neo-angiogenesis. Next to MSC, CDC and CSC are emerging, and the proof of principle of these cells in HF has been established. The near future will probably prove whether these cells have a definite place in this disease type.

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SUPPLEMENTAL MATERIAL

Content

MATERIAL AND METHODS

Search strategy

Medline (July 2002-December 2013) and the Cochrane Central Register of Controlled trials (CENTRAL) and the website of US Food and drug administration www.fda.gov were searched for relevant articles. The search included all randomized controlled trials regarding stem cell therapy for the treatment of and AMI, chronic heart failure or ischemic cardiomyopathy reported up to January 1st 2014. We also searched for relevant abstracts and presentations on this topic reported in major cardiology meetings. References in other articles were also investigated and included in the analysis whenever deemed appropriate. Websites, including *www.clinicaltrials.gov* and www.clinicaltrialregister.eu, were searched for ongoing trials and future trials. The following search strategy was applied: "stem cells", "progenitor cells", "mononuclear cells", "adipose tissue-derived regenerative cells", "mesenchymal stem cells', "cardiac-derived stem cells", "bone marrow", "vascular stromal fraction", "adipose stem cells", "mesenchymal-like stem cells", "skeletal myoblasts", "coronary artery disease", "myocardial infarction", "heart failure" "cardiac repair", and "myocardial regeneration". Only articles published in English were included. Limitations used in the search were the publication of the study within the last 10 years, limited to clinical trials and randomized controlled clinical trials.

Inclusion and exclusion of studies

Studies were included that met the following criteria: (1) randomized controlled trials with an appropriate control group who received standard therapy, (2) conducted in patients with an AMI that occurred less than 3 months before or patients with diagnosed heart failure or ischemic cardiomyopathie (3) using stem cells that were administered by intracoronary, intravenous injection or intramyocardial injection, (4) total of number of patients enrolled should exceed 10, (5) stem cells were derived from adipose tissue, heart, skeletal muscle, bone marrow (6) given in an allogeneic or autologous setting.

Only studies with a complete dataset and specified data on the amount of infused cells were included in this meta-analysis. Studies that described the combination of circulating progenitor cells (CPC) or CPC with granulocyte-colony stimulating factor (G-CSF) were excluded from this analysis to circumvent

the potential confounding effect of G-CSF therapy on LV function and dimensions, although G-CSF was previously proven ineffective as a mono-therapy for cardiac repair in AMI. When studies compared C-GSF and stem cells, only the patients in the control and stem cell arm were used in this analysis.

Data abstraction

Two reviewers (RdJ, JH) independently screened abstracts and reported their results in a standardized form. Data extracted from the articles were categorized in trial characteristics, functional outcome, scar size and safety. The following parameters were extracted from the articles: Left ventricular ejection fraction (LVEF), LV end-systolic volume (LVESV), LV end-diastolic volume (LVEDV), infarct size (MRI), perfusion defect (SPECT) and major adverse cardiac and cerebral events (MACCE) rates. MACCE was specified as: all-cause mortality, cardiac mortality, hospitalization for heart failure, in-stent thrombosis and restenosis, target vessel revascularization, ventricular arrhythmia, ICD implantation and stroke. Infarct size was expressed as the percentage of left ventricle infarcted (in %volume or mass). In the various studies, different imaging modalities have been used to determine left ventricular ejection fraction. Cardiac magnetic resonance imaging (MRI) was considered the golden standard. If more than one imaging modality was included, all data was extracted for subgroup analysis. For studies with more than 1 intervention arm (e.g. multiple doses) the weighted mean was calculated and applied for the main analysis.(SI-3) In trials with multiple follow-up time points, the primary endpoint was used in the main analysis.

Quality

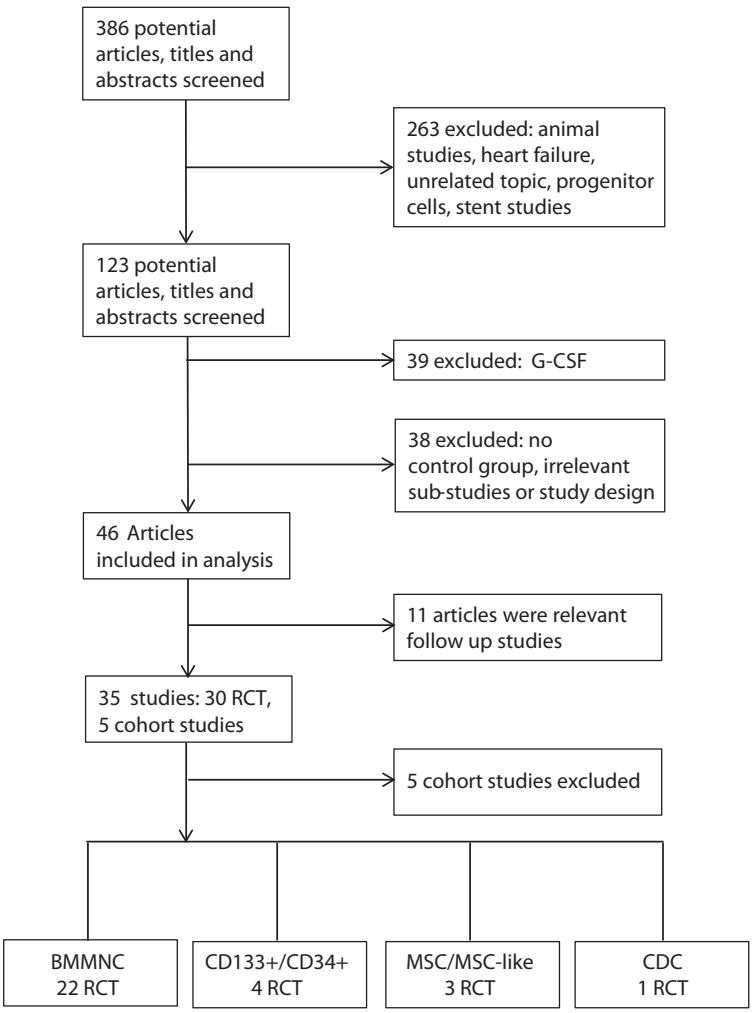
The methodological quality of randomized controlled trials was tested by the Jüni criteria.[1]

Data analysis

Left ventricular function was the primary endpoint of our analysis. In particular, we studied the difference in mean LV ejection fraction change (LVEF, from baseline to follow-up) between patients receiving stem cells and control treatment. We have applied inverse-variance weighting to combine the results from independent studies. Most studies reported mean LVEF ± one standard deviation (SD) at baseline and follow-up. The mean $LVEF_{change}$ was then determined as $LVEF_{follow\text{-}up}$ - $LVEF_{baseline}$, whereas the SD_{change} was estimated according to the method that is described by Hristov *et al*.[2] For studies that report standard errors of the mean (SEM), SDs were determined as SEM*√(sample size). In case interquartile ranges are reported, SDs are estimated as range/4. We applied a random effects model to obtain an overall estimate of the treatment effect, which we report as point estimate and 95% confidence interval (CI). Heterogeneity was analyzed with the I^2 statistic, and was defined as low (25%-50%), intermediate (50%-75%) or high (>75%).

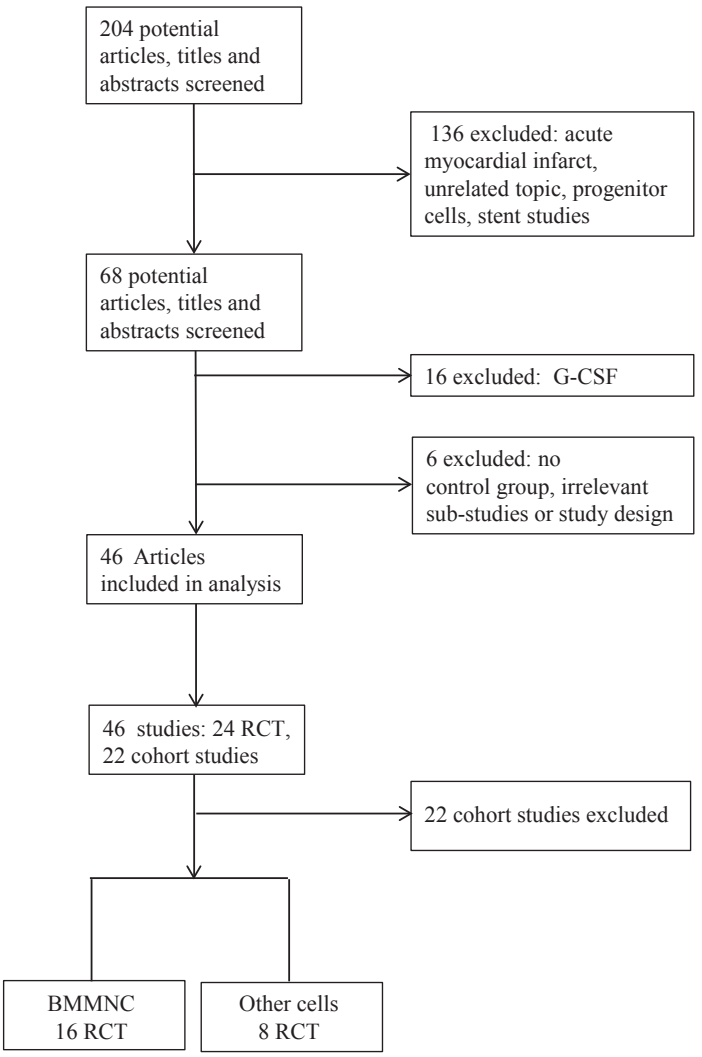
We have applied similar methodology to study several secondary endpoints, including (mean changes in) left ventricular end systolic (LVESV) and end diastolic (LVEDV) volumes, infarct size as measured by cardiac MRI, and perfusion defect as measured by SPECT. We applied the Mantel-Haenszel odds ratio to obtain an overall estimate of the odds ratio for MACCE, again assuming random effects.

All analyses were performed using Review Manager 5.2 analysis software (Rev Man, Version 5.2, Copenhagen, The Nordic Cochrane Centre, The Cochrane collaboration, 2012). We considered p-values <0.05 (two-sided) as statistical significant. Funnel plots were constructed to explore publication bias.



Supplemental Figure 1. Flow chart of search stem cell therapy in acute myocardial infarction

Supplemental figure S1: flowchart of search meta-analyses on stem cell therapy for the treatment of an acute myocardial infarction. G-CSF indicates granulocyte- colony stimulating factor; RCT: randomized controlled trial; BMMNC: bone marrow mononuclear cell.



Supplemental Figure 2. Flow chart stem cell therapy in heart failure patients

PART 2

Adipose Tissue-Derived Regenerative cells

CHAPTER 5

Clinical Study Using Adipose Derived Mesenchymal-like Stem Cells in Acute Myocardial Infarction and Heart Failure

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ABSTRACT

Adipose tissue represents an abundant, accessible source of regenerative cells that can be easily obtained in sufficient amount for therapy. Adipose-Derived-Regenerative-Cells (ADRC) are comprised of leukocytes, smooth muscles, endothelial cells and mesenchymal stem cells. In contrast to bone-marrow derived MSC, the abundance of adipose tissue in patients and the higher frequency per unit mass of regenerative cells allows for the isolation of cells in therapeutic meaningful amounts in less than 2 hours after donor tissue acquisition. Harvest of adipose tissue can thus follow primary PCI, allowing efficient treatment within 24 hours. This obviates the need for extensive cell culturing in GMP clean room facilities and makes ADRCs a promising and practical autologous cell source. In the following chapter, we will describe the liposuction procedure for stem cell harvest, two cell delivery techniques and pressure/volume loop analysis for the follow up of our patients enrolled in the clinical studies.

INTRODUCTION

The clinical utility of cardiovascular stem cell therapy passed the phase of proof-of-concept. After 14 years of research, this field is now entering phase III clinical trials to treat ischemic heart disease. Previous work in animal models shows the potential to improve tissue regeneration by neo-angiogenesis, and more recently neo-myogenesis. The promise of this therapy is high, with multiple disciplines working on cardiovascular ischemic models in the regenerative medicine field.[1]

In particular mesenchymal stem cells (MSC) appear to have many features that we specifically look for in an ideal stem cell for cardiovascular disease. Preclinical and clinical studies showed that these cells are highly proliferative in cell culture, and that they are capable of mediating cardiovascular regenerative effects [2,3], through paracrine mechanisms that target resident cardiomyocytes and vascular cells.[4] Long-term follow-up of patients, who received BM MSC stem cell therapy for the treatment of acute myocardial infarction, showed a sustained improvement of global LVEF by 3.9% and decreased infarct size at 5 year FU.[5]

The technique of isolating and subculturing autologous MSC stem cells is however logistically challenging. Patients first have to undergo bone marrow puncture(40 cc). The bone marrow aspirate is then transported to a GMP-certified facility, where the BM derived mesenchymal stem cell population is selected by plastic adherence-isolation.[6,7] For a therapeutic relevant amount, a bone marrow graft of 40 cc has to be sub-cultured for 6-12 weeks. The culturing of allografts for a prolonged period of time is inherently associated with an increased risk of graft failure and contamination.

One of the abundant sources of stem cells in adults are multipotent mesenchymal-like stem cells that have been isolated from subcutaneous adipose tissue. First identified by Zuk [8], these mesenchymal-like stem cells, act through their paracrine effects to improve angiogenesis, reduce ischemic-induced apoptosis, modulate inflammation and enhance progenitor cell recruitment and differentiation. One of the major benefits of adipose tissue is the ability to harvest ADRC in clinically relevant amounts that would not require additional cell culture to generate sufficient numbers of stem cells. [9,10] Within two hours after liposuction, from as little as 200 grams of lipo-aspirate, 40-60 million ADRC's can be isolated by simple centrifugation to separate the buoyant adipocyte fraction and the non-buoyant vascular stromal fraction. Feasibility and safety of ADRC therapy has been demonstrated in a multicenter, randomized, placebo-controlled trial in humans with STEMI (APOLLO) and Congestive heart failure (PRECISE).[11]

MATERIALS

To perform liposuction under aseptic conditions, use a Toomey cannula with a standard tip up to 15 cm long and a 4 to 6 mm inner diameter. For infiltration of the targeted liposuction regions in the peri-umbilical region use a Toomey syringe and a 14 gauge, blunt LAMIS infiltrator to inject tumescent fluid composition. We advise when removing fat in peri-umbilical region to do this symmetric, keeping the overall area of aspiration as small as possible (Note 1).

Liposuction

1. Check Hb and aPTT before initiation of the procedure for prevention or early detection of bleeding(Note 2)
2. Always employ monitoring of ECG vital signs and blood pressure (every 5 to 10 minutes) during procedure
3. Prepare a sterile field in around the peri-umbilical region
4. Anaesthetize the location of incision with a local injection Lidocaine (2%)
5. Make an approximately 0.5 cm stab skin incision
6. Infiltrate tumescent solution using a blunt LAMIS infiltrator and a volume ratio of 1:1 of tumescent solution (Note 3) to the selected fat for harvesting
7. After 20-30 minutes, the fat is harvested using the Toomey aspiration cannula attached to the 50cc syringe
8. After the syringe is filled (complete filling is not required), the syringe is capped and ready for transportation to isolation facility.
9. Continue liposuction until desire volume is achieved
10. Close surgical incision with appropriate suture
11. Apply standard elastic pressure corset immediately upon conclusion of tissue harvest
12. Check Hb and aPTT every hour for a minimum of 8 hours after the procedure

ADRC Isolation

The Celution™ system is essentially designed to automatically process lipo-aspirate tissue and separate the ADRCs from the adipose tissue by enzymatic digestion of the tissue using centrifugation to separate the non-buoyant ADRCs from the buoyant lipid adipocytes. The abundance of adipose tissue in human subjects and the higher concentration of adult regenerative cells per unit mass of adipose tissue allow for the isolation of an efficacious number of cells without having to sub culture them in a laboratory.

METHODS

Intracoronary Infusion

1. Place the therapeutic 7 Fr coronary guiding catheter into the culprit main vessel
2. Infuse 200 µg of Nitroglycerin through guiding catheter
3. Record fluoroscopy of target vessel in LAO and RAO projection with at least 90° difference to quantify by online QCA
4. Record TIMI flow of the culprit vessel
5. Place 0.014 inch soft tipped CFR wire (Volcano, Cordova CA) into the culprit vessel distal to stent edge.
6. Perform Coronary Flow Reserve measurement using adenosine continuous infusion at a rate of 140 µg/kg/min
7. Place over-the-wire the infusion catheter in the stent in the culprit vessel

8. Remove flow wire from microinfusion catheter
9. Attach infusion syringe to central lumen of infusion catheter (Note 4)
10. Start infusion (Note 5) of ADRC cells at 2,5 ml per min
11. After 30% of the total cell suspension volume is infused, the infusion pump is stopped and contrast dye is injected into the guiding catheter vessel to evaluate and record TIMI coronary flow
12. Repeat step 11 after 60% of total cell suspension volume has been infused
13. After completion of the cell injection, insert coronary flow wire though the microinfusion catheter
14. Perform Coronary Flow Reserve measurement using adenosine continuous infusion at a rate of 140 µg/kg/min
15. Measure maximal coronary flow during adenosine hyperemia (Note 6)
16. Remove flow wire
17. Inject 200 µg of Nitroglycerin through guiding catheter
18. Record coronary angiography of the culprit vessel with 2 views, with at least 60° difference to LAO recording for QCA analysis
19. Evaluate and record level TIMI coronary flow in target vessel

Intramyocardial Injection by use of the Helix catheter ™ (BioCardia Inc., San Carlos)

1. Place an 8 Fr Sheath into femoral artery artery
2. Perform LV gram in LAO and RAO projection (with at least 90° difference to the LAO recording)
3. Trace endocardial borders on transparencies overlaying the on-screen images during end diastole and end-systole of the LV gram cycle
4. Mark non-contractile myocardial segments on the transparent
5. Place a guidewire in left ventricle
6. Advance the BioCardia Morph steerable catheter over the wire into the left ventricle and remove the wire
7. Advance the Helix Infusion Catheter (HIC) through the Morph catheter. Advance the HIC perpendicular to the targeted endo-ventriculair wall under fluoroscopic guidance
8. Engage the myocardium by 3 turns clockwise of the Helic Catheter
9. Confirm intra myocardial engagement by flush of contrast through the HIC against the intra myocardial tissue
10. Start injection of 0.2 ml stem cell suspension by slow infusion over 30 seconds
11. After injection allow 30 seconds of dwelling time
12. Disengage the catheter by 3 counterclockwise turns
13. Repeat steps 7 to 12 until a total of 15 injections with 0.2 ml suspension are injected in the target zone

Pressure volume loop

1. Use a ≥7 Fr arterial and an ≥8 Fr venous sheath in the femoral artery/venae
2. Place a Swan-Ganz thermo-dilution catheter in the pulmonary artery
3. Place conductance catheter along the long axis of the left ventricle under fluoroscopic guidance
4. Optimize conductance catheter position based on on-line PV signals
5. Determine blood resistivity by drawing 5 ml blood into cuvette
6. Perform thermo-dilution CO measurements three times, record pressure-volume loops during CO measurements
7. Perform hypertonic saline injections (3x, 7 mL, 10% saline) via the distal port of the thermo-dilution catheter, record pressure-volume loops during injections (preferably during breath-holding at end-expiration)
8. Remove Swan-Ganz catheter
9. Place balloon occlusion catheter into inferior vena cava
10. Record positioning with cinegram without contrast
11. Record baseline pressure-volume loops during breath-holding at end expiration (approximately 10 sec)
12. Inflate balloon in vena cava position to reduce preload to LV and record pressure-volume loops during LV unloading until systolic pressure drops by approximately 20 mm Hg (preferably with breath-holding at end expiration)
13. Deflate balloon
14. Repeat once steps 11-13

NOTES

1. Patient must have the ability to undergo liposuction to obtain a minimum of 220 ml adipose tissue.
2. Liposuction must not be performed on patient that previously received any IIb/IIIa Glycoprotein Inhibitor within seven days preceding the liposuction, or those who have received any anticoagulant within 1 hour of liposuction and have an aPTT result of ≥ 1.8 times the control value
3. Prepare tumescent fluid with 1 mg epinephrine and 20 ml 2% Lidocaine in 500 ml Ringers lactate
4. Make sure no blood or air bubbles reside in connector, flush with small amounts of saline if necessary
5. For use in AMI, ADRCs suspended in a standard 20 ml/cc Lactated Ringers Solution may be infused into the coronary artery via an angioplasty catheter or micro-infusion catheter (TwinPass, Vascular Solutions, Mineapolis, USA)
6. To investigate the effect on hemodynamic changes during the measurements, both resting and hyperemic conditions should be measured twice

ACKNOWLEDGEMENTS

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CHAPTER 6

Long-term patient follow up after intracoronary infusion of adipose tissue-derived regenerative cells in patients with ST-segment elevation myocardial infarction: final results of the APOLLO trial

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ABSTRACT

Objectives APOLLO is a first-in-human, randomized, placebo-controlled trial to assess safety and feasibility of intracoronary infusion of adipose tissue-derived regenerative cells (ADRCs) after percutaneous coronary intervention (PCI) for acute myocardial infarction (AMI).

Background ADRC treatment following AMI showed favorable safety results and hints of efficacy at 6 months follow-up in a previous concise report. This is the first complete report of the APOLLO trial, and extends functional findings to 18 months, and clinical follow-up to 36 months.

Methods A total of 14 patients were randomized to receive intracoronary infusion of ADRCs (n=10) or placebo (n=4) within 24 hours following the primary PCI. The patients were monitored for procedural and postoperative safety, and the occurrence of adverse events with follow-up to 36 months. Functional analysis was performed by echocardiography, cardiac MRI, and nuclear imaging at baseline, 6 and 18 months.

Results Liposuction, but also intracoronary infusion of ADRC, was well tolerated in all patients. Through 36 months of follow-up, 2 ADRC and 1 placebo patient had experienced major adverse cardiac events requiring target lesion revascularization. No adverse events were associated with ADRC therapy. At 6 and 18 months, intracoronary ADRC infusion, but not placebo infusion, significantly improved perfusion defect, and also coronary flow reserve, while significantly decreasing the percentage of left ventricle infarcted.

Conclusions ADRC infusion proved to be safe during 36 months of clinical follow-up. Moreover, ADRC infusion was associated with improved perfusion and decreased infarct size through 18-month functional follow-up.

INTRODUCTION

Although regenerative cell therapy holds great promise for the adjunctive treatment of acute myocardial infarction (AMI) patients, the ideal progenitor cell still needs to be defined. Autologous adipose tissue-derived regenerative cells (ADRCs) can be readily obtained from subcutaneous adipose tissue in amounts that are sufficient for therapy.[1-3] ADRCs are similar to bone marrow-derived mesenchymal stem cells (MSCs) in their potential for differentiation and secretion of relevant growth factors and cytokines.[4-6] In contrast with the challenging and lengthy process required for deriving MSCs from bone marrow, the abundance of adipose tissue in patients and the higher frequency of regenerative cells per unit mass in that tissue can allow for isolation of a relevant number of cells in fewer than 2 hours from the time of donor-tissue acquisition. These factors of processing time and quantity with ADRCs eliminate the need for extensive *ex vivo* cell culturing and allow for treatment of AMI patients within hours after the primary PCI.

In vitro studies have shown that ADRCs secrete significant amounts of pro-angiogenic, anti-apoptotic and immunomodulatory factors, and that they can differentiate into spontaneously beating cells with cardiomyocyte features.[7-11] Also, several studies have demonstrated therapeutic efficacy of ADRCs in animal models of experimentally induced AMI and of chronic myocardial injury.[12-16]

The goal of the first-in-human "AdiPOse-derived stem ceLLs in the treatment of patients with ST-elevation myOcardial infarction" (APOLLO) trial was to extend the findings in pre-clinical investigations into the clinic and determine the long-term safety and feasibility of intracoronary infusion of ADRCs in patients within hours after successful PCI and stenting for acute ST-segment elevation myocardial infarction (STEMI). The earlier published concise report on the six-month follow up of the APOLLO trial showed a favorable safety profile and, among others, decreased infarct size and increased myocardial perfusion in ADRC-treated patients.[17] Here, we fully describe the trial, and extend the clinical follow up to 36 months. Also, several indices of cardiac function, remodeling, infarct size and myocardial perfusion have been included, thereby adding important, yet exploratory, parameters of efficacy.

METHODS

Study population and design

The APOLLO trial is a prospective, double-blind, randomized, placebo-controlled, phase I/IIa, first-in-man trial of the safety and feasibility of ADRC therapy via intracoronary infusion in the treatment of acute STEMI patients, successfully treated by PCI and stenting. The trial was conducted between November 2007 and May 2009. The trial was approved by the institutional and national review boards at the participating sites, and was conducted in accordance with the Declaration of Helsinki and ICH E6 Good Clinical Practice Guidelines. Written informed consent was obtained from all patients before trial enrollment.

Patients were eligible for enrollment in the trial, if they were 20 to 80 years of age and had been successfully treated with standard care (PCI and drug-eluting stent placement) within 2 to 12 hours of the acute onset of STEMI symptoms. All other inclusion and exclusion criteria are detailed in Table 1.

Table 1. Selected inclusion and exclusion criteria in the APOLLO study

Inclusion criteria
Clinical symptoms consistent with acute myocardial infarction (AMI) (pain, etc.) for a minimum of 2 and a maximum of 12 hours from onset of symptoms to percutaneous coronary intervention (PCI), and unresponsive to nitroglycerine
Successful revascularization of the culprit lesion in the major epicardial vessel within 2 to 12 hours of the onset of AMI symptoms
Area of hypo- or akinesia corresponding to the culprit lesion, as determined by left ventriculogram at the time of primary PCI
Left ventricular ejection fraction (LVEF) ≥30% and ≤50% at the time of successful revascularization.
Ability to undergo liposuction
Exclusion criteria
Prior MI, prior known cardiomyopathy, or prior hospital admission for congestive heart failure (CHF)
Significant valvular disease, need for mechanical intervention, or cardiogenic shock
Staged treatment of coronary artery disease, or other interventional or surgical procedures to treat heart disease (eg, valve replacement, PCI, or CABG) planned or scheduled within 6 months after the study procedure
Hemodynamic instability within 24 hours prior to randomization, defined as the presence of any of the following: · Systolic blood pressure <90 mmHg · Heart rate >100 bpm for more than 1 hour · Prior ventricular fibrillation or sustained ventricular tachycardia
Patients with increased bleeding risk including but not limited to: (a) those who have received any glycoprotein inhibitor within 7 days preceding the liposuction; or (b) those who have received any anticoagulant within 1 hour of liposuction or who have an aPTT result of ≥1.8 times the control value
Persistent atrial fibrillation
Neoplasia
Pacemaker, ICD, or any other contraindication for MRI
LVEF <30% or >50%
Moderate or severe COPD

Pre-enrollment screening of the post-PCI patients included, among others, 2D-TTE to confirm that post-AMI LVEF was between 30 and 50%, after which informed consent was obtained. Following enrollment, patients underwent a standard liposuction procedure to harvest approximately 200cc of lipoaspirate as described below. At this point, the patients were randomized 3:1 (by interactive voice-response system) to receive an injection of either autologous ADRC or placebo solution (lactated Ringer's solution with autologous peripheral blood making it indistinguishable from the study substance) via intracoronary infusion into the stented culprit coronary artery within 24 hours of the PCI.

In the immediate postoperative period, patients were monitored for arrhythmias using telemetry in the first 72 hours and Holter monitoring at week 1 through 3, and month 1 through 4, 6, 12, 18, 24, and 36. Per protocol, 2D-TTE, cardiac magnetic resonance imaging (CMR), and gated cardiac single photon emission computed tomography (SPECT) were scheduled 2 to 4 days post procedure and at 6 and 18 months. Coronary angiography and Doppler coronary flow measurements were performed before ADRC infusion, directly following ADRC infusion and at 6 month follow up. The imaging studies and

Holter recordings were analyzed by blinded core laboratories: Medstar Research Institute, Washington, DC, USA, for 2D echocardiography; Cardiovascular Core Laboratories, Boston, MA, USA, for CMR; Tufts New England Medical Center, Boston, MA, USA, for SPECT; and Agility Centralized Research Services, Bannockburn, IL, USA, for Holter monitoring.

Cell collection, preparation, and infusion

Adipose tissue was harvested by syringe-based lipoaspiration in a standard protocol-detailed procedure.[18] ADRCs were isolated from the lipoaspirate by use of the Celution® system (Cytori Therapeutics Inc., San Diego, CA), as previously described.[19] The Celution® system enzymatically digests the adipose tissue into a single cell suspension and uses differences in buoyancy to separate ADRCs from fat cells. The ADRCs are then further enriched and concentrated in suspension via series of washing and centrifugation steps within a procedure of approximately 120 minutes. Following randomization and blinding by the hospital pharmacist, the ADRC or placebo suspension was subsequently infused using a micro catheter (Twin Pass, Vascular Solutions, USA) placed in the culprit vessel at an infusion rate of 2 mL/min (~2.5 million ADRCs/min).

In order to ensure patient safety in this first-in-man application of ADRCs into coronary arteries, the current trial was originally conceived with a dose-escalation design, progressively employing doses of 20×10^6, 40×10^6, 60×10^6, and 80×10^6 ADRCs. The initial dose in the trial, up to 20×10^6, is a quarter of the maximum dose that was deemed safe and efficacious in preclinical porcine AMI studies.[14] When proof of concept was established with 20 million cells, and trial enrollment reached 14 patients, the trial steering committee and data safety monitoring board recommended that the original plan of dose escalation be discontinued in favor of a larger prospective, phase 2A/2B trial.

Endpoints

Standard safety assessments were performed at 1, 3, 6, 12, 18, 24, and 36 months. Safety endpoints included: the rate of major adverse cardiac and cerebrovascular events (MACCE; defined as the incidence of cardiac death, re-AMI, target site revascularization and/or stroke), the rate of serious adverse events (SAEs) and adverse events (AEs); and the occurrence of arrhythmic events as documented by the scheduled Holter recordings. Additional safety follow-up assessments included coronary flow reserve in the treated vessel before and after cell infusion, and differences and change from baseline in pro-brain natriuretic peptide type B levels (pro-BNP).[20] An independent, international Data Safety Monitoring Board (DSMB) and critical event committee (CEC) reviewed and adjudicated all MACCE and SAE events.

Efficacy endpoints included: measurement of the LV volumes and LVEF and of the change in LV volumes and LVEF from baseline, to 2 to 4 days post treatment, and 6 and 18 month follow up (2D-TTE), and from 2 to 4 days post treatment to 6 and 18 month follow up (CMR); determination of the change in percentage of LV infarcted and infarct mass (as measured by delayed-enhancement CMR (DE-CMR)); and determination of the change in perfusion defect from 2 to 4 days post treatment to 6 and 18 month follow up (SPECT).

Perfusion defects were expressed as the Visual Rest Score (VRS) and Total Severity Score (TSS), and are determined by the uptake of sestamibi into non-ischemic myocardium using a semi-quantitative, 17-segment scoring system in three short-axis slices. The VRS is defined as the weighted mean of the reader's visual perfusion scores over the target segments, whereas TSS represents an integration of the extent and the severity of the perfusion abnormality at rest by quantitative analysis. The latter represents the extent and severity of infarct or hibernating viable myocardium at rest.

Statistical analysis

APOLLO was primarily a safety and feasibility study, whereas first signs of possible efficacy were explored. All statistical tests were therefore considered exploratory, in the absence of prespecified hypotheses. All statistical tests are two-sided, and statistical significance is assessed with respect to a nominal p value ≤0.05. No prior sample size calculations were performed. The sample size was based on clinical judgment, with the goal of obtaining meaningful safety and feasibility information, while minimizing unnecessary patient exposure. Continuous variables are summarized by means and standard deviations. Categorical variables are summarized by counts and percentages of patients in the respective categories. All analyses and tabulations were performed using SAS software, version 8.2 or higher (SAS Institute Inc., Cary, NC, USA).

RESULTS

Patient population and baseline revascularization procedure

Between November 2007 and May 2009, 14 patients (12 men, 2 women, all Caucasian) were enrolled at the 2 study sites (Figure 1). Of the patients enrolled, 10 patients (61.0 ± 6.4 years [49 to 72 years]) were randomized to treatment with ADRCs; and 4 patients (55.0 ± 14.9 years [42 to 72 years]) were randomized to treatment with placebo solution. Table 2 summarizes the demographic

Table 2. Patient demographics and baseline characteristics (n = 13).

	ADRC (n = 9)	Placebo (n = 4)
Age (yrs)	61 ± 2.1	55.0 ± 7.5
Male sex (%)	78	100.0
Weight (kg)	86.2 ± 9.1	82.3 ± 12.5
Body mass index (kg/m^2)	27.5 ± 3.0	27.6 ± 3.3
Hypertension (%)	66.7	50
Smoking (%)	66.7	50
Diabetes (%)	20	25
CK-MB (μmol/l)	78.0 ± 3.9	92.0 ± 5.7
NT-proBNP (pmol/l)	250 ± 86	225 ± 116
Residual TIMI 3 coronary flow	100	100
LAD lesion	100	100

Data are reported as mean ± standard deviation

CK-MB = Creatine kinase-myocardial band

NT-proBNP = N-terminal pro-B-type natiuretic peptide

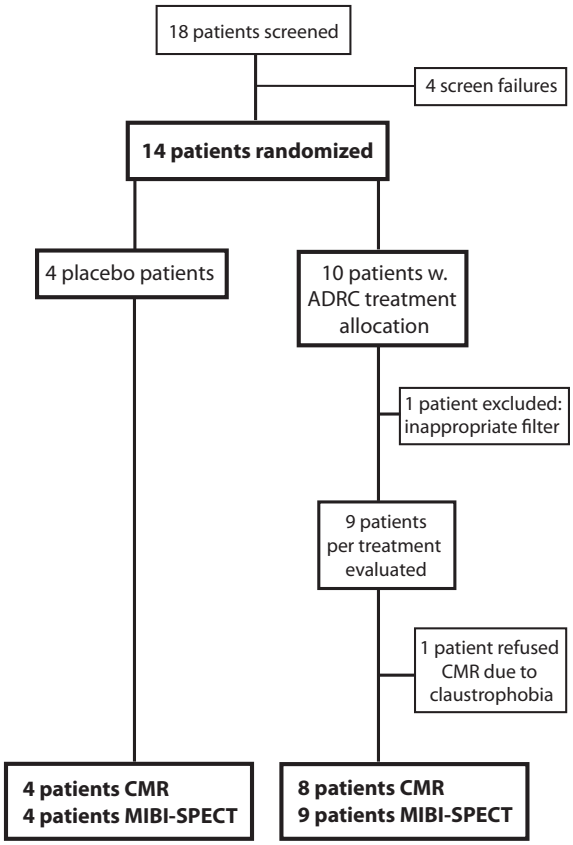

Figure 1. Flow chart of the APOLLO trial.

ADRC: adipose tissue-derived regenerative cells; CMR: cardiac magnetic resonance imaging; MIBI-SPECT: sestamibi single photon-emission computed tomography

and angiographic characteristics of both groups at baseline. All patients were admitted with an acute anterior myocardial infarction based on a proximal occlusion of the LAD.

Before enrollment and randomization, all patients were successfully revascularized. One patient presented with an acute myocardial infarction based on an in-stent thrombosis of a previously treated mid-LAD lesion 10 years before, and was treated by balloon angioplasty only. After enrollment, this patient was randomized to placebo and was included in safety, feasibility and efficacy analyses, although the balloon angioplasty of the previously implanted stent technically represented an inclusion criterion violation.

Adipose tissue harvest and intracoronary injection results

The syringe-based lipoaspiration procedure was well tolerated in all patients, although the first two enrolled patients experienced significant subcutaneous bleeding in the peri-umbilical area following lipoaspiration. After these 2 bleeding events, protocol amendments were made to exclude the use of glycoprotein IIb/IIIa inhibitors within 7 days preceding liposuction and to strengthen strict control

of anticoagulation by heparin after the primary PCI, permitting lipoaspiration only in patients with an aPTT-ratio ≤1.8. After these protocol amendments, there were no more serious bleeding events reported in the remaining patients. The mean amount of adipose tissue harvested was 210 ± 43.3 mL for the treatment group and 181.8 ± 41.5 mL for the placebo group. The intracoronary infusion required between 6 to 5 minutes to complete**.**

The 10 patients in the active treatment group received a mean dose of 17.3 x 10^6 ADRCs. For one of these patients, the use of an inappropriately sized filter (0.2 µm instead of 43 µm) during the intracoronary injection may have reduced the number of infused ADRCs. Therefore, data for this patient were therefore excluded from all feasibility outcome analyses.

SAFETY RESULTS

Bleeding events

In the first four patients enrolled, there were 2 TIMI grade major bleeding events, which were considered SAEs related to the liposuction procedure. In one of these patients, glycoprotein IIb/IIIa inhibition was initiated in the ambulance. In the other patient, a similar but less severe hematoma occurred at the liposuction site, probably associated with a prolonged aPTT (>240 seconds). After the protocol amendments related to the anticoagulation status of these post-PCI patients, the only

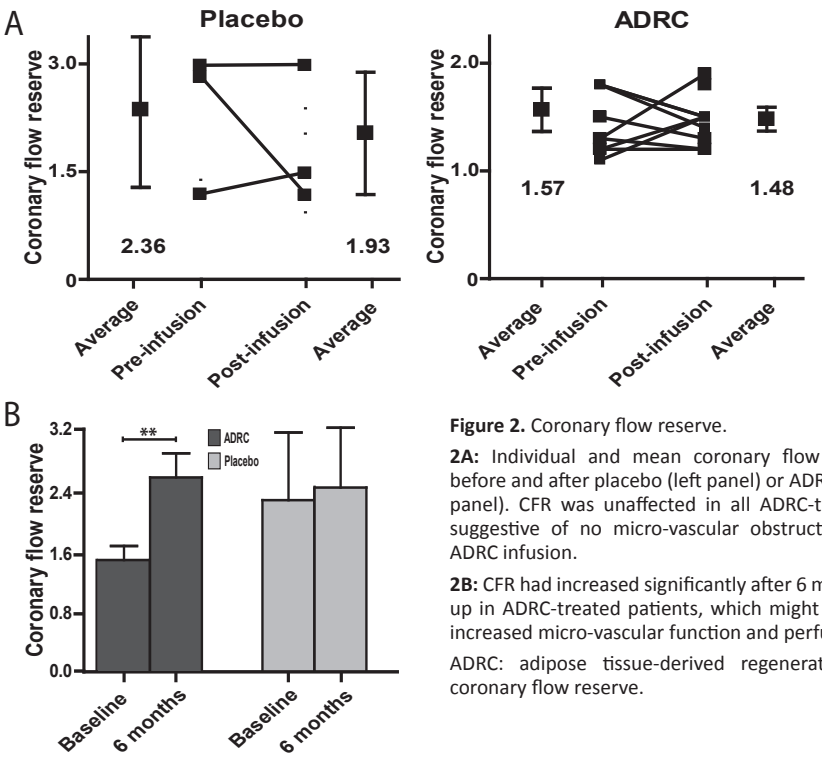

Figure 2. Coronary flow reserve.

2A: Individual and mean coronary flow reserve values before and after placebo (left panel) or ADRC infusion (right panel). CFR was unaffected in all ADRC-treated patients, suggestive of no micro-vascular obstruction due to the ADRC infusion.

2B: CFR had increased significantly after 6 months of follow-up in ADRC-treated patients, which might be indicative of increased micro-vascular function and perfusion.

ADRC: adipose tissue-derived regenerative cells; CFR: coronary flow reserve.

bleeding events that were reported were regarded as TIMI grade minor or minimal, and required no intervention.

Intracoronary infusion, TIMI flow and coronary flow reserve

Intracoronary infusion of ADRCs was successful and well tolerated in all patients, and did not result in any coronary flow impediment, as assessed by TIMI flow rate or quantified by coronary flow reserve (CFR). In both ADRC-treated patients, as well as controls, CFR remained unchanged before and after cell infusion (Figure 2A).

Interestingly, at six-month follow-up, the CFR had increased significantly by 60% in ADRC-treated patients from 1.57 ± 0.39 to 2.51 ± 0.74 (p= 0.031), as opposed to a non-significant increase of only 4% (from 2.37 ± 0.85 to 2.47 ± 0.72; p=0.93; Figure 2B) in the placebo group.

Table 3. Summary of MACCE*, Serious Adverse Events and Arrhytmias.

	Placebo (n=4)	ADRC† (n=9)	p-value
MACCE*			
Patients with at least 1 MACCE event	1 (25%)	2 (22%)	NS
Target lesion revascularization	1 (25%)	2 (22%)	NS
Serious Adverse Events (SAE)			
Patients with at least 1 SAE	2/4 (50%)‡	5/9 (56%)‡	NS
Cardiac	***1/4 (25%)***	***3/9 (33%)***	NS
Unstable angina pectoris	1 (25%)	1 (11%)	NS
Atrial fibrillation	0	1 (11%)	NS
Coronary artery stenosis	0	1 (11%)	NS
Non cardiac	***2 (50%)***	***2 (22%)***	NS
Non-cardiac chest pain	1 (25%)	0	NS
Neoplasms (pituitary tumour)	1 (25%)	0	NS
Peripheral artery disease	0	1 (11%)	NS
Pain in extremity	0	1 (11%)	NS
Post-procedural	***1 (25%)***	***1 (11%)***	NS
Bleeding post-liposuction	1 (25%)	1 (11%)	NS
Ventricular arrhythmia			
At least 1 NSVT§	2/4 (50%)	4/9 (44%)	NS
NSVT episodes/patient	2.8 ± 1.0	0.6 ± 0.3	0.048

Values are presented as n. *MACCE: major adverse cardiac or cerebral event; †ADRC: adipose tissue-derived regenerative cells; ‡some patients experienced more than one SAE; §NSVT: non-sustained ventricular tachycardia

Incidence of MACCE / SAE up to 36 months clinical follow-up

There were 3 MACCE events divided over two treated patients (2/9; 22%), and 1 placebo patient (1/4; 25%; p= NS). All MACCE were target vessel revascularizations (TVR). One patient in the treatment group presented with unstable angina pectoris 2 months following the index procedure, due to a thrombus proximal to the stent in the culprit vessel. In this patient, an anti-phospholipids syndrome was diagnosed as the probable cause of the repeated arterial thrombotic events. The other patient had an asymptomatic *de novo* lesion proximal to the stent in the target vessel at 6 months routine angiographic follow-up. Although asymptomatic, the fractional flow reserve was 0.67, and the patient was subsequently treated by direct stenting of the lesion. Also, one patient in the placebo group underwent TVR, after presentation with unstable angina pectoris more than two years following the index event. Ten SAEs were reported in 7 of the 14 patients over the 36 months follow-up – 6 events in 5 ADRC-treated patients, and 4 events in 4 of the placebo-treated patients. Two of these 10 SAEs were the TIMI grade major bleeding events associated with the liposuction procedure in 2 patients as described above. Six of the SAEs were considered to be related to the underlying disease. One SAE, a surgical excision of a benign pituitary tumor, was considered to be unrelated to either the treatment, or the underlying disease (Table 3). There were no deaths, strokes or repeat AMIs through the 36 month follow-up. There were no significant differences in MACCE-rate or SAEs between both groups.

BNP levels

At 6 months and 18 months follow-up, there was a continued decrease in pro-BNP. In the ADRC group, pro-BNP values decreased from 235.0 ± 240.9 pmol/L at baseline to 108.6 ± 158.2 pmol/L at 6 months (-32,3; p = 0.19) and to 39.4 ± 45.4 pmol/L at 18 months (- 195,6; p = 0.03). In the placebo group, pro-BNP values decreased from 224.8 ± 232.3 pmol/L, to 115.2 ± 175.4 at 6 months (-109,60; p = 0.30), and to 39.8 ± 43.0 pmol/L at 18 months (-185,0; p = 0.18). There was no significant difference between the groups at the different time points.

Holter monitoring for arrhythmia

No ventricular arrhythmias were reported in either treatment group between 1 and 4 days post-treatment by the continuous 72-hour telemetry and Holter monitoring. Following discharge, ambulatory 24 hour holter recordings were performed weekly for the first four weeks after the index

Table 4. Ventricular arrhythmia and ventricular ectopy.

	Placebo (n=4)	**ADRCs (n=9)**	**p-value**
Ventricular tachycardia (VT)			
At least 1 episode of VT	2/4 (50%)	4/9 (44%)	
Total episodes	11	7	0.44
Episodes/patient	2.8 ± 1.0	0.6 ± 0.3	**0.048**
PVC/patient/24 hour	616 ± 151	48.2 ± 10	**0.014**

Values are presented as n.; P-values are determined using two-tailed Chi-square tests when applicable

procedure, and subsequently every month afterwards until 6 months of follow-up, and also at 12, 18, 24 and 36 months of follow up.

ADRC therapy was associated with a significant reduction of ventricular ectopy and the number of ventricular arrhythmias. The number of premature ventricular contractions (PVC) per 24 hour holter recording was markedly reduced in ADRC-treated patients as compared to control patients (48 ± 10 PVC vs. 616 ± 151 PVC, p= 0.014). Moreover, a total of 7 episodes of non-sustained ventricular tachycardia (NSVT) were detected in 4 out of 9 ADRC-treated patients (44%), as compared to 11 episodes in 2 out of 4 placebo patients (50%). On average, each patient in the control group experienced 2.8 episodes of NSVT as opposed to only 0.6 episode of NSVT per ADRC-treated patient (p= 0.048). Holter data are summarized in table 4.

Efficacy results

Left ventricular Infarct size

Treatment with ADRCs resulted in a substantial and significant reduction of the percentage of LV infarcted at 6 months and 18 months follow-up, as opposed to a non-significant change in placebo controls. In the ADRC-treated patients, the percentage of LV infarcted decreased by -51.3% from baseline to 6 months (from 31.6 ± 15.1% to 15.4 ± 7.4%; p = 0.002), and by -38.2% from baseline to 18 months (19.5 ± 10.7%; p = 0.01). In the placebo-treated patients, the percentage of LV infarcted was -25.1% at 6 months (from 24.7 ± 18.3% to 18.5 ± 14.0%; p = 0.11), and -18.2% at 18 months (20.2 ± 15.9; p = 0.16; figure 3A).

Myocardial Perfusion

In the placebo group, the visual rest score (VRS) first deteriorated by +11.6% (15.0 ± 9.9 to 16.8 ± 8.7, P=0.73) from baseline to six months, but eventually remained unchanged at the 18 month time point (14.8 ± 10.2; p= 0.54). In contrast, treatment with ADRC resulted in a significant -36% reduction of the perfusion defect from 16.9 ± 5.9 to 10.9 ± 6.7 after six months (p=0.004), which sustained after 18 months (-33% to 11.3 ± 5.9; p= 0.007; Figure 3D).

The total summed score (TSS) showed similar results, with a minimal improvement from 11.3 ± 9.3 at baseline to 10.5 ± 5.7 after 6 months (p= 0.55), and to 10.8 ± 7.2 after 18 months (p= 0.30) in placebo patients. ADRC-treated patients however, exhibited a 35% improvement from 13.8 ± 4.9 to 9.0 ± 5.2 after 6 months (p= 0.004) and a sustained 36% improvement to 8.9 ± 3.6 (p< 0.001; **Figure 3E**) after 18 months. This represents a 3-fold reduction of the rest perfusion defect of the left ventricle in cell-treated patients at 18 months follow-up, as compared to control patients (p=0.238), suggestive of improved myocardial perfusion with sustained myocardial viability.

Left ventricular volumes and ejection faction

The parameters of left ventricular volumes and global LVEF by 2D-TTE and CMR are summarized in Table 5. When assessed by CMR, LV volumes increased equally in both groups. EDV in placebo patients

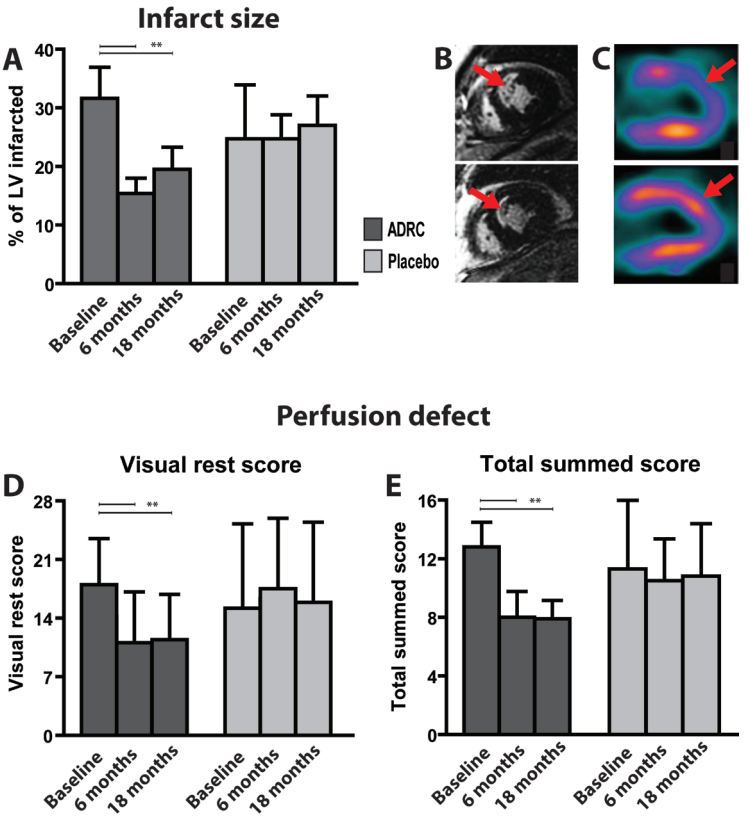

Figure 3. Infarct size and perfusion defect.

3A: The percentage of LV infarcted decreased by 50% after 6 months of follow-up. This effect sustained even after 18 months, whereas there was no change in the control group. **3B.** Representative example of the change in infarct size in ADRC-treated patients as assessed by DE-CMR. The top panel shows an infarct (red arrow) at baseline, the bottom panel shows the infarct in the same patient after 6 months. **3C:** Representative example of enhanced perfusion as seen in ADRC-treated patients as assessed by MIBI-SPECT imaging. The top panel clearly shows reduced perfusion in the anterior wall (red arrow) at baseline, which improved after 6 months (bottom panel). **3D:** Quantification of the enhanced perfusion by the visual rest score (VRS) and **3E:** total summed score (TSS). Both VRS and TSS improved significantly in ADRC-treated patients after 6 and 18 months, as opposed to no change in placebo patients.

ADRC: adipose tissue-derived regenerative cells; DE-CMR: delayed enhancement cardiac magnetic resonance imaging; MIBI-SPECT: sestamibi single photon-emission computed tomography. ** P< 0.01

increased by 18% from 171.5 ± 12.8 mL to 202 ± 60.5 mL (p= 0.76) and by 17% in ADRC-treated patients (from 166.5 ± 41.8 mL to 195.6 ± 45.1 mL; p= 0.01), whereas ESV increased from 88.5 ± 15.4 mL to 104.2 ± 55.9 mL (p= 0.65) and from 80.1 ± 33.0 mL to 100.0 ± 49.0 mL respectively (p= 0.021). LVEF slightly increased in placebo patients, as compared to a small non-significant decline in the treatment group (p= 0.47). Importantly, the differences between groups were not statistically significant.

When assessed by echocardiography, LV volumes progressively increased over the 18 months follow

up period in both groups, but more in the placebo than in the ADRC-treated group. More specifically, LV end-diastolic volume (EDV) increased by 43% in placebo controls (from 107.5 ± 19.3 mL at baseline to 153.7 ± 61.0 mL after 18 months; p= 0.22), whereas in the treatment group, EDV increased by 25% from 116.8 ± 25.3 mL to 146.4 ± 20.4 mL (p= 0.013). End-systolic volume (ESV) in placebo patients increased by 35% in placebo patients from 60.4 ± 7.6 mL to 81.5 ± 45.4 mL (p= 0.37), and by 15% in ADRC-treated patients (from 63.0 ± 17.9 to 72.4 ± 23.6 mL; p 0.20). LVEF significantly improved in ADRC-treated patients from baseline to 18 months follow up (46.1 ± 7.4% to 51.4 ± 9.6%; p= 0.014) as opposed to a non-significant change in placebo patients (43.5 ± 5.8% to 48.8 ± 8.2%; p= 0.25). However, the difference between groups was not statistically significant (p= 0.09).

DISCUSSION

The APOLLO trial is the first-in-man experience of intracoronary infusion of adipose tissue-derived regenerative cells (ADRCs) in the treatment of patients with ST-elevation AMI. The most important findings over 36 month follow-up are that the liposuction can be performed safely briefly following an AMI, whereas no MACCE or serious adverse events were related to the ADRC therapy. Also, ADRC therapy had no apparent pro-arrhythmogenic effects, but rather appeared to reduce the occurrence of ventricular arrhythmias and ectopy. Although exploratory, ADRC infusion seemed to result in a sustained improvement of the perfusion defect and a reduction of myocardial scar formation, whereas coronary flow reserve in the culprit vessel was significantly enhanced.

In the APOLLO study, limited liposuction in the acute phase of a myocardial infarction appeared to be well tolerated. Although two patients experienced significant bleeding, this was likely to be associated with anti-coagulation therapy in these particular cases. After modification of the protocol to monitor for normalization of the aPTT prior to the liposuction procedure, and to exclude patients with prior treatment with glycoprotein-IIbIIIa-inhibitors, the remaining patients were uneventful. However, post-liposuction bleeding may still represent a concern in the following studies and needs to be carefully monitored.

During cell infusion, coronary flow was monitored carefully by regular assessment of TIMI flow and by CFR analysis. Although intracoronary infusion of BM-derived and cell culture-expanded MSC has raised concerns in pre-clinical studies with respect to micro-vascular obstruction and myocardial infarction [21,22], the intracoronary infusion of freshly isolated ADRCs did not result in any detectable effect on coronary flow. The observed lack of vascular obstruction in the APOLLO patients corroborates the concept that direct isolation and infusion of ADRCs may circumvent the issues of microvascular plugging, although in the APOLLO trial a relatively low dose was applied.

No significant difference was observed between the occurrence of MACCE, SAE or AE in the ADRC-treated and placebo control group. Importantly, the independent DSMB considered no causal relationship between the MACCE and SAE events and ADRC therapy. Furthermore, ADRC therapy did not have any pro-arrhythmogenic effect. On the contrary, ADRC therapy was associated with a reduction of ventricular arrhythmias and ventricular ectopy. This is in line with other clinical and pre-

Table 5. Left ventricular ejection fraction and volumes

Echocardiography								
Treatment		**Baseline**	**2-4 days**	**6 months**	**p-value**	**18 months**	**p-value**	**p-value***
ADRC-treated	LVEF (%)	46.1 ± 7.4	52.2 ± 10.1	50.7 ± 11.3	0.13	51.4 ± 9.6	**0.014**	0.23
	LVEDV (mL)	117 ± 25	120 ± 25	133 ± 39	0.16	146 ± 20	**0.013**	0.76
	LVESV (mL)	63.0 ± 17.9	58.3 ± 22.3	69.4 ± 35.3	0.42	72.4 ± 23.6	0.20	0.32
Placebo	LVEF (%)	43.5 ± 5.8	51.2 ± 8.5	49.5 ± 13.7	0.36	48.8 ± 8.2	0.25	
	LVEDV (mL)	108 ± 19	119 ± 14	133 ± 34	0.10	154 ± 61	0.22	
	LVESV (mL)	60.4 ± 7.6	57.2 ± 6.3	76 ± 25.1	0.46	81.5 ± 45.4	0.37	
CMR								
Treatment		**Baseline**	**2-4 days**	**6 months**	**p-value**	**18 months**	**p-value**	
ADRC-treated	LVEF (%)		52.4 ± 13.6	57.0 ± 10.8	0.18	50.7 ± 15.7	0.47	0.87
	LVEDV (mL)		166 ± 42	193 ± 43	**0.010**	196 ± 45	**0.013**	0.76
	LVESV (mL)		80.1 ± 33	83.2 ± 30.8	**0.028**	100.2 ± 49	**0.021**	0.72
Placebo	LVEF (%)		48.0 ± 10.8	50.3 ± 5.6	0.69	50.1 ± 13.2	0.76	
	LVEDV (mL)		172 ± 13	201 ± 42	0.31	203 ± 61	0.43	
	LVESV (mL)		88.5 ± 15.4	100.9 ± 30.6	0.59	104.2 ± 55.9	0.65	

clinical observations in cell therapy studies, and might be correlated with the reduction of scar size and improved myocardial perfusion in ADRC-treated subjects.[23-25]

In the APOLLO trial, ADRC therapy was initiated within 24 hours following the primary PCI. This is in contrast to many previous clinical studies in which cell therapy was initiated in the sub-acute phase of the AMI.[26] However, one of the presumed working mechanisms underlying the beneficial effect of cell therapy is the prevention of cardiomyocyte loss by paracrine release of anti-apoptotic, pro-survival and immunomodulatory factors.[7,8,27] In line with this concept, cell therapy should be initiated as early as possible, when cardiomyocytes are at the highest risk of ischemia/reperfusion-induced apoptosis or necrosis, and the inflammatory response in the infarct area is most pronounced. The feasibility of ADRC therapy directly following reperfusion was demonstrated in various large animal models of AMI.[12,28] Although only 8 patients were analyzed with CMR in the treatment group, the significant and sustained reduction in infarct size may indeed suggest cardiomyocyte salvage evoked by the infused ADRC. A future phase III study will have to confirm these promising data in a larger patient cohort.

In addition to paracrine anti-apoptotic and immunomodulatory factors, ADRCs are known to secrete multiple pro-angiogenic factors [29-31]. As a result, ADRC transplantation in pre-clinical AMI studies consistently resulted in increased capillary density and improved perfusion in the infarct border zone, resulting in preserved cardiac function.[12,28,30] In our clinical study, significant and sustained improvement in both coronary flow reserve (+60%) and perfusion defect (-36%) were found at 6, and 18 months follow-up, as opposed to no change in the control patient group. Interestingly, in the landmark REPAIR-AMI trial, CFR was also profoundly enhanced [32], which seems to confirm the pro-angiogenic and reparative potential of infused regenerative cells in AMI patients. The observations in the current study indicate that ADRCs may promote (neo-)angiogenesis in the peri-infarct region resulting in improved myocardial perfusion, thereby possibly limiting ischemic damage and ultimately improving function.

In conclusion, ADRC therapy appears to be safe and feasible in the acute phase of an AMI. Although in these small patient numbers no statistically significant effect on global LV function was found, significant improvements through 18 months follow up in infarct size, perfusion defect, coronary flow reserve, and arrhythmia suggest a possible beneficial effect. The obvious major limitation of this phase I/IIa trial is the small sample size, although the study has been performed in a randomized, double-blind fashion with analysis of imaging and holter end-points by independent core laboratories.

Importantly, the possible beneficial effects are consistent with the findings in pre-clinical AMI studies, and concordant with the presumed pro-angiogenic, anti-apoptotic, and immunomodulatory working mechanism of ADRC therapy. ADRCs may thus represent an attractive adjunctive therapy to primary intervention of patients with a large AMI. However, further randomized, controlled trials are needed to confirm these promising results. The ADVANCE trial (ClinicalTrials.gov identifier: NCT01216995) is a prospective, randomized, double-blind, placebo-controlled, phase IIb/III clinical trial that will enroll up to 375 patients with STEMI in up to 35 centers in Europe. The primary endpoint of ADVANCE will be the reduction in infarct size at 6 months by DE-CMR expressed as a percentage of left ventricle infarcted. Completion of ADVANCE is expected in 2014.

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PART 3

Mesenchymal precursor cells

CHAPTER 7

Intracoronary Infusion of Allogeneic Mesenchymal Precursor Cells Directly Following Experimental Acute Myocardial Infarction Reduces Infarct Size, Abrogates Adverse Remodeling and Improves Cardiac Function

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ABSTRACT

Rationale Mesenchymal precursor cells (MPC) are a specific stro3+ sub population of mesenchymal stem cells (MSC) isolated from bone marrow. MPC exert extensive cardioprotective effects, and are considered to be immune-privileged.

Objective This study assessed the safety, feasibility and efficacy of intracoronary delivery of allogeneic MPC directly following acute myocardial infarction (AMI) in sheep.

Methods and results Initially, intracoronary delivery conditions were optimized in 20 sheep. These conditions were applied in a randomized study of 68 sheep with an anterior AMI. Coronary flow was monitored during MPC infusion and cardiac function was assessed using invasive hemodynamics and echocardiography at baseline and during 8 week follow up.

Coronary flow remained within TIMI III definitions in all sheep during MPC infusion. Global LVEF as measured by PV-loop analysis deteriorated in controls to 40.7±2.6% after eight weeks. In contrast, MPC treatment improved cardiac function to 52.8±0.7%. Echocardiography revealed significant improvement of both global and regional cardiac function. Infarct size decreased by 40% in treated sheep, whereas infarct and border zone thickness were enhanced. LV adverse remodeling was abrogated by MPC therapy, resulting in a marked reduction of LV volumes. Blood vessel density increased by >50% in the infarct and border areas. Compensatory cardiomyocyte hypertrophy was reduced in border and remote segments, accompanied by reduced collagen deposition and apoptosis. No micro-infarctions in remote myocardial segments or histological abnormalities in unrelated organs were found.

Conclusion Intracoronary infusion of allogeneic MPC is safe, feasible and markedly effective in a large animal model of AMI.

INTRODUCTION

Post myocardial infarction (AMI) left ventricular (LV) remodeling can lead to the clinical syndrome of heart failure, which is an increasing public health issue in the Western world. Although interventional and pharmacological therapy has improved over the past decades, the mortality and morbidity of heart failure is still considerable.[1, 2] Regenerative cell therapy to prevent adverse remodeling is one of the potential adjunctive therapies that has been under extensive investigation over the past few years, with promising results in phase I and II clinical trials.[3]

Although promising, the effects of unfractionated bone marrow (BM) mononuclear cells in these trials have been modest, and it has been suggested that mesenchymal stem cells (MSC) or their sub populations might be more effective.[4-6] Mesenchymal precursor cells (MPC) comprise a Stro-3 immune-selected, immature sub fraction of BM-derived MSC.[7] These MPC are multipotent cells with extensive proliferative potential, and secrete numerous anti-apoptotic, angiogenic factors, and growth factors. It was found that MPC display greater cardioprotective effects than conventional MSC that are selected by plastic adherence alone, which may be evoked by their potent paracrine activity, as well as more extensive multilineage differentiation potential.[8, 9] Interestingly, MPC are immune-privileged and can be transplanted to unrelated recipients, thereby creating the possibility of an allogeneic, “off-the-shelf” cell product, readily available during the acute phase of an AMI. Intramyocardial injection of MPC has been shown to improve cardiac function in small and large animal models of AMI.[9-12]

In contrast to intramyocardial delivery, intracoronary stem cell infusion is a simple, quick, cost-effective and reproducible delivery technique.[13, 14] It does not require specific infrastructure and is available in all interventional coronary cathlabs, while intracoronary infusion of an ”off-the-shelf” allogeneic stem cell product can be applied directly following revascularization of the AMI. More importantly, intracoronary infusion omits the risk of myocardial perforation and ventricular arrhythmia that has been associated with intramyocardial injection in infarcted tissue. However, intracoronary infusion of unselected and culture-expanded MSC has previously been associated with impeded coronary flow and micro infarctions in remote myocardial segments in large animal experiments.[15-18]

The primary aim of the current large animal study was to assess the feasibility of intracoronary MPC infusion, and to determine the optimal infusion conditions, while carefully monitoring coronary flow, arrhythmias, and other possible cell therapy related adverse effects. In addition, we investigated the potential efficacy of three different doses of MPC using functional (pressure-volume (PV-loop) analysis, echocardiography) and morphological (morphometry, histology) indices over an 8 week follow-up period.

MATERIALS AND METHODS

Experimental design

A total of 88 adult sheep were used in this study. All procedures were approved by the institutional animal welfare committee (University of Utrecht, Utrecht, the Netherlands). The study was divided into three distinct phases. In the first phase, the maximum tolerated dose and optimal MPC infusion rate were determined in non-infarcted sheep. In the second phase, we assessed the maximum tolerated MPC dose and optimal infusion rate in animals subjected to an anterior wall AMI. The targeting potential, and cell retention in the infarcted myocardial segment, as well as shedding of MPC to remote myocardial segments and organs, were analyzed in a cell tracking sub study. Finally, in the 3rd phase, the safety and efficacy of intracoronary infusion of three incremental doses of MPC directly following AMI over an 8 week follow-up period was assessed using optimized infusion conditions (see figure 1 for the study flow chart).

Mesenchymal Precursor Cells

MPC used in this study were ovine Stro-3 positive bone-marrow derived cells as previously described.[7, 19] The cells were frozen in cryoprotectant containing 7.5% DMSO and stored at -180ºC in vials at a final concentration of 25 million cells per vial. Before cell infusion, MPC were rapidly thawed, filtered through a 40 micron cell strainer, and suspended in 100 mL of lactated ringers' solution (LR) at a final concentration of 0.5 Mill MPC/mL (see online supplement for cell size measurements).

Phase 1 – Intracoronary MPC infusion in non-infarcted myocardium

A total of 12 sheep (45.2 ± 1.5 kg) were used in phase 1. To assess the optimal infusion rate and maximum tolerated dose, naïve sheep received an intracoronary infusion of incremental doses of MPC (25, 37.5 and 50 million) using an infusion rate of 1.25 or 2.5 million MPC/min (Figure 1A).

A Twin Pass® micro-catheter (Vascular Solutions, Minneapolis, USA) was placed in the proximal LAD and MPC were infused using an infusion pump (Alaris, San Diego, USA). Coronary flow was assessed regularly and troponin I (TnI) was determined at baseline, and 6 hours post cell injection (AccuTnI, Beckman Coulter, Brea, USA). After cell infusion, all animals received a subcutaneously implanted REVEAL DX® event recorder (Medtronic, Minneapolis, USA) to continuously monitor for potential arrhythmias. Two days following infusion, the animals were sacrificed and the heart, lung, liver, kidney and spleen examined by independent pathologists.

Phase 2 – Intracoronary MPC infusion and bio-distribution following AMI

To assess the optimal infusion rate and maximum tolerated dose in AMI, intracoronary MPC infusion was performed in an anterior AMI model in 8 sheep (62.8 ± 1.4 kg). Anterior wall AMI was induced by balloon inflation (Voyager Rx 3.0-3.5x12 mm, Abbott, Illinois, USA) in the mid LAD for 90 minutes. After 15 minutes of reperfusion, a Twin Pass® delivery catheter was positioned in the LAD at the location of prior balloon inflation. Subsequently, 50 million MPC were infused at a rate of 1 million MPC/

min (n=3) or 0.5 million MPC/min (n=3). Bio-distribution and myocardial retention was quantified using Indium^{111} labelling in two separate animals (see online supplement). The optimized and safe intracoronary infusion conditions that were found in phase 1 and 2 were subsequently applied in phase 3 of this study.

Phase 3 – Long-term safety effects and dose finding of intracoronary MPC infusion directly following AMI Induction of myocardial infarction and infusion of MPC

A total of 68 sheep (60.8 ± 1.7 kg) were used in phase 3 of the study (figure 1C). An anterior myocardial infarction was induced by LAD occlusion as described above. After reperfusion, the sheep were randomized by a blinded draw to receive an intracoronary infusion of 12.5, 25, or 37.5 million MPC or LR (control). The cells were infused at an infusion rate of 0.5 million MPC/min. After cell infusion, coronary flow was assessed and a subcutaneous event recorder was implanted. Also, blood was sampled for TnI measurement before AMI and 6 hours post cell or placebo injection.

Pressure–Volume loop analysis

In all animals, baseline PV-loop recordings were acquired directly following MPC or placebo infusion and at 8 weeks follow-up. Also, in a random subset of animals (n=12), pre-AMI PV-loop recordings were performed to obtain reference values of PV-loop parameters of non-infarcted sheep hearts (supplemental online table II). Off-line data analysis was performed by an investigator blinded for the treatment allocation of the individual animals (described in detail in the online supplement).

Echocardiography

In all animals, a transthoracic echocardiogram (TTE) was performed at baseline and directly following the AMI, but also at four and eight weeks follow up. LV volumes, LVEF, regional fractional area change (FAC) and regional systolic wall thickening were analysed off-line by an operator blinded for the treatment allocation of the individual sheep (see online supplement for a detailed description).

Necropsy and pathohistology (long-term safety)

At 8 weeks follow up, the animals were euthanized and routine necropsy was performed to screen for any gross anatomical abnormalities in lung, kidney, spleen, liver and gut, whereas biopsies of these organs were collected for further histological analysis. The heart was excised and prepared as described before, and stained using TTC (supplemental online figure I). Subsequently, the slices were carefully screened for micro-infarctions in remote myocardial segments. Biopsies of the infarct area and infarct border zone were randomly taken and processed for further histological analysis.

Histology samples of the liver, lung, spleen, and kidney were analyzed by an independent pathology core-lab (Druquest International, Leeds, USA) to screen for shedding of MPC and any remote adverse effects. A section of infarct and border zone tissue of each animal was analyzed by an independent and blinded pathologist, specialized in cardiac pathology (Erasmus University Medical Center, Rotterdam, the Netherlands) to screen for potential local adverse effects of MPC infusion.

Morphometry and histology parameters (efficacy)

Photographs of TTC-stained slices were taken. A blinded technician calculated the percentage of total

LV infarcted, and measured infarct and border zone thickness using automated image analysis software.

Collagen content, myocardial salvage index, cardiomyocyte size and cardiomyocyte density

Collagen content and cardiomyocyte size, and cardiomyocyte density were determined using Gomorri trichrome staining.

Blood vessel density

Blood vessel density was determined in the border zone, remote myocardial segments, and in the infarct area. Capillary density in the border zone and remote area was assessed by isolectin-B_4 staining. Arteriolar density in the infarct area was determined using smooth muscle actin staining.

Cardiomyocyte proliferation, apoptosis and cardiac stem cells

The amount of proliferating cardiomyocytes was quantified using Ki-67 staining, whereas the amount of apoptotic cardiomyocytes was assessed using a TUNEL assay. Resident cardiac stem cells were detected by cKit staining.

Statistical Analysis

Efficacy data are depicted as placebo (n=10) versus all MPC-treated (n=20) animals, unless otherwise stated. Continuous data are presented as mean ± standard error of the means. Comparisons of means (morphometry and histology) between groups were performed using a one-way ANOVA with Bonferroni correction for multiple comparisons when applicable. Differences of PV-loop and echocardiography derived parameters between treated animals and controls were analyzed by two-way ANOVA with repeated measures. The MPC-treated group was considered as a homogenous cohort, since ANOVA demonstrated no significant difference in relation to the MPC dose applied. A p-value of £ 0.05 was considered statistically significant. The final data set and statistical analysis were audited and approved by Medical Device Consultants, Inc (MDCI, Reston, Virginia, USA).

RESULTS

Phase 1 – MPC infusion in healthy animals

Coronary flow remained within TIMI 3 range in all animals. However, an infusion rate of 2.5 million MPC/min or infusion of 50 million cells resulted in an increase of TnI levels 6 hours following infusion, and micro infarcts in the LAD territory at 2 days follow-up. In contrast, an infusion rate of 1.25 million MPC/min permitted infusion of doses up to 37.5 million MPC without inducing myocardial necrosis (figure 2A+B). None of the animals experienced ventricular arrhythmias during the two-day follow-up. Analysis of H&E stained sections of all major organs rendered no differences between the sheep that received intracoronary infusion of MPC and healthy controls. More specifically, no shedding of the allogeneic MPC, or any MPC-related acute adverse effects were detected. No acute foreign body or anti-allogeneic response, defined as extensive or eosinophilic infiltrates, were found in any of the myocardial tissue specimens.

Phase 2- MPC infusion in infarcted myocardium

Infusion of MPC in the culprit artery directly following AMI at a rate of 1 million MPC/min resulted in sluggish flow after infusion of approximately 25 million MPC (n=3, figure 2C). After 25 million MPC, the coronary flow rapidly declined to TIMI grade 1/0 and flow velocities below 10 cm/sec. When applying a reduced infusion rate of 0.5 million MPC/min., sluggish coronary flow was only observed when the absolute dose exceeded 40 million MPC. As a result of these findings, an infusion rate of 0.5 million MPC/min and a maximal dose of 37.5 million MPC were adopted in phase 3 of this study.

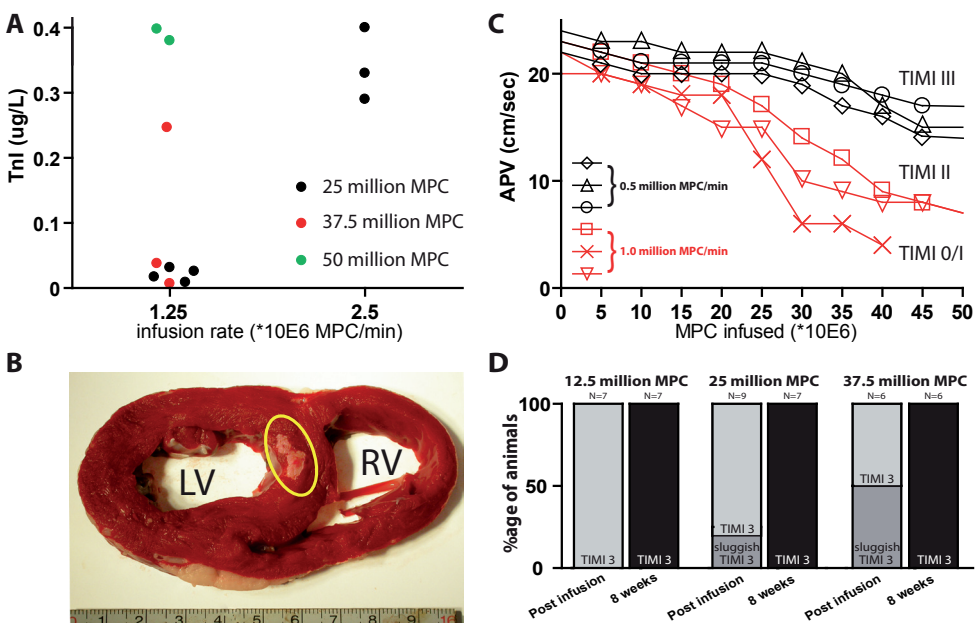


Figure 2. Effects of intracoronary infusion of MPC.

2A. TnI release six hours after intracoronary infusion of MPC in non-ischemic myocardium. A high infusion rate (right) resulted in significant TnI release in 3/3 animals, irrespective of the low dose infused. When a low infusion rate was adopted (left), infusion of 25 and 37.5 million MPC seemed safe, whereas infusion of 50 million MPC always evoked substantial myocardial necrosis. **2B.** Example of a septal myocardial infarct two days after infusion of 50 million MPC. **2C.** Effect of two different infusion rates on coronary flow in individual sheep directly after an acute myocardial infarction. A high infusion rate (red lines) results in an earlier and more abrupt flow impediment when compared to a low infusion rate (black lines). Coronary flow is depicted as APV and TIMI flow grade; **2D.** The effect on coronary flow of different doses of MPC, when infused directly following the AMI at 0.5 million MPC/min in phase 3 of this study and depicted by TIMI flow grade. Directly following infusion (grey bars), coronary flow was sluggish, but still within TIMI III definition, in 2/9 (22%) animals in the 25 million MPC group and in 3/6 (50%) in the 37.5 million MPC group. At sacrifice (black bars), coronary flow had always returned to normal. APV: average peak velocity; MPC: mesenchymal precursor cells; TIMI: thrombolysis in myocardial infarction; TnI: troponin I.

Phase 3 – Long-term safety effects and dose finding of intracoronary MPC infusion following AMI

Animal experiments

A total of 68 sheep were subjected to an anterior wall AMI by balloon occlusion in the mid LAD for 90 minutes. Due to ventricular fibrillation refractory to defibrillation, 34 sheep died during infarct induction. The surviving animals were randomized to placebo treatment (n= 12) or treatment with 12.5 (n= 7), 25 (n= 9), or 37.5 (n= 6) million MPC (Figure 1). Two animals in the control group and two animals in the MPC-treated group died during the 8 week follow-up (see below), resulting in 10 analyzable sheep in the control group and 20 in the MPC-treated group (divided in three dose cohorts treated with 12.5 (n=7), 25 (n=7), or 37.5 (n=6) million MPC).

In 6 control animals and 14 MPC-treated animals, serial TnI measurements were available. Both baseline (0.07 ± 0.02 vs. 0.05 ± 0.01; P= 0.89) and post-AMI (272.4 ± 36.6 vs. 297.1 ± 29.2, P= 0.66) measurements did not differ between placebo and cell-treated groups, suggesting a similar degree of injury in both groups.

Coronary flow during and after MPC infusion

MPC infusion was successful in all animals with TIMI grade 3 flow in all dose groups following MPC infusion (Figure 2D). However, infusion of 25 million MPC led to a transient sluggish flow in 2/9 animals (22%), whereas infusion of 37.5 million MPC resulted in sluggish flow in 3/6 (50%) animals. 'Sluggish flow' was defined as a visual difference in the rate of opacification between the culprit artery and reference vessel (circumflex artery), while antegrade flow remained within TIMI grade 3 definitions.[20] At 8 week follow up, coronary flow had normalized in all treated animals.

Death and ventricular arrhythmia analysis

Two sheep in the control group (2/12, 16%), and two sheep in the MPC-treated group (25 million MPC; 2/22, 9%; P= ns) died during the 8 week follow up period (supplemental online table I). Thorough analysis of the implanted event recorders demonstrated that the sheep in the control group died due to ventricular fibrillation within 12 hours after infarct induction. In the animals in the MPC group, fatal ventricular arrhythmia was excluded as the cause of death after analysis of Reveal® DX data. Subsequent necropsy and histo-pathological examination of the heart, lung, spleen, liver, and kidney by an independent pathologist remained inconclusive about the cause of death in these MPC-treated animals. Specifically, no signs of manifest heart failure were found in lungs, liver and spleen.

In addition to the two animals with a lethal arrhythmia in the control group, another control animal experienced a non-sustained ventricular tachycardia of 20 beats, three weeks following the index procedure.

Pressure–Volume loop analysis

All PV-loop derived data can be found in table II of the online supplement.

PV-loop derived LV ejection fraction and volumes

In control sheep, the global LVEF deteriorated from 44.2 ± 1.5% directly following AMI, to 40.7 ± 2.6% at eight weeks. MPC treatment markedly improved cardiac contractile function from 44.7 ± 1.0% to 52.8

± 0.7% (difference between groups: +12.1%; P<0.001; Figure 3B). No clear dose–effect relationship was observed between the different dose groups.

Following AMI, left ventricular (LV) volumes were comparable between all groups. However, MPC-treatment prevented cardiac remodeling at eight weeks follow-up. End-systolic volume (LVESV) in the MPC treatment group was 68.3 ± 1.8 mL as opposed to 102.8 ± 4.0 mL in the control animals (-34%, P<0.001; figure 3C). Likewise, end-diastolic volume (LVEDV) in the treatment group ameliorated by 16% (149.3 ± 4.1 mL vs. 178.0 ± 8.0 mL, P<0.001) as compared to controls (figure 3D). No significant dose–effect relationship was found between the different treatment groups.

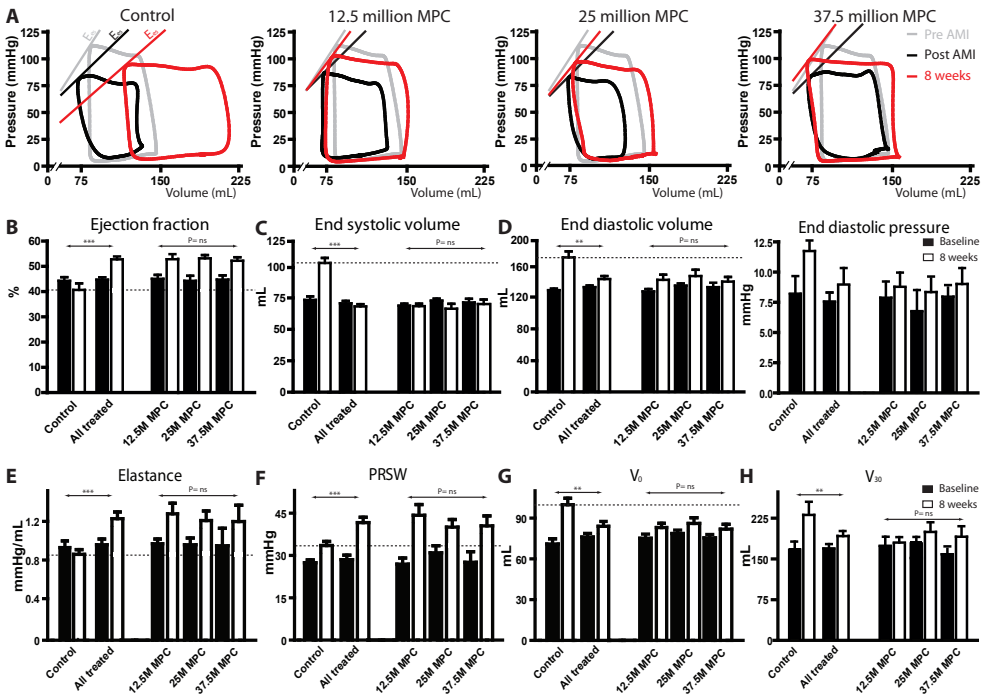


Figure 3. Pressure–volume loop analysis. **3**

A: Typical examples of PV-loops of individual animals in the four evaluated groups. The grey loop represents a normal PV-loop of a non-infarcted sheep heart, whereas the black loop represents the PV-relation briefly after an acute myocardial infarction. After eight weeks (red loop), the PV-loop in the control animal shows a rightward shift, indicating increased volumes, further decline of the end-systolic elastance (E_{es}), and increased end-diastolic pressure (filling pressure). In MPC-treated animals, left ventricular dimensions were preserved, whereas E_{es} returned to near baseline levels. **3B:** Left ventricular (LV) ejection fraction further deteriorated in control animals, but was enhanced by over 30% following MPC therapy. **3C/D:** LV volumes increased in the control group, indicative of LV remodeling. This remodeling process was abrogated by MPC therapy. **3E/F:** Pre- and afterload independent parameters of myocardial contractility, E_{es} and PRSW, were enhanced in MPC-treated sheep, as compared to controls. **3G/H:** V_0 and V_{30} are both points on the end-diastolic pressure–volume relation and represent diastolic function and capacitance.

MPC: mesenchymal precursor cells; ns: non significant ; PRSW: pre-load recruitable stroke-work; *P≤ 0.05 ; **P≤ 0.01 ; ***P≤ 0.001

PV-loop derived, load-independent indices of systolic function

LV contractile function is best reflected in the PV-loop derived pre- and afterload independent indices: end-systolic elastance (E_{es}) and pre-load recruitable stroke-work (PRSW). E_{es} and PRSW markedly improved over eight-week follow-up in MPC-treated animals, as opposed to no improvement in controls. In control animals, the baseline E_{es} was reduced to 0.96 ± 0.07 mmHg.mL and remained stable at 0.89 ± 0.05 mmHg.mL at 8 weeks. However, in the treatment group, E_{es} improved from 0.99 ± 0.06 post AMI to 1.26 ± 0.1 mmHg.mL (P= 0.003; Figure 3A/E). In line with these results, PRSW ameliorated to 41.6 ± 1.9 in MPC-treated animals eight weeks, as opposed to 33.5 ± 1.4 mmHg in controls (P= 0.008; Figure 3F).

PV-loop derived indices of diastolic function

Parameters that reflect LV stiffness, including E_{ed}, dP/dT-, and tau, were not significantly different between control and MPC-treated groups (table 1 of the online supplement). Nevertheless, there are indications that diastolic function had improved in MPC-treated animals. First, when corrected for end-diastolic volume, dP/dT- improved significantly, whereas there is a clear trend towards higher end-diastolic pressures in control animals (P= 0.08). Also, V_0 and V_{30} that reflect LV end-diastolic capacitance significantly improved in MPC-treated sheep. V_0 was reduced from 99.8 ± 4.7 mL in the control group to 83.9 ± 2.1 mL in the MPC-treated animals (P= 0.001, Figure 3G), whereas the V_{30} was enhanced from 241 ± 23.5 mL in the control group to 213 ± 8.2 mL in the MPC-treated group (P= 0.047; Figure 3H).

Echocardiography

All echocardiography measures can be found in table III of the online supplement. Global LV function, as depicted by LVEF, was comparable between groups before and directly following AMI (Figure 4A/B/C). Following AMI, LVEF decreased by 20% in both treatment and placebo groups. In the control group, LVEF gradually deteriorated further from 43.1 ± 1.2% following AMI to 37.3 ± 1.9% at 8 week follow-up. In contrast, LVEF improved by +21% to 47.7 ± 1.2% in sheep treated with MPC (P= 0.001; figure 4A), when compared to placebo animals, thereby corroborating the PV-loop data.

Echocardiography demonstrated that both LVEDV and LVESV were comparable at baseline and directly following AMI. Importantly, it confirmed PV-loop derived volumes at baseline and the improvement at 8 weeks follow up, as shown in figure 3. LVEDV and LVESV deteriorated in both groups over the 8 week follow-up period, but the increase in the placebo group was significantly greater than in the MPC-treated group. LVEDV increased to 182.9 ± 22.5 mL in control animals, and was reduced by 25% to 136.2 ± 12.0 mL in the treatment group (P= 0.037). LVESV improved by almost 40% from 115.8 ± 16.5 mL in placebo-treated animals to 71.3 ± 6.9 mL in MPC-treated animals (P= 0.042).

Regional function improved by MPC therapy, as FAC in the apex was 39% higher in cell-treated animals as compared to controls (41.4 ± 2.7% vs. 29.8 ± 2.0%; P= 0.027; figure 5A), and FAC was 30% higher in the mid-ventricle (46.7 ± 1.7 vs. 35.8 ± 2.6%; P= 0.007; figure 5B). FAC in the basal segments of the heart did not differ significantly between control and treated animals (figure 5C; P= 0.57). No clear dose–effect was found, and all three doses appeared to be equally effective.

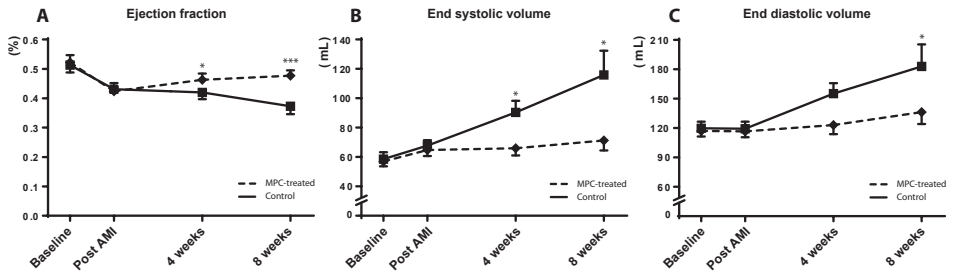


Figure 4. Global LV function and volumes measured by echocardiography. Intracoronary MPC infusion improves global LV function and volumes when compared to controls.

4A. Global LVEF deteriorated equally in treated and placebo animals after infarct induction, but was significantly enhanced by MPC therapy. **4B/C.** LV end systolic and diastolic volume in MPC-treated animals more or less stabilized after the ischemic insult of the infarct, whereas volumes in control animals further deteriorated. These data corroborate PV-loop derived data on cardiac function and volumes. No significant dose–effect was found. (LV) EF: (left ventricular) ejection fraction. * P≤ 0.05; ** P≤ 0.01; *** P≤ 0.001.

Also, regional contractility improved by MPC therapy. Systolic wall thickening was severely impaired in the apical and mid-ventricular anterior and antero-septal wall segments directly post AMI, whereas compensatory hypercontractility was present in a contra-lateral remote myocardial segment (Figure 5). Systolic wall thickening in the antero-septal segment of the apex improved from 0.5 ± 0.6% in control animals to 23.7 ± 4.2% in treated animals (P= 0.003; figure 5D) and in the anterior wall from 2.7 ± 4.3% to 24.9 ± 2.4% (P< 0.001; figure 5E). Both infarcted segments also markedly improved at the mid-ventricular level (anteroseptal segment: co 9.6 ± 5.5% vs. MPC-treated 39.1 ± 1.8%, P< 0.001; anterior wall: co 13.8 ± 3.6% vs MPC-treated 34.7 ± 1.9%; P< 0.001; figure 5F/G). No significant difference between both groups was found in the contralateral myocardial segment (P= 0.32 and 0.22 respectively; figure 5H/I).

Necropsy and histopathology analysis of tissue samples

During autopsy and macroscopic analysis, no signs of gross anatomical malformations, neoplasms or angiomas were detected in the heart, gut, liver, lungs, kidneys and spleen. This was confirmed by histological analysis by independent pathologists. In the TTC-stained slices of the heart, no signs of micro-infarctions in remote myocardial segments were found.

Infarct size and morphometry

The percentage of LV infarcted in control animals measured 18.4 ± 1.5% in placebo controls, and improved by 33% to 12.0 ± 0.7% in MPC-treated animals (figure 6A; P= 0.001). Also, the average infarct wall thickness in the mid ventricle was enhanced by 25% in treated animals, as compared to control animals (6.4 ± 0.2 vs. 8.0 ± 0.3 mm, P< 0.001; figure 6C). In control animals, the average border zone thickness was 8.5 ± 0.48 mm in the mid ventricle, whereas it improved to 10.5 ± 0.5 mm (P= 0.011) in MPC-treated animals respectively (figure 4D).

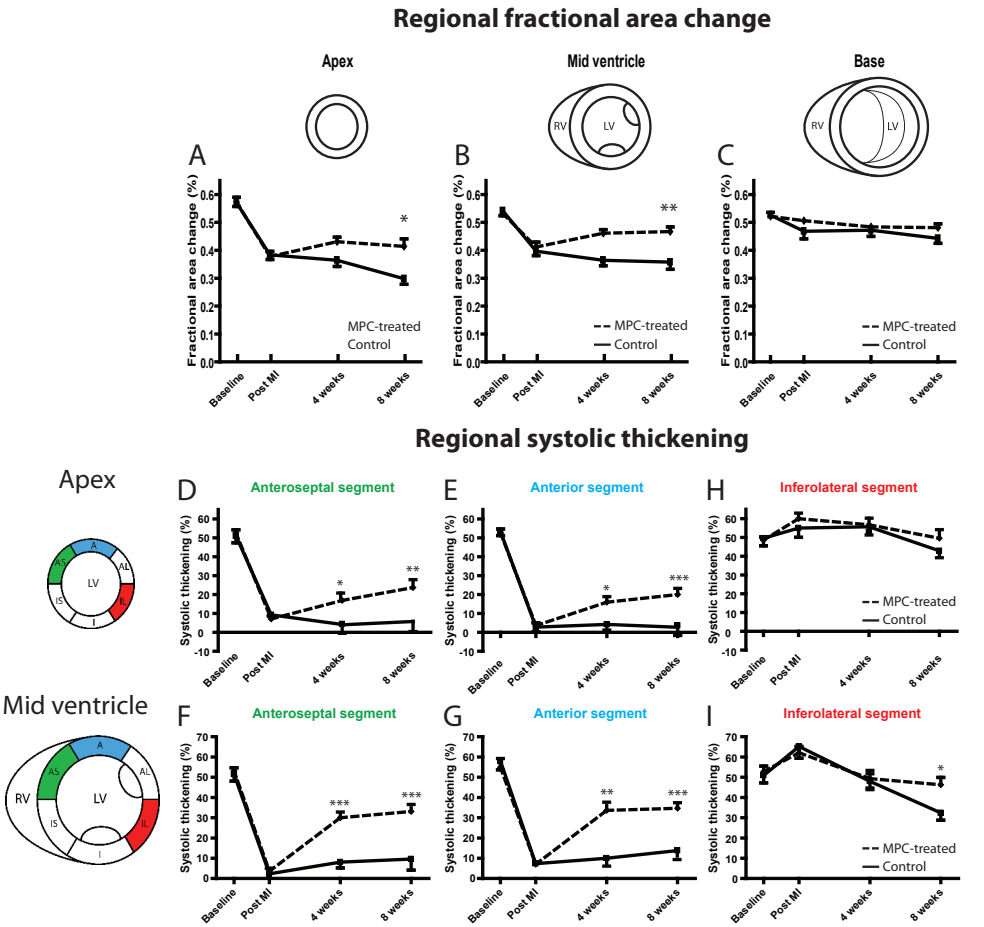


Figure 5. Regional cardiac function as assessed by echocardiography. Intracoronary MPC infusion improves regional function and contractility when compared to controls.

5A-C: regional cardiac function decreased comparably in both groups directly following the AMI, suggesting similar levels of injury. However, in MPC-treated animals, regional FAC was enhanced in the affected apical and mid-ventricular levels after 8 weeks, whereas the basal level did not show an improvement when compared to controls. **5D-G:** Anteroseptal and anterior systolic wall thickening decreased similarly at apical and mid ventricular levels after the AMI. Systolic wall thickening improved in MPC-treated animals as opposed to no improvement in placebo control animals. **5H/I:** Directly after the AMI, compensatory hypercontractility was seen in the contralateral myocardial segment in all animals, whereas it only improved significantly in MPC-treated animals at the mid-ventricular level after 8 weeks. AMI: acute myocardial infarction; MPC: mesenchymal precursor cells. *P≤ 0.05; **P≤ 0.01; ***P≤ 0.001.

Histology

Collagen content

MPC significantly reduced extracellular matrix deposition in all myocardial areas. Collagen content in the border zone decreased from 16.5 ± 2.1% in the control group to 7.4 ± 0.7% (P<0.001; figure 7A) in the treatment group, whereas collagen content in remote myocardial segments decreased from 2.1 ± 0.4% to 1.0 ± 0.2% (P=0.001; figure 7B).

Interestingly, also in the infarct area, the collagen content was significantly reduced in MPC-treated animals as compared to controls (figure 7C). This decrease in collagen content favored the amount of viable myocardium in infarct specimens, suggesting myocardial salvage. This was represented by a marked improvement in the myocardial salvage index from 0.29 ± 0.06 in controls to 1.30 ± 0.20 in MPC-treated sheep (P=0.002; figure 7D). No clear dose–effect relationship was present in the collagen deposition in all segments.

Blood vessel density, cardiomyocyte size and cardiomyocyte density

The number of capillaries in the border zone was enhanced by 58% from 1196 ± 87 capillaries/mm^2 in the control group to 1894 ± 105 in MPC-treated sheep (P<0.001; Figure 8A). Although not statistically significant, there appeared to be an incremental dose–effect relation in capillary density between the dose groups (12.5M: 1704 ± 144; 25M: 1953 ± 232; 37.5M: 2046 ± 144 capillaries/mm^2). The higher capillary density in the border zone resulted in a 35% increase of the capillary-to-cardiomyocyte ratio. In MPC-treated animals, each cardiomyocyte was supported by 1.39 ± 0.15 capillaries on average,

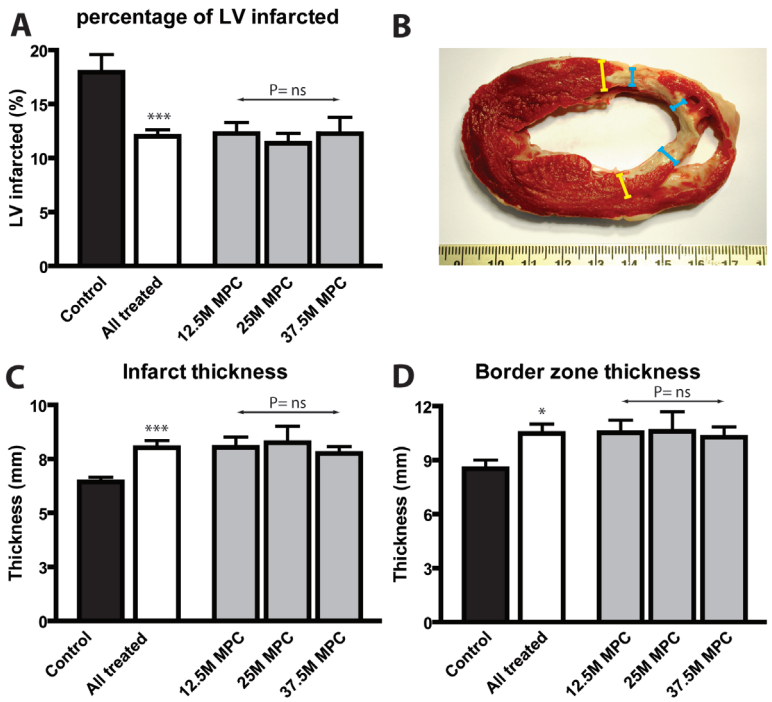


Figure 6. Infarct volume and morphometric analysis.

6A: Infarct size, calculated as the percentage of the total LV infarcted, significantly improved following MPC therapy. **6B:** Infarct thickness was measured in mid-ventricular slices at three sites in the infarct (blue lines) per slice, whereas the thickness of the border zone was assessed at both sides directly adjacent to the infarct (yellow lines). **6C:** Infarct wall thickness was enhanced by MPC therapy as compared to controls. **6D:** Border zone thickness increased in MPC-treated sheep.

LV: left ventricle; M: million; MPC: mesenchymal precursor cells; ns: non significant ; *P≤ 0.05 ; **P≤ 0.01 ; ***P≤ 0.001

whereas this was reduced to only 1.01 ± 0.11 in control animals (Figure 8B, P=0.012). In contrast, no difference between groups was found in the capillary-to-cardiomyocyte ratio in remote myocardial segments (figure 8B: controls: 1.81 ± 0.12 capillaries/cardiomyocyte vs. MPC-treated: 1.73 ± 0.19 capillaries/cardiomyocyte; P=0.856).

The arteriolar density in the infarct area was remarkably enhanced by MPC therapy. In the treatment group arteriolar density doubled, as compared to the control group (49.2 ± 4.1 vs. 21.7 ± 4.0 arterioles/mm^2, P<0.001; Figure 8C).

Post-AMI compensatory cardiomyocyte hypertrophy in border zone and remote areas was more pronounced in the control group as compared to the MPC-treated group. In the control group, cardiomyocyte size was 536 ± 42 µm^2 in the border zone, which was reduced by MPC treatment to 329 ± 45 µm^2 (P< 0.001; figure 8D). In the remote myocardial segment, cardiomyocyte size was 378 ± 32 µm^2 in the control group, and 252 ± 30 µm^2 in the MPC-treated group (P= 0.002; figure 8E). This effect on cardiomyocyte size was confirmed by a significant increase in cardiomyocyte nuclear density in both border en remote myocardial segments. In the border zone, the amount of cardiomyocytes was 1243 ± 102/mm^2 in control animals and increased by 62% to 2026 ± 185/mm^2 in MPC-treated animals (figure 8F; P<0.001). Interestingly, this effect was also found in remote segments, as cardiomyocyte nuclear density in MPC-treated animals was significantly higher than in controls (2645 ± 242/mm^2 vs. 1763 ± 122/mm^2, P<0.001).

Apoptosis, cardiomyocyte proliferation, and resident cardiac stem cells

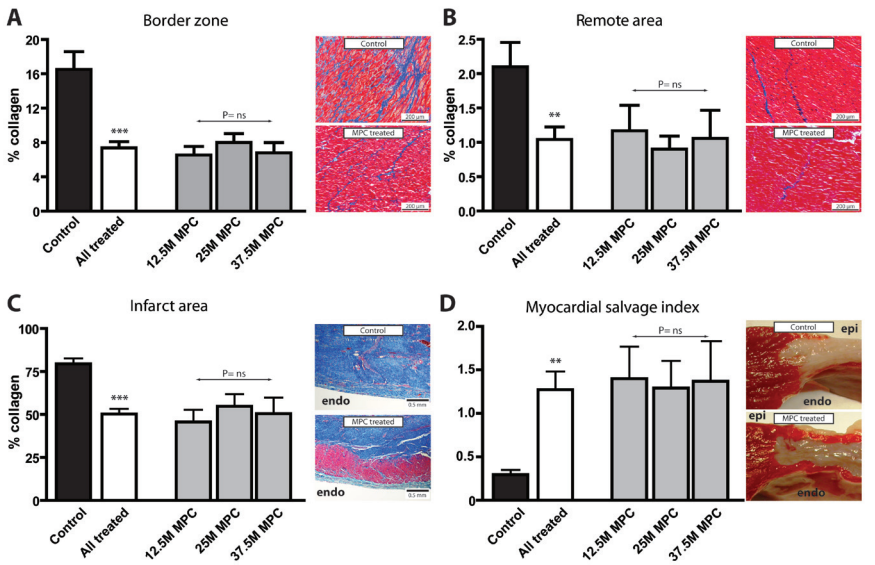


Figure 7. Collagen content and myocardial salvage index.

7A-C: Collagen content significantly decreased in the infarct, border zone and remote myocardial segments of MPC-treated animals as compared to placebo controls. **7D.** The myocardial salvage index represents the ratio of scar versus viable tissue in the infarct area. AMI: acute myocardial infarction; M: million; MPC: mesenchymal precursor cells; ns: non significant; endo: endocardial side; epi: epicardial side of the left ventricle; *P≤ 0.05 ; **P≤ 0.01 ; ***P≤ 0.001

Apoptotic, TUNEL-positive cardiomyocytes comprised 1.31 ± 0.15% of total cardiomyocytes in the border zone of control animals, and were reduced by 40% in MPC-treated animals to 0.77 ± 0.12% (figure 8G; P= 0.008). MPC therapy also had a favorable effect in remote myocardial segments by lowering the percentage of apoptotic cardiomyocytes from 0.63 ± 0.12% in controls to 0.43 ± 0.03% in MPC-treated animals (figure 8H; P= 0.037).

This effect on programmed cell death was accompanied by a small, but significant, increase in proliferating cardiomyocytes in the infarct border zone, but not in remote segments (figure 8 I/J). In MPC-treated sheep 1.38 ± 0.08‰ of cardiomyocytes were positive for Ki-67 as opposed to 0.97 ± 0.14‰ in placebo controls (P= 0.02).

Resident cardiac stem cells, defined as cKit+ cells, were rarely found in both remote segments and in the infarct border zone (0.071 ± 0.012‰ in controls vs. 0.098 ± 0.022‰ in treated animals; P= 0.30), and were not significantly increased by MPC therapy (figure 8K).

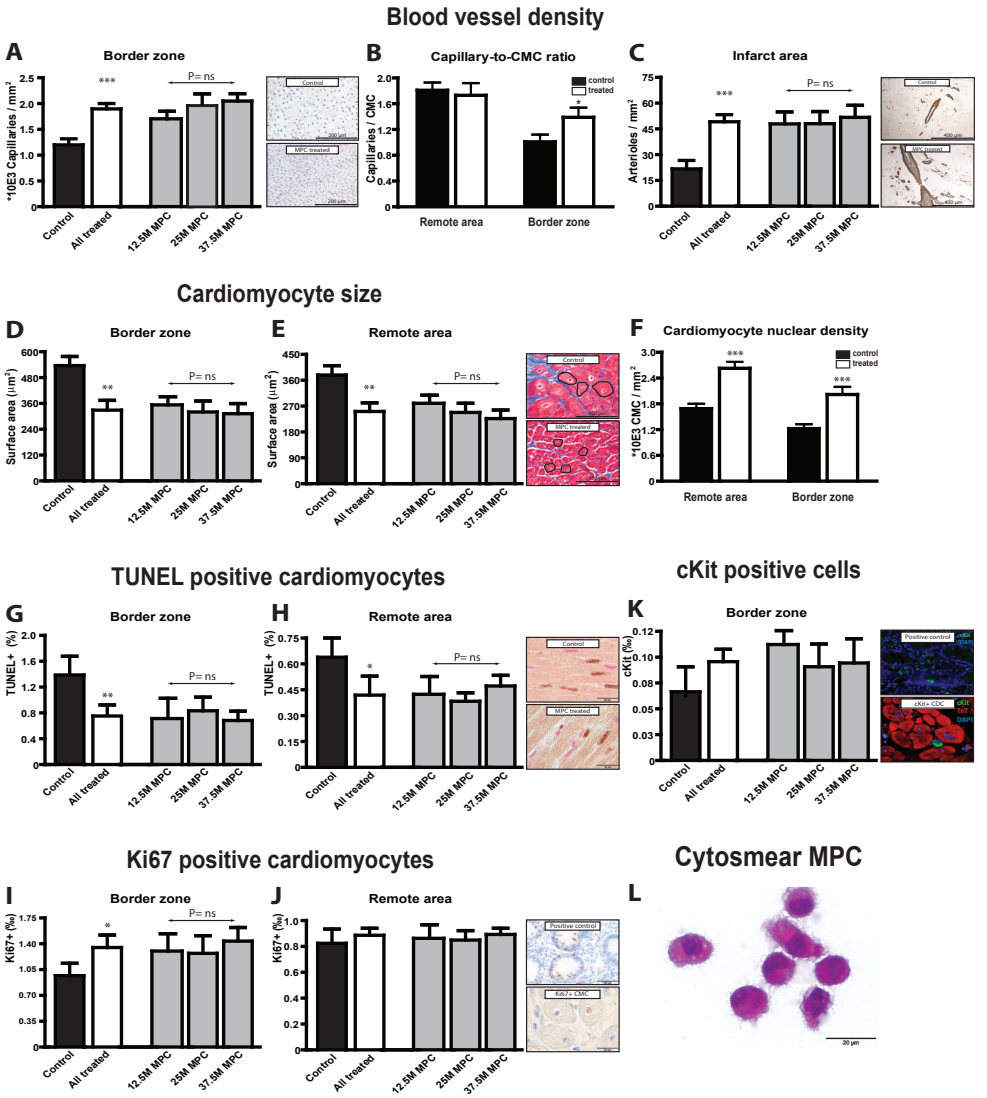
DISCUSSION

In the current study, we investigated a primitive sub population of bone marrow derived MSC. These Stro3+ cells were previously shown to possess potent cardioprotective and immunomodulatory properties *in vitro* and *in vivo*, and can be given to patients in an allogeneic setting. We found that, when certain conditions are adopted, intracoronary infusion of these MPC can be performed safely following AMI without adverse effects, impeding coronary flow, or micro infarctions in remote myocardial segments. Moreover, we demonstrate that intracoronary delivery of MPC prevents LV remodeling and improves residual cardiac function. The results of this study suggest that these effects are evoked by myocardial salvage and subsequent reduction of infarct size, accompanied by induced angiogenesis and reduced myocardial fibrosis.

Previous experience with intracoronary infusion of MSC

Previous studies showed that intracoronary infusion of non-selected MSC was associated with micro-vascular obstruction, coronary flow reduction and myocardial infarctions due to capillary plugging.[15-18] The prominent micro-vascular obstruction that was found in these previous studies might be explained by several factors. First, the size of non-selected MSC progressively increases during cell culturing and higher passages to well over 30 to 50 micrometer.[21] In contrast, MPC comprise an immature sub population of MSC with a median diameter of only 13 micrometer, even when expanded in cell culture (figure 8L and online supplement). As the diameter of capillaries does not exceed 6-10 micrometer, we believe that this small cell size facilitates intracoronary infusion.

Second, in previous studies, higher absolute doses of MSC were used, whereas relatively higher infusion rates were adopted than in the current study. For example, the study of Perin *et al.* infused 100 million MSC at a rate of 1 million cells per minute[15], whereas Freyman *et al.* infused 50 million cells at a rate of 1.5 million cells per minute.[17] In these studies, micro-vascular obstruction and no-flow phenomena are described. On the contrary, infusion of lower cell numbers did not hamper coronary flow in previous experiments. Valina and co-workers infused only 2 million MSC directly following AMI,



and Suzuki *et al.* infused 15 million MSC per coronary artery in hibernating myocardium, both without any flow-related side effects.[22, 23] Also, in a study by Johnston *et al.*, infusion of 10 million cardiosphere-derived cells (20 micrometer in diameter) following AMI was deemed safe, whereas 25 million cells or more caused significant infarctions.[24]

Safety and targeting efficiency of intracoronary MPC infusion

Figure 8. Blood vessel density, cardiomyocyte size, apoptosis, proliferation and cardiac stem cells.

8A: Capillary density was assessed in the border zone, revealing increased capillary densities in MPC-treated sheep. **8B:** The capillary-to-cardiomyocyte ratio was only enhanced in the perfusion territory of the culprit artery of MPC-treated sheep as compared to no change in placebo controls, or in remote myocardial segments. **8C:** In the infarct area, a doubling of arteriolar density of MPC-treated animals was observed. **8D/E:** Cardiomyocyte hypertrophy was markedly reduced in the border zones, as well as in remote myocardial segments of MPC-treated animals, when compared to controls. **8F:** This was confirmed by an increase in cardiomyocyte nuclear density in both border zone and remote areas, and is suggestive of delayed or abrogated adverse remodeling that typically precedes clinical heart failure. Together with the profound effect on cardiac function, it also strongly suggests cardiomyocyte regeneration. **8G/H:** MPC therapy reduced cardiomyocyte apoptosis in both border and remote myocardial segments, corroborating reduced adverse remodeling in MPC-treated sheep hearts. **8I/J:** MPC therapy stimulated cardiomyocytes in the infarct border zone to reenter the cell cycle, thereby increasing the number of proliferating cells and inducing endogenous repair. The top picture bordering the graph shows Ki67-positive cells in the gut that served as positive control. The bottom picture shows a Ki67-positive nucleus of a cardiomyocyte. **8K:** cKit staining revealed that the amount of resident cardiac stem cells did not increase in MPC-treated animals in both border and remote areas. The top picture bordering the graph shows cKit-positive cells in the gut that served as positive control. The bottom picture shows a cKit-positive cell (green) in a peri-vascular area of the myocardium (cardiomyocytes are red). **8L:** Microphotograph of MPC in suspension. MPC are considerably smaller than non immune-selected, cultured mesenchymal stem cells that reach sizes of well over 30 micron. M: million; MPC: mesenchymal precursor cells; ns: non significant; CMC: cardiomyocyte; *P≤ 0.05 ; **P≤ 0.01 ; ***P≤ 0.001

After several pilot experiments divided into two separate phases, we found that, following AMI, a low infusion rate of only 0.5 million MPC/minute permitted intracoronary infusion of 50 million cells without permanently compromising coronary flow, whereas higher infusion rates decreased the maximum tolerated dose. Interestingly, a marked difference between the maximally tolerated dose and infusion rates in animals with or without AMI was noted. This might be associated with increased vascular adhesion of the cells caused by increased expression of chemokines and cell adhesion factors by the activated endothelium following the ischemic insult.[25]

We hypothesize that a low infusion rate might enable the MPC to either pass through the capillary bed or to transmigrate into the peri-vascular tissue without aggregation or capillary occlusion. Indeed, the nuclear imaging retention sub study in two animals revealed that a significant number of MPC still resided in the heart two hours following intracoronary infusion, whereas epicardial coronary flow remained normal (see online supplement). Importantly, no micro-infarctions in, or shedding of MPC to, remote myocardial segments were detected by macroscopic inspection, microscopic analysis and nuclear imaging techniques. This demonstrates that intracoronary infusion of culture-expanded MPC is feasible and can be performed safely. Furthermore, MPC therapy did not have any pro-arrhythmogenic effect. On the contrary, the MPC-treated group showed a trend towards a reduction of ventricular arrhythmias. This might be correlated with the reduction of scar size and improved myocardial perfusion in MPC-treated animals.[26]

Also, no signs of tumorous growth or other focal abnormalities were detected in tissue samples of all major organs or sections of the infarct area by independent and blinded core lab histological analyses. These findings provide additional safety data, as shedding of the cells did not result in significant side-effects, engraftment or aberrant growth in the infused area or remote organs.

Proposed working mechanism of MPC therapy in AMI

The predominant working mechanism of MPC therapy in cardiovascular disease is generally considered to be through paracrine actions of the cells, as long-term engraftment and transdifferentiation into cardiomyocytes of MPC were found to be unlikely in previous studies, and can not account for the profound beneficial effect that has been found in numerous studies.[8-12, 27] Indeed, MPC are known to secrete significant amounts of relevant growth and angiogenic factors as stromal cell-derived factor (SDF)-1, hepatocyte growth factor (HGF)-1, insulin-like growth factor (IGF)-1, VEGF and IL-6. Importantly, the release of these factors exceeds the paracrine abilities of non-selected MSC, resulting in better cardioprotective properties of MPC when compared to MSC.[8, 9] Although actual cell engraftment and possible transdifferentiation of MPC into cardiomyocytes was not determined in the current study, we believe that it provides insightful data on the regenerative potential and working mechanism of post-AMI cell therapy using allogeneic MPC.

Cardiomyocyte salvage and reduced adverse remodeling

As the cells were administered directly following reperfusion of the AMI, we hypothesize that the therapeutic effect of the MPC is mainly exerted through the release of anti-apoptotic and pro-survival factors, thereby ascertaining cardiomyocyte salvage.[9, 27] In addition, the profound immunomodulatory actions of MPCs may preserve myocardial tissue and contribute to effective tissue healing with limiting scar tissue formation by ameliorating reperfusion injury or attenuating oxidative stress.[28, 29] The presumed efficacy of stem cell therapy within the first hours or days following an AMI was also suggested in two studies that used intracoronary delivery of MSC-like stem cells isolated from adipose tissue.[6, 22]

The reduction of infarct size in MPC-treated animals might have resulted in alleviated LV wall stress and reduced neurohumoral activation. This may then ultimately prevent interstitial fibrosis and compensatory cardiomyocyte hypertrophy in the non-infarcted myocardium and, on the long term, LV dilation.[1, 2] Moreover, in control animals, more apoptotic cardiomyocytes were found in both infarct-related and remote segments, which is a strong indication of ongoing adverse remodeling.[30] Indeed, the placebo-treated animals exhibited increased filling pressures and impaired filling rates, a rightward shift of the PV-relation (*i.e.* increased volumes), and more myocardial fibrosis and cardiomyocyte hypertrophy when compared to MPC-treated animals. These parameters are all part of the structural remodeling process that is generally progressive and precedes the clinical syndrome of congestive heart failure with poor prognosis.[1, 2]

Cardiomyocyte proliferation and resident cardiac stem cells

Recent studies have shown that also the postnatal heart contains resident stem cells.[31] Delivery of MSC to infarcted or hibernating myocardium can regenerate myocardium and improve cardiac function by stimulating these resident cardiac stem cells and cardiomyocytes to (re-)enter the cell cycle, thereby initiating cardiomyocyte generation or proliferation.[23, 31-33] In our study we found a marked difference in cardiomyocyte number and cardiomyocyte size in infarct border, as well as in remote myocardial segments of MPC-treated animals. We believe that this difference in part can be explained by initial myocardial salvage, resulting in subsequent reduction of compensatory cardiomyocyte hypertrophy and apoptosis, and eventually in abrogated adverse remodeling. However, the mere size

of the effect on contractile function and cardiomyocyte number, as well as the fact that also remote areas participate, may suggest syngeneic therapeutic working mechanisms induced by the infused MPCs. For instance, the current results indicate that in MPC-treated hearts, increased numbers of cardiomyocytes are in a proliferative state. These proliferating cardiomyocytes might comprise mature proliferating cardiomyocytes that have re-entered the cell cycle, but can also represent the end stage of differentiating cardiac stem cells. Although we found no significant effect on cardiomyocyte proliferation in remote segments after 8 weeks, based on evidence in previous pre-clinical studies[23, 32], we hypothesize that this is primarily due to the fact that this effect on cardiomyocyte proliferation may have been transient, and the current time point was too late to capture it. We pose that cardiac stem cell niches, which are primarily located in the apex and around the atria of the adult heart[31], might have been activated by the infused MPC, as was also suggested by Suzuki *et al*.[23] As the apical stem cell niche was probably depleted by ischemic damage, we hypothesize that cardiac stem cells originating from peri-atrial tissue might have migrated from the base of the heart to the apical, damaged area, thereby eventually not only regenerating the peri-infarct region, but also repopulating remote segments. It is plausible that increased cardiomyocyte number in basal remote segments, and enhanced proliferation in the apical peri-infarct region, are both late effects of this time-dependent cardiac stem cell activation and migration from base to apex. It should be noted however, that in contrast to previous studies[23, 33], we found no clear difference in the amount of cardiac cKit+ stem cells between treated and control animals, which again might be explained by the longer follow-up period of the current study. Also, the amount of resident cardiac stem cells in sheep myocardium was rather low, possibly caused by the fact that in previous studies[23, 33] mice, and juvenile pigs were used, whereas the current study was performed in adult sheep. We hypothesize that rodents and juvenile pigs may have more resident cardiac stem cells than adult animals, although direct comparative study data are still lacking. Importantly, the fact that MPC therapy also beneficially affects remote regions, which comprise >80% of the injured heart, might explain the profound effect on remodeling, and both global and regional cardiac contractile function that was found in MPC-treated animals.

Induced neo-capillary and arteriole formation

Beside the effect on infarct size, remodeling, and cardiomyocyte proliferation, we also found a marked increase in neo-capillary and arteriole densities in the infarct border zone and infarct area of MPC-treated animals. This increase in blood vessel density in the perfusion territory of the culprit artery suggests a pro-angiogenic potential of MPC therapy and is consistent with previous studies.[9-12, 28] Although we have not directly assessed myocardial perfusion using functional testing, these histologic data suggest improved myocardial perfusion and therefore oxygen and nutrient delivery in the (peri-) infarct region. This might in part explain the enhanced regional cardiac function and contractility that were found in this study.

Previous experience with MPC in acute myocardial infarction

In previous large animal studies that assessed the effect of MPC transplantation following AMI, MPC were injected intramyocardially. MPC transplantation was shown to attenuate LV remodeling and

improve cardiac function by enhancing vascular densities, and altering collagen dynamics.[11, 12] These studies also revealed that the low-dose groups (up to 75 million MPC) performed better than the groups that received higher doses (>200 million MPC), suggesting an inverse dose-response relation and a therapeutic threshold of MPC therapy. Accordingly, we found a marked therapeutic effect on both regional and global cardiac function at a relatively low dose range, although no clear correlation was found between efficacy and the cell dose applied. We speculate that higher doses of MPC may be effective, yet also lead to more microvascular obstruction that may counteract the therapeutic effect, whereas even lower doses might still be effective. In another study, See *et al.* for the first time compared MPC with conventional MSC, thereby showing the extensive cardioprotective and pro-angiogenic paracrine capacities of MPC that exceed the paracrine capacities of non-selected MSC.[9] They showed that paracrine actions were likely the predominant working mechanism of MPC therapy. The current study elaborates on these findings, but also adds to our understanding of the working mechanism of MPC therapy. We confirm that MPC exert cardioprotective effects, reduce fibrosis and increase blood vessel densities in infarct and infarct border zone, but also, for the first time, show effects on CMC proliferation with hints of stem cell activation. By determining optimal intracoronary delivery conditions in a relevant large animal model, we paved the way for clinical studies in the near future that use a protocol based on the results of the current study.

Advantages of allogeneic cell therapy

An allogeneic,"off-the-shelf", cell therapy product, originally derived from a young and healthy donor, has important advantages. It renders a laborious and potentially dangerous BM puncture, as well as the culturing steps in clean room facilities, unnecessary. In addition, the stem cell line ensures adequate quality control with inherent batch-to-batch consistency. Also, a negative correlation was found between the amount and functionality of progenitor cells, and age and cardiovascular risk factors.[34] This would make the use of allogeneic MPC in the typically elderly, cardiovascular patient population preferable over autologous cells. More importantly, the cell therapy can be initiated directly after the revascularization of the AMI, thereby maximally utilizing the anti-apoptotic and immunomodulatory capacities of the cells. Finally, intracoronary delivery of an "off-the-shelf" cell product can be easily performed in any interventional catheterization laboratory in the world, without the need for specific infrastructure or cell delivery techniques.

Clinical experience and prospects

Recently, the results from a clinical, phase IIa study, assessing the effect of intramyocardial injections of allogeneic MPC in 60 heart failure patients, were presented. Allogeneic MPC injections up to a dose of 150 million cells were shown to be safe and feasible without a clinically significant anti-allogeneic immune response. More importantly, MACCE rate, cardiac mortality and composite end points for heart failure were markedly decreased at 12 month clinical follow up. This study resulted in the preparations of a phase III study analyzing the therapeutic effect of MPC therapy via intramyocardial injections in 1,700 congestive heart failure patients.

Likewise, the robust effects of MPC therapy in the current large animal AMI study have led to the design of a multi-center, phase IIa/b, double blind, randomized and placebo-controlled clinical trial. The Allogeneic-Mesenchymal-precursor-cell-Infusion-in-myoCardial-Infarction (AMICI) trial, in which European, Australian and US sites will participate, is aimed to prove safety, feasibility and efficacy of MPC therapy in a minimum of 225 patients with ST-elevation AMI and will start enrollment in Q1 of 2013.

Limitations

Although the current randomized study was performed by blinded operators, and histopathology, PV loop, echocardiography and histology data were analyzed by blinded pathologists or technicians, it also has some limitations. First, the use of cardiac MRI would have supplied additional data on baseline infarct size, and might have rendered slightly more reliable analysis of LV volume. Due to logistical reasons these data are lacking, but we are confident that the combined echocardiography and PV-loop analysis provide adequate, and corroborating functional data on both regional and global LV function. Also, we used a non-atherosclerotic animal model without significant thrombus burden causing the AMI. In the real world, the dynamics of MPC following intracoronary infusion in patients with atherosclerotic and micro-vascular disease might be different and result in earlier flow-related effects. Hence, in the forthcoming AMICI trial, the highest dose tested in our pre-clinical study was omitted. Also, to prevent further loss of animals due to ventricular arrhythmias, all sheep were premedicated with amiodarone. Amiodarone treatment was continued throughout the 8 week follow up, which is different from the real life AMI treatment and might have clouded arrhythmia analysis.
Ideally, a control group of cultured, non-immune-selected MSC should have been part of this study. However, previous studies have shown that intracoronary infusion of comparable amounts of non-selected MSC would have resulted in microvascular obstruction and no-flow phenomena, which would have resulted in a substantial loss of animals. Also, the efficacy of MSC has been established before[35], which makes the addition of an extra group to this already large study obsolete.

CONCLUSION

Intracoronary infusion of allogeneic primitive mesenchymal precursor cells directly following an AMI is feasible and safe when certain conditions are adopted. It reduces infarct size and prevents subsequent adverse cardiac remodeling by cardiomyocyte salvage and stimulated cardiomyocyte proliferation and angiogenesis, thereby preserving cardiac function and dimensions. The findings of this study might extend the possible application of these cells from specialized cell therapy centers to virtually any interventional cath lab in the world. As MPC can be applied as an “off-the-shelf” product to all AMI patients, the target patient population is considerable, with over 800 primary percutaneous coronary interventions per million inhabitants in Europe alone.[36]

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SUPPLEMENTAL DATA

MATERIALS AND METHODS

Medication

All sheep were pre-treated with dual anti-platelet therapy (acetylsalicylic acid (Centrafarm, Etten-Leur, the Netherlands) 80 mg qd, clopidogrel (Sanofi-Aventis, Paris, France) 75 mg qd) and amiodarone (Centrafarm, Etten-Leur, the Netherlands) 400 mg qd for ten days prior to the index procedure. Before infarct induction, an intravenous bolus of 10 mg of metoprolol (AstraZeneca, London, United Kingdom) and 10,000 IU heparin (Leo pharma, Ballerup, Denmark) were administered. All sheep received eptifibatide (Merck, Whitehouse Station, USA; bolus of 180 µg/kg and 2 µg/kg/min) during the entire procedure.

Measurement of cell diameter of mesenchymal precursor cells

A vial of 25 million MPC was rapidly thawed, resuspended in a total volume of 25 mL of lactated Ringer's solution and washed twice. Upon the final resuspension step, cells were filtered through a 40 micron cell strainer to obtain a single cell suspension. Subsequently, several cytosmears were made, which were left to air dry. The cytosmears were fixed by submersion in methanol for 15 seconds, after which a common H&E staining was performed. The cytosmears were examined at 1000x magnification and pictures were taken. The diameter of a total of 500 MPC was measured using a routine in automated quantification software as mentioned elsewhere, and the mean and median size were calculated.

Phase 1 – Intracoronary MPC infusion in non-infarcted myocardium

A total of 12 sheep were used in phase 1. To assess the optimal infusion rate and maximum tolerated dose, naïve sheep received an intracoronary infusion of incremental doses of MPC (25, 37.5 and 50 million) using an infusion rate of 1.25 or 2.5 million MPC/min (Figure 1A).

A Twin Pass® micro-catheter (Vascular Solutions, Minneapolis, USA) was placed in the proximal LAD and MPC were infused using an infusion pump (Alaris, San Diego, USA). Coronary flow was assessed by visual estimation of TIMI coronary flow[1] at baseline, every five minutes during MPC infusion, and directly following MPC infusion, to evaluate microvascular obstruction as suggested by reduced antegrade coronary flow. Troponin I (TnI) was determined at baseline, 6 and 24 hours post cell injection (AccuTnI, Beckman Coulter, Brea, USA). TnI levels above 0.1 microgram/L were considered to be an indication of significant cardiomyocyte necrosis due to microvascular obstruction by the infused MPC. After cell infusion, all animals received a subcutaneously implanted REVEAL DX® event recorder (Medtronic, Minneapolis, USA) to continuously monitor for potential arrhythmias. After 48 hours, the animals were sacrificed, the hearts excised, and sectioned into five bread-loafed slices of 8-10 mm from apex to base. The sections were stained with 2,3,5-Triphenyltetrazolium chloride (TTC) to visualize

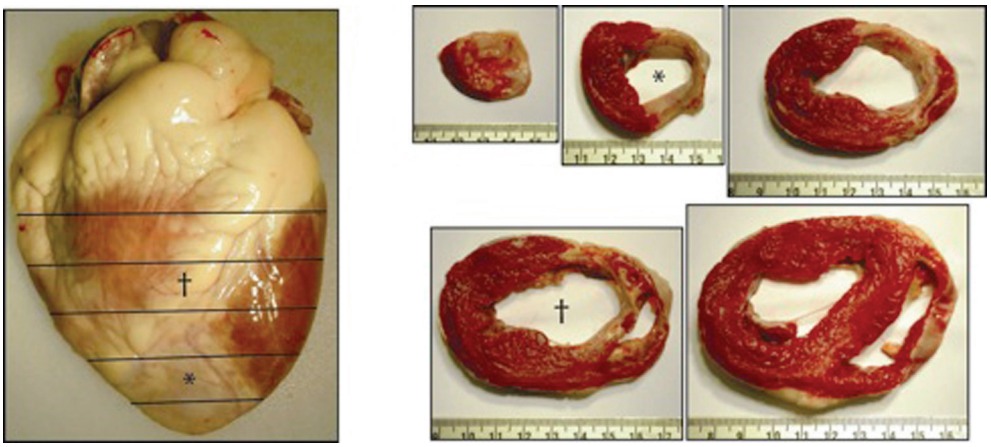

Figure 1. Post-mortem preparation of the heart and TTC staining.

After excision, the hearts were cut into five slices in a bread-loaf manner, after which the slices were stained by TTC. TTC staining turns viable myocardium red, whereas infarcted tissue remains white. This facilitates distinction between infarct area, border zones and remote myocardial segments. The slice marked with * represents the apical segment that was used for analysis, the slice marked with † represents the mid ventricular slice.

(micro-)infarctions. Samples were taken from the inferolateral wall (remote myocardial segment) and anteroseptal wall (target area), as well as from lung, liver, kidney and spleen for histological analysis by an independent pathologist (Erasmus University Medical Center, Rotterdam, The Netherlands) blinded to the individual treatment of the animals.

Phase 2 – Intracoronary MPC infusion and bio-distribution following AMI

To assess the optimal infusion rate and maximum tolerated dose in AMI, intracoronary MPC infusion was performed in an anterior AMI model in 8 sheep. Coronary flow was assessed by visual estimation of TIMI coronary flow, and quantified by intracoronary Doppler flow analysis using a Doppler flow wire

Table I. Coronary flow, ventricular arrhythmias and death

	Control group	Treatment group	p-value
Coronary flow			
Reduction of TIMI* flow	0/12	0/22	1.000
Death			
Ventricular fibrillation	2/12	0/22	0.144
Unknown	0/12	2/22	0.543
All	2/12	2/22	0.612
Ventricular arrhythmia			
Ventricular fibrillation	2/12	0/22	0.144
Ventricular tachycardia	1/12	0/22	0.371
All	3/12	0/22	0.059

P-values were determined using a two-sided Fischer's exact test.

*TIMI: thrombolysis in myocardial infarction

(Combowire®, Volcano, San Diego, USA) positioned between the first and second diagonal branch of the LAD, and expressed as the average peak velocity (APV; cm/sec; figure 1B). Bio-distribution and myocardial retention was quantified using Indium^{111} labeling in two separate animals (see below).
Anterior wall AMI was induced by balloon inflation (Voyager Rx 3.0-3.5x12 mm, Abbott, Illinois, USA) in the mid LAD for 90 minutes. After 15 minutes of reperfusion, a Twin Pass® delivery catheter was positioned in the LAD at the location of prior balloon inflation. Subsequently, 50 million MPC were infused at a rate of 1 million MPC/min (n=3) or 0.5 million MPC/min (n=3). The maximum tolerated dose of MPC was assessed by repeated Doppler flow measurements after infusion of every 5 million cells. In addition, TIMI flow was determined after infusion of every 10 million MPC. The optimized and safe intracoronary infusion conditions were subsequently applied in phase 3 of this study.

Phase 3 – Long-term safety effects and dose finding of intracoronary MPC infusion after AMI

Induction of myocardial infarction and infusion of MPC

A total of 68 sheep were used in phase 3 of the study (figure 1C). An anterior myocardial infarction was induced by LAD occlusion as described before. After reperfusion, the sheep were randomized by a blinded draw to receive an intracoronary infusion of 12.5, 25, or 37.5 million MPC or LR (control). The cells were infused via a Twin Pass® delivery catheter at an infusion rate of 0.5 million MPC/min. Coronary flow was assessed by coronary angiography before cell infusion, and every 15 minutes during cell infusion. Coronary angiographies were scored by a blinded interventional cardiologist at Thoraxcenter of the Erasmus University Medical Center in Rotterdam, to prevent bias. After cell infusion, a subcutaneous event recorder was implanted to monitor for ventricular arrhythmias during the 8 week follow-up. Eight weeks following AMI and MPC infusion, coronary angiography and TIMI flow grade assessments were performed and analyzed by independent and blinded investigators.

Nuclear labeling and imaging

MPC were labeled with Indium^{111} at 37°C for 20 minutes (20MBq; GE Healthcare, Pittsburgh, USA). After incubation, cells were washed three times with HANKS buffer (Invitrogen, Carlsbad, USA) and Indium^{111} uptake efficiency was measured with a dose calibrator (Veenstra, Joure, the Netherlands), whereas cell viability was assessed by trypan-blue counting. A total of 37.5 million MPC were infused following reperfusion of the culprit vessel at a rate of 0.5 million MPC/min. Animals were sacrificed two hours after MPC infusion and heart, lungs, liver, spleen and kidneys were excised. Subsequently, the organs, urinary catheter system and the infusion system (syringes, tubing, catheters) were scanned using a dual-head gamma camera (Philips, Best, the Netherlands) to quantify MPC bio distribution (acquisition of 5 minutes, 256x256 projection matrix). Bio distribution and retention were determinded using dedicated software (Pegasys, Philips, Best, the Netherlands) and expressed as percentage of the injected dose per organ.

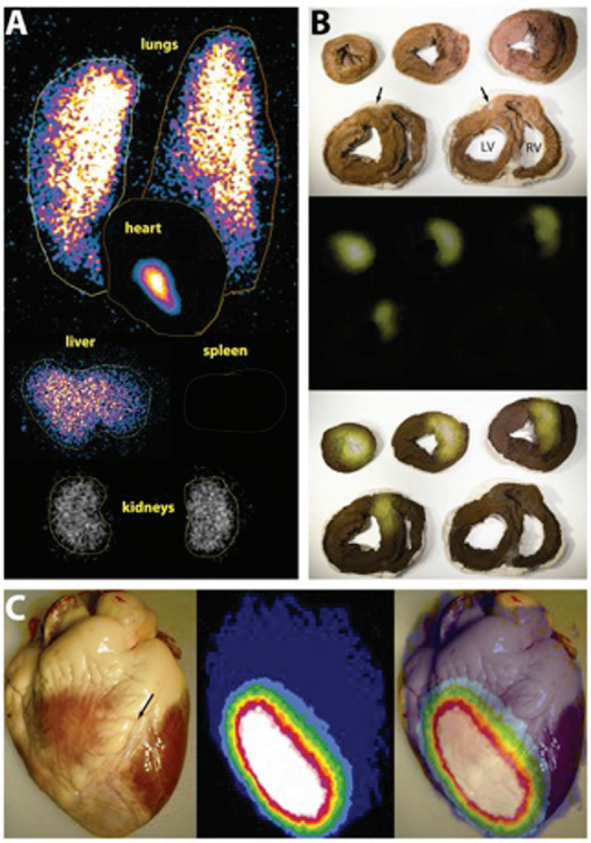

Figure 2. Biodistribution of MPC as assessed by Indium^{111} labelling.

3A. Ex vivo scanning of all major organs two hours following intracoronary infusion of 37.5 million MPC. 3B. Top picture: bread-loafed slices from apex to base; middle picture: MPC distribution in these slices; bottom picture: overlay. MPC were only targeted to the perfusion territory of the culprit artery (arrows), whereas no signs of activity were found in remote myocardial segments. 3C. Left picture: anterior view of the intact heart; middle picture: MPC distribution in the anterior wall and apex; right picture: overlay. Arrows: left anterior descending artery; MPC: mesenchymal precursor cells.

Pressure–volume loop calibration, parameters and analysis

The volume was calibrated by thermodilution and hypertonic saline dilution as previously described.[2,3] PV-loop measurements were performed at baseline and at eight-week follow-up and analyzed using customized software (Conduct NT 2.18, CD Leycom, Zoetermeer, The Netherlands). All values are based on the analysis of ten consecutive beats of sinus rhythm.

End-systolic elastance (E_{es}) was defined as the slope of the end-systolic pressure–volume relation (ESPVR). The E_{es} was calculated using a single-beat estimation method as previously described.[4,5] The V_0 of the end-diastolic pressure–volume relation (EDPVR), which represents the unstretched volume of the LV, was calculated using the following formula: (0.6-0.006*end diastolic pressure (EDP))*end-diastolic volume (EDV)).[6,7] The V_{30} is the theoretic point on the EDPVR where the pressure is 30 mmHg and was calculated using the following formula: $V_0 + (EDV - V_0)/(EDP/27.8)^{(1/27.6)}$.[6,7] End-diastolic stiffness (E_{ed}) was calculated as EDV/EDP and preload-recruitable stroke-work (PRSW) as stroke-work/EDV.[8]

Echocardiography

In all animals, a transthoracic echocardiogram (TTE) was made before the AMI and directly following the AMI, and at four and eight weeks follow up. Two-dimensional grey scale images at a frame rate of 60-90 frames/s were obtained from a parasternal position with a Philips iE33, equipped with a broadband S5-1 transducer (Philips Healthcare, Eindhoven, The Netherlands). Short axis views were recorded at three different levels (basal, mid ventricular and apical) and three consecutive cardiac cycles were acquired. These images were transferred to an Image Arena 4.1 (Tomtec Imaging Systems, Unterschleissheim, Germany) work station for offline analysis. The analysis of echocardiography data was performed by an independent operator, who was blinded for the treatment allocation of the sheep.

Table 2. Pressure–volume loop derived parameters.

	reference	**control**	**MPC***	**control**	**MPC***	**P value**
	Pre AMI†	Post AMI†	Post AMI†	8 weeks	8 weeks	
	n=12	n=10	n=20	n=10	n=20	
HF (beats/min)	70.1 ± 3.4	84.8 ± 4.0	76.5 ± 5.2	92.6 ± 10.0	69.9 ± 4.4	0.11
Volumes						
End systolic volume (mL)	56.1 ± 3.3	73.5 ± 2.8	71.1 ± 1.2	102.8 ± 4.0	68.3 ± 1.8	<0.001
End diastolic volume (mL)	138 ± 7.8	135 ± 2.0	137 ± 1.8	178 ± 8.0	149 ± 4.1	<0.001
Systolic function						
Ejection fraction (%)	63.5 ± 1.7	44.2 ± 1.5	44.7 ± 1.0	40.7 ± 2.6	52.8 ± 0.7	<0.001
Elastance (E_{es})	1.59 ± 0.12	0.96 ± 0.07	0.99 ± 0.06	0.89 ± 0.05	1.26 ± 0.1	0.003
PRSW‡ (mmHg)	47.7 ± 2.8	27.4 ± 1.0	28.5 ± 1.6	33.5 ± 1.44	41.6 ± 1.9	0.008
End systolic pressure (mmHg)	86.8 ± 4.5	70.5 ± 6.0	69.8 ± 3.8	90.5 ± 6.6	84.4 ± 3.7	0.51
Stroke volume (mL)	80.2 ± 5.7	65.9 ± 2.4	72.0 ± 3.0	71.4 ± 6.2	78.1 ± 1.9	0.41
Stroke work (mL.mmHg)	6511 ± 484	3707 ± 179	3954 ± 232	5970 ± 375	6331 ± 325	0.47
Cardiac output (L/min)	5.5 ± 0.3	4.8 ± 0.24	4.5 ± 0.3	6.2 ± 0.5	5.4 ± 0.3	0.22
dP/dtmax	1136 ± 90	980 ± 38	1027 ± 71	1091 ± 82	1155 ± 75	0.15
dP/dTmax / EDV	9.1 ± 0.7	7.3 ± 0.2	7.6 ± 0.6	7.5 ± 0.7	8.5 ± 0.4	0.042
tPER§ (msec)	165 ± 11	165 ± 12	147 ± 9	163 ± 22	165 ± 9	0.30
Diastolic function						
Stiffness	0.05 ± 0.01	0.09 ± 0.03	0.06 ± 0.01	0.04 ± 0.01	0.04 ± 0.00	0.22
V_0 (mL)	75.4 ± 4.1	71.0 ± 3.8	76.1 ± 1.1	99.8 ± 4.7	83.9 ± 2.1	0.001
V_{30} (mL)	167 ± 16	185 ± 15	185 ± 7	241 ± 24	213 ± 8	0.047
Tau	29.5 ± 1.5	37.7 ± 7.3	32.9 ± 2.1	27.0 ± 1.8	28.2 ± 0.9	0.27
End diastolic pressure (mmHg)	8.2 ± 1.5	11.0 ± 4.7	7.6 ± 0.9	9.7 ± 1.7	6.0 ± 1.0	0.08
dP/dtmin	-1102 ± 77	-818 ± 56	-823 ± 67	-1042 ± 114	-1126 ± 51	0.29
dP/dtmin / ESV	-12.5 ± 0.9	-11.1 ± 0.6	-11.2 ± 0.7	11.1 ± 1.0	15.3 ± 0.9	0.008
tPFR‖ (msec)	623 ± 42	525 ± 30	604 ± 36	505 ± 30	648 ± 52	0.023

*mesenchymal precursor cells; †acute myocardial infarction; ‡preload recruitable stroke work; §top peak ejection rate;

‖top peak filling rate

The endocardial border was traced at end-diastole and end-systole at each level. LV volumes were calculated using modified Simpson's rule: LV end diastolic volume (LVEDV)= (A_{bED}) * L/3 + (A_{mED}+A_{pED})/2) * L/3 + 1/3(A_{pED}) * L/3; LV end systolic volume (LVESV)= (A_{bES}) * L/3 + (A_{mES}+A_{pES})/2) * L/3 + 1/3(A_{pES}) * L/3, in which A_b is the area at basal level, whereas A_m and A_p are the areas at mid and apical level respectively. L is defined as the length of the ventricle from apex to base, and was set at 10 cm at baseline, based on cadaver measurements. L at 8 week follow up was measured by counting up the thicknesses of all post-mortem slices (see supplemental figure 1), and L at 4 week follow up was estimated by calculating the mean between baseline and 8 week follow up per animal. LVEF was calculated as follows: [(LVEDV-LVESV)]/LVEDV * 100. These estimations of LV volumes and EF were shown to correspond very well with radionuclide measurement techniques.[9]

Regional fractional area change (FAC) was calculated using the following formula: [(end-diastolic area) - (end- systolic area)]/(end-diastolic area). Also, regional systolic wall thickening was assessed in apical and mid-ventricular recordings. The local wall thickness was measured at end-diastole and systole in the infarct segments (anteroseptal and anterior wall) and one remote segment (inferolateral wall). Systolic wall thickening was subsequently determined by the following formula: [(end-systolic wall thickness) - (end-diastolic wall thickness)]/(end-diastolic wall thickness)].

Infarct volume and morphometry

After excision of the heart, the LV was isolated and cut into 5 slices from apex to base. To discriminate infarct tissue from viable myocardium, the slices were incubated in 1% triphenyltetrazolium chloride (TTC, Sigma-Aldrich Chemicals,Zwijndrecht, Netherlands) in 37 °C Sörensen buffer (13.6 g/L KH2PO4+17.8 g/L Na2H PO4·2H2O, pH 7.4) for 15 min. All slices were scanned from both sides and in each slide the infarct area was compared to total area using digital planimetry software. After correction for the weight of the slices, infarct size was calculated as a percentage of the LV. Infarct thickness was depicted as the average of three measurements from endocardial to epicardial border per slice, whereas the border zone thickness was the average thickness of viable myocardium measured directly adjacent to both sides of the infarct.

Immunohistochemical staining

Collagen content, myocardial salvage index and cardiomyocyte size

Collagen content was assessed using Gomorri trichrome staining. In short, sections of the infarct and border zone, as well as sections of the remote area were deparaffinized and submerged in Bouin's fixation solution (Sigma Aldrich, St. Louis, USA) at 56ºC for 15 minutes. Nuclei were stained with haematoxylin, after which the slides were submerged in Trichrome-LG solution (Sigma Aldrich, St. Louis, USA). After treatment with 0.5% acetic acid solution for one minute, slides were mounted in Entallan (Merck, Darmstadt, Germany).

Three random pictures were taken of each slide at 10x magnification, and collagen content was quantified using a customized software routine as mentioned before and depicted as percentage of

the total surface area. The myocardial salvage index was calculated by dividing the area of viable myocardium in the infarct by the area that was composed of collagen.

Also, cardiomyocyte size was measured in trichrome-stained sections of border zone and remote myocardial segments. Three random pictures were taken at 40x magnification and the average surface area of at least 10 cardiomyocytes per field of view was determined. Surface area was only assessed of transversely cut cardiomyocytes in which a nucleus was visible to assure measuring the surface area at the mid level of the cardiomyocyte.

Capillary and arteriolar density

The blood vessel density was determined in border zone, remote area and the infarct area. In the border zone and remote area, blood vessel density was quantified by counting the amount of capillaries per mm^2. Blood vessel density in the infarct area however, was determined by quantifying arterioles, which was necessitated by the disarray of capillaries and pronounced aspecific staining. In brief, sections of the infarct border zone and remote myocardial segment were deparaffinized, rehydrated, pre-treated with trypsin EDTA (Lonza, Verviers, Belgium) and stained for isolectin-B_4 (Bandeiraea simplicifolia Isolectin-B_4 peroxidase, Sigma Aldrich, St. Louis, USA; (20 mg/ml)). Sections of the infarct area were stained for smooth muscle actin (SMA; clone 1A4, Sigma Aldrich, St. Louis, USA; 1:100). All sections were blocked in methanol/H_2O_2 solution for 30 minutes and incubated overnight at 4ºC with isolectin-B_4 or SMA antibody solution. The slides for SMA staining were then washed and immersed in a secondary antibody (HRP-conjugated goat anti-mouse antibody, DAKO, Glostrup, Denmark) for 90 minutes. Subsequently, all slides were immersed in DAB solution (DAKO) for six minutes and finally mounted in Entellan. A technician blinded for the treatment allocation of the individual sheep took three random pictures of the border zone and remote myocardial segment or infarct area at 20x magnification after which capillaries and arterioles were quantified. Capillary density and arteriolar density were expressed as number per mm^2.

The micro-perfusion in the border zone and remote area was quantified as the number of capillaries per cardiomyocyte (capillary-to-cardiomyocyte ratio) and corrected for the collagen deposition in the extra-cellular matrix, and calculated using the following formula: [(capillaries/mm^2)/(cardiomyocytes/mm^2)*(1-collagen content)].

TUNEL, Ki67 and cKit staining

Paraformaldehyde-fixed, paraffin-embedded heart sections of 5 µm thick were used for TUNEL, Ki-67, and c-Kit staining. The amount of apoptosis was quantified using a “In situ cell death detection kit” (Roche, Basel, Swiss) per the manufacturer’s instructions. Most antibodies have been used successfully in swine, but not sheep, by other laboratories.[10,11] Antigen retrieval for Ki67 and cKit was done by boiling the slices for 30 minutes in 10mM citrate (pH 6). For Ki67 staining, sections were blocked in methanol/H_2O_2 solution for 30 minutes and incubated overnight at 4ºC with Ki67 antibody (clone MIB-1, DAKO; 1:100) solution**.** The slides were then washed and immersed in a secondary antibody (HRP-conjugated goat anti-mouse antibody, DAKO) for 90 minutes, after which they were immersed in DAB solution (DAKO). Pictures of multiple fields (400x) were used to quantify

Table 3. Echocardiographic volumes and ejection fraction

	Control	MPC-treated	p-value
LVEF BL (%)	51.3 ± 2.5	52.2 ± 2.5	0.77
LVEF post (%)	43.1 ± 1.2	42.4 ± 1.4	0.58
LVEF 4WFU (%)	42.0 ± 1.7	46.3 ± 0.9	***0.009***
LVEF 8WFU (%)	37.3 ± 1.9	47.7 ±1.2	***0.001***
ESV BL (mL)	58.5 ± 4.4	54.9 ± 3.3	0.51
ESV post (mL)	67.8 ± 3.7	62.8 ± 4.2	0.33
ESV 4WFU (mL)	90.4 ± 7.8	65.9 ± 4.8	***0.025***
ESV 8WFU (mL)	115.8 ± 16.5	71.3 ± 6.9	***0.042***
EDV BL (mL)	119.7 ± 6.7	115.1 ± 5.6	0.58
EDV post (mL)	119.3 ± 7.2	112.6 ± 5.9	0.39
EDV 4WFU (mL)	155.2 ± 10.6	123.0 ± 9.2	***0.018***
EDV 8WFU (mL)	182.9 ± 22.5	136.2 ± 12.0	***0.037***

BL: baseline; post: directly post myocardial infarction; LVEF: left ventricular ejection fraction; 4WFU/8WFU: 4 and 8 week follow up; ESV: end systolic volume; EDV: end diastolic function.

the frequency of Ki67 staining. cKit staining was performed by immersing the slides in cKit antibody solution (ab5506, Abcam, Cambridge, UK; 1:100) together with anti-cTnI (mouse monoclonal antibody clone 8I-7, Spectral diagnosis, 1:100) to detect myocyte filaments. Samples were posttreated with fluoroisothiocyanate (FITC) conjugated anti-rabbit and TRITC conjugated anti-mouse antibody (Dako). Nuclei were stained with DAPI (Vectashield). Multiple fields were photographed using an Olympus IX55 fluorescence microscope, after which cKit+ cells were quantified.

RESULTS

Measurement of cell diameter of mesenchymal precursor cells

Ovine MPC have a median diameter of 13 micron and a mean diameter of 13.2 ± 2.2 micron (supplemental figure 3)

Nuclear cell tracking experiments

In two separate sheep, bio-distribution was assessed following intracoronary infusion of 37.5 million

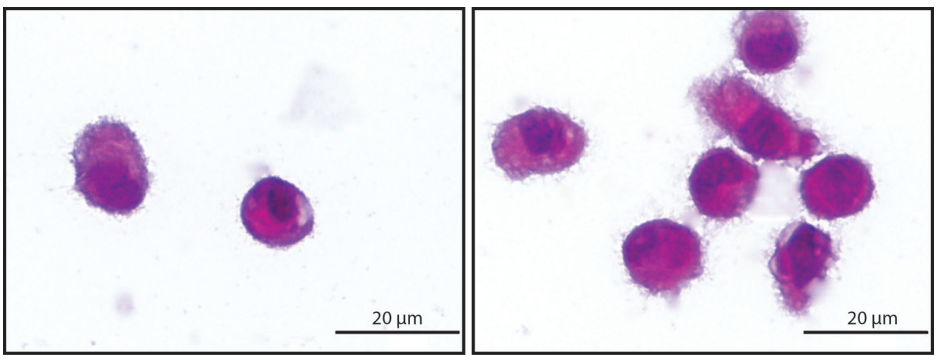

Figure III. Light photomicrographs of mesenchymal precursor cells at 1000x magnification.

Indium^{111} labeled MPC. The Indium^{111} labeling efficiency was 79.5 ± 7.5%, and cell viability exceeded 85% after the labelling procedure. *Ex vivo* quantification of Indium^{111} uptake in all major organs estimated a cell uptake in the heart of 40.8% of the total cell dose in sheep 1 and 53.5% in sheep 2, whereas lungs (13.7% and 6.9%), kidneys (2.3% and 1.7%), liver (5.6% and 2.6%), spleen (0.8% and 0.3%), and pericardium (0.3% and 0.1%) had limited uptake (figure 3A). The residual activity was predominantly detected in the infusion and urinary catheter systems. In the heart, the MPC were retained in the perfusion territory of the LAD (figure 3B/C), *i.e.* the anterior and anteroseptal wall. No activity was detected in remote myocardial segments (figure 3B).

DISCUSSION

Analysis of cardiac function

In this study, cardiac function and volumes were assessed using both PV-loop analysis and echocardiography. Invasive hemodynamics by analysis of the pressure-volume relation analysis renders reliable and reproducible quantification of the LV volumes, and thus LVEF, throughout the cardiac cycle.[12,13] Also, indices of intrinsic myocardial contractile function can be determined that are independent of pre- and afterload conditions, which are known to differ substantially between the acute phase of the AMI and at eight weeks follow up.[8,14] Echocardiography analysis showed comparable pre-AMI conditions between groups, provided supportive data on global and regional cardiac function, and corroborated PV-loop data. Importantly, PV-loop and echocardiography data were acquired and analyzed by separate technicians, who were blinded for the treatment allocation of the individual sheep.

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CHAPTER 8

Intracoronary infusion of allogeneic mesenchymal precursor cells in patients with anterior wall ST-elevation myocardial infarction: Rationale and design of the AMICI trial

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ABSTRACT

Allogeneic Mesenchymal Precursor cells (MPC; Revascor™; Mesoblast Inc.) comprise STRO-3 immune-selected stem cells, which are derived from donor adult bone marrow mononucleated cells. They may provide an "off-the-shelf" regenerative therapy for acute myocardial infarction (AMI) patients that can be directly applied following the primary PCI procedure.

The AMICI trial is a prospective, randomized, placebo-controlled, double blind clinical trial that will analyze the effect of intracoronary infusion of allogeneic mesenchymal precursor cells (MPC) in patients with an ST-elevation myocardial infarction. The therapy will be initiated directly following revascularization and standard therapy of the left anterior descending artery (LAD). Up to 225 patients with a first anterior wall AMI will be enrolled. After successful revascularization, the patients will be randomized in a 1:1:1 ratio to receive 12.5 or 25 million allogeneic MPC or placebo solution via intracoronary infusion. The primary safety endpoint is defined as the occurrence of MACCE at 30 days follow up. Patients will be further screened for coronary flow related side effects of MPC infusion during and following cell infusion. Primary efficacy endpoint is defined as the reduction in infarct size as quantified by delayed enhancement cardiac MRI. This study is initiated in Q1 2013.

Overall, In the AMICI study, we aim to determine the safety, feasibility and efficacy of intracoronary infusion of allogeneic MPC after AMI.

BACKGROUND

Despite improvements in the treatment of acute myocardial infarction (AMI), the incidence of heart failure is still rising.[1–3] Cardiomyocyte loss following AMI and subsequent scar tissue formation eventually leads to cardiac remodeling[4,5], which is characterized by expansion of the infarct area, progressive collagen deposition, and dilation of the left ventricle (LV).[6]

To prevent deterioration of cardiac function following AMI, stem cell therapy has raised considerable interest in the field of cardiology over the past 10 years, with over 2000 AMI patients treated worldwide with a form of stem cell therapy in several small-scale clinical studies.[7] In these studies, the effects of unfractioned bone marrow derived stem cells (BMC) have been investigated.[7–9] Recent meta-analyses that evaluated these studies concluded that intracoronary infusion of BMC is safe and feasible. Overall, there is a modest increase in ejection fraction of approximately 4% [7–9], whereas long-term follow up showed a decrease in clinical end points including: revascularization, hospitalizations for heart failure and recurrent AMI in some studies.[7,10,11] It is hypothesized that BMC have the ability to regenerate the myocardium predominantly by paracrine actions, thereby decreasing apoptosis and inducing neo-vascularization.[12]

As the effects of BMC on cardiac repair have been modest, the search for more potent cells is ongoing. [13,14] Mesenchymal precursor cells (MPC) comprise a Stro-3 immune-selected, immature sub fraction of BM-derived mesenchymal stem cells (MSC).[15] Both MSC and MPC have been shown to exhibit cardioprotective properties in preclinical studies that might exceed the properties of BMC.[13] MPC are multipotent cells with extensive proliferative potential, and secrete a vast array of anti-apoptotic factors, growth factors, pro-survival factors and immunomodulatory cytokines.[5,16–19] Importantly, it was found that MPC display greater cardio-protective effects than conventional MSC that are selected by plastic adherence alone, which may be evoked by their potent paracrine activity, as well as more extensive multilineage differentiation potential.[20,21] Due to an immunomodulatory effect on both the innate and adaptive immune system and a unique immunophenotype, MPC can be given in an allogeneic setting.[14,19,22] This renders the possibility of an 'off-the-shelf' mesenchymal cell product with several advantages: (1) painful harvesting procedures are no longer needed, (2) no delay in administration of cells after AMI, (3) high quality of the cell product with batch-to-batch consistency and thus (4) no variation in cell quality between patients.

The AMICI trial is the first clinical trial that will investigate the safety and feasibility, as well as functional implications of intracoronary administration of allogeneic MPC directly following primary percutaneous intervention for the treatment of acute myocardial infarction.

Investigational product

Revascor™ consists of STRO-3 mesenchymal precursor cells from the bone marrow of a healthy individual. STRO- 3 MPCs are isolated via STRO-3 based prospective immunoselection by magnetic activated cell sorting, as described before[15]. After selection, the STRO-3 MPCs are expanded in culture to produce a large number of pure, homogeneous, concentrated allogeneic MPC with defined

characteristics and minimal lot-to-lot variability. These expanded MPC have been shown to retain the MSC capacity for differentiation into bone, cartilage, and adipose tissue in vitro and regenerate bone in vivo. MPCs have established biological activity, consistent characterization, enhanced purity and potency and are immediately available for 'off-the-shelf' allogeneic use.

Preclinical experience with MPC and rationale of intracoronary infusion

The cardioprotective effects and potential of MPCs following intramyocardial injection have been investigated and recognised in several rodent and large animal preclinical studies.[21,23,24] In a recent large-scale, randomized study using a total of 88 adult sheep, the safety, feasibility and efficacy of intracoronary infusion of three different doses of MPC were investigated directly following AMI.[25] Although intracoronary infusion of stem cells has been associated with vascular plugging and the occurrence of myocardial infarctions, it was found that, when certain conditions are adopted, intracoronary infusion of doses up to 37.5 million MPC can be performed safely without compromising coronary flow. The cells had a favorable safety profile, as the treatment arm exhibited no ventricular arrhythmias, no cell therapy related side effects, and no histopathological abnormalities. More importantly, intracoronary MPC infusion reduced infarct size and prevented subsequent adverse cardiac remodeling by cardiomyocyte salvage and stimulation of angiogenesis, thereby preserving cardiac function and dimensions. In this study, we found no dose-effect relation, and all doses (12.5, 25, and 37.5 million MPC) seemed equipotent in preserving myocardial function.

METHODS AMICI TRIAL

Study objectives

The primary objective of the AMICI trial is to evaluate the safety and feasibility of intracoronary delivery of two different doses of allogeneic MPC in patients with ST-elevation myocardial infarction undergoing primary PCI of the LAD in a double-blind, randomized, and placebo-controlled setting. Also, the efficacy and optimal dosing of intracoronary delivery of MPC will be investigated.

Study overview and design

The AMICI study will be executed according to the declaration of Helsinki. The protocol for this study has been approved by the regional ethical committee of each participating hospital and by Dutch federal authorities. This study is a phase I/II, prospective, double blind, randomized, placebo-controlled trial that will take place in approximately 20 US, 30 European and 12 Australian centers. The trial has been registered at WHO trial database (EUCTR2010-020497-41-NL), European Clinical trial register (2010-020497-41) and at www.ClinicalTrials.gov (NCT01781390).

The data safety monitoring board (DSMB) will perform safety reviews of the initial 30, 60 and 90 patients at day 30 of their follow up. The DSMB will perform a conditional power analysis after the initial 60 patients have reached 6 months follow up. An interim analysis to assess futility and possible

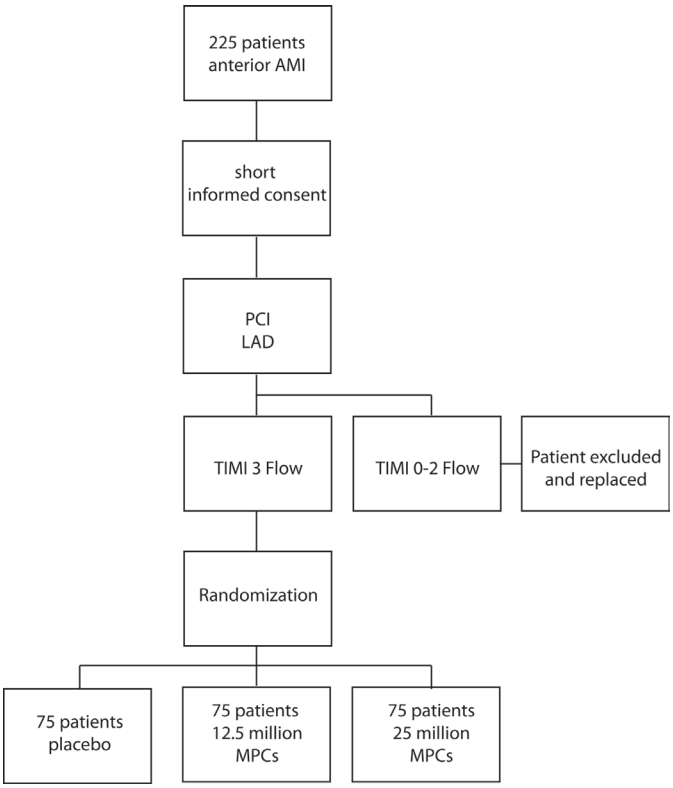

Figure 1. Flowchart of the inclusion of patients in the AMICI trial. Eligible patient and the cardiologist both sign a short informed consent form before primary PCI. When TIMI flow is 3 after reperfusion and stent implantation, the patient is blindly randomized to receive either a placebo solution, 12.5 million or 25 million MPCs. If TIMI flow is lower than TIMI 3, patients will be excluded from the study and replaced. AMI indicates: acute myocardial infarction; PCI: percutaneous coronary intervention; LAD: left anterior descending artery; TIMI: thrombolysis in myocardial infarction; MPCs: mesenchymal precursor cells.

sample size adjustments will also be performed by the DSMB when 120 subjects complete 6 months follow up. Flowchart 1 summarizes the design of this study.

Patient population

The study will enroll up to 225 patients undergoing percutaneous coronary intervention (PCI) of a de novo anterior wall AMI due to a lesion in, or occlusion of the left anterior descending (LAD) coronary artery. After successful revascularization of the LAD (defined as TIMI 3 flow and residual stenosis of less than 20%), eligible patients will be randomly assigned in a 1:1:1 ratio to receive 2 doses of MPCs (12.5 million; n=75 or 25 million; n=75) or placebo solution (n=75). All patients have to meet all of the inclusion and exclusion criteria as listed in table 1and 2. Qualifying patients will be approached before the primary PCI by the site investigator or interventional cardiologist and provided with an informed consent form for signature before PCI.

Table 1. Inclusion Criteria

Patients will be entered into this study only if they meet ALL of the following criteria:

- Willing and able to understand, sign, and date the Informed Consent Form
- Males or females ≥ 18 years.
- Clinical symptoms consistent with AMI between 2-12 hours from onset of symptoms PCI, unresponsive to Nitroglycerin.
- De novo anterior Acute Myocardial Infarct
- Successful revascularization of the culprit lesion in the LAD within 2-12 hours of the onset of AMI symptoms (defined as (1) primary or facilitated percutaneous coronary intervention (PCI) with stent implantation, resulting in TIMI 3 flow AND (2) residual stenosis of less than 20% by on-line QCA.
- If the subject or subject's partner is of childbearing potential (not amenorrhagic for the previous 24 months or not surgically sterile), the subject must be willing to use adequate contraception (hormonal pill, implant or intrauterine device, barrier methods only if used consistently) from the time of screening and for a period of at least 16 weeks after procedure.
- Negative urine pregnancy test
- Must be willing to return for required follow-up visits

Table 1 gives an overview of the key inclusion criteria of the AMICI trial

Table 2. Exclusion criteria

Patients will not be entered into this study if they meet ANY of the following criteria:

- Known prior MI, prior PCI LAD, CABG, prior known hypertrophic cardiomyopathy, or prior hospital admission for heart failure (HF).
- Significant valvular disease
- More than 12 hours between the onset of first symptoms of AMI and revascularization
- Unsuccessful revascularization of culprit artery defined as less than TIMI 3 flow or residual diameter stenosis of 20% by on line QCA analysis.
- Need for staged treatment of coronary artery disease, or other interventional or surgical procedures to treat heart disease (e.g., valve replacement, PCI or CABG) planned or scheduled within 6 months after the study procedure.
- Cardiogenic shock or hemodynamic instability
- History of persistent atrial fibrillation
- Malignancy within last 3 years from screening
- Acute or chronic bacterial or viral infectious disease
- Pacemaker, implantable cardioversion defibrillator (ICD) or any other contra-indication for cMRI
- Known history of Severe Chronic Obstructive Pulmonary Disease (COPD)
- Known history of sensitization to human leukocyte antigens
- Known allergy to DMSO, murine protein, bovine protein, aspirin, clopidogrel, prasugrel, and/or metallic stent
- Current participation in any other investigational trial
- Pregnant or lactating women
- Prior participation in any stem cell or any other investigational trial in the past 30 days
- Intent to participate in any other investigational drug or cell therapy study during the 2-year follow-up period of this study
- Any concurrent disease or condition that, in the opinion of the investigator, would make the patient unsuitable for participation in the study

Table 2 represents the key exclusion criteria of the AMICI trial

End points

The end points for this study are divided into safety endpoints, feasibility endpoints, and efficacy endpoints. All secondary endpoints are listed in table 3.

Table 3. Secondary endpoints

- Change in LVEF, LV end-diastolic and end-systolic volumes on MRI, MIBI-SPECT and 2D-echocardiography comparing baseline with the follow up
- Change in left ventricular wall thickness and thickening in all segments on MRI comparing baseline with the follow up
- Change in regional wall motion score index on MRI en 2D-echocardiography comparing baseline with the follow up
- Change in infarct size with late enhancement cardiac MRI comparing baseline with the follow up
- Change in perfusion defect, comparing baseline with the follow up
- Change in concentrations of NT-pro-BNP
- Score changes in the SF-36 and Seattle Angina questionnaire
- Occurrence within 24 months of a MACCE defined as cardiac death, myocardial infarction, target vessel revascularization, stroke, cardiac hospitalizations due to congestive heart failure requiring intravenous diuretics
- Occurrence of ventricular arrhythmia throughout the follow up period
- Change in CCS or NYHA classification
- Change in CFR or TIMI flow following intracoronary infusion of the MPC cell solution compared with the placebo.
- Change in reactivity of class I and II HLA with specificity
- Reaction on anti-bovine and anti-murine antibodies

Table 3 gives an overview of secondary endpoints used in the AMICI trial. LVEF indicates left ventricular ejection fraction; NT-pro-BNP: N-terminal-pro-brain-natriuretic-peptide; SF-36:short-form 36; MACCE: major adverse cardiac and cerebrovascular events; CCS: Canadian Cardiovascular Society; NYHA: New York Heart Association; CFR: coronary flow reserve; TIMI: thrombolysis in myocardial infarction; MPC: Mesenchymal progenitor cell; HLA: Human leucocyte antigen**Outcome measures of safety**

The primary safety end point is defined as the occurrence of major adverse cardiac or cerebral events (MACCE; cardiac death, myocardial infarction, target vessel revascularization, stroke, hospitalizations for heart failure requiring intravenous diuretics) during the 30 day period directly post treatment. Other safety end points are defined as the occurrence of MACCE, or any serious adverse events (SAE) and adverse events (AE) during the 24 month follow up. These clinical end points will be assessed throughout the hospitalization, as well as outpatient clinic visits at 2 weeks, and at 1, 3, 6, 12, 18 and 24 months after the procedure (table 4). To assess the occurrence of arrhythmias, patients will receive a 48 hour holter monitor at 2 weeks, 1, 3, and 6 months following MPC infusion. All data from the holter monitor will be send to and analyzed by Medpace LLC (Cincinnati, USA)

Outcome measures of feasibility

The primary feasibility endpoint is defined as the occurrence of a significant change in coronary flow during and post MPC infusion, as measured by coronary flow reserve (CFR) and TIMI flow assessments. CFR under adenosine will be obtained before and after the infusion procedure, whereas TIMI flow

Table 4. Summary of events

	Day 0	Day 2-4	14 days	1 month	3 months	6 months	12 months	18 months	24 months
Cardiac MRI		X				X	X		X
MIBI-SPECT		X				X	X		X
2D Echocardiography		X				X	X		X
NT-Pro-BNP	X			X	X	X	X		X
SF36	X			X	X	X	X		X
Seattle Angina Questionnaire	X			X	X	X	X		X
CFR	X								
TIMI	X								
MACCE	X	X	X	X	X	X	X	X	X
48 hour Holter		X	X	X	X	X			
CCS/NYHA	X	X	X	X	X	X	X	X	X
Class I en II HLA antibodies	X	X	X	X	X	X	X		X
Anti-murine and anti-bovine antibodies	X	X	X	X	X	X	X		X

Table 4 summarizes the patient follow up during 24 months. Cardiac function will be assessed by MRI, MIBI-SPECT and 2D-echocardiography 4 times during the study. The patients will visit the outpatient clinic 5 times during follow-up. Patients wellbeing and CCS/NYHA classification will be assessed additionally at 18 months follow-up during a telephone consult.
NT-pro-BNP indicates N-terminal-pro-brain-natriuretic-peptide; SF-36:short-form 36; MACCE: major adverse cardiac and cerebrovascular events; CCS: Canadian Cardiovascular Society; NYHA: New York Heart Association; CFR: coronary flow reserve; TIMI: thrombolysis in myocardial infarction; MPC: Mesenchymal progenitor cell; HLA: Human leucocyte antigen

will be assessed every 15 minutes during MPC infusion. CFR will be measured using a Combowire XT (Volcano, San Diego, USA) placed in the stented segment of the LAD. QCA of the culprit vessel will be obtained after stenting of the LAD prior to cell infusion.

Anti-bovine, anti-murine antibodies and anti-HLA antibodies

Blood will be sampled at baseline, 2 weeks, 1, 3, 6, 12 and 24 months post injection. Patients will be closely monitored for the development of anti-bovine and anti-murine antibodies, as in the process of cell culturing bovine and murine antigens are used that might lead to allergic reactions. Also, as MPC is an allogeneic cell product, patients will be monitored for the development of graft-versus-host disease, Class I and II antibodies and , leucocyte changes. All blood samples will be transported to and analyzed by ViraCore IBT Laboratories Inc.™ (Lee's Summit, USA).

Outcome measures of efficacy

The primary efficacy endpoint is defined as the change in relative infarct size as assessed by delayed enhancement cardiac MRI (DE-CMR) from baseline to 6 months follow up. Secondary efficacy endpoints (evaluated up to 24 months) will include CMR and 2D-echocardiography measures of infarct weight and size, left ventricular function and dimensions, wall thickness and thickening, wall motion

score and myocardial salvage index. Nuclear imaging (MIBI-SPECT) will provide data on left ventricular function and dimensions, and the perfusion defect at rest. NT-pro-BNP levels will be determined as a marker for the presence and severity of heart failure. To assess implications of the acute myocardial infarction on the patients wellbeing, impact of the disease on their life and severity of anginal state, patients will be evaluated using the Medical Outcome Study Short Form (SF-36), the Seattle Angina Questionnaire, New York Heart Association (NYHA) functional classification, Canadian cardiovascular society classification (CCS) and Killip classification . All secondary efficacy endpoints are listed in table 3.

Cardiac MRI

Baseline MRI procedure will take place 2-4 days after primary PCI and cell/placebo infusion.
Patients will undergo CMR at 2-4 days post myocardial infarction (baseline), 6, 12 and 24 months after the index procedure using 1.5 or 3.0 Tesla scanners. In brief, ECG-gated steady state free-precision images are obtained during breath hold in 4, 3, and 2 chamber cine. Short axis slices are obtained of the whole left ventricle for measurements of regional and global left ventricular function. Delayed enhancement images to determine infarct size (T1/T2 weighed images) are acquired 10 minutes after intravenous infusion (0.2 mmol/kg) of gadolinium-based contrast agent. Infarct size is calculated as total percentage infarct volume divided by total left ventricle tissue volume. Data analyses will be performed by an independent, blinded core lab (Imagepace LLC, Cincinnati, USA).

2D echocardiography

At baseline (2-4 days), 6, 12 and 24 months a transthoracic 2D ultrasound will be made to assess left ventricular ejection fraction, cardiac dimensions (LVEDV, LVESV), and regional wall motion score index. Images are obtained using a Phillips iE33 echo machine (Phillips, Eindhoven, The Netherlands), or a comparable device. Standard parasternal long axis (2-, 3-, 4-chamber view) and short axis (basal, mid-ventricular and apical) are acquired. The images will be analyzed by an independent, blinded core lab (Imagepace LLC, Cincinnati, USA). Left ventricular ejection fraction as well as cardiac dimensions will be obtained using parasternal 2 and 4 chamber view, using Simpson's method. A parasternal short axis view is obtained to calculate wall motions score index according to the 16 segments method.

MIBI-SPECT

Myocardial perfusion assessment by a MIBI-SPECT scan will be performed 2-4 days after AMI (baseline) and at 6, 12 and 24 months follow up, to obtain data about the perfusion defect at rest, LVEF and left ventricular end-diastolic and end-systolic volumes. Two hours before scanning TC-99-sestamibi will be intravenously administered, followed by scanning of the patient by a gamma camera. Analyses will be performed by Imagepace LLC (Cincinnati, USA).

The investigational product and placebo solution

Subjects will be randomized to receive approximately 12.5 million or 25 million allogeneic MPC

(in a bag of 4 cc) suspended in 100 cc of Ringers Lactate. The allogeneic MPCs are formulated in concentrations of 15 and 30 million nucleated cells in a 5-mL volume and are cryopreserved in 7.5% dimethyl sulfoxide (DMSO)/50% Alpha Modified Eagle's Medium (MEM) and 42.5% ProFreeze®. Placebo solution is sterile saline. The bags are stored in a tank with liquid nitrogen. The cells or placebo solution are rapidly thawed and dissolved in 100 cc of Lactated Ringers directly before infusion. The MPC dosages and placebo solution will be administered via direct intracoronary infusion via a micro-catheter (Twin Pass, Vascular Solutions, Minneapolis, USA) following the primary PCI at each site's cardiac catheterization suite.

Cell infusion Procedure

Directly after successful revascularization of the LAD, the cells will be administered by continuous infusion via a micro-catheter that is positioned in the stented segment of the culprit artery. The MPCs or placebo solution will be infused in 50 minutes at an infusion rate of 2cc/min (0.25×10^6 cells/min or 0.5×10^6 cells/min). Before MPC infusion, CFR is measured . During MPC infusion, the infusion will be stopped after every 33 cc (approximately every 15 minutes) and TIMI flow will be obtained. After completion of the cell infusion, CFR and TIMI flow will be determined again.

Sample size calculation

The study is planned for a sample of 150 treated subjects and 75 control subjects. In a reference study the infarct size reduction by MRI within each subject group was distributed with a standard deviation of 8.2.[26] If the true difference in the experimental and control means is 4.0 we will be able to reject the null hypothesis that the population means of the experimental and control groups are equal with a power of 0.84. The type I error probability associated with this test of the null hypothesis is 0.05. The sample size of 225 subjects is adequate to assess the primary endpoint of safety and feasibility utilizing descriptive statistics. Adjustment for approximately 10% early termination rate has been taken into consideration for the 225 subject sample size.

Current status

In total of 20 US, 30 European and 12 Australian sites will participate in the AMICI trial. The first patients are currently included.

DISCUSSION

The current phase I/II trial as outlined above, was designed to investigate the safety, feasibility and functional effects of intracoronary administration of MPC (Revascor™) in the treatment of an 'all-comers' population of large anterior wall AMI. The integrated phase I/II design of this study has several advantages. The primary safety analysis is performed 30 days after the inclusion of 30, 60, and 90 patients. However, each time the primary safety end point has been met, there is no delay in continuing the study. Also, all patients can be included in both long term safety and efficacy follow

up. Moreover, a new power analysis is performed after reaching the 6 month follow up of the first 60 and 120 patients, after which the number needed for reaching the primary efficacy end point can be adjusted downward, and fewer patients might be needed.

Another unique feature of the current study design is that we aim to include an "all-comers" patient population presenting with anterior wall AMI due to occlusion of the LAD. In contrast to several other stem cell studies, the amount of impairment of the left ventricular ejection fraction (LVEF) is not an in- or exclusion criterion. Although it is hypothesized that patients presenting with large myocardial infarcts benefit most from cellular therapy [7], assessing this baseline LVEF costs valuable time that is lacking in the current protocol. Moreover, in a previous study we found that all patients with a baseline LVEF below 50%, all presented with an anterior wall AMI.[27] Also, due to increasingly shorter door-to-balloon times in the Western world, patients with significantly impaired LVEF following their first AMI are getting rare. This implies that including only patients with an LVEF below 45-50% will slow down the inclusion of patients in this study considerably. By investigating this 'all comers' anterior wall AMI population, patients with both extensive and less extensive infarcts will be included, thereby providing valuable information on which patients benefit most from cellular therapy.

In most cell therapy studies to date, the predominantly used primary efficacy end point was left ventricular function as determined by LVEF. However, in these studies the overall effect of cell therapy on LVEF has been modest to none.[7] It is believed that current pharmacotherapy following AMI reduces the pace of LV remodeling, thereby preserving LVEF for a considerable time and exceeding the follow up time of these stem cell studies. We therefore believe that LVEF might not be the optimal end point in cell therapy studies. In contrast, in the recent Apollo trial that investigated the intracoronary infusion of adipose tissue-derived regenerative cells led to a significant decrease of infarct size in treated patients.[27] This finding was confirmed with preclinical data.[28] Therefore, in the AMICI trial, the change in infarct size from baseline to 6 months follow up as determined by DE-CMR is the primary efficacy end point. Although intravenous administration of mesenchymal stem cells (MSC) has been established before [14], the current study is the first large, multicenter study to assess the safety and efficacy of intracoronary infusion of a mesenchymal cell population. The MPC used in this study are a Stro3+ selected sub-population of bone marrow-derived MSC, and the cardioprotective effects of these cells were shown to exceed the cardioprotective effects of regular MSC in several pre-clinical studies.[20,21] In a recent randomized study in 88 sheep, it was found that intracoronary infusion of this investigational product had marked effects on LV remodeling and function, evoked by cardiomyocyte salvage and neo-angiogenesis.[28] It is hypothesized that the properties of MPC to secrete larger amounts of paracrine, cardioprotective factors, including SDF-1, result in less cardiomyocyte apoptosis following the ischemic insult. Also, MPC have the phenotype and characteristics of a vascular pericyte, and they secrete angiogenic factors including VEGF, SDF-1, HGF-1 and Ang-2. Intracoronary infusion of MPC in the culprit artery thereby stimulates (neo-)angiogenesis in the infarcted area, eventually leading to increased perfusion and preservation of cardiac function. Because myocardial salvage is believed to be the predominant working mechanism of cell therapy in AMI using MPC, in the AMICI trial the cells will be delivered directly following the primary PCI. Directly following MI, most cardiomyocytes are at

risk for ischemia-reperfusion injury, therefore the anti-apoptotic and cardioprotective properties of the MPC will be maximally utilized.

An allogeneic, ”off-the-shelf”, cell therapy product, originally derived from a young and healthy donor, has important advantages. It renders a laborious and potentially dangerous BM puncture, as well as the subsequent culturing steps in clean room facilities, unnecessary. In addition, the stable stem cell line ensures adequate quality control with inherent batch-to-batch consistency. Also, a negative correlation was found between the amount and functionality of progenitor cells, and age and cardiovascular risk factors.[29] This would make the use of allogeneic MPC in the typically elderly, cardiovascular patient population preferable over autologous cells. More importantly, the cell therapy can be initiated directly after the revascularization of the AMI, thereby maximally utilizing the anti-apoptotic and immunomodulatory capacities of the cells.

The safety of administrating allogeneic MSC to AMI patients was shown before.[14] As MSC, MPC lack the HLA II DR-domain; therefore no cytotoxic T-cell or natural killer cell response is provoked. Indeed, in a recent clinical phase IIa study, that assessed the safety and feasibility of intramyocardial injections of allogeneic MPC in 60 heart failure patients, allogeneic MPC injections up to a dose of 150 million cells were shown to be safe and feasible without a clinically significant anti-allogeneic immune response.[22] Importantly, the total cell dose injected in this study was 6 times higher as the maximum cell dose that is tested in the AMICI trial. Still, all patients in the AMICI trial are rigorously monitored for class I and II antibodies, C-reactive protein, anti-bovine and anti-murine antibodies. after 30 days, and 3 and 6 months following MPC infusion.

The AMICI trial is the first study to investigate the safety and feasibility of intracoronary infusion of allogeneic stem cells. Although intracoronary stem cell delivery is the preferred delivery method following AMI[13,30], it was deemed impossible to administer cultured stem cells intracoronarily, as several preclinical studies reported vascular plugging and the occurrence of myocardial infarctions following stem cell infusion.[31,32] However, MPC have some characteristics that might make them more suitable for intracoronary infusion than other cell types, the most important one being the average cell diameter of only 13 micron[28] Indeed, in the large animal study mentioned before, it was found that, when certain conditions are adopted, intracoronary infusion of MPC directly following AMI can be performed safely, without compromising coronary flow.[28] The favorable safety results of this preclinical study resulted in the design of the AMICI trial and dictated the infusion conditions that were adopted.

Implications

When intracoronary infusion of mesenchymal precursor cells has been proven to be safe and feasible and thereby results in functional improvements, this therapy can be a new therapeutic corner stone in the treatment of AMI and prevention of post AMI heart failure.

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PART 4

Encapsulated Mesenchymal Stem Cells

CHAPTER 9

Feasibility of intracoronary GLP-1 eluting CellBead™ infusion in acute myocardial infarction

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ABSTRACT

Background Cell therapy is a field of growing interest in the prevention of post acute myocardial infarction (AMI) heart failure. Stem cell retention upon local delivery to the heart, however, is still unsatisfactory. CellBeads were recently developed as a potential solution to this problem. CellBeads are 170 µm alginate microspheres that contain mesenchymal stem cells (MSC) genetically modified to express glucagon-like peptide-1 (GLP-1) supplementary to inherent paracrine factors. GLP-1 is an incretin hormone that has both anti-apoptotic and cardio-protective effects. Transplanting CellBeads in the post-AMI heart might induce cardiomyocyte salvage and ultimately abrogate adverse cardiac remodeling. We aimed to investigate the feasibility of intracoronary infusion of CellBeads in a large animal model of AMI.

Methods and results Four pigs were used in a pilot study to assess the maximal safe dose of CellBeads. In the remaining 21 animals, an AMI was induced by balloon occlusion of the left circumflex coronary artery for 90 minutes. During reperfusion, 60,000 CellBeads (n=11), control beads (n=4) or lactated Ringers' (n=6) were infused. Animals were sacrificed after two or seven days and the hearts excised for histological analyses.

Intracoronary infusion did not permanently affect coronary flow in any of the groups. Histological analysis revealed Cellbeads containing viable MSCs up to seven days. Viability and activity of the MSCs was confirmed by qPCR analysis that showed expression of recombinant GLP-1 and human genes after two and seven days.

CellBeads reduced inflammatory infiltration by 29% (P=0.001). In addition, they decreased the extent of apoptosis by 25% (P=0.001) after two days.

Conclusion We show that intracoronary infusion of 5 million encapsulated MSCs is safe and feasible. Also, several parameters indicate that the cells have paracrine effects, suggesting a potential therapeutic benefit of this new approach.

INTRODUCTION

Despite advances in pharmacotherapy and interventional cardiology, heart failure constitutes a growing patient population in the Western world (12). Acute myocardial infarction (AMI) is the major underlying etiology of congestive heart failure (14,28). Because an AMI leads to irreversible loss of cardiomyocytes, and cardiomyocytes have limited regenerative or compensatory capacity, loss of cardiomyocytes irrevocably leads to fibrosis and scar tissue formation (5). Subsequent adverse left ventricular remodeling can cause the heart to fail and lead to the clinical symptoms of heart failure with poor prognosis (6,7).

The cornerstone of the treatment of AMI patients, which is reperfusion in combination with medical treatment, only partly prevents scar tissue formation and remodeling of the damaged heart (8). The prevention of cardiomyocyte loss and remodeling has therefore been the topic of extensive research. The field of regenerative cellular therapy is an area of growing interest, in particular in AMI patients. In fact, small scale clinical cell therapy trials show promising results, using bone-marrow derived mononuclear cells (1,15).

The predominant working mechanism of post-AMI cell therapy is believed to be through the paracrine action of the grafted cells, resulting in myocardial salvage and neo-angiogenesis (13,20,25). The mesenchymal stem cell (MSC) is currently hypothesized to be the most potent, non-embryonic cell with respect to secretion of relevant paracrine growth factors, anti-apoptotic and pro-survival factors, as well as immunomodulatory cytokines, and have been shown to exert cardio-protective effects *in vivo* (3,16,19,30).

Also, a number of recent studies has shown that glucagon-like peptide-1 (GLP-1), one of the most potent incretin hormones, has potential beneficial action on the ischemic and failing heart (2,17,22,24). GLP-1 is a naturally occurring incretin with both insulinotropic and insulinomimetic properties, and has been shown to exert anti-apoptic actions. Interestingly, when added to standard therapy, GLP-1 infusion improved regional and global left ventricular function in a clinical study with AMI patients with severe systolic dysfunction after successful primary coronary intervention (PCI) (18). However, due to its very short half-life, a prolonged infusion of 72 hours with substantial side-effects for the patients was needed to achieve this beneficial effect.

One of the biggest challenges in the cell therapy field today is the poor retention of therapeutic cells upon local delivery in the heart, with retention rates as low as 1% after intracoronary delivery (10,11,27). Even though permanent engraftment of stem cells is not required to illicit the cardio-protective effect, it seems logical that the greater the number of cells that are retained in the injured myocardium and the longer they reside there, the more pronounced the potential beneficial effect may be. Despite wide-ranging efforts to increase cell retention using various delivery techniques, results are still unsatisfactory. A new concept of stem cell delivery has recently become available owing to advances in the field of biotechnology, as it is currently possible to encapsulate MSC in a biocompatible alginate shell (26,29). Alginate encapsulation of varying numbers of MSC results in so-called CellBeads™, available in discrete sizes between 150 and 600 µm.

CellBeads are made from a highly purified alginate material, which is used to encapsulate clusters of adult human MSCs. These MSCs have been genetically modified to secrete a proprietary recombinant GLP-1 fusion protein, which consists of two GLP-1 molecules bound by an intervening peptide. This form of GLP-1 is more stable than endogenous GLP-1, rendering a longer half-life and thus prolonged therapeutic potential. The alginate coating of the CellBeads is permeable to the GLP-1 fusion protein, allowing for continuous delivery, while protecting the MSCs from the patient's immune system. Also, oxygen and nutrients can freely pass through the alginate shell, which renders the MSCs viable for a long period of time. Thus, Cellbeads are potentially a unique, biological, long-term, local drug delivery platform that is capable of delivering GLP-1, or other therapeutic proteins, in addition to MSC-derived factors (VEGF, MCP-1, IL-6, IL-8, GDNF and NT-3) to any target tissue.
We hypothesize that when transplanted in the post-AMI heart, the synergystic effect of paracrine MSC-derived factors together with the cardio-protective GLP-1 peptide, might evoke myocardial salvage, reduce apoptosis and influence the inflammatory response in the acute phase of the AMI. In the long term, CellBead therapy may induce angiogenesis, and decrease post-AMI adverse cardiac remodeling. The aim of the current study was to evaluate the feasibility and safety of intracoronary delivery of 170 μm CellBeads in the acute phase of an AMI in a relevant large animal model.

METHODS

Experimental animals

All procedures were approved by the local animal welfare committee. A total of 25 female landrace pigs (Van Beek SPF pigs, Putten, The Netherlands, 69.2 ± 1.0 kg) were used in this study.

Experimental design.

This study was subdivided in two phases. In the first phase (phase 1), the feasibility of intracoronary injection of CellBeads was assessed in naïve, non-infarcted myocardium in four pigs. The goal of this phase was to determine the maximum dose of CellBeads to be delivered safely by continuously assessing coronary flow, or the occurrence of fatal arrhythmias upon CellBead infusion.
In phase 2, 21 pigs underwent an AMI by balloon occlusion, followed by intracoronary infusion of CellBeads to assess the feasibility and safety of CellBead infusion in AMI. Animals received intracoronary infusion of lactated Ringers' (LR) solution (n=6), alginate-only control beads (n=4) or GLP-1 expressing CellBeads (n=11). Coronary flow was assessed prior to, during and after CellBead infusion using coronary angiography, Doppler-aided coronary flow (phase 1) and coronary flow reserve (CFR; phase 2) measurements. Animals were sacrificed after two or seven days and the heart was excised for histological analysis (see figure 1 for study flow chart). In addition, the pigs received an event recorder to monitor for arrhythmic events.

Medication and anesthesia

The animals were pre-medicated for ten days with dual anti-platelet therapy (acetylsalicylic acid 80 mg qd and clopidogrel 75 mg qd) and anti-arrhythmic therapy (only in phase 2; amiodarone 400 mg qd). General anesthesia was induced and maintained with intravenous infusion of midazolam, sufentanil and pancuronium. Upon infarct induction and Cellbead infusion, all animals were therapeutically heparinized and received intravenous infusion of eptifibatide (bolus of 180 µg/kg and 2 µg/kg/min). A fentanyl plaster was applied after the procedure for analgesia. Dual antiplatelet and anti-arrhythmic therapy was continued until the sacrifice procedure.

Induction of the acute myocardial infarction

A myocardial infarction was only induced in phase 2 of the current study. Although in phase 1 the LAD was used to infuse CellBeads, we chose to switch to occlusion of the proximal left circumflex artery

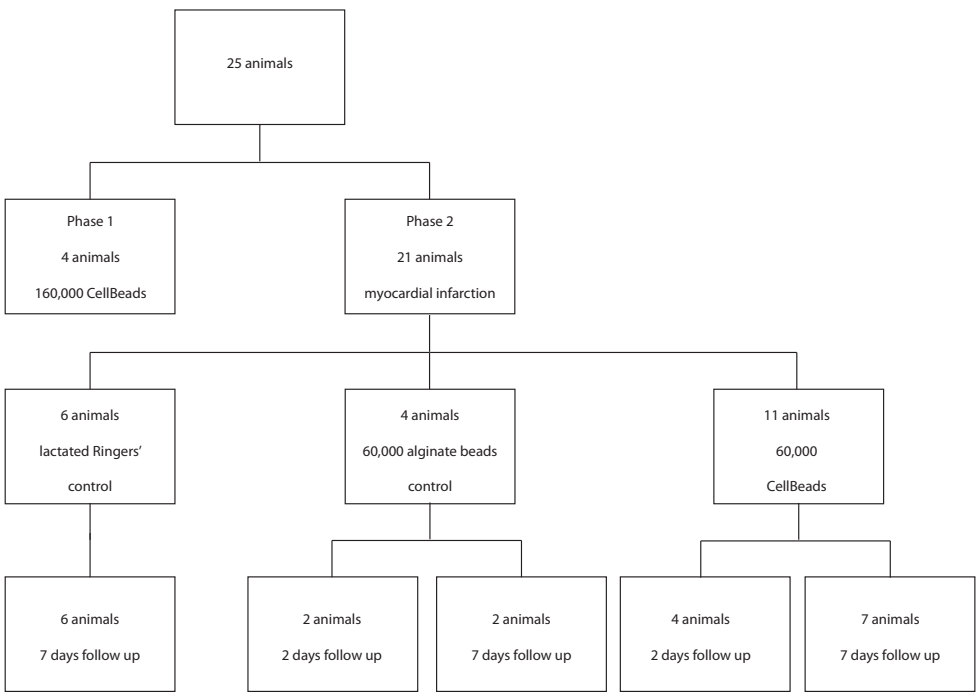


Figure 1. Study flow chart.

(LCX) for induction of the AMI due to an expected higher survival rate. Catheterisation of the left coronary system was performed via the right carotid artery. A left coronary angiogram was made to determine the optimal position for balloon occlusion, followed by inflation of an angioplasty balloon (Voyager Rx 3.5-4.0x12 mm, Abbott, Illinois, USA) for 90 minutes in the proximal LCX to induce an acute posterolateral myocardial infarction.

CellBead infusion and coronary flow assessment

CellBeads were thawed rapidly and diluted in a large volume of LR (150,000 Cellbeads in 250 mL of LR, rendering a concentration of 600 CellBeads per mL).

In naïve animals (phase 1), a micro-catheter (TwinPass, Vascular Solutions, Illinois, USA) was placed in the left anterior descending (LAD) coronary artery, distal to the first diagonal branch. The infusion rate in the first two animals was set at 4 mL/min or 2,400 CellBeads/min. In animals 3 and 4, the infusion rate was reduced to 2 mL/min or 1,200 CellBeads/min. During CellBead infusion, coronary flow was assessed regularly by coronary angiography, and depicted using the conventional TIMI flow grade nomenclature (23). Also, coronary flow was continuously measured during infusion using a Doppler flow wire (Combowire, Volcano, San Diego, USA) and expressed as average peak velocity (APV).

In phase 1, the maximum amount of CellBeads to be safely delivered without compromising coronary flow was found to be 60,000. Thus, in phase 2 (AMI animals), either 60,000 CellBeads (n=11), 60,000 control alginate beads (n=4), or LR alone (n=6) were infused intracoronarily. All animals received two 50 mL syringes, containing 30,000 beads or LR only each. These syringes were infused using a syringe pump (IVAC, Humberside, United Kingdom) at a constant flow rate of 2 ml/min (1,200 CellBeads/min). During infusion, setting of the beads was prevented by regular rocking of the syringe pump.

Beads were delivered after approximately 15 minutes of reperfusion, a TwinPass infusion catheter was placed in the proximal LCX. Coronary angiography was performed before infusion, at 25%, 50%, and 75 % of infusion and ten minutes after infusion of the beads, to determine TIMI flow grade. Also, coronary flow reserve (CFR) was measured before and after bead or LR infusion. CFR was determined by dividing the APV at maximal vasodilatation during adenosine infusion by the baseline APV. Six animals were sacrificed two days after the AMI and bead infusion (four animals that received CellBeads, two control beads), and 15 animals were sacrificed at day seven (seven Cellbeads, two control beads, six LR control; see figure 1). At sacrifice, TIMI flow grade was measured, after which the animals were terminated and the hearts excised for histological analysis. TIMI flow grade was determined by an independent cathlab technician, who was blinded to the treatment of the individual animals.

Event recorder

After the infusion procedure, all pigs were fitted with a REVEAL DX event recorder (Medtronic, Minneapolis, USA). The recorders were placed subcutaneously in the left thorax. The occurrence of arrhythmias was assessed prior to termination using a CareLink™ programmer (Medtronic, Minneapolis, USA).

Macroscopic and microscopic analysis

In phase two of this study, animals were euthanized at day two or seven and the hearts were excised and sectioned in five to six bread-loafed slices from the apex to the base. The slices were stained in 2,3,5-triphenyltetrazoliumchloride (TTC) to delineate the infarct area from non-infarcted myocardium. The non-infarcted myocardium was thoroughly screened for the presence of micro-infarctions. The

Table 1. Primer sequences used for qPCR

Recombinant GLP-1	
CM-1 forward	GTGAGCTCTTATCTGGAAGGCC
CM-1 reverse	AGATAAGAGCTCACATCGCTGG
Human household genes	
HPRT forward	AATGACCAGTCAACAGGGGAC
HPRT reverse	CCTGACCAAGGAAAGCAAAGTC
Porcine household gene	
HPRT forward	AATGACCAGTCAACGGGCGAT
HPRT reverse	CTTGACCAAGGAAAGCAAGGTT

Abbreviations: qPCR: quantitative polymerase chain reaction; HPRT: hypoxanthine-guanine phosphoribosyl transferase 1.

entire infarct area and biopsies of remote myocardial segments (anterior and anteroseptal wall) were taken and fixed in formalin, embedded in paraffin and sliced into 5 µm sections. All sections were stained by hematoxylin and eosin staining (H&E-staining) using a standard protocol. Sections were examined by a pathologist specialized in cardiac histopathology.

H&E stained sections of remote myocardial segments were investigated for signs of micro-infarctions or the presence of shedded CellBeads. Sections of infarct tissue of each study animal were investigated for the presence of CellBeads or control beads, inflammatory reactions and/or foreign body response. More specifically, the composition and surface area of the inflammatory infiltrate surrounding each CellBead or control bead was assessed and measured using an Olympus BX45 microscope with a reticle. We only investigated totally transverse cut beads and measured the distance from the bead to the periphery of the surrounding infiltrate.

TUNEL staining for apoptosis

Apoptosis was assessed using an 'In situ cell death detection kit' (Roche, Mannheim, Germany). Samples were counterstained with hematoxylin. At least 30 random pictures were taken from slides of animals that received CellBeads or control beads and were sacrificed at two or seven days. TUNEL and hematoxylin double positive nuclei were counted and expressed as positive cells/100 μm^2.

Quantitative PCR analysis of infarct specimens

For determination of recombinant GLP-1 expression in the target area, small samples of the infarct were taken of animals that survived two or seven days. RNA was extracted using RNA-Bee (Tel-test Inc., Friendswood, Texas, USA) according to the manufacturer's protocol. Quality and quantity of the RNA was verified on an Agilent 2100 Bioanalyzer (Agilent Technologies, UK), and reverse transcribed. Quantitative PCR (qPCR) analysis was performed using an iCycler iQ Detection System (Bio-Rad, the Netherlands). Primers were designed selectively for the recombinant GLP-1 dimer and not endogenous GLP-1, a porcine household gene (hypoxanthine-guanine phosphoribosyl transferase 1; HPRT) and a human household genes (HPRT). mRNA levels detected by qPCR were expressed relative to the porcine household genes. The primer sequences are provided in table 1.

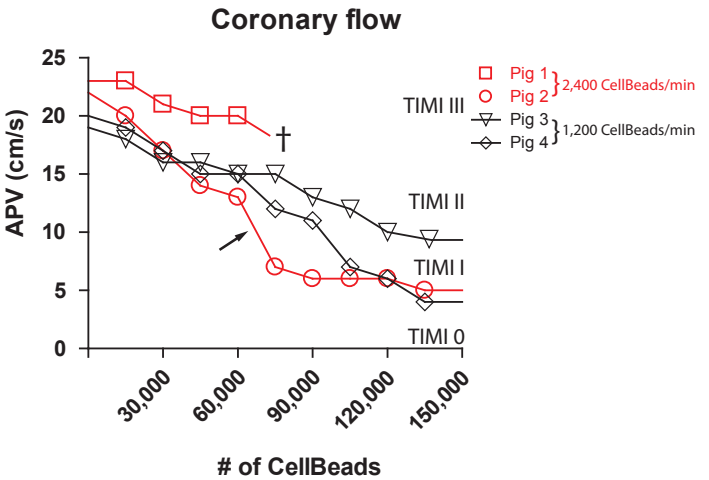


Figure 2. Change of coronary flow upon CellBead infusion in naïve, non-ischemic myocardium.

The individual points represent the APV that was measured every 15,000 CellBeads, whereas the corresponding TIMI flow is depicted on the right side of the graph. Intracoronary infusion of CellBeads reduces APV in a dose-dependent manner. An infusion rate of 2,400 CellBeads/min. caused fatal arrhythmia in one animal and a steep decline of coronary flow (both APV and TIMI flow) briefly after the infusion of 60,000 CellBeads (arrow). Reducing the infusion rate to 1,200 CellBeads/min. increased the amount of CellBeads that could be delivered before the APV dropped >50% from baseline. APV: average peak velocity.

Statistical analysis

The current study was designed as a pilot feasibility study, so no formal power calculations were performed. All statistical analyses were performed post hoc using SPSS 16 statistical software (IBM, Chicago, USA). An analysis of variance (ANOVA) was performed and a Bonferroni correction was used for pair wise comparison between means. Data are presented as mean ± SEM. P values ≤ 0.05 were considered significant.

RESULTS

Results of phase 1 (naïve animals)

All four experiments were a procedural success and it proved to be possible to infuse CellBeads selectively via intracoronary infusion. In the first pig, 75,000 CellBeads were administered without any sign of ischemia or reduction of flow as depicted by APV (Figure 2; delta-APV 5/23) However, after infusion of approximately 80,000 CellBeads several premature ventricular complexes occurred that ultimately progressed into ventricular fibrillation and death of the animal.

In pig 2, APV gradually decreased until the infusion of 60,000 CellBeads, after which a steep decline in APV occurred accompanied by a decrease in TIMI flow from TIMI grade III to I. Hence, we decided to decrease infusion rate in the remainder of the animals from 2,400 CellBeads/min. to 1,200 CellBeads/min. As a result, we found that in the remaining two animals the amount of CellBeads that

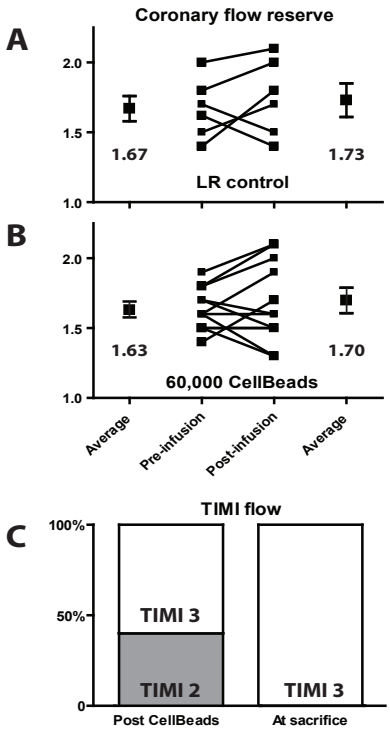


Figure 3. Coronary flow infusion in AMI pigs.

A. CFR remained unchanged after infusion of LR. **B.** Intracoronary infusion of CellBeads did not alter CFR. **C.** TIMI flow was slightly reduced in 40% of the animals that received intracoronary infusion of 170 µm beads, but had recovered completely at sacrifice.

could be delivered before the decline in APV (defined as >50% of baseline value) and TIMI flow was significant was higher (90,000 Cellbeads). Blood pressure remained stable in all four animals, showing no significant acute effect of CellBead infusion on cardiac performance. After reviewing the results of phase 1, we concluded that the maximal safe dose that can be infused in naïve/non-AMI pigs is 60,000 CellBeads. Therefore, in phase 2 the feasibility of intracoronary delivery of 60,000 CellBeads was evaluated in a pig LCX AMI model.

Results in porcine AMI model (phase 2)

Animal experiments

A total of 21 pigs were included in this phase of the study. Three animals experienced ventricular fibrillation during infarct induction, but all animals were successfully resuscitated. Animals received 60,000 CellBeads (n=11), 60,000 control beads (n=4) or LR (n=6). No lethal ventricular arrhythmias occurred during CellBead or control bead infusion, and all animals survived the dedicated follow-up time.

Coronary flow reserve

In control animals, average CFR before LR infusion was 1.67 ± 0.09 and 1.73 ± 0.12 after LR infusion (Figure 3A). In the pigs that received intracoronary infusion of CellBeads or control beads CFR remained unchanged with an average CFR of 1.63 ± 0.04 before infusion, and 1.70 ± 0.09 afterwards (p=ns; Figure 3B).

TIMI flow

All pigs had normal coronary flow after the induction of the AMI, defined as TIMI grade 3 flow. Infusion of 100 mL LR in control animals did not impede TIMI flow. Also, no effect on antegrade coronary flow was found in 60% (9/15) of the animals that received CellBeads or control beads. However, 40% (6/15) of the animals that received CellBeads or control beads experienced a slight reduction of coronary flow, resulting in sluggish flow (TIMI 2) in the culprit artery after the infusion of 60,000 beads (Figure 3C). The occurrence of sluggish flow was predominant during the infusion of the final 15,000 beads, as coronary flow remained unchanged until 45,000 of all 60,000 beads were infused.

Coronary flow returned to normal TIMI 3 flow at the day of sacrifice (day two or seven). There was no difference in coronary flow between animals that survived two days or seven days, or between animals that received CellBeads or control beads.

Analysis of arrhythmias

None of the animals, either control or CellBead-treated, experienced ventricular arrhythmias during the two or seven day follow-up period.

Histology

H&E staining

H&E stained sections of all myocardial segments were examined from all animals included in phase 2. No beads or micro-infarctions could be detected in remote myocardial segments. On the contrary, in infarcted tissue specimens, multiple beads were detected per section, as the majority of 100-200 µm arterioles contained one or more beads. As far as we could determine with conventional histological analysis of sections of the whole infarct area, the CellBeads were distributed equally throughout the infarct. CellBeads in pigs that survived two days as well as in pigs that survived seven days all contained viable MSCs, as was determined by the presence of basophilic nuclei (Figure 4B/C).

Two days after infarction, the cardiac tissue showed infiltration of predominantly neutrofilic granulocytes and loss of cardiomyocyte viability. After seven days, a clear fibrotic response was present with fibroblasts infiltrating the infarcted area. Also, there was a marked increase of the inflammatory infiltrate as compared to the two day animals, which is normal seven days post-AMI.

Inflammatory infiltration surrounding CellBeads

All the beads showed a thin rim of fibrin with neutrophilic granulocytes (Figure 5A). In addition, the beads were variably surrounded by a rim of lymphocytes and plasma cells. We noted that the control alginate beads showed significantly more inflammatory infiltrate directly surrounding the bead when compared to CellBeads (0.038 ± 0.004 mm^2 vs. 0.027 ± 0.004 mm^2, p= 0.003; Figure 5B/C) two days following the AMI.

At the seven day time-point, the natural healing process of the AMI caused such a background inflammatory infiltrate that measurements of the infiltrate surrounding the beads were not reliable. We therefore performed the analysis at the two day follow-up time-point after AMI only. Since this

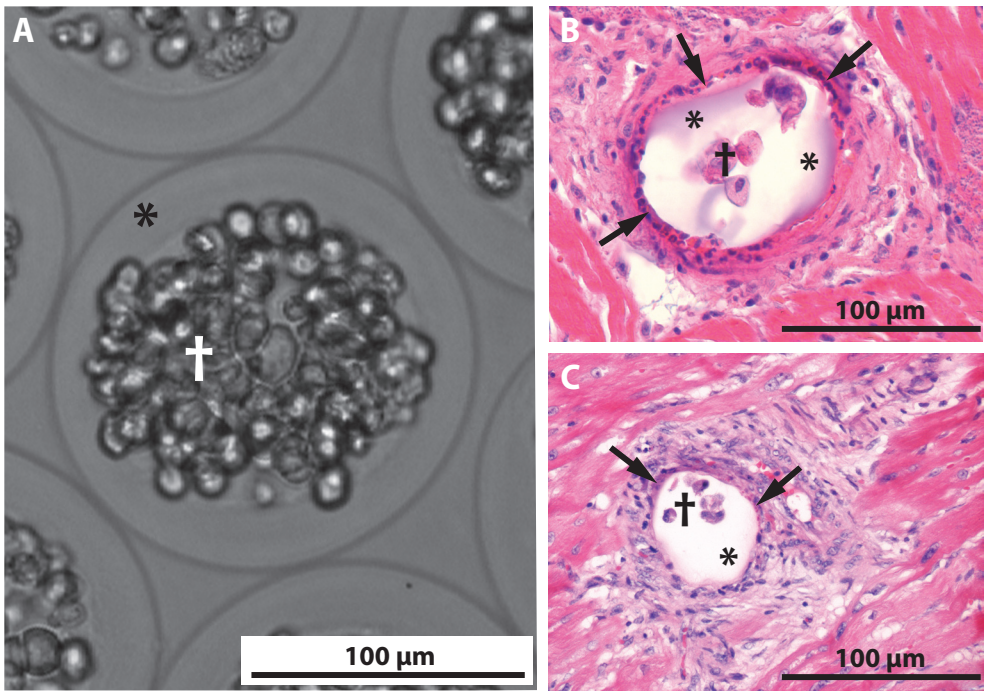


Figure 4. Photomicrograph of micro Cellbeads.

A. Light photomicrograph of a CellBead prior to infusion. The alginate shell (*) and MSC-containing core (†) can be appreciated. **B.** H&E stained section of a coronary arteriole completely comprised of one CellBead, two days after infusion. Only a thin rim of fibrin and granulocytes surrounds the CellBead (arrows). **C.** H&E stained section of a coronary arteriole containing one CellBead, seven days after infusion. Only a thin rim of fibrin and granulocytes surrounds the CellBead (arrows).

* Intact alginate; †.Mesenchymal stem cells.

inflammatory infiltrate after seven days was equally intense in the LR control animals, we considered this as a normal phase in the healing process.

Apoptosis

Apoptosis was significantly reduced in infarct areas of animals that received CellBeads as opposed to animals that received control beads. More specifically, in infarct tissue specimens of animals that received control beads, on average 7.5 ± 0.44 cells were apoptotic per 100 µm^2 after two days. This number was significantly reduced in animals that received CellBeads to 5.6 ± 0.36 per 100 µm^2 (p= 0.003; Figure 6). After seven days, the number of apoptotic cells had decreased to 3.3 ± 0.41 per 100 µm^2 in animals that received control beads as opposed to 2.3 ± 0.29 per 100 µm^2 in animals that received CellBeads (p= 0.34).

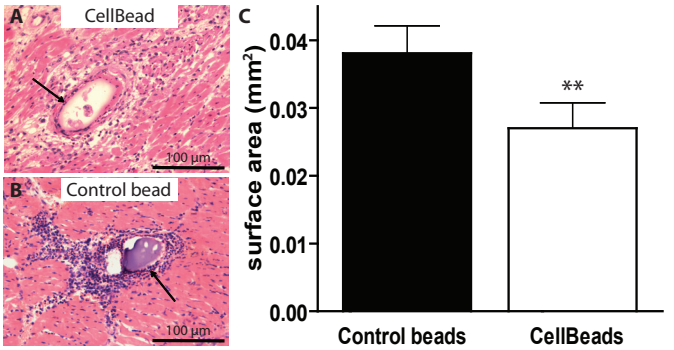
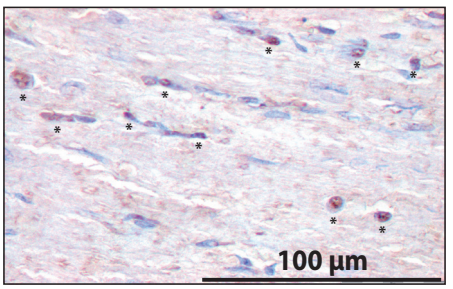


Figure 5. Peri-bead inflammatory infiltration after two days.

A. A thin rim of neutrophilic granulocytes is evident surrounding a single CellBead (arrow). **B.** A more pronounced inflammatory reaction around a control alginate bead (arrow). **C.** Comparison of inflammatory reaction by measuring the surface area of the inflammatory infiltrate reveals a significant decrease in inflammatory response to the CellBeads as compared to the control alginate beads (** p= 0.003).

Quantitative PCR analysis of infarct specimens

No expression of recombinant GLP-1 or human genes was found in the hearts of the control pigs. However, expression of recombinant GLP-1, together with the human household gene HPRT was found in infarct segments of animals that received GLP-1 expressing CellBeads, confirming the presence of CellBeads in the infarcted heart that contain viable and actively transcribing human MSC. Expression levels of recombinant human GLP-1 was comparable between specimens from animals that survived two and seven days (Figure 7).



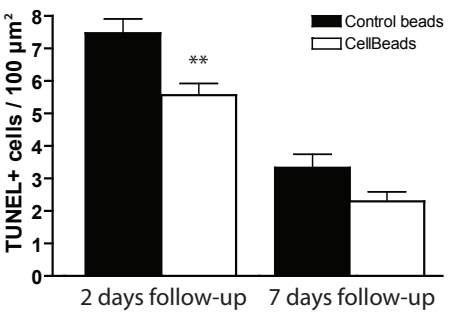


Figure 6. TUNEL staining for apoptosis.

(Above) Histological section of infarct tissue two days after the AMI, demonstrating apoptotic cardiomyocytes (*) throughout the tissue. (Below) Quantification of TUNEL positive cells revealed a significant difference in the amount of apoptosis in CellBead-treated tissue compared to controls after two days (** p= 0.003), but not after seven days (p=0.34).

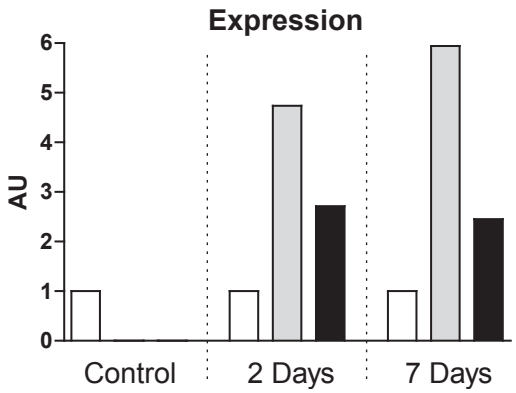


Figure 7. Quantitative PCR analysis of infarct samples.

qPCR revealed expression of both the human household gene (grey bar) and recombinant GLP-1 (black bar) as compared to porcine household gene (white bar) in animals that received CellBeads after two and seven days compared to no expression in control animals. The values of the porcine household gene was arbitrarily set to one.

DISCUSSION

The current study demonstrates for the first time that it is feasible to intracoronarily infuse alginate, MSC-containing micro-spheres in infarcted myocardium. Infusion did not permanently affect coronary flow, resulted in precise targeting of the infarct area, and MSCs remained viable for at least seven days. Interestingly, the inflammatory response to CellBeads was negligible in this xenogeneic transplantation model.

We found that intracoronary infusion of significant numbers of 170 µm particles did not severely hamper coronary flow. On the contrary, CFR remained unchanged in all animals that received 60,000 CellBeads or control beads, suggesting little or no micro-vascular obstruction. And although coronary flow as assessed by angiography was slightly decreased to sluggish flow in 40% of the animals, it always returned to normal flow within two or seven days follow-up. This suggests that, even though the coronary vasculature might have been partly obstructed by the beads, this obstruction was temporary, and that alternative collateral routes were found to perfuse the inflicted myocardium. Also, we hypothesize that infusion of limited amounts of obstructive beads into already infarcted myocardium will not increase ischemic damage. Importantly, we did not see any pro-arrhythmic effects of the infused beads. Although longer term follow-up is warranted to draw firm conclusions, these data add to the safety profile.

The current approach is the first that guarantees high stem cell retention. In fact, as 170 µm CellBeads are trapped in the arteriolar tree of the myocardium and can not pass the capillary bed, retention will approximate 100%. Because CellBeads contain ~80 MSCs per bead, infusion of 60,000 beads translates to almost five million delivered MSCs that remain in the targeted area for a substantial period of time. For reference, in cell therapy studies performed thus far, maximally 200 million bone-marrow derived mononuclear cells were transplanted (1,21). Of these cells, 0.001-0.01% comprises MSCs (4), rendering only 20,000 MSCs infused at the best. Retention rates of 1-10% results in negligible cell numbers as opposed to the approach investigated in the current study.

In addition, we show that a substantial amount of the MSCs in the CellBeads remain viable and

transcriptively active for at least seven days. In this period of time, the MSCs secrete MSC-derived soluble factors that have been shown to exert cardio-protective and pro-angiogenic effects (3,13,16,20,25). Also, our cells produce therapeutic amounts of GLP-1 that has beneficial, anti-apoptotic effects on the post-AMI heart (18,24). CellBeads are thus small factories of GLP-1 and MSC-derived factors that might have synergistic, favorable effects on cardiac scar tissue formation and adverse remodeling.

Indeed, in our experiments, infusion of CellBeads significantly reduced apoptosis in the infarct area after two and seven days when compared to control alginate beads. This is in line with one of the presumed working mechanisms of GLP-1, and suggests that GLP-1 is factually secreted into the area at risk of cardiomyocyte death following ischemia/reperfusion. The additive effect of GLP-1 remains hypothetical though, as in this pilot study the appropriate control group (MSC that do not express GLP-1) is lacking. However, qPCR analysis confirmed expression of recombinant GLP-1 and human genes in porcine tissue even after seven days.

The fact that there is a significant difference in inflammatory infiltration between CellBeads and control alginate beads is another direct validation of our paracrine hypothesis. Immunomodulatory cytokines like IL-6,IL-8 and IL-10 are secreted by the MSC and prevent the local acute immune reaction and rejection against these human-derived cells. In fact, we observed significantly less immune reaction against the CellBeads than against the biocompatible alginate control beads (9) that evoked some foreign body response in the highly inflammatory post-AMI environment.

There are two major limitations to this study. First, the short follow-up time of at most seven days does not rule out long-term safety issues. Second, the current study did not thoroughly investigate the biodistribution of the CellBeads. Although we hypothesize that retention of the CellBeads in the target area will approximate 100%, additional studies in the near future using nuclear imaging techniques will render more definite answers to this question. This study was designed as a feasibility study and was not aimed at investigating long-term effects and biodistribution. Nonetheless, the results of this pilot study are promising and future studies will focus on long-term safety effects and signs of efficacy. We are currently enrolling over 70 pigs in a large dose-finding and efficacy trial, in which we investigate three different doses of CellBeads versus three control groups. This study will answer questions concerning long-term safety, but also concerning the effect of CellBeads containing MSC that do not express GLP-1 and possible adverse effects caused by the CellBeads, by increased ischemic damage and vascular obstruction.

CONCLUSION

We show that intracoronary infusion of CellBeads is feasible and appears to be safe in a large animal model of AMI. CellBeads were successfully targeted to the infarct area and MSC remained viable and active for at least seven days. Also, several parameters indicate that the cells sort a paracrine effect, highlighting the potential for cardiovascular repair of this new therapy.

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CHAPTER 10

Intracoronary infusion of encapsulated GLP-1 eluting mesenchymal stem cells improves left ventricular function in a porcine model of acute myocardial infarction

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ABSTRACT

Background Engraftment and survival of stem cells in the infarcted myocardium remain problematic in cell-based therapy for cardiovascular disease. To overcome these issues, encapsulated mesenchymal stem cells (eMSC) were developed that were transfected to produce glucagon-like peptide-1, an incretin hormone with known cardioprotective effects, alongside MSC endogenous paracrine factors. This study was designed to investigate the efficacy of different doses of intracoronary infusion of eMSC in a porcine model of acute myocardial infarction (AMI).

Methods and Results One-hundred pigs were subjected to a moderate AMI (posterolateral AMI; n=50) or a severe AMI (anterior AMI; n=50), whereupon surviving animals (n=36 moderate, n=33 severe) were randomized to receive either intracoronary infusion of 3 incremental doses of eMSC or Ringers' Lactate control. Cardiac function was assessed using invasive hemodynamics, echocardiography and histological analysis.

A trend was observed in the moderate AMI model, whereas in the severe AMI model, left ventricular ejection fraction improved by +9.3% (p=0.004) in the best responding eMSC group, due to a preservation of left ventricular end-systolic volume. Arteriolar density increased 3-fold in the infarct area (8.4 ± 0.9/mm^2 in controls, versus 22.2±2.6/mm^2 in eMSC group; p<0.001). Although not statistically significant, capillary density was 30% higher in the border zone (908.1±99.7/mm^2 in control versus 1209.0±64.6/mm^2 in eMSC group; p=ns)

Conclusion Encapsulated MSC enable sustained local delivery of cardio-protective proteins to the heart, thereby enhancing angiogenesis and preserving contractile function in an animal AMI model.

Key words: Acute myocardial infarction, stem cell therapy, mesenchymal stem cells, GLP-1, percutaneous coronary intervention

INTRODUCTION

Regenerative stem cell therapy to promote cardiac repair has been a target of interest in the last 10 years to prevent heart failure after an acute myocardial infarction (AMI).[1,2] Various different stem cells have been investigated for their ability to repair the heart.[3] Mesenchymal stem cells (MSC) seem to be a potent candidate to date. The mechanism of action of MSC is primarily based on the release of paracrine factors to the myocardium.[4] However, retention and survival of stem cells in the myocardium after intracoronary infusion (IC) remains an issue in cell-based therapy, since only a limited number of surviving stem cells remain in the myocardium, thereby limiting the potential benefit of the therapy.[5–7] CellBeads™, that consist of alginate-encapsulated MSC (BTG International Germany GmbH, Alzenau, Germany), were developed to improve survival of cells in the myocardium, thereby elongating the release of cardio-protective proteins into the infarcted myocardium. These encapsulated MSC (eMSC) secrete endogenous paracrine factors that include vascular endothelial growth factor (VEGF), monocyte chemotactic protein-1 (MCP-1), interleukin (IL)-6, IL-8, glial-derived neurotrophic factor (GDNF) and neurotrophin-3 (NT-3) and are genetically modified to produce glucagon-like peptide-1 (GLP-1) fusion protein which comprises two GLP-1 molecules bound by an intervening peptide, extending its half life in vivo..[8–12] Amongst its beneficial effects in type 2 diabetes, GLP-1 has anti-apoptotic and cardio-protective properties.[13–16] Infusion of a GLP-1 analogue, exenatide, after AMI resulted in a reduction of infarct size, thereby improving cardiac function in a preclinical and clinical setting.[14–20] However, GLP-1 has a short half-life in vivo. Therefore infusion directly at target site or, in case of the GLP-1 eluting eMSC, production directly on-site could render a long-term release of GLP-1 especially due to the prolonged half-life of the GLP-1 fusion protein. Encapsulated MSC have a diameter of 170 micron resulting in entrapment in the coronary system following IC infusion..[10,21] The alginate shell surrounding the cells allows diffusion of oxygen and nutrients through the pores of the alginate shell into the MSC as well as diffusion of paracrine factors out of the bead. Moreover, the alginate shell protects the MSC against a host immune respons.[21]

Previously, IC infusion of up to 60,000 eMSC in naïve and infarcted porcine myocardium was well tolerated without any sign of microvascular obstruction.[10] The alginate-encapsulated MSC remain viable for at least 7 days, still secreting the recombinant GLP-1 and MSC paracrine factors.[10]

In this study, we aimed to explore the long term safety, feasibility, and efficacy of incremental doses of intracoronary delivered encapsulated MSC in a moderate and severe porcine AMI model. The primary endpoint in this study was cardiac function as assessed by echocardiography. The secondary endpoints were cardiac contractile function as measured by PV-loop analysis, infarct size, collagen density, capillary density, arteriole density, apoptosis and cardiomyocyte size.

MATERIALS AND METHODS

A total of 100 female Landrace pigs (Van Beek, Lelystad, The Netherlands; 70 ± 5 kg) were randomized in this study. All animal experiments were performed according to the 'Guide for care and the use of laboratory animals' and all experiments were previously approved by the institutional animal welfare committee of the University of Utrecht, Utrecht, The Netherlands. The efficacy of IC administered eMSC was investigated in a moderate size infarct (posterolateral AMI (LCx-model; study 1) and in the second phase in a severe anterior AMI model (LAD-model; study 2).

Experimental design

The design of this study is summarized in figure 1. Briefly, 100 female pigs underwent an AMI (50 in each group. Pigs that survived infarct induction (n=73; n=36 in the moderate infarct study and n=37 in the severe AMI study), were divided into 4 groups in each study to receive either IC infusion of eMSC or Ringers' Lactate control solution. The safety up to one week after IC infusion of GLP-1 eMSC was previously described.[10] The safety up to 8 weeks was investigated in this study and was defined as mortality, occurrence of ventricular arrhythmias and the occurrence of heart failure (fluid retention, need for treatment with heart failure medication). Cardiac function was assessed by echocardiography and pressure-volume-loop (PV-loop) analysis (in the severe anterior model only). Eight weeks after infarct induction animals were terminated and the hearts were excised for infarct size calculations and histological analysis.

Encapsulated Mesenchymal stem cells

The eMSCused in this study had an outer diameter of 170 µm and contained 75 human MSC stably lentivirally transfected to release a GLP-1 fusion protein, which comprises two GLP-1 molecules bound by an intervening peptide, giving it an extended half-life in vivo (CellBeads™, BTG Germany, Alzenau, Germany).[8,10,12] Animals were randomized to a treatment group 60 minutes after the onset of ischemia. Encapsulated MSC were then thawed and dissolved in 100 ml Ringers' Lactate (RL). A final concentration of 200 eMSC/mL to 600 eMSC/mL, depending on the dose, or 100 ml of RL were IC infused in 50 minutes at an infusion rate of 2 mL/min via a micro-catheter (Twin Pass catheter, Vascular Solutions, Minneapolis, USA) that was inserted through an 8F JL4 guiding catheter through a cannulated carotid artery. The eMSC were infused at the exact site of the previous occlusion 30 minutes after inducing reperfusion in both MI models. All solutions were color coded and administered in a blinded fashion.

Study 1: A moderate posterolateral infarct

All pigs were prepared, anesthetized, intubated and ventilated according to a standardized protocol described in the supplemental data section. An 8F sheath (Cordis, Miami, USA) was introduced into the carotid artery and an angiogram of the left coronary tree was acquired using an 8F JL4 guiding catheter (Boston scientific, Natick, USA). A posterolateral myocardial infarction was induced by

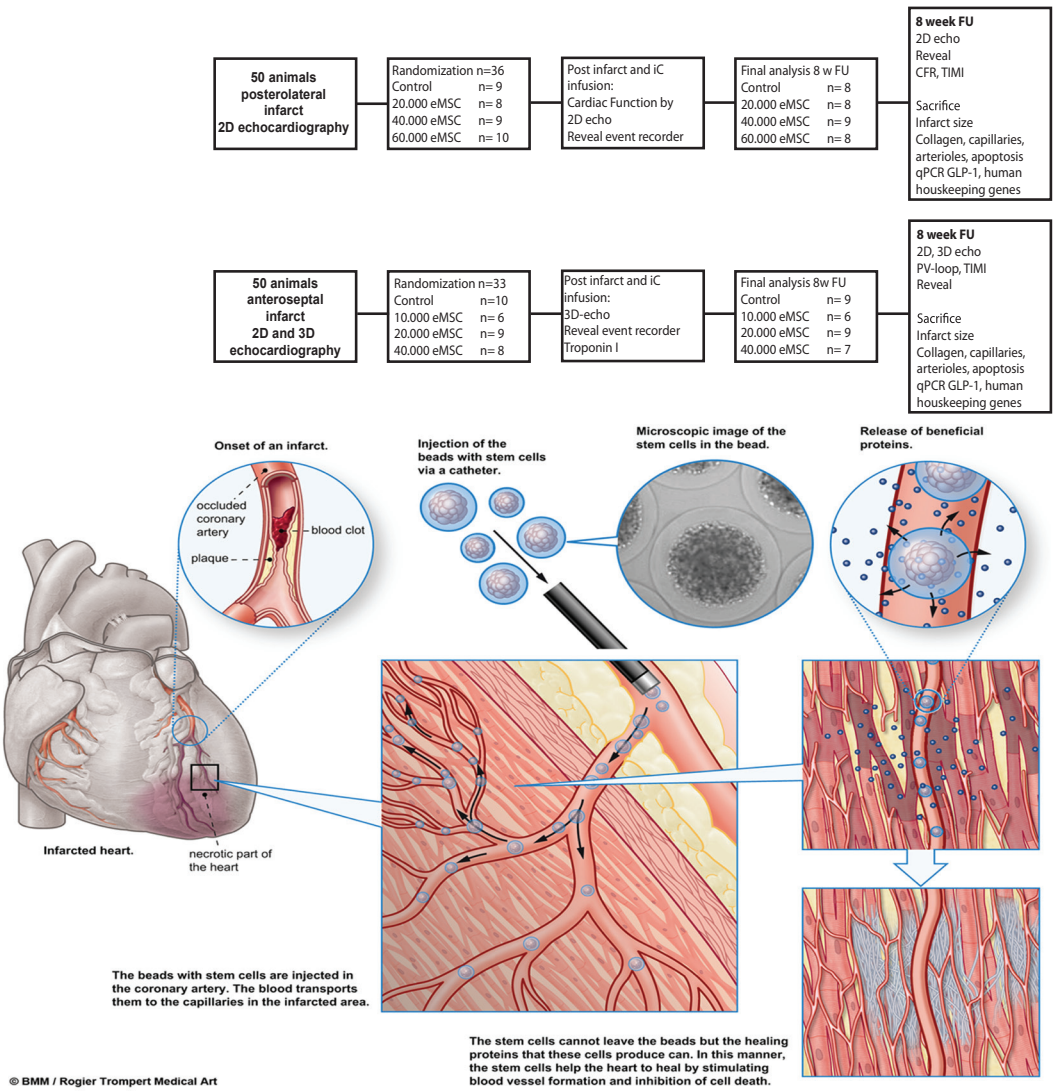

Figure 1: Study design.

Flowchart of both studies. B: image of intracoronary infusion of encapsulated mesenchymal stem cells (eMSC) via a micro-catheter into the target vessel. Following infusion, eMSC will be retained in the vascular bed, behaving like micro-factories that release paracrine factors for cardiac repair. The image of the formation of a blood clot reflects the human situation of an AMI, in the case of this study, myocardial infarction was induced by obstructing the coronary artery with a balloon or ligature. IC indicates intracoronary; LAD: left anterior descending artery; LCX; Left circumflex artery; PV-loop: pressure-volume loop; qPCR: quantitative polymerase chain reaction. This figure is used with permission of BMM, Rogier Trompert Medical Art.

inflation of an angioplasty balloon in the proximal LCx for 90 minutes (Trek, 3.5-4.0x12, Abbott, Illinois, USA). Animals were randomized into 4 groups: 20,000 eMSC (n= 8), 40,000 eMSC (n=9), 60,000 eMSC (n=10) or RL control solution (n=9) after 60 minutes of ischemia. The Thrombolysis in myocardial infarction (TIMI) flow in epicardial coronary arteries was registered pre-, during and post-infusion of eMSC to rule out potential microvascular obstruction (MVO). Both antegrade flow of contrast as well as outwash of contrast were observed. To further quantify MVO, coronary flow reserve (CFR) was measured before and after eMSC infusion and at 8 week follow-up (FU) (ComboWire, Vulcano, Zaventem, Belgium, see supplement). All animals received a Reveal™ event recorder for the detection of arrhythmic events (Medtronic, Tilburg, The Netherlands). Cardiac function was assessed using 2D-echocardiography at baseline, after infarct induction and at 8 week FU. Left ventricular ejection fraction (LVEF) and LV volumes derived from 2D-echocardiography were calculated by the modified Simpson rule (see supplement).[22] All analyses were performed by an investigator blinded for the therapy allocation.At 8 weeks (± 3 days) post MI, animals were anesthetized, intubated and ventilated according to institutional protocol (see supplement). The Reveal™ event recorder was interrogated. After assessment of cardiac function as described above, animals were terminated and the heart was excised.

Study 2: Severe anterior AMI model

A severe anterior AMI was induced by a sternotomy and ligation of the mid LAD distal to the first diagonal for 90 minutes using a prolene ligature. An open chest procedure was applied in this phase of the study to reduce peri-operative mortality. Previous studies that applied a closed chest LAD occlusion experienced a mortality rate of 40% due to VF during infarct induction, the animal welfare committee did therefore not allow a closed chest procedure and we had to change the protocol to an open chest procedure in which defibrillation directly on the heart was possible, thereby enhancing peri-procedural survival. Due to the open chest procedure, epicardial 3D-echocardiography was performed after reperfusion and animals with an LVEF higher than 45% were excluded from the study (n=4). The remaining animals were randomized into 4 groups: 10,000 eMSC (n=6); 20,000 eMSC (n=9); 40,000 eMSC (n=8) or RL buffer as control solution (n=10). Based on the results of study 1, we decided to infuse lower doses of eMSC in this study. A Twin pass catheter was then positioned in the target vessel and placebo or cell solutions were administered at a fixed infusion rate of 2 mL/min. TIMI flow was assessed before during and after infusion of eMSC or placebo solution. Three-D-echocardiography was performed in this study at baseline, after infarct induction and at 8 week FU. Due to the anatomical position of the porcine ribs, it is not possible to obtain clear 3D-echocardiography images in a close chest model nor is it possible to obtain 2D-echocardiographic images directly after open chest surgery. We therefore decided to perform 3D-echocardiography in this study at baseline, post AMI and at 8 week FU, alongside 2D-echocardiography at baseline and 8 week FU (see supplement). A Reveal™ recorder was implanted for the detection of arrhythmic events. At 8 weeks FU, functional measurements were performed, whereupon the heart was excised and processed as described above. Adding to the 2D and 3D-echocardigraphy data , PV-loop analysis was performed to obtain

data regarding cardiac contractility at week FU in this study. The exact protocol has been described elsewhere (supplemental methods).[23]

Tissue collection and infarct size analysis

At 8 weeks FU, the hearts were excised and processed for determination of infarct size and (see supplemental data), as previously described. [14,24]

Assessment of vascular density, collagen density and cardiomyocyte apoptosis

Paraffin embedded biopsies were sectioned into 5 µm slices (see supplement). Subsequently, the slides were stained for determination of vascular density, collagen density and cardiomyocyte apoptosis using appropriate antibodies (see supplement) Arterioles and capillaries were expressed as number per mm^2. cardiomyocyte apoptosis was expressed as percentage apoptotic cardiomyocytes per view.

Quantitative PCR analysis of GLP-1 and human housekeeping genes

Quantitative PCR analysis was performed to quantify expression of human GLP-1 and BNP (online supplement for detailed description).

STATISTICAL ANALYSIS

Continuous data are presented as mean ± standard error of the mean (SEM). The delta represents the difference between post AMI and 8 week FU and was first calculated for each animal individually whereupon the average per group was calculated. Comparison of means between groups was performed using a one-way-ANOVA, followed by Dunnett's test to detect differences between treatment groups and control. Changes over LVEF, LVEDV, LVESV and CFR over time were assessed using a linear mixed effects repeated measures model. P-values < 0.05 were considered significant.. All analyses were performed using IBM SPPS Statistics 20 (Chicago, USA).

RESULTS

Study 1: Moderate Posterolateral AMI model

Mortality and arrhythmias

One animal in the control group died of VF one day after the infarct procedure. Two animals in the 60.000 group died of VF, 1 and 5 days following the infarct procedures respectively. None of the animals needed to be treated for heart failure during follow-up and ventricular arrhythmias were not detected by the Reveal™ event recorder during the 8 week follow-up period in both groups.

Coronary flow

Infusion of the 170 µm eMSC did not visually impede antegrade coronary flow up to infusion of 60.000 eMSC since all animals exhibited TIMI 3 flow directly after infusion. Outwash of contrast was slower in 2/8 pigs in the 60.000 group and 1/9 animals in the 40.000 group but this was still within the definition of TIMI 3.[25] CFR did not change following eMSC infusion confirming that MVO did not occur to a

significant level directly following infusion (Figure 2). Based on these results, it was decided to omit CFR measurements in study 2. At 8 weeks follow-up, flow remained within TIMI 3 range in all groups. CFR did not change significantly over time within the groups (figure 2).

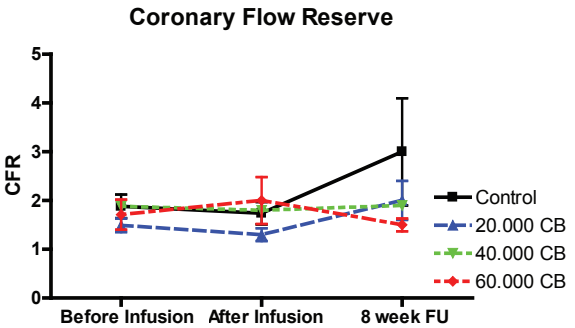

Figure 2: Coronary flow Reserve.

Coronary flow reserve after AMI before and after placebo or encapsulated MSC (eMSC) infusion and at 8 week FU.

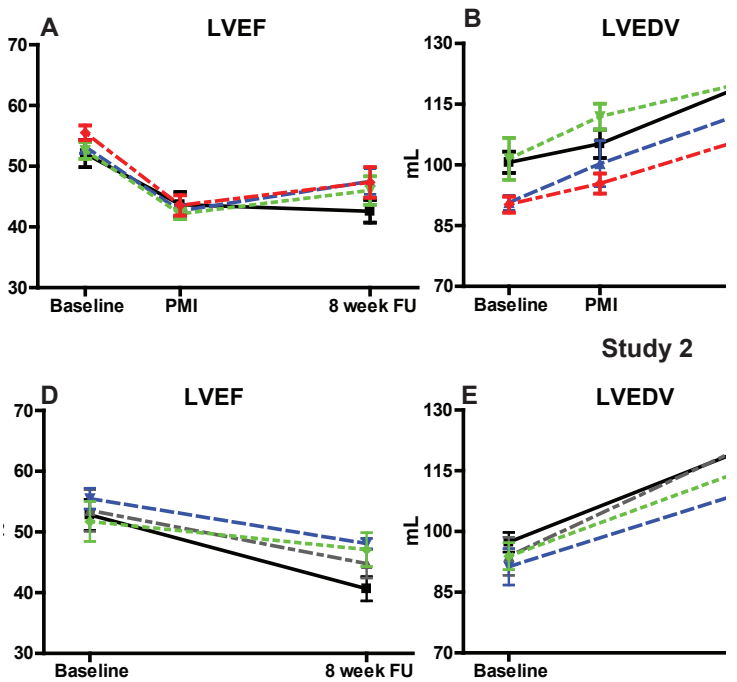

Figure 3: Cardiac function as measured by 2D-echocardiography.

A-C. Left ventricular ejection fraction (LVEF) and volumes in posterolateral AMI model over time. D-F LVEF and LV volumes over time in the anterior AMI model. eMSC: encapsulated mesenchymal stem cells; LVEDV: left ventricular end-diastolic volume; LVESV: left ventricular end-systolic volume; PMI: post myocardial infarct; FU: follow-up.

Left ventricular ejection fraction and left ventricular volumes

After infarct induction, no differences were observed on LVEF between groups, indicating comparable infarct size. At 8 weeks follow up, LVEF was a higher EF was observed in the 20,000 eMSC group opposed to control although this did not reach statistical significance. (+6%; p=NS; Figure 3; supplemental table 1).

Infarct size

Infarct size was only 9.6 ± 1.3% in the control group opposed to 7.6 ± 1.2% in the 20,000 group (p=NS). The 40,000 and 60,000 group however, showed similar infarcts sizes as the ringer lactate control (9.1 ± 1.2%; 9.3 ± 1.8% respectively; figure 4A)

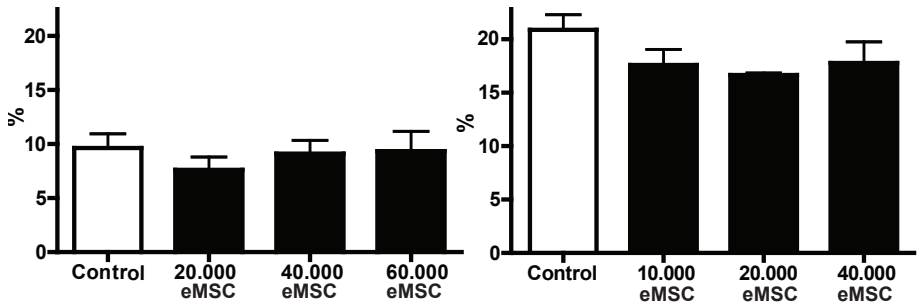

Figure 4: Infarct size.

A: infarct size in posterolateral infarct model in different groups . B: Although not statistically significant, infarct size in study 2 was approximately 20% in control and was reduced by 25% in the optimal dose group. eMSC: encapsulated mesenchymal stem cell.

Vascular density, collagen density and cardiomyocyte apoptosis

Capillary density in border and remote areas was not enhanced in all groups (Supplemental table III, supplemental figure I). However arteriolar density increased by almost 200% in the infarct area in the 20,000 group, this change was not statistically significant (supplemental figure I C and D, Supplemental table III), whereas eMSC did not enhance arteriole formation in the border and remote segments. Moreover collagen deposition and cardiomyocyte apoptosis were not affected (supplemental figure IE-F).

Study 2: Severe Anterior AMI model

Mortality and arrhythmias

One animal in the control group died of VF one day after the infarct procedure. None of the animals needed to be treated for heart failure during follow-up and ventricular arrhythmias were not detected by the Reveal™ event recorder (Figure 1). Outwash of contrast was decreased (TIMI 2) in 43% of the animals in the 40.000 group and none of the animals in the other groups directly post infusion and at 8 weeks FU.

Improvement in left ventricular ejection fraction

In this phase of the study both 2D-echocardiography and 3D-echocardiography were applied. On 3D-echocardiography, LVEF at baseline and following infarct induction was comparable between the groups (supplemental table I, figure 5). At 8 weeks follow up, LVEF increased by +0.2 ± 1.0% in the control group to 38.9 ± 1.5%. 20,000 eMSC improved LVEF by +9.3% to 44.7 ± 1.2 % (Anova: p=0.004; Control vs 20.000 p=0.011; supplemental table I). The increase in LVEF was mainly due to a preservation of LVESV, which decreased by -2.9 ± 4.8 ml (20,000 eMSC) compared to an increase of +18.1 ± 4.5 ml in control animals (figure 5). LVEF was comparable between 2D-echocardiography and 3D-echocardiography. LV volumes were 15 ml higher when measured by 2D-echocardiography. The same significant effect in LVEF was seen on 2D-echocardiography.

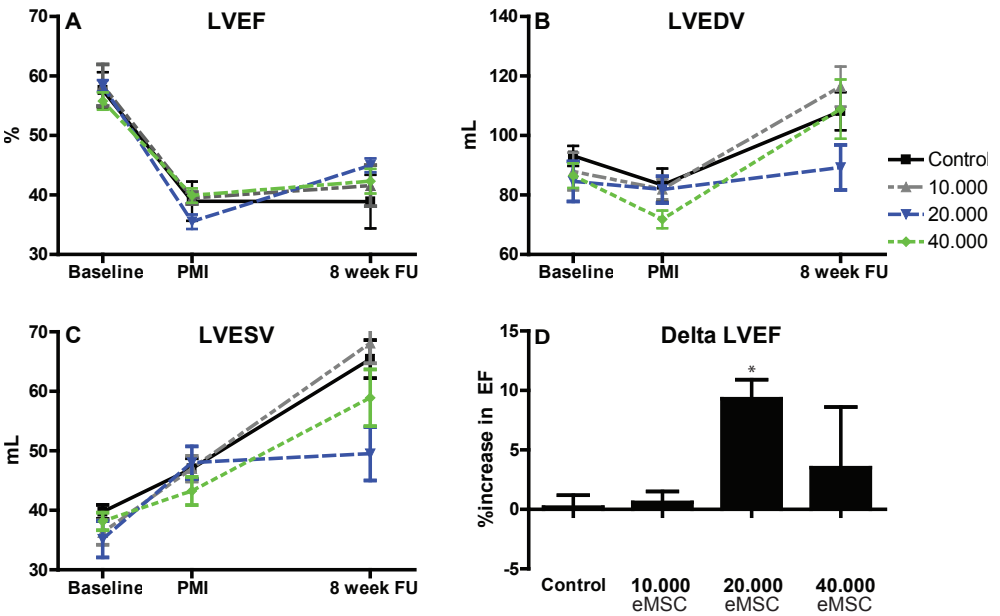

Figure 5: 3D-echocardiography anterior infarct model.

A: Left ventricular ejection fraction (LVEF) measured by 3D-echo over time. B: Left ventricular end-diastolic volume (LVEDV) over time. C: Left ventricular end-systolic volume (LVESV) over time. D: delta LVEF represents the absolute difference between LVEF at 8 weeks and directly following AMI. There is a remarkable 9.3% increase in LVEF in the 20.000 group. eMSC indicates encapsulated mesenchymal stem cells. *p=0.011 vs control

Cardiac contractility

End-systolic pressure volume relationship (ESPVR), which reflects systolic contractile function, was dramatically increased by 230% in all treatment groups as opposed to control animals (6.5 ± 1.3 mmHg/ml in 20,000 eMSC group vs. 1.9 ± 0.5 mmHg/ml in control; anova: p=0.03, p=0.007 vs control). No differences were observed in other PV-loop derived parameters (figure 6, supplemental table II).

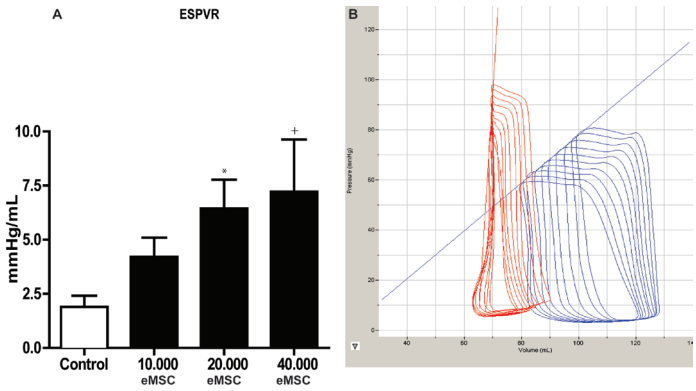

Figure 6: Pressure volume loop analysis of ESPVR.

A: End-systolic pressure volume relation (ESPVR) is enhanced in all encapsulated MSC (eMSC) groups. B: Actual vena cava occlusion of a control animal (blue) and 20.000 eMSC animal. *P=0.007 versus control, + P=0.03 versus control

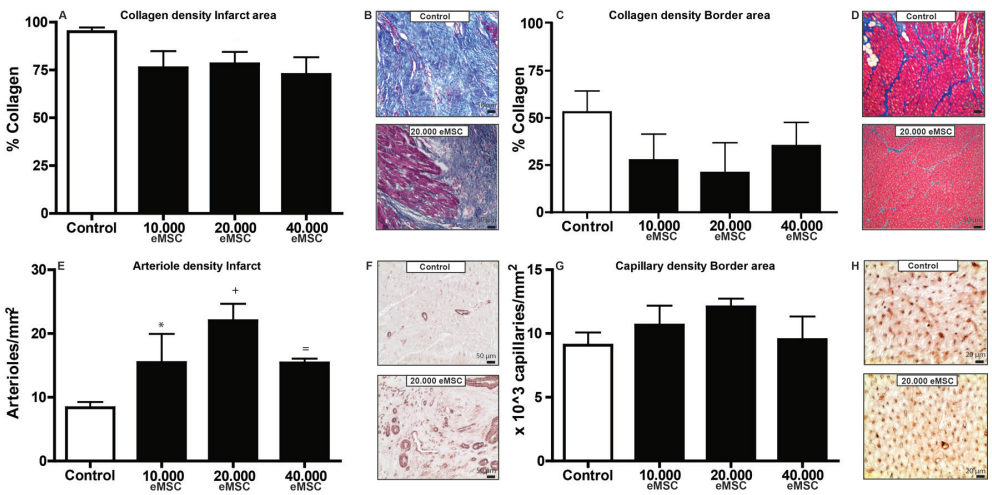

Figure 7: Angiogenesis.

A-D: Trichrome stain for detection of collagen. A: In the infarct area, no significant difference towards less collagen deposition was observed in all animals treated with encapsulated mesenchymal stem cells (eMSC; P=0.09). B: Example of Trichrome stain infarct area. Pictures are taken at a 10 times magnification. Blue represents collagen, pink viable myocardium. Collagen deposition is more dens in the animals in the control group opposed to animals in the 20,000 eMSC group. C: Collagen density border area was not enhanced in treated animals s. D: Example of Trichrome stain in border area. E-F: Arterioles were stained by smooth muscle actin stain. E: In the infarct area, arteriole density was enhanced in treated animals (*p=0.04; +p=0.0001, =p=0.002 vs control). F: Representative images of smooth muscle actin stain in infarct area in a control animal and in a 20,000 eMSC animal at 10 times magnification. G-H: Capillary density G: A trend in a decreased capillary density was observed in 20.000 eMSC group. H: Representative images of Isolectin stain in the border area of a control animal and a 20,000 eMSC animal respectively at 20 times magnification.

Infarct size

Although not significant, infarct size decreased by -20% in the 20,000 eMSC group (figure 4B, supplemental table III).

Vascular density and collagen density

Capillary formation was not enhanced in border areas, nor increased the capillary density in remote areas.

However, arteriolar density improved in the infarct area in all doses by +200-300% (22.2 ± 2.6/mm^2 versus 8.4 ± 0.9/mm^2; Anova: p<0.001.; figure 7; supplemental table III).

Collagen density was not reduced in all dose groups.

Cardiomyocyte apoptosis and hypertrophy

Cardiomyocyte apoptosis was not influenced by eMSC treatment, which might be related to the late time point.

However, the surface area of the cardiomyocytes in the infarct border zone of animals treated with 20.000 eMSC was almost 50% lower than in control and the other dose groups (417.1 ± 110.2 µm^2 in 20.000 group versus 823.1 ± 74.0 µm^2 in control ; p=0.009; figure 8). The same was observed in the remote myocardial segments.

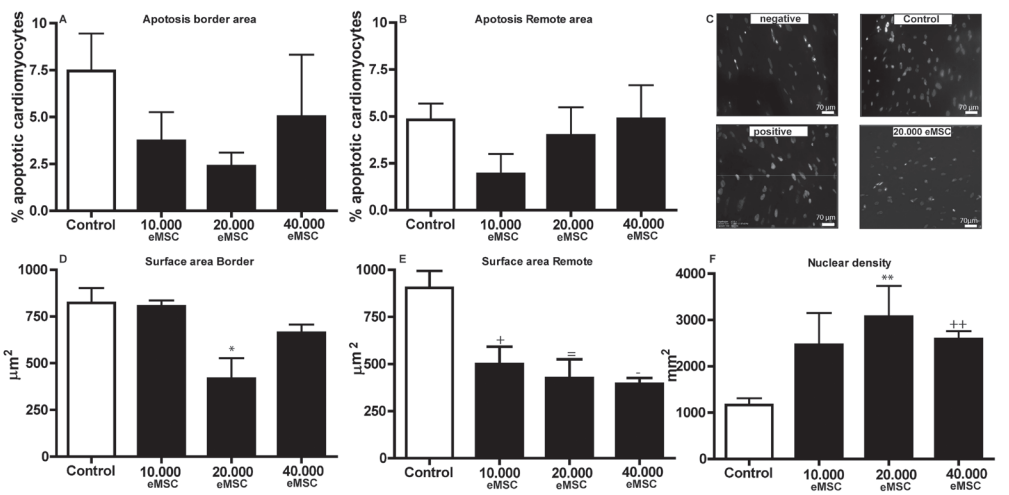

Figure 8: Cardiomyocyte apoptosis, cardiomyocyte size and cardiomyocyte nuclear density.

A-C: Cardiomyocyte apoptosis via TUNEL stain in the border area and remote areas. C: Example of Fluorescent TUNEL stain. Blue cells are alive, green cells are apoptotic.

D-F: Cardiomyocyte size in the border area was significantly lower in the 20.000 group (D). In the remote myocardial segments, the surface area was lower in all animals treated with eMSC (E). This finding corresponds with an increased nuclear density in the highest dose groups in remote areas (F). *p=0.009, +P=0.01, =p=0.006, -p= 0.0007, **p=0.03, ++p= 0.003 versus control . eMSC indicates encapsulated mesenchymal stem cells

Location and survival of encapsulated MSC

Encapsulated MSC were found on a regular bases in the infarct-border zone. No shedding was macroscopically and microscopically observed to remote myocardial segments or remote organs. Encapsulated MSC were still intact (Supplemental Figure II). Unfortunately, neither human housekeeping gene GAPDH nor human GLP-1 expression could be detected by qPCR, indicating that the MSC inside the beads do not elute paracrine factors at 8 week FU (supplemental figure II) and are most likely dead.

Surrounding the eMSC, a mild inflammatory response occurred . This response was comparable between the dose groups. Cells included in this response were macrophages and granulocytes.

DISCUSSION

In the current study, we investigated the efficacy and the optimal dose of encapsulated immunoprotected MSC in a porcine model of AMI. This is the first time that the efficacy of microencapsulated MSC was investigated for treatment of AMI. In the moderate infarct study, only a trend towards an improvement of cardiac function was observed. In the severe infarct study, LVEF was remarkably improved following eMSC infusion. Moreover, contractility was enhanced and neovascularization occurred.

Safety

In line with our previous results, eMSC infusion did not show side-effects in a preclinical setting.[10] Previous studies suggested a reduction in TIMI flow and CFR following intracoronary infusion of mesenchymal stem cells.[6,26,27] In this study, CFR did not significantly decrease directly after infusion, nor at 8 week FU, indicating that MVO is not significant. Studies that report MVO after infusion of MSC, usually apply high doses , which could eventually result in obstruction. Several other preclinical and clinical studies have shown that under carefully controlled and monitored conditions, e.g. slow infusion rate and low cell number, infusion of MSC or MSC like stem cells or cardiospheres is well tolerated.[24,28,29] In the case of eMSC the same assumption could be made.

None of the animals developed heart failure during the follow-up period, nor increased the incidence of arrhythmic events, indicating no side-effects of eMSC therapy were observed during 8 week FU period.

Efficacy

When given IC, 20,000 alginate encapsulated MSC significantly improve LVEF after a severe AMI. In the moderate infarction model we detected a trend, which was further explored in a more severe AMI model. As expected, the treatment effect turned out to be more pronounced in animals with more severe AMI, presumably due to a larger pharmacological window in the anterior model. This corresponds with a large meta-analysis by Jeevanantham *et al* who concluded that patients with a LVEF blow 43% benefit more from cell-based cardiac repair.[1] However, another meta-analysis failed to show a more prominent treatment effect of BMMNC therapy in patients with lower LVEF.[3] If a treatment effect is already noticeable in a small infarct, the benefit will be expanded in a large infarct.

In absolute terms, IC infusion of eMSC improved LVEF by +9.3% in severe anterior AMI model. The most effective dose in our study was 20,000 eMSC, whichequals 1.6 million MSC, and is several orders of magnitude less than used in most preclinical trials using cultured MSC. However, eMSC are better retained into the myocardium than unprotected stem cells culminating in prolonged protein release. This complicates direct comparison. Infusing less than 20,000 eMSC was not effective, suggesting that this dose is the lower limit. On the other hand, 40,000 eMSC were not superior to lower doses, which does not necessarily preclude a dose dependent effect but could also reflect unfavorable effects of incremental MVO undetectable by our CFR measurements or the adverse effects of a possible xenogenic immune response which could be more severe in the high dose group.

Clinical applicability

Before possible translation of this product to a clinical treatment strategy, several issues should be addressed in future studies. First, lentiviral transduction for cell based gene therapy remains subject to debate since insertional mutagenesis and thus tumorgenicity might be of increased risk.[30] This could influence the clinical applicability of the transduced eMSC. However, the MSC are contained by the alginate and are unable to migrate to the host's tissue. Furthermore, the alginate exceeds the lifespan of the cells, with no exception observed in our study, making the risk for possible tumorgenicity rather low, in our opinion. Second, as described in previous studies, an increased inflammatory response around the eMSC has been detected, which can most likely be attributed to a xenogenic reaction. Future studies should determine if this response is due to xenogenecity or a direct result of e.g. cell death inside the alginate microsphere. If the latter is the case then this would also influence the clinical applicability of this product since excessive inflammation is detrimental to infarct healing post-MI.[31]

Working mechanism of encapsulated GLP-1 eluting MSC

Several preclinical studies showed that MSC therapy after AMI enhanced the formation of arterioles and capillaries by a release of paracrine factors.[24,32] MSC inside the alginate beads also have a pro-angiogenic effect.[33] Moreover, eMSC also improved angiogenesis in a hind-limb ischemia model and in porcine interposition grafts.[11,33,34] This pro-angiogenic effect might explain the observed preservation of cardiac function.[33,34]

Moreover, eMSC a trend towards reduced fibrosis and improved myocardial viability in the infarct zone was observed, which could result in a reduction in total infarct size.[4,24,35] However, this effect was not statistically significant in our study, most likely based on limited numbers of animals. The trend towards less fibrosis could be explained by the secretion of immunomodulatory cytokines by MSC. IL-6 has been shown to increase the lifespan of neutrophils in the hostile post-AMI environment and improves healing of the infarct wound.[36] In addition, MSC trigger the transition of classical M1 to anti-inflammatory M2 macrophages, thereby enhancing infarct healing via increased angiogenesis.[36] Alongside pro-angiogenic factors and immunomodulatory cytokines, MSCs secrete anti-apoptotic factors that improve cell survival.[24,35] Additionally, eMSC in this study were transfected to secrete recombinant GLP-1. Exenatide, a GLP-1 analogue, has shown to reduce infarct size and improve cardiac function in a pig AMI model.[14] Moreover, in 2 recent clinical trials in which exenatide was

injected in AMI patients (TIMI 0-1 flow) before PCI, infarct size was reduced by 50%, indicating that Exenatide prevents reperfusion mediated cell death.[15,16] As eMSC are infused within 30 minutes after reperfusion, the effect on infarct size could be related to limitation of reperfusion damage.

Next to its anti-apoptotic effects, GLP-1 has been shown to directly improve cardiac contractility by increasing intracellular cyclic-AMP concentrations.[37] This latter effect could be responsible for the significant ESPVR improvement in all eMSC groups. The observed preservation in contractility could also be explained by strengthening of the heart's matrix by the infusion of alginate thereby preventing LV remodeling as previously was shown.[38,39] However, in these studies liquid alginate was used that diffuses through the vessel wall.[38] Although eMSC cannot leave the vessel lumen, they seem to provide structural support to the myocardium. The heart contains a population of resident cardiac stem cells.[40] It is hypothesized that MSC can activate these stem cells in order to stimulate infarct repair following an ischemic event. Suzuki *et al* concluded from a porcine study, in which MSC were IC injected following AMI that MSC stimulate endogenous cardiac stem cells to home to the site of injury and differentiate into cardiomyocytes.[32] In addition to this, the MSC in their study differentiated into cardiac stem cells. Moreover, cardiomyocytes were stimulated to proliferate in animals that were treated with MSC. All these effects combined resulted in an increased cardiomyocyte nuclear density. In our study, cardiomyocyte nuclear density is increased in border and remote areas possibly suggesting that eMSC stimulate myocardial salvage which, in turn, results in a reduction of compensatory hypertrophy. Myocardial salvage in this study is based on a reduction of apoptotis by GLP-1 and most likely proliferation of adult cardiomyocytes. It was recently shown, that IL-6 secreted by MSC-like stem cells, stimulated cardiomyocyte proliferation.[41] As the eMSC produce IL-6, this would be the proposed working mechanism of cardiomyocyte proliferation in this study. Differentiation of MSC inside the beads could not have contributed to the increased number of cardiomyocytes, because MSC do not leave their shell. Activation of endogenous stem cells also remains a proposed mechanism of action. As was shown by Suzuki *et al* and Houtgraaf *et al* activation of resident cardiac stem cells is not detectable after 6 weeks following transplantation, the c-kit stain was omitted from current protocol.[24,32] Moreover, cardiomyocyte proliferation could be not detected at 8 week FU in a previous study that used MSC.[24] Therefore ki-67 stain was not executed.

Study limitations

Despite our best efforts, this study has some limitations. First, cardiac function was not assessed by golden-standard cardiac MRI. However, echocardiographic measurements have shown excellent correlations with MRI. We are therefore confident that our findings would be corroborated by MRI.[42]

In the two sub-studies we used slightly different protocols. In the more severe anterolateral model we used an open chest procedure to minimize high peri-procedural mortality following mid LAD occlusion. The advantage of this open chest procedure enabled us to use epicardial 3D-echocardiography alongside transthoracic 2D-echocardiography in the anterior model. Although the two methods have shown to be in good concordance with one another, values should not be directly compared. Therefore, the 2 methods were both shown in the second phase. [43,44]

Second, In this study we did not observe an increased MVO, as assessed by CFR, after eMSC infusion.

However, variability in heart rate and arterial blood pressure might interfere with reliable CFR measurement. Since we did not do CFR measurements under atrial pacing, these parameters might have varied in the present study, thereby possibly influence the sensitivity of the CFR measurements. Third, we did not include all possible controls including eMSC not transduced to produce GLP-1, empty beads or MSC only. This decision was based on our previous pilot data where we found beneficial effects on apoptosis and inflammatory response of eMSC opposed to empty alginate beads without MSC. Moreover, in a proof of concept study by Wright *et al.,* IC infusion of macro-eMSC resulted in preserved LVEF and reduced infarct size when compared to empty alginate beads without cells and encapsulated human MSC that were not transduced to express GLP-1. [11] Since both the animal model and the eMSC preparation differed in this study, no firm conclusion can be drawn on the superiority of GLP-1 eluting eMSC used in the current study compared to non-transduced eMSC. Furthermore, a side by side comparison between eMSC and MSC would have been very interesting. However, in this phase of the study, we decided that we first wanted to evaluate the efficacy of this new product before we performed a side-by-side comparison. Moreover, we did not know the optimal eMSC dose. If we would like to compare the efficacy of eMSC end MSC, the amount of MSC should be comparable between the groups. Here, the MSC are derived from humans. This makes sense as we aimed to test the efficacy of the clinical product. Further research is needed to test whether allogeneic encapsulated mesenchymal stem cells are equal or even have a superior effect. As the GLP-1 analogue produced by the eMSC consists of a GLP-1 fusion protein with a short half-life, a direct comparison with native GLP-1 or GLP-1 analogues with prolonged half-life would be difficult to interpret.
There is no sign of degradation of the encapsulated MSC at 8 weeks FU. More research is needed to investigate how and when the encapsulated MSC are degraded.

CONCLUSIONS

IC infusion of encapsulated GLP-1 eluting MSC resulted in a preserved LVEF in a porcine AMI model and is well tolerated up to 8 weeks FU. Infusion of eMSC improves cardiac function by preservation of end-systolic volume and cardiac contractility. Encapsulated MSC are able to prevent cardiomyocyte hypertrophy, enhance adaptive neovascularization, thereby limiting left ventricular remodeling. Importantly, encapsulated MSC are an interesting platform that enables sustained paracrine delivery of MSC proteins and recombinant proteins to the damaged myocardium.

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SUPPLEMENTAL DATA

Content

DETAILED MATERIAL AND METHODS

Medication and Anesthesia

All animals were pre-medicated starting ten days prior to infarct induction with dual anti-platelet therapy ((acetylsalicylic Acid: 300 mg loading followed by 80 mg qd (Centrafarm, Etten-Leur, The Netherlands) and clopidogrel 75 mg qd(Sanofi-Aventis, Paris, France)) and anti-arrhythmic therapy (amiodarone, loading dose of 1200 mg followed by 800 mg qd; Sanofi-Aventis, Paris, France). Dual antiplatelet and anti-arrhythmic therapy was continued during follow up.

General anesthesia was induced with 0.4 mg/ kg midazolam (Actavis, Zug, Switzerland), 10 mg/kg ketamine (Narketan,Vétoquinol, Lure Cedex, France) and 1 mg of Atropine (Pharmachemie BV., The Netherlands) and maintained with intravenous infusion of midazolam 0.5 mg/kg/h, sufentanil 2.5 µg/kg/h (Janssen-Cilag B.V., Tilburg, The Netherlands) and pancuronium 0.1 mg/kg/h (Inresa, Battenheim, Germany). Upon infarct induction and Cellbead infusion, all animals were therapeutically heparinized with 2 doses of 5000 IE (Leo pharma, Ballerup, Denmark) IV and received intravenous infusion of eptifibatide (bolus of 180 µg/kg and 2 µg/kg/min (GlaxoSmithKline BV, Zeist, The Netherlands).

A fentanyl plaster 25 µg/h (Janssen-Cilag B.V., Tilburg, The Netherlands) was applied before and after the first procedure for analgesia. The animals were treated with one doses of Augmentin intravenously (1000/100 mg, Sandoz, Holzkirchen, Denmark) before the infarct procedure.

All animals in received meloxacam 2 times 0.5 mg/kg daily (Produlab-Pharma B.V. Raamsdonksveer, The Netherlands) for two days after index procedure for additional analgesia.

Coronary flow

In the first study, coronary flow was assessed after myocardial infarct,after the complete infusion of eMSC and 8 weeks following infusion by coronary flow reserve measurements (CFR). Every 25cc infusion was stopped and TIMI flow was assessed according to the TIMI criteria.[1] During CFR measurements, Average peak velocity (APV; cm/sec) was assessed in normal conditions after AMI and

during maximum vasodilation following injection of 140 mcg/kg/min adenosine IV. Peak APV was assessed and CFR was calculated.

Echocardiography

Two-dimensional grey scale images at a frame rate of 60-90 frames/s were obtained in parasternal position using a Philips iE33 (Phillips, Einhoven, The Netherlands), equipped with a broadband S5-1 transducer. Transthoracic echocardiographs were acquired at baseline, post myocardial infarct and eight weeks follow-up in the posterolateral infarct model and at baseline and 8 week FU in the anterior infarct model. A long axis view and three levels of short axis view (basal, mid ventricular and apical levels) were obtained by acquiring three successive cardiac cycles.

Echocardiographic data was transferred to and analyzed using Image Arena 4.1 (Tomtec Imaging Systems, Uterschleissheim, Germany). This analysis was performed by an investigator blinded for the treatment groups. Regional left ventricular function was assessed by measuring the change in LV cavity area (fractional area change) during systole and diastole at basal (mitral valve level), mid ventricular (papillary muscle level) and apical level. Left ventricular volumes were then calculated by the modified Simpson rule: LV end diastolic volume (LVEDV)= (A_{bED}) * L/3 + $(A_{mED}+A_{pED})/2)$ * L/3 + 1/3(A_{pED}) * L/3; LV end systolic volume (LVESV)= (A_{bES}) * L/3 + $(A_{mES}+A_{pES})/2)$ * L/3 + 1/3(A_{pES}) * L/3, in which A_b is the area at basal level, whereas A_m and A_p are the areas at mid and apical level respectively.[2] L is defined as the length of the ventricle from apex to base. The length was obtained of 4 chamber view 3D echo in severe AMI study at baseline and follow-up. In the LCx study, the length of the ventricle in the long axis view was used. LVEF was calculated following standardized formula: ((LVEDV-LVESV)/LVEDV)*100.

3D-echocardiography

In study 2, epicardial 3D-echocardiographs were acquired with X-3 transducer using the iE33 ultrasound machine (Philips, Eindhoven, The Netherlands). The 3D-transducer (X-3, Philips, Eindhoven, The Netherlands) was wrapped in a sterile sleeve. A pocket of gel was positioned under the transducer, to bring the complete apex a vu. The transducer was positioned directly epicardially on the apex of the heart. We positioned the transducer in all animals in the same direction so we obtained a 4 chamber view. The depth and sector size were adjusted to fit the complete ventricle. In each pig, the data sets were acquired in real time using 7 consecutive cardiac cycles (full volume analysis).

The images were offline analyzed using QLab 10.1 (3DQ advanced) analysis software. The tracing of the ventricle was perfomed by semi-automatic border detection as described before by Soliman and coworkers. [3] Briefly, LV quantification starts by proper 4-chamber view and orthogonal views. The end-diastolic volume and end-systolic frames are identified and on both frames and the apex, anterior, lateral, inferior and septal mitral annulus is identified. Qlab 10.1 automatically traces the endocardial border. Traces that are unsatisfactory can be manually adjusted. The ejection fraction is calculated by the Qlab software as (LVEDV-LVESV)/LVEDV*100.

PV-loop measurement and analysis

In the severe infarct model, a 7F tetrapolar admittance catheter (7.0 VSL Pigtail, no lumen, Transonic, Scisence, London, Canada) was introduced in the left ventricle via the aortic valve, and the pigtail tip of the catheter was positioned in the apex. This catheter measures admittance magnitude and phase in combination with pressure. The catheter exists of 7 electrodes, that divide it into 4 segments. The largest segment that was positioned into the LV was used for the measurements. The catheter was connected to the ADVantage sytem (Transonic, Scisence, London, Canada) for real-time data assessment.[4–6] PV loop measurements were performed at eight weeks follow up. To obtain data regarding contractility, a caval vein occlusion was performed. The thorax was opened, the inferior vena cava was located whereupon a prolene suture was placed around it. During breath hold, the inferior caval vein was occluded by the suture until pressure drop was >50%. The suture was released and blood flow to the heart was restored. In this study, contractility parameters and relaxation parameters were used in the analysis. LVEF and LV-volumes were assessed by 2D-echocardiography and 3D-echocardiography. Systolic PV-loop-derived parameters that were assessed and analysed in current study were: dP/dt max (maximum increase in pressure/s), end-systolic-pressure-volume-relationship (ESPVR), pre-recruitable stroke work (PRSW). Diastolic parameters that were determined were: Tau (an isovolumic relaxance constance), dP/dt min (maximum relaxation over time), end-diastolic pressure volume relationship (EDPVR).

Tissue collection and infarct size calculations.

At 8 weeks follow-up, the left ventricle was separated from the right ventricle and sliced into 5-6 slices of approximately 1 cm thickness. Slices were incubated in 1% triphenyltetrazolium chloride (Sigma-Aldrich Chemicals, Zwijndrecht, the Netherlands) in 37°C 0.9%NaCl for 15 min to discriminate infarct tissue from viable myocardium followed by inspection for the presence of potential ectopic micro-infarctions in non-infarcted segments. Slices were weighed and photographed for infarct size calculations as described before [14,23]. Biopsies of infarct area, border zone and remote areas were obtained and embedded in paraffin for further histological analysis. Additional biopsies were snap frozen for RNA retrieval and qPCR analysis. The liver, spleen, lungs and kidneys were excised and macroscopically analyzed for abnormalities, whereupon multiple random biopsies were taken to exclude shedding of eMSC to remote organs. Biopsies were embedded in paraffin, cut and stained with Hematoxilin and Eosin and microscopically evaluated to a technician blinded for the allocated therapy and evaluated regarding the occurrence of eMSC in remote organs.

Immuno-histochemical stainings

Collagen content, cardiomyocyte size and myocardial salvage index

Collagen content in infarct, border and remote myocardial segments was assessed via trichrome stain. Briefly, all sections were deparaffinised, fixated in Buoin's fixative (Sigma-Aldreich, St. Louis, USA) at 56º for 15 minutes, Nuclei were stained with haematoxylin for 3 minutes. The slides were submerged in Trichrome-AB solution for 5 minutes after which they were treated with 0.5% acetic acid for 1

minute. Slides were mounted with Entallan (Merck, Darmstadt, Germany). Three random pictures were made at a 10X magnification and collagen content was calculated as percentage collagen of total surface area using automated analysis software (Clemex, Quebec, Canada).
To calculate cardiomyocyte size, 3 random pictures of all slides of the border area and remote area were obtained at 40X magnification. The surface area was determined by the automated analysis software. Only transversely cut cardiomyocytes containing a nucleus were analysed.

Capillary and arteriole density

Arteriole density was obtained in infarct, border and remote myocardial segments, using alpha smooth muscle actin (SMA, clone 1a4, Sigma-Aldreich, St Louis, USA). Endogenous peroxidase activity was blocked by 3% Methanol/H_2O_2 solution for 30% and incubated with SMA 1:1000 overnight. Subsequently, the slides were incubated with secondary HRP-conjugated goat-anti-mouse dilution 1:200 (DAKO, Glostrup, Denmark) for 90%. All slides were immersed in DAB solution for 2 minutes (DAKO, Glostrup, Denmark) and mounted with Entallan. A technician that was blinded for the dose groups took 3 random pictures at 10 times magnification. Arterioles per pictures were counted and expressed as number per mm^2.
Capillary density was only assessed in border en remote areas, because almost all capillaries are destroyed after AMI. All sections were dewaxed and pre-treated with trypsin EDTA (Lonza, Verviers, Belgium). Endogenous peroxidase activity was blocked as described above. All slides were incubated with Isolectin B_4 (Bandeiraea simplicifolia Isolectin B_4, Dako, Glostrup, Germany) diluted 1/50 overnight. After that the slides were immersed in DAB solution at mounted with Entallan. Photographs were taken at 20X magnification and number per mm^2 was calculated.

Apoptosis

Apoptosis was quantified in all regions using In-Situ cell detection kit-FITC labelled (second study) or In-situ cell detection kit-HRP labelled (first study) (Roche, Basel, Swiss). The manufacturer's instructions were followed. After mounting with Vectashield with DAPI, 3 random photographs at 40X magnification were made, using un Olympus IX55 fluorescence microscope and number of apoptotic cells were counted. Apoptosis is depicted as percentage of apoptotic cells.

Quantitative PCR

The expression of human household genes, GLP-1 and BNP as markers for hypertrophy were investigated using qPCR analysis. RNA was isolated from snap frozen biopsies of infarct area, border zone and remote myocardial using RNA-Bee™ RNA Isolation Solvent (Tel-Test Inc., Friendswood, USA) according to the manufacturer's protocol. CDNA was created using Bio-Rad iScript™ cDNA Synthesis Kit (Bio-Rad, Veenendaal, The Netherlands), according to the manufacturer's instructions. The primers that were investigated can be found in the table below. qPCR was performed using SensiMix™ SYBR & Fluorescein Kit (Bioline, Boston, USA) and expression was detected by Bio-Rad MyiQ System Software of MyiQ™ Optical Module. The threshold cycle (Ct) values from interested genes were normalized to

porcine Beta Actin expression(housekeeping gene) or human GAPDH (GLP-1).

Primers for qPCR	
Porcine housekeeping gene	
B actin forward	AAGAGCTACGAGCTGCCCGAC
B actin reverse	GTGTTGGCGTAGAGGTCCTTC
Human housekeeping gene	
GAPDH forward	GCTCATTTCCTGGTATGACAAC
GAPDH reverse	GAGGGTCTCTCTCTTCCTCTT
Recombinant GLP-1	
CM-2 forward	GTGAGCTCTTATCTGGAAGGCC
CM-2 reverse	AGATAAGAGCTCACATCGCTGG
BNP	
BNP forward	GCAGCAGCCTCTATCCTCTC
BNP reverse	TCCTGTATCCCTGGCAGTTC

RESULTS

Brain Natriuretic Peptide (BNP) analysis

Blood was sampled at baseline and at 8 week follow up. The clinical chemical laboratory of University medical center in Utrecht and in Rotterdam, both tried to measure BNP levels using an ELISA. Unfortunately, this was not successful, therefore BNP levels were detected using qPCR analysis. There were no differences in qPCR levels of BNP between groups.

Necropsy

There were no signs of anatomical malformations by macroscopic analysis of the heart, liver, spleen, lungs and kidneys. There were no signs of macro-infarcts in remote areas of the heart.

Left ventricular weight

No differences were observed regarding left ventricular weight at 8 week follow-up in all groups in both studies.

Supplemental figure I: Histology study I: moderate infarct model

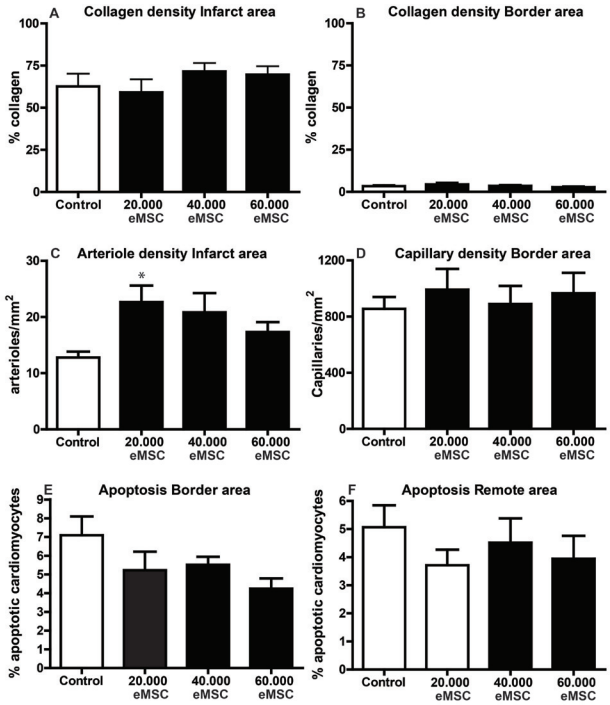
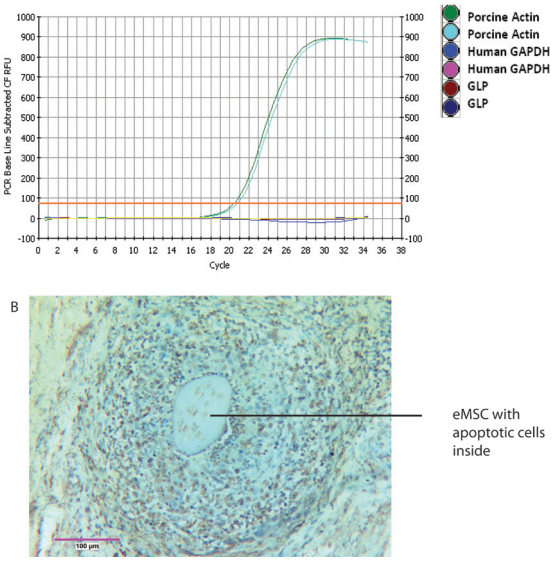


Figure I: Fibrosis, vascular density and apoptosis.

A-B: Collagen density in infarct and border area in the first phase of the study. C: Arteriole density increased in infarct area in all dose groups opposed to control. D: Capillary density. E-F: Cardiomyocyte apoptosis in border and remote segments respectively. eMSC indicates encapsulated mesenchymal stem cells



Supplemental figure II. eMSC at 8 week FU

A; Example of qPCR reaction using porcine housekeeping gene actin, human housekeeping gene GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) en GLP-1 (Glucagon-like peptide-1) in a border sample of an animal treated with eMSCs. There was no signal detected of GLP-1 or human housekeeping genes.

B. TUNEL stain of an eMSC at 8 week FU. Cell density inside the eMSC is low and the cells are positive (brown) for apoptosis, indicating that the MSC have died during the 8 week FU.

Supplemental table I: Volumes and ejection fraction assessed by 2D and 3D echocardiography.

		Intermediate infarct			
2D-echo	**Control**	**20.000 CB**	**40.000 CB**	**60.000 CB**	**P -value ANOVA**
LVEF Baseline	51.2 ± 0.6	52.0 ± 0.2	52.7 ± 0.6	56.3 ± 0.5	NS
LVEDV Baseline	102.2 ± 1.0	90.7 ± 0.7	101.5 ± 2.2	90.2 ± 0.8	NS
LVESV Baseline	50.0 ± 1.0	43.5 ± 0.4	48.4 ± 1.5	39.4 ± 0.5	NS
LVEF PMI	43.7 ± 0.8	42.6 ± 0.4	42.1 ± 0.3	43.6 ± 0.7	NS
LVEDV PMI	105.2 ± 1.4	100.3 ± 2.0	111.9 ± 1.1	95.4 ± 1.0	NS
LVESV PMI	59.3 ± 1.2	59.0 ± 1.0	55.7 ± 1.0	55.7 ± 1.0	NS
LVEF 8W FU	42.6 ± 0.8	47.5 ± 0.7	46.0 ± 0.8	47.3 ± 1.0	0.09
LVEDV 8W FU	123.4 ± 3.5	116.1 ± 1.4	122.0 ± 2.7	109.2 ± 2.3	0.08
LVESV 8W FU	70.8 ± 2.2	61.2 ± 1.4	69.5 ± 2.2	55.5 ± 2.1	0.09
		Severe infarct			
2D-echo	**Control**	**10.000 CB**	**20.000 CB**	**40.000 CB**	**P-value ANOVA**
LVEF Baseline	52.8 ± 2.6	54.6 ± 3.5	55.5 ± 1.7	51.7 ± 3.3	NS
LVEDV Baseline	97.4 ± 2.4	93.9 ± 4.7	91.3 ± 4.5	93.9 3.3	NS
LVESV Baseline	40.6 ± 5.3	43.6 ± 3.9	40.8 ± 2.8	45.0 ± 3.4	NS
LVEF 8W FU	40.6 ± 2.0	44.7 ± 2.4	48.1 ± 0.9	47.1 ± 2.8	0.04
LVEDV 8W FU	123.8 ± 3.7	125.2 ± 3.5	112.5 ± 3.8	118.5 ± 8.6	NS
LVESV 8W FU	70.3 ± 3.4	70.4 ± 3.8	58.6 ± 3.0	61.8 ± 8.3	NS
3D-echo	Control	10.000 CB	20.000 CB	**40.000 CB**	**P-value ANOVA**
LVEF Baseline	57.6 ± 1.0	58.5 ± 1.8	58.4 ± 1.0	55.8 ± 1.4	NS
LVEDV Baseline	93.1 ± 3.4	87.7 ± 7.9	84.6 ± 6.7	86.5 ± 4.1	NS
LVESV Baseline	39.7 ± 1.2	36.3 ± 2.5	35.1 ± 3.0	38.1 ± 1.5	NS
LVEF PMI	38.9 ± 1.1	41.9 ± 1.5	35.3 ± 1.2	39.9 ± 1.3	NS
LVEDV PMI	83.4 ± 5.5	81.9 ± 4.1	81.8 ± 4.5	71.8 ± 2.9	NS
LVESV PMI	51.0 ± 3.6	47.0 ± 2.6	52.0 ± 2.7	43.2 ± 2.4	NS
LVEF 8W FU	38.9 ± 1.5	41.6 ± 1.7	44.7 ± 1.2	42.3 ± 2.1	0.032
LVEDV 8W FU	108.1 ± 6.4	116.4 ± 5.8	89.2 ± 7.1	99.2 ± 13.1	NS
LVESV 8W FU	65.4 ± 3.2	68.0 ± 4.1	49.5 ± 4.2	58.9 ± 8.9	0.03
Delta LVEF	0.2 ± 1.0	0.6 ± 0.9	9.3 ± 1.6	3,5 ± 5,1	0.004
Delta LVEDV	30.9 ± 8.2	38.9 ± 8.1	6.4 ± 7.5	21.9 ± 11.3	NS
Delta LVESV	18.1 ± 4.5	20.8 ± 3.9	2.9 ± 4.8	12.8 ± 7.7	NS

Supplemental table I represents echocardiographic derived volumes and ejection fraction. 2D-echocardiographic volumes are calculated by the modified Simpson method in both studies and 3D-echocardiography in study 2. P-values are corrected using a post-hoc Bonferroni correction. LCx indicates left circumflex artery;; LVEF: left ventricular ejection fraction; LVEDV: left ventricular end-diastolic volume; LVESV: left ventricular end-systolic volume; PMI: post myocardial infarct; 8W FU: eight week follow-up; LAD: left anterior descending artery; delta: indicates the absolute difference between post myocardial infarct measurement and 8 week FU. eMSC indicated encapsulated MSC

Supplemental table II: PV-loop parameters

Parameter		Study 2: severe Infarct				
Systolic Parameters	**measure**	**Control**	**10.000 eMSC**	**20.000 eMSC**	**40.000 eMSC**	**P-value**
dP/dt + 8 week FU	mmHg/s	974.6 ± 161.7	1417.1 ± 113.2	1071.4 ± 135.3	1226.4 ± 136.2	NS
ESPVR 8 week FU	mmHg/mL	1.9 ± 0.5	5.2 ± 1.0	6.5 ± 1.3	5.2 ± 1.7	0.03
PRSW 8 week FU	mmHg	59.7 ± 10.2	72.4 ± 13.2	56.7 ± 7.8	51.2± 17.7	NS
Diastolic Parameters						
Tau 8 week FU	ms	68.8 ± 5.8	55.0 ± 4.0	67.9 ± 6.8	47.7 ± 2.1	NS
dP/dt - 8 week FU	mmHg/s	-624.2 ± 129.7	-1051 ± 82.8	-634.5 ± 94.3	-851.0 ± 66.8	NS
EDPVR Baseline	mmHg/ml	0.184 ± 0.07	0.027 ± 0.015	0.0168 ± 0.028	0.02 ± 0.003	NS

Supplemental table SIII represents some admittance derived parameters. End-systolic pressure volume relationship (ESPVR) is significantly better in all animals indicating a preserved contractility. PRSW indicates; pre-recruitable stroke work. EDPVR: end-diastolic pressure volume relationship.

Supplemental table III. Histological analysis

Study 1: Moderate infarct						
parameter	**measure**	**Control**	**20,000 eMSC**	**40,000 eMSC**	**60,000 eMSC**	**P- value**
Infarct size	%	9.6 ± 1.3	7.6 ± 1.2	9.1 ± 1.2	9.3 ± 1.8	NS
Collagen density						
Infarct	% collagen	62.6 ± 7.6	59.1 ± 7.8	71.5 ± 5.0	69.6 ± 5.0	NS
Border	% collagen	3.4 ± 0.6	4.4 ± 1.1	3.5 ± 0.7	2.8 ± 0.6	NS
Remote	% collagen	2.0 ± 0.3	1.4 ± 0.2	1.8 ± 0.3	1.7 ± 0.5	NS
Arteriole density						
Infarct	arterioles/mm^2	12.8 ± 1.1	22.7 ± 2.9	20.8 ± 3.4	17.3 ± 1.8	NS
Border	arterioles/mm^2	14.7 ± 1.5	18.3 ± 3.3	16.7 ± 1.5	14.2 ± 1.3	NS
Remote	arterioles/mm^2	10.1 ± 0.9	10.4 ± 2.5	11.2 ± 1.7	9.6 ± 2.3	NS
Capillary density						
Border	capillaries/mm^2	855.5 ± 83.7	992.0 ± 147.4	889.3 ± 128.9	966.7 ± 145.2	NS
Remote	capillaries/mm^2	1131 ± 196.2	890.7 ± 142.6	1140.0 ± 1160.4	1157.0 ± 196.5	NS
Cardiomyocyte apoptosis						
Border	% apoptosis/view	7.0 ± 0.7	5.2 ± 0.9	5.5 ± 0.4	4.2 ± 0.5	NS
Remote	% apoptosis/view	5.4 ± 0.6	3.7 ± 0.5	4.5 ± 0.9	3.9 ± 0.7	NS
Study 2: Severe infarct						
		Control	**10.000 eMSC**	**20.000 eMSC**	**40.000 eMSC**	**P-value**
Infarct size	%	20.5 ± 1.4		16.7± 0.2		NS
Collagen density						
Infarct	% collagen	95.1 ± 2.1	76.1 ± 8.7	78.2 ± 6.2	72.6 ± 9.0	0.09
Border	% collagen	9.2 ± 3.1	4.7 ± 1.3	3.2 ± 1.3	5.2 ± 1.6	0.04
Remote	% collagen	7.6 ± 2.6	2.5 ± 0.7	3.2 ± 0.6	1.8 ± 0.9	0.05
Arteriole density						
Infarct	arterioles/mm^2	8.4 ± 0.9	15.5± 4.4	22.2 ± 2.6	15.4 ± 0.6	0.004
Border	arterioles/mm^2	6.6 ± 0.4	6.0 ± 0.4	9.7 ± 1.4	7.2 ± 2.1	NS
Remote	arterioles/mm^2	3.8 ± 0.5	4.2 ± 0.3	5.7 ± 1.1	3.7 ± 0.8	NS
Capillary density						
Border	capillaries/mm^2	908.1 ± 99.6	1066.0 ± 152.0	1209 ± 64.6	951.3 ± 181.5	NS
Remote	capillaries/mm^2	1131.0 ± 119.9	899.8 ± 76.8	1232.0 ± 130.9	837.3 ± 137.5	NS
Cardiomyocyte apoptosis						
Border	% apoptotis/view	7.5 ± 2.0	3.7 ± 1.5	2.4 ± 0.7	5.0 ± 3.3	NS
Remote	% apoptotis/view	4.8 ± 0.9	1.9 ± 1.0	4.0 ± 1.5	4.8 ± 1.8	NS

Supplemental table III. Overview of histological analysis. NS indicates not significant. eMSC: encapsulated mesenchymal stem cells

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PART 5

Methods in experimental cardiology

CHAPTER 11

Cardiac function in a long term follow-up study of a moderate and severe porcine model of chronic myocardial infarction

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Submitted

ABSTRACT

Background Novel therapies need to be evaluated in a relevant large animal model that mimics the clinical course and treatment in a reasonable time frame. To reliably assess therapeutic efficacy, knowledge regarding the translational model and the course of disease is needed.

Methods Landrace pigs were subjected to a transient occlusion of the proximal left circumflex artery (LCx), (n=6) or mid left anterior descending artery (LAD), (n=6) for 150 min. Cardiac function was evaluated before by 2D-echocardiography or 3D-echocardiography and pressure-volume loop analysis. At 12 weeks follow-up the heart was excised for histological analysis and infarct size calculations.

Results Directly following AMI, LVEF was severely reduced compared to baseline in the LAD group (-17.1± 1.6%, p=0.009) compared to only a moderate reduction in the LCx group (-5.9± 1.5 %, p=0.02) and this effect remained unchanged during 12 week follow-up.

Conclusion Two models of chronic MI, representative for different patient groups, can reproducibly be created through clinically relevant ischemia-reperfusion of the mid-LAD and proximal LCx.

INTRODUCTION

The treatment of patients suffering from myocardial infarction (MI) is aimed at the preservation of cardiac function. Most treatment regimens directly target pathways that limit infarct size or reduce adverse remodeling, thereby preventing progression into heart failure (HF).[1–3] To fully determine the possible effect of such therapies, thorough testing in clinically relevant animal models is needed.[4,5] Since large animal models enable clinical treatment regimens, delivery route and identical function-related measurements, they are considered to withhold greater translational value than small animal models and are therefore superior for efficacy testing.[6–10]

Importantly, due to optimized logistical and diagnostic health care, the complaint-to-needle-time has decreased considerably in the western world.[11] This has resulted in a large proportion of patients with a relatively preserved left ventricular function after MI.[12] These patients could, however, still benefit from therapy that further confines the amount of damage directly post-MI. To test the efficacy of such therapies, an animal model that closely resembles the clinical course of disease in a mildly damaged heart is mandatory. At the same time, therapy optimization has also resulted in an increased incidence of HF, since patients survive with severely deteriorated cardiac function.[13] This patient group would greatly benefit from therapy that improves cardiac function. For this purpose, an animal model that closely resembles the progression into HF after MI in patients with a severely damaged heart is needed.

To create both a moderate and a severe model of chronic myocardial infarction that resemble these different patient-groups, ischemia can be induced in several ways. This includes permanent ligation, progressing occlusion by placement of ameroid constrictors, a bottleneck stent model, coiling or infusion of ethanol in the target coronary artery.[14–18] However, most of these models do not mimic current clinical reality, where most patients are revascularized within relatively short time after occlusion. Since revascularization of the target vessel remains the cornerstone treatment in MI patients, animal models have to simulate this situation. Even more so since myocardial wound healing and other molecular mechanisms are substantially different after revascularization compared to those during persistent ischemia.[19,20] Moreover, the chronic coronary occlusion precludes intracoronary infusions, the easiest and fastest technique for myocardial delivery of therapeutics. Hence, models of ischemia/reperfusion, in which coronary flow is restored after an ischemic period, seem most suited to validate novel therapies for the treatment of MI.

In most ischemia/reperfusion models, the left anterior descending artery (LAD) is occluded, which is associated with a high mortality rate during infarct induction and reperfusion due to ventricular fibrillation (VF). [8,21] As an alternative for LAD occlusion, the left circumflex artery (LCx) can be occluded. The LCx is responsible for approximately 20% of the blood supply to the left ventricle and an occlusion-reperfusion model of this artery may result in a lower mortality rate.[21] However, it is not fully elucidated to what extent animals that are subjected to LCx ischemia-reperfusion develop cardiac dysfunction during long term follow-up. Moreover, no thorough sequential investigation of cardiac (dys)function and ventricular dilatation has been performed in either model to the best of our knowledge. Therefore, this study was designed to investigate the long-term effect of myocardial infarction on cardiac function in two clinically relevant large animal models of MI: a severe mid-LAD

ischemia-reperfusion and a moderate proximal LCx ischemia-reperfusion model. In the present study we investigated the course of both global and regional cardiac function decay in the two models combined with mortality, infarct size and histological analysis.

Objectives

To determine the long-term course of cardiac function in a moderate LCx and a severe LAD ischemia-reperfusion model for future validation of novel therapeutic strategies. We hypothesized that LAD ischemia-reperfusion will result in fast development of cardiac dysfunction representing a good model for severely affected patients with cardiac dilatation and signs of heart failure. Secondly, we hypothesized that the LCx ischemia-reperfusion model leads to a moderately affected heart representing a large patient group that has been adequately revascularized after an initial ischemic event but could still benefit from additional therapy.

MATERIAL AND METHODS

All procedures were approved by the local animal welfare committee of the University of Utrecht (Utrecht, The Netherlands; Permit number 2011.II.04.068). A total of 12 female specific pathogen free (SPF) landrace pigs were included (65.1 ± 1.0 kg; Van Beek, Lelystad, The Netherlands). The pigs were housed in the experimental animal facility at the University of Utrecht (Utrecht, The Netherlands) in a group prior to procedure and individually after the AMI (to prevent hostile behavior after infarct procedure). The animals were checked by a veterinarian upon arrival at the facility and daily scored by a bio-technician and once a week by a veterinarian during follow-up. Six animals were randomized to undergo ischemia-reperfusion of the LCx and 6 animals were randomized to undergo LAD ischemia reperfusion (Figure 1).

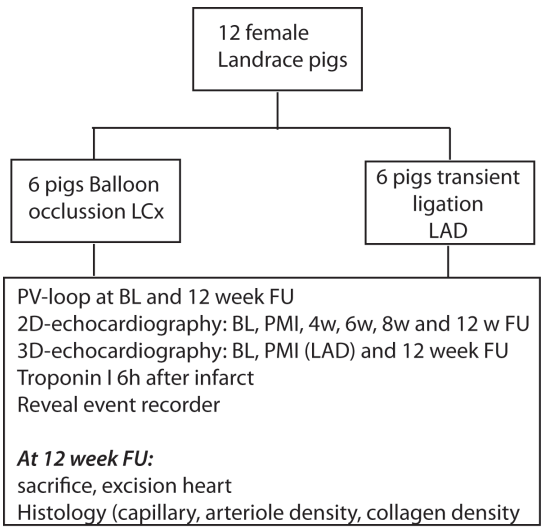

Figure 1. Study design.
a; example of balloon occlusion of the left circumflex artery (LCx). b; Example of ligation of the left anterior descending artery (LAD). BL: Baseline before infarct induction; PMI: post myocardial infarct, w: week

Medical treatment before infarct induction

All animals received dual anti-platelet therapy (Acetyl Salicylic Acid 80 mg/d: Ratiopharm, Haarlem, The Netherlands; clopidogrel 75 mg/d: Apothecon, Barneveld, The Netherlands) and anti-arrhythmic drugs (Amiodarone, 1200 mg loading dose, 800 mg qd; Sanofi-Aventis, Paris, France) starting 10 days prior to infarct induction up until sacrifice at 12 weeks FU,.

Anesthesia protocol

General anesthesia was induced with an intramuscular injection of 0.5 mg/ kg midazolam (Actavis, Zug, Switzerland), 10 mg/kg ketamine (Narketan,Vétoquinol, Lure Cedex, France) and 1 mg of Atropine (Pharmachemie BV, The Netherlands) and maintained with intravenous infusion of midazolam 0.5 mg/kg/h, sufentanil 2.5 µg/kg/h (Janssen-Cilag B.V, Tilburg, The Netherlands) and pancuronium 0.1 mg/kg/h (Inresa, Battenheim, Germany). Upon infarct induction, all animals were therapeutically heparinized with 2 doses of 5000 IE (Leo pharma, Ballerup, Denmark). All pigs received a Fentanyl patch (25mg, Janssen-Cilag,Tilburg Netherlands) and meloxacam (Boehringer-Ingelheim, Alkmaar, The Netherlands) 0.5 mg/kg/d, as post-surgery analgesia .

Infarct procedure

Animals were randomized before the start of the procedure via sealed envelopes. Cardiac function at baseline was quantified by 2D-echocardiography and pressure-volume (PV)-loop analysis. Blood was sampled before AMI and 6 hours after for Troponin I analysis. All animals received a Reveal™ event recorder (Medtronic, Tilburg, The Netherlands).

Balloon occlusion of the LCx

An 8F sheath was inserted in the carotid artery and an 8F guiding catheter (JL 3.5-4.0, Boston Scientific Nederland B.V., Nieuwegein, The Netherlands) was positioned at the ostium of the left main coronary artery. An angioplasty balloon (Trek 3,5-4,0 x12, Abbott) was inflated (8-14 bar) for 150 min in the proximal LCx (figure 1). After balloon inflation, the guiding catheter was carefully retracted to enable normal blood flow through the non-occluded part of the left coronary system. Position was verified every 15 minutes to ensure appropriate occlusion of the LCx. After the procedure catheters were removed and the wound was closed.

Open chest ligation of the LAD

An antero-septal myocardial infarct was induced during an open chest procedure in order to reduce peri-procedural mortality due to VF. The thorax was opened via sternotomy. The infarct was induced by a transient ligation of the mid LAD after the first diagonal for 150 min. All pigs subjected to LAD ischemia-reperfusion underwent a 3D epicardial echocardiography before and after infarct induction.

Follow-up

During the 12 week FU, a 2D-echocardiography was performed at 4 and 8 weeks FU under induction

medication as described above. Twelve weeks after infarct induction the animals were anesthetized, and 2D and 3D epicardial echocardiography were performed followed by invasive PV-loop measurements. The animals were sacrificed and the hearts were excised for infarct size determination and histological analyses.

Echocardiography

Echocardiography was performed using a Phillips iE33 echocardiography machine (Phillips, Eindhoven, The Netherlands). 2D images were obtained of the parasternal long axis and parasternal short axis at basal, mid-ventricular and apical level. The 2D echo was repeated after infarct induction in the animals with LCx infarction. This was not possible in the LAD group because of fluid and air in the chest after open chest procedure. Additionally, a 3D epicardial echocardiogram was performed in the LAD ischemia reperfusion before and after myocardial infarction as previously described.[22] In both groups, animals underwent additional 2D-echocardiography at 4 and 8 weeks following infarct induction, using a mild sedation of Midazolam, ketamine and atropine as described earlier. At sacrifice, both animals in the LCx and LAD group underwent transthoracic 2D and an epicardial 3D echocardiography. Images were analyzed using Velocity Vector Imaging (VVI, Siemens Medical solutions, USA). The end-diastolic and end-systolic volumes were calculated by the modified Simpson rule (LV end diastolic volume (LVEDV)= (A_{bED}) * L/3 + $(A_{mED}+A_{pED})/2)$ * L/3 + 1/3(A_{pED}) * L/3; LV end systolic volume (LVESV)= (A_{bES}) * L/3 + $(A_{mES}+A_{pES})/2)$ * L/3 + 1/3(A_{pES}) * L/3, in which A_b is the area at basal level, whereas A_m and A_p are the areas at mid and apical level respectively and L is the length of the ventricle) [23]. LVEF was calculated by: ((LVEDV-LVESV)/LVEDV)*100.

3D-echocardiographs were analyzed offline using Qlab 10.1 software (Phillips, Eindhoven, The Netherlands) as described before [24]. One full volume analysis was used and the end diastolic frame was selected. Markers were placed at the base of anterior, posterior, inferior, lateral side and at the apex. The left ventricle was automatically traced by the software. All frames were checked for correct tracing and manually corrected if needed. The same was repeated for the end systolic phase.

Strain analysis

Strain is defined as the total deformation of the myocardium during 1 cardiac cycle [25,26].Radial and circumferential strain were analyzed on the 2D-echocardopgraphy short axis views via speckle tracking (VVI, Siemens Medical solutions, USA) as previously described.[25] Strain was analyzed according to the 17-segment echocardiography model. Figure 2 provides a schematic overview of the radial strain and circumferential strain and the 17-segment echocardiography model. In the LAD model anterior and antero-septal segments were analyzed, whereas the inferior and infero-lateral segments were used as reference segments in this group. In the LCx group, inferior and inferio-lateral segments were mostly affected and anterior segments were used as reference segments. Moreover, global strain was calculated by the software. Strain is represented as percentage of left ventricular deformation.

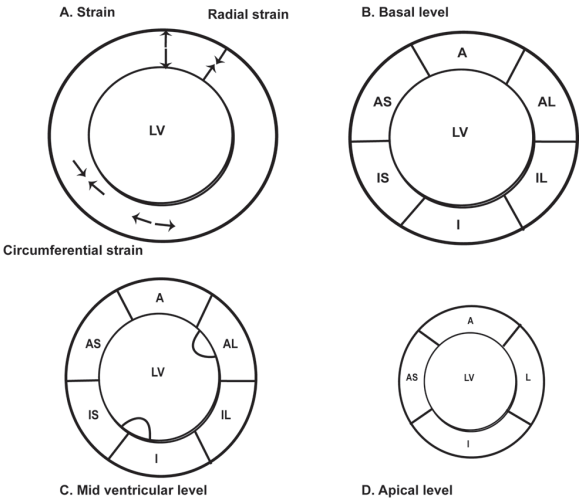

Figure 2: Schematic overview of Strain and 17-segment echocardiography model.

a; schematic overview of strain analysis. Radial strain represents thickening (systole, arrows outwards) and thinning (diastole, arrows facing each other) of the myocardium during 1 cardiac cycle. Circumferential strain: elongation and shortening (arrows facing each other) of myocardial muscle fibers. b; schematic overview of myocardial segments on short axis view at basal level (mitral valve level). A indicates anterior wall; AL: antero-lateral wall; IL: infero-lateral; I: inferior; IS: infero-septal; AS; antero-septal. C: Schematic overview of short axis view of myocardial segments at mid-ventricular level (papillary muscle). D: Schematic view of the apex. The apex only consists of 4 segments. L indicates lateral wall.

Pressure volume loop analysis

A 7F conductance catheter (CD Leycom, Zoetermeer, The Netherlands) was placed under fluoroscopic guidance in the apex of the left ventricle as previously described [27]. Pressure Volume loops (PV loops) were assessed during apnea to avoid pressure/volume changes due to mechanical ventilation. Calibration was performed as previously described [28]. PV-loops were performed to obtain data of systolic indices (End-systolic pressure, stroke volume, end systolic pressure volume relations, stroke work, dP/dt max, prerecruitable stroke work), and diastolic indices (end diastolic pressure, tau, dP/dt min and end diastolic pressure volume relations). Analyses were performed using Conduct NT analyses software version 16.1 (CD Leycom, Zoetermeer, The Netherlands).

Troponin I levels

Troponin I levels were quantified using a standardized ELISA protocol of the Clinical chemistry laboratory of the University of Utrecht

Infarct size calculations and histological analysis

After the heart was excised, the atria were removed and the ventricles were cut into 5 slices of approximately 1 cm thickness. The heart was stained using 5% Tetrazolium chloride solution for 10-15 minutes at 37º. The slices were photographed and infarct size was calculated using automatic

computer assisted image analysis software (Clemex, Quebec, Canada) as described before [29]. Infarct size was calculated as percentage of the total LV area.

Biopsies were taken from the infarct area, the infarct border zone and the remote myocardial segments followed by embedding into paraffin for further light microscopical analysis. Collagen content in infarct, border and remote myocardial segments was assessed by trichrome stain. Briefly, all sections were deparaffinised, fixed in Buoin's fixative (Sigma-Aldrich, St. Louis, USA) at 56º for 15 minutes, Nuclei were stained with haematoxylin for 3 minutes. The slides were submerged in Trichrome-AB solution for 5 minutes after which they were treated with 0.5% acetic acid for 1 minute. Slides were mounted with Entallan (Merck, Darmstadt, Germany). Three random pictures were made at a 10X magnification and collagen content was calculated as percentage collagen of total surface area using automated analysis software (Clemex, Quebec, Canada).

Arteriole density was quantified in infarct, border and remote myocardial segments, using alpha smooth muscle actin immunohistochemistry analysis (SMA, clone 1a4, Sigma-Aldrich, St Louis, USA). Endogenous peroxidase activity was blocked by 3% methanol/H_2O_2 solution for 30% and incubated with SMA 1:1000 overnight. Subsequently, the slides were incubated with secondary HRP-conjugated goat anti-mouse antibody dilution 1:200 (DAKO, Glostrup, Denmark) for 90%. All slides were immersed in DAB solution for 2 minutes (DAKO, Glostrup, Denmark) and mounted with Entallan. A technician that was blinded for group allocation took 3 random pictures at 10 times magnification. Arterioles per field of view were counted and expressed as number per view.

Capillary density was only assessed in border en remote areas, because almost all capillaries are destroyed after AMI. All sections were deparaffinised and pre-treated with trypsin EDTA (Lonza, Verviers, Belgium). Endogenous peroxidase activity was blocked as described above. All slides were incubated with Isolectin B_4 (Bandeiraea simplicifolia Isolectin B_4, Dako, Glostrup, Denmark diluted 1/50) overnight. Subsequently, the slides were immersed in DAB solution and mounted with Entallan. Photographs were taken at 20X magnification and number of capillaries per mm^2 was calculated.

Statistical analysis

All data were analyzed using SPSS Statistics 20 (IBM statistics, Chicago, USA). All data are presented as mean ± SEM. Comparisons between groups at a single time point (histology, infarct size, comparison of 12 week FU data) were analyzed using Student's t-tests or Mann Whitney U tests. For sequential data, a two-way repeated measures ANOVA was applied. Correlations were analyzed by the Pearson's correlation coefficient. $P<0.05$ was considered statistically significant.

RESULTS

Mortality and safety

All animals that underwent LCx occlusion survived initial infarct induction (100%) compared to 5/6 animals in the LAD group (83 %; p=NS). This animal died of ventricular fibrillation during infarct induction, resistant to defibrillation. Fifty percent of the animals in the LCx group needed defibrillation during infarct induction with an average of 7.8 times, as compared to 5/6 animals in the LAD group

with an average of 12.2 times defibrillation. During the 12 week FU period, none of the animals died or were treated for heart failure. No ventricular arrhythmias were recorded on the Reveal™ detector during the 12 weeks FU.

Cardiac function by echocardiography

Both LVEF and LV volumes before infarct induction were comparable between the LCx and LAD group. Post-AMI LVEF decreased with 5.9% to 50.8±1.6 % (p=0.03) in the LCx group and with 17.1% to an average of 39.4±1.6% in the LAD group (p=0.009). Following AMI, LVESV increased with 5.6± 2.0 mL (p=NS) in the LCx group and with 17.8±6.7 mL (p=0.01) in the LAD group (*figure 3)*. There was no significant change in LVEDV following infarct induction in either of the groups. *Table 1* depicts all cardiac dimensions in both groups.

Table 1. Echocardiography data.

Echocardigraphy	LCx	p-value to BL *	LAD	p-value to BL*	p-value between groups#
LVEF Baseline	56.7 ± 2.0		56.4 ± 1.5		NS
LVEDV Baseline	80.8 ± 6.6		68.3 ± 3.7		NS
LVESV Baseline	35.3 ± 4.3		30.1 ± 4.0		NS
LVEF PMI	50.8 ± 1.6	0.03	39.4 ± 1.6	0.009	0.02
LVEDV PMI	83.7 ± 4.3	NS	79.2 ± 5.8	NS	NS
LVESV PMI	40.9 ± 1.1	NS	47.9 ± 3.7	0.01	0.09
LVEF 4 weeks	48.6 ± 1.0	0.02	40.3 ± 0.7	<0.001	0.04
LVEDV 4 weeks	88.6 ± 4.4	NS	96.5 ± 4.3	0.01	0.06
LVESV 4 weeks	45.5 ± 2.0	0.07	57.7 ± 2.8	0.01	0.03
LVEF 8 weeks	51.4 ± 1.0	NS	39.9 ± 1.0	<0.001	0.04
LVEDV 8 weeks	95.8 ± 5.9	NS	97.4 ± 7.8	0.06	NS
LVESV 8 weeks	46.5 ± 3.4	0.06	58.5 ± 5.0	0.01	0.07
LVEF 12 weeks	49.3 ± 0.8	0.06	41.7 ± 1.5	<0.001	0.004
LVEDV 12 weeks	91.4 ± 4.9	NS	104.1 ± 5.7	0.02	0.03
LVESV 12 weeks	46.0 ± 2.4	0.06	60.7 ± 4.1	<0.001	0.04

Table 1 represents all data and p-values for echocardiography. The post myocardial infarct values of the LAD study are measured with 3D echocardiography. LCx indicates left circumflex artery; LAD: left anterior descending artery. LVEF: left ventricular ejection fraction; LVEDV: left ventricular end-diastolic volume; LVESV: left ventricular end-systolic volume. BL: baseline before infarct, PMI: post myocardial infarct. * repeated measures Anova (values compared to baseline), # student's t-test.

At 12 weeks FU, 2D-echocardiography revealed a decreased LVEF of 7.9% to 49.3±0.8% (p=0.06) in the LCx group whereas LVEF of the LAD group declined with 14.7% to 41.7±1.5 % (p <0.001). LVESV increased non-significantly in the LCx group with 10.7mL to 46.0±2.4 mL (p=0.06), where the LAD group did show a significant increase of 30.6 mL to 60.7±4.1 mL (p<0.00,1). No significant dilatation as measured by LVEDV was seen in the LCx group (+10.4mL to 91.4±4.9 mL, p=0.09). LVEDV in pigs subjected to LAD occlusion, however, was significantly increased during follow-up (+35.8mL to

104.1±5.7 mL, p= 0.02). At 12 weeks FU, 3D-echocardiography confirmed the 2D-echocardiography measurements (*figure 3*). In the LCx group, LVEF was 50.0±2.4% as opposed to 39.2±1.9% in the LAD group. LVEDV in the LCx group at 12 weeks FU was 98.5±2.9 mL as compared to 126.1±3.5 mL in the LAD group.

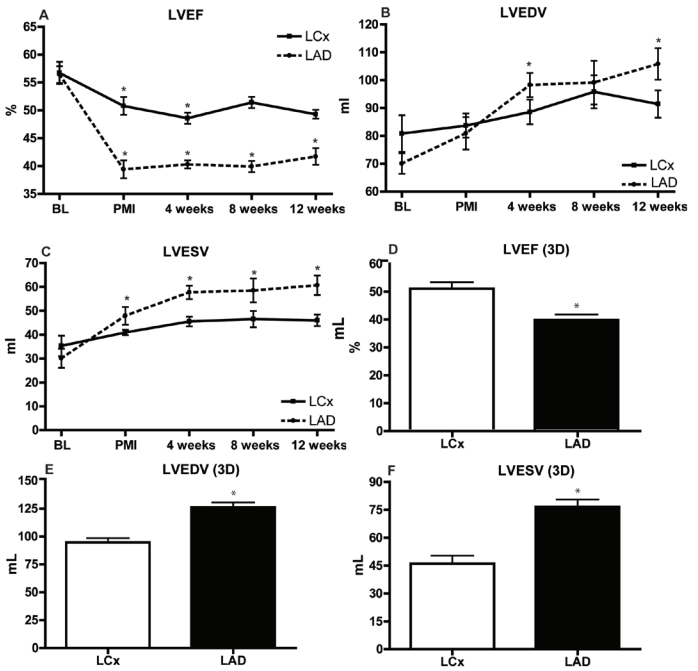

Figure 3. Echocardiography.

a-c: LVEF and LV volumes on echocardiography over time. a: Left ventricular ejection fraction (LVEF) over time. b: Left ventricular end-diastolic volume (LVEDV) over time. c: Left ventricular end-systolic volume (LVESV) over time. d-f: 3D-echocardiography at 12 week FU. d: LVEF; e: LVEDV and; f: LVESV at 12 weeks FU as measured by 3D-echocardiography. LCx indicates left circumflex artery; LAD: left anterior descending artery.*P<0.05

Radial and circumferential strain

Decreased strain indicates deteriorated contractile function of the myocardium. In the LCx group, no changes in radial strain were observed in the infarcted inferior segment (*figure 4*).

For the LAD-group, radial strain was significantly attenuated in the anterior segment of the apex during FU and kept progressively declining (42.8±4.8% at baseline and 5.6±7.7% at 12 week FU; p=0.008; figure 4). In this group, total apical radial strain at 12 week FU also decreased to 20.0±4.5% opposed to 45.1±4.1% at baseline (p=0.04). The total ventricular strain at 12 week FU was significantly lower in this group at 12 week FU (48.6±2.0% at baseline and 30.1±3.8 % at follow-up; p=0.03. These findings corroborate with the LVEF echocardiography data. Circumferential strain was significantly reduced in the LAD group in the apex and at mid-ventricular level of the heart throughout the 12 week FU compared to baseline (*figure 4*).

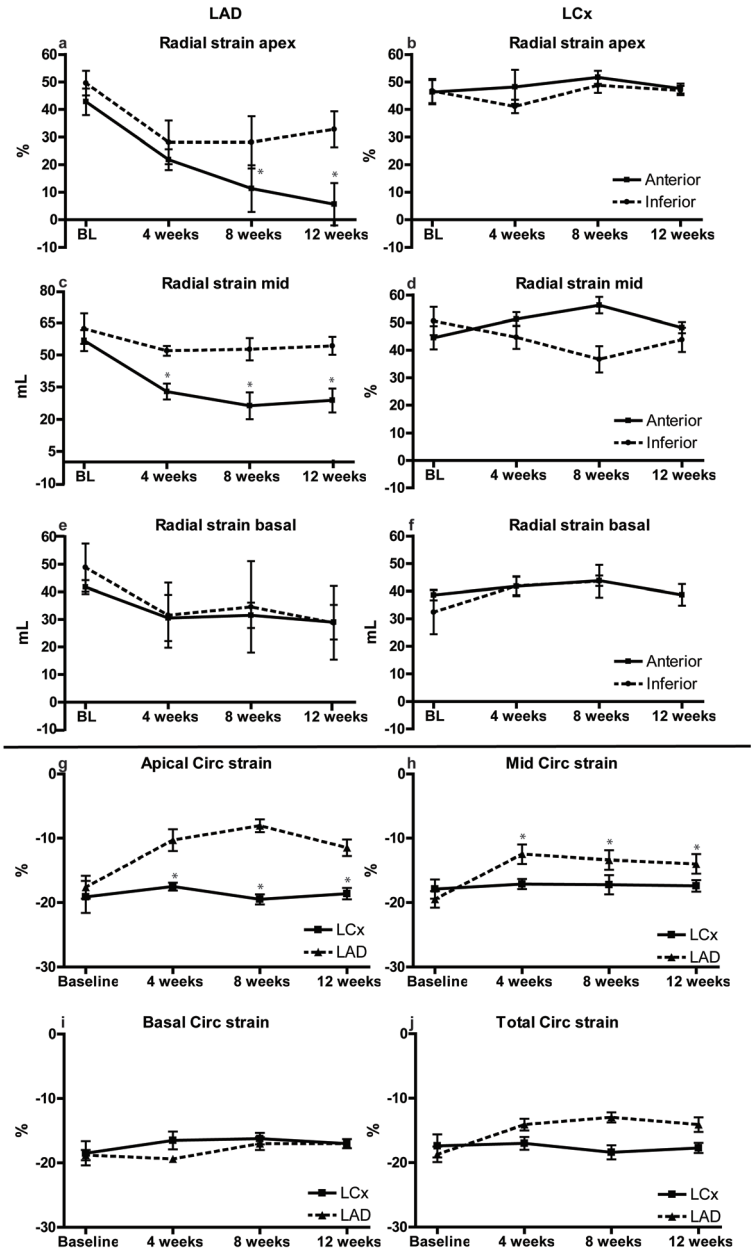

Figure 4. Radial strain an circumferential strain.

A-f: Radial strain anterior and inferior segments. a-b: Radial strain apex, inferior and anterior segments per group. Strain in the anterior section of the LAD study is significantly lower during follow-up than at baseline. C-d: Radial strain mid ventricular level, inferior and anterior segments per group. E-f: Radial strain basal level, inferior and anterior segments per group. G-j: Circumferential strain. g: Apical circumferential strain over time; h: Circular strain at mid ventricular level over time. i; Circumferential strain at basal level over time. j; Total ventricular circumferential strain over time. LCx indicates left circumflex artery; LAD: left anterior descending artery; BL: baseline. *p<0.05 within the LAD group.

PV-loop analysis, Infarct size, Troponin and Histology

Invasive real-time PV-loop analysis was also performed at baseline and 12 weeks FU. dP/dt – (relaxation of the ventricle) and ESPVR (derivative of contractility) were significantly decreased in the LAD group but not in the LCx group (*figure 5*). Indices of diastolic dysfunction, Tau and tPFR (time to peak filling rate) and systolic indices tPER (time to peak ejection rate) increased in both groups. Troponin I levels 6 hours after infarct induction in the LCx group were lower than in the LAD group (358.6 ± 79.9 ug/L vs. 560.0 ± 79.9 ug/L, P=0.02; *figure 5*). Post AMI decrease in cardiac function and Troponin levels 6 hours after infarct correlated significantly (R= -.763; P=0.028). The average infarct size in the LAD group was significantly higher than in the LCx group (23.4 ± 2.1% in the LAD group versus 9.5 ± 1.7% in the LCx group, *figure 5*).

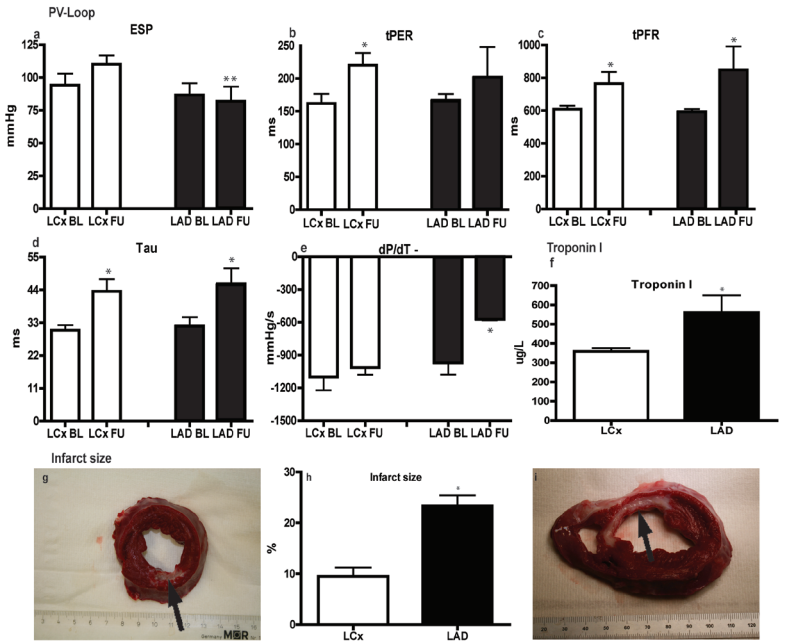

Figure 5. Pressure-Volume loop analysis, troponin I and infarcts size.

A-e: Pressure-volume loop analysis. a; End-systolic pressure (ESP) is significantly lower at follow-up in the LAD group; b: Time to peak ejection rate (tPER); C: Time to peak filling rate (tPFR); d: Tau: Relaxation constant; e: Increase in pressure over time (dP/dt +). F: Troponin I levels 6 hours after myocardial infarction are higher in the LAD group. g-I; Infarct size. g: example of infarct by left circumflex artery infarct (LCx) ischemia- reperfusion model. Viable myocardium is red, infarct is depicted in white(arrow); h: Infarct size in left anterior descending artery (LAD) group is significantly higher; i: example of an LAD infarct. LCx indicates left circumflex artery; LAD: left descending anterior artery; BL: baseline; FU: follow-up. *P<0.05

There were no differences in collagen density, capillary density and arteriole density in all myocardial segments (*figure 6*; table 2). A trend towards an increase in arteriole density was observed in the LAD group (p=0.09)

Table 2. Histological analysis

Parameter	measure	LCx	LAD	P-value
Collagen density infarct area	%	89.2 ± 4.0	89.2 ± 4.2	NS
Collagen density border area	%	5.1 ± 1.5	5.5 ± 1.3	NS
Collagen density remote area	%	4.4 ± 1.1	3.0 ± 0.7	NS
Arteriole density infarct area	arterioles/view	11.2 ± 1.5	10.1 ± 1.9	NS
Arteriole density border area	arterioles/view	5.1 ± 1.1	9.2 ± 1.4	NS
Arteriole density remote area	arterioles/view	3.5 ± 0.5	5.4 ± 0.9	NS
Capillary density border area	capillaries/mm²	713.9 ± 74.3	714.4 ± 85.5	NS
Capillary density remote area	capillaries/mm²	470.5 ± 48.0	376.4 ± 37.9	NS

Overview of histological parameters. NS indicates not significantly different between de groups.

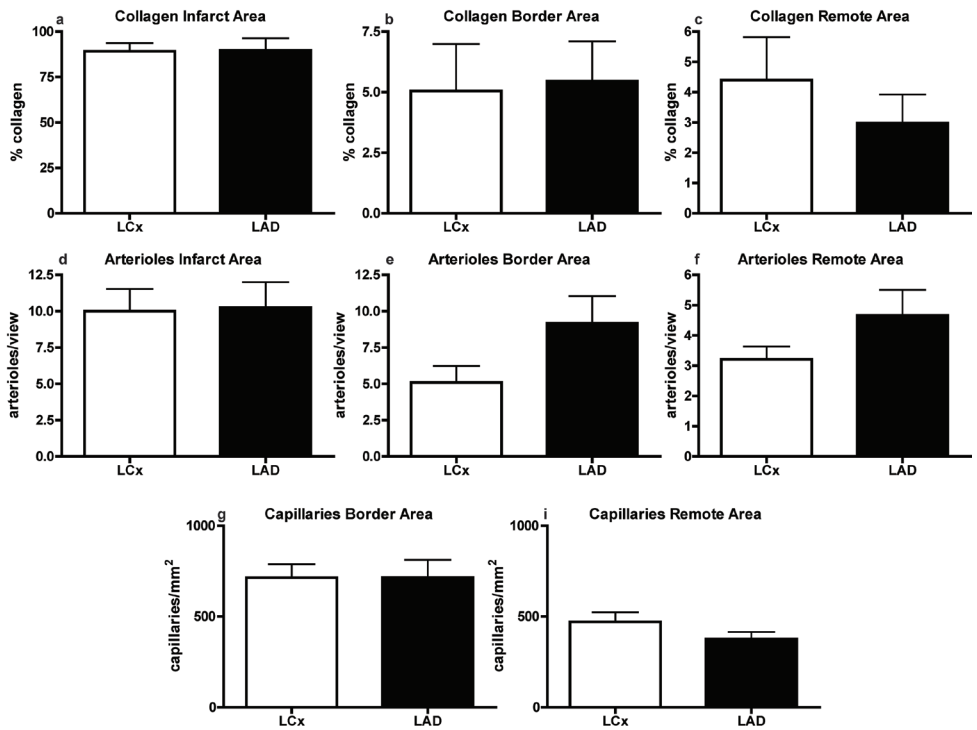

Figure 6. Histology.

A-H: There were no statistical differences on histological parameters in both groups. LCx indicates left circumflex artery; LAD: left descending anterior artery. P=NS

DISCUSSION

The potential effects of novel therapeutics that confine the damage after MI depend on the severity of the ischemic event.[3] To determine which patient group (e.g. moderately or severely affected patients) benefits most from such therapeutics, their efficacy has to be tested in large animal models that resemble the clinical course and severity as closely as possible.[4,6,21] Therefore, the current study investigated cardiac function over time in two clinically relevant porcine models of ischemia-reperfusion: a severe LAD and a moderate LCx ischemia-reperfusion model. Here, we demonstrated that occlusion of 150 minutes of the LAD resulted in overt deterioration of cardiac function and progressive cardiac dilatation. Therefore, this model is most suitable for testing therapeutics that focus on the prevention of adverse remodeling post-MI in severely affected patients. On the other hand, occlusion of the LCx resulted in limited effects on cardiac contractility and dilatation, resembling the clinical course of adequately revascularized MI patients with only limited cardiac damage. Thus, this model can be used to test compounds that reduce infarct size directly post-MI but is less suitable for prevention of adverse remodeling.

As hypothesized, the two models showed a marked difference in response to ischemia-reperfusion. First, in the LCx model none of the animals died which was, most likely, based on fewer episodes of VF. Presumably this is due to the involvement of the septum in the infarct area in the LAD model combined with a smaller area at risk after occlusion of the LCx. This difference in the area at risk also resulted in a large difference in infarct size, which was approximately 23% of the LV in the LAD model compared to 10% in the LCx model. In turn, the larger infarct size in the LAD model resulted in a profound decrease in LVEF culminating in adverse remodeling and cardiac dilatation. The decrease in LVEF occurred to a lesser extent in the LCx group and this did not result in any cardiac dilatation post-MI. In concordance with the functional echocardiography data, myocardial strain was decreased in the affected myocardial segments in the LAD group, whereas strain was preserved in the affected segments in the LCx group. To the best of our knowledge, this is the first study that has studied myocardial strain when comparing different MI models and these findings show that the two models are not only different regarding the global cardiac function but also differ on a regional contractile level. Despite the significant differences in systolic function in the LAD group, no differences between the groups were observed regarding diastolic dysfunction. In both studies, diastolic dysfunction worsened relative to baseline measures, which suggests that these measurements are very robust and are not directly influenced by infarct size or dilatation in the current study.

In a previous study of Suzuki in which the mid-LAD was occluded for 60 minutes followed by reperfusion, a comparable mortality (16%) and decrease in cardiac function was observed following infarct induction.[18] However, in that study LVEF decreased transiently and recovered significantly at 14 days and 28 days follow-up to 47%. This is most likely due to the occlusion time. A 60-minute occlusion period presumably leads to reversible cell damage, with a large fraction of myocardial stunning/hibernation that is known to resolve spontaneously within days to weeks.[30] In our study, LVEF did not recover during FU after the initial decrease post-MI in either model. In the LAD model this simultaneously occurred with increased LVESV and LVEDV, suggesting progression into heart failure.

This provides a larger therapeutic window for experimental therapies that target adverse remodeling and prevent cardiac dilatation in this model compared to spontaneously recovering models. The same holds true for the LCx model. Again, therapeutic efficacy is easier to test in a model in which outcome is not affected by confounding factors such as spontaneous recovery that may exceed and hence mask the therapeutic effect. Importantly, our findings in both the LAD model and the LCx model on cardiac function and infarct size correspond with chronic myocardial ischemia models.[15,16] This indicates that 150 minute occlusion followed by reperfusion results in the same amount of myocardial damage but more closely resembles the clinical course and treatment of MI. Moreover, in our models, intracoronary therapy to limit myocardial damage is still possible.

Despite our best efforts, the study has some limitations. First, we assessed cardiac function by 2D-echocardiography during follow-up and obtained 3D echocardiogram directly post-MI (LAD-model only) and at sacrifice (both models). Due to the anatomical position of porcine ribs, it is not possible to obtain a transthoracic 4 chamber view that would be needed for 3D- echocardiography. Although cardiac MRI is considered the gold standard for cardiac function and volumes, due to logistic reasons, it was not feasible to obtain sequential cardiac MRI data in our study. We are confident that our echocardiography data are reproducible and depict the true cardiac function following both ischemia-reperfusion models. Second, in one group we performed a closed chest balloon occlusion model and in the other group an open chest model was applied. The latter was chosen in order to be able to perform epicardial defibrillation in the LAD model to improve survival in the LAD model. Since the study was not designed to directly compare these two models, a similar infarct induction was, however, not essential.

To conclude, the current study showed that it is feasible to create two very distinctive models of chronic myocardial infarction to test novel therapeutics post-MI for the possible treatment of different patient groups. Reperfusion after 150 min ischemia of the LAD leads to severe cardiac dysfunction and the development of heart failure over a limited period of time, whereas ischemia-reperfusion of the LCx culminates in stable, moderate cardiac dysfunction. Since these models closely resemble the clinical course and treatment of MI and enable intracoronary therapy administration, they are preferable to models with persistent coronary occlusion. This study adds to refinement of preclinical studies which hopefully will result in improved translational medicine and a reduction of animals needed in preclinical research.

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CHAPTER 12

Admittance based pressure volume loop measurements in a porcine model of chronic myocardial infarction

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ABSTRACT

Objective The aim of this study was to validate admittance based pressure volume (PV) loop measurements for the assessment of cardiac function in a porcine model of chronic myocardial infarction.

Background The traditional PV-loop measurement technique requires hypertonic saline injections for parallel conductance correction prior to signal conversion into volume. Furthermore, it assumes a linear relationship between conductance and volume. More recently, an admittance based technique has been developed, which continuously measures parallel conductance and uses a non-linear equation for volume calculation. This technique has not yet been evaluated in a large animal myocardial ischemia model.

Methods Eleven pigs underwent invasive PV measurements with the admittance system (AS) and the traditional conductance system (CS) followed by 3D-echocardiography (3DE). After baseline measurements, pigs were subjected to 90 minute left anterior descending artery occlusion followed by the same measurements at 8 weeks follow-up.

Results In the healthy heart, the AS showed good agreement with 3DE for LV volumes and a reasonable correlation for ejection fraction (EF) ($R=0.756$, $p=0.007$). At follow-up, an increase in end systolic volume (ESV) was observed with 3DE (+15.4±14.4mL, $p=0.005$) and the AS (+34.6±36.1mL, $p=0.010$). EF measured with 3DE (-13.2±5.2%, $p<0.001$) and the AS (-20.3±11.2%, $p<0.001$) significantly decreased.

Conclusion The AS can be used to quantitatively monitor the cardiac function changes induced by myocardial infarction and provides comparable results as 3DE, rendering it a useful tool for functional testing in large animal cardiac models.

INTRODUCTION

Functional cardiac parameters are considered the gold standard to evaluate therapeutic efficacy in experimental and clinical cardiovascular studies. Hence, means for reliable cardiac function measurement are essential to assess efficacy of compounds that reduce myocardial ischemia/reperfusion injury or modulate post-infarct remodeling. Several techniques are available for left ventricular (LV) function assessment, predominantly using LV volumes as a surrogate of cardiac function. This includes Magnetic Resonance Imaging (MRI) and echocardiography. MRI is considered to be the most precise technique to measure LV volumes, but it remains expensive, time consuming and does not allow intra-ventricular pressure measurements for real time pressure-volume relation calculations. The latter also holds true for echocardiography, which provides only limited information on fundamental aspects of ventricular contraction and relies on the use of geometric assumptions. [1–3] Invasive Pressure Volume (PV) measurements (PV-loops) combine real time pressure and ventricular volume measurements to determine pressure volume relationships. This provides researchers with more detailed information about systolic and diastolic function that is unique for cardiac assessment with PV studies. [4–6]

To accurately determine these myocardial characteristics, a precise estimation of LV volumes is needed. The classical PV systems determine LV volumes by converting measured conductance signals to volume after correcting for parallel conductance by hypertonic saline injection. [7–9] Recently, a new technique has been designed to render hypertonic saline calibration unnecessary. [4,10–12] This admittance system (AS) continuously measures the phase angle to determine myocardial conductance during the cardiac cycle. Since myocardial (parallel) conductance is continuously measured, the effects of ventricular wall movement on the relative contribution of myocardial conductance to total (measured) conductance can be taken into account. Traditional systems do not allow correction for wall movement since early studies suggested that parallel conductance does not vary throughout the cardiac cycle. [8] However, increasing evidence emerges that the attribution of parallel conductance does vary, especially in the diseased heart. This theoretically leads to overestimating or underestimating conductance during diastole or systole respectively, affecting ventricular volume calculation.[5,13] Another key difference between the classical conductance and the novel admittance technique is the conversion of the measured signals into volumes. While traditional systems assume a constant, linear relationship between conductance and volume using Baan's equation[7], the AS is based on a continuous nonlinear relationship, incorporated into Wei's equation. [14,15]

Though the AS was validated in small animal models[12,15], data from large animal studies are scarce. Recently, the admittance technique was tested in the healthy porcine heart, where it showed good agreement with transesophageal three-dimensional echocardiography.[16] However, the animals used in that study were only half the weight of average humans (34.4kg) and accordingly had smaller LV volumes compared to humans. [3] Moreover, future use in experimental and clinical studies requires thorough testing of the system's ability to discriminate between normal and diminished cardiac function. In the current study, we investigated this by comparing admittance based PV-loops with a

classical conductance system (CS) results and 3-dimensional echocardiography (3DE) as a reference standard. The latter is a well-established technique for the assessment of LV volumes that has recently been proven to correlate very well with MRI and thermodilution, the gold standards for LV volume assessment. [1–3,17,18]

MATERIALS AND METHODS

All animal experiments were approved by the institutional animal welfare committee and were executed conforming to the 'Guide for the Care and Use of Laboratory Animals'.
A total of 11 female landrace pigs were used in this study. Pigs (body weight 73.1±5.3 kg) were subjected to left ventricular invasive measurements with both the CS and AS, followed by epicardial 3DE in the healthy heart. After these measurements, pigs were subjected to myocardial infarction followed by invasive PV measurements and 3DE 8 weeks later.

Pressure volume loop measurements in the naïve myocardium

Animals were anesthetized with 10 mg/kg ketamine, 0.4 mg/kg midazolam and 0.5 mg/kg atropine. Anesthesia was maintained with 0.5 mg/kg/h midazolam, 2.5 μg/kg/h sufentanyl and 0.1 mg/kg/h pancuronium. Pre- and post-operatively, animals received a fentanyl patch (25μ/h) and post-operatively a single injection 2mg/kg meloxacam. Venous and arterial access was obtained by placement of a 7F sheath in the jugular vein and an 8F sheath in the carotid artery after the blood vessels were surgically exposed. The thorax was opened via medial sternotomy and a snare around the inferior vena cava for preload reduction was placed. A conductance catheter was inserted into the left ventricle through the sheath in the carotid artery (CD Leycom, Zoetermeer, the Netherlands). A 6F catheter was placed in the pulmonary artery through the sheath in the jugular vein for hypertonic saline injections. After data acquisition, the PV catheter was removed and the admittance catheter was inserted (Transonic Scisense, London, Canada). After removal of the admittance catheter, epicardial 3DE was performed. All catheters were placed under fluoroscopic guidance.

Infarct induction and PV measurements in the diseased heart

Directly following baseline measurements, pigs were subjected to myocardial infarction (MI). The heart was exposed by opening the pericardium. This was followed by placement of a ligature around the left anterior descending (LAD) artery just distal from the origin of the first diagonal artery. The LAD was subsequently closed for 90 minutes. After reopening the vessel and observation for approximately 3 hours, the sternum was closed and the animals were weaned from anesthesia. If any cardiac arrhythmias occurred during the procedure, animals were epicardially defibrillated with 5-20 Joules. Eight weeks after infarct induction, pigs were again anesthetized and measurements were performed as described above after lateral sternotomy due to the presence of adhesions of the heart to the sternum after primary surgery. After these measurements pigs were sacrificed by rapid bleeding and the heart was excised for the determination of infarct size.

Conductance Technique

A 7F conductance catheter (*CA-71103-PL*, CD Leycom, Zoetermeer, The Netherlands) was connected to the Sigma acquisition system (CD Leycom, Zoetermeer, The Netherlands). After ex vivo calibration, the catheter was placed in the left ventricle via a sheath in the carotid artery under fluoroscopic guidance. On average, 5 conductance segments were positioned in the left ventricle depending on the size of the heart. After insertion, baseline recordings were obtained during apnea at a rate of 250 samples/second. To correctly determine absolute LV volumes, 5 mL of 10%NaCL was directly injected into the pulmonary artery. Injections were repeated three times per animal each during simultaneous beat recording and apnea. Cardiac output required for data analyses was derived from the stroke volume (SV) measured by 3DE. Blood resistivity was assumed to be constant in all animals (150 Ω*cm). Subsequently, preload was reduced by temporary inferior caval vein occlusion during apnea. End systolic pressure volume relations (ESPVR) were derived from the recorded beats during vena cava occlusion that were performed three times per animal. All recordings were analyzed offline using Conductance NT 16 Software (CD Leycom, Zoetermeer, The Netherlands).

Admittance technique

For the admittance based technique, a 7F tetra-polar catheter (7.0 VSL Pigtail/no lumen, Transonic Scisense, London, Canada) was used that measures admittance magnitude and phase in combination with pressure. It contains 7 platinum electrodes dividing it into 4 selectable segments. The largest segment inside the LV was used for absolute volume assessment. The catheter was connected to the ADVantage system™ (Transonic SciSense, London, Canada) linked to a multi-channel acquisition system (Iworx 404), required for real-time data acquisition. Again, the catheter was inserted via the sheath in the carotid artery under fluoroscopic guidance. After insertion, the admittance catheter measures blood and parallel conductance separating both based on phase angle with a rate of 200 samples/second. A baseline scan was performed to determine the end diastolic and end systolic blood conductance required for absolute volume calculations. The external stroke volume (SV) that is required for analyses was derived from echocardiographic measurements. Blood resistivity was assumed to be constant in all animals (150 Ω*cm). Baseline PV measurements and caval occlusions were performed under apnea as described above. Data were analyzed offline using Iworx analysis software (Labscribe V2.0).

Three-dimensional echocardiography

3DE was performed in all animals using a Philips iE33 machine with an X3-1 transducer (Philips, Eindhoven, The Netherlands). The 3D-transducer (X-3, Philips, The Netherlands) was wrapped in a sterile sleeve. A pocket of gel was positioned under the transducer, to bring the complete apex in view. The transducer, together with the gel pocket, was positioned directly epicardially on the apex of the heart. The depth and sector size were adjusted to fit the complete ventricle. All data sets were acquired in real time using 7 consecutive cardiac cycles (full volume analysis).

The images were analyzed offline using QLab 10.1 (3DQ advanced) analysis software. The tracing of the ventricle was performed by semi-automatic border detection as described before. [19] Briefly, end-

diastolic and end-systolic frames are identified and on both frames the apex, anterior, lateral, inferior and septal mitral annulus are identified. The endocardial border is automatically traced. Ejection fraction is calculated by the Qlab software as (EDV-ESV)/ EDV*100 *(figure 1a).*

Infarct size

After the animals were sacrificed, the hearts were excised. The LV was then cut into 5 equal slices from apex to base and incubated in 1% triphenyltetrazolium chloride (Sigma-Aldrich Chemicals, Zwijndrecht, the Netherlands) in 37°C 0.9%NaCL for 15 min to discriminate infarct tissue from viable myocardium in 10/11 animals.

Statistical Analysis

All data are expressed as mean ± standard deviation unless mentioned otherwise. Both, echocardiographic data and PV measurements were separately analyzed by two different researchers blinded to the outcome of the other technique. PV-Loop specific data (e.g. ESPVR, ESP, HR) were compared using a paired Student's t-test after log transformation in SPSS 20.0. End diastolic volume (EDV), end systolic volume (ESV) and ejection fraction (EF) at baseline and follow-up measured by the three methods were compared using a two-factor repeated analysis of variance (ANOVA). Baseline LV volumes for the AS and CS were separately compared with 3DE measurements using a paired Student's t-test. For follow-up measurements, a paired t-test was performed including infarct size as a covariate for EF. Follow-up and baseline measurements of the same system were compared using a paired Student's t-test for EDV, ESV and EF. Delta EDV, ESV and EF between baseline and follow-up were compared among the different systems using a repeated measures ANOVA. Correlations at baseline and follow-up were tested using Pearson's correlation test. The limits of agreement (1.96*SD = 95% confidence interval) of both PV systems compared to 3DE were determined by Bland-Altman analysis. A two-tailed F test was used to compare the size of the limits of agreement of the AS and the CS. All statistical analyses were performed in SPSS statistics version 20.0. A two-sided P-value of <0.05 was regarded statistically significant in all analyses.

RESULTS

LV volumes at baseline and follow-up were measured in 11 pigs with good quality of measurements *(figure 1)*. There were no significant differences in heart rate and pressures between measurements, indicating that measurements were performed during steady state *(table 1)*. At follow-up, the AS' ESPVR slope showed a non-significant increase, accompanied by a significant rightward shift of V_0 (+55±52, p=0.013) *(figure 1b, table 1).*

After absolute volume calibration, EDV, ESV and EF were measured by the two systems. Both, baseline and follow-up data were compared with 3D-echocardiographic findings by two-factor repeated measures ANOVA. There was a significant interaction between method (3DE, AS, CS) and time point (baseline, follow-up) for EDV (p=0.036) and ESV (p=0.007) and a trend towards an interaction for EF (p=0.062) indicating that cardiac volume assessment across the two time points differed for the three methods (*table 2*).

Table 1. Cardiac parameters measured *in vivo* in the healthy porcine heart using the AS and CS.

Cardiac Parameters	AS Baseline	CS Baseline	AS Follow-up	CS Follow-up
Heart Rate (bpm)	58±15	62±15	63±12	61±10
ESP (mmHg)	117.8±21.5	110.8±23.1	92.9±23.2	89.1±18.0
EDP (mmHg)	10.5±3.2	11.7±6.5	8.6±3.9	10.4±4.5
ESPVR (slope)	2.56±2.43	2.34±1.27	3.63±4.87	3.05±2.50
V_0	-21±20	-21±61	34±52*	-14±23

All values are presented as means ± standard deviations. ESPVR – End Systolic Pressure Volume Relationship. * Significant difference vs. baseline AS. Differences with p-value <0.05 were regarded significant.

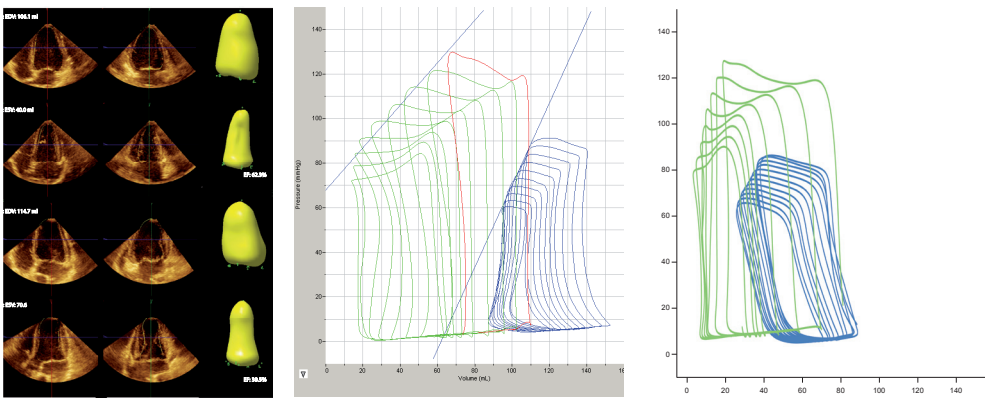

Figure 1. Methods of LV volume assessment

LV volumes were determined by 3 different modalities. **A**. End diastolic and end systolic LV volume assessment with subsequent calculations of EF at baseline (upper half) and at follow-up (lower half). **B.** Admittance based pressure volume loops at baseline (red) with preload reduction in the healthy heart (green) and after myocardial infarction (blue). The lines depicted represent the ESPVR. Note that the slope of the ESPR at follow-up compared to baseline hardly changes while a shift of V_0 can be observed. **C.** Conductance based pressure volume loops with preload reduction in the healthy heart (green) and after myocardial infarction (blue).

In the naïve myocardium, none of these parameters differed between admittance, conductance and 3DE (*supplemental table 1, figure 2 and 3*). Moreover, EF derived from the AS and 3DE correlated well at baseline (R=0.756, p=0.007) *(table 3)*. However, at 8 weeks follow-up after myocardial infarction, significant differences were observed. The AS overestimated both, EDV (+28.7±41.4mL, p=0.028) and ESV (+31.3±35.4mL, p=0.014) compared to 3DE. In turn, this overestimation resulted in a significant underestimation of EF (-10.9±10.2%, p=0.009) *(supplemental table 2, figure 2 and 3*).

For the CS, the opposite was found. Both, EDV (-11.3±23.4, p=0.021) and ESV (-15.4±14.7, p=0.018) were significantly underestimated using this technique *(supplemental Table 2, figure 2 and 3)*. This culminated in an overestimation of EF (+10.2±13.4, p=0.037) compared to 3DE. Furthermore, infarct size correlated significantly with EF measured by 3DE at follow-up (R=-0.698, p=0.025, *figure 4*). Therefore, infarct size was added as a covariate in the analysis when EF at follow-up was compared

among the different methods. After controlling for infarct size, the differences for EF between the AS and 3DE remained, whereas for the CS the difference in EF compared to 3DE was no longer significant *(supplemental table 3).*

Table 2. Two-way repeated measures ANOVA between method used, time point of actual measurement and interaction between time point and measurement.

Source	EDV	ESV	EF
Method	F(2,20)=6.9 p=0.005	F(2,20)=8.8 p=0.002	F(2,20)=9.9 p=0.001
Time	F(1,10)=0.7 p=0.063	F(1,10)=5.9 p=0.036	F(1,10)=23.9 p=0.001
Method*Time	F(2,20)=3.9 p=0.036	F(2,20)=6.4 p=0.007	F(2,20)=3.2 p=0.062

Differences between EDV, ESV and EF for the method used (3DE, AS or CS) the timepoint measured (baseline or follow-up) and the interaction between time point and method. *p-values <0.05 were regarded significant. Data were analyzed with a two-factor repeated measures ANOVA. Corrected EF = differences in EF corrected for infarct size.

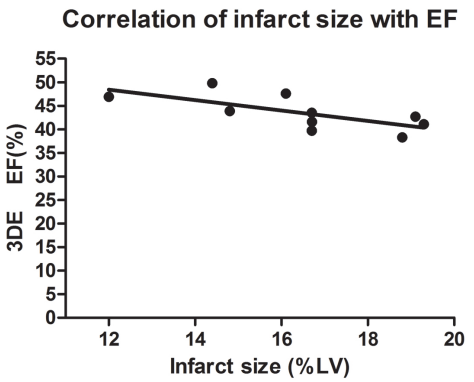

Figure 2. Left ventricular volume assessment in the healthy and infarcted porcine heart

Volumes were measured by 3D-echocardiography and invasive PV-measurements with the admittance and the conductance technique in the healthy and infarcted porcine heart (n=11). **A**. Baseline End Diastolic Volume **B**. Baseline End Systolic Volume **C**. Baseline Ejection Fraction **D**. End Diastolic Volume at follow-up **E**. End Systolic Volume at follow-up **F**. Ejection Fraction at follow-up. Data are presented as mean ± 95%CI. * p<0.05; **p>0.05 after correction for infarct size.

To determine the general agreement of the two systems with 3DE as a reference standard, both in the healthy heart and after 8 weeks of follow-up, Bland-Altman analyses were performed *(figure 3*). Next, the limits of agreement of the different PV systems versus 3DE were compared to determine differences in variance. The limits of agreement of baseline PV-loop measurements of both systems did not differ significantly. In the post infarction remodeled heart, however, the admittance system versus 3DE showed larger limits of agreement for both EDV (±37.2, p<0.05) and ESV (±34.8, p<0.05) when compared with the limits of agreement of the conductance system versus 3DE for EDV (±13.8) and ESV (±18.1) (*figure 3*).

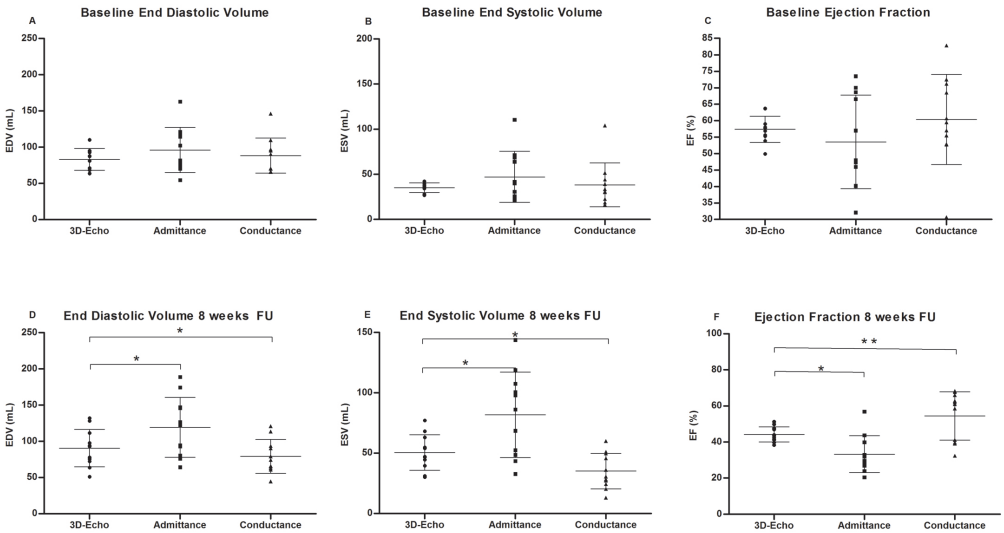

Figure 3. Bland-Altman analysis of left ventricular volume assessment in the healthy and infarcted porcine heart

Comparison of 3D-echocardiography (set as reference standard) and invasive PV measurements with both the admittance and the conductance technique (n=11) in the healthy and infarcted porcine heart. **A**. Baseline End Diastolic Volume **B**. Baseline End Systolic Volume **C**. Baseline Ejection Fraction. **D**. End Diastolic Volume at follow-up **E**. End Systolic Volume at follow-up **F**. Ejection Fraction at follow-up. Data are presented as mean ± 95%CI. + = significant overestimation vs. 3D-echo; - = significant underestimation vs. 3D-echo before correction for infarct size; # = significantly larger limits of agreement versus conductance technique, p<0.05.

Table 3. Correlation between 3DE, the AS and CS in the healthy porcine heart for EDV, ESV and EF.

LV Parameters	3DE AS	3DE CS	AS CS
EDV (mL)	0.339	0.539	0.517
ESV (mL)	0.361	0.341	0.486
EF (%)	0.756*	0.309	0.300

Numbers shown are the correlation coefficients (R). *Significant correlation between two systems (p=0.007).

Table 4. Correlation between 3DE, the AS and CS in the infarcted porcine heart for EDV, ESV and EF.

LV Parameters	3DE AS	3DE CS	AS CS
EDV (mL)	0.466	0.847*	0.240
ESV (mL)	0.252	0.247	-0.56
EF (%)	-0.050	-0.019	-0.007

Numbers shown are the correlation coeffecients (R). *Significant correlation between two systems (p=0.005).

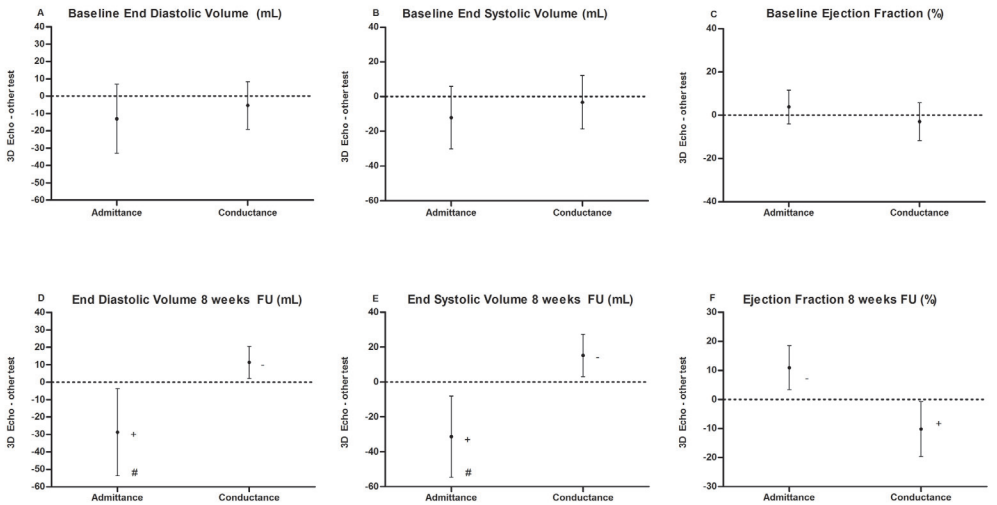

Figure 4. Correlation between infarct size and 3DE

Correlation plot showing a significant correlation between infarct size and EF determined at follow-up by 3DE. R=0.698, p=0.025 (n=10).

Cardiac function: Baseline versus follow-up

Any particular system for cardiac function assessment can be considered valid when a true change in cardiac function can be detected reliably. Therefore, the primary aim of this study was to compare baseline and follow-up measurements with the AS. To monitor the degree of cardiac function changes, we used 3DE as reference. 3D-echocardiographic LV volume measurements confirmed a decline in cardiac function after MI with a significant increase in ESV (+15.4±14.4 mL, p=0.005) and a decrease in EF (-13.2±5.2%, p<0.001) (*table 5*), but did not reveal any significant cardiac dilatation after MI. The AS detected a significant decrease in ESV (+34.6±36.1mL, p=0.010) and EF (-20.3±11.2%, p<0.001). Similar to the echocardiographic measurements, the AS indicated no significant EDV changes after MI. Finally, delta EDV, ESV and EF were compared among the different systems. Only delta ESV (+34.6±36.0 mL vs. -3.2±32.9 mL, p=0.030) between the AS and CS significantly differed, whereas no significant differences between 3DE and the two PV techniques were observed (*table 5).*

Table 5. Differences in LV volumes between follow-up and baseline measurements for 3DE, the AS and CS.

LV Parameters	3DE	AS	CS
EDV (mL)	7.6±24.0	23.3±44.8	-9.1±37.8
ESV (mL)	15.4±14.4*	34.6±36.1*	-3.2±32.9†
EF (%)	-13.2±5.2*	-20.3±11.2*	-6.0±21.0

All values are presented as mean differences ± standard deviations. *Difference of Paired T-test for measurements at follow-up vs. baseline for the same system. †Difference between delta measurements vs. the AS. Differences with p-value <0.05 was regarded significant.

DISCUSSION

To evaluate therapeutic efficacy of treatments for ischemic heart disease, a careful assessment of cardiac function is mandatory. Therefore, it is important to evaluate techniques for functional testing under conditions that reflect its application field as close as possible. The traditional conductance system has been used in many different studies to assess cardiac function.[6,20,21] However, this system structurally overestimates LV volumes due to its inherent inability to separate parallel conductance from blood conductance.[5,7] This can be overcome by hypertonic saline injection. However, this premises a constant parallel conductance and a linear relationship between conductance and volume, which might be too simplified and hence imprecise.[5,13,14] The admittance based system used in our study has been validated and implemented in multiple murine studies for the determination of cardiac function[10,15,22], where it showed to be more accurate than the CS. [15] Recently, the AS has been used to measure LV volumes in moderately sized healthy pigs. In the study by Kutty (2013), the admittance system significantly overestimated LV volumes with a good estimation of EF in the healthy porcine heart. Furthermore, a good correlation was found between LV volumes measured by the AS and 3DE, based on repeated measurements in the same animals.[16] Moreover, in larger LVs, the AS showed a trend towards a poorer agreement with 3DE.[16] More importantly, the effect of regional ischemia and post-infarction remodeling has not yet been evaluated. Therefore, the aim of our study was to test the ability of the AS to reliably monitor cardiac function in a human sized large animal model of chronic myocardial infarction.[3] To the best of our knowledge, our study is the first to evaluate the AS in a large animal ischemic heart disease model.

In concordance with Kutty (2013), EF was similar at baseline and correlated significantly between AS and 3DE, whereas EDV and ESV did not. The lack of correlation for the latter two could be due to larger LV volumes in our study, which is supported by the observation that larger hearts showed a poorer agreement of 3DE and the AS in the mentioned study. [16] Furthermore, Bland-Altman analyses revealed modest limits of agreement at baseline. These data suggest that the AS can accurately and reliably measure cardiac function in the healthy porcine *in vivo* heart. Interestingly, our study reveals prominent differences at 8 weeks follow-up. At this time point, the AS overestimated LV volumes resulting in an underestimation of EF. Also, Bland-Altman analyses at follow-up showed significantly larger limits of agreement for the AS than for the CS for LV volumes, which tended to underestimate volumes. These discrepancies between baseline and follow-up measurements for the AS compared to 3DE could largely explain the interactions found between method and time point. Moreover, infarct size significantly correlated with EF measured by 3DE at follow-up. Therefore, infarct size was used as a covariate in the comparison of EF at follow-up between the different methods. After controlling for infarct size, EF measurements at follow-up remained different between the AS and 3DE indicating that the found differences between 3DE and the AS are independent of the actual infarct severity.

This combination of volume over- or underestimation for the AS and CS respectively, combined with the lack of correlation of both systems with 3DE and infarct size, suggest that PV-measurements in the infarcted heart might be less accurate and reliable than in the healthy heart, supporting the importance

of choosing appropriate models in the testing of novel technology. Indeed, studies on agreement and correlation between different imaging techniques (e.g. MRI, echocardiography) and volumes measured by PV methods both in small and large animal models are inconsistent. [15,23–28] It should be noted however, that 3DE moderately underestimates absolute LV volumes compared to MRI.[1,3], which could partly explain the AS' overestimation of both EDV and ESV at follow-up. Nevertheless, this cannot be held responsible for the significant interaction between method and timepoint.

Moreover, given the limited variation of the size of pigs used in this study, the overall variation of data -even at baseline- is considerable for all three systems. This could indicate that the reproducibility of the different techniques is not optimal. Recently, the reproducibility of 3DE for the measurement of EF has been established. However, the inter-observer variability was considerably higher than for MRI.[3] To the best of our knowledge, the reproducibility of the AS has not been well established, although Kutty (2013) recently showed that repeated measurements for the same animal in the healthy heart were very similar with the AS system. In the current study, reproducibility in form of repeated measurements was not investigated and future studies should assess whether the reproducibility of the AS is influenced by MI.

Furthermore, we examined the differences in volume measurements with the AS between baseline and follow-up. Importantly, EDV, ESV and EF measured with the AS at follow-up compared to baseline showed comparable changes as with 3DE. Also, comparison of delta EDV, ESV and EF between the AS and 3DE did not reveal any differences. This indicates, although induction of MI influences its accuracy, the AS' ability to determine functional and volume changes after MI. One of the classical features of post-infarction PV measurements is a decline of the ESPVR slope.[29,30] In our study however, we were unable to detect such changes with both systems. This can likely be ascribed to the fact that the previous ex vivo preload reducing experiments were performed under idealized conditions that do not necessarily represent the in vivo physiology following post MI remodeling, especially when dealing with regional ischemia. Therefore, deviations from the traditionally observed decline in the ESPVR slope may occur with a typical shift in V_0, as observed by us and others. [6,24,31,32]

The main limitation of the current study is that measurements in animals inherently have to be performed under general anesthesia. This could potentially influence the overall outcome since different levels of anesthesia might influence results. Additionally, the sternotomy could interfere with conductance signals resulting in less accurate measurements.
Furthermore, the sequence of measurements was not random, meaning that LV function was first measured with the CS and only thereafter with the AS in all animals. Although it is imaginable that catheter insertion and extraction could influence cardiac function on a short-term basis, it is probably not sufficient to explain the differences found in LV volumes. In this perspective, hypertonic saline injections could also temporarily alter the resistivity of blood in the LV. Since the circulatory volume of the animals exceeds the amount of hypertonic saline injected by far, it seems unlikely that the

admittance measurements were influenced, also considering the time between the injections and the insertion of the admittance catheter. Finally, the 3DE recordings and the PV measurements were not simultaneously performed. Although no major hemodynamic changes between the measurements were observed, the observed discrepancies could partly be due to moderate changes between the consecutive measurements.

In conclusion, LV volumes in the healthy heart can be accurately and reliably measured by the AS with a good correlation for EF with 3DE. However, post-MI remodeling influences these measurements, making PV-loop computations less accurate. Nevertheless, like 3DE, the AS can successfully identify a decrease in cardiac function in a porcine model of chronic myocardial infarction. These data show that the admittance based technique is valid for the assessment of cardiac function in large animal models. Given the lack of correlation between echo- and PV-loop-based volume measurements with the current systems, future studies ideally should combine the strength of either echocardiographic or MRI based volume measurements with PV-loop specific parameters as functional endpoints in cardiac large animal models.

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SUPPLEMENTAL TABLES

Table 1. Cardiac parameters measured *in vivo* in the healthy porcine heart using the AS and CS.

Cardiac Parameters	AS Baseline	CS Baseline	AS Follow-up	CS Follow-up
Heart Rate (bpm)	58±15	62±15	63±12	61±10
ESP (mmHg)	117.8±21.5	110.8±23.1	92.9±23.2	89.1±18.0
EDP (mmHg)	10.5±3.2	11.7±6.5	8.6±3.9	10.4±4.5
ESPVR (slope)	2.56±2.43	2.34±1.27	3.63±4.87	3.05±2.50
V_0	-21±20	-21±61	34±52*	-14±23

All values are presented as means ± standard deviations. ESPVR – End Systolic Pressure Volume Relationship.

* Significant difference vs. baseline AS. Differences with p-value <0.05 were regarded significant.

Table 2. Two-way repeated measures ANOVA between method used, time point of actual measurement and interaction between time point and measurement.

Source	EDV	ESV	EF
Method	F(2,20)=6.9 p=0.005	F(2,20)=8.8 p=0.002	F(2,20)=9.9 p=0.001
Time	F(1,10)=0.7 p=0.063	F(1,10)=5.9 p=0.036	F(1,10)=23.9 p=0.001
Method*Time	F(2,20)=3.9 p=0.036	F(2,20)=6.4 p=0.007	F(2,20)=3.2 p=0.062

Differences between EDV, ESV and EF for the method used (3DE, AS or CS) the timepoint measured (baseline or follow-up) and the interaction between time point and method. *p-values <0.05 were regarded significant. Data were analyzed with a two-factor repeated measures ANOVA. Corrected EF = differences in EF corrected for infarct size.

Table 3. Correlation between 3DE, the AS and CS in the healthy porcine heart for EDV, ESV and EF.

LV Parameters	3DE AS	3DE CS	AS CS
EDV (mL)	0.339	0.539	0.517
ESV (mL)	0.361	0.341	0.486
EF (%)	0.756*	0.309	0.300

Numbers shown are the correlation coefficients (R). *Significant correlation between two systems (p=0.007).

PART 6

Discussion and Summary

CHAPTER 13

General discussion and future prospectives

DISCUSSION

Over the past decade stem cells have been a hot topic in many different fields of medicine. The idea that stem cells can regenerate tissue that is destroyed, set high hopes to cure diseases that were incurable before. In cardiology, stem cell therapy is mostly investigated to treat ischemic heart disease, which includes an acute myocardial infarction (AMI) and heart failure (HF). It has been over 10 years since the first patients was treated with the first generation of stem cell therapy, but still to date many questions remain unanswered. Are we using the correct cell type? What is the appropriate time point to give stem cells? How many cells do we need to give? Are we using an appropriate isolation protocol? What is the optimal injection route? How can we optimize cell therapy? Does every disease need the same cell? And so on. In this chapter, the results obtained in the thesis are summarized and placed into context to existing literature. The main findings of this thesis are described in table 1.

In chapter 3, a random-effects meta-analysis was performed in which the effects of bone marrow-derived mononuclear cells (BMMNCs) were investigated for the treatment of an AMI. Intracoronary infusion of BMMNCs improved LVEF by only +2.10%. This effect is limited, but what has to be kept in mind is that even small improvements in LVEF can be fruitful. For instance, primary percutaneous coronary intervention (PCI) only improves LVEF by 4%.[1,2] This moderate effect on LVEF has resulted in a massive decrease in cardiac mortality. This could indicate that the effect that is observed in the meta-analysis could also reduce major adverse cardiovascular and cerebral events (MACCE) rates. However, no significant reductions in MACCE rates were noted in the meta-analysis. This in contrast with another meta-analysis by Jeevanantham *et al* [3] who reported a reduction in MACCE rates, including all-cause mortality, after BMMNC treatment. The main difference between their meta-analysis and our meta-analysis is that they pooled the data of HF and AMI patients for MACCE calculations. This could cloud the true potential for BMMNC therapy for both diseases. One possible reason why no effect is detected on MACCE in our meta-analysis, is that most studies were not designed to show any effect on MACCE. Most trials were only phase I/II trials and were therefore underpowered to detect any effect on MACCE. Moreover, the median follow-up duration was only 6 months which might be too short to draw conclusions regarding MACCE rates. Another explanation could be that BMMNCs are not beneficial for cardiac repair. This statement is fortified by the finding that intracoronary infusion of BMMNCs did not improve LVEF when only studies are pooled that used MRI for analysis of cardiac function. As MRI is still the golden standard for detecting LVEF and LV volumes, it could be stated that, when no benefit is detected using this imaging modality, the therapy is not effective at all.

Newer generations of stem cells that were applied in clinical trials for the treatment of an AMI were also investigated in our meta-analysis, autologous enriched cells (CD34+/CD133+ cells), mesenchymal and mesenchymal-like stem cells (autologous and allogeneic combined) and cardiosphere-derived cells (CDC). Only a limited number of trials investigated the effects of these cells to date, so no clear conclusion can be drawn regarding superiority of one of these cell types. Mesenchymal stem cells (MSC) showed an improvement in LVEF of almost 5%, but due to a number of trials and a limited

number of patients treated with MSCs, no significant differences opposed to BMMNCs on cardiac function could be observed. However, a reduction was detected in the number of episodes of ventricular tachycardia and ventricular fibrillation (VT/VF) and implantable cardioverter defibrillator (ICD) implantations. MSC must be further investigated in larger clinical trials before superiority can be detected. The same accounts for the other new generations of cell-based repair, like the CDC.

As mentioned above, Jeevanantham did find beneficial effects of BMMNC transplantation on MACCE events when they pooled the data of HF and AMI patients. We did not see any effect on MACCE in AMI patients, so could it be concluded that the effects on MACCE only occur in HF patients? To answer this question, a meta-analysis of BMMNC therapy in HF patients was performed as described in chapter 3. Overall, LVEF improved by almost +4% in this patient population after BMMNC transplantation. Moreover, when data was corrected for MRI studies, the improvement on LVEF was still +3%. The effects of this improvement were noted on the clinical outcome of HF patients. BMMNC transplantation was associated with a decrease in all-cause mortality. It could thereby be concluded that the effect on all-cause mortality in the meta-analyses by Jeevanantham *et al.* is based on HF patients. The other MACCE events that were improved in their meta-analysis were not improved in our meta-analysis. The benefit on cardiac mortality, recurrent AMI and hospitalizations for HF in their study could be the effect of combining the AMI and HF patients. Moreover in Jeevanantham *et al.* cohort studies were included. We omitted cohort studies from the analysis, because they tend to overestimate treatment effects.

Based on our meta-analyses, BMMNC do not seem to be the best candidate for cardiac repair, at least in AMI patients. The moderate effects that were found could be related to the limited treatment effect of the cell itself but could also be associated to the differences in trial design. There is, for example, still no consensus regarding the optimal time point for injection nor for the dose of cells. In our meta-analysis regarding the AMI population, also timing and cell dose were investigated. Cell dose did not influence outcome, but we concluded that patients benefit the most when injection of BMMNCs was performed within 7 days after AMI. Thus far, it was believed that cell therapy should be initiated 3-10 days after the AMI, based on findings in phase I studies, logistical issues, and the assumption that in the first 72 hours, the infarct territory encompasses a too hostile environment for the infused cells. However, others argued that stem cells should be infused as soon as possible to prevent cardiomyocyte loss by secreted anti-inflammatory, pro-survival and anti-apoptotic paracrine factors.[4] This hypothesis was recently supported by preclinical and clinical evidence.[5,6] Due to logistical reasons injection of BMMNC is often impossible within 24 hours, rendering in a delay between infarct and infusion of cells. Hence, the effects of BMMNC could be limited due to the late timing of the stem cell transplantation.

Table 1. Main Findings of this thesis

- Stem cell therapy for cardiovascular disease has been investigated in many preclinical and clinical trials in the last decade
- First generation stem cells, including the Skeletal myoblast and bone marrow mononuclear cells are not effective for cell-based cardiac repair
- Injection of skeletal myoblasts results in a higher incidence of ventricular arrhythmias due to the lack of electromechanical coupling between the skeletal myoblasts and the cardiomyocytes
- Infusion of bone marrow mononuclear cells in AMI patients does not lead to an improvement in cardiac function nor improvement in MACCE events
- Injection of bone marrow mononuclear cells in HF patients increased ejection fraction by 3% and resulted in decrease in all-cause mortality
- Mesenchymal stem cells and mesenchymal progenitor cells have a better profile for cardiac repair than bone marrow mononuclear cells
- Mesenchymal stem cells and mesenchymal precursor cells can be given in an allogeneic setting
- Infusion of Mesenchymal precursor cells in an animal AMI model is safe and results in a preservation of left ventricular function and dimensions
- The primary mechanism of action of mesenchymal precursor cells is based on the release of paracrine factors that prevent apoptosis, improve wound healing and enhance angiogenesis
- Retention of stem cells in the myocardium following an event is minimal, to improve retention mesenchymal stem cells were encapsulated in an alginate shell
- Intracoronary infusion of Encapsulated mesenchymal stem cells (CellBeads) is safe and feasible up to 90,000 beads
- Intracoronary infusion of 20,000 encapsulated mesenchymal stem cells preserves left ventricular function and dimensions
- The primary mechanism of action of encapsulated mesenchymal stem cells is based on the release of paracrine factors by the stem cells which resulted in a reduction of apoptosis, thereby limitation of infarct scar size and angiogenesis
- A porcine model of ischemia-reperfusion of the left anterior descending artery results in a more severe infarct and larger reduction in cardiac function than ischemia-reperfusion of the left circumflex artery, making LAD occlusion more suitable for preclinical research
- Admittance PV-loop is superior to conductance PV-loop in assessing cardiac volumes and ejection fraction when compared to 3D-echocardiography.

Another restricting factor of BMMNC therapy is their expression profile of paracrine factors, which is limited for cardiac repair.[7] Currently, more potent cells have emerged that secrete factors that are more beneficial for heart repair, like the MSC and the MSC-like adipose tissue-derived regenerative cells (ADRCs). This in combination with limited efficacy of BMMNCs has resulted that less clinical trials are exploring BMMNCs but are shifting towards new generations of stem cells.

A meta-analysis on preclinical studies using stem cell therapy for ischemic heart disease concluded that MSC are more beneficial for cardiac repair.[8] BMMNCs improve LVEF in preclinical trials by +5% whereas MSC improve LVEF by +10%.[9] MSCs could be more potent for cardiac repair due to their expression profile of paracrine factors and the possibility of transdifferentiation into cardiomyocytes.[7,10–12] Moreover they home to the site of injury following infusion thereby enhancing retention of the cells in the myocardium.[13] In addition, they can be easily isolated via plastic adherence.[11] The disadvantage is that the fraction of MSCs in a bone marrow biopsy is very limited. Only 0.001%-0.01% cells in the adult bone marrow are MSC.[11] To obtain therapeutic doses, they need to be culture-expanded following isolation. Culturing of bone marrow MSCs could take up to 2 months rendering infusion of autologous MSC in AMI patients not useful. However, they are suitable for the application in HF patients in whom timing of stem cell injection is a less important issue.

Adipose tissue-derived regenerative cells

Next to isolation out of the bone marrow, MSCs can be derived from multiple tissues including adipose tissue.[11] ADRCs consist of a population of endothelial progenitor cells and mesenchymal-like stem cells. ADRCs can be isolated out of 200 ml of fat tissue using a specialized Cytori Cellution™ device and specialized enzymes that were described in chapter 5. The optimal injection route in AMI patients is intracoronary injection, whereas in HF patients intramyocardial injection via NOGA™ is preferred. The Apollo trial (chapter 6), was the first-in-man clinical trial that investigated the safety and feasibility of intracoronary infusion of ARDCs in the treatment of ST-elevation AMI in 14 patients (n=10 ADRCs group versus 5 in the control group). The most important findings over 36 month follow-up were: 1) liposuction can be performed safely briefly following an AMI, and 2) no MACCE or serious adverse events occurred that were related to the ADRC therapy. Moreover, ADRC therapy had no apparent pro-arrhythmogenic effects, but rather appeared to reduce the occurrence of ventricular arrhythmias and ectopy.

In the Apollo trial, liposuction was performed as soon as possible following AMI to return the ADRCs within 24 hours after the ischemic event to the patient. Although two patients experienced significant bleeding following liposuction, most likely related to anti-coagulation therapy, liposuction in the acute phase of a myocardial infarction appeared to be well tolerated. The patients who had a bleeding complication were treated with glycoprotein IIb/IIIa inhibitors during PCI, prior to liposuction. The protocol was adjusted and patients that received this anti-coagulant were excluded. Moreover aPTT ratio should be normalized before the liposuction procedure. However, this issue should be closely monitored in future trials.

The ADRCs in the Apollo trial were intracoronary injected within 24 hours after revascularization. Although intracoronary infusion of BM-derived and cell culture-expanded MSC has raised concerns in pre-clinical studies with respect to micro-vascular obstruction and myocardial infarction [21,22], the intracoronary infusion of freshly isolated ADRCs did not result in any detectable effect on coronary flow as measured by TIMI flow and CFR analysis. This could be associated with the low dose that was used in this study and the filtration of the ADRC suspension prior to infusion.

The feasibility of ADRC therapy directly following reperfusion was demonstrated in various large animal models of AMI.[14,15] The effects in the preclinical trials were related to the paracrine effects of the ADRCs that resulted in neo-angiogenesis, immunomodulation and cardiomyocyte salvage.[14,16–18] Although only 8 patients in the treatment group were analyzed with cardiac MRI, and the study was not powered for efficacy, the significant and sustained reduction in infarct size may indeed suggest cardiomyocyte salvage evoked by the infused ADRC. Moreover, significant and sustained improvement in both coronary flow reserve (+60%) and perfusion defect (-36%) were found at 6, and 18 months follow-up, as opposed to no change in the control patient group what could be related to (neo)-angiogenesis in the peri-infarct region resulting in improved myocardial perfusion, thereby possibly limiting ischemic damage and ultimately improving function.

Although ADRCs isolation and infusion has proven to be safe within 24 hours following AMI and showed hints of efficacy, the true treatment effect in clinical trials remains unclear to date. The results of the Apollo trial have resulted in the design of the ADVANCE trial which is a prospective, randomized, double-blind, placebo-controlled, phase IIb/III clinical trial that will enroll up to 216 patients with STEMI in up to 35 centers in Europe (ClinicalTrials.gov identifier: NCT01216995).

Autologous ADRCs seem to be promising for the treatment of ischemic heart disease, however, there are some issues related to ADRC treatment. First, the cells need to be isolated from the patient directly following an ischemic event, what can result in additional discomfort and risks for the patient. Moreover, the isolation process is time consuming and every center that wants to use this therapy needs to have access to a laboratory and an isolation device and trained personnel that is available 24/7. Second, the cells are isolated from a, in most cases, elderly patient. It has been proven that cell quality decreases with aging and illness, rendering in less efficacy of the cells.[19]

These issues can be avoided by using MSCs. As described above, autologous bone marrow MSCs are less suitable for application in AMI, because they need to be culture expanded, which could take up to 2 months. Next to their excellent expression profile for cardiac repair, MSC are immune-privileged cells. This is achieved by several immunological features of MSC: 1) lack of expression of MHC class II antigen, and low levels of MHC class I; 2) lack of co-stimulatory molecules and CD40, CD80, and CD86; 3) secretion of immuno-modulatory factors including nitric oxide, heme-oxygenase I, and interleukin-6; 4) suppress innate immune cells via direct cell-cell contact, but also 5) suppress T-cell proliferation and alter naïve T-cells into an anti-inflammatory state.[12,20] This makes them suitable for allogeneic

transplantation without the need of immunosuppressive drugs. An allogeneic cell product renders a painful and time-consuming procedure of cell harvesting and isolation unnecessary. Moreover the cells are obtained from a healthy donor, thereby enhancing cell quality and the MSC can be directly infused following a coronary event. Also allogeneic MSC can be easily used in every catheterization suite worldwide, whereas no specialized personnel is needed.

Mesenchymal precursor cells

Mesenchymal precursor cells (MPCs) are an Stro-3 immune-selected immature subpopulation of bone marrow-derived MSCs.[21] These MPC are multipotent cells with extensive proliferative potential, and they secrete numerous anti-apoptotic, angiogenic factors, and growth factors, such as stromal cell-derived factor (SDF)-1, hepatocyte growth factor (HGF)-1, insulin-like growth factor (IGF)-1, VEGF and IL-6.[22–24] It was found that MPC display greater cardioprotective effects than conventional MSC that are selected by plastic adherence alone.[22,24] Moreover, MPCs are immune-privileged, like MSCs, which makes them suitable for allogeneic transplantation.[23–26]

In part 4 of this thesis, MPCs are investigated for the treatment of an AMI. Chapter 7 described a preclinical study in which MPCs were intracoronary administered in a sheep AMI model. Initially, safety and optimal dose of intracoronary delivered MPCs were investigated in pilot study, using 20 sheep. In this study it was concluded that intracoronary infusion of MPCs was safe up to a dose of 37,5 million cells when infusion rate was low. Previous studies showed that intracoronary infusion of non-selected MSC was associated with micro-vascular obstruction, coronary flow reduction and myocardial infarctions due to capillary plugging.[27–30] This effect could be related to; 1) the size of non-selected MSC (up to 30-50 μmeter in diameter) that progressively increases during cell culture[31] and 2) higher absolute doses of MSC and high infusion rates.[9,14,27,29] The size of the MPCs used in our study was only 13 micrometer, even when expanded in cell culture.

This truly distinguishes MPC from MSC, and, for the first time, enables intracoronary infusion of such cells in the culprit artery following AMI. Moreover, in our MPC study, cell dose was approximately 50-75% lower opposed to other preclinical studies that investigate MSCs. Also infusion rate was only maximal 375.000 cells per minute compared to 1-2 million cells per minute in other preclinical trials.[14,27,29] We hypothesized that a low infusion rate might enable the MPC to either pass through the capillary bed or to transmigrate into the perivascular tissue without aggregation or capillary occlusion. This was confirmed after a nuclear imaging retention sub study in two animals that revealed a significant number of MPC still in the heart two hours after intracoronary infusion, whereas epicardial coronary flow remained normal. Retention in this study was 40% opposed to 3% retention following intracoronary infusion as was found in the study by Hou *et al*.[32]

When the optimal conditions for MPC transfer were found, the efficacy of intracoronary infusion of MPCs was explored in a randomized study of 68 sheep with an anterior AMI. We demonstrated that

intracoronary delivery of MPC prevents LV remodelling and improves residual cardiac function. The results of this study suggest that these effects are evoked by myocardial salvage and subsequent reduction of infarct size, accompanied by induced angiogenesis and reduced myocardial fibrosis due to paracrine actions of the MPCs. No clear dose response relation was observed between groups and all groups showed a comparable improvement in cardiac function.

Encapsulated MSC

As described above, retention of stem cells is low following intracoronary infusion after AMI[32] and intracoronary infusion of MPCs is associated with a higher retention rate. As the effects of stem cell therapy are related to the release of paracrine factors by the cells, it was hypothesized that the beneficial effects of stem cell therapy are improved when cells are retained in the myocardium for a longer period of time. To extend the release of paracrine factors in the myocardium, MSC were encapsulated in an alginate shell (CellBeads). Encapsulated MSCs (eMSC) that are genetically modified to produce glucagon-like-peptide-1. An incretin hormone that has cardioprotective effects in preclinical and clinical trials, alongside MSC paracrine factors.[33–37]

The safety and efficacy of eMSC therapy was investigated in a pilot study, described in chapter 9. First the maximal safe dose was investigated in the naïve myocardium. It was shown that after 90.000 eMSC, coronary flow impeded. This number is much lower than the number of MPCs that can be infused before flow reduces. This is related to the size of the eMSC (170 micron). However, it seems that the same rule could be applied for eMSC as for stem cells in general: when the number is low and the infusion rate is slow, cells or eMSC can be intracoronary infused (via continuous infusion) without obstruction of coronary flow.

Moreover, it was also shown in the pilot study in chapter 9 that eMSC were viable up to 1 week, still secreting GLP-1 and therefore also other therapeutic paracrine factors. The results of the pilot study resulted to in the development of a large porcine dose-finding study which was described in chapter 10. In phase I of this study, 3 doses of eMSC or placebo solution were administered in 50 pigs in which an intermediate infarct was created by occlusion of the LCx. Infusion of eMSC showed only a trend towards an improvement in LVEF in this study. No other functional benefits were detected. However, infarct size due to an LCx occlusion was, even in control animals, only 10% of the left ventricle. When the infarct is small, the therapeutic effects of a therapy could be underestimated. In the second phase of this study, a more severe AMI was induced by occlusion of the LAD for 90 minutes, also in 50 pigs, whereupon the pigs were also subjected to 3 doses of eMSC or placebo control. 20.000 eMSC showed a significant improvement in LVEF and a reduction in LVESV. The other eMSC doses failed to show improvement in cardiac function. Moreover infarct size was also reduced in the best responding eMSC group. Although other parameters, like infarct size and apoptosis were not statistically significant in the other dose groups, all eMSC treated animals showed a significant effect on neovascularization and End-Systolic pressure volume relationship (ESPVR) on PV-loop.

Mechanisms of action of CellBeads and mesenchymal precursor cells

The primary hypothesized mechanism of action of both eMSC and MPCs is based on the release of paracrine factors by the cells. Additionally eMSC are transfected to produce glucagon-like peptide-1 (GLP-1) alongside the MSC's endogenous paracrine factors including vascular endothelial growth factor (VEGF), monocyte chemotactic protein-1 (MCP-1), interleukin (IL)-6, IL-8, glial-derived neurotrophic factor (GDNF) and neurotrophin-3 (NT-3). [33,38–41] MPCs are known to secrete stromal cell derived factor-1 (SDF-1), hepatocyte growth factor-1 (HFG-1), Insulin-like growth factor-1 (IGF-1), VEGF and IL-6. There is considerable overlay between the expression profile of paracrine factors by the eMSC and MPCs, but are the results comparable between both stem cell products?

In both studies, the cell products were administered directly following reperfusion of the AMI, we therefore hypothesized that the therapeutic effect of MPC and eMSC is mainly exerted through the release of anti-apoptotic and pro-survival factors, thereby enhancing cardiomyocyte salvage.[24,42] In addition, the immunomodulatory actions of MPCs and eMSCs may preserve myocardial tissue and contribute to effective tissue healing with limiting scar tissue formation by reducing reperfusion injury or attenuating oxidative stress.[43] Moreover, IL-6, both secreted by the eMSC and MPCs, increases the lifespan of neutrophils in the hostile post AMI environment and improves healing of the infarct wound. [44] In addition, MSC trigger the transition of classical M1 to anti-inflammatory M2 macrophages, further improving infarct healing by increased angiogenesis. [44] Moreover, the reduction of infarct size in in both studies, might have resulted in alleviated LV wall stress and reduced neurohumoral activation. This may then ultimately prevent interstitial fibrosis and compensatory hypertrophy in the non-infarcted myocardium and, on the long term, LV dilation.[45,46] This was confirmed in the MPC study by the fact that in control animals, more apoptotic cardiomyocytes were found in both infarct-related and remote segments, which is a strong indication of enhanced and ongoing adverse remodeling.[47] Indeed, the placebo-treated animals exhibited increased filling pressures and impaired filling rates, a rightward shift of the PV-relation (*i.e.* increased volumes), and more myocardial fibrosis and cardiomyocyte hypertrophy when compared to MPC-treated animals.

Opposed to the MPC study, in which apoptosis was reduced in border and remote areas in MPC treated animals, apoptosis was only reduced in the border area in animals in the optimal encapsulated MSC group. Moreover no differences were observed in PV-loop derived parameters that were improved in the MPC study. This difference could be related to the difference in paracrine factors that are released by the cells, but regarding apoptosis it would be expected that eMSC infusion was related to a larger reduction in cardiomyocyte apoptosis due to the secretion of GLP-1, although this effect could have faded over time, due to a decrease in GLP-1 release by the MSC.[34,37] Several clinical studies investigated exenatide, a GLP-1 analogue, for the treatment of an AMI. Patients who were treated with exenatide infarct size was reduced by 50% .[35,36] It was therefore stated that exenatide protected against reperfusion damage. The difference between eMSC and these studies is that eMSC, and thus GLP-1, are infused in the reperfusion phase what could have altered the outcome.

Beside the effect on infarct size and remodeling, there was also a marked increase in neo-capillary and arteriole densities in the infarct border zone and infarct area of encapsulated MSC and MPC treated animals. This increase in blood vessel density in the perfusion territory of the culprit artery suggests a pro-angiogenic potential of MSC and MPC therapy, related to the release of VEGF. This finding and is consistent with previous studies that used MSC for cardiac repair.[22–25] Moreover, eMSC also improved angiogenesis in a hind-limb ischemia model and in porcine interposition grafts.[48,49] Although we have not directly assessed myocardial perfusion using functional testing, these histologic data suggest improved myocardial perfusion and therefore oxygen and nutrient delivery in the (peri-)infarct region. This pro-angiogenic effect might partly explain the observed preservation of cardiac function.

As described above, MSC are capable to transdifferentiate into cardiomyocytes in vivo and in vitro.[11] In the MPC study we did not investigate transdifferentiation, based on the results of an earlier study in which MPCs did not transdifferentiate after intramyocardial injection following AMI.[25] However, recent studies have shown that delivery of MSC to infarcted or hibernating myocardium can improve cardiac function by stimulating resident cardiomyocytes to re-enter the cell cycle, thereby initiating cardiomyocyte proliferation.[50,51] Indeed, in the MPC study we found a small, but significant effect on cardiomyocyte proliferation in the infarct border zone, indicating that MPC enhance endogenous repair. Moreover, we investigated, whether MPC therapy might stimulate, or increase numbers of, resident cardiac stem cells, as has been suggested in some previous studies with MSC.[51,52] In contrast to those studies, the number of resident cardiac stem cells in sheep myocardium seemed to be rather low. This might very well be explained by the fact that in those previous studies mice, and juvenile pigs were used, whereas the current study was performed in adult sheep. It seems plausible that juvenile animals have more resident cardiac stem cells than adult animals, although direct comparative study data are lacking. No difference in cKit+ cells was found in both border zone and remote myocardial segments between treated and control animals, which might be explained by the long follow-up period in the current study. In a study by Suzuki *et al.* in which MSC were intracoronary infused directly following AMI and pigs were terminated after 2 and 6 weeks, the most pronounced effect of cell therapy on cKit+ cells was found after 2 weeks and the effect declined after 6 weeks.

Transdifferentiation of MSC was not hypothesized as potential working mechanism of eMSC. The MSC do not leave their shell and the space inside the CellBeads limits them from proliferation and possibly also differentiation. Proliferation of endogenous cardiac stem cells was not investigated in the CellBeads study. This was based on the results of the MPC study, where the follow-up was also eight weeks and no effects were seen on proliferation of endogenous stem cells.

From both studies, it can be concluded that eMSC and MPC are beneficial for cardiac repair. Although a side-by-side comparison was not performed, could we made a statement which product, MPC or eMSC, is more beneficial for cardiac repair? First, both studies show that intracoronary infusion of eMSC and MPCs is safe. Encapsulated MSC will occlude small coronary arterioles due to their size of

170 µm in diameter. Their working mechanism is based on retention and survival of the cells in the target area, but this will result in obstruction of small vessels, what eventually will lead to occlusion and ischemia.

Although no effect is observed on antegrade TIMI flow and CFR, directly following infusion, it might be a possibility that there is microvascular obstruction. The outwash of contrast was slower in animals that were treated with more than 40.000 eMSCs, which could indicate some microvascular obstruction. MPCs are only 13 µm in diameter and many more are needed before arterioles are occluded. Moreover MPCs can deform and pass the capillary system. This results in a decreased number of cells in the target area but it does not lead to permanent blockage of the coronary microvasculature.

Both studies show a comparable effect in LVEF by approximately +10% in animals treated with MPCs or 20.000 eMSC. This effect is comparable with the effects of MSC in a meta-analysis on preclinical studies.[9] The difference between the studies is the number of cells that were needed to obtain this effect. In the MPC study, there was no clear dose response relationship. All groups performed equally. So it could be stated that 12.5 million cells is enough to prevent deterioration of cardiac function. In the encapsulated MSC study, only 20.000 eMSC were beneficial, what equals 2 million cells. This could indicate that they are more potent, most likely due to the elongated local release of paracrine factors. But there is a fine line between the effective eMSC dose and the ineffective doses; 10.000 eMSC (equals 1 million cells) were too few to show any effect on cardiac repair, whereas 40.000 eMSC (4 million cells) did not show any effect, most likely based on microvascular obstruction. This was in a preclinical setting, in animals without atherosclerosis. The question remains whether 20.000 eMSC are safe to infuse in the coronary system of a patient with this disease. Moreover, the effects on histology were more pronounced in the MPC study than in the eMSC study. This can be related to the product, MPCs are shown to secrete more and a larger amount of paracrine factors than MSCs[24], but it could also be related to the animal model. Sheep were used in the MPC study opposed to pigs in the eMSC study. In addition, eMSC were transplanted in a xenogeneic setting and MPCs in an allogeneic setting. Although the MSC inside the alginate shell should be protected from a host immune response, an immunological reaction can occur related to leakage of dead cell material out of the shell into the surrounding tissue what could have limited their effects. To test this hypothesis eMSC with porcine MSC should be investigated.

MPCs are now ready to enter the clinic for the treatment of an AMI. The have already been intramyocardially injected in ischemic heart failure, without any side-effects and recently the first AMI patients are included in the AMICI trial (discussed below). Encapsulated MSC are not ready to enter de clinic yet. The optimal dose is now known, but the viability of the MSC inside the beads is unknown. In the pilot study it was concluded that they were alive one week following infusion, based on qPCR analysis and histological analysis, but at 8 weeks follow-up no human genes were detected on qPCR, indicating that the cells were dead.[53]

Currently, the survival of cells is investigated in a mouse skin model and in a porcine model AMI model. In this porcine study, also porcine eMSC are infused after AMI in one group and compared to human eMSC in the other group. This design was chosen to investigate the hypothesis whether the MSCs have a better survival rate in a allogeneic setting opposed to the xenogeneic setting. Adding to that, eMSC were originally developed as a biodegradable, and at 8 weeks they are not degraded. Even after 6 months in a mouse model, the eMSC did not show any sign of degradation. First it has to made clear when and how encapsulated MSC degrade for safety issues. But overall, the eMSC concept remains interesting for the delivery of cells, cytokines, growth factors, drugs and so forth, to the heart or any other organ.

Both studies using either eMSC or MPC were executed in an animal model for AMI is used. It is important to use an appropriate model in the preclinical setting that is reproducible and representative for the clinical setting. In the MPC study, the sheep model was applied. This was related to the MPCs that were isolated from the bone marrow of a sheep. However, in most preclinical cardiology studies with large animals, a porcine is used. The porcine heart shows a lot of similarities with a human heart.[54] In the first phase of the eMSC study, a posterolateral infarct was induced, but this resulted in a small infarct size. When the infarct size is small the true potential benefit of your therapy can be underestimated. Therefore, a LAD occlusion model was chosen in de second phase of the eMSC study. In **chapter 11**, the differences on cardiac function and infarct size were investigated between these 2 different ligation sites in the heart. It was concluded that LAD occlusion resulted in a lower post-AMI LVEF, a decrease in myocardial strain and a larger infarct size opposed to LCx occlusion. This makes the LAD a more appropriate model to investigate AMI and post-AMI HF and the new therapeutic strategies that are developed to improve function. On the other hand, peri-procedural mortality is lower in the LCx model. Moreover in western society to date, door-to-balloon time is very short. This results in a smaller infarct size and therefore it also needs to be investigated whether new therapeutic strategies are also beneficial in a population with small infarcts. The main conclusion should be that for every disease and every new therapeutic strategy, the appropriate animal model has to be selected.

In addition to the correct model, the assessment of, in this case, cardiac function to evaluate therapeutic efficacy, needs to be accurate and reproducible. Therefore, it is important to evaluate techniques for functional testing under conditions that reflect its application field as close as possible. The golden standard for the assessment of cardiac function is cardiac MRI.[55] In preclinical research cardiac AMI is often out of reach due to logistical reasons and additional costs. In clinical research MRI is not always possible due to patient related factors or also additional costs. 3D-echocardiography corresponds with cardiac MRI in the assessment of LVEF and LV volumes.[56] Pressure-volume loop (PV-loop) analysis is a hemodynamic measurement that measures other cardiac parameters alongside LVEF and LV-volumes, and could provide additional information above 3D-echocardiography. Currently, there are two different PV-loop methods: conductance and admittance. The traditional conductance system (CS) has been used in many different studies to assess cardiac function. [57–59] However, this system structurally overestimates LV volumes due to its inherent inability to separate parallel conductance

from blood conductance. [60,61] This can be overcome by hypertonic saline injection. However, this assumes a constant parallel conductance and a linear relationship between conductance and volume, which might be too simplified and hence imprecise. [61–63] The admittance based system (AS) used in the study in chapter 12 has been validated and implemented in multiple murine studies for the determination of cardiac function [64–66], where it showed to be more accurate than the CS. [65] Recently, a good correlation was found between LV volumes measured by the AS and 3DE, based on repeated measurements in the same healthy animals.[67] Moreover, in larger LVs, the AS showed a trend towards a poorer agreement with 3DE.[67] More importantly, the effect of regional ischemia and post-infarction remodeling has not yet been evaluated. Therefore, the aim of the study described in chapter 12 was to test the ability of the AS and the CS system to reliably assess LV volumes and function in a human sized large animal model of chronic myocardial infarction and compare this with 3D echocardiography.[56]

It was found in **chapter 12** that, in concordance with a study of Kutty *et al*., LVEF was similar at baseline and correlated significantly between AS and 3DE, whereas LVEDV and LVESV did not.[67] The lack of correlation for the latter two could be due to larger LV volumes in our study, which is supported by the observation that larger hearts showed a poorer agreement of 3DE and the AS (in the mentioned study). [67] Interestingly, the study reveals prominent differences at 8 weeks follow-up. At this time point, the AS overestimated LV volumes resulting in an underestimation of LVEF. Moreover, infarct size significantly correlated with LVEF measured by 3DE at follow-up. Therefore, infarct size was used as a covariate in the comparison of LVEF at follow-up between the different methods. After controlling for infarct size, LVEF measurements at follow-up remained different between the AS and 3DE indicating that the found differences between 3DE and the AS are independent of the actual infarct severity.

This combination of volume over- or underestimation for the AS and CS respectively, combined with the lack of correlation of both systems with 3DE and infarct size, suggests that PV-measurements in the infarcted heart might be less accurate and reliable than in the healthy heart, supporting the importance of choosing appropriate models in the testing of novel technology. Indeed, studies on agreement and correlation between different imaging techniques (e.g. MRI, echocardiography) and volumes measured by PV methods both in small and large animal models are inconsistent. [65,68–73] It should be noted however, that 3DE moderately underestimates absolute LV volumes compared to MRI[56,74], which could partly explain the AS' overestimation of both LVEDV and LVESV at follow-up.

These data show that the admittance-based technique is valid for the assessment of cardiac function in large animal models. Given the lack of correlation between echo- and PV-loop-based volume measurements with the current systems, future studies ideally should combine the strength of either echocardiographic or MRI based volume measurements with PV-loop specific parameters as functional endpoints in cardiac large animal models.

THE FUTURE OF STEM CELL THERAPY

It was concluded from the meta-analysis in **chapter 3** that BMMNCs are not effective for cardiac repair when data is pooled for MRI studies only. However, most studies to date were designed as safety and feasibility studies and were underpowered to show efficacy. Currently larger trials are emerging in the clinic that are well-powered to detect differences on cardiac function and clinical outcome. The BAMI study, a phase III clinical trial, funded by the European Union, which aims to include 3,000 AMI patients, will render definite answers if BMMNCs are capable of preserving left ventricular function.
As stated before, new cells are entering the arena of cell-based cardiac repair. MSC and mesenchymal-like stem cells, are currently taking over clinical trials, as is described in chapter 2. The positive results of the Apollo trial have resulted in the development of the ADVANCE trial. In addition, the sheep MPC study has resulted in the design of the phase I/II AMICI trial in which 225 AMI patients will be treated with allogeneic MSC as was described in **chapter 8**.

In the ADVANCE trial, inclusion of patients is limited to the level of CK-MB or Troponin T/I which is related to infarct size. Patients with larger AMI are included in this study, whereas the AMICI is based on an all-comers population. Although it is hypothesized that patients presenting with large myocardial infarcts benefit most from cellular therapy[3], our meta-analysis in this thesis revealed that infarct size did not predict outcome (chapter 3). In addition, assessing this baseline LVEF takes valuable time that is lacking in the AMICI protocol. Also, due to increasingly shorter door-to-balloon times in the Western world, patients with significantly impaired LVEF following their first AMI are getting rare. This implies that including only patients with an LVEF below 45-50% will slow down the inclusion of patients in this study considerably. By investigating this all-comers anterior wall AMI population, patients with both extensive and less extensive infarcts will be included, thereby providing valuable information on which patients benefit most from cellular therapy.

The primary endpoint of the AMICI trial is the change in infarct size between baseline and 6 months follow-up. It is believed that current pharmacotherapy following AMI reduces the pace of LV remodeling, thereby preserving LVEF for a considerable time and exceeding the follow up time of these stem cell studies. It is therefore believed that LVEF might not be the optimal end point in cell therapy studies. Because myocardial salvage is believed to be the predominant working mechanism of cell therapy in AMI, the cells will be delivered directly following the primary PCI. Directly following MI, most cardiomyocytes are at risk for ischemia-reperfusion injury, therefore the anti-apoptotic and cardioprotective properties of the MPC will be maximally utilized.

Alongside efficacy, also safety and clinical outcome are part of both trials. Both trials will help to get inside whether MSC have a place in the clinic.
Next to MSC, many different cell types are investigated in clinical and preclinical trials to date, but the puzzle of mending broken hearts is not yet solved. Cardiosphere-derived cells (CDC) are promising cells that have the ability to transdifferentiate to cardiomyocytes and are currently evaluated in the

ALLSTAR clinical trial in which AMI patients with significant cardiac dysfunction will receive allogeneic CDC. Primary outcome will be change in infarct size additionally to safety endpoints. The first trial that implemented CDC used autologous CDC. A shift towards allogeneic cells is noticeable in the field due to reasons that are mentioned before.

Newer generations of cells, like the embryonic stem cells (ESC) and induced pluripotent cells (iPS) are currently investigated in preclinical trials. There are still a lot of concerns related to ESC. First, ethical issues and second, the ESC can become every cell in the body and is therefore teratogenic. Third, ESC are not immune-privileged rendering additional immunosuppressive therapy necessary. iPS are more promising as they lack ethical issues and immunology issues. It will probably take a decade to optimize ESC or iPS therapy, therefore the most promising cells in the near future will be the allogeneic cells: MSC, MPC or the CDC.

This thesis was set up to investigate new options for cell-based cardiac repair. Can we conclude after this thesis that stem cells mend broken hearts? Currently, stem cell therapy seems to be promising for heart repair, but many questions remain unanswered. With newer generations of cells types, hopes are high hopes that damage after an AMI can be limited or reversed, but more preclinical and clinical research is needed before stem cell therapy becomes a cornerstone in the treatment of patients with ischemic heart disease.

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CHAPTER 14

Summary

SUMMARY

Cardiovascular disease is still the number 1 cause of mortality in western societies. About half of the cardiovascular deaths are related to coronary artery diseases. Acute myocardial infarction (AMI) which is due to an occlusion of a coronary artery is the most common coronary artery disease. Treatment of an AMI consists of acute revascularization in combination with optimal pharmacological treatment. Despite advances in treatment options, heart failure that develops following an AMI cannot be prevented. Therefore, the search for new therapeutic strategies is ongoing. In the past decade stem cell therapy has emerged as a new strategy to treat an AMI and prevent the heart to fail.

In **chapter 1**, a general introduction of stem cell therapy is given and the aims and outline of the thesis are described. **Chapter 2** consists of a concise review of stem cell therapy to date. It gives an up-to-date overview of stem cells applied in preclinical and clinical setting for the treatment of an AMI and HF.

In **Chapter 3,** a random effects meta-analysis regarding stem cell therapy for the treatment of an AMI was performed. The main analysis was aimed at the first generation bone marrow mononunclear cells (BMMNC) and their effect on cardiac function and clinical outcome. Overall, intracoronary infusion of BMMNC resulted in an improvement in left ventricular ejection fraction (LVEF) of 2.10%, but when data was pooled and only studies that used MRI derived measurements were employed, the effects on LVEF diminished. Moreover, no effects of BMMNC therapy were found on clinical outcome. This was in contrast with existing literature in which data of AMI and HF patients was combined which could have distorted the outcome in AMI patients. New generations of stem cells are entering the field of cell-based cardiac repair and are slowly taking over the place of BMMNC. Also these cells were added in the meta-analysis, but still no superiority of newer generations could be found which could be related to the small number of clinical trials with these cells to date.

To investigate if BMMNC treatment is beneficial in HF, a random effects meta-analysis was performed regarding this topic in **chapter 4**. It was concluded from this meta-analysis that BMMNC therapy increased LVEF by almost +4% and even when studies were pooled for MRI solely, the effects on cardiac function remained. Moreover, BMMNC therapy gave a reduction in all-cause mortality in HF patients. However, the trials performed to date were not powered to detect differences in clinical outcome and therefore true beneficial effects need to be further investigated in well powered randomized clinical trials. Moreover new cells emerged that have more potential to repair the heart, like mesenchymal stem cells.

Part 3 describes the role of adipose tissue-derived regenerative cells (ADRCs) that are mesenchymal-like stem cells for the treatment of an AMI. In **chapter 5**, the isolation and injection protocol of ADRCs is described. Significant numbers of ADRCs can be isolated out of as little as 200 ml adipose tissue that can be obtained without any serious adverse events. After isolation, the cells can be administered via intracoronary infusion or intramyocardial injection. **Chapter 6** consists of the design and the results

of the first-in-man Apollo trial in which the first patients were treated with intracoronary infusion of autologous ADRCs within 24 hours following AMI. It was concluded that this was safe and feasible in the acute phase following AMI. Although in these small patient numbers no statistically significant effect on global LV function was found, significant improvements through 18 months follow up in infarct size, perfusion defect, coronary flow reserve, and arrhythmia suggest a possible beneficial effect. The obvious major limitation of this phase I/IIa trial is the small sample size, although the study has been performed in a randomized, double-blind fashion with analysis of imaging and holter end-points by independent core laboratories.

An advantage of MSC is that they can be given in an allogeneic setting due to their immune-privileged profile. Allogeneic transplantation has some benefits above autologous transplantation. It renders painful harvesting procedures unnecessary, it can be directly given following an event and the cells are derived from a healthy donor. Mesenchymal precursor cells (MPCs) are an immature subpopulation of MSC that also have the same abilities. In **chapter 7**, allogeneic MPCs are investigated in a sheep model of anterior AMI. In the first part of this study, infusion parameters were optimized and the maximum dose of MPCs was determined. MPC therapy was safe when the infusion rate was maintained low and the maximum of infused cells was 37.5 million. In the second part the efficacy and mechanism of action were investigated in 68 female sheep, that were subjected to an anterior AMI whereupon MPCs or placebo solution were infused. Overall MPC infusion resulted in preservation of left ventricular dimensions and volumes. Moreover infarct size was reduced, angiogenesis was enhanced and more proliferating cardiomyocytes were detected. Nonetheless no effects were seen on activation of endogenous cardiomyocytes. The promising results of this preclinical trial have resulted in the AMICI study of which the design is described in **chapter 8**. In this first in man trial, allogeneic MPCs will be intracoronary administered following AMI in 225 patients. The primary endpoint is change in infarct size as measured by cardiac MRI between baseline and 6 months follow-up. The study is initiated in 2013 and the first patients are included.

Currently, the proposed working mechanism of stem cell therapy is based on the release of paracrine factors by the stem cells. Unfortunately, retention and survival of stem cells in the myocardium is poor. To prolong the release of beneficial paracrine factors to the myocardium, mesenchymal stem cells were encapsulated in an alginate shell (CellBeads). The encapsulated human MSC are chosen based on their expression profile of paracrine factors. The MSC inside the beads are immortalized and transfected to produce glucagon-like peptide-1 (GLP-1) which has cardioprotective effects. Encapsulated MSC (eMSC) are intracoronarily infused whereupon they get stuck in the pre-capillary coronary bed where they behave like 'micro-factories' for the production of paracrine factors and their fusion protein GLP-1. In **chapter 9,** the safety and feasibility was investigated of intracoronary infusion of an incremental dose of encapsulated MSC. In the first phase of the study 4 pigs were subjected to infusion of up to 160,000 eMSC. It was found that coronary flow impeded when more than 90,000 eMSC were infused. This value was the cut-off value in part 2 of this study in which 21 pigs were randomized to receive a control solutions, 60,000 eMSC without cells (empty beads) or 60,000 eMSC following an acute myocardial infarction. The animals were sacrificed at 2 and 7 days and hearts were

explanted for histological and qPCR analysis. It was concluded that intracoronary infusion of eMSC is safe and feasible. Encapsulated MSC were targeted in the infarct area and no shedding to remote myocardial segments or remote organs occurred. The MSC inside the beads remained viable for 7 days and they were still secreting GLP-1 fusion protein.

The promising effects of the pilot study resulted in the design of a large preclinical study in which 100 pigs were subjected to an AMI and infusion of eMSC or placebo solution as described in **chapter 10**. In the first study, 50 pigs underwent a posterolateral infarct whereupon the pigs were randomized to receive 20,000 eMSC, 40,000 eMSC, 60,000 eMSC or placebo control. Cardiac function was assessed by echo and pressure-volume loop analysis. 8 weeks after infarct induction and eMSC treatment, animals were sacrificed and the hearts were processed for further analysis. Only a trend was observed on LVEF in the 20,000 eMSC group. Infarct size calculations revealed that even in control animals, infarct size was only 10%. When infarct size is limited, the treatment effect of a product could be underestimated. In the second phase of this study, an anterospetal infarct was induced in 50 pigs whereupon the animals were randomized to receive 10,000 eMSC, 20,000 eMSC, 40000 eMSC or a control solution. The rest of the study was comparable with the first study. It was found that the intracoronary infusion of 20,000 eMSC resulted in an significant improvement in LVEF and a preservation in LV end-systolic volume. Moreover end-systolic pressure volume relationship, a measure for cardiac contractility, was improved in all animals that were treated with eMSC, which could indicate strengthening of the myocardium by the eMSC. On histological analysis, arteriole density was improved in all eMSC treated animals and capillary density was improved in the 20,000 eMSC group. This effect could be due to the release of paracrine factors that enhance neo vessel formation. Also, infarct size was lower in animals treated with 20,000 eMSC, most likely due to a decrease in apoptosis in the early phase after the AMI by the release of anti-apoptotic GLP-1. Noteworthy, no effect was detected in the 10,000 and 40,000 CellBeads group. Overall, encapsulated MSC are an interesting new platform to deliver stem cells and therapeutic proteins to the heart following an ischemic event.

To translate preclinical research to the clinic, appropriate animal models need to be applied that are reproducible and representative for the human situation. Acute myocardial infarct and heart failure are dynamic processes that cannot be mimicked by computer models. **Chapter 11** described the comparison between ischemia-reperfusion at two different sites in the porcine heart. In one group, the left circumflex artery was occluded for 150 minutes, in the other group an anterospetal occlusion was made by occlusion of the left descending coronary artery for 150 minutes. Cardiac function was assessed by echocardiography during at several time points during follow-up. An anteroseptal infarct resulted in a significant decrease in LVEF and an increase in LV volumes. Moreover myocardial strain deteriorated in in the LAD animals. This rendered the conclusion that an anteroseptal infarct is more appropriate for preclinical investigation of ischemia-reperfusion and new therapeutic options for AMI and HF than the LCx model.

Assessment of cardiac function in preclinical trials has to be performed by modalities that are consistent, easy to use and precise in assessing cardiac function. Cardiac MRI is still the golden standard in the assessment of cardiac function and volumes, but MRI analysis in the preclinical

research is often not feasible due to logistic reasons and the costs related to the procedure. In **chapter 12** two PV-loop systems that work via admittance and conductance respectively, are compared to each other and to 3D-echocardiography in their assessment of LVEF and LV volumes in the healthy heart and infarcted heart. It was concluded that admittance based PV-loop showed a good correlation with 3D-echocardiography in the healthy heart, but correlated less in the infarcted heart. Conductance PV-loop performed less opposed to admittance PV-loop.

In **Chapter 13**, the findings of this thesis are evaluated and placed into the context of existing literature. Furthermore, future perspectives on the potential role of stem cell therapy for the treatment of AMI of HF are described.

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Renate

LIST OF PUBLICATIONS

1. ***R. de Jong***, J.H. Houtgraaf, S. Samiei, E. Boersma, H.J. Duckers. Intracoronary stem cell infusion following acute myocardial infarction: A meta-analysis and update on clinical trials. Circ Cardiovasc Interv. 2014;7:156-67

2. ***R. de Jong****, G.P.J. Van Hout*, J.E. Vrijenhoek, L. Timmers, H.J. Duckers, I.E. Hoefer. *Admittance-based pressure-volume loop measurements in a porcine model of chronic myocardial infarction.* Experimental Physiology, 2013;98(11):1565-75. (*contributed equally to this work)

3. J.H. Houtgraaf; ***R. de Jong***; K. Kazemi; T.I.G. van der Spoel; D. de Groot; I. Hoefer; G. Pasterkamp; M.L. Geleijnse, S. Itescu; F. Zijlstra, P. Serruys; H.J. Duckers. *Intracoronary infusion of allogeneic mesenchymal precursor cells directly after experimental acute myocardial infarction reduces infarct size, abrogates adverse remodeling, and improves cardiac function.* Circulation Research, 2013; 113: 153-166.

4. Panfilov IA, **d*e Jong R***, Takashima S, Duckers HJ. *Clinical study using adipose-derived mesenchymal-like stem cells in acute myocardial infarction and heart failure.* Methods Mol Biol. 2013;1036:207-12.

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8. K. Larsen, C. Cheng, D. Tempel, S. Parker, S. Yazdani, W.K. den Dekker**,** J.H. Houtgraaf, ***R. de Jong***, S. Swager-ten Hoor, E. Ligtenberg, S. Rowland, F. Kolodgie, P. W. Serruys, R. Virmani, and H.J. Duckers. *Capture of circulatory endothelial progenitor cells and accelerated re-endothelialization of a bioengineered stent in human ex vivo shunt and rabbit denudation model.* Eur Heart J. 2012;33(1):120-8.

9. Arslan F, Houtgraaf JH, Keogh B, Kazemi K, ***de Jong R***, McCormack WJ, O'Neill LA, McGuirk P, Timmers L, Smeets MB, Akeroyd L, Reilly M, Pasterkamp G, de Kleijn DP. *Treatment with OPN-305, a humanized anti-Toll-Like receptor-2 antibody, reduces myocardial ischemia/reperfusion injury in pigs.* Circ Cardiovasc Interv. 2012;5(2):279-87.

10. Cheng C, Haasdijk R, Tempel D, van de Kamp EH, Herpers R, Bos F, Den Dekker WK, Blonden LA, ***de Jong R***, Bürgisser PE, Chrifi I, Biessen EA, Dimmeler S, Schulte-Merker S, Duckers HJ. *Endothelial cell-specific FGD5 involvement in vascular pruning defines neovessel fate in mice.* Circulation. 2012;125(25):3142-58.

PUBLICATIONS SUBMITTED/IN PREPARATION

1. J.H. Houtgraaf, ***R. de Jong,*** W.K. de Deker, I. Panfilov, B.M. van Dalen, F. Zijlstra, P.W. Serruys, H.J. Duckers. *Intracoronary infusion of adipose tissue-derived regenerative cells in patients with ST-elevation myocardial infarction: first complete and final results of the APOLLO trial.* ***Submitted***
2. ***R. de Jong***, G.P.J. van Hout, J.H. Houtgraaf , K. Kazemi,; I. Hoefer, H.J. Duckers. *Intracoronary infusion of encapsulated GLP-1 eluting mesenchymal stem cells (CellBeads™) improves left ventricular function in a porcine model of acute myocardial infarction.* ***Submitted***
3. ***R. de Jong*****,** G.P.J. van Hout, J.H. Houtgraaf, S. Takashima, G. Pasterkamp, I. Hoefer, H.J. Duckers. Cardiac function in a long term follow-up study of a moderate and severe porcine model of chronic myocardial infarction. ***Submitted***
4. ***R. de Jong*****,** J.H. Houtgraaf, T. Henry, S. Itescu, H.J. Duckers. *Intracoronary infusion of allogeneic mesenchymal precursor cells in patients with anterior wall ST-elevation myocardial infarction: Rationale and design of the AMICI trial*. ***In preparation***
5. ***R. de Jong*****,** J.H. Houtgraaf, S. Takashima, F. Zijlstra, P. Serruys , H.J. Duckers. *Cell-based cardiac repair: what the clinician needs to know.* ***Submitted***
6. ***R. de Jong,*** J.H. Houtgraaf. *First generation stem cell therapy for ischemic heart disease: a review,meta-analysis and future prospectives.* ***Provisionally accepted***

CURRICULUM VITAE

Renate de Jong was born on the 23th of March 1985 in Rotterdam. She started her pre-university training at Comenius College in Capelle aan den IJssel, from which she graduated in 2003. In the same year she began to study Medicine at Erasmus University Medical Center in Rotterdam. In the final year she performed research at the Molecular Cardiology department of the same institution, regarding the topic Cell-based cardiac repair. She obtained her degree in Medicine in 2009. In the same year, she started as a PhD candidate at the same department as her research internship. The topic of her research was Advanced cell-based cardiac repair.

In March 2014 she started as resident at the Cardiology department of Erasmus University Medical Center. During her PhD, she developed a special interest in physiology, whereupon she decided to apply for the position of resident anesthesiology instead of cardiology. From October 1st 2014, she will start her anesthesiology training at Erasmus University Medical Center in Rotterdam.

PhD PORTFOLIO

COURSES

2012 Scientific English writing course, Rotterdam, The Netherlands

2012 PhD course Vascular Biology (Dutch Heart Foundation, Papendal, The Netherlands)

2011 Basic Science Course European Society of Cardiology, Nice, France

2011 Masterclass English speaking, Rotterdam, The Netherlands

2011 Coeur Course congenital heart disease

2010 PhD course Cardiac function and adaptation course (Dutch Heart Foundation, Papendal, The Netherlands)

2010 Laboratory Animal Science (article 9), Utrecht, The Netherlands

2010 Good clinical practice (GCP course), Rotterdam, The Netherlands

2010 Coeur Course Cardiovascular pharmacology

2009 Basis cursus klinische onderzoekers (BROK cursus), The Netherlands

PRESENTATIONS

AWARDS

2013 Best abstract Wetenschapsmiddag Erasmus MC, Rotterdam, The Netherlands

2013 Best abstract Utrecht stem cell conference, Utrecht, The Netherlands

2013 Best abstract Cardiovascular Conference, Noorwijkerhout, Netherlands

2011 Best Poster at the 8th International Conference on Stem Cell Therapy and Cardiovascular Innovations, Madrid, Spain

ORAL PRESENTATIONS

2014 Dutch-German meeting 2014, Groningen, The Netherlands

2014 Research Master Infection and Immunology, Erasmus MC (Invited), Rotterdam, The Netherlands

2013 Wetenschapsmiddag Erasmus MC, Rotterdam, The Netherlands

2013 BioMedical Materials Annual meeting, Ermelo, The Netherlands

2013 Cardiovascular Conference, Noordwijkerhout, The Netherlands

2013 Research Master Infection and Immunology, Erasmus MC (Invited), Rotterdam, The Netherlands

2012 BioMedical Materials Annual meeting, Ermelo, The Netherlands

2012 Cardiovascular Research school Erasmus MC (Coeur), Rotterdam, The Netherlands

2011 American Heart Association Scientific Sessions. Orlando, FL, USA

2011 BioMedical Materials Annual meeting, Ermelo, The Netherlands

2011 Cardiovascular Research school Erasmus MC (Coeur), Rotterdam, The Netherlands

2010-2013 Staff lunch presentations once a year

POSTER PRESENTATIONS

2013 American Heart Association Scientific session, Dallas, TX, USA

2013 Utrecht stem cell Conference, Utrecht, The Netherlands

2013 BioMedical Materials annual meeting, Ermelo, The Netherlands

2012 Sixth International Conference on cell therapy for cardiovascular repair, New York, USA (2 posters)

2012 PhD course Vascular Biology course, Papendal, The Netherlands

2011 8th International Conference on Stem Cell Therapy and Cardiovascular Innovations, Madrid Spain

2011 Basic Science course of the European Society of Cardiology, Nice, France

2011 BioMedical Materials Annual Meeting, Ermelo, The Netherland

2010 Scientific Sessions American Heart Association, Chicago, IL, USA

2010 PhD course Cardiac function Course Papendal, The Netherlands

STUDENTS

2013 Tirza Hendrik (Master student Infection and Immunology)

2012 Laura Buter (MBO histology)

2012 Deborah Hubert (MBO histology)

2012 Gerard Marchal (HBO, zoology)

2011 Ilona Boons (MBO histology)

2011 Maaike de Witte (MBO histology)

2010 Janita snikkers (MBO histology)

2010 Bob Jespers (MBO histology)

2010-2011 Kushan Kazemi (Medical student)

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