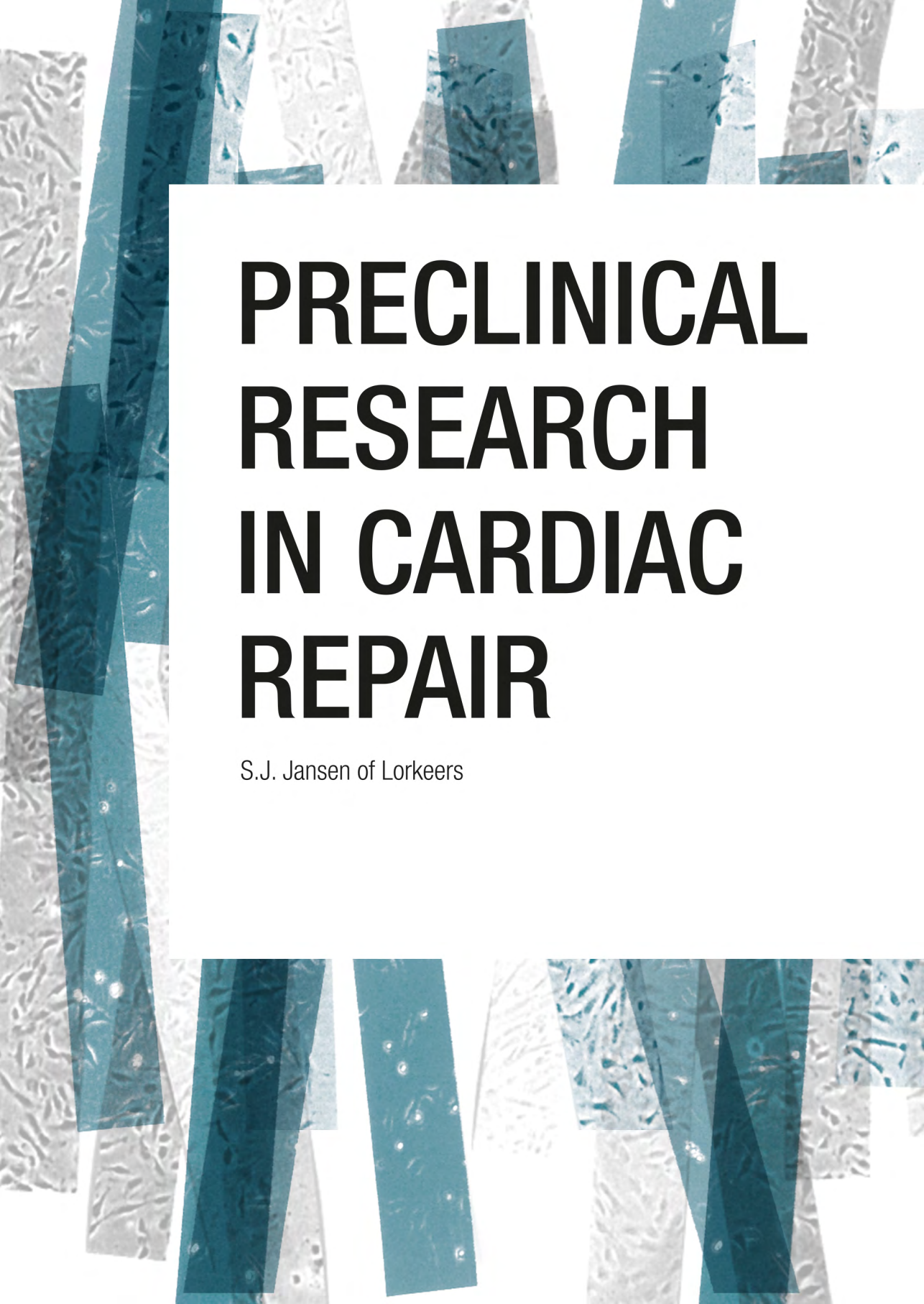

PRECLINICAL RESEARCH IN CARDIAC REPAIR

S.J. Jansen of Lorkeers

PRECLINICAL RESEARCH IN CARDIAC REPAIR

Sanne Jansen of Lorkeers, 2015, Utrecht

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PRECLINICAL RESEARCH IN CARDIAC REPAIR

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geboren op 23 maart 1986 te Raalte

Promotor: Prof. dr. P.A. Doevendans
Co-promotor: Dr. S.A.J. Chamuleau

The first principle is that you must not fool yourself
and you are the easiest person to fool

Richard Feynman

CONTENTS

CHAPTER 1
INTRODUCTION

Ischemic heart disease

With 7,4 million deaths per year ischemic heart disease (IHD) accounts for 13,2% of mortality worldwide.[1] IHD covers grossly three manifestations of the disease: acute coronary syndrome (including myocardial infarction), chronic ischemic heart failure and refractory angina pectoris. The acute myocardial infarction (AMI) is characterized by obstruction of blood flow to the myocardium, usually caused by rupture of an atherosclerotic plaque. Development and improvement of therapy for acute myocardial infarction (*i.e.* percutaneous coronary intervention) has resulted in a reduction in mortality from approximately 20% in the late 1980s to 5% in 2008.[2,3] However, patients surviving the initial event often suffer from heart failure later on.

The initial ischemic event causes irreversible damage by insufficient supply of oxygen and nutrients, leading to apoptosis and necrosis of the myocardium. Restoration of blood flow and subsequent reinstatement of oxygen accelerates generation of reactive oxygen species, further damaging to the heart. The next damaging component of AMI involves inflammation, initiated by the injured myocardium.[4–6] After the initial event remodeling of the heart takes place by dilation, hypertrophy and scar formation in order to account for changes in pressure and volume load and to preserve cardiac output.[4] Remodeling is complete when the distending forces are counterbalanced and this process may take up to months.[7] Although remodeling initially restores cardiac function, it eventually gives rise to chronic ischemic heart failure (CIHF). Decrease in mortality from AMI contributes to significant increase in CIHF morbidity. This growing group of patients is responsible for a great burden of disease, additional healthcare costs and the increase in mortality by IHD from 6 million per year in 2000 to 7,4 million in 2012.[1]

Current treatment of CIHF consists mainly of pharmacological therapy with or without resynchronization- and defibrillation devices.[8] Angiotensin converting enzyme inhibitors, diuretics, beta-blockade and mineralocorticoid/aldosterone receptor antagonists are the fundamentals in CIHF therapy. These drugs, however, effectuate symptom relieve, but are no real remedy for heart failure. Today the only way to really *cure* heart failure is by heart transplantation and this option is considered for patients suffering from end stage heart failure. The growing number of patients on the waiting list is disproportionate to the constant low number of organ donors each year, which is a serious threat for this group of patients.[9]

Since current therapies for ischemic heart disease are limited, there is a great need for new therapies with curative potential. Regenerative medicine has taken off in this field and efforts have been put in different approaches, like stem cells, growth factors or microRNAs.

Cardiac regeneration

The paradigm of the heart being a post-mitotic organ was challenged in the beginning of this century, substantiated by the presence of a Y-chromosome in cardiomyocytes of a transplanted female donor heart in a male recipient.[10] At the same time, Beltrami et al. showed presence of cell division in infarcted hearts.[11] Despite the presence of stem cells and prove for an annual myocyte turnover of 1% per year, the heart is unable to repair itself after a damaging event.[12] Transplantation of autologous or allogeneic stem cells into the heart underwent a fast and dynamic development.

The potential of cell therapy varies with the stage of disease.[13] In the acute setting, delivery of stem cells may serve as 'damage control' by modulation of the inflammatory response, while 'damage elimination' by regeneration and prevention of adverse cardiac remodeling may be achieved in the setting of CIHF. In the chronic setting, proposed mechanisms of effect are vasculogenesis and cardiomyogenesis leading to decreased infarct size, regain of healthy myocardium and amelioration of cardiac function and quality of life.[14,15] Rather than regeneration by true differentiation of stem cells, a more likely mechanism of action is through paracrine signaling.[16,17] Paracrine signaling, executed via excretion of growth factors and exomoses, is thought to reduce the inflammatory response, promote vasculogenesis, and stimulate endogenous (cardiac) stem cells.[18]

Different cell types have been tested in clinical setting the past decades, from myoblasts in the beginning, to bone marrow mononuclear cells, mesenchymal stem cells and cardiac stem cells today. Myoblasts were the first cells used in patients[19], but were rapidly deserted because of moderate results and safety concerns (*i.e.* life threatening arrhythmias).[20,21]

Bone marrow mononuclear cells (BMCs) are the most frequently used cell type since these are abundant, easy to obtain via bone marrow biopsy and *ex-vivo* handling is convenient. The first-in-man study using BMCs was published in 2002[22] and meta-analyses show an estimated mean increase in ejection fraction of 3-4%.[23–25]

Mesenchymal stem cells (MSCs) are mononuclear cells that can be isolated from bone marrow, adipose tissue and peripheral blood. Since MSCs are multipotent, expandable and stated to be immunoprivileged, they hold great potential for cardiac cell therapy.[26,27] Many preclinical studies have been performed, and several clinical trials show promising results by MSCs.[28–30]

Next, the heart itself forms a source for cardiac stem cells. Grossly, three different cell types can be isolated based on c-kit or sca-1 markers, or by growing cardiospheres from cardiac biopsies.[31–33] Despite different isolation procedures and mixed results, individual cardiac cell lines (including, c-kit+, cardiosphere derived cells, and sca1+ cells) turned out to share high similarity in gene expression profile.[34] C-kit positive cardiac stem cells and cardiosphere derived cells are extensively tested in pre-clinical models and the first clinical trial shows promising results.[35–37] Induced pluripotent stem cells and embryonic stem cells may serve as potent cell lines in the future, but have not yet been investigated in clinical trials.[38]

Replacement, reduction and refinement

Animal models can provide a great tool to get insight in physiology and pathology and to test safety and efficacy of new therapies. They allow us to search for new diseases treatments without endangering patients. However, to enable the process of transition from bench to bedside, and to prevent needless suffering of patients, solid transparent and clinically relevant animal research is needed. Besides, animal welfare is a genuine concern and animal research is often seen as a necessary evil.

Back in 1959, Russel and Burch introduced the so called "3Rs" to remove 'inhumanity' in animal science.[39] Replacement, reduction and refinement represent the three pillars in animal research ethics and implementation of the 3Rs is a legal requirement in Europe.[40]

Replacement is seen as the ultimate goal of the 3Rs and implicates the substitution of 'conscious' animals by 'insentient' material.[39] The replacement of pigs by crash test dummies in automobile safety tests by General motors in 1993 is an appealing example.

In a more scientific perspective, one may think of replacement of animals by computer models mimicking human physiology, cell culture systems, post mortem tissue etc. A specific example in the field of healthcare research is the use of organoids, which are small tissues grown from individual patient cells or specific tumor cells.[41] This method may even eliminate the need for large groups study subjects since organoids are the perfect model for individual drug testing. This technique not only saves lives of laboratory animals and patients, but is also less time- and costs-consuming than *in vivo* experiments.

Reduction involves the optimal use and reporting of experimental animals and statistical test, in order to reduce the number of animals needed. Appropriate experimental design, statistical analysis techniques and power calculation are key means of minimizing the use of animals in research.[43,44] Power calculation provides the number of subjects needed to be able to legitimately accept or reject the null hypothesis. It is obvious that using more subjects than needed leads to excessive use of laboratory animals. But using too few animals may conceal significant existing effects and (false) negative studies are more prone to stay unpublished resulting in repetition of research.

As described in the series 'increasing value, reducing waste' by the Lancet in 2014, so called "waste" in animal research can be declined by minimizing the risk of bias through improving study design and conduct.[45] Examples to optimally perform and use animal experiments are randomization of animals, using standardized methods, making research protocols publically available before conducting the research (also for meta-analyses) and publication of all results.

Refinement is the R with the broadest potential. The most obvious implementation of 'refinement' is minimizing stress, relieving pain and optimizing natural behavior of animals. For example, animals housing should resemble their natural habitat and for most animals cage enrichment is mandatory. However, refinement may also be achieved by refining the specific experiments. For example, standardized research protocols and experienced personnel will lead to shorter operation duration, less errors and less discomfort.

Outline of the thesis

The fundamental backbone of the present thesis is to appeal to more standardized and focused preclinical experiments in cardiac repair. Additionally, efforts should be made to reduction and refinement of animal studies. First, lessons from published preclinical experiments are presented, since many of these studies have been performed so far. However, we often forget that having a close look at pooled data may reveal interesting views on specific research questions. Also several methods mandatory for conducting preclinical research on cardiac repair are presented, in order to show transparent protocols and provide standardization in the field. Sharing protocols of study design, planned meta analysis and specific research techniques will definitely be of incremental value for both validity and reproducibility.

PART ONE

Lessons from preclinical research

The first part of the thesis mainly consists of meta-analyses of preclinical studies. Previously published preclinical studies are used to answer clinically relevant questions and to generate hypotheses for future (pre)clinical trials. Parameters for risk of bias in preclinical studies,

and their role in failure and success of translational research are discussed as well.
In **chapter 2**, we contemplate the significance of publication bias in preclinical research and we oppose upfront registration of preclinical studies as a potential for reduction of the number of animals used. In **chapter 3** a protocol is described for the conduction of a meta-analysis about anti-inflammatory compounds for myocardial infarction. The meta-analysis itself is presented in **chapter 4**, where the effect of anti-inflammatory compounds on infarct size was estimated, and potential explanations for translational failure of these therapies towards clinical application are discussed. **Chapters 5** and **6** include meta-analyses of large animal studies concerning stem cell therapy for MI. In **chapter 5**, the mean effect of stem cell therapy is estimated and the human relevance in terms of therapy specific parameters is discussed. In **chapter 6**, focus is put on a specific and important research issue in the field of cardiac repair, *i.e.* if *allogeneic* is at least as good as compared to *autologous* cell therapy in an even larger and updated data set.

PART TWO
Methods in preclinical research
In the second part, methods of large animal studies in cardiac repair are described. By standardizing study methodology and outcome assessment, within-study and between-study variation is declined contributing to reduction and refinement of animal use.
Chapter 7 provides a standardized method to create an ischemic heart failure pig model including the functional measurements. In view of the use of non-autologous cell products, the feasibility of immunosuppression by cyclosporin and the effect of cyclosporin on cell products are discussed in **chapter 8**.
In line with the proposed working mechanisms, cardiac function is the primary *in vivo* outcome parameter in cardiac regenerative therapy. Left ventricular volumes and ejection fraction are used as surrogates for cardiac function and prognosis, usually measured by the gold standard MRI, echocardiography or pressure volume loop (PVloop) measurements. **Chapters 9** and **10** describe new ways of measuring outcome parameters in cardiac regenerative therapies. In **chapter 9** admittance based PV loop measurements are validated and compared to MRI and echocardiography. Since one of the major goals of cardiac regeneration is to establish proper perfusion of the myocardium by improving (micro-) vasculature, hyperemic microvascular resistance index (HMR) is introduced as a new outcome measure for myocardial microvasculature in **chapter 10**.
In the final chapter of this thesis, **chapter 11**, the previous chapters converge in a randomized, blinded, placebo controlled large animal study. Human cardiomyocyte progenitor cells (CMPCs) are tested in an immunosuppressed pig model of chronic ischemic heart disease, as described in **chapter 7** and **8**. Cardiac function is measured by MRI, echocardiography and admittance based PV loop, and myocardial microvasculature is determined by HMR, as described in **chapter 9** and **10**. By implementing lessons learned and methods described in the previous chapters, this study will offer a solid piece of evidence to serve as a key factor in the translational axis of CMPCs from bench to bedside testing.

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PART ONE
LESSONS FROM PRECLINICAL RESEARCH IN CARDIAC REPAIR

CHAPTER 2

ALL PRECLINICAL TRIALS SHOULD BE REGISTERED IN ADVANCE IN AN ONLINE REGISTRY

European Journal of Clinical Investigation 2014;4:80-86

S.J. Jansen of Lorkeers, P.A. Doevendans, S.A.J. Chamuleau

In this short report, we contemplate the seriousness of publication bias in both clinical and preclinical studies. We therefore promote the online registration of preclinical studies, just as is it implemented for clinical trials.

Publication bias in clinical studies

Publication bias is a major concern in clinical and preclinical trials. The declaration of Helsinki - Ethical Principles for Medical Research Involving Human Subjects, requires trial registration for all studies concerning human subjects.[1] It says *'Every clinical trial must be registered in a publicly accessible database before recruitment of the first subject.'* Not even half of the registered clinical trials are being published.[2–4] Unpublished results appear to be concerning smaller sample sizes,[3] Phase I/II studies and studies funded by industry. [2,4] The number of unpublished trials was highest in the USA and China.[3] Positive or statistically significant studies have higher probability of getting published than non-significant results.[5] As an exception, studies funded by the National Institute for Health Research Health Technology Assessment Programme (HTA), are published in 98% of the cases.[4] Explanation can be found by the fact that a part of the grant will only be provided after publication of the data. Furthermore the HTA provides a platform for publication of all supported research, the online journal called 'Health Technology Assessment'.

Parties involved in publication bias are authors, journal editors and research sponsors. According to several studies by prominent researchers,[6–8]authors are the largest contributors of publication bias, because of 'lack of interest' especially when results are negative or non-significant. Next, journal editors have difficulties getting negative or non-significant papers reviewed by external reviewers and tend to select papers for publication that are most exciting or papers that make the journal 'look good'.[6] Fortunately, publication of 'negative results' is becoming more convenient since the raise of open access journals and special sections or journals for negative results.

Publication bias in preclinical studies

For preclinical trails, no statement like the declaration of Helsinki exist. The European Union did publish directives for protection of laboratory animals based on 'replacement, reduction and refinement of animal use', the 3 Rs.[9] Every EU member is obliged to have a national committee for the protection of laboratory animals and for promoting the 3Rs.[9] All animal research should be approved by a committee, before starting the project. In spite of these committees, the lack of online and advanced registration of preclinical studies could lead to even lower publication rates, compared to clinical trials.

Consequences of publication bias

The goal of publishing clinical and preclinical research results is to share knowledge within the field, achieve progress in medical science and eventually improve patient healthcare. These goals need contentious assessment of previous results and performed research. Systematic reviews and meta-analyses are very helpful for summarizing available clinical or preclinical data. Meta-analyses can give evidence in favour of treatments not incorporated in daily practice, or even against treatments that are incorporated in daily practice.[10] In case of preclinical data, meta-analyses can help transition of therapies from bench to bedside.[11] A mean effect size of a specific therapy can be calculated and heterogeneity in included

studies can be used to find predictors for this effect size. This will give guidance for the design of clinical studies. Publication bias is detrimental, since it skews the mean outcome of meta-analyses.[12,13] In animal studies of stroke, publication bias relatively overestimates the efficacy of interventions by 31.1%.[12] This bias causes erroneous interpretation of results and unjustified transition of interventions towards clinical trials. Another serious result of publication bias is the lack of sharing both negative and positive results with other researchers in the field. Lack of publication of (negative) studies contributes to repetition of research and corresponding costs. In view of the '3 Rs', reduction will not be optimally practised and superfluous use of laboratory animals will be facilitated .

Registration of preclinical trials

To improve transition of research from preclinical setting towards clinical application and to reduce the number of laboratory animals used, effort should be made to reduce publication bias. We believe that the first step for preclinical studies is a convenient and easy one: prospective registration of preclinical trials.

We propose that the registration includes at least a description of the study (including the interventions), a power calculation, number of animals to be included in the study, the duration of the follow up and the primary and secondary outcomes. The study proposal which is submitted mandatory to the national committee for protection of laboratory animals can be submitted in a 1 to 1 order to the registry. We are aware of the fear for sharing scientific ideas beforehand. Therefore, protocols should be uploaded to a secured environment, only available to the authors themself and the independent surveillant of the system. All protocols will be disclosed after finishing the project, publication of the results or at a set timepoint (e.g. 3 years). Amendments and expansions of protocols like changes in primary and secondary outcomes should be tracked as well. Similar to the platform of the HTA and the sections of negative results in journals, the online registry should provide a platform for disclosing results as well. Authors themselves contribute to publication bias because of lack of interest, but publication of at least the results (without any additional introduction of discussion) is necessary for interpreting all available data in a specific field. The combination of mandatory registration and the warranty of publication of results, will contribute to higher publication rates. By this means, no repetitive animal studies need to be conducted and the field will be able to get a true and reliable impression of the effect size.

As it is for clinical trials, prospective registration of preclinical trials should be a requirement for journals publishing the results of these studies. We believe that at least a European registry is feasibly to start with, when the national committees for protection of laboratory animals of the EU collaborate . This European collaboration should guarantee, audit and regulate the system. (proposed registry: www.preclinicaltrials.eu) However, the registry should be open for any animal study conducted in any country.

Conclusion

In conclusion, we would appeal to the European Union to provide central registration of all preclinical trials in an online public database, to improve transition from bench to bedside and to reduce the number of laboratory animals used, through optimizing publication rate. Improvement of the publication rate is gained by registration itself as well as by providing a platform for reporting the main results.

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CHAPTER 3

ANTI-INFLAMMATORY COMPOUNDS TO REDUCE INFARCT SIZE IN LARGE ANIMAL MODELS OF MYOCARDIAL INFARCTION: A META-ANALYSIS

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ABSTRACT

Targeting the inflammatory response after myocardial infarction (MI) could potentially prevent infarct expansion, resulting in a preservation of cardiac function. Despite extensive testing in large animal models of MI, anti-inflammatory therapeutics are not incorporated in daily clinical practice. Methodological review of the literature describing the effects of anti-inflammatory compounds in large animal models of MI may provide useful insights into the reasons for the translational failure from preclinical to clinical studies. Moreover, systematic review of these preclinical studies may allow us to determine which anti-inflammatory agents have the greatest potential to successfully treat MI in the clinic and guide which pre-clinical setting seems most appropriate to test these future treatment strategies in. The current systematic review protocol provides a detailed description of the design of this systematic review of studies investigating the effects of anti-inflammatory compounds in large animal models of MI.

GENERAL

The outline of this protocol is based on the Systematic Review Protocol For Animal Intervention Studies.[1]

Title of the review
Anti-inflammatory compounds to reduce infarct size in large animal models of myocardial infarction: a meta-analysis

Authors (name and contribution)
G.P.J. van Hout and S.J. Jansen of Lorkeers are shared first author and are responsible for the study design, title/abstract screening, full text screening, data extraction and manuscript preparation. G.P.J. van Hout is responsible for the data-analysis.
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Stage of review at time of protocol submission

Review stage	Started	Completed
Preliminary searches	Yes	Yes
Piloting of the study selection process	Yes	Yes
Formal screening of search results against eligibility criteria	Yes	No
Data extraction	No	No
Risk of bias (quality) assessment	No	No
Data analysis	No	No

BACKGROUND

Cardiac damage after myocardial infarction (MI) induces a sterile immune response that leads to infarct expansion after the initial ischemic event.[2–4] Due to the release of pro-inflammatory mediators from the damaged myocardium post-MI, circulating cells are drawn to the infarcted tissue to clear out dead cardiac resident cells and promote infarct healing. Paradoxically, these circulating cells are known to also target viable tissue and greatly amplify the initial inflammatory response, thereby inducing infarct enlargement regardless of the application of revascularization therapies in patients suffering from MI.[5,6] Therefore, targeting the immune response could potentially prevent infarct expansion, resulting in a preservation of cardiac function and a prevention of heart failure in patients suffering from ischemic heart disease. [7]

Before effectiveness of new anti-inflammatory therapeutics can be tested in clinical trials, novel compounds are commonly tested in large animal models of MI. These large animal models have shown to possess additive translational value due to comparable hemodynamics, similar heart size and corresponding coronary physiology.[8–10] Moreover, large animal models enable clinical treatment regimens, delivery route and identical function-related measurements and therefore could provide an evidence base for clinical trial design. [11–15] Over the last 5 decades, numerous pharmacological therapies that target the inflammatory response have been tested in large animal models of MI. Unfortunately, none of these treatments have made it past clinical trials into daily practice.

Methodological review of the preclinical literature describing the effects of anti-inflammatory compounds in large animal models of MI may provide useful insights into the reasons for the translational failure from preclinical to clinical studies. Systematic review of these preclinical studies may also allow us to determine which anti-inflammatory agents have the greatest potential to successfully treat myocardial infarction in the clinic and guide which pre-clinical setting seems most appropriate to test these future treatment strategies in.

Objectives of this SR

Specify the disease/health problem of interest

The mortality due to myocardial infarction (MI) has decreased over the past 30 years. This can be mainly attributed to optimized revascularization therapy.[16,17] However, MI still accounts for a large amount of cardiovascular deaths worldwide and is expected to increase again due to an increased prevalence of obesity and diabetes in the western world.[18] Moreover, as more patients survive, the prevalence of heart failure – a direct consequence of MI – dramatically increases.[19] These high mortality numbers, combined with the societal and economic burden of heart failure, call for improved treatment after acute MI.

There is considerable heterogeneity regarding the way myocardial infarction is applied in the different studies performed during the past few decades (e.g. open/closed chest, permanent/temporary ligation). In the current systematic review we have included studies that satisfy the following definition: "An intervention that leads to permanent or temporary total occlusion of a coronary artery, disabling blood flow for a period long enough (>30min) to induce permanent damage to the myocardium."

Specify the population/species studied

In this systematic review we will focus on large animal models. We specifically want to study this group of animals because the cardiac physiology and anatomy (e.g. hemodynamics, coronary anatomy) combined with the immune response post-MI is considered relatively similar to humans. Also, route of drug administration and treatment regimens in large animal models allow protocols that resemble clinical treatment. In this review, we focus on four species: pigs, dogs, sheep and goats because these species are mostly used and widely applied for the validation of novel anti-inflammatory treatments for MI.[10,13,20]

Specify the intervention/exposure

The intervention applied for the treatment of MI should in this case be a pharmacological treatment with an anti-inflammatory drug. Any route of drug delivery or treatment regimen of anti-inflammatory compounds (IM/IV/SC/PO) is possible. Studies were included in the analyses if the interventions applied met the following criteria:

1. A compound that is FDA-approved for its anti-inflammatory mechanisms of action. AND/OR
2. A compound that directly targets a (recently discovered) mechanisms, that plays a proven role in inflammation.

In this perspective it is possible that certain compounds may have multiple mechanisms of action and pleiotropic effects besides being anti-inflammatory. To exclusively investigate anti-inflammatory compounds, we chose to exclude interventions that have relevant and evident pleiotropic effects. Also, if the mechanism of action was unclear regardless of the effect on inflammatory parameters, these interventions were excluded. Finally, the treatment had to be solely pharmacological. According to these criteria, the following interventions were excluded from analysis:

Stem-cells, biomaterials, pro-inflammatory compounds, statins, ACE-inhibitors, βblockers, calcium-channel antagonists, adenosine analogues, prostacyclin analogues, L-arginine analogues, endothelin-1 analogues, thromboxane A2-antagonists, omega-3 fatty acids, gene therapy, anesthetics, extracorporal treatments, aspirin in dosages <6.25mg/kg/day (anti-platelet therapy), flavonoids, flavonols, map-kinase inhibitors.

Specify the control population
The control population is a group of animals that receive the above-defined MI without any additional (anti-inflammatory) treatment and is preferably placebo controlled. If studies use multiple control groups, the control group that resembles to the interventional group the most in terms of vehicle use and administration route was chosen.

Specify the outcome measures
Primary outcome measures:
Myocardial infarct size (IS) measured as a percentage of the area at risk (AAR)
Secondary outcome measures:
1. Myocardial IS measured as a percentage of the total left ventricle (LV)
2. Left ventricular ejection fraction (LVEF)
3. Myocardial scar thinning given in millimetres or as a ratio, divided by the opposite, non-infarcted wall
4. Left ventricular end diastolic volume (LVEDV) and left ventricular end systolic volume (LVESV) in mL
5. Mortality after therapy administration

State your research question
What is the effect of anti-inflammatory compounds on mortality, infarct size, cardiac function and myocardial scar thinning in large animal models of myocardial infarction, when compared to placebo-treated large animal models of MI?

METHODS

Search and study identification

Identify literature databases to search
Pubmed and Embase

Define electronic search strategy
Pubmed:
Myocardial ischemia[Mesh] OR Myocardial infarction[tiab] OR Myocardial infarctions[tiab] OR Myocardial infarct[tiab] OR Myocardial infarcts[tiab] OR Myocardial ischemia[tiab] OR Myocardial ischemias[tiab] OR Myocardial ischaemia[tiab] OR Myocardial ischaemias[tiab] OR Myocardial reperfusion injury[Mesh] OR Myocardial reperfusion[tiab] OR (Myocardial[tiab] AND Reperfusion injury[tiab]) OR Coronary occlusion[tiab] OR Coronary occlusions[tiab]
AND

Large model[tiab] OR Large animal[tiab] OR Swine[Mesh] OR Swine[tiab] OR Pigs[tiab] OR Pig[tiab] OR Porcine[tiab] OR Suidae[tiab] OR Hog[tiab] OR Minipig[tiab] OR Minipigs[tiab] OR Dogs[Mesh] OR Dogs[tiab] OR Dog[tiab] OR Canis[tiab] OR Canine[tiab] OR Hound[tiab] OR Sheep[Mesh] OR Sheep[tiab] OR Ovis[tiab] OR Ovine[tiab] OR Goats[Mesh] OR Goat[tiab] OR Goats[tiab] OR Capra[tiab] OR Capras[tiab]

AND

Anti-inflammatory agents[Mesh] OR Anti-inflammatory agents[Pharmacological Action] OR Anti-inflammatory[tiab] OR Antiinflammatory[tiab] OR Anti inflammatory[tiab] OR Inflammation[tiab] OR Inflammatory[tiab] OR Pro-inflammatory[tiab] OR Immunosuppressive agents[Mesh] OR Immunosuppressive agents[Pharmacological Action] OR Immunosuppression [Mesh] OR Immunosupressive [tiab] OR Immuno-supressive [tiab] OR Immuno suppressive [tiab] OR Immunosuppressant [tiab] OR Immune suppressant [tiab] OR Immunosupression[tiab] OR Antioxidants[Mesh] OR Antioxidants[tiab] OR Antioxidant[tiab] OR Anti-oxidant[tiab] OR Free radical scavenger[tiab] OR Radicals[tiab] OR Radical[tiab] OR Metalloproteinase[tiab] OR Complement system proteins[Mesh] OR Complement[tiab] OR Cyclosporins[Mesh] OR Cyclosporine[tiab] OR Adrenal cortex hormones[Mesh] OR Adrenal cortex hormones[pharmacological action] OR corticosteroids[tiab] OR corticosteroid[tiab] OR cyclooxyenase[tiab] OR COX[tiab] OR Anti-inflammatory agents, non-steroidal [Mesh] OR NSAID[tiab] OR Cox-2[tiab] OR NSAIDS[tiab] OR Leukocytes[Mesh] OR Leukocyte[tiab] OR Leukocytes[tiab] OR Neutrophil[tiab] OR Neutrophils[tiab] OR Monocyte[tiab] OR Monocytes[tiab] OR Macrophage[tiab] OR Macrophages[tiab] OR Cytokines[Mesh] OR Cytokine[tiab] or Cytokines[tiab] OR Interleukin[tiab] OR Interleukins[tiab] OR Chemokine[tiab] OR Chemokines[tiab] OR Integrins[Mesh] OR Integrin[tiab] OR Integrins[tiab] OR Toll-like receptors[Mesh] OR TLR[tiab] OR Toll-like receptor[tiab] OR Toll-like receptors[tiab] OR TLRs[tiab] OR Inflammasome [tiab]

Embase:
'ischemic heart disease'/exp OR 'myocardial infarction':ab,ti OR 'myocardial infarctions':ab,ti OR 'myocardial infarct':ab,ti OR 'myocardial infarcts':ab,ti OR 'myocardial ischemia':ab,ti OR 'myocardial ischemias':ab,ti OR 'myocardial ischaemia':ab,ti OR 'myocardial ischaemias':ab,ti OR 'myocardial reperfusion':ab,ti OR 'coronary occlusion':ab,ti OR 'coronary occlusions':ab,ti OR (myocardial:ab,ti AND reperfusion:ab,ti AND injury:ab,ti)

AND

'swine'/exp OR 'dog'/exp OR 'sheep'/exp OR 'goat'/exp OR 'large model':ab,ti OR 'large animal':ab,ti OR swine:ab,ti OR pigs:ab,ti OR pig:ab,ti OR porcine:ab,ti OR suidae:ab,ti OR hog:ab,ti OR minipig:ab,ti OR minipigs:ab,ti OR dogs:ab,ti OR dog:ab,ti OR canis:ab,ti OR canine:ab,ti OR hound:ab,ti OR sheep:ab,ti OR ovis:ab,ti OR ovine:ab,ti OR goat:ab,ti OR goats:ab,ti OR capra:ab,ti OR capras:ab,ti

AND

'antiinflammatory agent'/exp OR 'immunosuppressive agent'/exp OR 'immunosuppressive agent'/exp OR 'corticosteroid'/exp OR 'anti-inflammatory':ab,ti OR 'antiinflammatory':ab,ti OR 'anti inflammatory':ab,ti OR 'anti-inflammatory drugs':ab,ti OR 'anti-inflammatory drug':ab,ti OR 'inflammation':ab,ti OR 'inflammatory':ab,ti OR 'pro inflammatory':ab,ti OR 'immunosupressive':ab,ti OR 'immuno supressive':ab,ti OR 'immuno suppressive':ab,ti OR 'immunosuppressant':ab,ti OR 'immune suppressant':ab,ti OR 'immunosupression':ab,ti OR 'radicals':ab,ti OR 'radical':ab,ti OR 'metalloproteinase':ab,ti OR 'complement':ab,ti OR 'cyclosporin':ab,ti OR 'corticosteroids':ab,ti OR 'corticosteroid':ab,ti OR 'cyclooxyenase':ab,ti OR 'cox':ab,ti OR 'nsaid':ab,ti OR 'cox 2':ab,ti OR 'nsaids':ab,ti OR 'leukocyte':ab,ti OR 'leukocytes'/exp OR leukocytes:ab,ti OR neutrophil:ab,ti OR neutrophils:ab,ti OR monocyte:ab,ti OR monocytes:ab,ti OR macrophage:ab,ti OR macrophages:ab,ti OR cytokines:ab,ti OR cytokine:ab,ti OR chemokines:ab,ti OR chemokine:ab,ti OR interleukin:ab,ti OR interleukins:ab,ti OR integrin:ab,ti OR integrins:ab,ti OR tlr:ab,ti OR 'toll-like receptor'/exp OR 'toll-like receptor':ab,ti OR 'toll-like receptors':ab,ti OR 'tlrs':ab,ti OR 'inflammasome':ab,ti

Identify other sources for study identifications
None

Define search strategies for these sources
Not applicable

Study selection procedure
Define screening phases
1. Title/Abstract screening phase
2. Full-text screening phase

Specify number of observers per screening phase
2 observers per reference per phase: G.P.J. van Hout and S.J. Jansen of Lorkeers

Study selection criteria
Type of study design
Inclusion criteria: Controlled study / Cohort study
Exclusion criteria: Other study types including non-controlled studies and case reports.

Type of animal/population
Inclusion criteria: Pigs, dogs, sheep and goats subjected to MI as defined previously. Co-medication that potentially influences infarct size (e.g. platelet inhibitors/βblockade) is no exclusion criteria as long as the control group also receives the co-intervention.
Exclusion criteria: Transgenic animals. All other species of animals different from the ones previously mentioned or animals from the same species but different disease models. *In vitro* and *ex vivo* studies are also excluded.

Type of intervention e.g. doses, time, frequency
See the previously stated definition of the intervention. The timing, delivery route and frequency of the anti-inflammatory compounds are no exclusion criteria for this systematic review.

Outcome measures
Inclusion criteria, studies are included in the analysis if the reported at least one of the following outcome measures:
1. Primary outcome measurement: Myocardial infarct size (IS) measured as a percentage of the area at risk (IS/AAR)
2. Myocardial IS measured as a percentage of the total left ventricle (IS/LV)
3. Left ventricular ejection fraction (LVEF)
4. Myocardial scar thinning given in millimetres or as a ratio, divided by the opposite, non-infarcted wall

Exclusion criteria, studies are excluded in absence of the following outcome measures:
1. The absence of inclusion criteria 1 to 4: IS/AAR, IS/LV, LVEF or myocardial scar thinning.

Language restrictions
None.

Publication date restrictions
None.

Other
Inclusion criteria: Full text original papers only, meaning no congress abstracts.
Exclusion criteria: Congress abstracts.

Sort and prioritize your exclusion criteria per selection phase
Selection phase title/abstract:
1. No MI
2. No anti-inflammatory compound
3. No original data (e.g. review)
4. No large animals
5. Not *in vivo*

Selection phase Full Text
1. No full-text publication
2. No MI
3. No anti-inflammatory compound
4. No original data
5. No large animals
6. Not in vivo
7. No correct endpoints (EF, IS/AAR, IS/LV or scar thinning)
8. Lack of control group

Study characteristics to be extracted

Study ID
First author, corresponding author, journal, publication year, source of funding.

Study Design characteristics
Number of animals per groups and experimental groups.

Animal model characteristics
Species, gender, age, weight, location of infarct, method of induction of injury (e.g. ligation, balloon occlusion), model (I/R or permanent), anaesthetics, ventilation, duration of occlusion, surgical procedure (closed chest, lateral sternotomy, medial sternotomy), co-morbidity.

Intervention characteristics
Name compound, treatment group (e.g. NSAID, free radical scavenger, corticosteroids) dosage, time of delivery, number of administrations, bolus vs. continuous administration, duration of delivery, duration of follow-up, route of delivery, co-treatment,.

Outcome measures
Method of functional outcome assessment, method of infarct size determination.
Outcome data for any of the following:
1. Myocardial infarct size (IS) measured as a percentage of the area at risk (AAR)
2. Myocardial IS measured as a percentage of the total left ventricle (LV)
3. Left ventricular ejection fraction (LVEF)
4. Myocardial scar thinning given in millimetres or as a ratio, divided by the opposite, non-infarcted wall
5. Left ventricular end diastolic volume (LVEDV) and left ventricular end systolic volume (LVESV)
6. Mortality after therapy administration

Risk of bias assessment
Scoring for the risk of bias is performed based on the CAMARADES checklist.[21]
- Publication in a peer reviewed journal
- Reporting of random allocation
- Reporting of blinding of the operator
- Reporting of blinded assessment of outcome
- Use of comorbid animals
- Reporting of a sample size calculation
- Reporting of compliance with animal welfare regulations
- Reporting of a potential conflict of interest

Data collection

- Infarct size: IS/AAR in %
- Infarct size: IS/LV in %
- LVEF in %
- Scar thinning in millimetres and/or ratio
- LVESV and LVEDV in mL
- Mortality: number of animals that died after therapy administration

Methods of data extraction/retrieval

Primarily we extract data from the result section of the manuscript. When data is unavailable in text or tables, data will be extracted electronically from graphs. In absence of these data in graphs, authors will be contacted through e-mail. In case of no response after 2 weeks, we will exclude the study from the analysis.

Data-analysis and -synthesis

Data combination

Data will be combined in a systematic review with a forest plot followed by a meta-analysis.

Specify when data combination is appropriate

Based on our inclusion- and exclusion criteria, we expect the models and outcome measures to be uniform enough to pool data for a combined analysis for each separate outcome measurement. Anti-inflammatory compounds have been tested for several decades in large animal models of MI and we therefore expect to include over one hundred different studies. We chose 25 as a cut off for number of studies to be included in order to allow reliable determination of publication bias and meta-analysis.[22]

In the current systematic review, we also aim to determine whether study related parameters influence outcome (e.g. surgical approach, time of compound delivery and type of anti-inflammatory compound). In our opinion, direct comparison of these subgroups is feasible when groups contain at least 5 independently conducted studies. Meta-regression will be used to explore heterogeneity between included studies. Significant predictors will be further investigated by supgroup analysis. If sufficient studies are included, within subgroup analyses will also be performed for different treatment groups of anti-inflammatory compounds (*i.e.* NSAIDS, free radical scavengers) to determine if study related parameters influence outcome in these particular groups of anti-inflammatory compounds. The number of parameters tested by meta-regression is based on the number of included studies per outcome measure and will be 1 parameter for every 10 studies. For the primary outcome measurement (infarct size) no correction will be applied ($p<0.05$ will be regarded as significant). For all the secondary outcome measures, a bonferroni correction will be applied, based on the number of parameters tested.

If meta-analysis seems feasible

Specify the effect measures to be used

We expect the reporting of the selected outcome measurements to be very uniform since our primary outcome measures (IS/AAR, IS/LV, LVEF, scar thinning) are reported on a relative

scale (ratio/percentage) in the vast majority of cases. For this reason it is assumable we will be able to use the raw difference in means for these parameters. For each study group we will extract the reported mean combined with either the standard deviation or standard error of the mean, depending on the parameter provided by the different studies.

Effect measures used for each individual parameter:
- Mortality – Odds ratio
- IS/AAR – Raw difference in means
- IS/LV – Raw difference in means
- LVEF – Raw difference in means
- Scar thinning – Raw difference in means or Standardized difference in means, depending on the uniformity of the data
- LVEDV and LVESV – Standardized difference in means

Specify which study characteristics will be analysed as possible sources of heterogeneity
1. Treatment group (NSAIDs, corticosteroids, immunosuppressives, free radical scavengers, complement inhibition, cytokine/chemokine inhibitors, integrin/selectin/cell surface inhibitors, others)
2. Ischemia-reperfusion/permanent occlusion
3. Occlusion duration
4. Timing of therapy
5. Timing of assessment
6. Year of publication
7. Animal species
8. Surgical procedure (sternotomy vs. no sternotomy)
9. Location of injury (which coronary artery was occluded)
10. Gender
11. Randomization
12. Blinding
13. Methods of infarct/function measurement
14. English/non-English articles

Specify subgroups and comparisons of interests
Subgroup analysis will be performed for significant predictors of outcome, based on meta-regression. No sensitivity-analysis will be performed.

Method of analysis
Because of anticipated heterogeneity between included studies, a random effects model will be appropriate. We will also determine the extent of heterogeneity in our dataset by assessing the Tau^2 and I^2 statistic.

Method for assessing risk of publication bias
We will assess the risk of publication bias through the construction of a Funnel Plot and a subsequent Egger's regression for testing symmetry in funnel plot and detecting small study bias. Duval and Tweedie's 'trim and fill analysis' will be performed to identify missing studies.

OTHER

Possible limitations

1. Inclusion of studies is based on LVEF, IS and scar thinning which means that studies that do not report this will be excluded, enabling possible selection bias.
2. Our definition of 'anti-inflammatory treatment' is based on working mechanism and clinical usage of certain compounds, requiring arbitrary choices. In our study, possible anti-inflammatory therapies with unknown working mechanisms or major pleiotropic effects are excluded. On the other hand, compounds that will be included in the final analysis could, to some extent, also have pleiotropic effects possibly clouding the sole effect of inhibiting the inflammatory response post MI.
3. In the current study design we have not taken into account the commercialization potential of certain drug types. While clinical testing of certain compounds may seem to be very promising based on this systematic review, it should be noted that we have not taken the economic position (e.g. patent, costs) into consideration, which is also essential for eventual drug development

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CHAPTER 4

ANTI-INFLAMMATORY COMPOUNDS REDUCE INFARCT SIZE AFTER MYOCARDIAL INFARCTION: A META-ANALYSIS OF LARGE-ANIMAL MODELS

Submitted

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ABSTRACT

Background

The development of novel therapies is required to confine cardiac damage and prevent heart failure (HF) in patients after myocardial infarction (MI). For this purpose, numerous anti-inflammatory drugs have been tested in experimental large animal studies. However, translation of these therapeutic strategies into clinical practice has proven to be difficult. To identify promising drug groups and better understand which factors underlie the failure of transition towards the clinic, we performed a meta-analysis of large animal studies on the cardioprotective effects of anti-inflammatory drugs post-MI.

Methods and Results

Meta-analysis of the 183 included studies revealed a significantly reduced infarct size (IS) as a ratio of the area at risk (12.7%; 95%CI 11.1%–14.4%, $p<0.001$) and also a reduced IS as a ratio of the left ventricle (3.9%;95%CI 3.1%–4.7%, $p<0.001$). No difference in mortality was observed. Effect size depended on the type of drug used ($p<0.001$), was higher when outcome was assessed early after MI ($p=0.013$) and where studies included only male animals ($p<0.001$). Mortality in treated animals was higher in studies that blinded the investigator during the experiment ($p=0.041$).

Conclusions Treatment with anti-inflammatory drugs leads to smaller infarct size in large animal MI models. Effect size depends on the type of drug used, which enables identification of promising compounds for future clinical testing. Timing of outcome assessment, sex and study quality are significantly associated with outcome and may explain part of the translational failure in clinical settings.

INTRODUCTION

Myocardial infarction (MI) and its consequences remain one of the greatest burdens of disease worldwide.[1,2] Optimized medical care has resulted in reduced acute mortality but patients surviving MI often develop diminished cardiac function and heart failure (HF).[3] The progression from MI to HF occurs through adverse remodeling, a complex mechanism involving infarct expansion, myocardial scar thinning and left ventricular geometrical adaptation.[4] Accumulating evidence indicates that adverse remodeling arises from an exaggerated inflammatory response that is initiated during ischemia and early reperfusion.[5,6] The damaged myocardium attracts inflammatory cells towards the site of injury where these cells secrete cytokines, proteases and oxygen free radicals. This results in degradation of the extracellular matrix and clearing of necrotic cardiac resident cells, enabling scar formation in a later phase of cardiac wound healing.[7] This inflammatory effect is essential to stabilize the scar tissue.[8] However, it is outweighed by the acute effects of inflammatory cells on infarct expansion and cardiac function worsening early after ischemia in the reperfusion phase.[9–11]

Indeed, multiple clinical studies have shown that elevated numbers of circulating neutrophils, monocytes and cytokines in the acute setting are associated with adverse remodeling, the development of heart failure and a worse overall prognosis.[12–17] Attenuation of this inflammatory response is therefore a promising strategy to limit infarct size and preserve cardiac function post-MI.

Development of such anti-inflammatory strategies and transition from bench to bedside, requires their testing in clinically relevant animal models.[18] Both temporal and spatial development of tissue damage post-MI, along with inflammation-related signaling pathways, differ in small animals compared to larger mammals, including humans.[19–21] Large animal models more closely resemble human anatomy, hemo- and pharmacodynamics and enable clinical treatment regimens, delivery routes and functional read-outs.[22–24]

Since the early 1970s, many anti-inflammatory compounds have been shown to have efficacy in reducing reperfusion damage and post-MI remodeling in large animal models.[25] However, none have proved successful in clinical trials and entered routine clinical practice.[26,27] The reasons for this lack of success – or which class of anti-inflammatory drug has greatest efficacy in large animal MI models –are not clear.

The interpretation of findings from individual studies to give an overview of the utility of this therapeutic approach is challenging because these studies use diverse experimental set-ups, animal species, timings of therapy and induction of MI. We therefore performed a systematic review and meta-analysis of large animal studies testing anti-inflammatory compounds after MI. In addition to providing an unbiased research summary, our purpose was to identify promising drug groups for future clinical testing. Moreover, we considered that meta-regression may enable us to better understand sources of heterogeneity, to identify the influence of study design factors which may be important for translational success.[28]

Methods

The protocol for this systematic review and meta-analysis has been published.[29] We searched PubMed and Embase on May 1st 2014 for studies using anti-inflammatory compounds in large animal models of MI. Our inclusion criteria were: controlled study

design, and the use of large animal models (defined as either pigs, dogs, sheep, goats) of myocardial infarction (coronary obstruction >30 minutes). Anti-inflammatory compounds were defined as either having FDA approval for anti-inflammatory effects or as directly targeting pivotal inflammatory mechanisms.[29] Abstract publications were excluded, as were case reports and studies lacking a control group. There were no language restrictions. Studies were screened in two phases (title/abstract followed by full text) by two independent researchers (GvH and SJ). In case of disagreement, consensus was achieved by discussion in all cases.

The primary outcome for meta-analysis was infarct size as percentage of the area at risk (IS/AAR). Secondary outcomes were infarct size as percentage of the left ventricle (IS/LV), mortality, left ventricular ejection fraction (EF), left ventricular end diastolic volume (LVEDV), left ventricular end systolic volume (LVESV) and wall thickness as ratio of the opposing wall (WT). Data were extracted from included studies and added to the 'Collaborative Approach to Meta-Analysis and Review of Animal Data from Experimental Studies' (CAMARADES) online international database.

Statistics

Because we included a variety of studies using different compounds in different animal models, any overall estimate of efficacy is of limited interest. Due to this anticipated heterogeneity, we performed a random effect meta-analysis. We used a raw difference in mean (RMD) analysis for the outcome parameters IS/AAR, IS/LV, EF and WT. For mortality, we used odds ratios.[30] Where individual studies reported outcome for several treatment groups (*i.e.* dose response studies), the size of the control group was adjusted to reflect this, as previously described.[28,30]

To determine whether the observed results were confounded by publication bias, we used three approaches: visual inspection of funnel plots, Egger's regression analysis for small study effects and Tweedie and Duval's trim and fill.[31,32] Funnel plotting can be used to visually identify studies with small precision that overstate the effect of an intervention and are consistent with the presence of small study publication bias. Egger's regression statistically assesses the presence of publication bias of small studies in a funnel plot by determining whether the regression line and its 95% CIs for precision versus standardized effect size intersect at the origin of the graph. Trim and fill analysis non-parametrically attempts to correct for funnel plot asymmetry by identifying and imputing theoretical missing studies. This enables recalculation of the overall treatment effect in the absence of publication bias.[30–32]

We explored heterogeneity using meta-regression and tested which parameters were significantly associated with outcome. We planned to include 15 parameters as potential sources of heterogeneity that are relevant in either a clinical or translational perspective, provided that we had at least 10 times that number of experimental comparisons.[30] We tested parameters in 3 separate categories: therapeutic characteristics (e.g. timing of therapy, drug group), model characteristics (e.g. species, sex, surgical approach) and risk of bias (e.g. reporting of blinding and randomization). Compounds were pooled in drug groups, based on working mechanism (Supplemental Table 1). We only performed meta-regression for parameter values reported in at least 5 independent groups for comparison.

Meta-regression is more conservative than stratification of heterogeneity. We set the statistical threshold for our predefined primary outcome measure at $p<0.05$, and for secondary outcome measures we applied a Bonferroni correction to give a critical value of p of 0.017. All analyses were performed using Stata version 11 (StataCorp LP, Texas, USA).

RESULTS

We found 2,530 results in PubMed and 2,778 in Embase. After merging and removal of duplicates, 4,105 unique publications remained. We excluded 3,570 studies in the first phase (title/abstract) of screening. In the next phase (full text screening), we included 183 publications in the database (Supplemental Figure 1). These 183 publications reported 219 experimental comparisons for the primary outcome IS/AAR, reporting outcome from 3331 animals (1,839 treated and 1,492 control). Included studies predominantly used occlusion of the left anterior descending coronary artery (140 experiments) rather than the circumflex coronary artery (75 experiments); 4 used both. Most experiments used both male and female animals (both n=101, male n=60, female n=13, unknown n=45) and used dogs (dog n=163, pig n=54, sheep n=2). Random allocation of animals, blinded outcome assessment and blinding of the operator was reported in 143 (65%), 64 (29%) and 21 (10%) of the 219 comparisons respectively. For the secondary outcomes, 88 studies reported IS/LV (138 experiments) and 97 reported mortality (143 experiments). LVEF was reported in 21 studies (31 experiments) and the analysis for WT included 11 studies (16 comparisons).

Primary outcome

For the primary outcome, treatment led to an absolute reduction in the infarct size as a percentage of the area at risk of 12.7% (95% CI 11.1% – 14.4%, $p<0.001$) (Figure 1A). There was substantial heterogeneity between studies (I^2 80.9%, Tau^2 104.5). Visual inspection of the funnel plot and Egger regression suggests a small study bias (Figure 1C and D). This was confirmed by Tweedie and Duval's trim and fill, with 12 imputed missing studies and a corrected efficacy of 11.4% (95%CI 9.6% – 13.1%) (Figure 1C).

Secondary outcomes

Treatment with anti-inflammatory compounds was also associated with improved secondary outcome measurements. There was an absolute difference in mean IS/LV of 3.9% (95%CI 3.1% - 4.7%, $p<0.0001$) in favor of treated animals (Figure 1B). EF was higher in treated animals compared to control animals (RMD 3.4, 95% CI 0.8 – 6.1, $p<0.001$). No difference in mortality was observed (OR 0.96, 95% CI 0.79 –1.17, $p= 1.0$). Wall thickness as a percentage of the opposing wall was lower in treated animals, but of note, this outcome was predominantly assessed in studies testing the effect of non-steroid anti-inflammatory drugs (NSAIDs) (11 out of 16) (WT RMD 16.3% 95%CI 8.5% – 24.0%, $p<0.001$) (Supplemental Figure 2). Only a small subset of studies reported LVEDV and LVESV and the extent of the dataset was too small to enable meta-analysis.

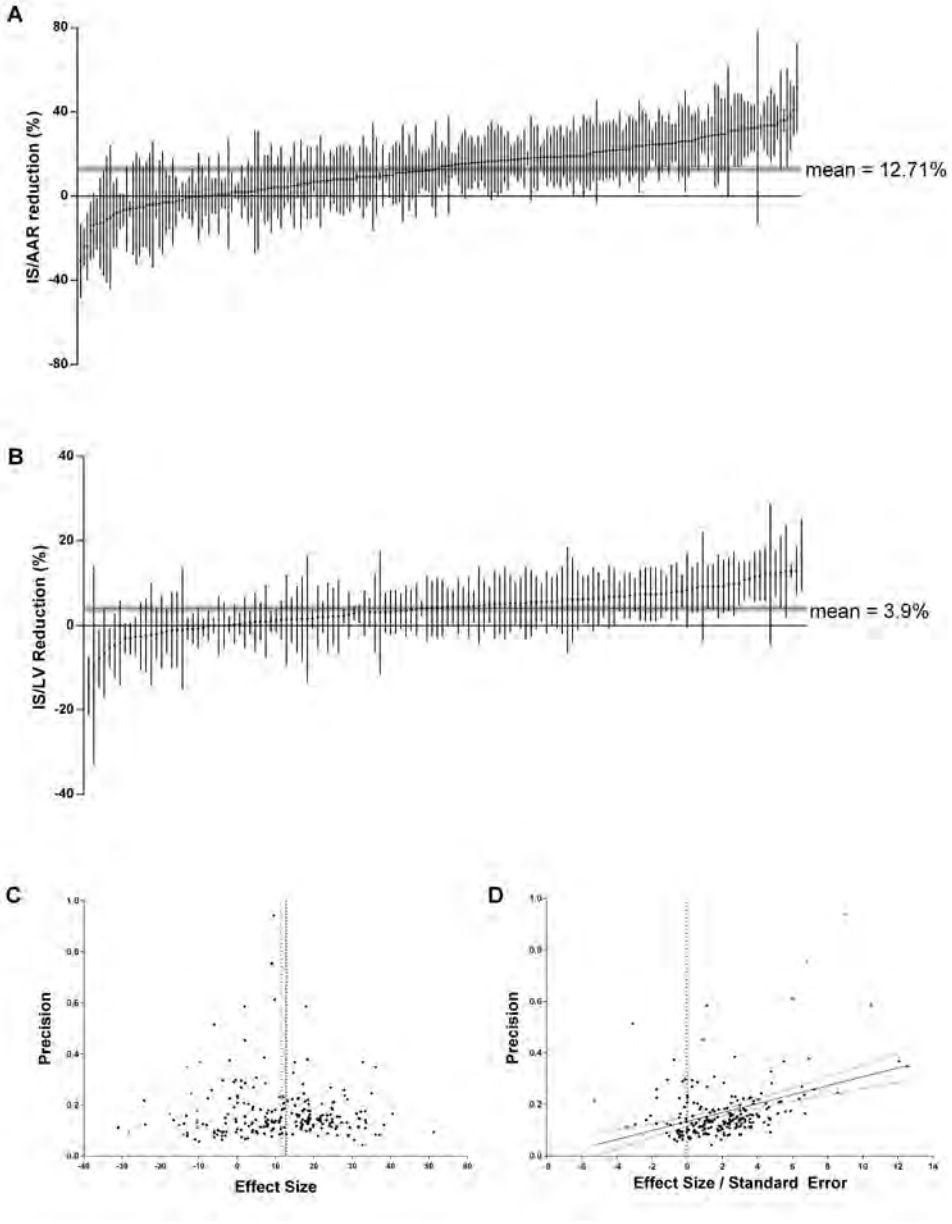

Figure 1. Timberplots and publication bias
Timberplot of the effect of anti-inflammatory compounds on IS/AAR (A) and IS/LV (B). Dots and vertical lines represent the mean and 95% CI of individual studies. Horizontal gray bar represents the 95%CI of the mean effect size. C. Funnel plot of includes studies for the primary outcome IS/AAR. Red dots represent theoretically missing studies, identified by Tweedie and Duval's trim and fill. Vertical dotted line represents the mean effect size with (red) or without (black) correction. D. Egger's regression, showing the regression line and the 95% CI. IS/AAR=infarct size as percentage of area at risk, IS/LV=infarct size as percentage of the left ventricle.

Meta-regression

We sought possible sources of heterogeneity using meta-regressions with effect on IS/AAR, IS/LV and mortality as the dependent variable. The number of comparisons reporting EF and WT were insufficient for meta-regression. Drug class was a predictor of efficacy for IS/AAR and IS/LV endpoints ($p<0.001$, $p=0.002$) but not for mortality, although the point estimates for different drug classes were similar (Figure 2).

For all 3 outcome measures, leukotriene inhibitors and cell adhesion molecule (CAM) inhibitors performed best, showing greater reduction in infarct size and lower mortality. The effect of anti-inflammatory compounds in improving IS/AAR and IS/LV was less apparent at later times of outcome assessment, both when measured in a categorized fashion ($p=0.013$ IS/AAR, $p=0.001$ IS/LV) (Figure 3A and 3B) and on a continuous scale ($p=0.016$ IS/AAR, $p=0.007$ IS/LV). Furthermore, the sex of the animals used appeared to be important; for both IS/AAR and IS/LV, greater effects were seen in experiments using male animals than in those with female animals or a mixed population (IS/AAR $p=0.002$, IS/LV $p=0.002$) (Figure 4). Additionally, the effect on IS/LV was larger in temporary occlusion models compared to permanent occlusion models ($p=0.014$) (Figure 5A).

Measures taken to preserve the internal validity of studies influenced reported outcomes. When outcome assessment was performed in a blinded fashion there was no significant difference in efficacy for IS/AAR ($p=0.075$).

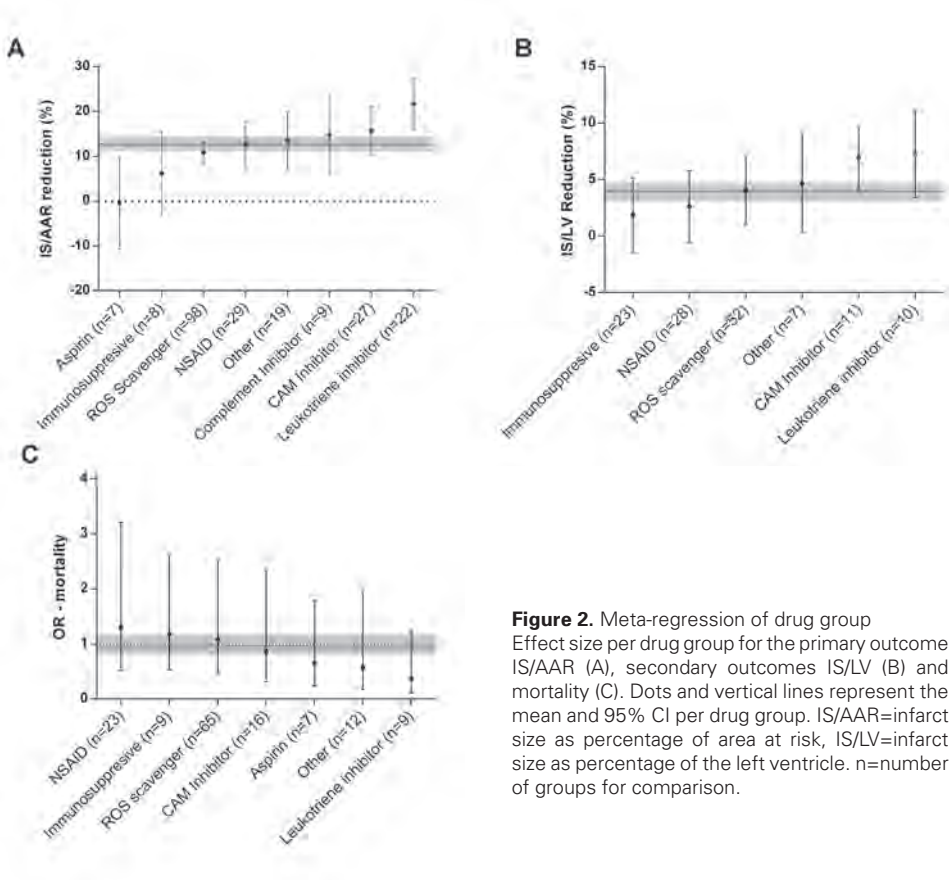

Figure 2. Meta-regression of drug group
Effect size per drug group for the primary outcome IS/AAR (A), secondary outcomes IS/LV (B) and mortality (C). Dots and vertical lines represent the mean and 95% CI per drug group. IS/AAR=infarct size as percentage of area at risk, IS/LV=infarct size as percentage of the left ventricle. n=number of groups for comparison.

However, blinding of the operator performing the intervention was associated with increased mortality in the treatment group (p=0.041) (Figure 5B and C).
To assess if sources of heterogeneity could also be identified in specific drug groups, we performed meta-regression for sex and time of assessment for the primary outcome IS/AAR for each of the 4 largest drug groups (NSAIDs (n=29), CAM inhibitors (n=27), Leukotriene inhibitors (n=22) and reactive oxygen species (ROS) scavengers (n=98) (see Supplemental Table 1 for classification). For time of assessment, results were similar to those found for the complete dataset was observed, but only time of assessment had sufficient power in the subgroup of ROS scavengers to reach statistical significance (p=0.015) (Supplemental Figure 3).

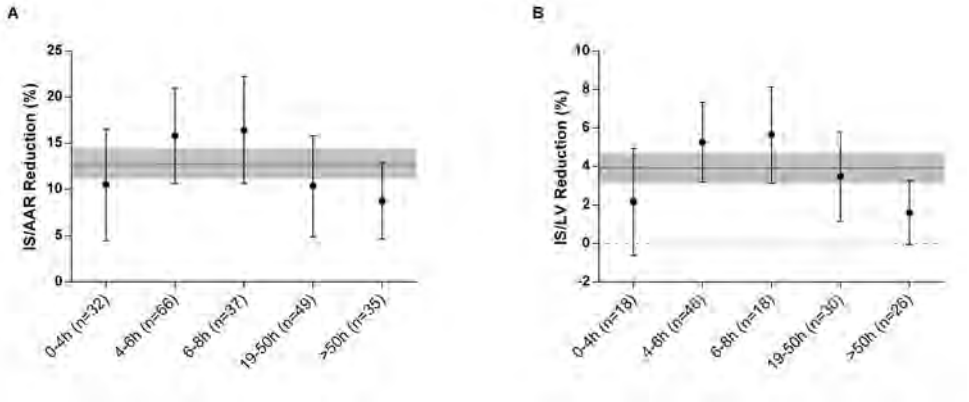

Figure 3. Meta-regression of Meta-regression of timing of outcome assessment
Effect size per timing interval after occlusion of the coronary artery, for the primary outcome IS/AAR (A) and secondary outcome IS/LV (B). IS/AAR=infarct size as percentage of area at risk, IS/LV=infarct size as percentage of the left ventricle. n=number of groups for comparison.

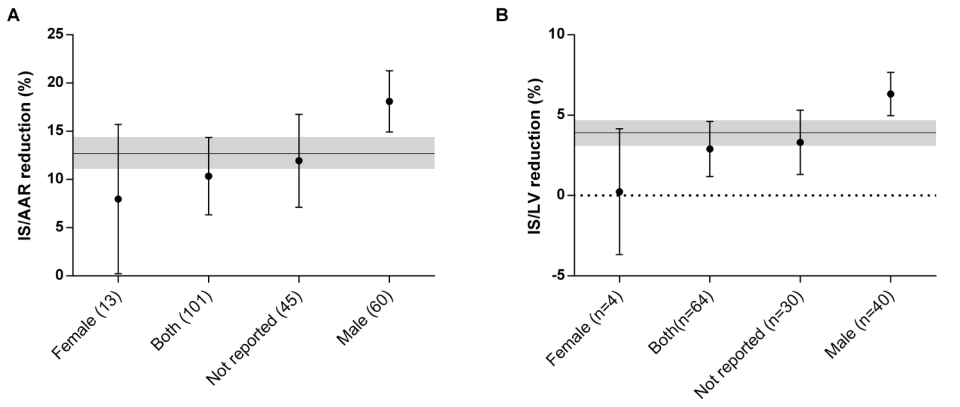

Figure 4. Meta-regression of sex
Effect size per sex for the primary outcome IS/AAR (A) and secondary outcome IS/LV (B).
IS/AAR=infarct size a percentage of area at risk, IS/LV=infarct size as percentage of the left ventricle. n=number of groups for comparison.

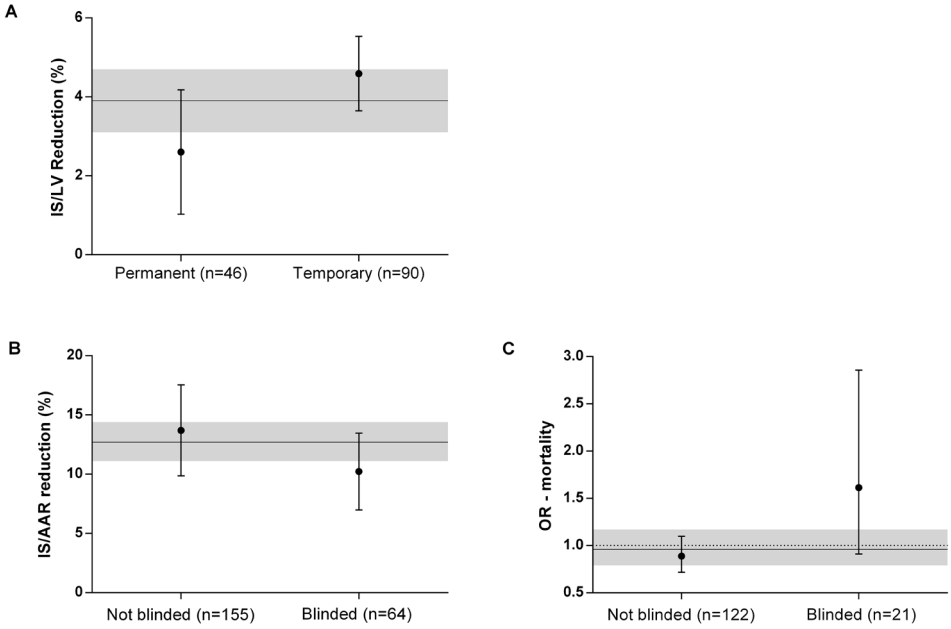

Figure 5. Meta-regression of infarction model and blinded outcome assessment
Meta-regression of model of infarct creation (permanent versus temporary occlusion of the coronary artery) on the secondary outcome IS/LV (A). Meta-regression of the effect of blinded versus non-blinded outcome assessment on the primary outcome IS/AAR (B) and secondary outcome mortality (C). IS/AAR = infarct size as percentage of area at risk, IS/LV = infarct size as percentage of left ventricle. N = number of groups for comparison.

DISCUSSION

Efficacy of anti-inflammatory compounds

Both clinical and preclinical studies suggest that the post-MI inflammatory response orchestrates cardiac wound healing and is accountable for infarct expansion.[17,33] Our systematic review and meta-analysis of data from more than 180 large animal studies, including over 3,300 animals subjected to myocardial infarction, confirms that treatment with anti-inflammatory compounds reduces infarct size, thereby decreasing the risk of adverse remodeling.[4]

Since inflammation is a complex multifactorial process, the pharmacological interventions used in different large animal studies are very heterogeneous. Our meta-regression suggests that inhibition of different inflammatory pathways has different effects on infarct size. For the first time, our analysis allows an assessment of the relative efficacy of these approaches in large animal MI models. Our findings suggest that treatment with for instance leukotriene inhibitors is more effective than NSAIDS or immunosuppressive drugs, and may therefore have greater prospects for clinical use. To the best of our knowledge, no clinical trials with compounds from the first group have been commenced in the setting of MI and therefore may be promising candidates to make their way into clinical application.

Clinical translation and preclinical study design

Although comparison of the effect of different drug groups may be useful to select agents for future clinical testing, none of the compounds evaluated in the included experimental studies, that have also been tested in clinical trials, have been implemented in daily clinical practice.[34] This may imply critical discrepancies between the field of large animal studies of MI and clinical trials, leading to a presumable overestimation of the effect in large animal models.

A possible explanation for this overestimation is publication bias. Previous reports have shown that the effect of compounds in animal research may be overestimated due to underreporting of small, neutral studies.[35,36] Our analysis also presents evidence of small study bias, and after correction for this bias, the overall effect of anti-inflammatory compounds is estimated to be 10% lower than the observed effect.

Next, both internal and external validity of pre-clinical studies are important factors in translational medicine. Lack of blinding of both the operator and outcome assessment pose threats to internal validity.[37,38] Less than a third of the included studies in our systematic review reported blinding of the induction of ischemia or outcome assessment. Moreover, we observed an effect of investigator blinding; while there were no significant effects for infarct size reduction the best estimate of efficacy was lowest in high quality studies; and in studies reporting mortality, treatment was associated with significantly higher mortality in high quality studies. This underlines the importance of high methodological quality to ensure internal validity.

In the perspective of external validity in large animal research, studies should resemble the clinical situation as closely as possible with regard to study design to allow for extrapolation of study outcome to the clinical situation.[38,39] Some aspects are inherent to preclinical studies and cannot be easily altered (e.g. comorbidity, etiology of MI, anesthesia) and are thus an overall limitation of large animal MI models. However, other aspects (e.g. timing of assessment and timing of therapy) can resemble the clinical situation. Our study shows that these aspects influence outcome, since infarct size reduction in the included large animal studies is highest during the first hours post-MI and this effect is attenuated when the time of assessment is extended. Any effect limited to the first hours post-MI only, is of no clinical relevance and fails to be detected in clinical trials given their long term follow-up.

Similarly, we observed sex to be a significant predictor of outcome. Since most studies used both male and female animals, translational failure is not necessarily attributable to the difference in effect size. However, the more pronounced effect in studies using male animals only, emphasizes the importance of external validity. Although the exact mechanism behind this phenomenon remains unidentified, differences between male and female subjects regarding outcome after MI have been observed in clinical and preclinical studies. (30–33) To the best of our knowledge, this is the first study showing that attenuating the inflammatory response in male subjects is more efficacious compared to inhibiting this response in female subjects, a phenomenon that should be taken into account when designing large animal MI studies.

Meta-analysis of translational studies

Although our analysis shows significant effects of anti-inflammatory treatments on infarct size, it should be kept in mind that meta-analyses cannot reveal causal relationships. Hence, we do not intend to provide mechanistic insights with our study. Furthermore, the strength of a meta-analysis depends on the strength and quality of included studies. For example, reporting of several functional outcomes in pre-clinical models varies between studies, disallowing pooling of these data. Also, reporting of mortality is considered to be less rigorous compared to clinical studies, which may introduce bias into the dataset.

In contrast to meta-analyses of clinical studies, heterogeneity in pre-clinical studies can be of great additional value, since parameters responsible for heterogeneity can be used as predictors for success and can help design future clinical and pre-clinical studies. The extent of our dataset was insufficient for a multivariate meta-regression analysis, therefore we cannot completely rule out co-linearity of the different sources of heterogeneity (Supplemental Table 2).

Conclusion

Our systematic review and meta-analysis of large animal MI studies show reduced infarct size in animals treated with anti-inflammatory compounds compared to control animals. This study reveals differences between the inhibitory effect of different inflammatory pathways and leukotriene inhibitors seem to be the most promising candidate to make their way to clinical application. Moreover, we stress the use of clinically relevant animal models and study designs to increase the translational value of pre-clinical research.

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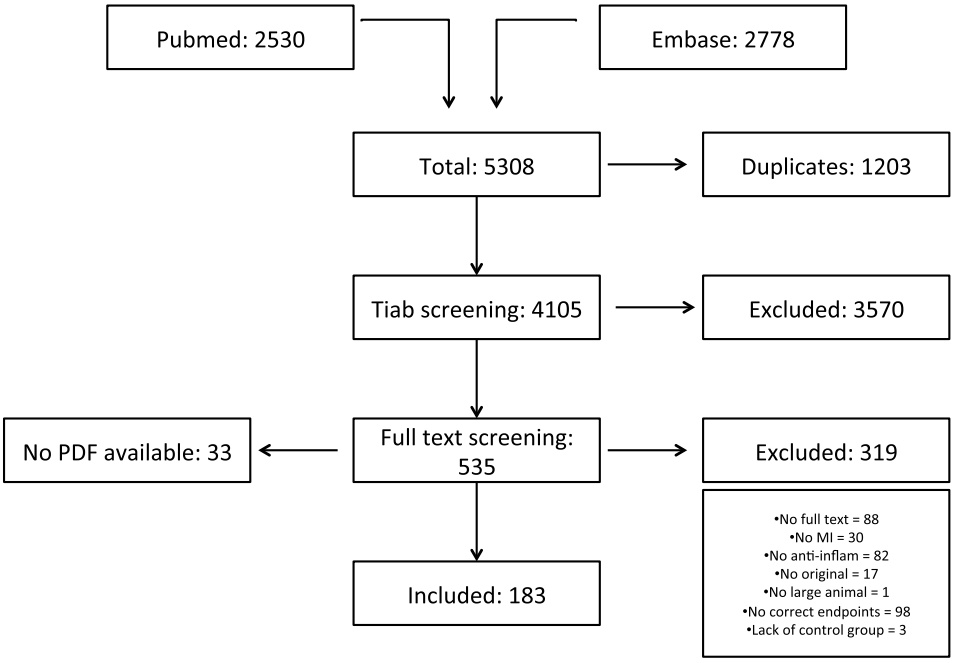
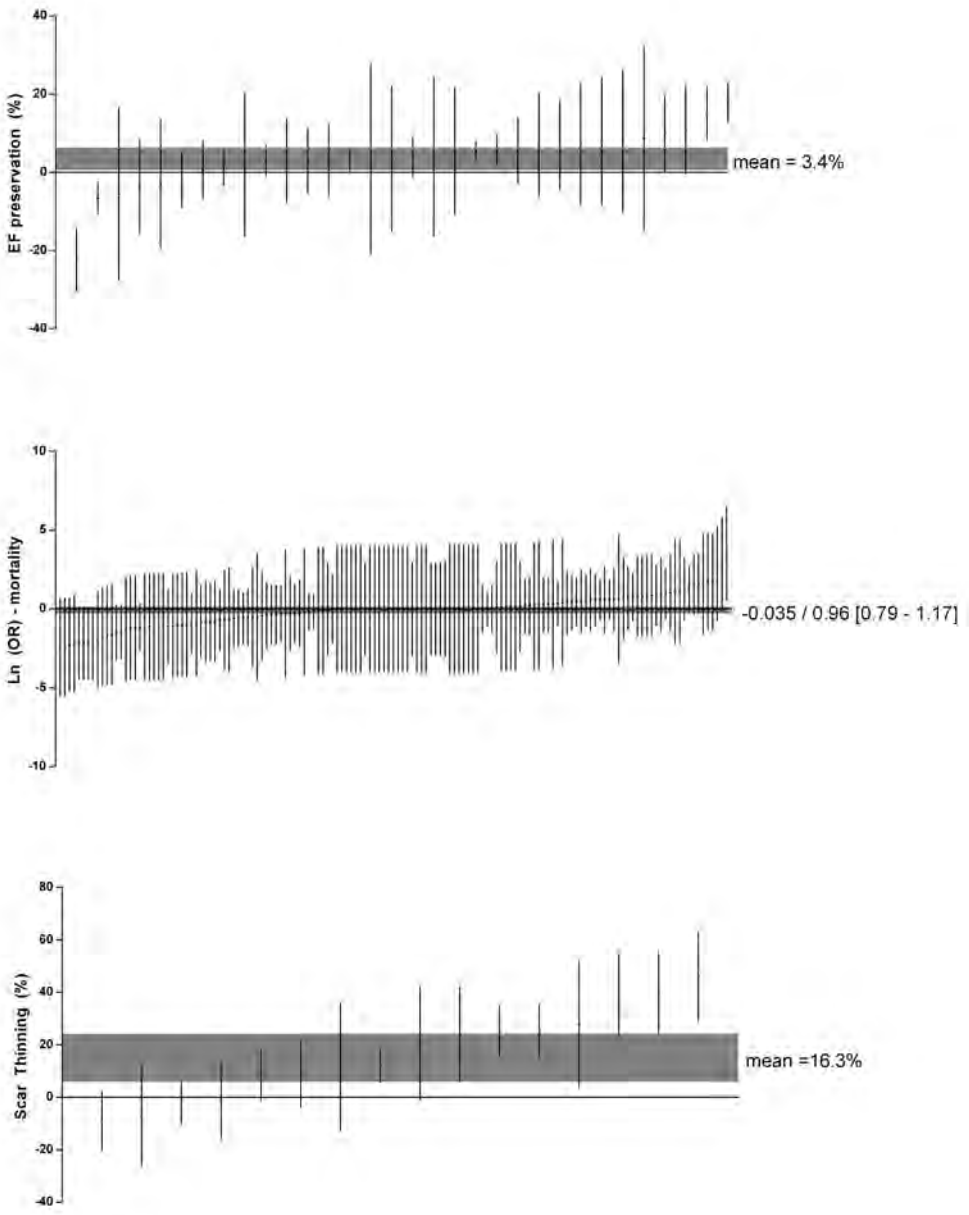
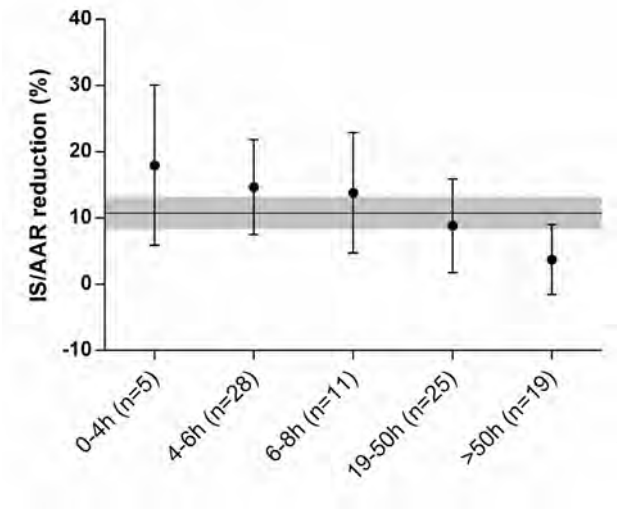
SUPPLEMENTARY MATERIAL



Supplemental Figure 1. Flow chart of inclusion/exclusion



Supplemental Figure 2. Timberplot of the effect of anti-inflammatory compounds
(A) Ejection fraction, (B) mortality , (C) scar thinning. Vertical lines representing 95% CI of individual studies. Horizontal line and gray bar represents the mean and 95%CI of the mean effect size. EF = Left ventricular ejection fraction.



Supplemental Figure 3.
Meta regression of timing of outcome assessment within the ROS scavengers. Dots and vertical bars represent the mean effect size and 95% CI per timing interval on IS/AAR. Horizontal line and gray bar represent the overall mean and 95% CI. IS/AAR = infarct size as a percentage of the area at risk. N = number of groups for comparison.

Supplemental Table 1. Classification of drugs

ROS Scavenger	CAM inhibitor	other	Leukotriene inhibitor	Complement inhibitor	NSAID	Immunosuppressive	Aspirin
2-octadecylascorbic acid	Anti-CD18	Acetaminophen	AA-861	Anti-C5a MAb	Azapropazone	Cyclosporin A	Aspirin
Allopurinol	Anti-ICAM-1 MAb	Adiponectin	BW755C	C1-INH	Celecoxib	Dexamethasone	
Allopurinol + SOD	Anti-Mo1 (clone 904)	Chemically Modified Tetracycline-3	CI-922	C5a receptor antagonist ADC-1004	Flurbiprofen	Etanercept	
alpha-Tocopherol	Anti-P-selectin Ab + Anti-ICAM-1 MAb	Chymase INH (TY51469)	Ebselen	Mirococept	Ibuprofen	Methylprednisolone	
Ascorbic Acid	Anti-P-selectin Antibody	Colchicine	Glutathione	N-Acetylheparin	Indomethacin		
Ascorbic Acid + Desferrioxamine	Bb15-42	L-659,989	LY233569	Tyrosine sulfate	Naproxen		
Ascorbic Acid + Desferrioxamine + N-Acetylcysteine	CI-959	o-Desulfated Heparin	LY255283				
Bucillamine	CY 1503	OPN-305	Nafazatrom				
Catalase	MAb to ICAM-1	Poloxamer 188	ONO-1078				
Deferoxamine	MHM.23	Rolipram	REV-5901				
Desferrioxamine	Neutrophil Antibody	RP 59227 (Tulopafant)					
Dimethylsulfoxide	NPC 15669	TG100-115					
Dimethylthiourea	PLM-2						
H290/51	R15.7						
Hydrogen Sulfide	rPSGL-Ig						
Methionine	Sialyl Lewis x-OS						
N-2-Mercaptopropionyl Glycine							
N-Acetylcysteine							
N-tert-butyl-alpha-phenylnitrone							
Oxypurinol							
PEG-SOD + Catalase							
Polyethylene glycol-conjugated SOD							
SC-52608							
SOD							
SOD + Catalase							
Trolox							
Trolox + Ascorbic Acid							
U7006F							
Vitamin E							
Vitamin E + Vitamin C							

Supplemental Table 2. Crosstabs of the number of groups for comparison for the 3 significant predictors on outcome (Sex, drug group and timing of outcome assessment)

A.		**Sex**			
		Both (n=101)	Female (n=13)	Male (n=60)	not reported (n=45)
Druggroup	Aspirin (n=7)	6	0	0	1
	Complement Inhibitor (n=9)	6	0	1	2
	ROS Scavenger (n=98)	39	3	29	27
	Immunosuppressive (n=8)	1	4	2	1
	CAM Inhibitor (n=27)	17	2	5	3
	Leukotriene inhibitor (n=22)	7	0	14	1
	NSAID (n=29)	17	0	4	8
	Other (n=19)	8	4	5	2

B.		**Timing of assessment**				
		0-4h (n=32)	4-6h (n=66)	6-8h (n=37)	19-50h (n=49)	>50h (n=35)
Druggroup	Aspirin (n=7)	3	0	3	1	0
	Complement Inhibitor (n=9)	7	1	1	0	0
	ROS Scavenger (n=98)	5	30	16	28	19
	Immunosuppressive (n=8)	3	5	0	0	0
	CAM Inhibitor (n=27)	6	11	2	5	3
	Leukotriene inhibitor (n=22)	2	6	10	4	0
	NSAID (n=29)	2	9	1	6	11
	Other (n=19)	4	4	4	5	2

C.		**Timing of assessment**				
		0-4h (n=32)	4-6h (n=66)	6-8h (n=37)	19-50h (n=49)	>50h (n=35)
Sex	Both (n=101)	16	38	11	18	18
	Female (n=13)	4	1	0	8	0
	Male (n=60)	3	14	23	14	6
	Not reported (n=45)	9	13	3	9	11

CHAPTER 5

HUMAN RELEVANCE OF PRE-CLINICAL STUDIES IN STEM CELL THERAPY; SYSTEMATIC REVIEW AND META-ANALYSIS OF LARGE ANIMAL MODELS OF ISCHEMIC HEART DISEASE

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ABSTRACT

Aims

Stem cell therapy is a treatment strategy for ischemic heart disease in patients. Meta-analysis of randomized human trials showed <5% improvement in left ventricular ejection fraction (LVEF). Meta-analysis of available pre-clinical data of ischemic heart disease could provide important clues to design human clinical trials.

Methods and results

Random-effects meta-analysis was performed on pig, dog or sheep studies investigating the effect of cardiac stem cell therapy in ischemic cardiomyopathy (52 studies; N=888 animals). Endpoints were LVEF and death. Ischemia/reperfusion infarction was performed in 23 studies and chronic occlusion in 29 studies. Pooled analysis showed a LVEF difference of 7.5% at follow-up after cell therapy vs. control (95% confidence interval (CI), 6.2% to 8.9%; P<0.001). By exploratory multivariable meta-regression significant predictors of LVEF improvement were: cell type (bone marrow mononuclear cells (BM-MNC) showed less effect than other cell types, e.g. mesenchymal stem cells; P=0.040) and type of infarction (left anterior descending artery 8.0% vs. left circumflex artery 5.8%; P=0.045). Cell therapy was not associated with increased mortality (P=0.68). Sensitivity analysis showed trends towards more improvement with higher cell number ($\geq 10^7$), chronic occlusion models and late injections (>1 week). After follow-up of 8 weeks the effect of cell therapy decreased to 6%.

Conclusion

This meta-analysis showed that large animal models are valid to predict outcome of clinical trials. Our results showed that cell therapy is safe and led to a preserved LVEF. Future trials should focus on cell types other than BM-MNC, large infarction and strategies to obtain sustained effects overtime.

INTRODUCTION

Coronary heart disease is a major public and economic health problem leading to more than 7 million deaths worldwide each year.[1, 2] Myocardial infarction (MI) is characterized by loss of cardiomyocytes, scar formation, ventricular remodeling and it can develop into end-stage heart failure. Optimal pharmacologic treatment and coronary reperfusion therapy have led to improved survival of patients with coronary artery disease, even if current medical therapies cannot replace dysfunctional cardiomyocytes. Cell therapy has emerged as a potential therapeutic strategy. The ultimate goals of cell therapy are myocardial regeneration and revascularization, thus, re-establishing synchronous contractility and bioelectrical conductivity to achieve overall clinical improvement of cardiac function without severe adverse effects.

Many large animal studies in acute MI and ischemic cardiomyopathy have been performed, mostly with heterogeneous design and conflicting outcomes. These pre-clinical results led to the initiation of clinical trials and showed at best marginal results.[3] Nevertheless, pre-clinical studies are mandatory to assess risk of a new therapy and predict safety, feasibility and efficacy. Moreover, they address unresolved issues regarding clinical cell therapy (*i.e.* choice of cell type, cell number, method of delivery, time of delivery and follow-up after cell transplantation), which questions have been outlined by the task force of the European Society of Cardiology on stem cell repair of the heart.[4] Therefore, large animal models are valid and relevant for clinical practice, and have important clues regarding these yet unanswered questions. Similar to the human cardiac stem cell therapies, large number of animal studies have been performed including relative small number of animals. We hypothesize, that meta-analysis of these pre-clinical data might be helpful to design future clinical studies similarly to the meta-analysis of human cardiac stem cell trials.

We performed a systematic overview of the pertinent literature including a quantitative meta-analytical pooling of the data to assess the effects of stem cell transplantation in large animals with acute or chronic ischemic cardiomyopathy. A pre-specified sub-analysis is performed to focus on aforementioned unresolved issues.

METHODS

Eligibility criteria

Acute MI or chronic ischemic cardiomyopathy models in large animals were screened. Randomized controlled (RCT) and cohort trials investigating the effect of stem cell therapy on cardiac function as determined by left ventricular ejection fraction (LVEF) were analyzed. In addition, a placebo or sham operated control group had to be included in the study. Trials that only investigated transfected or genetically engineered stem cells altering cell behavior, or studies using conditioned medium were excluded, but studies using reporter genes (solely for stem cell imaging purposes) were included. Reviews, editorials, comments, reports from scientific sessions and discussions were excluded.

Search Strategy

A Pubmed search was performed (January 1980 to March 2010) using the following search terms: "(*pig OR porcine OR swine OR canine OR dog OR sheep OR ovine) AND (stem cells OR progenitor cells OR bone marrow) AND (myocardial infarction OR heart failure OR coronary artery disease OR cardiac repair OR myocardial regeneration)".* Only English and published reports were included. The retrieved studies were carefully examined to exclude potentially duplicate or overlapping data. The complete search strategy is available on request.

Data abstraction

Two reviewers (TS and SJ) independently screened abstracts and the resulting manuscripts were approved by a third reviewer (SC). The following information was extracted from the complete manuscripts of the qualified studies: basal characteristics of the study, LVEF, end-diastolic volume (EDV), end-systolic volume (ESV) and mortality. If necessary, data were estimated from graphics or recalculated by available data: LVEF was recalculated as follows: (EDV-ESV)/EDV *100%. Accordingly, standard deviations (SD) were determined or recalculated from standard errors. Volume data were recalculated for body-weight. For final analysis we preferably used MRI data. Alternatively, data derived by echocardiography, nuclear imaging, left ventricle angiography or pressure-volume (PV) loops respectively, were used in absence of MRI data. In case of missing data, corresponding authors were contacted. Thirty-six emails were sent and 18 authors responded. Standard guidelines[5] for quality assessment of clinical trials could not be completely applied in these pre-clinical experiments. Therefore, we used modified criteria to assess selection, performance and detection bias: randomization (yes/no), adequate allocation (y/n), adequate method of randomization (y/n), blinding of the operator (y/n) and blinding of the functional analysis (y/n).

Data analysis

Our primary outcome was difference in mean LVEF (reported in %) at follow-up between control and treated animals. Secondary endpoints were difference in EDV and ESV (reported as volume in mL) at follow-up and mortality after treatment. In case of multiple measurements over time, data measured at the longest duration of follow-up were used for analysis. A random-effect model was applied. Continuous variables were reported as weighted mean differences with 95% confidence intervals (CI) between the cell-treated animals and control groups. In case of dichotomous data, the pooled estimate of effect was presented as odds ratio (OR) with 95% CI. In case of multiple experimental groups next to one control group within one study, the number of animals in the control group was divided equally by the number of experimental groups. Details of enrolled subgroups are provided in data Supplemental Table 1. Unadjusted P values are reported throughout, with hypothesis testing set at the 2-tailed 0.05 level. Heterogeneity was considered significant at $P<0.10$[6]. Inconsistency was estimated by using the I^2 statistic; values of 25%, 50% and 75% were considered low, moderate and high inconsistency, respectively.[7]

Based on clinical scenario a multivariate analysis was performed for: MI model (ischemia/reperfusion or chronic occlusion); location of infarct-related artery (left anterior descending artery (LAD) or left circumflex artery (LCX)); type of animal (pig, dog, sheep); cell type;

number of cells injected; method of cell delivery (retrograde coronary transvenous injection, surgical, intracoronary (IC), and trans-endocardial (TE) delivery); timing of cell therapy after acute MI and follow-up after cell therapy. Furthermore, from a clinical point of view the following subgroup analyses were performed: MI model (ischemia/reperfusion or chronic occlusion); type of infarction (LAD or LCX); cell type (bone marrow mononuclear stem cells (BM-MNC) or mesenchymal stem cells (MSC)); number of cells injected ($<10^7$, 10^7-10^8, 10^8-10^9, or $\geq 10^9$), timing of cell therapy after acute MI (≤ 1 day, 1-7 days, ≥ 7 days) and follow-up after cell therapy (1-4 weeks, 5-8 weeks, 9-12 weeks, >12 weeks). A Funnel plot was drawn for LVEF to explore publication bias. A power-analysis for future studies in ischemic heart disease was performed. All analyses were performed with Review Manager version 5 (The Nordic Cochrane Center, Købehvn Denmark) and SPSS 17.0; SPSS, Chicago, IL.

RESULTS

Included study characteristics

The electronic database search identified 304 articles, among which 52 articles were eligible for review (34 RCT and 18 cohort studies; see Figure 1). In total 1251 animals were described in the included articles but 888 animals met our inclusion criteria and were analyzed. Characteristics of the enrolled studies are depicted in Table 1. Most studies used a porcine model (41 studies). In 23 studies ischemia/reperfusion was used as a MI model. Myocardial infarction was mainly induced in the left anterior descending coronary artery (38 studies), but site of ligation/constriction of the vessel (proximal, mid or distal) varied. Ten different cell types have been studied. In most cases surgical or IC delivery was performed. Timing of cell therapy after induction of MI was <1 day (15 studies), 1-7 days (11 studies) or >7 days (26 studies). Median and interquartile range of time to follow-up imaging was 6 weeks (4-8 weeks). Functional endpoints were assessed by MRI (18 studies), echocardiography (23 studies), nuclear imaging (5 studies), left ventricle angiography (4 studies) or PV-loop (2 studies). Volume data were reported in 25 studies and mortality in 32 studies.

Quality of included studies

Data supplement Table 2 shows the methodological quality of the enrolled studies. Blinded analysis of LVEF was performed in 12 RCT and 10 cohort studies. The operator was blinded in 5 studies. One article reported the method of randomization. Thirty-six studies (69%) were published in journals with an impact factor ≥3.0.

Meta-analyses

Pooled analysis showed a LVEF difference of 7.5% at follow-up after cell therapy vs. control (95% CI, 6.2% to 8.9%; P<0.001) with significant heterogeneity (p<0.01) and inconsistency (I^2: 77%) (Figure 2). At follow-up, mean LVEF after cell transplantation and control was 56% and 48%, respectively. Consistently an ESV difference of -7.4 mL (95% CI, -12.9 mL to -1.8 mL; P=0.01) and EDV difference of -5.3 mL (95% CI, -12.7 mL to 2.1 mL; P=0.16) was found with significant heterogeneity (p<0.001 for both) and inconsistency (I^2 >90% for both). Overall, no significant difference in LVEF at baseline between the control group and

cell treated group was found (P=0.31); however only 69 % of the studies reported these baseline data. No significant differences were found in mortality after cell transplantation: 9.5% (36/380) in cell treated group vs. 8.4 % (21/251) in the control group (OR 1.13 [0.63 to 2.02], I^2=0%, P=0.68). The majority of deaths were due to arrhythmias (data not shown).

Sensitivity-analyses

A multivariable meta-regression analysis showed that cell type (P=0.040) and type of infarction (P=0.045) are the only independent significant predictors of LVEF improvement. A trend was observed (Figure 3) towards more improvement of cell therapy regarding: anterior infarction with LAD as infarct-related artery, high cell number (≥10^7) and late injections (>1 week after MI). BM-MNC showed less effect than MSC. In addition, less benefit was observed in ischemia/reperfusion MI models compared to chronic MI models.

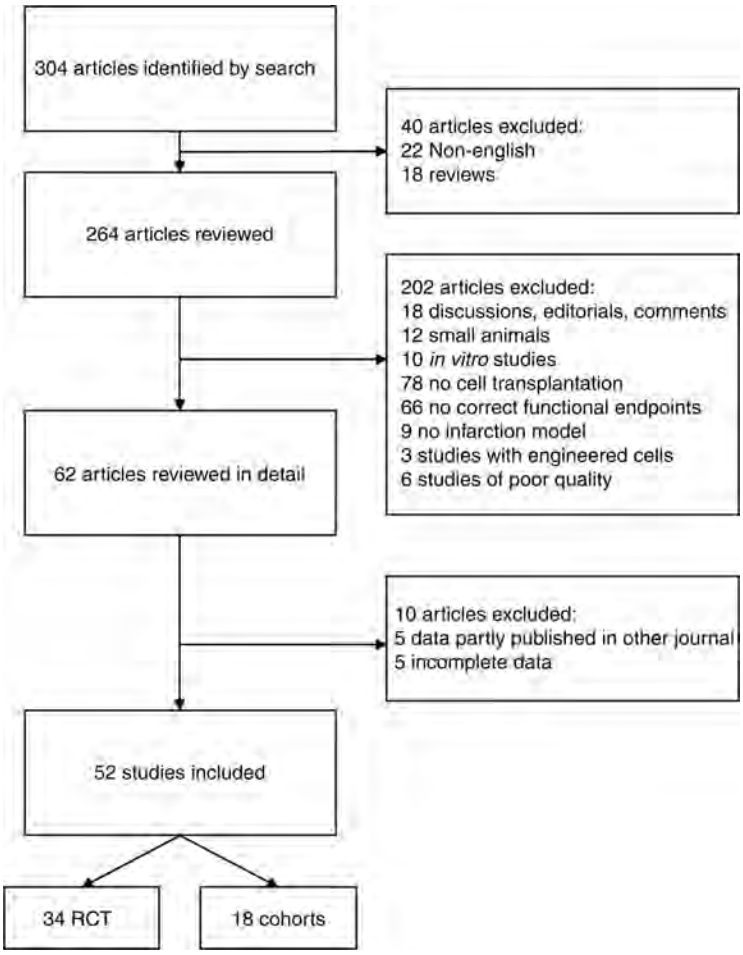

Figure 1. Flowchart
Overview of enrolled studies on cell therapy in large animals with acute myocardial infarction and chronic ischemic cardiomyopathy. RCT= Randomized controlled trial.

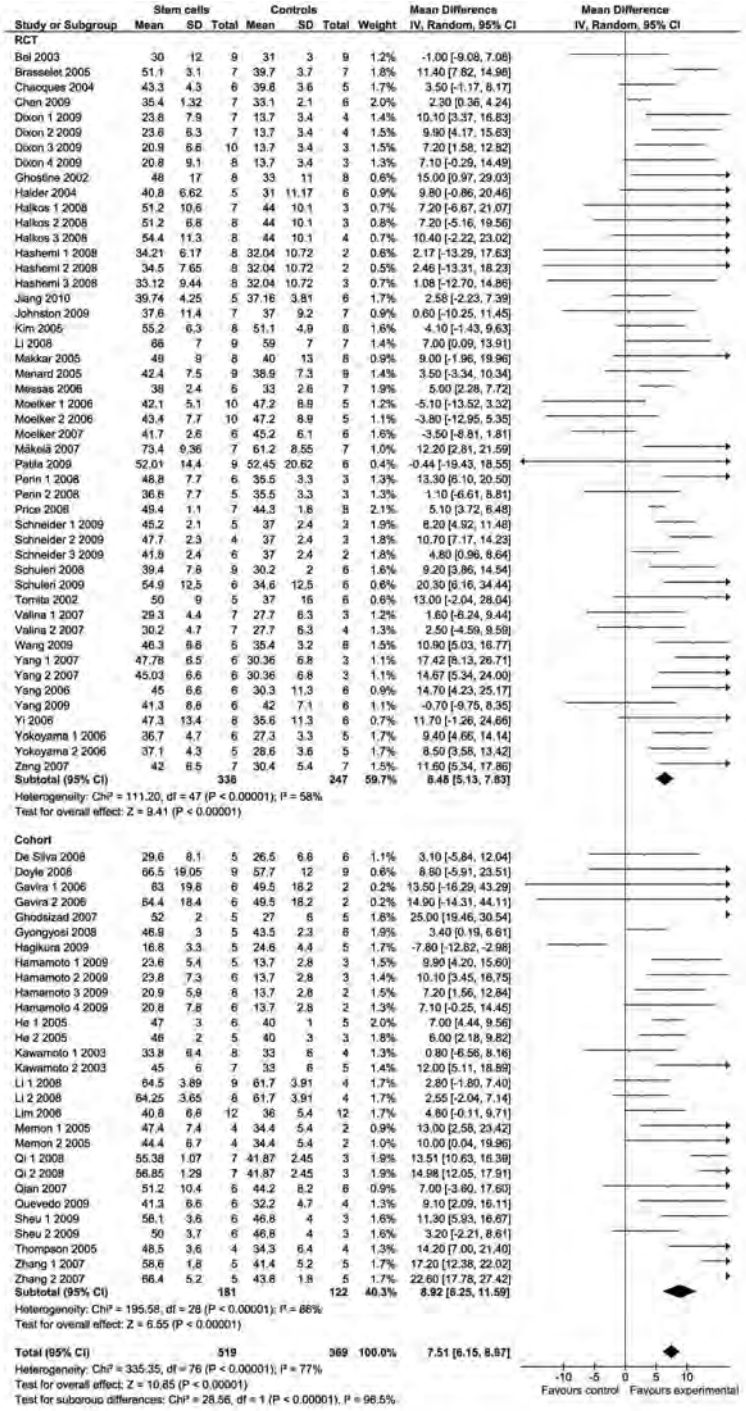

Figure 2. Forest plot
Showing the impact of stem cell therapy on LVEF improvement compared to controls. RCT= Randomized controlled trial; 95% CI= 95% Confidence interval; WMD= Weighted mean difference.

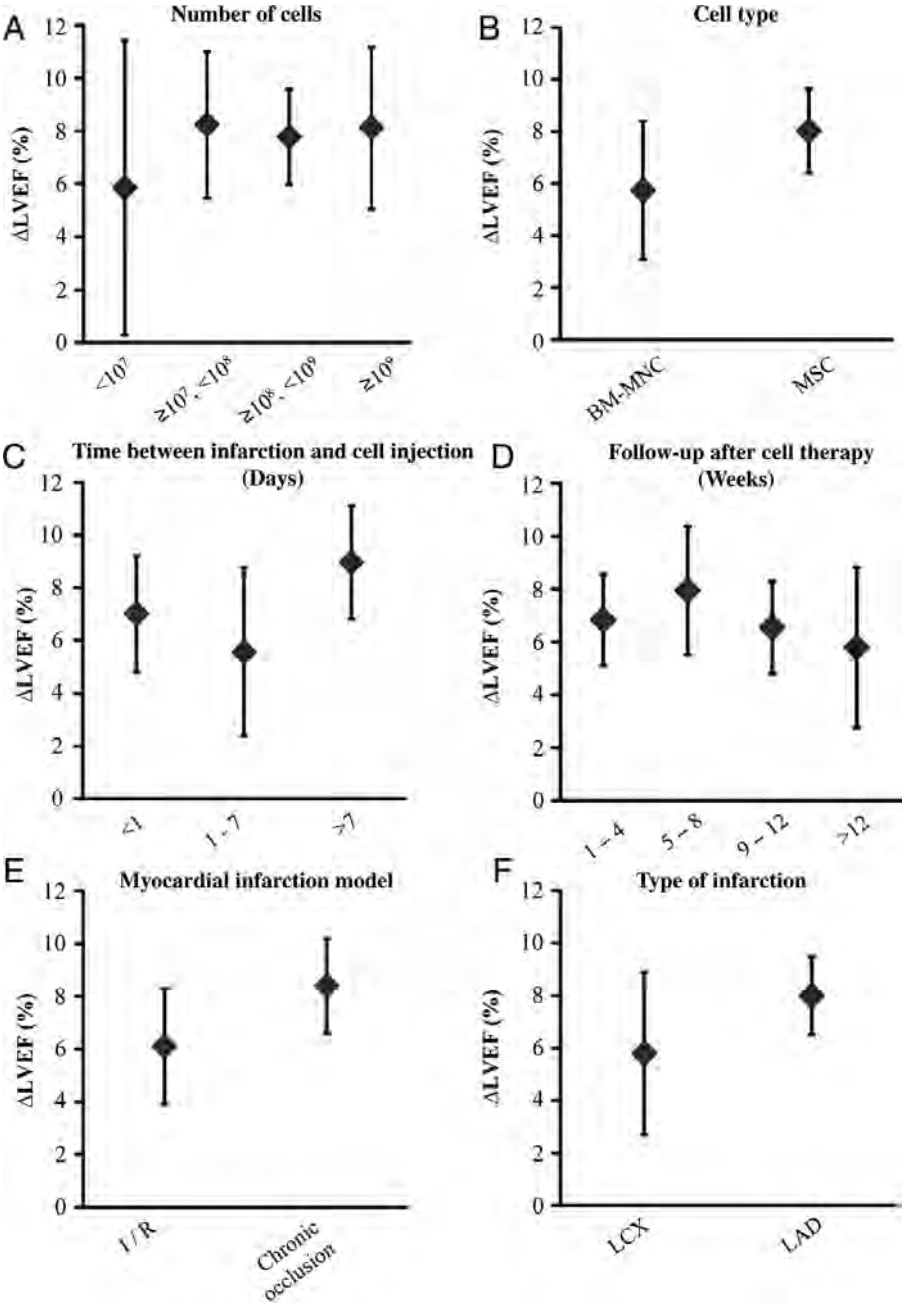

Figure 3. Sensitivity analysis
By visual inspection a trend is seen towards more improvement of cell therapy compared to control regarding: (A) high cell number (≥107) (P=0.52), (B) other cell types than bone marrow (P=0.040), (C) late injections (>1wk) (P=0.68), (E) chronic occlusion model (P=0.70) and (F) LAD infarction (P=0.045). After 8 weeks follow-up (D) the effect of cell therapy fades away (P=0.11). LVEF= Left ventricular ejection fraction; LAD= Left anterior descending artery; LCX= Left circumflex artery; I/R= ischemia/reperfusion; BM-MNC= Bone marrow mononuclear stem cells; MSC=Mesenchymal stem cells; P values are derived from the multivariate analysis.

During follow-up the effect of cell therapy appeared to decline over time. No trend in LVEF improvement was observed regarding animal model (P=0.49) and route of cell delivery (P=0.90). The funnel plot for LVEF suggests a lack of publication bias as values were evenly distributed around the overall estimate (Figure 4).

Power calculation

Based on our results we performed a sample size calculation for future studies in ischemic heart disease. To obtain a power of at least 80% in a two-sided two-sample t-test with an alfa of 0.05, 11 animals needed to be included in each group to detect a significant difference of 8% in LVEF.

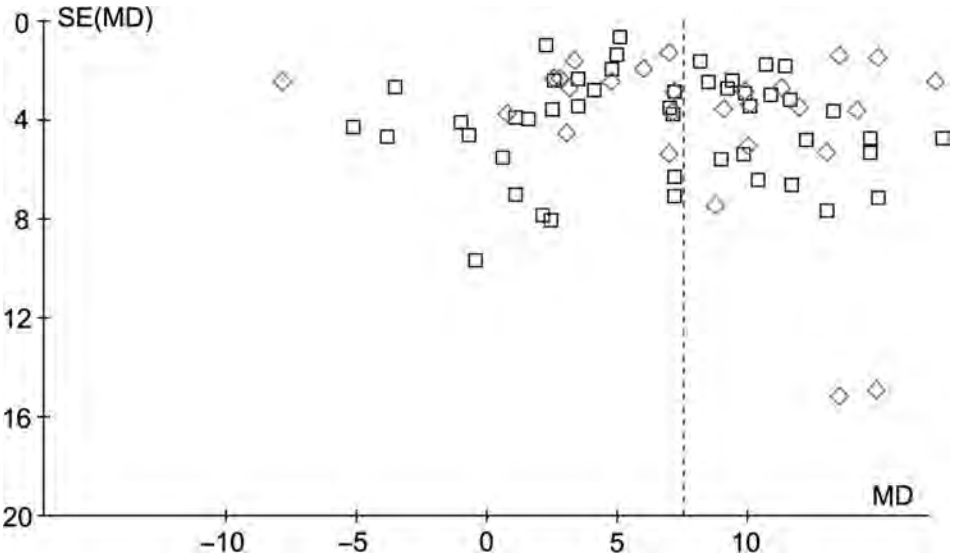

Figure 4. Funnel plot for LVEF improvement
Blue dotted line shows the overall estimated mean difference. The red diamond shaped color displays cohort studies and black squares displays RCTs. No evidence for publication bias was found. SE=Standard Error; MD= Mean difference.

DISCUSSION

The current analysis comprises data of 52 published pre-clinical studies involving large animals treated with stem cells in order to investigate the effects of cell therapy for ischemic heart disease. The main findings are: (1) Cell therapy improves LVEF by 7.5% due to a significant decrease in ESV; (2) There is no increased mortality after cell treatment; (3) Cell type and type of infarction are important predictors of functional outcome; (4) Sensitivity analysis suggests that MSC, LAD infarction, chronic occlusion MI models, a higher number of cells (≥10^7) and cell injection at least one week after MI have a beneficial effect on LVEF; (5) No effect on animal type and route of delivery was found.

Table 1:Study Characteristics

Author	N	Type of animal	Study design	Type of infarction	MI model	Cell type	Number of cells	Route of delivery	Timing of cell therapy after MI*	Follow up (weeks)
Bel et al.[24] 2003	18	Sheep	RCT	LCX	No I/R	BM-MNC	$4.2*10^8$	Surgical	21d	8
Brasselet et al.[25] 2005	14	Sheep	RCT	LAD	No I/R	Skeletal myoblasts	$2.4*10^8$	RCV	14d	8
Chacques et al.[26] 2004	11	Sheep	RCT	LAD	No I/R	Skeletal myoblasts	$7.0*10^7$	Surgical	21d	12
Chen et al.[27] 2009	13	Pig	RCT	LCX	No I/R	MSC	$4.0*10^7$	Surgical	42d	4
De Silva et al.[28] 2008	11	Pig	Cohort	LAD	I/R	BM-MNC	$1.0*10^9$	IC	4d	6
Dixon et al.[29] 2009	46	Sheep	RCT	LAD	No I/R	MSC	$2.5*10^7$-$4.5*10^8$	Surgical	1h	8
Doyle et al.[30] 2008	18	Pig	Cohort	LCX	I/R	EPC	$3.0*10^7$	IC	2d	8
Gavira et al.[31] 2006	16	Pig	Cohort	LAD	No I/R	Skeletal myoblasts	$4.0*10^8$	TE/Surgical	56d	12
Ghodsizad et al.[32] 2007	10	Pig	RCT	LCX	No I/R	USSC	$1.3*10^7$	Surgical	1h	8
Ghostine et al.[33] 2002	16	Sheep	RCT	LCX	No I/R	Skeletal myoblasts	$4.2*10^8$	Surgical	14d	17
Gyöngyösi et al.[34] 2008	11	Pig	RCT	LAD	I/R	MSC	$7.1*10^6$	TE	16d	1.5
Hagikura et al.[35] 2009	10	Pig	Cohort	LAD	No I/R	MNC	$5.0*10^6$	Coronary sinus venous infusion	4h	4
Haider et al.[36] 2004	11	Pig	RCT	LCX	No I/R	Skeletal myoblasts	$3.0*10^8$	Surgical	21d	6
Halkos et al.[37] 2008	33	Pig	RCT	LAD	I/R	MSC	$3.9*10^7$-$3.7*10^8$	IV	1h	12
Hamamoto et al.[38] 2009	35	Sheep	Cohort	LAD	No I/R	MSC	$2.3*10^7$-$4.4*10^8$	Surgical	1h	4-8
Hashemi et al.[39] 2008	31	Pig	RCT	LAD	I/R	MSC	$2.4*10^7$-$4.4*10^8$	TE	3d	8-12
He et al.[40] 2005	19	Dog	Cohort	LAD	No I/R	Skeletal myoblasts	$3.6*10^8$-$5.4*10^8$	Surgical/TE	7d	10
Jiang et al.[41] 2010	11	Pig	RCT	LAD	I/R	MSC	$1.0*10^7$	IC	1h	13
Johnston et al.[42] 2009	14	Pig	RCT	LAD	I/R	CDC	$1.0*10^7$	IC	28d	8
Kawamoto et al.[43] 2003	24	Pig	Cohort	LCX	No I/R	MNC/EPC	$1.0*10^7$	TE	28d	4
Kim et al.[44] 2005	16	Pig	RCT	LAD	No I/R	USSC	$1.0*10^8$	Surgical	28d	4
Li et al.[45] 2008	16	Pig	RCT	LAD	I/R	BM-MNC	$3.5*10^8$	IC	1h	4
Li et al.[46] 2008	25	Pig	Cohort	LAD	No I/R	MSC/BM-MNC	$6.2*10^5$-$4.7*10^7$	IC	2h	4
Lim et al.[47] 2006	24	Pig	Cohort	LAD	I/R	MSC	$1.0*10^7$	IC	3d	4
Mäkelä et al.[48] 2007	14	Pig	RCT	LCX	I/R	BM-MNC	$1.0*10^8$	Surgical	1h	3
Makkar et al.[49] 2005	16	Pig	RCT	LAD	No I/R	MSC	$2.0*10^8$	Surgical	28d	8
Memon et al.[50] 2005	12	Dog	Cohort	LAD	No I/R	Skeletal myoblasts/ BM-MNC	$3.0*10^6$-$1.0*10^8$	Surgical	14d	4

Menard et al.[51] 2005	18	Sheep	RCT	LCX	No I/R	ESC	$3.0*10^7$	Surgical	14d	4
Messas et al.[52] 2006	13	Sheep	RCT	LCX	No I/R	Skeletal myoblasts	$2.5*10^8$	Surgical	56d	8
Moelker et al.[53] 2006	30	Pig	RCT	LCX	I/R	BM-MNC	$5.0*10^8$	IC	7d	4
Moelker et al.[54] 2007	12	Pig	RCT	LCX	I/R	USSC	$1.0*10^8$	IC	7d	4
Patila et al.[55] 2009	15	Pig	RCT	LCX	No I/R	Skeletal myoblasts	$2.0*10^6$	Surgical	24d	4
Perin et al.[56] 2008	17	Dog	RCT	LAD	I/R	MSC	$1.0*10^8$	TE/IC	7d	2
Price et al.[57] 2006	15	Pig	RCT	LAD	I/R	MSC	$3.2*10^8$	IV	1h	13
Qi et al.[58] 2008	20	Pig	RCT	LAD	I/R	MSC	$1.0*10^8$	IC	5d	4-8
Qian et al.[59] 2007	12	Pig	Cohort	LAD	I/R	MSC	$1.0*10^9$	IC	7	6
Quevedo et al.[60] 2009	10	Pig	Cohort	LAD	I/R	MSC	$2.0*10^8$	IC	84d	12
Schneider et al.[61] 2009	23	Pig	RCT	LCX	No I/R	MSC/BM-MNC	NA/$1.7*10^7$	TE	14d	4
Schuleri et al.[62] 2008	15	Pig	RCT	LAD	I/R	MSC	$2.0*10^8$	TE	2d	8
Schuleri et al.[63] 2009	12	Pig	RCT	LAD	I/R	MSC	$2.0*10^8$	Surgical	111d	12
Sheu et al.[64] 2009	18	Pig	Cohort	LAD	No I/R	BM-MNC	$3.0*10^7$	Surgical	1h	13
Thompson et al.[65] 2005	8	Pig	Cohort	LAD	No I/R	BM-MNC	$3.0*10^8$	RCV	35d	8
Tomita et al.[66] 2002	11	Pig	RCT	LAD	No I/R	MSC†	$1.0*10^8$	Surgical	28d	4
Valina et al.[67] 2007	21	Pig	RCT	LAD	I/R	MSC/ADSC	$2.0*10^6$	IC	1h	4
Wang et al.[68] 2009	12	Pig	RCT	LAD	I/R	MSC	$5.0*10^7$	Trans coronary injection	1h	4
Yang et al.[69] 2009	12	Pig	RCT	LAD	I/R	MSC	$3.0*10^7$	Surgical	1h	6
Yang et al.[70] 2006	12	Pig	RCT	LAD	No I/R	MSC	$5.0*10^6$	IC	28d	4
Yang et al. [71] 2007	18	Pig	RCT	LAD	I/R	MSC	$5.0*10^6$	IC	28d	4
Yi et al.[72] 2006	14	Pig	RCT	LAD	No I/R	MSC	$8.0*10^7$	TE	28d	4
Yokoyama et al.[73] 2006	21	Pig	RCT	LAD	No I/R	BM-MNC	$3.2*10^9$	Coronary sinus venous infusion	6h or 14d	4
Zeng et al.[74] 2007	14	Pig	RCT	LAD	No I/R	MSC	$5.0*10^7$	Surgical	1h	4
Zhang et al.[75] 2007	20	Pig	Cohort	LAD	No I/R	HPC	$5.0*10^7$	IC	14d	4

ADSC= Adipose tissue derived stem cells; BM-MNC= Bone marrow mononuclear cells; CDC= Cardiosphere derived cells; EPC=Endothelial progenitor cells; ESC= Embryonic stem cells; HPC=Hematopoietic progenitor cells; IC= Intracoronary infusion; I/R= Ischemia/reperfusion; IV= Peripheral intravenous; LAD= Left anterior descending artery; LCX= Left circumflex artery; MI=Myocardial infarction; MNC= Peripheral mononuclear cells; MSC= Mesenchymal stem cells; N= number of animals (treated group and control group); NA= Not applicable; RCT=Randomized controlled trail; RCV= Retrograde coronary transvenous injection; TE= Trans-endocardial injection; USSC= Unrestricted somatic stem cells. *timing in days (d) or hours (h); †MSC cultured with 5-azacytidine.

Safety and efficacy of cardiac stem cell therapy in pre-clinical trials

Safety of cell therapy is still an important issue,[8] regarding the no reflow after intracoronary cell injections and myocardial perforation by intramyocardial application. In spite of cell delivery- associated adverse events, human trials did not report increased mortality. Similarly, the present meta-analysis of pre-clinical trials showed no significant difference in death in animals receiving cell transplantation compared to controls, although only 32 studies (61%) addressed this issue.

Although global LV function improves after cell therapy, no significant difference in EDV (-1.92 mL) was documented, indicating that cell therapy led to increase in contractility, but did not prevent ventricular remodelling. Similar result was observed in two clinical meta-analyses.[3, 9] This could be due to the relative short-term follow-up (less <4 months) of the enrolled studies in our analysis. However, it is possible that structural myocardial changes and effects on diastolic filling occur after 4 months.

Transplantation of higher number of cells ($\geq 10^7$) appears to have a more pronounced impact on improvement in LVEF. Our results are in agreement with clinical meta-analysis in that significant effect on LVEF may only be achieved when infusing doses are higher than 10^8 cells.[9] Moreover, meta-regression analysis showed that choice of cell type is an important predictor for LVEF. Subanalysis revealed a trend towards larger benefit in case of transplantation of MSC, as compared to BM-MNC. Scarce evidence is available that these cell types can regenerate new cardiomyocytes *in vivo*. This suggests the stimulation of an endogenous regenerative capacity of the heart upon cell transplantation, by release of growth factors, cytokines and other paracrine molecules by the transplanted and host cells, enhancing angiogenesis and reducing apoptosis.[10-12]

Unfortunately, no complete data on infarct size were reported. However, our meta-regression analysis showed that type of infarction is an important significant independent predictor for clinical outcome. In detail, LAD-related anterior wall infarction showed more benefit after stem cell therapy compared to LCX infarction (LAD 8.0% vs. LCX 5.8%). Interestingly, there was no important difference in ratio of MI model for LAD and LCX infarction. Therefore, the observed effect may be caused by a greater degree of expansion after LAD infarction[13] leading to lower LVEF and a higher risk for mortality therefore more benefit from cell therapy is expected in this patient group. Indeed, post hoc analyses of the REPAIR-AMI trial database and a clinical meta-analyses suggested that the effects of bone marrow cells were significantly higher in the subgroup with a baseline LVEF<49% who may have a tendency to develop heart failure.[14, 15] Ischemia/reperfusion MI models were associated with less improvement in LVEF compared to chronic occlusion models (LVEF; 6.3% vs. 8.3%) although there was a higher incidence of permanent ligation animal studies. No reliable insight in baseline LVEF was available between these groups. In theory, percutaneous ischemia/reperfusion models are considered most reliable for translational research as it mimics more closely the clinical practice of primary percutaneous coronary intervention. Timing of injection is important with more pronounced benefit if applied 7 days after MI. Our findings are comparable to clinical studies.[3, 16] In the acute setting (<24 hours) cellular retention and survival is likely influenced by the local hostile microenvironment. In large animals, the effect of cell therapy fades away 8 weeks after cell injection. This phenomenon is in accordance with initial observations in patient studies.[17]

This finding should trigger researchers towards novel applications and strategies of stem cell therapy (e.g. slow release agents, genetic engineering of stem cells, or repetitive injections overtime).

Effect of study design on study outcome

An overall beneficial effect of cardiac stem cell therapy has been observed in this analysis. However this effect appears to be more pronounced in cohort studies as compared to RCT (LVEF; RCT 6.5% vs. Cohorts 8.9%). It is conceivable that cohort studies are designed for practical reasons and might systematically overestimate the effect of cell therapy.
The capability of animal studies to predict human clinical outcome have been questioned by some authors.[18, 19] However, the results from the animal RCT studies are comparable to clinical meta-analyses.[3, 20] (RCT; LVEF 6,5% vs. 4%) indicating that ischemic large animal models are relevant for translational purposes.

Recommendations for future translational stem cell research

In view of clinical practice it is mandatory that pre-clinical studies are performed according to high standards. In our opinion, the following items should be reported in pre-clinical studies for establishing standards for translational stem cell research with in mind the clinical horizon: randomized study design; blinded functional analysis; number of animals used in the study protocol must be clear and include the measured data before treatment and at the follow-up, mortality after treatment and during follow-up.
Over the next few years, adequately powered large animal studies and clinical trials should focus on transplantation of $\geq 10^7$ stem cells, other cell types than BM-MNC and later time point of injection (>1 week after MI). To maintain the beneficial effect on LVEF over time, repeated cell injections or the use of biomaterials to enhance survival of transplanted cells should be evaluated. No difference between species was observed and we therefore recommend the use of pigs to evaluate the effect of cell therapy since many studies are available for comparison. We suggest the use of IC in the setting of acute MI and TE for chronic MI since no difference was found in our meta-analysis between these transplantation techniques.
Meta-analyses of animal studies are not common, yet they are recommended in several settings[21] and can often guide research and clinical endeavours.[22] Performing pre-clinical meta-analysis may also be attractive to evaluate the effect of other therapies to design future (pre-)clinical trials.

Limitations

Limitations of meta-analysis are well known.[23] In particular, in our study the diversity in animal type, incidence of permanent occlusion, delivery method, time of injection after MI, follow-up after cell therapy and number of cells may play a role in the observed outcomes in the present study. However, multivariate analysis (used as an exploratory tool) showed no differences, but should be used with caution to generate new hypothesis. Heterogeneity may be present due to the extremely sensitive end-points chosen (all continuous: LVEF, EDV and ESV). By using random-effect analysis the risk of finding erroneous estimates is minimized. Although various imaging modalities have been used to measure our endpoints. Univariate analysis showed no significant difference between these techniques (P=0.44).

Our analysis was based on study outcomes and we did not have access to individual data. Accordingly we provided mean values. As some studies did not report all data necessary for the analysis, effort was made to contact corresponding authors to complete the database: only 5 studies were finally excluded due to incomplete data.
To date, numerous human clinical trials have already been conducted in order to assess the efficacy and safety aspects of cardiac stem cell therapy.[3] Obviously, differences exist between large animal models and clinical practice. Healthy young large animals differ from older patients with long standing coronary artery disease, and frequently comorbidities (e.g. diabetes, hypertension, renal failure) are present. Consequently, many patients are routinely treated with other drugs (e.g. ACE-inhibitors, beta-blockers, anti-diabetic medication), in contrast to research animals. Furthermore, autologous stem cells extracted from young large animals are 'fresh' whereas cells from patients are 'aged'. Finally, duration of follow-up is relatively short in animal studies. Despite these differences we have shown that pre-clinical data are highly relevant to predict outcome for clinical trials.

Conclusion

To the best of our knowledge, this is the first systematic review and meta-analysis in large animal models to evaluate the effect of cell therapy in ischemic heart disease. This analysis showed that large animal models are valid to predict outcome of clinical trials. Moreover, the results showed that cardiac cell therapy is safe, led to an improved LVEF and revealed important clues for designing (pre-) clinical trials.

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SUPPLEMENTARY MATERIAL

Table 1. Details of the subgroups of the enrolled studies

Author	Number of Groups	Subgroups
Dixon et al.(1) 2009	4	2.5*107 7.5*107 2.2*108 4.5*108
Halkos et al.(2) 2008	3	3.9*107 1.1*108 3.7*108
Hamamoto et al.(3) 2009	4	2.3*107 7.2*107 2.1*108 4.4*108
Hashemi et al.(4) 2008	3	2.4*107 2.4*107 4.4*108
He et al.(5) 2005	2	Surgical TE
Kawamoto et al.(6) 2003	2	MNC EPC
Li et al.(7) 2008	2	MSC BM-MNC
Memon et al.(8) 2005	2	Skeletal myoblasts BM-MNC
Moelker et al.(9) 2006	2	Bone marrow BM-MNC
Perin et al.(10) 2008	2	IC TE
Qi et al. (11)2008	2	Labeled MSC Unlabeled MSC
Schneider et al.(12) 2009	3	Autologous MSC Allogeneic MSC BM-MNC
Sheu et al.(13) 2009	2	Infarct- relative arterial approach Noninfarct- relative arterial approach
Valina et al.(14) 2007	2	MSC ADSC
Yang et al.(15) 2007	2	Infarct- relative arterial approach Non-infarct relative arterial approach
Yokoyama et al.(16) 2006	2	AMI group OMI group
Zhang et al.(17) 2007	2	Rentrop score=0 Rentrop score =1

ADSC= Adipose tissue derived stem cells; AMI= Acute myocardial infarction; BM-MNC= Bone marrow mononuclear cells; EPC=Endothelial progenitor cells; IC= Intracoronary infusion; MNC= Peripheral mononuclear cells; MSC= Mesenchymal stem cells; TE= Trans-endocardial injection; OMI= Old myocardial infarction.

Table 2. Methodological quality of the included studies according to the Jüni guidelines

Author	RCT	Adequate allocation	Method of randomization described	Operator blinded	Analyst blinded
Bel et al.(18) 2003	Y	N	N	N	N
Brasselet et al.(19) 2005	Y	N	N	N	Y
Chacques et al.(20) 2004	Y	N	N	N	N
Chen et al.(21) 2009	Y	N	N	N	Y
De Silva et al.(22) 2008	N	N	N	Y	Y
Dixon et al.(1) 2009	Y	N	N	N	N
Doyle et al.(23) 2008	N	N	N	N	Y
Gavira et al.(24) 2006	N	N	N	N	Y
Ghodsizad et al.(25) 2007	Y	N	N	N	Y
Ghostine et al.(26) 2002	Y	N	N	N	N
Gyöngyösi et al.(27) 2008	Y	N	N	N	Y
Hagikura et al.(28) 2009	N	N	N	N	N
Haider et al.(29) 2004	Y	N	N	N	Y
Halkos et al.(2) 2008	Y	N	N	Y	Y
Hamamoto et al.(3) 2009	N	N	N	N	Y
Hashemi et al.(4) 2008	Y	N	N	N	N
He et al.(5) 2005	N	N	N	N	Y
Jiang et al.(30) 2010	Y	N	N	N	N
Johnston et al.(31) 2009	Y	N	N	N	N
Kawamoto et al.(6) 2003	N	N	N	N	Y
Kim et al.(32)2005	Y	N	N	N	N
Li et al.(33) 2008	Y	N	N	N	N
Li et al.(7) 2008	N	N	N	N	N
Lim et al.(34) 2006	N	N	N	N	N
Mäkelä et al.(35) 2007	Y	N	N	Y	Y
Makkar et al.(36) 2005	Y	N	N	N	Y
Memon et al.(8) 2005	N	N	N	N	N
Menard et al.(37) 2005	Y	N	N	N	N
Messas et al.(38) 2006	Y	N	N	N	Y
Moelker et al.(9) 2006	Y	N	N	N	N
Moelker et al.(39) 2007	Y	N	N	N	N
Patila et al.(40) 2009	Y	N	N	N	Y
Perin et al.(10) 2008	Y	N	N	N	Y
Price et al.(41) 2006	Y	N	N	N	N
Qi et al. (11)2008	Y	N	N	N	N
Qian et al.(42) 2007	Y	N	N	N	N
Quevedo et al.(43) 2009	N	N	N	N	N

Table 2. *Continued*

Schneider et al.(12) 2009	Y	N	N	N	Y
Schuleri et al.(44) 2008	Y	N	N	N	Y
Schuleri et al.(45) 2009	Y	N	N	Y	N
Sheu et al.(13) 2009	N	N	N	N	Y
Thompson et al.(46) 2005	N	N	N	N	Y
Tomita et al. (47)2002	Y	N	N	N	N
Valina et al.(14) 2007	Y	Y	Y	Y	Y
Wang et al.(48) 2009	Y	N	N	N	N
Yang et al.(49) 2009	Y	N	N	N	N
Yang et al.(50) 2006	Y	N	N	N	N
Yang et al.(15) 2007	Y	N	N	N	N
Yi et al.(51) 2006	Y	N	N	N	N
Yokoyama et al.(16) 2006	Y	N	N	N	N
Zeng et al.(52) 2007	Y	N	N	N	N
Zhang et al.(17) 2007	N	N	N	N	N

RCT= Randomized trial; Yes=Y; No=N.

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CHAPTER 6

SIMILAR EFFECT OF AUTOLOGOUS AND ALLOGENEIC CELL THERAPY FOR ISCHEMIC HEART DISEASE: SYSTEMATIC REVIEW AND META-ANALYSIS OF LARGE ANIMAL STUDIES

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ABSTRACT

Rationale

In regenerative therapy for ischemic heart disease, use of both autologous and allogeneic stem cells have been investigated. Autologous cell can be applied without immunosuppression but availability is restricted and cells have been exposed to risk factors and aging. Allogeneic cell therapy enables pre-operative production of potent cell lines and immediate availability of cell products, allowing 'off the shelf' therapy. It is unknown which cell source is preferred with regard to improving cardiac function.

Objective

We performed a meta-analysis of pre-clinical data of cell therapy for ischemic heart disease.

Methods and Results

We conducted a systematic literature search to identify publications describing controlled pre-clinical trials of unmodified stem cell therapy in large animal models of myocardial ischemia. Data from 82 studies involving 1415 animals showed a significant improvement in mean left ventricular ejection fraction (LVEF) in treated compared to control animals (8,3 %, 95% CI 7,1 – 9,5, p <0.001). Meta-regression revealed a similar difference in LVEF in autologous (8,8% 95% CI 7,3 – 10,3 n= 981) and allogeneic (7,3% 95% CI 4,4 – 10,2 n= 331) (p=0,3) cell therapies.

Conclusion

Autologous and allogeneic cell therapy for ischemic heart disease show a similar improvement in LVEF in large animal models of myocardial ischemia, compared to placebo. These results are important for the design of future clinical trials.

INTRODUCTION

Stem cell therapy for ischemic heart disease has been of great interest for more than a decade. Clinical meta-analyses show that stem-cell therapy is associated with an improvement in left ventricular ejection fraction (LVEF) of 3-4%.[1–4] This is accompanied by an improvement in exercise capacity and quality of life. The increase in LVEF is promising, but effort should be put into strategies to further improve the magnitude of effect. The European Society of Cardiology urge researchers to focus on unsolved issues in cardiac repair strategies, including the type of cell used.[5] Consequently, the field has shifted from bone marrow mononuclear cells to mesenchymal stem cells and more recently to cardiac stem cells.[6–9]

The vast majority of clinical trials have used autologous stem cells, an attractive approach since no immunologic problems are encountered.[10] Two important drawbacks of autologous cell therapy are exposure of cells to the patient's risk factors; and the limited availability. Patient's lifelong exposure to risk factors contributing to ischemic heart disease (*i.e.* age, diabetes and smoking), may impair the potential of autologous stem cells.[11–13] Restricted availability is present since selection and culturing of sufficient potent cells is cumbersome and time-consuming. This limitation is especially important in the acute setting of myocardial ischemia.

Allogeneic cell therapy enables preparatory production of potent cell lines, immediate availability and allows 'off-the-shelf' therapy. However, immunological matters have to be taken into account. Where immunosuppression is required this carries risk for the patient (opportunistic infections, risk for malignancies) and might affect the potential of stem cells.[14–17] Features of allogeneic and autologous cell sources are summarized in Table 1.

To help inform the design of future clinical trials we set out to establish whether, in large animal models, allogeneic cell therapy is associated with the same magnitude of effect as autologous cell therapy. To do this end we carried out a meta-analysis of pre-clinical data.

Table 1. Features of autologous and allogeneic cell sources

	Advantage	Disadvantage
Autologous	• No immunological issues	• Cell exposure to risk factors • Restricted (immediate) availability
Allogeneic	• Production of potent cell lines • Immediate availability	• Immunological issues

METHODS

A meta-analysis was performed for safety and efficacy of stem cell therapy for cardiac repair in large animal models of myocardial ischemia. Differences in effect size for autologous and allogeneic stem cell were explored by meta-regression.

Methods for selection of studies are extensively described in van der Spoel et al.[18] In brief, a systematic search was performed in the electronic databases Pubmed and Embase on January 15th 2013. (see Supplemental material for search strategy) Inclusion criteria were: reporting of an original study in English language peer reviewed journals, the use of large

animal myocardial ischemia models (dogs, sheep, pigs), the use of stem cells, the use of a proper control group and reporting of left ventricular ejection fraction (LVEF) as outcome measure. Exclusion criteria were studies not published in full (e.g. meeting abstracts) and the use of cells modified to enhance cell function.

Results were screened independently by two researchers (SJ and JE). Consensus of inclusion was achieved in all cases by discussion. Reference lists of included studies were checked for additional relevant publications.

Publication details including animal model, functional endpoints, mortality, cell characteristics, quality parameters, and general study information were extracted. The primary functional endpoint was LVEF and the secondary endpoints were left ventricular end diastolic volume (EDV), left ventricular end systolic volume (ESV) and safety, presented as mortality after cell therapy. Data were entered in the online international database of the working group 'Collaborative Approach to Meta Analysis and Review of Animal Data from Experimental Studies' (CAMARADES).

For LVEF, data at the end of the experiment were extracted since baseline data and/or change from baseline was not reported in several studies. For safety, only mortality occurring after stem cell therapy was included in the database. Mortality during induction of myocardial infarction, and thus before actual treatment, was not included in this analysis. Risk of bias for included articles was established based on the CAMARADES scoring system.[19] Included parameters for quality were reporting of randomisation, allocation concealment (meaning blinding of the operator to the given therapy), blinded assessment of outcome, compliance with animal welfare regulations and statement of potential conflict of interest.

Statistics

For LVEF a raw difference in mean analysis was performed.[20] Data are reported as an absolute difference in mean LVEF at follow up between treated and control groups, with 95% confidence interval (CI) or standard error of means (SEM). Because of difference in animal size, and consequential difference in cardiac volumes, we performed a standardized difference in mean analysis for both ESV and EDV. Safety was evaluated by estimating the odds ratio of mortality in treated and control groups.

The presence of publication bias was evaluated using funnel plot and Egger regression and trim and fill was used to correct for this bias. Funnel plot asymmetry can be used to identify a preponderance of imprecise studies overestimating treatment effects that is consistent with publication bias. Egger regression is a formal statistical test where in a symmetrical funnel plot the regression line and its 95% CIs for precision versus standardized effect size pass through the origin of the graph.[21] Trim and fill is a non-parametric test which attempts to impute the theoretical 'missing' studies that cause funnel plot asymmetry and recalculates the overall treatment effect in absence of publication bias.[22]

Where different treatment groups were reported within the same study (*i.e.* different cell types or cell numbers), the number of animals in the control group was divided by the number of treatment groups served. We assigned weight of studies based on inverse variance. We anticipated substantial heterogeneity and so used a random effects model. Differences in effect size for cell source (autologous, allogeneic, xenogeneic) were explored by random effects meta-regression. For meta-regression the number of covariates included was statistically limited to 10. Based on clinical interest, we explored the impact of the

the following 9 parameters next to cell source: type of ischemia (permanent ischemia vs. ischemia/reperfusion), infarct location (left circumflex coronary artery (LCX) vs. left anterior descending coronary artery (LAD)), cell type (bone marrow mononuclear cells (BMMNC), mesenchymal stem cells (MSC), cardiac stem cells (CSC), cell dose ($<10^7$, 10^7-10^8, 10^8-10^9, $>10^9$), delivery method (intracoronary, intramyocardial injections, transendocardial injections), timing of treatment (<1 day, 1-7 days, >7 days), randomisation, blinding of operator and total quality score. All analyses were performed using Stata version 12 (StataCorp LP, Texas, USA).

RESULTS

The search identified 459 publications in PubMed and 168 in EMBASE. After merging, 595 unique publications were screened. After excluding 513 publications (Supplemental material Figure 1) 82 articles could be included in our analysis. No additional studies could be added by screening the reference list of included studies. The 82 articles contained 125 groups for comparisons of the primary outcome (67 comparisons for EDV, 59 for ESV and 74 for mortality). A total of 1415 animals were included, 832 in treatment groups and 583 control animals. The vast majority investigated cell therapy in pigs (67 studies, n=1141) (dogs 5 studies, 64 animals; sheep 10 studies, 210 animals). See Supplemental Material Table 1 for specific characteristics per included study (including first author, year of publication, animal species, number of animals, location of infarct, type of injury, cell type, dose, cell source, delivery method, timing of treatment and method of endpoint assessment, LVEF of control and treatment group and effect size).

Risk of bias in included studies

Visual inspection of the funnel plot suggests symmetry. (Figure 1a) However, using Egger regression the 95% CIs of the regression line do not pass through the origin, suggesting asymmetry of the funnel plot, consistent with potential publication bias.(Figure 1b) Where we tried to correct for publication bias using trim and fill, this test did not identify any theoretical missing studies.

Internal validity was examined by scoring studies for randomisation, allocation concealment (meaning blinding of the operator for the treatment), blinded assessment of outcome, reporting of compliance with animal welfare regulations and a statement of potential conflict of interest. Randomisation was reported in 61%, allocation concealment in 11%, blinded assessment of outcome in 42%, compliance with animal welfare regulations in 74% and a statement of conflict of interest was reported in 4% of the included studies (Supplemental material Figure 2). The total quality score is the total number of positive scored parameters, with a minimum of 0 and a maximum score of 5. The median quality score was 2.

Meta-analysis

Overall, treatment showed an absolute difference in LVEF between treated and control animals of 8,3% (95% CI 7,1 – 9,5% SEM 0,6 p< 0,0001) in favour of cell treated animals. (Figure 2) Increased LVEF can be explained by a significant decrease in EDV (standardized difference in mean 0,60, 95%CI 0,32 -0,90% SEM 0,14). There is no significant difference

in ESV for treated and control animals (standardized difference in mean 0,76 95%CI 0,44 -1,1% SEM 0,16). Cell therapy did not lead to increased therapy-related mortality. Odds ratio for mortality is 1,1 (95% CI 0,7 – 1,6). See Supplemental Material Figure 3 for the timber plot of mortality.

Observed heterogeneity for the primary endpoint LVEF was higher than would be expected from sampling error alone (tau^2 = 31,4, I^2 = 79%.). We used meta-regression to explore potential contributions to this heterogeneity of parameters chosen *a priori (*type of ischemia, infarct location, cell type, cell dose, delivery method, timing of treatment, randomisation, blinding of operator and total quality score).

Cell source

Autologous cells were compared to allogeneic and xenogeneic cell sources. Of 125 comparisons, 85 groups received autologous cells, 30 received allogeneic and the remaining 10 comparison groups received xenogeneic cells. No significant difference in effect size was found between different cell sources by meta-regression. Subgroup analyses revealed

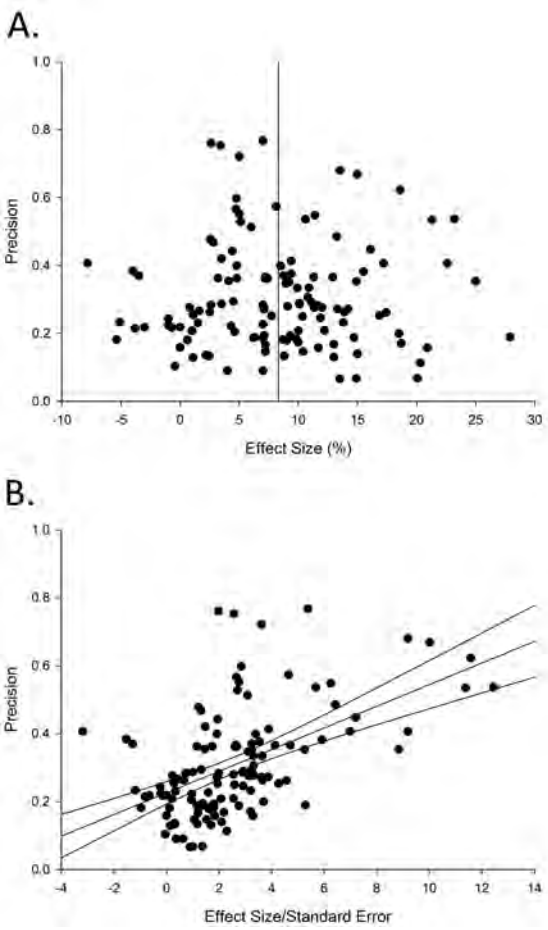

Figure 1. Publication bias
Funnel plot (a) and Egger regression (b) of left ventricular ejection fraction, showing potential evidence for publication bias.

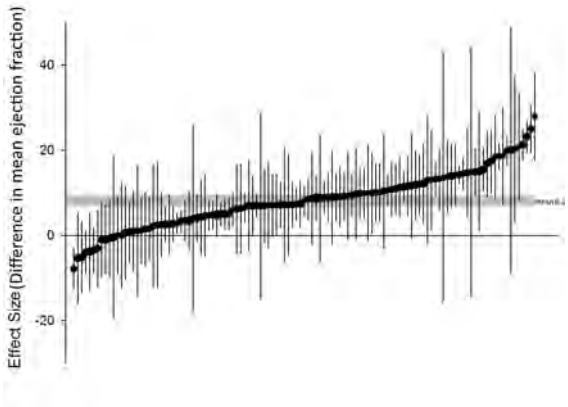

Figure 2. Timber plot
Timber plot of differences in mean left ventricular ejection fraction (LVEF) between treated and placebo animal. The vertical error bars represents the 95% confidence intervals of individual studies. The gray bar represents the 95% confidence interval of the mean difference in LVEF

mean difference in LVEF for autologous cells 8,8 (95% CI 7,3 – 10,3), for allogeneic 7,3 (95% CI 4,4 – 10,2) and 7,1 (95% CI 2,4 – 11,7) for xenogeneic cell therapy (differences not significant) (Figure 3A). Cell source had also no impact on ESV or EDV.

In most cases (25 out of 30 comparisons, 300 out of 352 animals) the allogeneic cells used were MSCs. Only one study using allogeneic cells, used immunosuppression.[23] For studies using xenogeneic cells, 8 out of 10 used immunosuppression, which is too few to perform meta-regression. Cyclosporin was the immunosuppressant of choice (doses ranging from 5 to 500 mg/kg/day). One group added methylprednisolone (125 mg/day).[24]

Because of the abundance of studies using MSCs in the allogeneic group (300 out of 352 animals), a post hoc regression analysis was performed to explore differences in cell source for MSCs alone. As with the overall analysis no effect of cell source on effect size by MSCs was seen for LVEF (autologous 8,6% (95% CI 5,6 – 11,7); allogeneic 7,4% (95% CI 5,3 – 9,6) and xenogeneic 5,8% (95% CI -3,3 – 14,9); p=0,4; tau^2 = 13,7; I^2 = 64% (Figure 3B)) and for ESV and EDV. Because bone marrow derived cells were all autologous, except for 1 study, no meta-regression for cell source could be performed for bone marrow derived cells alone.

Meta-regression for other parameters

Meta-regression could be performed including all 125 comparisons per parameter, except for administration route (110) and dose (121). Meta-regression showed 'myocardial infarction model' to be the only significant predictor for a difference in LVEF (Table 2), with a the largest effect seen in permanent occlusion models compared to ischemia/reperfusion (difference in mean LVEF 9,8% (95% CI 8,3 – 11,4) and 6,2% (95% CI 3,8 – 8,6) respectively (p=0,004)). All other clinical parameters were no predictors for effect size. We performed a post hoc subgroup analysis for method of outcome assessment. The majority of studies used echocardiography (n=62), MRI (n=35) or SPECT (n=9). The difference in LVEF between treated and untreated animals was 8.7% (95%CI 7.4 – 10.3) for echocardiography, 6.7% (95%CI 4.5 – 8.9) for MRI and 10.1% (95%CI 4.6 – 15.6) for SPECT, without any differences between methods (p=0.2)

For total quality score and for randomisation and allocation concealment, no statistically significant differences between groups were observed. (Table 2 and Supplemental material Figure 2B)

Table 2. Results from meta-regression of parameters other than cell source

Parameter					
Type of injury	Permanent (n=765)	Temporary (n=650)			
	9,8 ± 0,8	6,2 ± 1,2			p=0,004
Dose	< 1E7 (n=109)	1E7 - 1E8 (n=626)	1E8 - 1E9 (n=604)	> 1E9 (n=40)	
	4,8 ± 2,4	8,6 ± 0,9	8,3 ± 1,3	12,3 ± 3,5	NS
Time of administration	< 1 day (n=452)	1-7 days (n=335)	> 7 days (n=628)		
	7,7 ± 1,6	8,5 ± 1,8	8,3 ± 1,9		NS
Cell type	BMMNC (n=248)	MSC (n=536)	CSC (n=64)		
	7,6 ± 1,3	8,0 ± 0,7	5,2 ± 4,1		NS
Route of delivery	Intracoronary (n=355)	Surgical (n=610)	Transendocardial (n=264)		
	7,0 ± 1,5	9,2 ± 0,9	8,9 ± 1,8		NS
Location of infarct	LAD (n=1128)	LCX (n=287)			
	8,8 ± 0,7	6,3 ± 1,5			NS
Blinding of operator	Non Blinded (n=685)	Blinded (n=730)			
	7,7 ± 0,9	8,9 ± 1,2			NS
Randomisation	Non randomized (n=879)	Randomized (n=536)			
	9,3 ± 1,0	7,7 ± 1,3			NS

Data presented as difference in mean LVEF (mean ± SEM) between treated and placebo per subgroup. BMMNC Bone marrow mononuclear cells, MSC Mesenchymal stem cells, CSC Cardiac stem cells, LAD Left anterior descending coronary artery, LCX Left circumflex coronary artery. NS Not significant.

DISCUSSION

This meta-analysis of 82 studies, including 1415 large animals, shows that 1) autologous and allogeneic cell therapy for myocardial infarction exhibit similar effect size, 2) cell therapy provides an overall significant difference in mean LVEF of 8,3% and a significant decrease in EDV and 3) cell therapy appears safe.

Autologous vs. allogeneic cell therapy

To the best of our knowledge, no direct comparative preclinical study of autologous and allogeneic cell therapy for myocardial ischemia has been reported. However, safety and efficacy of both autologous and allogeneic MSC therapy in ischemic cardiomyopathy has been compared in the POSEIDON study (Percutaneous Stem Cell Injection Delivery Effects on Neomyogenesis).[25] Overall, both end systolic and end diastolic volumes decreased and a non-significant decrease in LVEF of 2,0% was observed, without any difference between autologous and allogeneic cell sources. Authors concluded that both allogeneic and autologous cell therapy is safe and demonstrates potential regenerative activity. No increased antibody response was seen in patients receiving allogeneic MSCs.

Immunological issues are of great interest in allogeneic cell therapy for cardiac repair. Alloreactivity depends on presenting foreign peptides to T-cells by MHC-complexes on antigen-presenting cells. Immunosuppression (*i.e.* tacrolimus, cyclosporin, HLA matching)

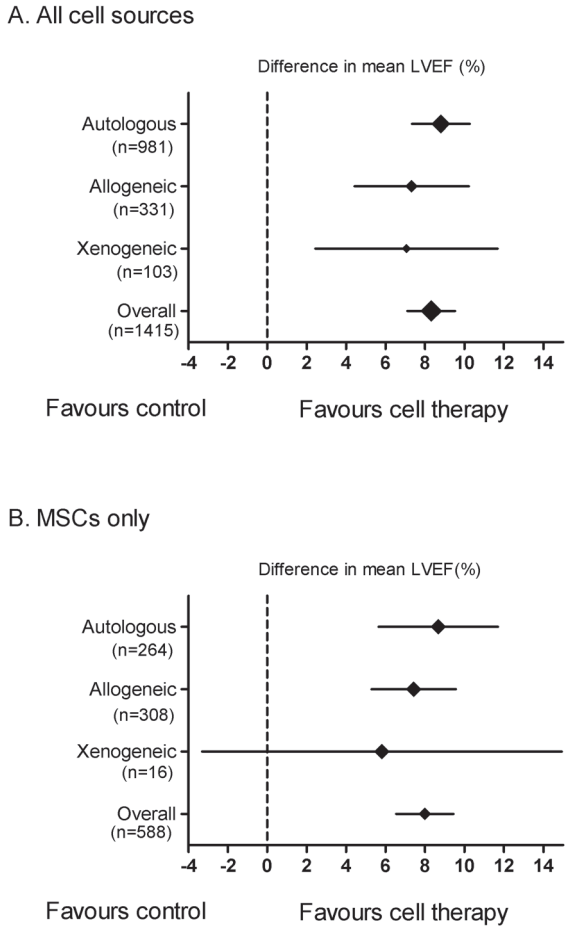

Figure 3. Comparison of cell source Effect size and 95% confidence intervals of different cell sources based on meta-regression, for Meta-regression of all celltypes (A) and for mesenchymal stem cells alone (B).

might be needed to improve cell engraftment over time.[26,27] Cardiac repair by cell therapy is more often thought to act via paracrine signalling, rather than true regeneration by differentiation of transplanted cells.[28,29] Prolonged presence of transplanted cells, and thus (sustained) immunosuppression, might not be essential for cardiac repair by paracrine effects. We hypothesize that the mechanism of action and the need for immunosuppression differs for various stages of disease, treatment goals and cell types.

MSCs are the most commonly used cell type in clinical and pre-clinical setting (pre-clinical see Figure 2), and are often regarded as the ideal and 'universal' cell type.[30] In our analysis, 88% of animals treated with allogeneic cells received MSCs (*i.e.* 300 of 352 see Figure 3). None of them received immunosuppression. Immunosuppression for MSCs might be redundant, since these cells are considered by some to be 'immuno privileged'.[31] However, MSCs do interact with the immune system, play a role in immunomodulation[32,33], and even elicit immune responses *in vivo*.[34] We performed a post hoc meta-regression of cell source for MSCs alone to establish whether there was any evidence of immunoprivileged properties for MSCs in cardiac repair. Interestingly, allogeneic MSCs appeared to be as

effective as autologous MSCs, suggesting either that MSCs do not elicit an immune response, or that their mechanism of action does not require resistance to immune attack and clearance. We were unable to perform post-hoc analysis on immunosuppression within the allogeneic subgroup, since only one study used immunosuppression.[23] In this study, allogeneic PMultistem cells were surgically injected in a model of acute myocardial infarction, with and without cyclosporin immunosuppression. LVEF was significantly increased after cell therapy and this effect was independent of the presence of cyclosporin; further, cyclosporin did not increase cell engraftment. The authors speculated that rejection mechanisms may have limited activity in these models, or that apoptosis of some transplanted cells might itself have immunosuppressive consequences.

Non-cardiac meta-analyses of stem cell therapy

Four meta-analyses concerning stem cell therapy in animal models in other areas of medicine are found in literature.[35–38] Lees et al. conducted a meta-analysis of pre-clinical data of stem cell therapy for experimental stroke (119 studies, 2704 animals).[35] In contrast to our analysis, differences in effect size between cell sources are observed. For functional outcome efficacy was higher for allogeneic cells, but autologous cells did better for infarct volume. Immunosuppression by cyclosporin had a positive effect on functional outcome, but not on infarct volume. The need for sustained survival of cells and the requirement of integration of transplanted cells in experimental stroke is questioned in this paper as well. In the meta-analysis of Antonic et al. about cell transplantation in traumatic spinal cord injury, only one of the included 156 articles used autologous stem cells.[38] Overall, allogeneic cells improve motor and sensory outcomes. Any kind of immunosuppression significantly decreased efficacy, where cysclosporin combined with methylprednisolone performed even worse than cyclosporine alone. Authors suggest that in their analysis, the beneficial effect of immunosuppressants is outweighed by other factors (*i.e.* stem cell biology, intrinsic repair mechanisms).

Oliveri et al. investigated the locomotor recovery by MSCs in rat models of traumatic spinal cord injury.[37] In this meta-analysis of 83 studies including 15668 rats, 57% of studies used non-autologous cells. In these studies 28% of cells were administered in combination with immunosuppression, predominantly cyclosporin. Cell source was a significant predictor for improved outcome as autologous and allogeneic cells performed better than xenogeneic and syngeneic cells. These differences are not further discussed by the authors. Immunosuppressive status in allogeneic or xenogeneic cell therapy, was no significant predictor for locomotor outcome. Authors describe the anti-inflammatory, immunomodulatory and anti-apoptotic properties of MSs. The lack of contribution of immunosuppression is explained by the hypo-immunogenic properties of MSCs and the absence of long-term engraftment. Wang et al. analysed 21 pre-clinical studies, including 382 animals, concerning MSC therapy for renal impairment, but did not address differences in cell source and immunological issues.[39]

Translatability of pre-clinical studies

Large animal models are generally used in medicine for development and validation of new therapies, but their usefulness has been questioned. The CAMARADES working group aims to provide evidence to inform translational medicine and investigates the translatability

of *in vivo* studies using systematic approaches, including meta-analyses.[40,41] Poor quality and in particular flaws in internal and external validity turn out to be significant predictors of outcome, affecting translation towards clinical practice.[35,40,42] The relative high effect size compared to clinical studies[1–4] and the dominance of positive studies might imply presence of flaws in validity or presence of publication bias against negative results. In our assessment of publication bias, Egger regression suggests asymmetry in the funnel plot, but trim and fill did not identify missing studies. This is consistent with previous data that suggests trim and fill may lack statistical power compared to Egger regression.[40] Further, asymmetry in the funnel plot may be caused by other factors than publication bias which is a limitation of these methods.

Publication bias is a serious problem in both clinical and pre-clinical studies,[40,43] and impedes transition from pre-clinical towards clinical studies, by skewing the expected effect size. It is known from pre-clinical studies in stroke, that publication bias causes an relative overestimation of effect size of 31.1%.[40] Largest contributors to publication bias are authors or researchers not willing to put effort in publishing negative results and editors who tend to select papers that are most exciting.[44,45] Therefore, we call for registration of pre-clinical trials upfront[46] and for tendency of editors to accept neutral or negative results for publication.

Flaws in internal and external validity can partly be solved by randomisation and blinding. In the current analysis, the quality of included studies was considered low. However, the reported prevalence of randomisation and blinding are substantially higher than observed in most other systematic reviews of preclinical data, but we consider this still not to be of sufficient quality to be robust. Interestingly, randomisation and blinding were no significant predictors for effect size, nor was total quality score. (Supplemental material Figure 2B) This may be a limitation of using reported study quality as a proxy for how experiments were performed; too few studies detail the methods used to blind or randomise to allow detailed analysis of their susceptibility to bias. It is entirely plausible that some studies were performed in a blinded and randomised manner but this was not explicitly stated by the authors, or the vice versa. We believe that providing empirical evidence of the poor reporting of measures to reduce the risk of bias will encourage the field to report both the performance of these measures and also details of the methods used.

By adding 30 new studies, we are able to reproduce the significant increase in LVEF we found in the previous meta-analysis.[18] The slight increase in effect size (7,3% in the previous analysis, 8,3% in this analysis) might imply that pre-clinical research is improving and focusing on the right issues. Based on statistics, the number of parameters included in the current meta-regression was limited to 10. Therefore we were not able to analyse other relevant issues, like animal species or duration of follow up. Fortunately, we included several parameters in the sensitivity analysis in the previous analysis where we showed no difference in animal species and duration of follow up.[18]

Limitations

In this meta-analysis we used the best available evidence to assess differences in the effects of autologous and allogeneic cell therapy for myocardial infarction. This exploration of the literature is a post-hoc analysis of the data and as such is considered hypothesis generating rather than confirmatory.

We identified a number of limitations in the preclinical studies included in this review, and subsequently this meta-analysis should be interpreted with caution. LVEF is considered the golden standard outcome measure and the reporting of other outcomes was less robust. Infarct size, for example, was reported in a small subset of studies, and the methods used to assess infarct size and the units in which they were presented differed greatly between these studies. Therefore, we were unable to include infarct size as one of the outcome parameters. In addition, the reporting of mortality appears to be less rigorous in preclinical studies compared to clinical studies; mortality was reported in only 74 of the 125 comparisons included. Studies that did report mortality did not show a difference between treatment groups, but this may be an artefact of limited reporting.

A notable feature of these animal data is the limited generalisability to humans in a clinical setting. Patients suffering from ischemic heart disease are usually old, and exposed to several risk factors, in contrast to the young healthy animals often used to model the disease. This might explain the larger effect size in our analysis, compared to that reported in clinical data.[1–4] Moreover, the lack of exposure of autologous cells to risk factors in a preclinical setting is discordant to the autologous cells of a patient in a clinical setting. Therefore, we hypothesise a greater difference in effect sizes between pre-clinical and clinical studies where autologous cell therapy is used compared to allogeneic cell therapy. We are unable to provide empirical evidence of the added value of immunosuppression in allogeneic cell therapy, as almost all allogeneic studies were performed without immunosuppression. However, LVEF is improved by allogeneic cell therapy compared to placebo, suggesting that allogeneic cell therapy can be performed without immunosuppression.

Conclusion

In pre-clinical studies of cell therapy for cardiac repair, allogeneic cells are associated with a similar magnitude of effect as autologous cells. The majority of these allogeneic cells were MSCs. Based on the logistical and practical advantages of allogeneic cell sources and our data presented here, we support future clinical trials of MSCs for cardiac repair to focus on allogeneic cell therapy, without the use of immunosuppressive therapy.

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SUPPLEMENTARY MATERIAL

Detailed methods
Search used in electronic databases:
((pig OR porcine OR dog OR canine OR sheep OR ovine)
AND
(stem cells OR progenitor cells OR bone marrow))
AND
(myocardial infarction OR heart failure OR coronary artery disease OR cardiac repair OR myocardial regeneration)

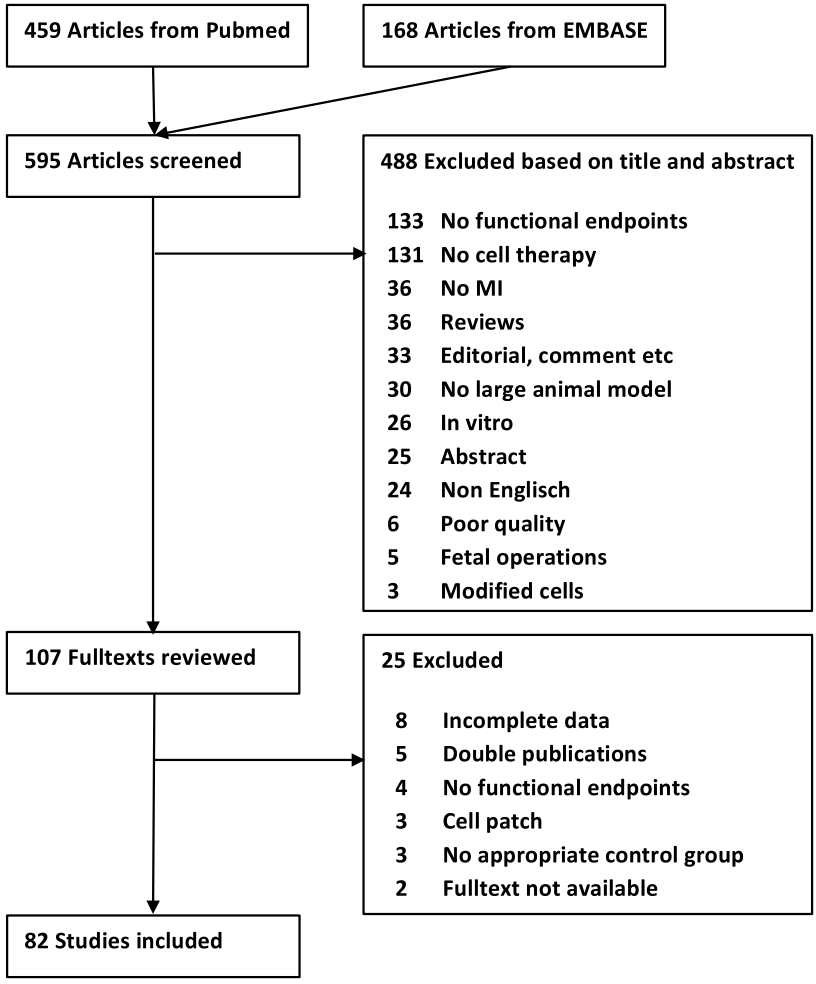

Figure 1. Flow chart of excluded and included studies

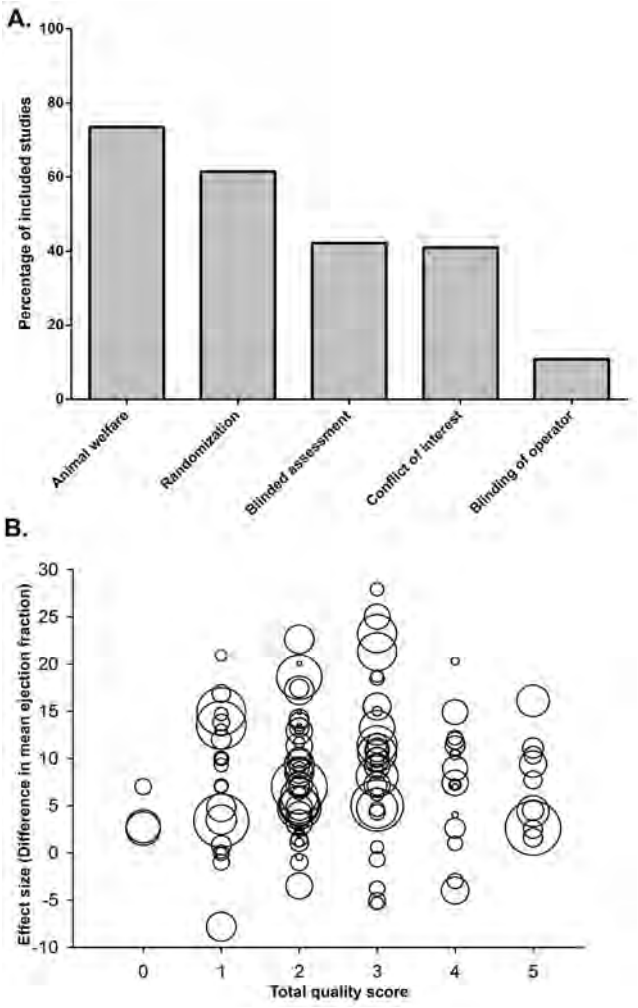

Figure 2. Quality of included studies
A. Quality of included studies presented as percentage of studies reporting individual parameters. B. Bubble plot of the meta-regression for total quality score (out of 5), where each study is plotted against its quality score. Larger bubbles represent more precise studies, based on inverse standard error.

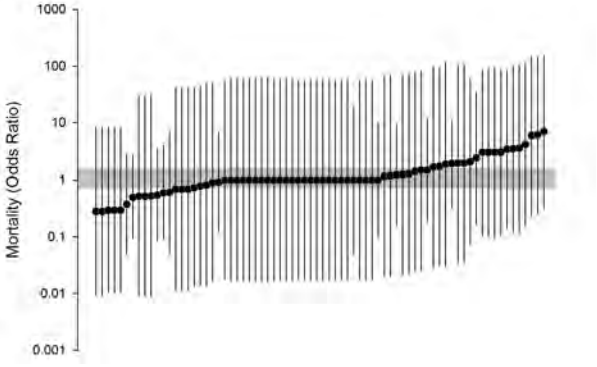

Figure 3. Mortality
Timber plot of odds ratio of mortality in cell treated and placebo animals per study. Vertical error bars represents 95% confidence intervals of individual studies. The grey bar represents the 95% confidence interval of the mean odds ratio.

Table 1. Study characteristics of included studies

First author	Year	Animal	N (control)	N (treatment)	Location	Type of ischemia	Cell type	Dose	Cell source	Delivery route	Timing of treatment (days)	Endpoint assessment
Moelker	2006	Pig	10	10	LCX	Ischemia/Reperfusion	BMMNC	5,0E+08	Autologous	Intracoronary	7	MRI
				10			Bone marrow	5,0E+08				
Moelker	2007	Pig	6	6	LCX	Ischemia/Reperfusion	USSC	1,0E+08	Xenogeneic	Intracoronary	7	MRI
Niccoli-Asabella	2011	Pig	4	7	LAD	Permanent	BMSC	5,0E+07	Autologous	Intramyocardial	≤1	SPECT
Pätilä	2009	Pig	6	9	LCX	Permanent	Skeletal myoblast	2,0E+06	Autologous	Intramyocardial	14	MRI
Peng	2011	Pig	5	5	LAD	Ischemia/Reperfusion	MSC	1,6E+08	Autologous	Intracoronary	7	Echocardiography
Perin	2008	Dog	6	5	LAD	Ischemia/Reperfusion	MSC	1,0E+08	Autologous	Intracoronary	7	Echocardiography
				6						Transendocardial		
Price	2006	Pig	8	7	LAD	Ischemia/Reperfusion	MSC	3,2E+08	Allogeneic	Intravenous	≤1	Left ventriculography
Qi	2008	Pig	6	7 c	LAD	Ischemia/Reperfusion	MSC	1,0E+08	Autologous	Intracoronary	5	MRI
				7 d								
Qian	2007	Pig	6	6	LAD	Ischemia/Reperfusion	BMMNC	1,0E+09	Autologous	Intracoronary	7	MRI
Quevedo	2009	Pig	4	6	LAD	Ischemia/Reperfusion	MSC	2,0E+08	Allogeneic	Transendocardial	84	MRI
Rigol	2010	Pig	4	6	LAD	Ischemia/Reperfusion	ADSC	2,3E+07	Autologous	Transendocardial	7	Intracardiac echo
			4	5				1,6E+07		Intracoronary		
Sato	2011	Pig	5	8	LAD	Permanent	MSC	1,0E+07	Autologous	Intraventricular vein	28	Left ventriculography
Schneider	2009	Pig	8	6	LCX	Permanent	BMMNC	1,7E+07	Autologous	Transendocardial	14	Echocardiography
				4			MSC	unknown	Allogeneic			
				5			MSC	unknown	Autologous			
Schuleri	2008	Pig	6	9	LAD	Ischemia/Reperfusion	MSC	2,0E+08	Autologous	Transendocardial	2	MRI
Schuleri	2009	Pig	6	6	LAD	Ischemia/Reperfusion	MSC	2,0E+08	Autologous	Intramyocardial	111	MRI
Schuleri	2011	Pig	8	8	LAD	Ischemia/Reperfusion	MSC	2,0E+08	Autologous	Intramyocardial	84	MRI
Sheu	2009	Pig	6	6 b	LAD	Permanent	BMMNC	3,0E+07	Autologous	Intramyocardial	≤1	Echocardiography
				6 a								
Silva	2005	Dog	6	6	LAD	Permanent	MSC	1,0E+08	Allogeneic	Intramyocardial	30	Echocardiography
Simioniuc	2011	Pig	7	7	LAD	Permanent	Placental MSC	1,0E+07	Xenogeneic	Intramyocardial	≤1	MRI
Thompson	2005	Pig	4	4	LAD	Permanent	BMMNC	3,0E+08	Autologous	Transvenous injections	28	Left ventriculography
Tomita	2002	Pig	6	5	LAD	Permanent	BMSC	1,0E+08	Autologous	Intramyocardial	28	SPECT
Valina	2007	Pig	7	7	LAD	Ischemia/Reperfusion	MSC	2,0E+06	Autologous	Intracoronary	≤1	SPECT
				7			ADSC					
Wang D.	2011	Pig	10	12	LAD	Ischemia/Reperfusion	MSC	3,0E+08	Allogeneic	Intracoronary	≤1	Left ventriculography
Wang X.	2009	Pig	6	6	LAD	Ischemia/Reperfusion	MSC	5,0E+07	Allogeneic	Transcoronary	≤1	MRI
Williams	2012	Pig	5	5	LAD	Ischemia/Reperfusion	CSC/MSC	2,0E+08	Xenogeneic	Intramyocardial	14	MRI
				5			CSC	1,0E+06				
				5			MSC	2,0E+08				

Quality score	EDV(ml) control	EDV(ml) treatment	EDV Effect Size (ml)	ESV(ml) control	ESV(ml) treatment	ESV Effect Size (ml)	EF(%) control	EF(%) treatment	Effect Size
3	128 ± 23.3	133.8 ± 26.2	5.8 ± 13.31	70 ± 20	66 ± 12	-4 ± 9.72	47,2 ± 8,9	43,4 ± 7,7	-3,8 ± 4,67
		116.4 ± 14.5	-11.6 ± 11.38		78	8 ± 10.95		42,1 ± 5,1	-5,1 ± 4,29
2	124.4 ± 8.9	151.1 ± 11.1	26.7 ± 5.81	66.1 ± 7	87 ± 10.4	20.9 ± 5.12	45,2 ± 6,1	41,7 ± 2,6	-3,5 ± 2,71
2	9825 ± 1.7	87.7 ± 6.9	-10.55 ± 2.74	6.75 ± 3.3	43 ± 2.6	-20.75 ± 1.92	32,3 ± 2,6	50,9 ± 2,5	18,6 ± 1,61
2	102.54 ± 55.85	130.73 ± 94.77	28.19 ± 38.96	49.22 ± 38.7	63.16 ± 53.5	13.94 ± 23.83	52,45 ± 20,62	52,01 ± 14,4	-0,44 ± 9,69
4	69.89 ± 7.64	46.31 ± 6.18	-23.58 ± 4.39	19.68 ± 3.29	11.84 ± 2.96	-7.84 ± 1.98	68,63 ± 5,62	79,98 ± 5,86	11,35 ± 3,63
2							35,5 ± 3,3	36,6 ± 7,7	1,1 ± 3,94
								48,8 ± 7,7	13,3 ± 3,68
2							44 ± 4	49 ± 3	5 ± 1,81
1							41,87 ± 2,45	56,85 ± 1,29	14,98 ± 1,50
	112.33 ± 3.93	102.5 ± 4.6	-9.83 ± 2.37	62.5 ± 1.05	46.67 ± 3.88	15.83 ± 1.53		51,2 ± 10,4	7,0 ± 5,4
1	66.7 ± 5.3	63.3 ± 7.8	-3.4 ± 3.85	38.5 ± 7.3	31.5 ± 10.3	-7 ± 5.15	44,2 ± 8,2	51,2 ± 10,4	7,0 ± 5,41
2							32,2 ± 4,7	41,3 ± 6,6	9,1 ± 3,58
1	34.1 ± 5.9	38.4 ± 13.9	4.3 ± 6.40	16.6 ± 3.2	17.7 ± 2.9	1.1 ± 1.99	51 ± 8	51 ± 12	0 ± 6,32
	27.3 ± 3.9	32 ± 4.8	4.7 ± 2.90	13.8 ± 1.3	16.2 ± 2.7	2.4 ± 1.37	49 ± 2	49 ± 10	0 ± 4,58
3	30.8 ± 10.9	35.1 ± 13.6	4.3 ± 6.85				40,3 ± 6,8	44,6 ± 9,4	4,3 ± 4,50
3	63.6 ± 8.3	59.3 ± 4.8	-4.3 ± 5.45	40.00 ± 8.10	34.51 ± 4.68	-5.49 ± 5.32	37,1 ± 2,4	41,8 ± 2,4	4,7 ± 1,77
		57.7 ± 13.6	-5.9 ± 8.49		30.18 ± 13.29	9.83 ± 8.29		47,7 ± 2,3	10,6 ± 1,87
		58.2 ± 10.2	-5.4 ± 6.83		31.89 ± 9.99	-8.11 ± 6.67		45,2 ± 2,1	8,1 ± 1,74
3							30,2 ± 2	39,4 ± 7,8	9,2 ± 2,84
4							34,6 ± 12,5	54,9 ± 12,5	20,3 ± 8,84
2	65.1 ± 2.8 *	53.5 ± 4.6 *	-11.6 ± 5.24	48.3 ± 3.7 *	36.4 ± 4.1 *	-11.9 ± 5.52	27,8 ± 1,9 *	41,7 ± 3,3 *	13,9 ± 3,81
2							46,8 ± 4	50,0 ± 3,7	3,2 ± 2,76
								58,1 ± 3,6	11,3 ± 2,74
2							28,16 ± 13,22 *	48,22 ± 6,53 *	20,06 ± 14,74
3	68.5 ± 4.5 *	75.58 ± 9.2 *	7.08 ± 10.24	24 ± 1.7 *	31.33 ± 7.6 *	7.33 ± 7.79	64,9 ± 1,5 *	59,5 ± 5,3 *	-5,36 ± 5,51
2							34,3 ± 6,4	48,5 ± 3,6	14,2 ± 3,67
2							37 ± 16	50 ± 9	13 ± 7,67
5							27,7 ± 6,3	29,3 ± 4,4	1,6 ± 3,76
								30,2 ± 4,7	2,5 ± 3,81
2							40,76 ± 6,48	49,52 ± 6,1	8,76 ± 2,70
3							35,4 ± 3,2	46,3 ± 6,6	10,9 ± 2,99
1	106.1 ± 21.17	95 ± 14.9	-11.1 ± 17.70	74.38 ± 20.52	53 ± 7.91	-21.38 ± 16.28	30 ± 4,61	43,8 ± 5,4	13,8 ± 4,31
		109.2 ± 21.96	3.1 ± 19.11		67.2 ± 19.19	-7.18 ± 18.06		39,3 ± 8,36	9,3 ± 5,17
		105.1 ± 23.99	-1 ± 19.60		67.4 ± 22.46	-6.98 ± 18.80		37,1 ± 8,32	7,1 ± 5,16

Table 1. Continued

First author	Year	Animal	N (control)	N (treatment)		Location	Type of ischemia	Cell type	Dose	Cell source	Delivery route	Timing of treatment (days)	Endpoint assessment
Wojakowski	2012	Pig	5	5		LAD	Ischemia/Reperfusion	BMMNC	unknown	Autologous	Intracoronary	≤1	Echocardiography
Yang Y.	2009	Pig	6	6		LAD	Ischemia/Reperfusion	MSC	3,0E+07	Autologous	Intramyocardial	≤1	MRI
Yang Z.	2006	Pig	6	6		LAD	Permanent	MSC	5,0E+06	Autologous	Intracoronary	28	SPECT
Yang Z.	2007	Pig	6	6		LAD	Permanent	BMSC	5,0E+06	Autologous	Intracoronary	28	SPECT
Yang K.	2011	Pig	5	10	e	LAD	Ischemia/Reperfusion	MSC	1,4E+08	Autologous	Intracoronary	10,5	MRI
				10	f								
Yi	2006	Pig	6	8		LAD		MSC	8,0E+07	Autologous	Transendocardial	28	Echocardiography
Yokoyama	2006	Pig	5	6		LAD	Permanent	BMMNC	3,2E+09	Autologous	Coronary venous	≤1	PV loop
			5	5								14	
Yu	2010	Pig	6	6		LAD	Permanent	MSC	1,0E+07	Autologous	Intracoronary	28	Echocardiography
Zeng	2007	Pig	9	7		LAD	Permanent	pMultistemcell	5,0E+07	Allogeneic	Intramyocardial	≤1	MRI
				6	g								
Zhang S.	2012	Pig	4	4	h	LAD	Permanent	EPC	5,0E+07	Autologous	Intramyocardial	28	Echocardiography
			5	5	i							28	
			5	4	h							14	
			5	4	i							14	
			4	3	h							≤1	
			5	4	i							≤1	
Zhang S.	2007	Pig	5	5	h	LAD	Permanent	BMSC	5,0E+07	Autologous	Intracoronary	14	Echocardiography
			5	5	j								

LAD Left anterior descending coronary artery, LCX Left circumflex coronary artery, BMMNC Bone marrow mononuclear cell, MSC Mesenchymal stem cell, EPC Endothelial progenitor cell, USSC Unrestricted somatic stem cell, PBMNC Peripherial blood mononuclear cell, BMSC Bone marrow stromal cell, CDC Cardiac stem cell, CSph Cardiosphere derived cell, ADSC Adipose derived stem cell. EDV (ml), ESV (ml) and EF(%) for control and treated groups is presented as mean ± SD. Effect size is presented as mean ± SEM. Quality score out of 5. * = Mean ± SEM. Subgroups: a Infarct related artery, b non-Infarct related artery, c unlabeld cells, d labelled, e GFP labelled, f dual labelled, g Cyclosporin treated animals, h Rentrop score 0, i Rentrop score 2, j Rentrop score 1.

Quality score	EDV(ml) control	EDV(ml) treatment	EDV Effect Size (ml)	ESV(ml) control	ESV(ml) treatment	ESV Effect Size (ml)	EF(%) control	EF(%) treatment	Effect Size
3							39,60 ± 0,57 *	52,84 ± 1,98 *	13,24 ± 2,06
3	67.2 ± 6.6	65.2 ± 5.8	-2 ± 3.59	39.2 ± 7.3	38.5 ± 8.6	-0.7 ± 4.61	42 ± 7,1	41,3 ± 8,8	-0,7 ± 4,62
1							30,3 ± 11,3	45 ± 6,6	14,7 ± 5,34
2							30,36 ± 2,76 *	47,78 ± 2,64 *	17,42 ± 3,82
5	69.86 ± 7.64	48.68 ± 6.09	-21.18 ± 5.20	19.68 ± 3.29	13.47 ± 2.48	-6.21 ± 2.22	38,63 ± 5,62	46,35 ±5,61	7,72 ± 3,97
		46.31 ± 6.18	-23.55 ± 5.21		11.84 ± 2.96	-7.84 ± 2.28		48,98 ± 5,86	10,3 ± 4,01
2							35,6 ± 10,4	47,3 ± 13,4	11,7 ± 6,36
2	111.4 ± 7.1	95.6 ± 7.1	-12.8 ± 4.30				27,3 ± 3,3	36,7 ± 4,7	9,4 ± 2,42
	110.7 ± 8.6	101.4 ± 7.1	-9.3 ± 4.99				28,6 ± 3,6	37,1 ± 4,3	8,5 ± 2,51
1							35,94 ± 2,31 *	52,80 ± 3,21 *	16,86 ± 3,95
3							30,4 ± 5,4	42,0 ± 6,5	11,6 ± 3,53
								41,2 ± 5,0	10,8 ± 3,26
5	53.4 ± 5.2	42.8 ± 6.38	-10.6 ± 4.28	35.8 ± 5.8	26.3 ± 4.8	-9.5 ± 3.76	34,3 ± 4,5	38,8 ± 5,1	4,5 ± 3,40
	52.5 ± 3.8	41.7 ± 5.3	-10.8 ± 2.92	33.2 ± 5.4	25.3 ± 3.5	-7.9 ± 2.88	36,7 ± 1,7	39,3 ± 2,4	2,6 ± 1,32
	48.4 ± 4.6	37.4 ± 7.4	-11 ± 4.23	31.6 ± 1.8	23.6 ± 3.2	-8 ± 1.79	34,0 ± 3,0	45,1 ± 6,3	11,1 ± 3,42
	48.4 ± 8.4	29.1 ± 3.7	-19.3 ± 4.19	30.7 ± 6.8	13.7 ± 2.6	-17 ± 3.31	36,9 ± 3,6	53,0 ± 3,1	16,1 ± 2,23
	51.3 ± 3.9	40.6 ± 7.2	-10.7 ± 4.59	33.6 ± 1.5	24.9 ± 3	-8.7 ± 1.89	34,6 ± 1,1	39,0 ± 3,8	4,4 ± 2,26
	48.8 ± 7.6	36.1 ± 5.3	-12.7 ± 4.31	29.8 ± 3.1	20.1 ± 1.9	-9.7 ± 1.68	35,4 ± 1,7	44,8 ± 5,1	9,4 ± 2,66
2							41,4 ± 5,2	58,6 ± 1,8	17,2 ± 2,46
							43,8 ± 1,8	66,4 ± 5,2	22,6 ± 2,46

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PART TWO
METHODS IN PRECLINICAL RESEARCH FOR CARDIAC REPAIR

CHAPTER 7

MYOCARDIAL INFARCTION AND FUNCTIONAL OUTCOME ASSESSMENT IN PIGS

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ABSTRACT

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

Video Link

The video component of this article can be found at http://www.jove.com/video/51269/.

INTRODUCTION

Acute myocardial infarction (AMI) and its long-term sequelae such as chronic heart failure (CHF) profoundly impact patient prognosis and quality of life, let alone the high cost restraints imposed on our available healthcare resources.[1] The prevalence of CHF in the western world is estimated at 1-2%, of which ~60% of cases are the consequence of AMI as primary cause.[2] In the USA alone, about 5.7 million patients suffer from CHF accounting for approximately $30 billion in annual healthcare costs in 2008, with a predicted triplicate in costs rising to $97 billion annually in 2030.[1] Taken together, these numbers make a strong argument for the development of new cardioregenerative treatments that, for swift translation, rely on a reproducible and reliable large animal myocardial infarction model that accurately mimics the human scenario.

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

Protocol

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

1. Medication, Anesthesia, Venous Access, and Intubation

Medication and Anesthesia

Premedication

1. Start amiodarone 150 mg/kg 10 days prior to surgery to prevent arrhythmias. Continue amiodarone in a dose of 100 mg/kg from the day of the procedure until day 28. Reduce the dosage to 50 mg/kg on day 29 and continue until the end of the study.
2. Start anti-platelet therapy, 1 mg/kg clopidogrel from day 3 prior to surgery and 4.5 mg/kg acetylsalicylic acid 1 day before surgery. Continue clopidogrel 1 mg/kg per day and acetylsalicylic acid 80 mg/day.
3. Start pain medication 1 day prior to surgery by Fentanyl patch, 25 µg/hr for ~70 kg pigs. Continue the use of Fentanyl patches for 24 hr post-operatively to ensure adequate pain medication. Also, daily monitor for signs of putative discomfort (*i.e.* behavior, breathing, gait, mobility, *etc.*) to increase pain medication.
4. Fast the animal for 12 hr, maintain unlimited access to water.

Anesthesia

1. To prevent unnecessary stress and discomfort, sedate the pig in its stable by intramuscular injection of a mixture of midazolam 0.4 mg/kg, ketamine 10 mg/kg, and atropine 0.014 mg/kg.
2. Obtain venous access by cannulating the ear vein with an 18 G i.v. cannula. Induce anesthesia by intravenous administration of 5 mg/kg sodiumthiopental, and give 1,000/100 mg amoxicillin/clavulanic acid to prevent infections. The pig will receive 1,000/100 mg amoxicillin/clavulanic acid the day after surgery as antibiotic treatment.
3. Intubate the pig by using an endotracheal tube (size 8.5 for pigs weighing ~70 kg). If necessary perform balloon-ventilation with a frequency of 12/min and transport the pig to the operating theater.
4. At arrival on the operation theater, immediately start mechanical positive pressure ventilation with FiO2 0.50, 10 ml/kg tidal volume, and a frequency of 12/min under continues capnography.
5. Start balanced anesthesia by continues intravenous infusion of a combination of midazolam 0.5 mg/kg/hr, sufentanil 2.5 microg/kg/hr and pancuronium bromide 0.1 mg/kg/hr. During the entire operation, continuously monitor ECG, arterial blood pressure, temperature and capnography to measure depth of anesthesia. For example, if a sinus tachycardia is present, check if pain medication and/or anesthesia are adequate.
6. Infuse 4.3 mg/kg of amiodarone in 500 ml venofundin 6% intravenously.
7. Monitor heart rhythm by a 5 leads ECG.
8. Shave and clean the neck
9. Insert a Foley catheter.

2. Transthoracic Echocardiography

1. Place the animal in the right lateral position. In landrace pigs such as Dalland pigs, only parasternal views (long and short axis) can be obtained. Due to the shape of the thorax, apical view acquisition is not feasible.
2. Orient and obtain parasternal long axis view in 2D (B-mode). Determine the LV dimensions at end diastole and end systole in M-mode.
3. Rotate the echo probe 90° clockwise whilst maintaining its parasternal position to acquire the LV short axis views at the levels of the mitral valve, papillary muscle and apex. The short axis view of the papillary muscle and apex may require placement of the echo probe one or two intercostal spaces lower relative to the position for the mitral valve short axis view.

3. Surgical Preparation and Vascular Access

1. Disinfect the surgical areas with iodine 2% and use sterile surgical drapes to cover the nonsterile parts of the pig.
2. Make a medial incision in the neck. Pass the linea alba to minimize muscle damage and bluntly approach the carotid artery and internal jugular vein next to the trachea.
3. Carefully isolate the carotid artery and internal jugular vein. Make sure the vagal nerve is undamaged. Place Vicryl 2-0 sutures around both vessels to gain vessel control.

Achieve arterial access by cannulating the internal carotid artery with an 8F sheath using the Seldinger technique. Fix the sheath to the artery, make sure the artery is not fully occluded by the suture. Venous access can be acquired by cannulating the jugular vein with a 9F sheath also using the Seldinger technique. Before securing the sheath make sure the vein is ligated.

4. Alternatively, the femoral artery can also be used for arterial access.
5. Administer 100 IE/kg heparin immediately after inserting the sheaths to inhibit thrombus formation.
6. For a stable and constant arterial pressure measurement, cannulate one of the smaller arteries in one of the hind limbs by making a small incision above the artery. The artery is found just under the skin, pulsations can be felt through the skin. Isolate the artery from its surrounding tissue. Place 2 Vicryl 2-0 sutures around the vessel, 1 proximal and 1 distal. Ligate the distal side and insert an 18 G i.v. cannula and secure tightly connect the pressure.

4. Invasive Pressure Volume Loop Analysis

1. Insert a Swann-Ganz catheter (SG) via the previously placed sheath in the internal jugular vein.
2. Connect a cardiac output device to the part of the SG that culminates in the proximal lumen.
3. Inject 5 ml of 0.9% saline into the proximal lumen of the SG and measure cardiac output; repeat this three times and average the indices.
4. Calibrate the PV system by using the previously determined cardiac output.
5. Insert the 7F conductance catheter *via* the carotid artery into the left ventricle under fluoroscopic guidance.
6. Select the largest segment present in the LV for volume measurements and perform a baseline scan under apnea.
7. After volume calibration is completed, record 10-15 beats under apnea.
8. Remove the SG and place a balloon catheter in the inferior caval vein at the level of the diaphragm under fluoroscopic guidance.
9. Perform preload reduction by inflating the balloon under apnea and record the corresponding PV-Loops.

5. Intracoronary Pressure and Flow Measurement

1. Dilute nitroglycerin in a concentration of 100 µg/ml and dilute adenosine in a concentration of 30 µg/ml.
2. Position an 8F guiding catheter in the ostium of the left coronary artery.
3. Place the combined pressure/flow wire in the proximal part of the left coronary artery.
4. Administer 200 µg of nitroglycerin intracoronary and normalize the distal pressure (Pd), measured by the wire, with the arterial pressure.
5. Place the wire in the mid part of the left anterior descending artery (LAD).
6. Start measuring baseline pressure and flow. Induce hyperemia by administering 60 µg of adenosine intracoronary, flush with 2 ml of saline
7. and measure hyperemic pressure and flow. Wait for the flow to restore to baseline values. Repeat the measurement twice.

8. Infuse another 200 µg nitroglycerin intracoronary and repeat steps 1.6 and 1.7 for the left circumflex coronary artery.

6. Induction of MI

1. Place the intracardiac defibrillation catheter in the right ventricle using the venous sheath. The distal electrodes should be in the apex of the ventricle, the proximal electrodes in the atrium and/or superior caval vein. Connect the catheter to the defibrillator and set it to 50 J.
2. Measure the diameter of the LAD distal from the second diagonal (D2) in AP and LAO 30° view.
3. Choose an angioplasty balloon with a diameter according the diameter of the LAD distal from the D2 (Figure 1).
4. Position a guidewire through the guiding catheter distally in the LAD.
5. Advance the balloon catheter over the guidewire. Place the balloon distal from the D2.
6. Administer 30 IE/kg heparin.
7. Inflate the balloon until the pressure matches the right diameter of the LAD.
8. Check total occlusion of the LAD by angiography (Figure 1).
9. Cover the sterile working field and the wound in the neck with sterile drapes cloths. Free the chest from any coverage to make it available for chest compressions or transthoracic defibrillation.
10. Check the pressure in the balloon during the next 90 min and restore the pressure if necessary.
11. In case of ventricular fibrillation:
    1. Immediately start chest compressions with a frequency of 100/min.
    2. Administer 300 mg amiodarone intravenously as a fast bolus (~1 min).
    3. Start intracardiac defibrillation, give shocks of 50 J.
    4. After 5 unsuccessful shocks, restart chest compressions. Change intracardiac defibrillation to transthoracic defibrillation and shock with 150 J. In case of unsuccessful shock, change to 200 J.
    5. If necessary, administer another dose of 150 mg amiodaron and/or 1 mg adrenalin. Repeat adrenalin twice with intervals of 3-5 min, when necessary.
    6. Continue chest compressions, interspersed with transthoracic defibrillation

Finishing the Surgical Procedure (for Long Term Follow Up)

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

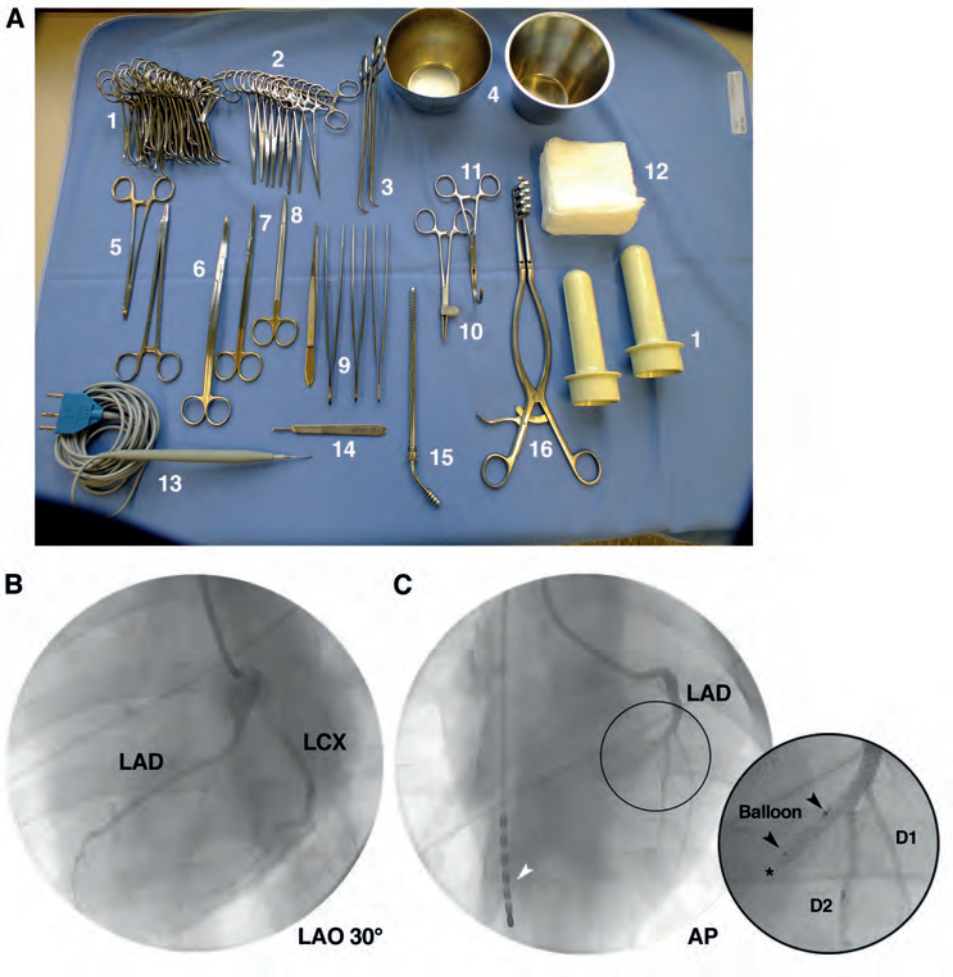

Figure 1. MI model based on LAD balloon occlusion
(A) Standard surgical equipment with: 1) towel clamps; 2) mosquitos; 3) dissecting forceps; 4) round container; 5) needle holders (fine and rough); 6) Klinkenberg scissor; 7) dissecting scissors (straight and curved); 9) forceps (De-Bakey, fine and rough);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

7. Cardiac Magnetic Resonance Imaging

1. Place the animal on the MRI table head first in the supine position under continuous anesthesia.
2. Place a dedicated phased-array cardiac coil over the chest of the animal.
3. For image planning obtain scout images in short axis and two-chamber long axis views.
4. Acquire ECG-gated steady-state free precession (SSFP) cine of short axis (from apex to base of LV) and two chamber long-axis views.
5. Late gadolinium enhancement (LGE) can be acquired using an inversion recovery 3D-turbo-gradient-echo-technique 15 min after doubledose i.v. bolus injection of a gadolinium based contrast agent.
6. Perform offline analysis with validated software of functional parameters. Assess left-ventricular ejection fraction (LVEF), LV mass, end diastolic volume, end systolic volume, stroke volume, cardiac output, and scar mass.

8. End of Study and Infarct Size

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

REPRESENTATIVE RESULTS

Mortality and Infarct Size

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

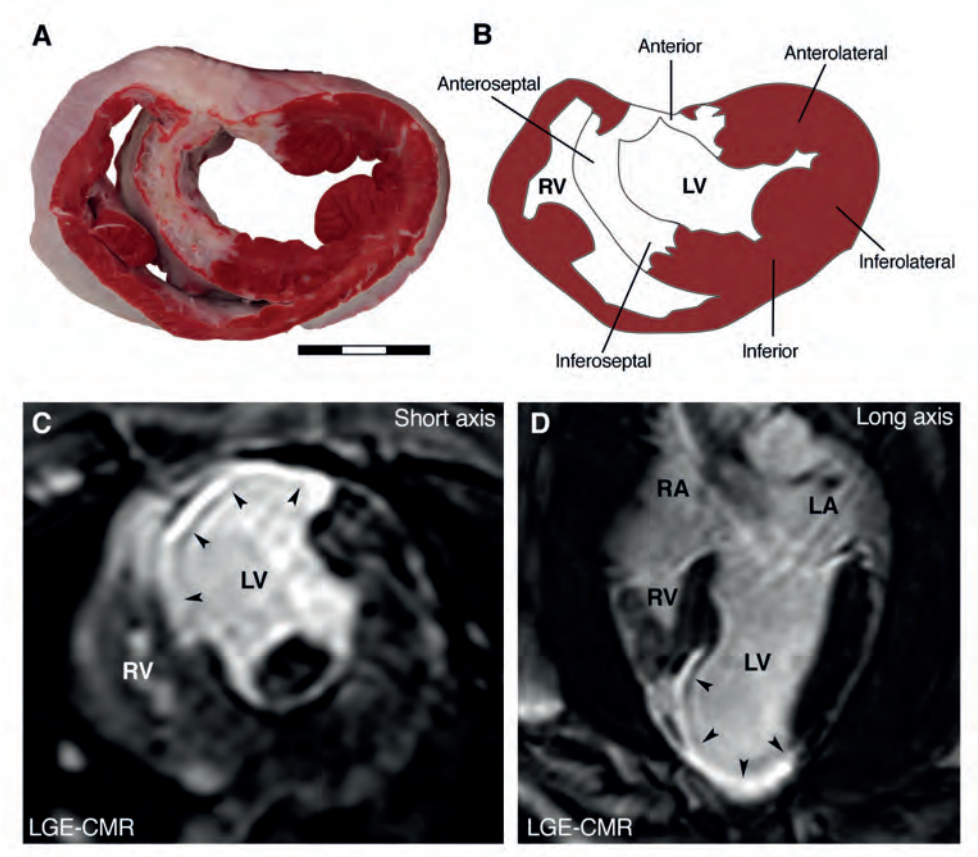

Figure 2. Infarct size after MI
(A) The 90 min balloon occlusion of the LAD leads to extensive myocardial damage and scar formation (white color), visualized by TTC staining at 1 month follow up. (B) Schematic infarct distribution shows that the infarction is located in the anterior, anteroseptal and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

Cardiac Function and Remodeling

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

Coronary flow and pressure parameters

Angiogenesis and formation of new capillaries are often regarded as important treatment goals in ischemic heart disease.[8] Assessment of microvascular resistance can be indirectly based on the combined measurement of intracoronary pressure and flow velocity.

Representative pressure and flow velocity measurement under normal conditions and maximal hyperemia is shown in Figure 4. Four weeks after MI, the hyperemic microvascular resistance should be increased in the infarct related coronary artery (LAD) compared to the baseline situation.[8]

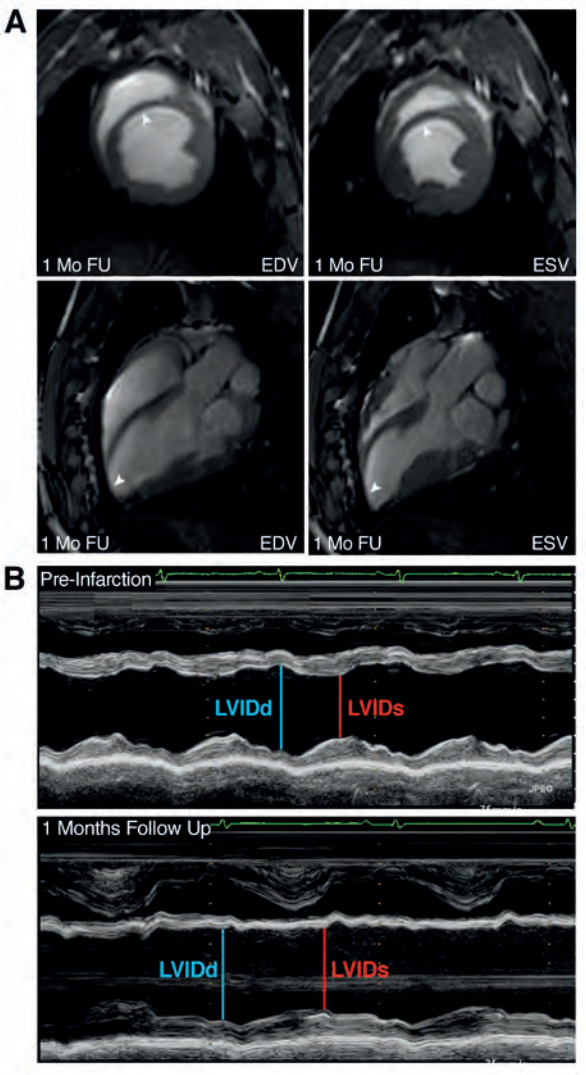

Figure 3. Assessment of cardiac function in ischemic MI models (A) Representative CMR cine-loop images at end diastole and end systole showing functional impairment of the infarct scar segments. (B) M-Mode image of 2D parasternal long axis by echocardiography, showing LV dilatation (increase in LVIDd)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

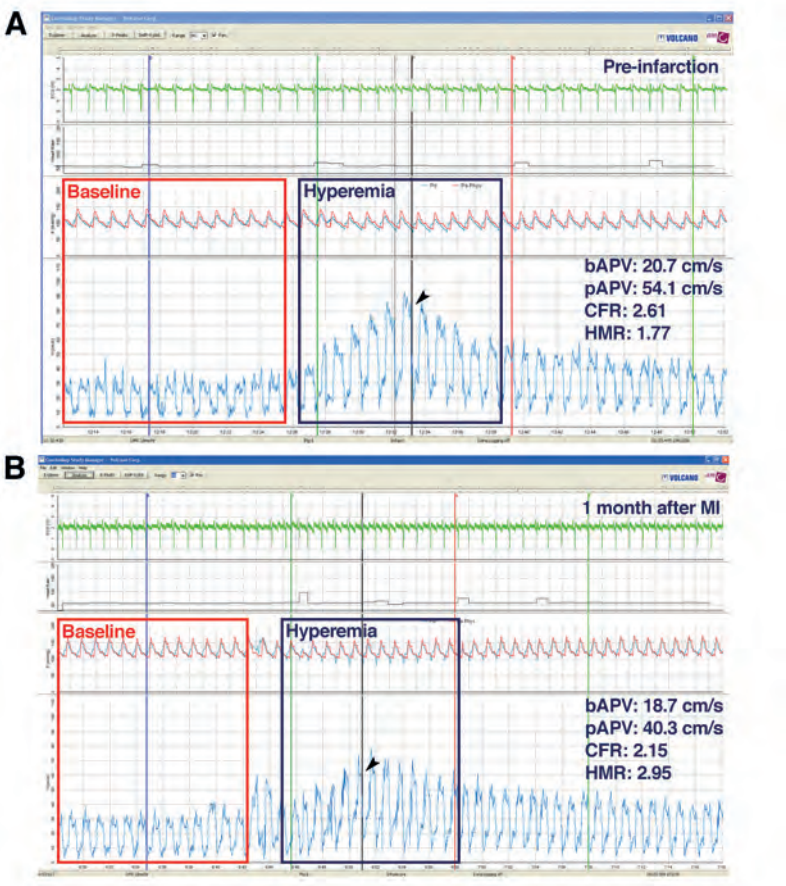

Figure 4. Intracoronary pressure and flow velocity derived parameters
Intracoronary pressure and flow velocity recordings using the Combowire showing (A) reference values prior to MI with high response to hyperemia (black arrowhead). (B) 1 month after MI, the infarct related artery (LAD) has a decreased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

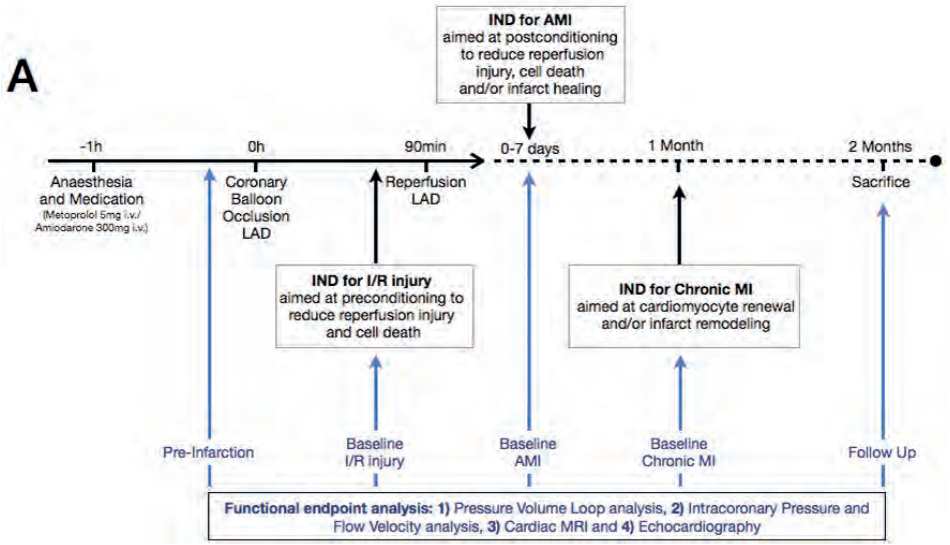

Figure 5. Overview of different study designs
Schematic of multiple possible study designs to validate investigational new drugs (INDs) in various stages of MI using this LAD MI pig model. Dependent on the chosen phase of MI that is under investigation, functional analysis can be performed just prior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

DISCUSSION

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

The success of the described protocol outlined here is dependent on the myocardial ischemia as the most critical phase of the protocol. Correct placement of the balloon distal to the second diagonal branch of the LAD is crucial for reaching adequate infarct size whilst ensuring a high survival rate. Based on this MI model, a ~15% mortality rate was observed, while extensive mid and apical segments of the anterior, septal and inferior walls were infarcted as seen on CMR and TTC staining (Figures 2A and 2B). The duration of ischemia can be tailored according to the desired infarct size. Although we have used Landrace pigs in this protocol, minipigs (*i.e.* Göttingen minipigs) usually require longer durations of myocardial ischemia (*e.g.* 150 min occlusion).

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

local improvement.[10] Therefore, the proposed measures of outcome used in this model shed light on different aspects of MI and post-MI remodeling thereby providing the investigators the means to accurately assess the efficacy of new therapies on multiple levels.

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

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

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

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CHAPTER 8

CYCLOSPORIN IN CELL THERAPY FOR CARDIAC REGENERATION

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ABSTRACT

Stem cell therapy is a promising strategy in promoting cardiac repair in the setting of ischemic heart disease. Clinical and pre-clinical studies have shown that cell therapy improves cardiac function. Whether autologous or allogeneic cells should be used, and the need for immunosuppression in non-autologous settings, is a matter of debate. Cyclosporin A (CsA) is frequently used in pre-clinical trials to reduce cell rejection after non-autologous cell therapy. The direct effect of CsA on the function and survival of stem cells is unclear. Furthermore, the appropriate daily dosage of CsA in animal models has not been established. In this review we discuss the pros and cons of the use of CsA on an array of stem cells both *in vitro* and *in vivo.* Furthermore, we present a small collection of data put forth by our group supporting the efficacy and safety of a specific daily CsA dosage in a pig model.

Cardiac regenerative therapy

Stem cell therapy for cardiac repair

Stem cell therapy (or progenitor- or precursor cell therapy) has emerged as a promising therapy for cardiac repair. Despite the presence of endogenous cardiac stem cells,[1,2] the heart's ability to self-renew is inadequate for compensating the extensive ischemic injury.[3] In the acute setting, delivery of stem cells may modulate the post-inflammatory response, while regeneration and prevention of further cardiac remodelling may be achieved in a more chronic phase. Apart from differentiation of stem cells into cardiomyocytes, a more likely mechanism of action is through paracrine signalling.[2–6] Paracrine signalling may reduce the inflammatory response, promote vasculogenesis, and stimulate endogenous (cardiac) stem cells.[7] Stem cell therapy has successfully been investigated for the recovery of cardiac function in ischemic heart disease in clinical and pre-clinical setting.[8–10] Although these results are promising, low delivery efficiency and engraftment rates (≤10%) should be emphasized.[5,11–14] Mechanical washout and/or loss, cell death,[15] and redistribution to other organs[12] play a role. Additionally, in non-autologous therapy, cell rejection may cause even lower engraftment, due to decreased survival of transplanted cells in the hostile environment.

Allogeneic versus autologous stem cells

Allogeneic cell therapy enables prior preparation of the right cell type and immediate 'off-the-shelf' therapy, but may require immune suppression to avoid cell rejection. Autologous cell therapy lacks immunologic concerns, but is associated with low cost-effectiveness, logistic concerns and lifelong exposure of cells to ageing, comorbidity and risk-factors.[3,4,16] A meta-analysis of pre-clinical trials showed no difference in effect size between autologous and allogeneic cell therapy for cardiac repair, irrespective of immunosuppressive therapy.[17] This underscores the potential paracrine working mechanism of cell therapy and might even imply that immunosuppression is not necessary.

The use of mesenchymal stem cells (MSCs) for allogeneic cell therapy may obviate the need for immune suppression due to the MSC's proposed immunomodulatory effect and apparent immune –privileged state.[18–20] The immunosuppressive capability of MSCs can even be enhanced by pharmacological agents like cyclosporine (CsA).[21,22] Conflicting

studies, however, have shown that MSCs are indeed immunogenic and provoke an immune response.[23,24] Thus, the potential role of immunosuppressive drugs cannot be ignored for MSCs as well. The need of immunosuppression in clinical application of allogeneic cells for cardiac regeneration is unknown, as is the role of CsA in this setting. An overview of pre-clinical data might be elucidating and guiding for future clinical studies.

Alloreactivity

Alloreactivity depends on foreign peptide presentation by Major Histocompatibility Complex (MHC) on antigen presenting cells and detection by T cells.[25] Immunomodulation for prevention of alloreactivity should therefore act on T cell suppression. T cell suppressors include calcineurin inhibitors, corticosteroids, antimetabolites, and target-of-rapamycin inhibitors. As CsA, a calcineurin inhibitor, is most often used in pre-clinical trials of allogeneic cell therapy, it will be the focus of this review. Little information exists on the pharmacokinetics and subsequent correct dosage of CsA in large animals.

Cyclosporin

Mechanism of action of CsA

CsA suppresses T cell activity by forming a complex with the intracellular receptor cyclophillin. This CsA-cyclophillin complex subsequently binds to calcineurin A, inhibiting its phosphatase activity.[26–30] Inhibition of calcineurin A blocks activity of nuclear factor of activated T cells (NFAT). The inhibition of the calcineurin/NFAT pathway reduces IL-2, IL-4, and interferon-γ production,[31–35] leading to cell cycle arrest, DNA and RNA inhibition, and inhibition of protein synthesis and growth factor production of T cells.[36]

In clinical practice, CsA is used as an immunosuppressor in organ transplantation, bone marrow transplantation and inflammatory diseases like rheumatoid arthritis and psoriasis. CsA is known for its interaction with several other pharmacological agents and its serious adverse effects. The most often described adverse effect is nephrotoxicity.[37]

CsA in clinical practice

In clinical application of CsA, dosages differ based on indication.[38] Defining the correct daily CsA dosage and therapeutic serum levels in organ transplantation proves to be challenging as a fine balance must be found between organ rejection (underdose) and toxicity (overdose).[39–41] Large intra- and interindividual differences in absorption, distribution, metabolism, and elimination are observed and no true definitions of cell rejection and toxicity exists.[42] Nevertheless, serum concentrations exceeding 300 ng/mL are associated with toxicity, nephrotoxicity in particular, while lower levels were linked to organ rejection.[39]

In renal transplant patients, an initial serum level of 250-400 ng/mL and a maintenance level of 100-250 ng/mL are achieved with dosages of 4-6mg/kg/d and 2-6 mg/kg/day respectively.[43,44] In a survey carried out among 87 "European Group for Blood and Marrow Transplantation" centers, the median daily oral CsA maintenance dose was 10mg/kg/d, with a range of 2-12.5mg/kg/d.[45] Most target serum concentration ranges were 100-500 ng/mL.

CsA in Cardiology

In the field of cardiology, CsA has been investigated for reduction of infarct size after myocardial ischemia. In a meta-analysis of animal models of myocardial ischemia/

reperfusion, cyclosporine was associated with a smaller infarct size compared to control.[46] The proposed mechanism is prevention of opening of the mitochondrial permeability transition pores, which is triggered by the oxygen-derived free radicals coupled with Ca^{2+} during reperfusion. However, in a third of the included studies, there is no effect of CsA and there was evidence for publication bias, based on funnel plotting. The direct effect of CsA on myocardial infarct size, on top of the effect of CsA on stem cells, must be emphasized, in a setting of stem cells therapy for acute myocardial ischemia.

In order to identify studies of the effect of CsA on stem cells *in vitro* and *in vivo* in the setting of myocardial infarction, we conducted a search in the Pubmed database on August 1st 2013, using the search terms (((((((ciclosporin[Title/Abstract]) OR cyclosporin[Title/Abstract]) OR ciclosporine[Title/Abstract]) OR cyclosporine[Title/Abstract]) OR Neoral[Title/Abstract]) OR Sandimmune[Title/Abstract]) OR CsA[Title/Abstract])) AND ((((((“stem cells”[Title/Abstract]) OR “stem cell”[Title/Abstract]) OR progenitor*[Title/Abstract]) OR “bone marrow cells”[Title/Abstract]) OR “bone marrow derived cells”[Title/Abstract]) OR pluripotent[Title/Abstract])

Table 1. *In vitro* effects of CsA on different cell types

Positive Effect

Reference	Cell type	CsA concentration	Conclusion
Chen at al.[58]	Rat MSC	0.5-5 μM (0.6-6 μg/mL)	Reduced apoptosis after hypoxia/reoxygenation.
Fujiwara et al.[47]	Mouse/Human iPSC	1–3 μg/mL	Enhanced cardiac differentiation
Hunt et al.[49]	Mouse NPC	0.1 μg/mL - 0.5 μg/mL	Enhanced proliferation, enhanced survival
Perry et al.[50]	Mouse HSPC / BMC	0.1 μg/mL - 100 μg/mL	Enhanced proliferation in low dose, inhibition in high dose
Sachinidis et al.[51]	Mouse ESCs	1μM (1.2μg/mL)	Enhanced cardiac differentiation.
Yan et al.[56]	Mouse ESC	1–3 μg/mL	Enhanced cardiac differentiation.

Negative Effect

Reference	Cell type	CsA concentration	Conclusion
Byun et al.[52]	Rat MSCs	50-500 nM (0.06-0.6 μg/mL)	No effect on proliferation.
Davies et al.[53]	Porcine MNCs	0.001-0.2 μg/mL	Decreased proliferation, differentiation, migration of smooth muscle and endothelial outgrowth cells
Guo et al.[57]	Rat NSC	0.5 μg/mL - 5 μg/mL	Decreased proliferation.
Poncelet et al.[54]	Swine MSC	0.2 μg/mL	Reduced proliferation
Song et al.[55]	Mouse MSC	1 10 μM (1.2-12 μg/mL)	Reduced proliferation
Yang et al.[59]	Human EPC	0.01-10μg/mL	Reduced proliferation, increased apoptosis, reduced angiogenesis

iPSC= induced pluripotent stem cell, HSPC= hematopoietic stem/progenitor cell, BMC= Bone marrow cells, EB= embryonic bodies, ESC= embryonic stem cell, NPC= neural precursor cell, MSC= mesenchymal stem cell, MNC= mononuclear cell, EPC= endothelial progenitor cell, NSC= neural stem cell.

In vitro experiments

Aside from both a (nephro)toxic and immunosuppressive effect, CsA may have a direct effect on stem cells. In several experiments, stem cells were incubated with varying concentrations of CsA and proliferation,[47–57] survival,[49] apoptosis,[58,59] differentiation,[53,55,57] migration,[53] and angiogenesis[59] were analysed. Table 1 summarizes the studies that revealed a positive effect (enhanced differentiation, proliferation, survival or decreased apoptosis) or negative impact (reduced differentiation, proliferation, survival or increased apoptosis) of CsA on different cell types, compared to control.

As can be seen in Table 1, numerous studies have shown a pro-proliferative effect of CsA on stem cells. A calcineurin/NFAT pathway-independent effect has been proposed,[47,49,56] because of the absence of a significant effect when using other calcineurin/NFAT inhibitors such as Tacrolimus and 11R-VIVIT.[56]

Apoptosis

Reduced apoptosis and increased survival, as seen in Chen et al. and Hunt et al, are explained by prevention of mitochondrial dysfunction[49,58] and promotion of bcl-2, an anti-apoptotic factor.[58] However, Hunt et al. showed decreased cell viability and decreased cell proliferation at higher concentrations (≥5µg/mL).[49] Yang et al. reported increased apoptosis of endothelial progenitor cells by all concentrations tested (0.1 – 10 µg/ml), and attributed this to decreased NO generation, which promotes EPC apoptosis.[59]

A dose dependent effect is endorsed by Perry et al. showing enhanced proliferation in dosages until 1 µg/mL and decreased proliferation in higher dosages.

Proliferation

Different studies showed both anti- and pro-proliferative effects of CsA. The mechanism behind the anti-proliferative effect remains unclear. Disruption of the calcineurin pathway may influence the fate of stem cells.[57] Other studies showed or stated the reduced proliferation to be caused through alteration in the NOS/NO pathway by CsA, where a decrease in NO production causes decrease in proliferation and differentiation.[55,59,60]

The pro-proliferative effect is ascribed to inhibition of IFN-β[50], decreased cell-adhesion by decreased cyclophilin and subsequently decreased matrix-metalloproteinase production.[49]

Cell types

Incubation of CsA with MSCs seems to negatively influence stem cell growth. Two studies showed reduced proliferation,[54,55] one study showed no effect on proliferation,[52] and only one study showed a positive effect in the form of reduced MSC apoptosis.[58] Two studies used embryonic stem cells (ESCs)[51,56] and both revealed a positive pro-proliferative effect of CsA, suggesting ESCs may benefit from the presence of CsA.

In vivo experiments

We identified only 4 studies investigating the role of CsA in cell therapy in an animal model of ischemic heart disease. Stem cells were transplanted into the ischemic myocardium following ligation of the left anterior descending artery, with or without CsA.[61–64] Immune cell infiltration, cell survival, contractile performance, and/or ejection fraction (EF) were monitored. Table 2 summarizes the positive and negative effects (positive: enhanced

survival or engraftment, reduced immune reaction, improved cardiac function; negative: decreased survival or engraftment, immune reaction, less improved cardiac function) of CsA on non-autologous stem cells *in vivo.* Guo et al. investigated immune cell kinetics, myoblast survival and cardiac function after myocardial injection of human myoblasts, human myoblast + CsA, rat myoblasts and rat myoblasts + CsA, in rats one week after MI. CsA caused less infiltration of immune cells, more myoblast survival and improved cardiac function, compared to cells alone. The observed improvement in cardiac function was largely attributed to the reduced infiltration of macrophages and CD4+/CD8+ cells, causing prolonged myoblast survival.[61] However, 10 minutes after injection, stated as baseline, two thirds of the injected cells was already lost. After one day, only 10% of the numbers of cells at baseline were still present in all groups.

Westrich et al. compared intramyocardial syngeneic and allogeneic MSCs injections in rats, both with and without CsA, 5 days after MI. Cell survival of syngeneic cells was higher than allogeneic, regardless of use of CsA. However, cell survival per cell source was higher in CsA treated groups compared to cells only, explained by the anti-inflammatory and anti-apoptotic effect of CsA.[62]

In contrast, Chiavegato et al. showed the activity of macrophages and dendritic cells remains unaffected by CsA and graft cell rejection still occurs.[63] In this study, human amniotic fluid-derived stem cells (AFS) were injected in the myocardium of 3 groups of rats: healthy animals; 20 minutes after MI; and athymic animals. Authors were surprised by the results because of their previous experience with engraftment and differentiation

Table 2. *In vivo* effects of CsA on different non-autologous cell types, compared to control

Positive effect						
Reference	**Cell type**	**Animal model**	**N=**	**CsA dosage (mg/kg/d)**	**Administration**	**Conclusion**
Guo et al.[61]	Human SkM	Rat	208	10	Intraperitoneal	Reduced immunocyte infiltration, enhanced cell survival, improved EF
Westrich et al.[62]	Rat MSC	Rat	12	10	NA	Enhanced cell survival
Negative or neutral effect						
Reference	**Cell type**	**Animal model**	**N=**	**CsA dosage (mg/kg/d)**	**Administration**	**Conclusion**
Chiavegato et al.[63]	Human AFS	Rat	42	5	Intramuscular	Unsuccessful inhibition of cell rejection
Zeng et al.[64]	Swine pMultistem	Swine	30	15	Oral	No effect on engraftment and cardiac function.

SkM=skeletal myoblasts, MSC=mesenchymal stem cells, AFS=amniotic fluid derived stem cells, MNC= mononuclear cell, pMultistem=bone marrow-derived adherent stem cells, EF=ejection fraction.

of human amniotic MSCS in immune competent rats after MI and engraftment of human 'amnion cells' in neonatal swine and rats. Because of the rejection in athymic rats and rats treated with CsA, a T-cell independent mechanism is proposed, where natural antibodies, complement factors, NK cells or macrophages may play a role.[63]
Zeng et al. demonstrated that injection of allogeneic pMultistem cells (porcine derived multipotent adult progenitor cells) in pigs immediately after MI, led to improvement in cardiac function, irrespective of CsA treatment. Engraftment rate was also not affected by CsA and only 0.35% of the cells could be detected after 4 weeks. Differentiation of present cells into cardiomyocytes was limited and infarct size was not affected. Therefore, paracrine signalling was proposed as the main mechanism of effect.[64]

Endogenous recruitment
Hunt al al. showing increased proliferation and survival *in vitro* (Table 1), also investigated the effects of CsA *in vivo* in healthy mice.[49] Authors observed an increased number of neurospheres from endogenous neural progenitors in CsA treated animals compared to control animals. Furthermore, an increased number of neural stem cells was observed. This recruitment of endogenous progenitor cells is confirmed by Wang et al.[65] In this study, authors show endothelial progenitor cell mobilization by CsA in a hind limb ischemia mouse model. Proposed mechanism is by decreased activity of CD26, which causes an increase in the level of chemo attractants for progenitor cells (like stroma-derived factor-1α). In contrast to this data, Davies et al. showed in a porcine cardiac transplant model, that endothelial and smooth muscle outgrowth colony numbers decreased after treatment with CsA in sham operated as well as transplanted animals.[53] In the sham operated + CsA treated animals, the number of colonies increased to above baseline levels at 4 weeks. In sham alone, without CsA, the
endothelial colony numbers remained stable. This detrimental effect *in vivo* is in accordance with the effects shown by Davies et al *in vitro* (Table 1).

Target CsA levels in large animals
The therapeutic range and the subsequent recommended daily dosage of CsA in large laboratory animals are largely unknown. The pig serves as an excellent model for cardiac and pharmacological studies due to its similar physiology and anatomy compared to man.[66] Nevertheless, Frey et al. demonstrated that pigs require 2 times higher intravenous doses and up to 4-6 times higher oral doses of CsA in order to reach the same serum levels as in humans.[67] In accordance with Frey et al., Cibulskyte et al. demonstrated that an oral dosage of 30 kg/mg/d in pigs is needed to reach blood concentration levels of 475 ng/ml. However, at such high dosages acute nephrotoxicity was observed within 4 weeks. A dosage of 15mg/kg/d was stated to be safe and caused a trough concentration of 338 ng/ml.[68]

Dosing and safety in a pig model of myocardial infarction

Rationale
In order to address the problem of the inconsistency in recommended CsA dosages in the setting of cardiac repair, we performed a small study investigating serum levels and animal safety after administration of CsA in a myocardial ischemia/reperfusion pig model.

Methods

Open chest LAD occlusion was performed for 90 minutes in a porcine model (n=5, mean weight 32.5±2.1 kg at start of study). CsA was orally administered twice daily (15mg/kg/d), and venous blood was collected at different time points through a peripherally inserted central line, tunneled to the back. The mean follow-up was 44.6 days. We determined the target range to be between 100-300 ng/mL, based on levels for safety and efficacy suggested by others.[39,43,53]

Results

CsA serum levels were within the therapeutic range in 81.7% of all cases (49/60)(Fig. 1). The mean CsA levels (and the upper and lower limit) per pig were: pig 1, 182 ng/mL (280-85); pig 2, 177ng/mL (344-108); pig 3, 182ng/mL (261-81), pig 4, 167ng/mL (287-50); pig 5, 153ng/mL (319-30). To investigate the safety of the given CsA dosage and the significance of the determined therapeutic range, serum values were analyzed and compared to existing reference values (Table 3). None of the serum levels exceeded the reference values. In conclusion, our data suggest that the administration of 15 mg/kg/d twice a day in a pig model of myocardial infarction is feasible and represents a safe dosage that ensures adequate CsA serum concentration values with 82% of the values within the determined therapeutic range.

Conclusion

This essay summarizes current knowledge on the role of CsA in non-autologous stem cell therapy. CsA has been reported to exert inconsistent effects on stem cells both *in vitro* and *in vivo. In vitro,* a positive effect was observed through enhanced proliferation, enhanced survival, or reduced cell apoptosis. At the same time, different studies using a similar methodology reported negative effects, namely reduced proliferation, angiogenesis, differentiation and migration and increased apoptosis. In both the positive and negative studies, approximately the same range of CsA concentration was used. Interestingly, enhanced proliferation was reported ,while cell viability was drastically reduced at higher concentrations,[48–50] suggesting CsA may only have a beneficial effect up to a certain concentration.

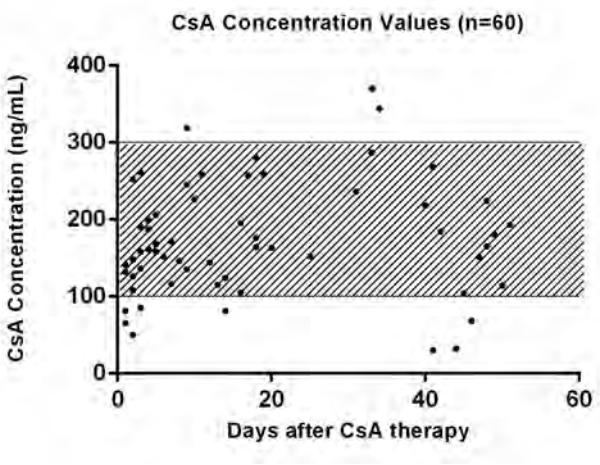

Figure 1. CsA concentration values of all 5 pigs (n=60)
Therapeutic range determined at 100-300 ng/mL.

Table 3. Mean serum value per pig throughout the follow-up period

	Reference		Pig 1	Pig 2	Pig 3	Pig 4	Pig 5	Mean
Creatinine	1.2-2.0	mg/dL	1.7	1.4	1.1	1.5	1.6	1.5±0.23
AST	14-56	IU/L	26.1	46.2	52.6	43.2	44.0	42.4±9.8
ALT	5-78	IU/L	37.6	59.8	51.9	50.2	51.2	50.1±8.0
Hb	9.0-16.2	g/dL	9.0	11.1	10.2	10.4	11.2	10.4±0.9
WBC	6.3-21.1	103/mL	14.2	15.4	19.6	13.9	18.5	16.3±2.6
BUN	8-24	mg/dL	11.1	10.1	7.6	10.0	9.4	9.6±1.3
Sodium	133-153	meq/dL	NA	141.3	135.4	139.3	143.6	139.9±3.5
Potassium	3.1-6.2	meq/dL	NA	4.3	4.4	4.4	4.4	4.4±0.1

Number of measurements per pig: pig 1 n=19; pig 2 n=10; pig 3 n=9; pig 4 n=8; pig 5 n=5.
AST=Aspartate Transaminase, ALT=Alanine Aminotransferase, Hb=Hemoglobin, WBC=White Blood Cells, BUN=Blood Urea Nitrogen.

In vivo, similar conflicting results were observed. In both the positive and negative studies, CsA dosage was fairly equal. Studies focused on both cell engraftment and cardiac function, but little is known on the link between engraftment rate and cardiac function.

The conflicting results in both *in vivo* and *in vitro* data, maybe explained by heterogeneity in included studies. Methodology of included studies are quite similar, but cell type and cell source are different. From these data, the exact role of CsA in animal models cannot be fully elucidated.

Non-autologous cells will eventually be rejected, with or without immune suppression.[4] A large proportion of the observed beneficial effect of stem cell therapy is thought to be through indirect pathways. Cell rejection may not be a matter of concern as long as enough time has passed for these paracrine effects to occur.[4] At the same time, avoiding cell rejection and hence prolonging graft survival may contribute to overall improvement in cardiac function.

We have also presented data showing that oral administration of CsA twice a day in a pig model of myocardial infarction is feasible and safe and blood concentrations remain within the therapeutic range. As others have suggested, the daily dose in our pig study is higher than in humans.

In conclusion, there is no evidence to clearly demonstrate whether CsA, or immune suppression in general, is essential and beneficial in non-autologous cell therapy. Regardless of this limited knowledge, the issue of immune suppression is one of great importance and thus should be further elucidated.

Limitations

First, the effect of CsA on endogenous stem cell properties was not discussed. Since one of the suggested mechanisms of effect of cell therapy is paracrine signaling, this might be of importance. Second, we focused on animal models of myocardial infarction. The effect of, and need for CsA, in other disease models might be different. Finally, no reliable meta-analysis could be performed, because of the large variability of cell type, CsA dosages and measures of effect size.

Future directions

Although CsA is commonly used as an immunosuppressant in organ transplantation, its contribution to cell therapy for cardiac regeneration remains unclear. If immune suppression is deemed redundant, future studies should focus on the impact of cell rejection (and low engraftment) on cardiac function following cell transplantation.

On the other hand, if immune suppression is a requirement for cell engraftment in non-autologous stem cell therapy, future experiments should focus on the effect of CsA on cardiac function after cell therapy in a myocardial ischemia large animal model. Furthermore, a dose-response experiment could shed light on the fine balance between successful cell engraftment and animal safety.

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CHAPTER 9

ADMITTANCE BASED PRESSURE VOLUME LOOPS VERSUS GOLD STANDARD CARDIAC MAGNETIC RESONANCE IMAGING IN A PORCINE MODEL OF MYOCARDIAL INFARCTION

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ABSTRACT

Background

A novel admittance based pressure volume system (AS) has recently been developed and introduced. Thus far, the new technique has been validated predominantly in small animals. In large animals it has only been compared to three-dimensional echocardiography (3DE) where the AS showed to overestimate left ventricular (LV) volumes. To fully determine the accuracy of this device, we compared the AS with gold standard cardiac magnetic resonance imaging (CMRI).

Objective

The aim of this study was to directly compare LV volume measurements obtained by the AS with CMRI in a porcine model of chronic myocardial infarction (MI).

Methods

Fourteen pigs were subjected to 90 minutes closed chest balloon occlusion of the left anterior descending artery. After 8 weeks of follow-up, pigs were consecutively subjected to LV volume measurements by the AS, CMRI and 3DE under general anesthesia.

Results

The AS overestimated end diastolic volume (EDV; +20.9±30.6mL, p=0.024) and end systolic volume (ESV; +17.7±29.4mL, p=0.042) but not ejection fraction (EF; +2.46±6.16%, p=NS) compared to CMRI. Good correlations of EDV (R=0.626, p=0.017) and EF (R=0.704, p=0.005) between the AS and CMRI were observed. EF measured by the AS and 3DE also correlated significantly (R=0.624, p=0.030).

Conclusion

After subjection of pigs to MI, the AS very moderately overestimates LV volumes and shows accurate measurements for EF compared to CMRI. This makes the AS a useful tool to determine cardiac function and dynamic changes in large animal models of cardiac disease.

INTRODUCTION

Cardiac function is a main endpoint in efficacy testing of novel therapeutics in clinical and translational cardiovascular research.[1,2] This requires objective and reliable tools for its determination. Many current methods use left ventricular (LV) volumes as a surrogate of cardiac function. Apart from volume assessment, invasive pressure-volume (PV) measurements (PV-Loops) provide researchers with more specific information on both systolic and diastolic myocardial function and dynamic changes which are not measurable with most imaging modalities.[3–5] However, reliable analysis of various PV-Loop parameters depends on adequate LV volume calibration, which requires accurate LV volume assessment.

Recently, a novel method of measuring PV loops has been introduced. This admittance based PV Loop system (AS) differs from the classical conductance system in several ways. First, the new method does not assume a constant contribution of parallel conductance during the cardiac cycle.[6,7] Secondly, it differs in the way parallel conductance is separated from blood conductance making the new method less time-consuming and the system easier to use. Finally, the AS applies a non-linear relationship between conductance and volume for the conversion of blood conductance into volume.[7–9] This novel method has been extensively validated in small animal models.[10–16] Only recently, the AS has been evaluated in large animal models.[17,18] The large animal studies demonstrated good performance, but show that the AS moderately overestimates EDV and ESV. In both studies, however, three-dimensional echocardiography (3DE) was used as a reference standard. 3DE has been proven to be superior to the more conventional two-dimensional echocardiography for volume measurements and is relatively inexpensive and useful for quick and non-invasive assessment of LV volumes.[19–22] However, 3DE has been observed to be less precise in estimating EF compared to cardiac magnetic resonance imaging (CMRI), the gold standard for volume determination.[19,20] 3DE is also known to consistently underestimate both EDV and ESV and this underestimation increases in diseased hearts.[23,24] Therefore, direct comparison with CMRI is especially mandatory after post-infarction remodeling. Hence, the aim of this study was to compare the AS derived EDV, ESV and EF to CMRI derived measurements in a closed chest porcine model of chronic myocardial infarction.

MATERIAL AND METHODS

All animal experiments were approved by the institutional animal welfare committee of the UMC Utrecht and were executed conforming to the 'Guide for the Care and Use of Laboratory Animals'. A total of 14 specific pathogen free female landrace pigs were evaluated in this study (Van Beek Lelystad, the Netherlands). Pigs (body weight 79.1±5.9 kg) were subjected to myocardial infarction followed by invasive PV measurements, CMRI and 3DE at 8 weeks follow-up. Before any procedural intervention, animals were housed in pairs. Because of health concerns, animals were housed individually after myocardial infarction until the end of the study. Pigs were fed twice a day, water was available *ad libitum*.

Infarct induction

All animals were pre-treated with amiodaron for 10 days (1200mg loading dose, 800mg/day maintenance), clopidogrel for 3 days (75 mg/day) and acetylsalicylic acid for 1 day (320 mg loading dose, 80 mg/day maintenance). All medication was continued until the end of the study. Animals were anesthetized in their cage with an intramuscular injection of 10 mg/kg ketamine, 0.4 mg/kg midazolam and 0.5 mg/kg atropine. Anesthesia was maintained with intravenous infusion of 0.5 mg/kg/h midazolam, 2.5 µg/kg/h sufentanyl and 0.1 mg/kg/h pancuronium and pigs were mechanically ventilated. Pre-operatively, animals received a fentanyl patch (25µg/h). Arterial access was obtained by introduction of an 8F sheath into the carotid artery after surgical exposure. A coronary angiogram of the left coronary tree was acquired using an 8F JL4 guiding catheter (Boston scientific, Natick, USA). The diameter of the left anterior descending artery (LAD) was measured directly distally to the second diagonal artery. An adequately sized balloon was placed distal from the second diagonal branch and inflated for 90 minutes. After reperfusion and observation for approximately 2 hours, the surgical wound was closed and animals were weaned from anesthesia. Animals were defibrillated in case of ventricular fibrillation (VF).

Invasive PV-Loop measurements

Admittance based PV-loop measurements were performed as recently described.[17] In short, animals were again anesthetized according to the protocol described above 8 weeks after infarct induction. Arterial access was obtained by introduction of an 8F sheath into the carotid artery. The 7F tetra-polar admittance catheter (7.0 VSL Pigtail/no lumen, Transonic Scisense, London, Canada) was inserted into the left ventricle through the sheath in the carotid artery under fluoroscopic guidance. The catheter 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. 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) required for volume calibration and analysis was derived from CMRI measurements. Blood resistivity was assumed to be constant in all animals (150 Ω*cm). All measurements were performed during apnea. Data were offline analyzed using Iworx analysis software (Labscribe V2.0).

Cardiac magnetic resonance imaging (CMRI)

Immediately after PV measurements, pigs were transported to the CMRI scanner. CMRI images were obtained with a 3T CMRI scanner (Achieva TX, Philips Healthcare). Animals were placed on the CMRI table in a supine position, under continuous anesthesia. A 32-channel receiver coil was placed over the chest. ECG-gated steady-state free precesion cine imaging was obtained in a short axis and a two-chamber long axis view (Voxelsize acquisition = 2*2.1mm, recon voxelsize = 1.25*1.25mm, Slice thickness = 8mm, Bandwith = 1243Hz, echo time = 1.62ms, repetition time = 3.2ms, balanced gradient echo readout = 20. Offline imaging analysis was performed in Qmass MR 7.4 enterprise solutions (Medis medical imaging systems BV, Leiden).

Three-dimensional echocardiography

3DE was performed with a X3-1 transducer on an iE33 ultrasound device (Philips, Eindhoven, The Netherlands) directly after CMRI as previously described.[17] After medial sternotomy, a gel-filled flexible sleeve was placed directly 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. Ventricle tracing was performed by semi-automatic border detection as described before (Gain 50%, compression 50%, frame rate = 20-30 frames/second, 1 dataset = 7 beats).[25] Because of incomplete capture of the left ventricle on echocardiographic recordings, 2 out 14 pigs were excluded from the analysis.

Infarct size

Animals were sacrificed by exsanguinations under anesthesia. The hearts were excised and the LV was then cut into 5 equal slices from apex to base. 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.

Statistical analysis

All data are expressed as mean ± standard deviation unless stated otherwise. CMRI data and PV measurements were separately analyzed by two different researchers blinded to the outcome of the other technique. End diastolic volume (EDV), end systolic volume (ESV) and ejection fraction (EF) measured by the AS and 3DE were compared with CMRI values using a paired Student's t-test. Correlations were tested using Pearson's correlation test. The limits of agreement (1.96*SD = 95% confidence interval) of the AS compared to CMRI were determined by Bland-Altman analysis. 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

At 8 weeks follow-up after MI, LV volumes were assessed in 14 pigs with both the AS and CMRI (Figure 1A and 1B). 7 out of 14 animals showed at least one episode of VF. Left ventricular end systolic pressure (122±27mmHg) and end diastolic pressure (13±8mmHg) were in a physiological range during the experiment. Heart rate did not show any significant difference between AS measurement and CMRI measurements (54±18 vs. 58±16 bpm, p=NS). Moreover, heart rate correlated significantly (R=0.747, p=0.002) between AS and CMRI derived measurements, indicating that both measurements were performed under a steady state.

3DE vs. CMRI

To test the degree of LV volume underestimation by 3DE compared to CMRI, we measured LV volumes with 3DE in 12 out of 14 animals (Figure 1C). EDV (79.1±14.9mL (3DE) vs. 165.3±24.8mL (CMRI), p<0.001) and ESV (45.8±10.6mL (3DE) vs. 98.6±15.2mL (CMRI),

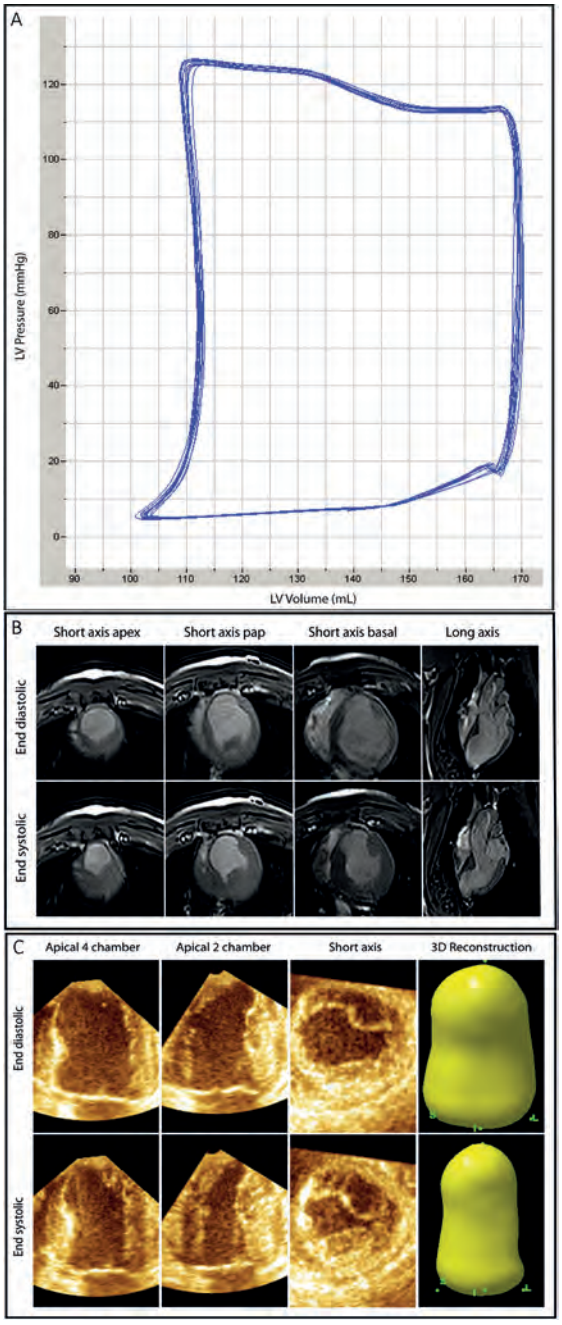

Figure 1. Different techniques for LV volume measurements
EDV, ESV and EF were measured with 3 different modalities. A. Representative recordings of PV Loops with the AS. 10 beats were recorded during apnea. B. Representative images of a pig subjected to CMRI measurements at the end of systole and diastole. 4 different views are depicted. Short axis at the apex, papillary muscle (pap) and at a basal level combined with a long axis view. C. Representative images 3DE measurements at the end of systole and diastole. Three different views are depicted. Apical 4 chamber, apical 2 chamber and a short axis view, together resulting in a 3D reconstruction of the left ventricle.

p<0.001) were underestimated by 3DE compared to CMRI as expected (Figure 2A and 2B, Table 1). EF measured by MRI was slightly overestimated by 3DE (41.9±9.23% (3DE) vs. 40.0±7.0% (CMRI), p=0.018), with an almost perfect correlation (R=0.982, p<0.001) (Figure 2C and 3C, Table 2). We also determined whether LV size influenced the accuracy of the measurements by 3DE. Indeed Bland-Altman analyses revealed a trend towards a lower accuracy for EDV, ESV and EF in the larger sized hearts (Figure 4A – 4C).

AS vs. CMRI

Next we determined whether EDV, ESV and EF measured with the AS deviated from the CMRI values. In the present study, the AS significantly overestimated both EDV (186.2±39.2mL (AS) vs. 165.3±24.8mL (CMRI), p=0.024) and ESV (116.4±29.7mL (AS) vs. 98.6±15.2mL (CMRI), p=0.042) compared to CMRI (Figure 2A and 2B, Table 1). However, EF measured by the AS was not significantly different from MRI derived EF (37.5±8.6% (AS) vs. 40.0±7.0% (CMRI), p=NS) (Figure 2C, Table 1). Importantly, we observed a good correlation of EDV (R=0.626, p=0.017) and EF (R=0.704, p=0.005) between the AS and CMRI (Figure 3A and 3B, Table 2) and a good correlation of EF between AS and 3DE (R=0.624, p=0.030) (Figure 3D). Subsequently, the limits of agreement of the AS opposed to CMRI were determined by Bland-Altman analysis. Both EDV and ESV measured by the AS showed a moderate to good agreement and EF showed a good to excellent agreement with CMRI (Figure 4). The mean percentage inter-method difference was 12% for EDV, 17% for ESV and 6% for EF. No particular trend was detected between the accuracy of measurements and increasing values for ventricular volumes by CMRI.

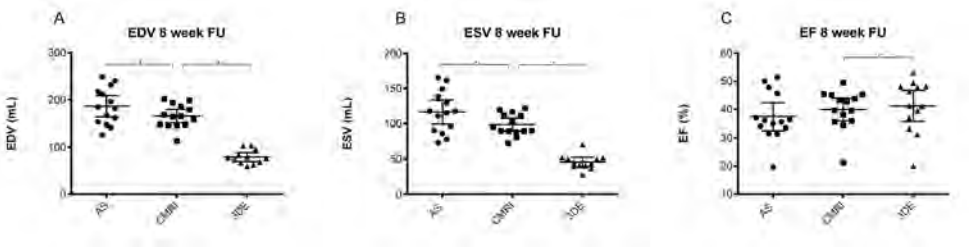

Figure 2. Measured LV volumes by the AS, CMRI and 3DE in the infarcted porcine heart
LV dimensions were obtained by the three different modalities. A. EDV 8 weeks after MI. B. ESV 8 weeks after MI. C. EF 8 weeks after MI. Data are presented as mean ± 95%CI. * p<0.05. LV = left ventricular; AS = admittance based PV system; CMRI = cardiac magnetic resonance imaging; 3DE = three-dimensional echocardiography.

Table 1. Assessment of LV dimensions in the infarcted porcine heart with the AS, CMRI and 3DE

LV Parameters	AS	CMRI	3DE
EDV (mL)	186±39	165±25	79±15
ESV (mL)	116±30	99±15	46±11
EF (%)	37.5±8.6	40.0±7.0	41.9±9.2

All values are presented as mean ± standard deviations. N=14 for AS and CMRI measurements, n=12 for 3DE measurements.

Correlation with infarct size

As LV volumes are often measured as surrogates of cardiac function, it is essential to test whether the volume measurements of the various systems reflected the actual extent of myocardial damage. Therefore, infarct size was determined at 8 weeks follow-up. Mean infarct size was 13.8±2.9%. We found a good correlation of CMRI based ESV (R=0.685, p=0.007) and EF (R=-0.608, p=0.021) with infarct size (Figure 5A and 5B, Table 3). For 3DE, a similar correlation for ESV (R=0.659, p=0.020) and EF (R=-0.650, p=0.022) compared to infarct size was found, indicating that both systems' measurement reflect the decay in cardiac function after myocardial infarction (Figure 5C and 5D). With the AS, however, we failed to establish a significant correlation between either ESV or EF with infarct size.

Table 2. Correlations between the AS, CMRI and 3DE for EDV, ESV and EF in the infarcted porcine heart

LV Parameters	CMRI vs. AS	CMRI vs. 3DE	3DE vs. AS
EDV (mL)	0.626*	0.160	0.235
ESV (mL)	0.271	0.320	-0.111
EF (%)	0.704*	0.982*	0.624*

Numbers shown are the correlation coefficients (R). *Significant correlation between two techniques (p<0.05).

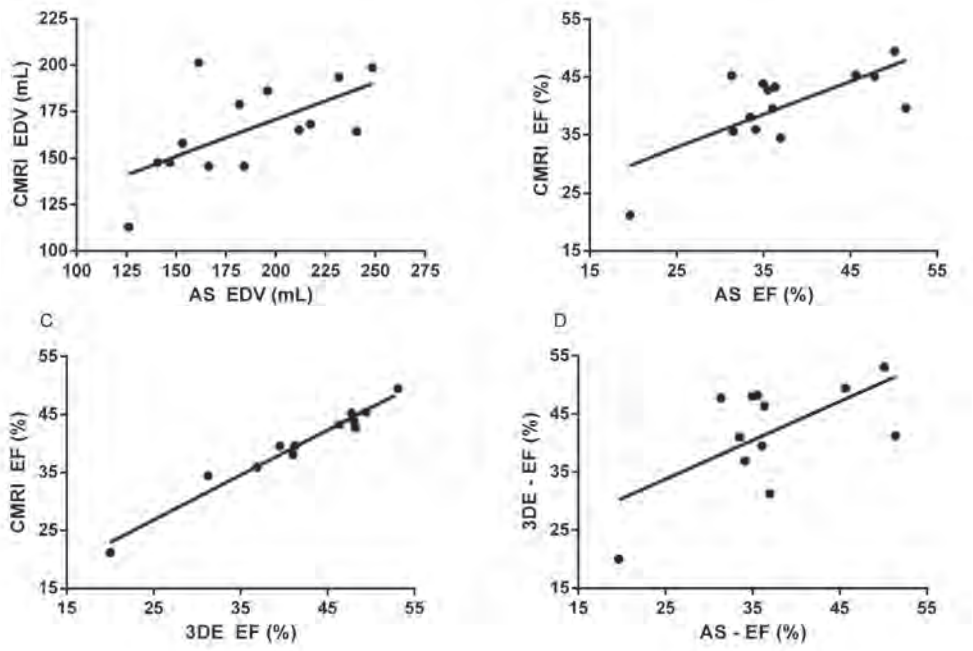

Figure 3. Correlation plots of LV volumes between the AS, CMRI and 3DE in the infarcted porcine heart A. EDV correlates significantly between the AS and CMRI. R=0.626 p=0.017, n=14. B. EF correlates significantly between the AS and CMRI. R=0.704, p=0.005, n=14. C. EF correlates significantly between CMRI and 3DE. R=0.982, p<0.001, n=12. D. EF correlates significantly between 3DE and the AS. R=0.624, p=0.030, n=12. LV = left ventricular; AS = admittance based PV system; CMRI = cardiac magnetic resonance imaging; 3DE = three-dimensional echocardiography.

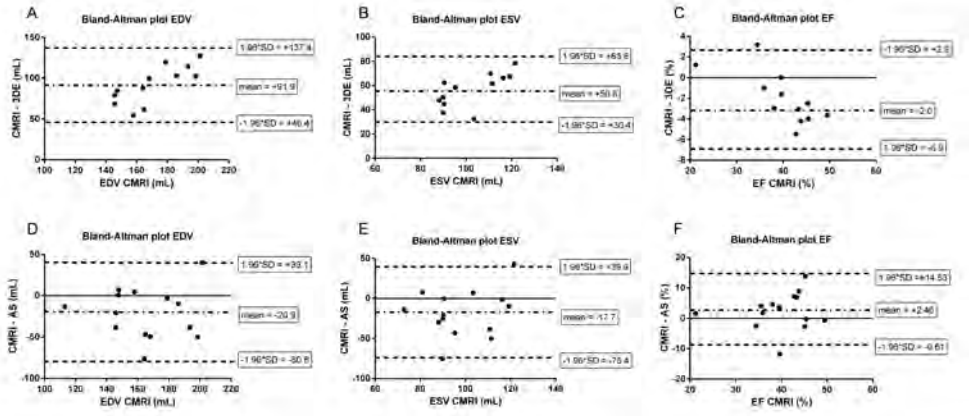

Figure 4. Bland-Altman plots of LV volumes in the infarcted porcine heart
Bland-Altman plots of 3DE (A to C) and the AS (D to E) with CMRI as reference standard. The green line represents the mean. The red lines represent the limits of agreement. A. End diastolic volume. B. End systolic volume. C. Ejection fraction. AS = admittance based PV system; CMRI = cardiac magnetic resonance imaging; 3DE = three-dimensional echocardiography.

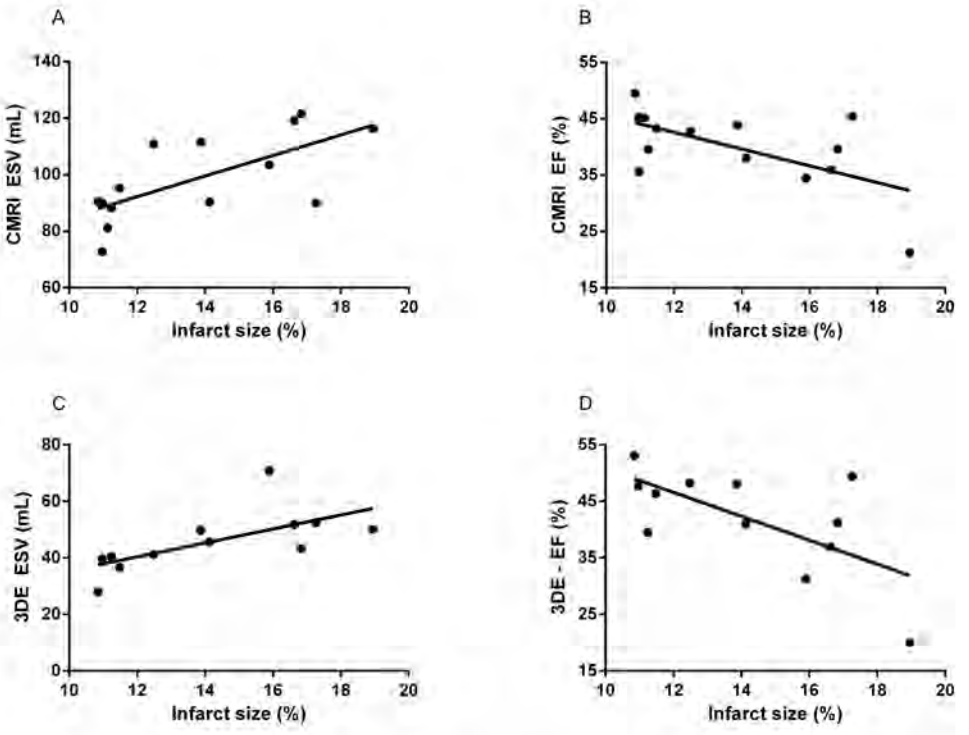

Figure 5. Correlation plots of LV volumes with infarct size in the infarcted porcine heart
Significant correlations of LV dimensions of CMRI and 3DE with infarct size. A. ESV (CMRI) correlates significantly with infarct size. R=0.685, p=0.007, n=14. B. EF (CMRI) correlates significantly with infarct size. R=-0.608, p=0.021, n=14. C. ESV (3DE) correlates significantly with infarct size. R=0.659, p=0.020, n=12. D. EF (3DE) correlates significantly with infarct size. R=0.650, p=0.022. CMRI = cardiac magnetic resonance imaging; 3DE = three-dimensional echocardiography.

Table 3. Correlations between ESV and EF for the AS, CMRI and 3DE and infarct size (IS) in the infarcted porcine heart

LV Parameters	AS vs. IS	CMRI vs. IS	3DE vs. IS
EDV (mL)	-0.146	0.239	0.365
ESV (mL)	-0.072	0.685*	0.659*
EF (%)	-0.174	-0.608*	-0.650*

Numbers shown are the correlation coefficients (R). *Significant correlation between infarct size and the LV parameter ($p<0.05$).

DISCUSSION

Real-time PV loops provide researchers with more detailed information on cardiac function than most imaging modalities.[3,26] Although some of these parameters are volume-independent, many PV-loop parameters depend on accurate determination of LV volumes. Traditional PV-systems have shown to accurately estimate LV volumes but require additional calibration steps that are susceptible to technical error.[4,9,27] The novel AS is easier to use and does not require these calibration steps that could potentially increase variability.[11]

To the best of our knowledge, this is the first study to directly compare the AS with CMRI, the gold standard for *in vivo* LV volume assessment. Using CMRI as a reference standard in a closed chest porcine model, the AS accurately measures EF with only modest overestimation of EDV and ESV. The AS shows small mean percentage inter-method differences and a better accuracy for EDV, ESV and EF than 3DE in this study. Furthermore, our study shows that there is a good correlation for EDV and EF between AS and CMRI and a good correlation for EF between AS and 3DE. Both CMRI and 3DE are reflecting the extent of cardiac damage since ESV and EF of the two methods correlate well with infarct size, even though scar remodeling has already occurred after such an extensive follow-up period and infarct size analysis has become less representative due to possible scar shrinkage and resorption.[28] Presumably this phenomenon causes the lack of correlation between infarct size and EDV measured by any method in our study.

Very recently the performance of the AS in a large animal model has also been investigated by us and others.[17,18]. Here, an overestimation of EDV and ESV at baseline was found. This overestimation was more pronounced after MI and led to an underestimation of EF in the diseased heart compared to the reference standard.

Both large animal studies were however limited by the use of 3DE as the reference standard. It has recently been shown that EDV and ESV are increasingly underestimated by 3DE in more diseased left ventricles, which is in line with our findings in the present study (Figure 4a-4c).[23,24] The underestimation observed in our study is slightly more severe compared to clinical data. Mor-Avi et al. found an underestimation for EDV of -67±46mL when comparing 3DE to CMRI in a heterogeneous population of patients with both healthy and failing hearts.[24] In the present study we only performed measurements in infarcted hearts, which presumably is the reason for this relatively severe underestimation. These data suggest that comparison of the AS with CMRI is warranted to fully determine the accuracy of this novel technique.

The current study has several limitations. First of all, measurements were performed under general anesthesia. Since different levels of anesthesia could influence cardiac performance, anesthesia could be a confounding factor in comparing the AS with CMRI despite a consistent heart rate. Secondly, during CMRI, arterial pressure was not registered. Although heart rates did not indicate a change in hemodynamic state, theoretically a difference in arterial pressure between data assessment with the different systems could occur, possibly influencing the outcome. In this study CMRI was preceded by measurements with the AS. Ideally this should be the other way round since the more invasive procedure could influence measurements with the less invasive procedure. Unfortunately, this was proven to be impossible due to logistical difficulties. Nevertheless, measurements with the AS only take up 15 to 20 minutes and the surgical intervention to reach the carotid artery is only minor without any substantial blood loss. Swine used in the present study had relatively large hearts compared to averagely sized human hearts.[20] We failed to observe a trend in decreased accuracy towards the larger sized hearts, so this is unlikely to be of major influence. In the current study we did not perform measurements with a classical conductance system. Therefore, no conclusion can be drawn on the possible superiority of either one of the systems above the other. Lastly, we did not take the effect of the sternotomy during 3DE on physiologic parameters into account. Animals underwent sternotomy after AS and CMRI measurements so the direct comparison of these two methods is not affected by this limitation.

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CHAPTER 10

ASSESSMENT OF CORONARY MICROVASCULAR RESISTANCE IN THE CHRONIC INFARCTED PIG HEART

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ABSTRACT

Background

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

Objectives

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

Methods

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

Results

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

Conclusion

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

INTRODUCTION

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

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

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

METHODS

Animals and Study Design

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

Myocardial infarction

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

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

Intracoronary pressure and flow velocity assessment

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

Analysis of pressure-/flow velocity derived indices

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

Pressure-Volume loop protocol

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

Histology

Four animals, that served as control treated animals in a larger study[20], were euthanized 8 weeks after MI by exsanguination under general anesthesia (see SI Figure 2). After excision of the heart, the left ventricle was cut into five slices from base to apex and incubated in 1% triphenyl-tetrazolium chloride dissolved in phosphatase buffered saline (PBS) at 37°C for 15 minutes. Next, the slices were washed in PBS and photographed digitally (Sony Alfa

55). Snap frozen tissue samples from the infarct zone and remote area (septal wall) were embedded in Tissue-Tek (Sakura) and 7 µm cryosections were prepared on a microtome (Leica). Sections were dried for 30 min at room temperature (RT) and fixed in acetone. Subsequently, slides were incubated with 0.1% Triton X-100 (Sigma) in PBS with 1% bovine serum albumin (BSA), blocked in 10% goat serum, incubated overnight at 4°C in 1% goat serum with primary antibodies against α-smooth muscle actin (αSMA) (1:1500, Mouse monoclonal, Clone 1A4, Sigma) and CD31 (1:100, rabbit polyclonal, Abcam) and then incubated for 1 hour at RT with secondary antibodies (Invitrogen). Slides were mounted in Fluoromount (Southern Biotech) and fluorescence images were acquired on an Olympus DP71 microscope. For image analysis, the number of arterioles (defined as aSMA-positive vessels > 20 µm and < 300 µm) and wall thickness (defined as the wall thickness of the aSMA-positive cell layer) was measured in 10 different fields/section in the infarct zone and the border zone at x40 magnification using ImageJ (1.44g). The number of arterioles was expressed per 1.0mm^2.
The density of capillaries in the infarct region and remote area was assessed by staining with an antibody against CD31 (1:100, mouse monoclonal, AbD Serotec, Raleigh, NC, USA). Endogenous peroxidase in the cryosections was blocked with 0.15% hydrogen peroxide in acetone for 15 min at RT. The 2° antibody used was horse anti-mouse, biotinylated (1:200) (Invitrogen) for 60 min. at RT followed by a streptavidin-HRPO in PBS (1:1000) for 60 min. at RT. The chromogen 3-amino-9-ethylcarbazole (AEC) (Sigma) was used to visualize capillaries. The slides were counterstained with hematoxylin for identification of nuclei. The number of capillaries (defined as one to three cells spanning the CD31-positive vessel circumference) was determined by counting 10 fields/section in the infarct zone and the peri-infarct border zone at x40 magnification. The number of capillaries was expressed per 1.0 mm^2.

Statistics

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

RESULTS

Effect of chronic MI on coronary pressure and flow velocity

The mean coronary diameter at baseline was 3.3 ± 0.7 mm and 3.1 ± 0.8 mm in the LCX and LAD, respectively, and did not change over time. Coronary pressure- and flow-velocity derived indices at baseline and follow-up are shown in Table 1. HMR in the infarct related artery (LCX) was significantly higher at follow up (Figure1A; 2.4±1.1 mmHg/cm/s) compared to baseline (Figure1A; 1.9±0.6 mmHg/cm/s; p=0.03), indicating an increase in regional microvascular resistance in the area of interest. The increased HMR in the LCX was based on an impaired peak coronary flow response to distal vasodilation at four weeks compared to baseline (Figure1B; 42.5±11.4 cm/s versus 53.0±17.3 cm/s, respectively; p=0.05). The distal intracoronary pressure did not show any change between four weeks after MI and

baseline (Figure1C; 92.4±21.2 mmHg versus 92.4±19.1 mmHg; p=0.50). Coronary flow velocity at rest was also impaired four weeks after MI compared to baseline (Supplemental Figure1). As a result, the CFVR remained unchanged following MI compared to baseline values (Supplemental Figure1). The reference artery (LAD) did not show any change in coronary pressure and velocity derived indices (Figure1A-C), indicating a stable and unchanged ability to regulate flow velocities to myocardial demand.

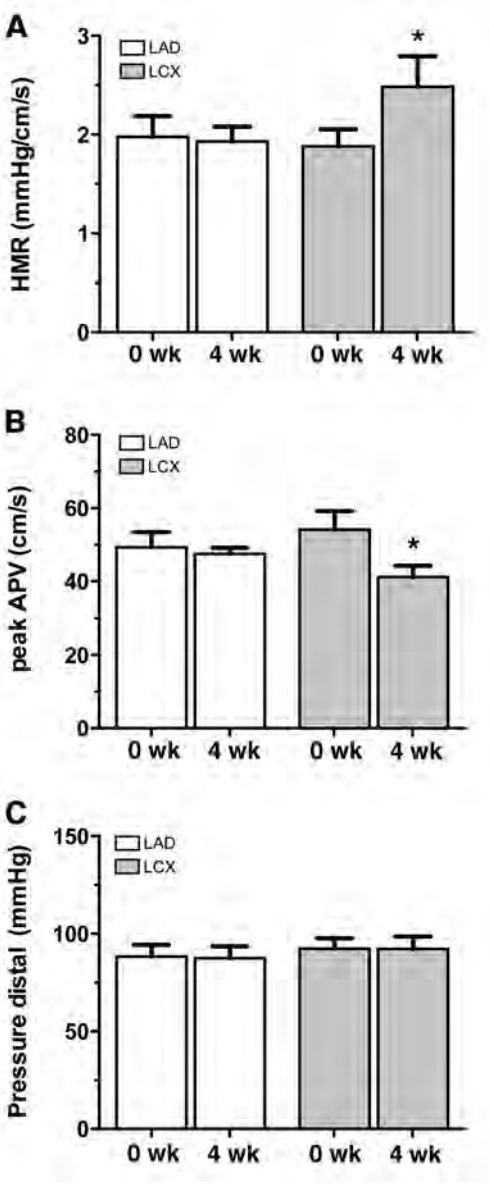

Figure 1. Coronary pressure-/flow derived assessment of microvascular circulation
A. Combined pressure and peak hyperemic flow were used to calculate HMR in both the reference artery (white bars) and the infarct related artery (grey bars). Four weeks after MI, HMR in the LCX was increased (* denotes p = 0.03). B. The peak APV was decreased in the LCX at four weeks after MI compared to baseline (* denotes p = 0.05). C. Intracoronary pressure measured by the Combowire did not change throughout the study. Error bars represent SEM.

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

To examine the role of myocardial vascular density in microvascular resistance, we quantified the CD31+ capillaries (Figure2A,B) and the αSMA+ positive arterioles (Figure2 C,D) in both the infarct area and the remote area. The number of CD31+ capillaries was reduced in the scar tissue (Figure2E; 658±207 per mm^2) compared to the remote area

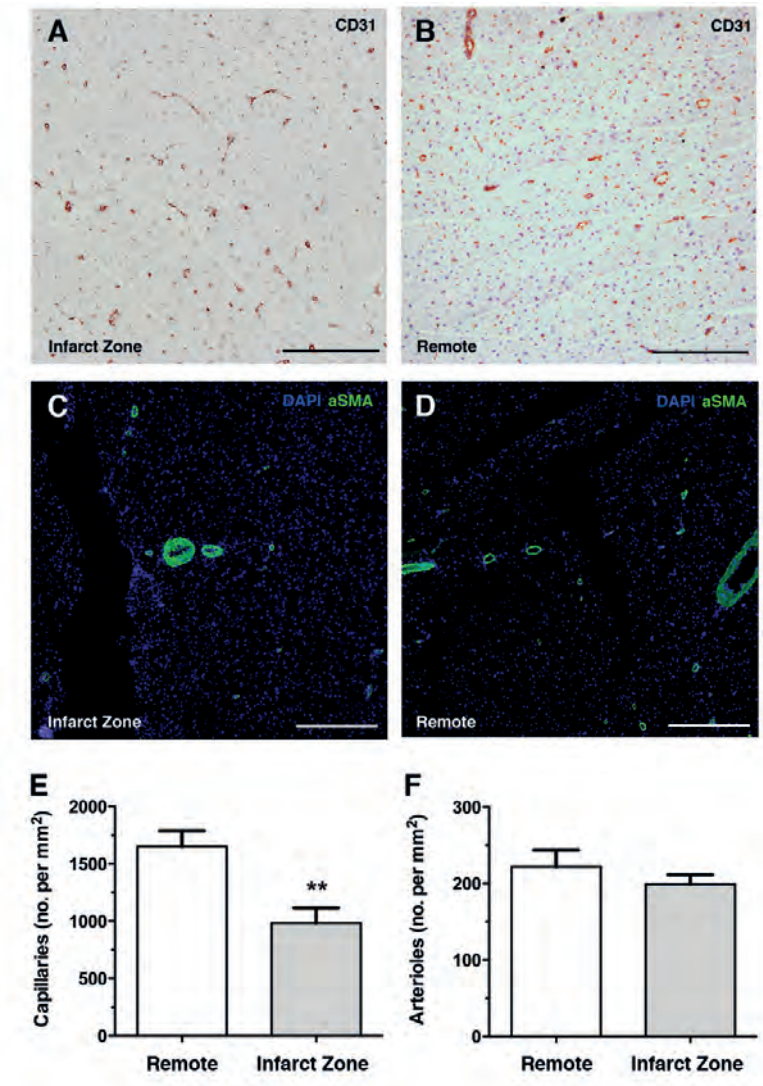

Figure 2. Altered vascular density of the scar tissue impairs microvascular resistance
Representative photographs showing microscopic fields of transversally oriented cardiomyocytes with CD-31 positive capillaries (A,B) or immunofluorescent-labeled αSMA (C,D). A. Peri-infarct zone in the LCX vascularized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

(Figure2E; 1650±304 per mm^2, p=0.0009). Regarding the number of arterioles, there was no change in the infarct area (Figure2F; 199±30 per mm^2) compared to the remote area (Figure2F; 222±52 per mm^2, p=0.369). Thus, these results indicate that the balance between capillaries and larger arterioles is shifted in the infarct scar on the basis of a decreased number of capillaries per number of arterioles.

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

Next, we examined the morphology of the αSMA+ arterioles to elucidate the role of adverse remodeling as a mechanism for impaired coronary response to hyperemia. Strikingly, the αSMA+ arterioles within the infarct zone were characterized by a pronounced thickening of the αSMA+ cells forming a dense layer surrounding the CD31+ endothelial cells compared to the remote area where αSMA+ cells appeared as a small rim surrounding the arterioles (Figure3A-B). The average thickness of the αSMA+ cells per arteriole was increased in the infarct area (Figure3E; 36±14 µm) compared to the remote area (Figure3E; 15±4 µm; p=0.002).

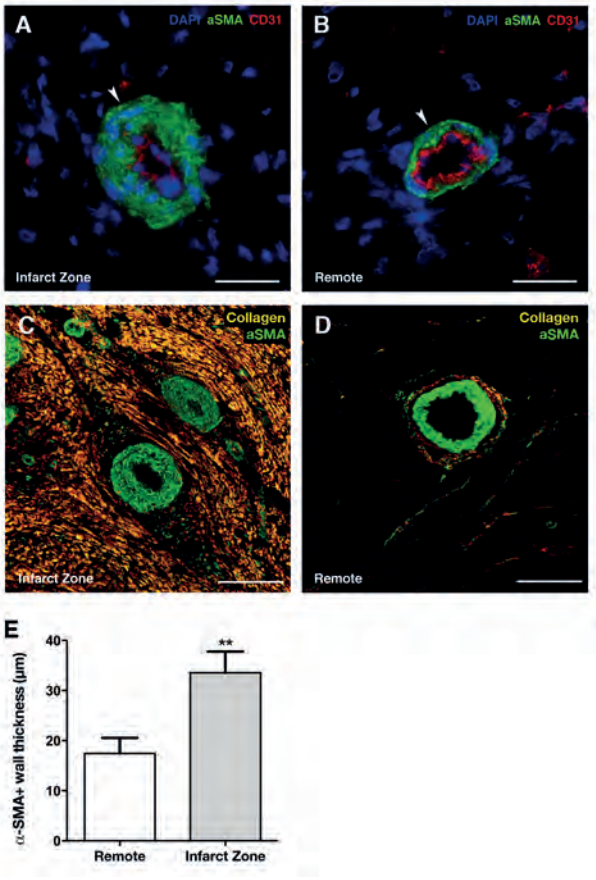

Figure 3. Structural changes in arterioles and extracellular matrix increase microvascular resistance
Representative photographs showing microscopic fields of co-staining with immunofluorescent-labeled αSMA (green signal), CD-31 (red signal) and nuclei counterstained with 4',6-diamino-2-phenylindole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

Furthermore, when combining the αSMA+ fluorescent images with the polarized light microscopy of picrosirius stained collagen, arterioles appeared entrapped in between the dense collagen fibers (Figure3 C) in the infarct zone. In contrast, the remote area revealed a small layer of collagen surrounding the arterioles dispersed in between the viable myocardium (Figure3 D). Altogether, these findings indicate that both adverse remodeling of the αSMA+ vessels and altered extracellular matrix composition could be potential underlying mechanisms leading to impaired coronary flow response and increased hyperemic microvascular resistance.

Table 1. Parameters before and 4 weeks after MI

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

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

DISCUSSION

Since the 1990s, intracoronary pressure and flow velocity measured with sensor-tip guidewires have been introduced as a novel approach for assessment of coronary hemodynamics.[21] The use of guidewire-based assessment of coronary hemodynamics in the cardiac catheterization laboratory is well established to guide clinical decision-making.[15] Yet, this powerful tool has thus far not been implemented as a functional endpoint in large animal models of ischemic heart disease and in particular those designed to validate new angiogenic therapies such as growth factors/cytokines, stem cell therapy or gene therapy. Here, we have shown that intracoronary pressure-/flow velocity could successfully detect an increased hyperemic microcirculatory resistance in a porcine model of chronic MI. Potential mechanisms pertaining to this increased microvascular resistance are three-fold. First, capillary density in the infarct scar tissue was reduced two-fold while larger arterioles were present in similar numbers as in the remote area. Second, arterioles within the infarct zone were characterized by a marked thickening of the αSMA+ cell layer. Third, infarct zone arterioles were dispersed in between dense collagen fibers that could hamper the dilatory capacity of these vessels to hyperemic stimuli. Collectively, these data indicate a dual origin for the increase in hyperemic microvascular resistance, both on the level of arterioles as well as on the level of capillaries.

The role of capillaries in microvascular dysfunction

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

Microvascular dysfunction and arteriolar remodeling in the infarct zone

Another finding of the present study is the contribution of increased arteriolar wall thickness on the curtailed coronary flow. This phenomenon has been observed previously in patients with arterial hypertension.[26] Furthermore, although in a distinct patient population, increased arteriolar wall thickness in heart allografts was linearly associated with an increase in hyperemic microvascular resistance.[27] Coronary arterioles tend to structurally adapt to a

wide variety of pathophysiological situations. In pigs that underwent a gradual coronary stenosis, a range of structural changes and impaired response to bradykinin-1 in arterioles distant to a stenosis was observed.[28] Additionally, type-2 diabetes induced similar narrowing of coronary arteriolar lumen by increase in wall thickness.[29] Given the role of arteriolar dilation during hyperemic peak flow, it is conceivable that this arteriolar remodeling negatively affects their ability to relax and hence impair their vasodilatory capacity and thereby increases vascular resistance.

The effect of the extracellular matrix on the microvascular resistance

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

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

Study Limitations

When extrapolating the current findings to an equivalent patient population of chronic MI, it should be kept in mind that several determinants of increased HMR have not been incorporated in this animal model, such as age, diabetes or reduced pressure due to atherosclerosis and/or subsequent formation of thrombus and/or thrombotic emboli- in the epicardial vessels.[31]

Secondly, it should borne in mind that the use of adenosine has a few practical limitations that precludes an unbiased estimate of the maximal coronary flow. We cannot rule out that dysfunction of vessels and thereby improper reaction on adenosine might also contribute to the increased HMR as well. In coronary artery disease and after myocardial infarction, α-adrenergic vasoconstriction occurs and adenosine does not dissolve this α-adrenergic vasoconstriction.[32]

Thirdly, regarding mechanisms of increased microvascular resistance, undoubtedly, numerous processes in the chronic infarct can account for an increase in microvascular resistance, such as mechanical stresses and strain of the microvessels during myocardial contraction and relaxation or neurologic and/or metabolic dysregulation on vascular alpha-tones.

Finally, morphometric analysis of arterioles may be influenced by fixation of the myocardial tissue. However, we did not observe any signs of arteriolar remodeling in the healthy myocardium, suggesting a dominant role for the chronic infarct as a causal factor.

Acknowledgements

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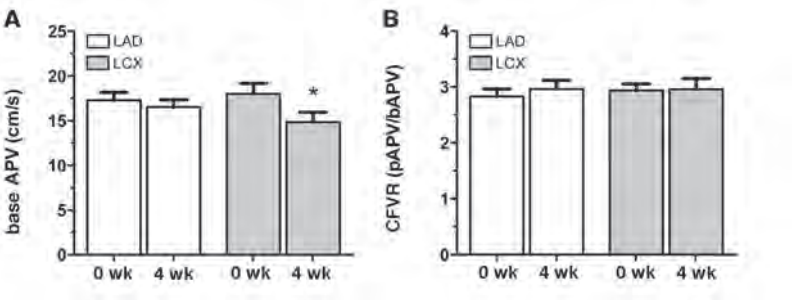
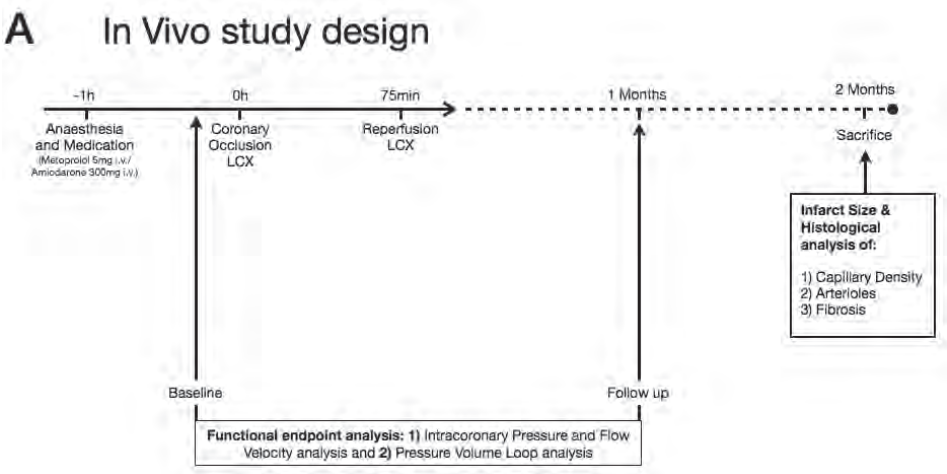
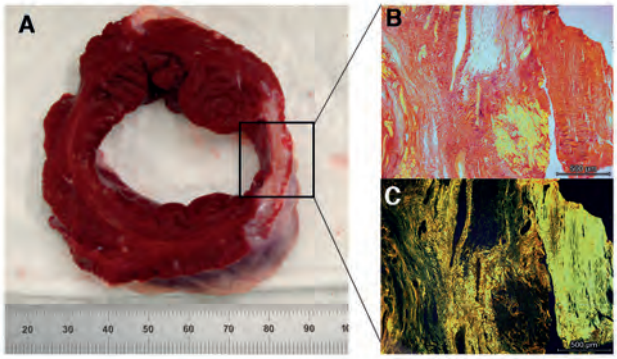
SUPPLEMENTARY INFORMATION



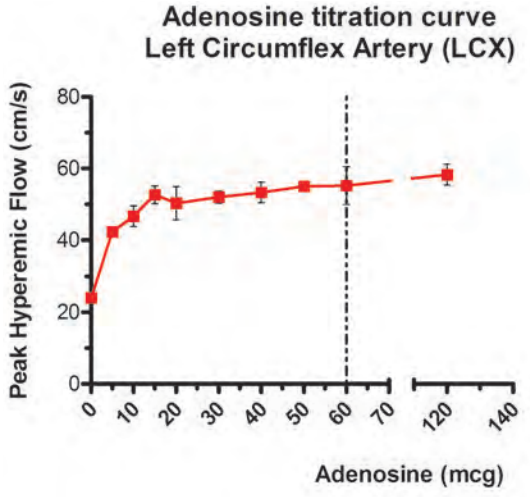
Supplementary Figure 1. Assessment of Coronary Flow Velocity Reserve
(A) At four weeks follow up, coronary flow velocity under base condition was decreased. (B) As a result, the ratio between peak and base APV, reflected by the CFVR, remained similar throughout the follow up duration.



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



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



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

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CHAPTER 11

XENOTRANSPLANTATION OF HUMAN CARDIOMYOCYTE PROGENITOR CELLS DOES NOT IMPROVE CARDIAC FUNCTION IN A PORCINE MODEL OF CHRONIC ISCHEMIC HEART FAILURE RESULTS FROM A RANDOMIZED, BLINDED, PLACEBO CONTROLLED TRIAL

Submitted

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ABSTRACT

Background

Recently cardiomyocyte progenitor cells (CMPCs) were successfully isolated from fetal and adult human hearts. Direct intramyocardial injection of human CMPCs (hCMPCs) in experimental mouse models of acute myocardial infarction significantly improved cardiac function compared to controls.

Aim

Here, our aim was to investigate whether xenotransplantation via intracoronary infusion of fetal hCMPCs in a pig model of chronic myocardial infarction is safe and efficacious, in view of translation purposes.

Methods and Results

We performed a randomized, blinded, placebo controlled trial. Four weeks after ischemia/reperfusion injury by 90 minutes of percutaneous left anterior descending artery occlusion, pigs (n=16, 68.5 ± 5.4 kg) received intracoronary infusion of 10 million fetal hCMPCs or placebo. All animals were immunosuppressed by cyclosporin (CsA). Four weeks after infusion, endpoint analysis by MRI displayed no difference in left ventricular ejection fraction, left ventricular end diastolic and left ventricular end systolic volumes between both groups. Serial pressure volume (PV-)loop and echocardiography showed no differences in functional parameters between groups at any timepoint. Infarct size at follow-up, measured by late gadolinium enhancement MRI showed no difference between groups. Intracoronary pressure and flow measurements showed no signs of coronary obstruction 30 minutes after cell infusion. No premature death occurred in cell treated animals.

Conclusion

Xenotransplantation via intracoronary infusion of hCMPCs is feasible and safe, but not associated with improved left ventricular performance and infarct size compared to placebo in a porcine model of chronic myocardial infarction.

INTRODUCTION

The heart has regenerative capacity as it harbours a pool of cardiac stem cells.[1] However, this is clearly not sufficient to repair the damage caused by myocardial infarction (MI) to prevent the development of heart failure. The number of stem cells available might just be too little. *Ex vivo* expansion and reapplication of cardiac stem cells to the injured heart was proposed, however isolation of these cardiac stem cells remains challenging. Our lab succeeded in isolating fetal and adult cardiomyocyte progenitor cells (CMPC) from mouse and human hearts based on the stem cell antigen Sca-1.[2] The Sca-1-like positive human CMPCs (hCMPCs) from fetal and adult hearts showed differentiation into spontaneously beating cardiomyocytes after stimulation with 5-azacytidine and TGFβ[3] These hCMPC-derived cardiomyocytes have functional gap junctions, enabling metabolic and electrical coupling of cells.[3] Additionally, hCMPCs can also be differentiated into endothelial cells and smooth muscle cells by exposure to vascular endothelial growth factor (VEGF).[2–4]
Intra-myocardial injection of hCMPCs in a mouse model of acute MI led to engraftment of 3,5% of cells, differentiation towards coupled cardiomyocytes, increased vascular density, and to improved cardiac function. [5] These promising results led to the current large animal study, as a next step towards potential clinical application of hCMPCs. Since the greatest burden of ischemic heart disease is based on chronic ischemic heart failure, hCMPCs were tested in a chronic disease model.
In the current study, fetal hCMPCs were intracoronary administered in a porcine model of chronic ischemia/reperfusion injury (I/R). We hypothesized that this strategy is safe, improves left ventricular performance and reduces infarct size compared to placebo.

METHODS

Experimental design

All animal experiments were executed in conformance with the 'Guide for the Care and Use of Laboratory Animals'. The experiment was evaluated and approved by the Animal Experiments Committee of the Utrecht University, the Netherlands (permit number 2012. II.09.145). Animals were subjected to I/R, randomized to fetal hCMPC or placebo infusion 4 weeks later and endpoint analyses were performed 4 weeks after stem cell injection (see study protocol, Figure 1). All animals were immunosuppressed by Cyclosporin A (CsA) to facilitate xenogeneic cell treatment. Based on a power calculation (estimated effect 7.5% [6], standard deviation of 5%, a power of 0.9 and alpha of 0.05) 8 pigs per group were needed. Animals that died before endpoint analysis were supplemented. Animals were randomized after surviving the initial I/R, using a computer based random order generator. Cell and placebo infusion as well as data analysis were performed in a blinded fashion (investigators, technicians and animal caretakers). Deblinding was performed after collecting and analysing all data.
The primary endpoint of this study was defined as left ventricular ejection fraction (EF) at the end of follow-up, measured by magnetic resonance imaging (MRI). Secondary endpoints were left ventricular end diastolic volume and left ventricular end systolic volume (EDV and ESV) measured by MRI, infarct size measured by *ex vivo* gross macroscopy after incubation

with triphenyltetrazolium chloride (TTC) and late gadolinium enhancement (LGE) MRI, functional parameters serially measured by pressure volume (PV-)loop and echocardiography, coronary microvascular function by intracoronary pressure- and flow measurements and vascular density and fibrosis on histology.

Cell isolation

Cells were isolated from fetal human heart tissue (derived after elective abortion with informed consent) and cultured as described before. [2] Shortly, tissue was minced, incubated with collagenase and grinded through a cell strainer. Cells were incubated with anti-Sca-1 microbeads and separated using a MiniMACS magnet (Miltenyi Biotec, Leiden, the Netherlands). hCMPCs were collected and dissolved in growth medium containing endothelial growth medium (EGM-2, Cambrex, CC-4176), FBS, penicillin/streptomycin, non-essential amino acids and bFGF. After attachment of the cells, cells were split after +/- 3 days at 80-90% confluence in a 1:6 fashion. All pigs received hCMPCs of passage 5-7 from the same donor.

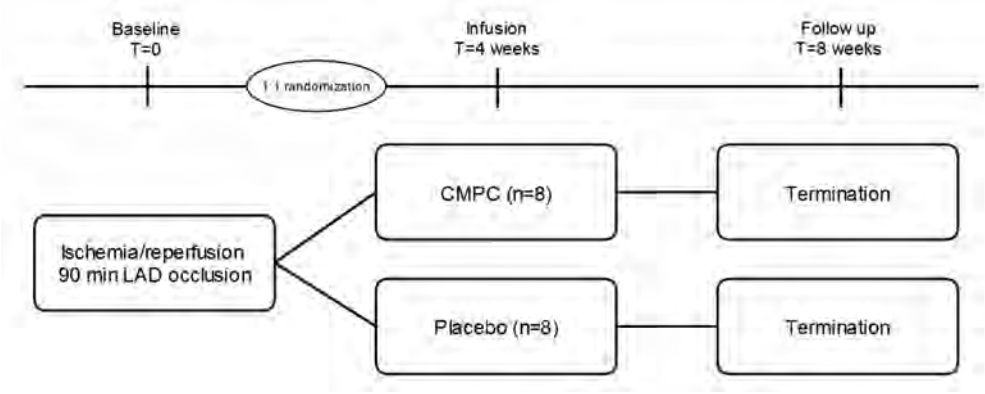

Figure 1. Study protocol

Animal experiment

A comprehensive description of the protocol is also available at http://www.jove.com/video/51269.[7] Female landrace specific pathogen free pigs (n=19) (van Beek SPF Varkensfokkerij B.V. Lelystad, the Netherlands), weighing 68.5 ± 5.4 kg (Upper and lower limit 60.6 – 82.0 kg) at baseline, were pre-treated with amiodaron for 10 days (1200 mg/day for 10 days, 800 mg/day maintenance), clopidogrel for 3 days (75 mg/day) and acetylsalicylic acid for 1 day (320 mg loading dose, 80 mg/day maintenance) and a fentanyl patch (25µg/h) for 1 day. One day before cell or placebo delivery, cyclosporin (CsA) was started. Based on clinical organ transplantation protocol, pigs received a loading dose of 800 mg, then 400 mg b.i.d. for 1 week and 200 mg b.i.d for the remaining 3 weeks by oral administration (Neoral drink, 100mg/ml, Novartis Pharma bv). At the day of surgery, one dosage was i.v. infused as 200 mg in 100mL over 2 hours (Sandimmune 50mg/ml, Novartis Pharma bv). All medication, except for the fentanyl patch, was continued until the end of follow up. Pigs received fiber rich pellets (Abdiets animal nutrition, product 2755, Woerden,

The Netherlands) twice a day and water was available ad libitum. Animals were kept fasted the day of surgery (except for medication).
Anaesthesia was obtained by intramuscular injection of 10 mg/kg ketamine, 0.4 mg/kg midazolam and 0.5 mg/kg atropine in the cage. Pigs were intubated and transported to the operating theatre. Maintenance anaesthesia consisted continues infusion of 0.5 mg/kg/h midazolam, 2.5 µg/kg/h sufentanyl and 0.1 mg/kg/h pancuronium. Other perioperative medication consisted of 300 mg amiodaron, amoxicillin + clavulanic acid 750/75 mg and heparin (100 IE/kg after positioning the sheaths and 50 IE/kg every 2 hours). Pigs were mechanically ventilated with a positive pressure ventilator with FiO2 0.5, 10ml/kg tidal volume and a frequency of 12/minute under continuous capnography. Arterial access was achieved by cannulating the internal carotid artery with an 8F sheath. Venous access was achieved by cannulating the jugular vein with a 9F sheath. An additional arterial line was inserted in a small peripheral artery in the hind limb for continuous stable arterial pressure registration.

Echocardiography

Pigs were positioned in the right lateral position. Parasternal short axis images were obtained at 3 levels (mitral valve, papillary muscle and apex) during 5 beats per level (iE33 ultrasound device Philips, Eindhoven, The Netherlands). Short axis images were analysed offline using Xcelera R2.L1 (Philips Healthcare, Best, The Netherlands) and fractional area shortening (FAS), fractional shortening (FS) and septal wall thickening (WTsept) at the level of the mitral valve, papillary muscle and apex (mitral, pap, apex respectively) were calculated. Because of apical dilatation of the left ventricle after infarct, the modified Simpsons rule was not applicable and no reliable volumes could be calculated.

Pressure volume loop measurement

Admittance based PV-loop measurements were performed as recently described. [8] Cardiac output (CO) was measured three times by thermodilution and stroke volume was calculated out of three measurements. The 7F tetra-polar admittance catheter (7.0 VSL Pigtail/no lumen, Transonic Scisense, London, Canada) was inserted into the left ventricle through the arterial sheath under fluoroscopic guidance. Inferior caval vein occlusion was performed using an 8F Fogarty occlusion catheter (62080814F, Edwards Lifesciences). All measurements were performed during apnea. Data were offline analysed using Iworx analysis software (Labscribe V2.0).

Intracoronary pressure and flow velocity assessment

Intracoronary pressure and flow were assessed by positioning a Combowire in the left anterior descending artery (LAD) and the left circumflex coronary artery (LCX) acting as a control subsequently. Intracoronary pressure and flow measurements, together with arterial pressure and ECG, were recorded using the ComboMap system (Volcano Corporation). An intracoronary bolus of 200 µg nitroglycerin was administered to prevent coronary spasms. Three successive measurements were recorded in rest and during hyperaemia, achieved by intracoronary administration of 60 µg adenosine. Data were analysed offline, using AMC Volcano Studymanager (versus 6.0, Borland Software Corporation and Delphi versus 2010, Embarcadero, San Francisco, CA, USA).

Ischemia/reperfusion injury

After baseline measurements, the diameter of the LAD, distal to the second diagonal branch (D2) was measured in anterior-posterior and left anterior oblique 30° view. Before positioning the balloon, an intracardiac defibrillation catheter was placed in the right ventricle. A PCI-balloon catheter with a suitable diameter was positioned distal from the D2 and inflated for 90 minutes. In case of ventricular arrhythmias, chest compressions were started immediately, amiodaron 300mg was infused intravenously and pigs were defibrillated intracardiac with 50J. In case of multiple unsuccessful shocks, defibrillation was switched to transthoracic defibrillation with 150-200J.

Cell infusion

Ten million cells were resuspended in 10mL of phosphate buffered saline (PBS), one hour before intracoronary infusion. Cell suspension or placebo (10mL of PBS) was handed to the animal technician in a covered sterile syringe so that the personnel at the operating theatre remained blinded for the treatment allocation. An over-the-wire PCI balloon catheter (Apex, Boston Scientific), was positioned distal to the D2, according to the position of the PCI catheter during infarct creation. The balloon was inflated until pressure matched the coronary diameter. 10mL of cell suspension or placebo was infused in three sessions consisting of 30 seconds infusion of 3.3mL during 2 minutes balloon occlusion followed by 3 minutes of reperfusion. Intracoronary pressure and flow measurements were repeated, 30 minutes after the last reperfusion period, to detect possible flow restrictions caused by cell or placebo infusion.

MRI

At the end of follow up, cardiac MRI and all other functional measurements were performed. All MR studies were performed on a 3T MRI scanner (Achieva TX, software release 3.2; Philips Healthcare, Best, The Netherlands). Both cine images and LGE images in short axis and a two-chamber long axis orientations were obtained under continuous anesthesia and mechanical ventilation. LGE images were obtained at least 15 minutes after injection of 0.2 ml/kg gadobutrol (Gadovist, Bayer Healthcare). Offline imaging analysis for functional measurements was performed in Qmass MR 7.4 enterprise solutions (Medis medical imaging systems BV, Leiden) and by Segment version v1.9R 3293 (Medviso AB, Lund, Sweden) for infarct size. Infarct size at the end of follow-up was analysed based on the 2SD method.

Ex vivo

Infarct size

Animals were sacrificed by exsanguination under general anesthesia. The hearts were excised, washed and cooled with running tap water and the LV was then cut into 5 equal slices from apex to base. Slices were incubated in 1% TTC (Sigma-Aldrich Chemicals, Zwijndrecht, the Netherlands) in 37°C 0.9%NaCl for 15 min to discriminate infarcted tissue from viable myocardium. The infarcted area was calculated as percentage of the left ventricle.

Histology

Sections of infarct zone, border zone and remote area of all pigs were isolated and fixated in 4% formalin for at least 1 week and then embedded in paraffin. Sections of 5 µm were

stained for endothelial cells by lectin (Lectin fom Bandeiraea simplicifolia, Sigma Aldrich L5391) and for fibrosis by picrosirius red (Sirius red F3B, BDH and picrin acid, Boom. Cat. 12388).
For vascular density, 5 random pictures per slide with 40x magnification were taken from the border zone area. The number of vessels was counted manually by two independent observers (S.J. and J.G.) and the absolute number of lectin positive vessels per field were averaged. For assessment of fibrosis, 3 random pictures per slide (slides for infarct zone, border zone and remote area) were taken using polarized light during one session. Pictures were analysed in Cell^p (version 5.0 Olympus) for mean grey value and percentage of fibrotic area by using the same settings for all slides.

Cyclosporin assay

The effect of CsA on the cells was tested by a migration assay, sprouting matrigel assay and by testing cellular growth factor release. Based on the pig serum levels of CsA, *in vitro* assays were executed to analyze performance of CMPCs in presence of CsA. CMPCs were maintained in 0.1% gelatin coated plates with standard CMPC culture medium. For all *in vitro* experiments low passages were used (passage 8-15). For all assays, standard culture medium was used as a negative control. Three different concentrations of CsA were used as experimental conditions (50 ng/mL, 150 ng/mL and 300 ng/mL). See supporting information for detailed description of the methods.

Statistical analysis

Data are reported as mean ± standard deviation. Comparisons of serial measurements over time within one group or for all animals combined were performed by paired t-tests. Comparisons between both groups were made by independent t-tests. Differences between conditions in cyclosporin assays were tested by one-way ANOVA. The statistical software used is IBM SPSS statistics, version 20.0 (IBM Corporation, Armonk, NY, USA).

RESULTS

A total of 19 pigs were used in this study. In 9 pigs VF occurred during occlusion, of which 8 converted successfully by defibrillation and amiodaron infusion. A total of three pigs died; one pig died of VF during LAD occlusion, one pig died by periprocedural complications before cell/placebo infusion and one animal died one day after placebo infusion by unknown cause. All other 16 animals were included in this study.

Safety

Two hours after cell infusion during short coronary occlusion, high sensitive troponin I (TnI) levels were 545 ± 460 ng/L n=8 after cell infusion and 623 ± 764 ng/L n=8 after placebo infusion. In comparison, mean TnI levels 3.5 hours after the start of 90 minutes balloon occlusion, were 1147822 ± 584559 ng/L (n=16), confirming the induction of a large MI. Intracoronary pressure and flow measurement (see below) showed no evidence for intracoronary embolization or obstruction in CMPC treated animals 30 minutes after infusion. None of the CMPC treated animals died by arrhythmias, or any other cause.

Left ventricular performance

MRI

At the end of follow-up EF, EDV and ESV did not differ between groups. EF in CMPC treated animals was 40.6 ± 4.2 % versus 38.3 ± 8.6% in placebo animals (p=0.52). EDV was 172.7 ± 19.0 ml in CMPC treated animals and 150.0 ± 27.1 ml in placebo (p=0.07). ESV was 102.6 ± 13.3 ml in CMPC treated and 91.5 ± 14.9 ml in placebo animals (p=0.14). (Figure 2)

PV-loop

Serial functional measurements were performed by PV-loop. Because of technical failure, we could not complete data analysis of all animals at all time points. For EF, ESV and EDV we could include 7 out of 8 cell treated animals and all 8 placebo animals. For end systolic pressure volume relationship (ESPVR), end diastolic pressure volume relationship (EDPVR) and V0, we could include 6 animals per group.

Induction of MI caused a significant decrease in EF over 4 weeks (-8.7 ± 11.6% from 54.8 ± 8.7 to 46.1 ± 9.5 p=0.01) and an increase in ESV (+40.6 ± 62.3 ml from 85.3 ± 27.1 to 125.9 ± 49.1 p=0.02), and trend towards an increased EDV (+38.1 ± 73.6 ml, from 186.7 ± 37.6 ml to 224.7 ± 50.9 p=0.07), confirming successful creation of a chronic MI model. No significant differences between both groups were observed at pre-infusion time point (EF p=0.34, EDV p=0.40, ESV p=0.27). (Figure 3)

For all parameters (EDV, ESV, EF, ESPVR, EDPVR and V0), no differences between both groups were found at any time point except for EDPVR at follow up. EDPVR was 0.004 ± 0.002 for cell treated animals and 0.008 ± 0.004 for placebo animals (p=0.04).

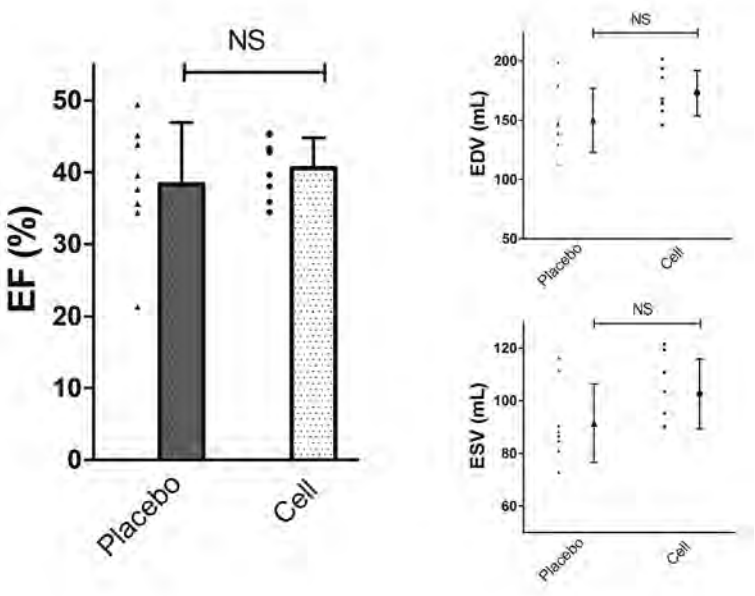

Figure 2. Functional measurements by MRI of cell- and placebo treated animals at follow up
Individual data and mean with SD, n=8 per group. EF Left ventricular ejection fraction, EDV End diastolic volume, ESV End systolic volume.

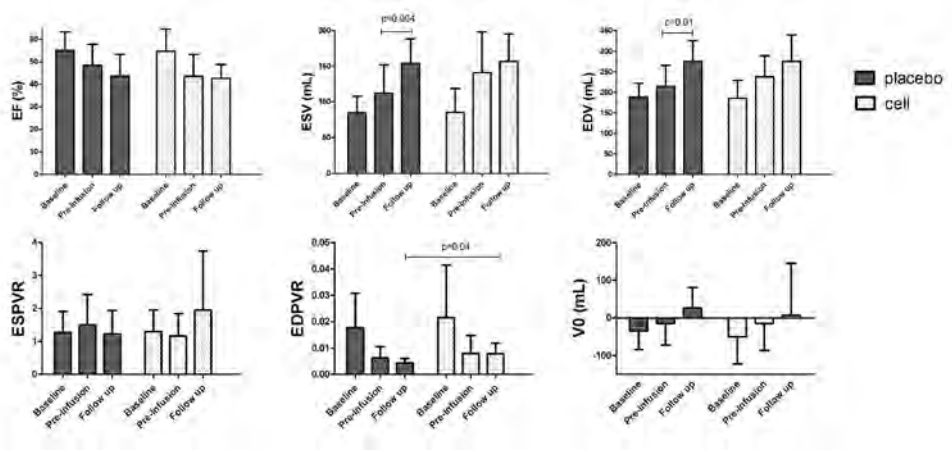

Figure 3. Functional outcome measurements by PV loop
Mean and SD for PVloop parameters. EDV End diastolic volume, ESV End systolic volume, EF Left ventricular ejection fraction, ESPVR End systolic pressures volume relationship, EDPVR end diastolic pressure volume relationship. Placebo n=8, cell n=7 for ESV, EDV and EF, n=6 per group for ESPVR, EDPVR and V0.

Visual appearing trends in functional outcomes are observed in Figure 3 (and Supporting Table 2). However, EF did not increase after cell or placebo infusion (p=0.85 and p=0.17 respectively) EDV and ESV did not change after cell treatment (p=0.25 and p=0.58 respectively), but both significantly increased after placebo infusion (p=0.01 and p=0.004), suggesting further deterioration after placebo infusion compared to cell treatment. No statistical significant differences in ΔEDV and ΔESV were found between cell treated animals and placebo animals.

Echocardiography

WTsept and FAS at the apical level significantly decreased after MI (WTsept -0.34 ± 0.23 p < 0.001, FAS -0.08 ± 0.11 p = 0.02), confirming successful apical infarct creation. A trend towards decrease of FS at the apical level was also observed (-0.50 ± 0.11 p=0.08) in both groups.
For FAS, FS and WTsept, no statistical significant differences were identified between groups at any time point. A trend was observed for a higher mean FAS (mean of three levels) at follow up in cell treated animals compared to placebo animals (cell treated 0.49 ± 0.06 placebo 0.44 ± 0.04 p=0.08). Next, no differences in decrease or increase of any other echocardiographic parameter after hCMPC or placebo infusion was observed. (Figure 4 and Supporting Table 3)

Intracoronary pressure and flow

Hyperemic microvascular resistance is a parameter for assessing the microvascular bed and is calculated as the ratio of intracoronary pressure (Pd) and hyperemic average peak flow velocity (pAPV). It is known from previous studies that HMR increases after I/R, accompanied by decreased pAPV and a constant Pd.[9] At the time point of cell infusion, measurements in one animal failed because of technical problems.
HMR in the LAD region significantly increased after I/R as expected (p=0.03), Pd in the LAD significantly increased (p=0.03) and pAPV in the LAD tended towards a decrease of

(p=0.10). All parameters in the control region remained stable over time. Thirty minutes after cell or placebo infusion, HMR was significantly decreased in all animals (2.7 ± 1.0 mmHg/cm/s pre-infusion, 2.2 ± 0.6 mmHg/cm/s post-infusion, p = <0.001), without any differences between hCMPC and placebo treated animals (ΔHMR -0.50 ± 0.48 mmHg/cm/s for hCMPC treated, -0.56 ± 0.66 mmHg/cm/s for placebo treated animals, p=0.21). Decrease in HMR was accompanied by an increase in pAPV (41.5 ± 11.0 cm/s pre-infusion, 48.9 ± 11.7 post-infusion, p=0.01), without differences between groups (p=0.7), both advocating against intracoronary (micro-)embolization or obstruction by cell infusion. Decreased HMR and increased pAPV can be explained as a hyperemic response after short ischemia during infusion. Neither cell-, nor placebo infusion caused a change in microvascular parameters over time.

Infarct size

For LGE, infarct size was 17.5 ± 3.8% and 18.0 ± 4.5% of the left ventricular mass for cell treated and placebo animals respectively at follow up. For TTC, infarct size in the cell treated group was 14.6 ± 2.5% of the left ventricle, and 13.8 ± 3.9 in the control group. There was no statistical difference in infarct size between both groups at termination of follow-up, for both modalities. (Figure 5)

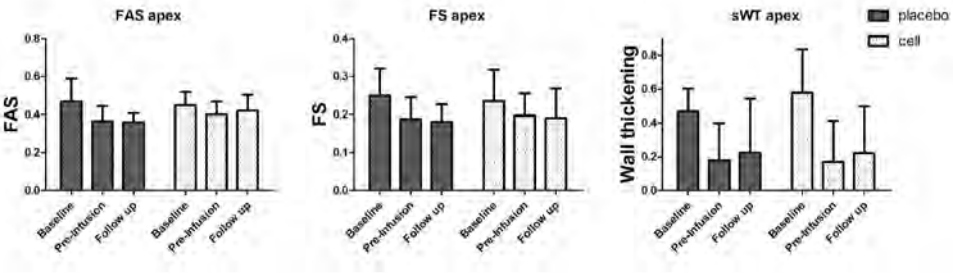

Figure 4. Functional measurements by echocardiography
Mean and SD, n=8 per group. FAS Fractional area shortening, FS Fractional shortening, sWT Septal wall thickening at the level of the apex.

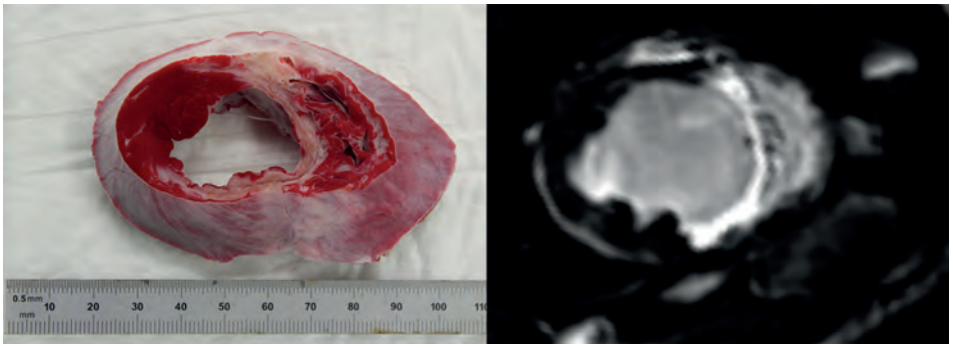

Figure 5. Infarct size
Representative pictures of TTC stained heart slice and LGE image of the same animal.

Histology

For fibrosis, there were no differences between both groups. A significant higher vascular density was observed in the placebo group compared to the hCMPC animals. (Table 1, Figure 6)

Cyclosporin

CsA did not affect functioning of CMPCs *in vitro* with regard to migration capacity, angiogenesis and growth factor secretion. See supporting information for results and figures.

Table 1. Fibrosis

		CMPC (n=8)	Placebo (n=8)
% Fibrosis	Infarct	39.1 ± 12.2	40.0 ± 16.0
	Border	1.6 ± 1.2	2.9 ± 2.3
	Remote	6.5 ± 7.8	2.8 ± 2.3
Grey value	Infarct	27.0 ± 8.9	28.5 ± 12.7
	Border	1.2 ± 0.7	2.0 ± 1.4
	Remote	4.3 ± 4.3	2.6 ± 2.2
Vascular density		33.9 ± 10.1	48.3 ± 10.1 *

* p=0.01 between CMPC and placebo.

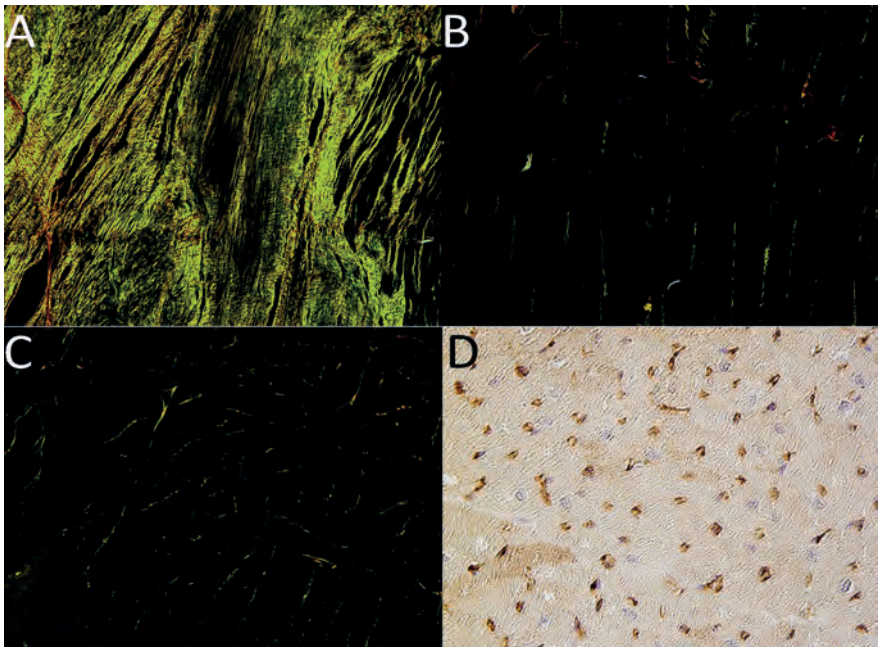

Figure 6. Histology
Representative pictures of picrosirius red staining of infarct area (A), borderzone (B) and remote area (C) and lectin staining of the borderzone (D). Table presents mean ± SD percentage fibrosis and mean grey Infarct size

DISCUSSION

In this pre-clinical model of chronic MI, we show that xenotransplantation via intracoronary infusion of fetal hCMPCs is feasible and safe, but we did not find a benefit in the current model on cardiac function, infarct size, fibrosis and vascular density. The non-significant difference in EF between both groups of 2.3% measured by the golden standard MRI is lower than the effect size of 7.5% as documented in a recent meta-analysis and used for power calculation.

We believe that these data in the field of cardiac regeneration are of great importance. It complements the preclinical evidence needed for successful translation of cardiac regeneration by cardiac cell therapy from bench to bedside. We present a solid and sound piece of evidence since this study is conducted by clinical standards in terms of blinding, randomization and outcome assessment.

Preclinical studies of cardiac cell therapy

The neutral results presented in the current study, are partly in line with other large animal studies with cardiac stem cells for MI (table 2). Over the last decade, five different cardiac stem cell sources have been identified[10] and two of them have been extensively tested in large animal studies. Clonogenic, multipotent and self-renewing cardiac stem cells, based

Table 2. Overview of studies of cardiac derived stem cells in pig models

	N	Route	Source	Immuno suppression	Dose	Timing	Follow up	Result
CDC								
Johnston et al.[31] *Circulation 2009*	17	IC	Autologous	NA	1,0E+07	4 wk	8 wk	No EF or IS improvement
Lee et al.[32] *JACC 2011*	29	IM	Autologous	NA	1,0E+07	4 wk	8 wk	No functional improvement on echo. EF improved on LV angiography
Malliaras et al.[33] *Circulation 2013*	10	IC	Allogeneic	No	1,25E+07	2-3 wk	8 wk	EF preservation, IS reduction
Kanazawa et al.[34] *Circ. Heart Fail. 2015*	14	IC	Allogeneic	No	8,5-9E+06	30 mins	2 days	No difference in EF, IS reduction
Yee et al.*[35] *PLoS ONE 2014*	22	TE	Allogeneic	No	1,5E+08	8 wk	8 wk	No functional improvement, IS reduction
CSC								
Williams et al.[22] *Circulation 2013*	10	IM	Human	Cyclosporin	1,0E+06	14 days	1 mnth	Restored EF, IS reduction
Bolli et al.[23] *Circulation 2013*	21	IC	Autologous	NA	5,0E+05	3-4 mnth	1 mnth	Improved EF, IS reduction

IM intramyocardial injections, IC intracoronary infusion, TE transendocardial injections, EF ejection fraction, IS infarct size. * used cardiospheres instead of CDCs.

on the C-kit epitope (CSCs)[11,12] and cluster-forming cells, positive for C-kit and Sca-1, called cardiosphere derived cells (CDCs) [13] were used in these studies. Both cell types have gone through the process of pre-clinical investigation and progressed from bench to bedside testing. In a recent study, expanded cells upon different cardiac progenitor cell isolation procedures were compared (including, c-kit+, cardiospheres, and sca1+ cells), and it was suggested that these cells have highly similar transcriptional profiles.[14] Therefore, it is important to closely look at particular study design aspects of the study to look for possible explanations

Study design considerations

A multitude of parameters in the research field of cell therapy for cardiac repair must be considered when conducting pre-clinical or clinical studies, like timing of therapy, administration route, cell source, cell number, etc. In a meta-analysis including all large animal studies on cell therapy for MI, none of these parameters showed significant impact on effect size.[15] Additionally, no ruling about these issues was provided based on previous CSC studies (Table 2). Therefore, we made upfront decisions about all these variables based on experience and clinical relevance.

Treatment of acute myocardial infarction is greatly improved over the last decades, but patients surviving the initial episode often suffer from chronic ischemic heart failure. The current animal model was designed to represent this group. A direct comparison of efficacy of stem cells in acute versus chronic ischemic heart disease has never been performed, but safety is at least similar in acute and chronic patients.[23] Table 2 shows negative and positive results of cardiac cell therapy in both acute and chronic settings. We administered cells via intracoronary infusion, which is considered as efficient as other routes with regard to cell retention [24–26] and functional improvement [27,28] but is less invasive then intramyocardial injections and less time consuming compared to transendocardial delivery.

We investigated efficacy of infusion of 10 million cells. Table 2 show both positive and neutral results from CDCs in comparable doses. For CSCs, promising results are found by infusion of 0.5 and 1 million cells in smaller pigs (1.5 ± 0.8kg and 35-40 kg respectively, compared to 68.5 ± 5.4 kg in the current study).[21,22] Roughly, we applied 4 times higher indexed doses (37.000/kg versus 140.000/kg) of cells. These data show that relative under-dosing was certainly not anticipated for in the present study.

Xenogenecity

The impact of xenogenicity is important since human cells were administered in a porcine model. Alloreactivity (and xenoreactivity) depends on foreign peptide presentation by the major histocompatibility complex (MHC) on antigen presenting cells and detection by T cells.[29] In clinical practice, alloreactivity is suppressed by T cell suppressors like CsA, and this strategy was applied accordingly in the present study.

Next to immunosuppression, the effect of CsA includes prevention of apoptosis [30] and protection of the myocardium in the setting of acute myocardial infarction.[31]. Since CsA was administered at 4 weeks after MI until the end of follow-up, we believe it did not affect the outcome by that cause. Adequate dosing by oral administration of CsA is safe in pigs[32], but not much is known about the pharmacodynamics. We did not observe differences in

serum levels of leukocytes, nor on renal function between hCMPC and placebo treated animals 30 minutes and 4 weeks after treatment, all receiving CsA. Also, we did not find a correlation between serum CsA levels at follow up and effect size.
There is no consensus about the effect of CsA on stem cells *in vitro* and *in vivo*.[32] In this respect, we tested the effect of different levels of CsA on migration, angiogenesis and growth factor expression of hCMPCs *in vitro* (See supporting information). No significant effect of CsA was noted. Taken together, we assume that our results are not confounded by the chosen CsA regimen.

Internal and external validity

Internal validity has major impact on effect size, as it is known that blinded and randomized studies produce smaller effect sizes compared to non-blinded and non-randomized reports.[33] Based on clinical standards, our pre-clinical study has been performed in a randomized, blinded (both for intervention and outcome assessment) and placebo-controlled manner.
The animal model is a surrogate for human disease. We included large pigs (68.5 ± 5.4 kg), as the heart corresponds with human (coronary) anatomy and size, to increase external validity (the translatability towards other models or populations). However, age and co-morbidity are not accounted for in this model.
Our standardized way of infarct creation caused a decrease in EF of 8.7% based on PV-loop measurements, despite high TnI levels and a mean infarct size of 17.8%. It is known from clinical studies that patients with a low baseline EF benefit most from cell therapy.[34,35] In retrospect, the anticipated effect size of 7.5% (difference in EF at follow up between cell and placebo treated animals on MRI) might have been too high. Power calculation was based on difference in EF at follow up, measured by MRI and not for the other functional measurement modalities (echocardiography and PV-loop), which might thereby be underpowered to report significant differences.

Conclusion

In this randomized, blinded placebo controlled preclinical study, xenotransplantation via intracoronary infusion of fetal human CMPCs in a pig model of chronic I/R injury appeared to be feasible and safe, but showed no significant improvement with regard to cardiac function or infarct size.

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SUPPLEMENTARY METHODS

Histology

For staining, slides were deparafinated by 2x10minutes ultraclear, 2x5 minutes 99% EtOH, 2x5 minutes 96% EtOH and 5 minutes 7% EtOH. Slides were washed three times with dH2O for 5 minutes. For picrosirius staining, all slides were stained at once for 30 minutes in filtered picrosirus red. Slides were washed twice 0.2N HCl, then in dH2O for 5 minutes and dehydrated (quick 70% EtOH, 2x quick in 96% EtOH, 2x 5 minutes 99% EtOH, 2x10 minutes Ultraclear), mounted with entallan and covered with coverslips.
For lectin, deparafinated slides were incubated with 3% H2O2 for 20 minutes and with 1% BSA in PBS for 15 minutes. Slides were incubated over night with Lectin (1:50 in PBS). After washing twice with PBS, slides were stained with DAB+ chromogen in DAB+ substrate buffer (Dako K3467) for a maximum of 4 minutes. Slides were washed with running tap water, dehydrated as above, mounted with entellan and covered with coverslips.

Cyclosporin assays

Migration assay

A migration scratch assay was performed to compare migratory capacity. CMPCs were plated in 0.1% gelatin coated 6 well plates in medium with additional CsA in three concentrations. At 90% confluency a scratch was placed vertically in the middle of the well using a pipet tip, after which medium was changed and replaced with either fresh medium or medium containing CsA. Pictures of the initial scratches were immediately taken. After 3 and 5 hours new pictures were taken of the scratched area. Analysis of migration capacity was performed using the ImageJ software.

Sprouting matrigel assay

Angiogenesis µ-slides (Ibidi) were used to quantify matrigel tube formation. Slide chambers were coated with 10 µl ECMatrixTM (Millipore). CMPCs were seeded on the matrigel in 50 µl, with or without CsA. Tube formation was imaged after 13 hours and quantified using the Angioquant software in Matlab.

Growth factor antibody array

Conditioned medium was collected from the CMPCs after 9 days of culture in low serum medium without any additional growth factors, with or without CsA. Human Angiogenesis Array G1 (RayBiotech) was performed on the conditioned medium according to the provided instructions. Three conditions were tested; normal medium, 150 ng/mL and 300 ng/mL. Next, we tested culture medium from a different CMPC cell line as a positive control.

RESULTS

Migration of CMPC was the same for all conditions (Supplemental Figure 1). The migration was 51.0 ± 9.8 % for the normal medium and 42.8 ± 10.7%, 50.2 ± 10.1% and 57.5 ± 8.5% in the presence of CsA (50ng/mL, 150 ng/mL and 300 ng/mL respectively) (p=0.95) For angiogenesis, the number of segments per area, total length per area and length per

segment were calculated. For all 3 measures, no difference exists between conditions (Supplemental Table 3, Figure 2).
Conditioned medium of CMPCs cultured for 9 days contained Angiogenin (ANG), CXCL 1, 2and3 (GRO), Il-6, Il-8, CCL2 (MCP1), CCL5 (RANTES) and metallopeptidase inhibitor 1 (TIMP1). Presence of CsA did not affect growth factor secretion by CMPCs (Supplemental Figure 3).

Supplemental Table 1. Angiogenesis

	medium	50 µg/L	150 µg/L	300 µg/L	P-value
Total number/area	1.26 ± 0.23	1.10 ± 0.09	1.05 ± 0.25	1.00 ± 0.25	0.49
Total length/area	86.0 ± 15.0	70.6 ± 12.0	63.1 ± 15.9	64.5 ± 12.8	0.25
Length/number	67.9 ± 1.8	64.1 ± 5.6	60.1 ± 1.8	64.9 ± 4.6	0.18

Supplemental Table 2. Functional outcome measured by PV loop

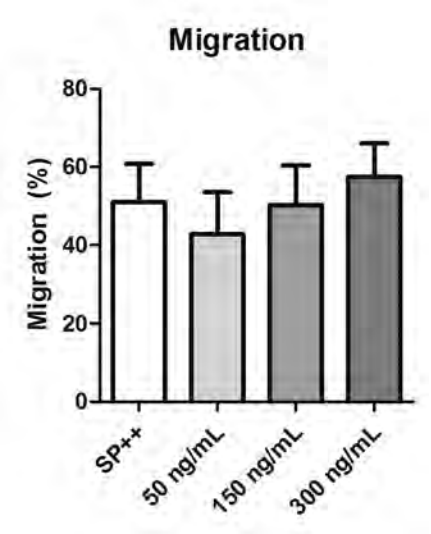
	Cell			Placebo		
	Baseline	Pre-infusion	Follow up	Baseline	Pre-infusion	Follow up
EDV (ml)	185.1 ± 43.7	237.1 ± 51.4	275.5 ± 64.1	188.1 ± 34.3	213.9 ± 51.3	274.9 ± 51.0
ESV (ml)	85.6 ± 33.3	141.2 ± 57.0	157.0 ± 38.3	84.9 ± 22.9	112.5 ± 39.9	154.2 ± 34.3
EF (%)	54.6 ± 9.8	43.5 ± 9.8	42.7 ± 6.0	55.0 ± 8.3	48.4 ± 9.3	43.7 ± 9.7
SW	1.3 ± 0.4	0.9 ± 0.8	1.6 ± 0.8	1.3 ± 0.4	0.9 ±0.7	1.7 ± 0.4
ESPVR	1.3 ± 0.6	1.2 ± 0.7	1.9 ± 1.8	1.3 ± 0.6	1.5 ± 0.9	1.2 ± 0.7
EDPVR	0.022 ± 0.020	0.008 ± 0.007	0.007 ± 0.004	0.017 ± 0.013	0.006 ± 0.004	0.004 ± 0.002*
dPdT+	1559.3 ± 196.3	1483.9 ± 414.0	1667.1 ± 324.9	1544 ± 210.4	1575.9 ± 182.3	1316.9 ± 97.9
dPdT-	-1231.3 ± 195.9	-1213.1 ± 379.4	-1305.5 ± 274.7	1388.4 ± 224.7	-1223.0 ± 248.8	-1077.7 ± 269.2
V0	-49.9 ± 71.8	-14.4 ± 71.5	-6.9 ± 139.0	-34.7 ± 49.0	-14.5 ± 57.5	-26.8 ± 54.3
Tau	45.8 ± 9.6	55.2 ± 22.4	53.4 ± 10.2	40.2 ± 8.4	48.4 ± 7.5	60.6 ± 14.8

EDV End diastolic volume, ESV End systolic volume, EF Left ventricular ejection fraction, SW Stroke work, ESPRV End systolic pressure volume relationship, EDPVR End diastolic pressure volume relationship, dPdT+ maximum pressure rise, dPdT- Minimum pressure rise, V0 Theoretical volume at zero pressure. * p=0.03 Cell treated animals compared to placebo. For EDV, ESV and EF n=7 for cell treated animals, n=8 for placebo treated animals. For SW, ESPRV, EDPVR, dPdT+, dPdT-, V0 and Tau n=6 per group.

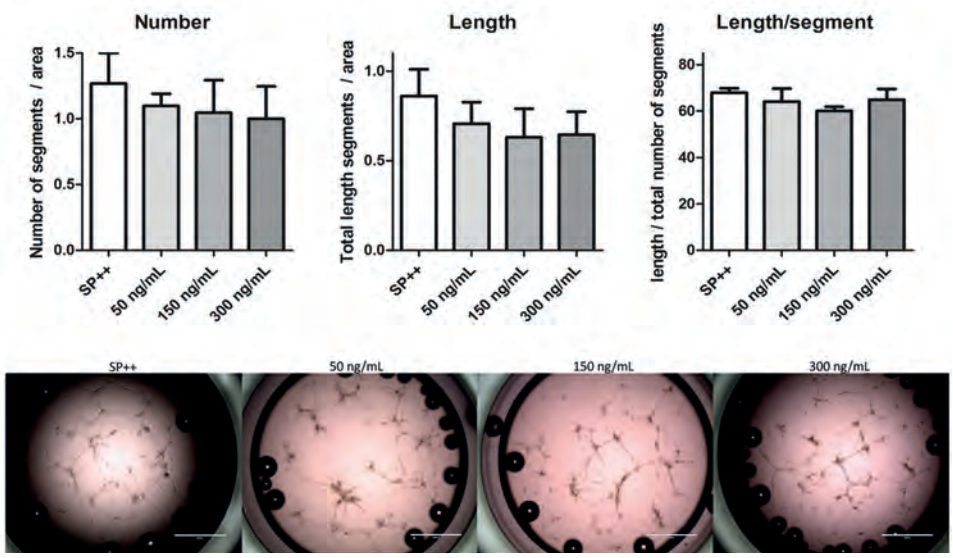
Supplemental Table 3. Functional outcomes measured by echocardiography

	Cell			Placebo		
	Baseline t=0 (n=8)	**Pre-infusion (n=8)**	**Follow up (n=8)**	**Baseline t=0 (n=8)**	**Pre-infusion (n=8)**	**Follow up (n=8)**
sWT mitral	0,18 ± 0.12	0.23 ± 0.12	0.27 ± 0.20	0.29 ± 0.20	0.28 ± 0.11	0.14 ± 0.16
sWT pap	0.36 ± 0.13	0.37 ± 0.15	0.35 ± 0.24	0.39 ± 0.15	0.35 ± 0.32	0.42 ± 0.41
sWT apex	0.58 ± 0.26	0.17 ±0.24	0.22 ± 0.27	0.47 ± 0.14	0.18 ± 0.21	0.23 ± 0.32
sWT mean	0.37 ± 0.13	0.26 ± 0.10	0.28 ± 0.13	0.38 ± 0.14	0.27 ± 0.16	0.26 ± 0.21
FS mitral	0.23 ± 0.06	0.29 ± 0.05	0.29 ± 0.05	0.28 ± 0.05	0.30 ± 0.04	0.27 ± 0.06
FS pap	0.23 ± 0.05	0.28 ± 0.05	0.27 ± 0.04	0.24 ± 0.05	0.30 ± 0.04	0.25 ± 0.06
FS apex	0.24 ± 0.08	0.20 ± 0.06	0.19 ± 0.08	0.24 ± 0.05	0.19 ± 0.06	0.18 ± 0.05
FS mean	0.23 ± 0.03	0.25 ± 0.04	0.25 ± 0.04	0.26 ± 0.05	0.24 ± 0.05	0.23 ± 0.04
FAS mitral	0.49 ± 0.09	0.55 ± 0.05	0.57 ± 0.05	0.54 ± 0.08	0.55 ± 0.07	0.53 ± 0.07
FAS pap	0.46 ± 0.08	0.47 ± 0.06	0.49 ± 0.08	0.48 ± 0.09	0.478 ± 0.12	0.44 ± 0.07
FAS apex	0.45 ± 0.07	0.40 ± 0.07	0.42 ± 0.08	0.47 ± 0.12	0.37 ± 0.08	0.36 ± 0.05
FAS mean	0.47 ± 0.03	0.42 ± 0.17	0.49 ± 0.06*	0.49 ± 0.08	0.47 ± 0.07	0.44 ± 0.04*

sWT Septal wall thickening, FS Fractional shortening, FAS Fractional area shortening at levels of the mitral valve (mitral) papillary muscle (pap), apex and mean of three levels (mean). N=8 per group.
*p=0.08 for cell treated animals compared to placebo.

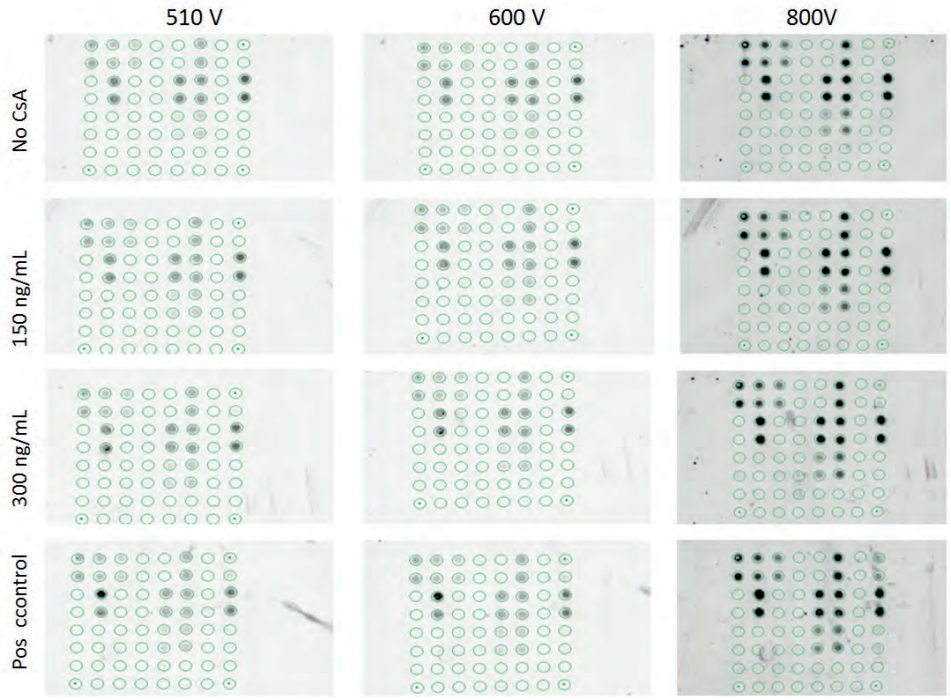


Supplemental Figure 1. Quantification of cell migration



Supplemental Figure 2. Sprouting matrigel assay
A. bar plot of angiogenesis parameters. B. representative pictures of matrigel assays for all 4 conditions.

	A	B	C	D	E	F	G	H
1	POS1	POS2	POS3	NEG	NEG	ANG	EGF	ENA-78
2	POS1	POS2	POS3	NEG	NEG	ANG	EGF	ENA-78
3	bFGF	GRO	IFN-γ	IGF-1	IL-6	IL-8	Leptin	MCP1
4	bFGF	GRO	IFN-γ	IGF-1	IL-6	IL-8	Leptin	MCP1
5	PDGF-BB	PLGF	RANTES	TGF-β1	TIMP1	TIMP2	THPO	VEGF-A
6	PDGF-BB	PLGF	RANTES	TGF-β1	TIMP1	TIMP2	THPO	VEGF-A
7	VEGF-D	NEG	NEG	NEG	NEG	NEG	NEG	NEG
8	VEGF-D	NEG	NEG	NEG	NEG	NEG	NEG	NEG



Supplemental Figure 3. Array map and pictures of the array of 4 conditions at three different intensities. Positive control meaning different cell line without Cyclosporin

CHAPTER 12

SUMMARY AND GENERAL DISCUSSION

PART ONE

In medical research, animal models serve as surrogates for human disease which has both advantages and disadvantages. Importantly, it prevents human suffering. Further advantages are mainly financial, since animal studies are cheaper and less time consuming than clinical research and logistical, since animals are more easily accessible compared to healthy volunteers or patients. Also, the (genetic) propinquity of animals, diminishes the 'noise' within one study leading to a decline in the number of study subjects required to find significant results. On the other hand, one should consider the translatability of a uniform collection of animals towards a diverse patient population, threatening external validity of preclinical studies.
Preclinical research has to be transparent and accessible to be of any additional value to medical research. However, as described in **chapter 2** publication bias is a major concern. The absence of relevant data (which often includes negative or neutral results) in the scientific literature results in repetition of research and accompanied superfluous use of animals and financial sources. Besides, incomplete scientific evidence contributes to translational failure, and potentially puts patients at unacceptable risks. In **chapter 2** we support upfront registration of preclinical research in an online registry, like it is implemented for clinical trials.

Availability of data is also important in view of performing systemic reviews and meta-analyses. Meta-analysis offers a technique to summarize data from different studies and to produce an overall estimate of effect. The aim of a meta-analysis of clinical data is often to increase the power by increasing the number of subjects and finding a 'true mean' in that particular population. For that purpose, data has to be as homogeneous as possible. In the field of cell therapy for cardiac repair, different cell types are administered, cells are stored and cultured in different conditions, administered at different time points etcetera. Meta-analyses of these data should therefore be considered to be hypothesis *generating* instead of hypothesis *testing*.[1,2]
On the contrary, meta-analyses of preclinical studies thrive by heterogeneity. An overall estimate of effect can be found, but the clinical relevance of this 'true mean' has to be questioned. Meta-analyses of heterogeneous preclinical studies can be of great additional value when this heterogeneity is used to find predictors of success.[3]
This tool was used to explain translational failure of anti-inflammatory compounds for myocardial infarction (MI) in **chapter 3 and 4**. Inflammation plays an important role in the process of ischemia and reperfusion in the course of MI and anti-inflammatory therapy appears to be a favorable approach in protecting the myocardium from further damage. Many anti-inflammatory compounds have been tested in preclinical studies with promising results, but none is implemented in daily clinical practice. By pooling all large animal data present in the literature, meta-analysis revealed a significantly improved infarct size by anti-inflammatory compounds compared to control. Methodological characteristics influencing therapy effectiveness which may be accountable for the hampered transition to the clinic were also identified. For example, the effect of anti-inflammatory therapies was strongest when measured in the first hours after therapy administration. Clinically relevant follow-up duration showed a significantly lower effect, underlining the importance

of the use of adequate and clinically relevant methods in preclinical research to warrant external validity.

Meta-analysis was also applied in **chapter 5** to describe the human relevance of preclinical research of cell therapy for cardiac repair. Apart from the overall effect of stem cells on left ventricular ejection fraction (LVEF), we searched for possible predictors of effect and the similarity with clinical studies. LVEF significantly improved by cell therapy, due to a decrease in end diastolic volume, which was concordant with the first meta-analysis of clinical studies.[4] We therefore concluded that pre-clinical models for cell therapy for MI were of clinical relevance. A sensitivity analysis suggested cell type, infarct model and cell number to be significant predictors for LVEF improvement, and these were interpreted as important parameters for future clinical and preclinical studies. Related predictors are also identified in some of the multiple clinical meta-analyses,[5,6] but interpretation of the plentiful data without getting 'lost in meta-analyses' is difficult.[1]
In the developing field of stem cell therapy for cardiac repair, important questions arose from a clinical point of view, like the issue whether to use allogeneic or autologous cells. As described in **chapter 6** allogeneic cell therapy enables 'off-the-shelf' therapy and selection and pre-preparation of the most potent cell line. However, immunologic hurdles have to be taken into account. Autologous cells are exposed to the patient's risk profile (*i.e.* comorbidity, smoking, age) hampering cell quality,[7–9] but transplantation can be performed without immunologic concerns. The impact of type of cell source on the efficacy of therapy has never been investigated in animal studies, and only one clinical trial investigated this issue.[10] Hare and colleagues compared autologous and allogeneic mesenchymal stem cell (MSC) therapy and concluded that both approaches were save and were associated with improved end diastolic and end systolic volumes. Both cell sources had no significant effect on LVEF and no differences between cell sources were observed. In **chapter 6,** meta-analysis of the abundant preclinical data was used to investigate the impact of cell source on efficacy of cell therapy in 1415 large animal models of MI. The vast majority of included studies used autologous cells. Allogeneic cells were usually MSCs. Meta-regression revealed no difference between cell sources. It must be emphasized that by meta-analysis this hypothesis was generated without the use of any additional laboratory animal and the results can be of great importance for the design of future clinical and preclinical studies. This information might be sufficient to justify the up to 30 recruiting clinical trials using allogeneic cells for ischemic heart disease.[11] Importantly, this larger analysis confirmed the significant effect on LVEF (8.3%) as found in chapter 5 (7.3%).

In the first part of this thesis, hypotheses for future clinical and preclinical studies on cardiac repair are formed, including *i*) cell therapy effectuates a significant effect on cardiac function in large animal MI models; *ii)* allogeneic cell therapy is associated with a similar magnitude of effect as autologous cell therapy; *iii*) the pig MI model for cardiac cell therapy serves as relevant model for human disease. Furthermore lessons were learned about the importance of internal and external validity in view of successful progression on the translational axis from bench to bedside, and solving the issue of publication bias. Internal validity is jeopardized by several forms of bias, like selection bias, performance bias and detection

bias. Risk of bias with regard to internal validity is easily reduced by randomization, allocation concealment (*i.e.* blinding of the operator) and blinded outcome assessment.[12,13] In **chapters 4 and 6** it is shown that reporting of quality parameters in large animal studies is low. Randomization of animals was reported in ~60% of the studies, blinded outcome assessment in 30-40% and blinding of the operator was reported in only 1 of out 10 studies. Failure of reporting of this relevant information must be distinguished from failure of actual blinding and randomization. The National Centre for the 3Rs developed the ARRIVE guidelines in order to improve standards of reporting of animal studies and minimize unnecessary studies. These guidelines are endorsed by almost 500 journals.[14] Flaws in external validity result in low predictive value of preclinical studies for clinical studies. Methodology of preclinical studies should be of clinical relevance in terms of disease model, timing of administration and follow up in order to be of translational value. This is emphasized in the first part, and methodology of preclinical trials is extensively described in the second part of the thesis as discussed below.

Open and transparent publishing is imperative for reliable preclinical research and translation to clinical studies, but also contributes to the 3Rs. Several journals encourage reporting of all data, including neutral or negative results (*i.e.* The Journal of Negative Results in Biomedicine, the Journal of Articles in Support of the Null Hypothesis and PLOS ONE). F1000 Research also promotes sharing neutral and negative results and introduces a different approach in transparent publishing.[15] Immediate publication online, an open and transparent reviewing process (by publishing reviewers and authors comments on the journal website) based on scientific soundness and publication of updated versions of the article are key parts of a complete novel way of transparent publishing. Besides, F1000 Research enables sharing of small findings, data articles, study protocols, scientific posters etcetera. The major objective of researchers not to put time and effort in writing full research articles because of negative results is overcome in this way.[16] Another approach called 'registered report' is offered by the Wiley journal 'Cortex'. This innovative way of publishing divides the review process in two phases. In the first phase the methodology and proposed analyses is submitted. If the reviewers decision is positive, publication of the future results in the second phase is guaranteed when the authors stick to the previously published methods, regardless whether they confirm or reject the hypothesis.[17] Prospective registration of systematic reviews is offered for clinical and preclinical studies (by PROSPERO[18] and Evidence Based preclinical medicine[19] respectively) and thereby provides insight in on-going reviews and helps prevent duplication of work. An example of a protocol of a systemic review and meta-analysis is presented in **chapter 3.** The transparency achieved by pre-registration of trials and systematic reviews contributes to several quality aspects of research as authors are required to stick to a pre-approved protocol and analysis plan,. First, it will reduce publication bias. Second, it will prevent arguable but common practices that promote false discoveries and translational failure, including, illegitimate outlier exclusion, and selective reporting of results.[20] Third, by requiring an a priori power analysis, false negative results will be greatly reduced. It is of great value that journals encourage researches to perform and publish sound and solid work instead of solely cutting-edge research.

PART TWO

In the second part of this thesis, several methodological studies regarding animal models and outcome measurement in cardiac regenerative medicine are presented. Methods described in these chapters may serve as protocols for future testing of cardiac regenerative therapies in a preclinical setting. In our opinion, upfront validation and publication of methodology contributes to standardized research, and *reduction* and *refinement* of animal studies. Based on the methodology described in chapters 7, 8, 9 and 10, and the considerations in the first part of this thesis, a randomized blinded placebo controlled preclinical study was conducted in order to test the efficacy of fetal human cardiomyocyte progenitor cells (CMPCs) in a pig model of MI, as described in **chapter 11**.

The pig model was used in accordance with **chapter 7** where the creation of the pig model of MI was described and visualized. Furthermore, methodology for functional outcome assessment like cardiac MRI, echocardiography, pressure-volume loop (PVloop) and intracoronary pressure- and flow measurements are described. Modest changes to the protocol were applied by introducing immunosuppression in view of application of human cells in a pig model. As shown in chapter 6, efficacy of non-autologous cells is not inferior to autologous cell sources. Most xenogeneic studies used an immunosuppressant in order to prevent rejection by the immune system. **Chapter 8** supports the use cyclosporine A (CsA) as an immunosuppressant in pigs. Administration of CsA was proven to be safe, but the exact role of CsA on stem cell potency could not be addressed by reviewing the literature.

Cardiac MRI is the golden standard for measuring EDV, ESV and LVEF[21] acting as surrogate endpoints for cardiac function and prognosis. In **chapter 9** we correlate admittance based PV loop to the golden standard MRI and 3D echocardiography. Along with volume measurements, PVloop enables invasive measurement of more specific information on both systolic and diastolic function and dynamic changes which are not measurable with most other imaging modalities.[22] In our study admittance PVloop moderately overestimates volumes in diseased hearts, but measures LVEF accurately compared to MRI. Next, since one of the proposed mechanisms of cell therapy is improvement of myocardial perfusion by vasculogenesis or angiogenesis, examination of the myocardial microvasculature is proposed. In **chapter 10** hyperemic microvascular resistance (HMR) by simultaneous intracoronary pressure and flow measurement as an index for microvasculature is introduced. MI led to a significant increase in HMR after 4 weeks which is confirmed in **chapter 11**, but HMR could not be restored by infusion of CMPCs.

The study described in **chapter 11**, served as a translational step between *in vitro* results and small animal studies on the one hand and a first-in-man trial (FIM) on the other hand.[23–25] Both the results of part 1 (adequate study design) and 2 (standardized methodologies) ultimately came together in this preclinical study. We showed no benefit of CMPCs on any of the outcome measures which makes us reconsider a FIM.

Preclinical perspective of cardiac stem cells

Other CSC, *i.e.* c-kit positive cardiac stem cells (CSCs) and cardiosphere derived cells (CDCs) were tested in pig models (see **chapter 11** Table 2).[26–32] Human and autologous CSCs were tested in two non-randomized, non-blinded studies, using small numbers of

cells.[31,32] Human CSCs proved to be efficacious but a combination of 1 million human CSCs and 200 million human mesenchymal cells was associated with a significant higher effect on cardiac function and infarct size than either cell type alone.[32] In another publication, serial measures of functional data showed that autologous CSCs weren't able to improve cardiac function compared to placebo. However, the conclusion as stated by the authors is that '*intracoronary infusion of autologous CSC improves regional and global LV function and promotes cardiac and vascular regeneration',*[31] which is based on comparison of cell-to vehicle treated animals at follow up. Study quality regarding internal validity (*i.e.* randomization and blinding) of the studies involving CDCs can be considered higher compared to the CSCs studies. In general, CDCs seem to have no effect on cardiac function. However, infarct size was reported to be decreased by CDCs in all studies. In order to be able to properly interpret these results, information on primary outcomes and power calculations should be provided. Were studies conducted to test the hypothesis that cell therapy would result in improved cardiac function, and did absence of results make the authors choose for presenting decreased infarct size as the main outcome? Or was infarct size the primary outcome from the very beginning and was the study underpowered to find significant results on cardiac function? This again emphasizes the value of upfront registration or publication of study protocols. In the same manner, the words '… cardiosphere-derived cells preserve function...' in the heading of Lee et al. should be interpreted with caution. Cardiac function measured by echocardiography did not show any benefit of cell treatment. Left ventricular angiography, which is known to be inferior to echocardiography and overestimates effect size compared to other imaging modalities, shows a significant difference in delta LVEF between placebo and CDC treated animals.[6,33] Despite these concerns, both CSCs and CDCs are already tested in open-label phase I safety trials (SCIPIO NCT00474461 and CADUCEUS NCT00893360 respectively) and promising results of short term follow up has been published already.[34,35]

Conclusion

Doctors, patients, researchers and laboratory animals all benefit from proper, honorable and conscientious preclinical research. Moreover, frank and honest publishing is mandatory for interpretation of the current literature. By presenting (protocols of) meta-analyses, study protocols and methodology, this thesis contributes to these aspects and to the 3Rs of replacement, reduction and refinement. Furthermore this thesis summarizes the strengths and weaknesses of previous conducted preclinical research in cardiac repair. This evidence, supported by methodology described and implemented in the second part of this thesis, was used to help design our preclinical study of CMPCs for cardiac repair. Our blinded and randomized study showed no benefit of intracoronary infused human CMPCs on cardiac function in an immunosuppressed pig model of MI. Therefore, further transition of CMPCs on the translational axis towards clinical application is not justified, at this moment.

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APPENDIX

NEDERLANDSE SAMENVATTING
LIST OF PUBLICATIONS
DANKWOORD
CURRICULUM VITAE

NEDERLANDSE SAMENVATTING

Dit proefschrift beschrijft zowel methodologisch als praktisch preklinisch onderzoek naar regeneratieve therapie binnen het veld van cardiale ischemie.

DEEL ÉÉN

In hoofdstuk 2 wordt gepleit voor het publiceren van al het preklinische onderzoek, ook als deze de getoetste hypothese ontkrachten (zogenaamde negatieve studies). Het voorstel is om preklinische studies vooraf te registreren zoals dat ook gebruikelijk is voor klinische studies. Op deze manier zal herhaling van studies, met gepaard gaande proefdieren, kosten en tijd, kunnen worden voorkomen. Registratie en publicatie van studies dragen bij aan de volledigheid van de (medisch) wetenschappelijke literatuur, waardoor een reëler beeld kan worden verkregen van de effectiviteit van nieuwe therapieën op het moment dat zij de overgang naar klinische studies maken.
Als deze wetenschappelijke literatuur compleet is, kan systematisch onderzoek en meta-analyse van de data helpen bij het vormen en toetsen van hypotheses voor toekomstige klinische en preklinische studies.1 Op deze manier kan belangrijke nieuwe informatie worden verkregen, zonder daarvoor extra proefdieren op te offeren.
In hoofdstuk 3 & 4 wordt deze techniek gebruikt om het falen van translatie van anti-inflammatoire middelen als therapie voor ischemisch hartlijden te onderzoeken. Inflammatie speelt een belangrijke rol in het proces van een myocardinfarct en in preklinische studies worden veelbelovend resultaten behaald met anti-inflammatoire middelen. Echter, geen van deze middelen is geïmplementeerd in de dagelijkse klinische praktijk. Meta-analyse van de literatuur toont aan dat anti-inflammatoire middelen de infarctgrootte significant verkleinen in grote proefdieren en dat onder andere het geslacht van de proefdieren en tijdstip van de uitkomstmeting invloed hebben op dit effect.
Meta-analyse werd ook toegepast in hoofdstuk 5, waar het effect van celtherapie in grote proefdieren wordt beschreven. Celtherapie verbetert de ejectiefractie en het eind diastolisch volume van het linker ventrikel. De resultaten zijn in dezelfde orde van grootte als die van klinische meta-analyses.
In het continue in beweging zijnde veld van celtherapie voor cardiale regeneratie, ontstaan klinisch relevante vraagstukken zoals vraag of allogene of autologe cellen de voorkeur genieten. Zoals beschreven in hoofdstuk 6 maakt het gebruik van allogene cellen 'off-the-shelf' therapie mogelijk waarbij vooraf selectie en productie van de meest potente cellijn kan plaatsvinden. Echter, er moet rekening worden gehouden met immunologische verschillen tussen donor en ontvanger. Autologe cellen kunnen zonder immunologische bezwaren worden gebruikt, maar deze zijn levenslang blootgesteld aan het risicoprofiel van de patiënt (o.a. comorbiditeit, roken, leeftijd). In hoofdstuk 6 wordt door middel van meta-analyse geen verschil gezien in de effectiviteit van allogene en autologe stamcellen in grote proefdierstudies.
In de beschreven meta-analyses laten we tevens zien dat methodologische kwaliteit een voorspeller is van de effectgrootte. We pleiten daarom voor correcte en solide methodologie van proefdierstudies met betrekking tot randomisatie en blindering maar ook voor het

gebruik van klinisch relevante proefdiermodellen. Op deze manier wordt de wetenschappelijke waarde van preklinisch onderzoek vergroot en kan het dienen als basis voor toekomstige preklinische en klinische studies.

DEEL TWEE

In het tweede deel van dit proefschrift wordt een aantal methodologische studies met betrekking tot diermodellen en uitkomstmaten in cardiale regeneratieve geneeskunde gepresenteerd. De methoden die in deze hoofdstukken worden beschreven kunnen dienen als protocollen voor toekomstige dierstudies naar regeneratieve therapieën. Validatie en publicatie van methodiek draagt bij aan gestandaardiseerd onderzoek en daardoor aan vermindering en verfijning van dierproeven. Op basis van de hoofdstukken 7, 8, 9 en 10, en de overwegingen in het eerste deel van dit proefschrift, werd een gerandomiseerde, geblindeerde en placebo gecontroleerde preklinische studie uitgevoerd naar de effectiviteit van foetale humane cardiomyocyte progenitorcellen (CMPC) in een varkens model, zoals beschreven in hoofdstuk 11.
De methodologie voor het creëren van een varkensmodel voor chronisch ischemisch hartfalen door middel van 90 minuten occlusie van de linker coronair arterie wordt uitgebreid beschreven en gevisualiseerd hoofdstuk 7, net als de methodologie voor functionele uitkomstmetingen (o.a. cardiale MRI, echocardiografie, pressure-volume loops (PVloop) en intracoronaire druk- en flowmetingen).
Bij het gebruik van humane of allogene cellen in een proefdiermodel, kan het gebruik van immunosuppressie worden overwogen. Hoofdstuk 8 ondersteunt het gebruik cyclosporine A (CsA) als een immunosuppressivum in varkens. Toediening van CsA aan varkens na cardiale ischemie is veilig, maar de precieze rol van CsA op de kwaliteit en effectiviteit van stamcellen kon niet worden vastgesteld.
De doelen van regeneratieve therapie voor ischemisch hartlijden zijn grofweg herstel van het beschadigde hartweefsel, verbeteren van de (micro)circulatie van het hart het verbeteren van de pompfunctie. Cardiale MRI is de gouden standaard voor het meten van volumina en de linker ventrikel ejectie fractie als surrogaat eindpunten voor de hartfunctie en prognose. In hoofdstuk 9 correleren we ‘admittance based PV-loop’ aan de gouden standaard MRI en 3D-echocardiografie. Behalve het accuraat meten van volumina, maakt PV-loop invasieve meting van systolische en diastolische functie en dynamische veranderingen mogelijk die niet meetbaar zijn met de meeste andere beeldvormende technieken. In deze studie laten we zien dat admittance based PV-loop volumina matig overschat in gedilateerde harten, maar dat de linker ventrikel ejectie fractie nauwkeurig wordt gemeten in vergelijking MRI.
In hoofdstuk 10 introduceren we hyperemische microvasculaire weerstand index (HMR) als uitkomstmaat voor de kwaliteit van de microvasculatie van het myocard. Deze index kan worden gemeten door middel van gelijktijdige druk- en flowmeting in de coronairarterie. We laten zien dat de HMR significant toeneemt, 4 weken na een myocardinfarct. Dit wordt bevestigd in hoofdstuk 11, maar dit kon niet worden hersteld door intracoronaire infusie van CMPCs.

De resultaten uit het eerste deel (adequaat studie design) en het tweede deel (gestandaardiseerde methodologie) van dit proefschrift komen samen in het laatste hoofdstuk. Het in hoofdstuk 11 beschreven onderzoek naar de effectiviteit van CMPCs in een varkensmodel voor chronisch ischemisch hartlijden, dient als een translationele stap tussen de in vitro resultaten en kleine dierstudies enerzijds en een first-in-man trial anderzijds.2,3 De resultaten van deze studie laten zien dat het toedienen van CMPCs veilig is, maar dat er geen effect is op de functionele eindpunten in vergelijking met een placebo. Dit weerhoudt ons er op dit moment van om verder te gaan in het proces van translatie en de CMPCS te testen in een first-in-man-trial.

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DANKWOORD

Dit proefschrift is tot stand gekomen met de hulp van heel veel mensen. Bij deze zou ik iedereen die op wat voor manier dan ook heeft geholpen willen bedanken.

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CURRICULUM VITAE

Sanne werd geboren op 23 maart 1986 te Raalte en groeide samen met haar zus op in een liefdevol gezin. In 2004 rondde ze haar Gymnasium af aan het Carmel College Salland te Raalte. Omdat ze werd uitgeloot voor de studie geneeskunde startte ze met Biomedische Wetenschappen aan de Universiteit Utrecht. Tijdens deze studie werd haar interesse voor het biomedische onderzoek gewekt, maar haar wens geneeskunde te studeren bleef. Na het behalen van haar bachelordiploma werd zij in 2008 aangenomen tot de selectieve 4-jarige geneeskunde- en onderzoeksmaster 'SUMMA'. Deze rondde ze met succes af in 2012. Al tijdens het eerste jaar van SUMMA deed ze haar eerste onderzoekervaring op bij de Cardiologie in het UMC Utrecht onder enthousiaste supervisie van dr. Chamuleau. De publicatie die hieruit voortvloeide vormde het fundament voor haar latere promotie-onderzoek. Na het behalen van haar masterdiploma, zette Sanne haar onderzoek voort in de vorm van promotieonderzoek op het gebied van regeneratieve therapie voor het hart onder supervisie van professor Doevendans en dr. Chamuleau in het UMC Utrecht. Tijdens haar promotieonderzoek zette ze zich ook in voor meer transparantie en betere kwaliteit van proefdieronderzoek. Eind 2015 zal ze starten met de cardiologie opleiding in Isala te Zwolle onder supervisie van dr. Ramdat Misier. Sanne woont samen met Pieter en werd op 2 oktober 2014 moeder van Lena.