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DISSERTATION

The study of stem cells in cardiac surgery: from cell type selection to optimization for transplantation

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Abbreviations and Acronyms

	General
RCTs	randomized controlled trials
ECMO	extracorporeal membrane oxygenation
HFrEF	heart failure with reduced ejection fraction
LVAD	left ventricular assistant device
MI	myocardial infarction
MRI	magnetic resonance imaging
RR	risk ratio
	Clinical indicator
6MWD	6-minute walk distance
BNP	brain natriuretic peptide
HF-MACEs	heart failure-related major adverse cardiac events
LVEDD	left ventricular end-diastolic diameter
LVEDV	left ventricular end-diastolic volume
LVEDVI	left ventricular end-diastolic volume index
LVEF	Left ventricular ejection fraction
NT-proBNP	N-terminal brain natriuretic peptide
NYHA	New York heart association
SAEs	serious adverse events
	Cell type
ALDH	aldehyde dehydrogenase
ATMSCs	adipose tissue-derived mesenchymal stem cells
ATSVF	adipose tissue-derived stromal vascular fraction
BMMNCs	bone marrow-derived mononuclear cells
BMMSCs	bone marrow-derived mesenchymal stem cells
CB-MSCs	cord blood-derived mesenchymal stem cells
CDCs	cardiosphere-derived cells
CSCs/CPCs	cardiac stem cells/cardiac progenitor cells
ESCs	embryonic stem cells
iPSC-CMs	induced pluripotent stem cell-derived cardiomyocytes
iPSCs	induced pluripotent stem cells
MDSC	muscle-derived stem cells
STRO-3^{+} MPCs	stromal precursor antigen-3 positive mesenchymal precursor cells
UCMSCs	umbilical cord mesenchymal stem cells
	Delivery route
CABG	coronary artery bypass grafting
ICI	intracoronary infusion
IMI	intramyocardial injection

IVI	intravenous infusion
PCI	percutaneous coronary intervention
TESI	transendocardial stem cell injection
	In-vitro experiment
FBS	fetal bovine serum
CS	control serum
HFS	heart failure serum
AcS	acute stress
HP	hypoxia preconditioning
RT-PCR	reverse transcription-polymerase chain reaction
	Gene name
ATM	ataxia telangiectasia-mutated
ATR	ataxia telangiectasia and Rad3 related
Tp53	Tumor protein p53
Bax	Bcl-2 Associated X
Bcl-2	B-cell lymphoma 2
Apaf1	apoptotic protease activating factor 1
DFFA	DNA fragmentation factor subunit alpha
DFFB	DNA fragmentation factor subunit beta
CCNA	cyclin-A
CCNB	cyclin-B
CCND	cyclin-D
CCNE	cyclin-E
CDC	cell division cycle
CDK	cyclin-dependent kinase
RB	retinoblastoma
CDKN	cyclin-dependent kinase inhibitor

Abstract (English)

Aims: The goal of this project was to evaluate the effectiveness of stem cells in heart failure to optimize them for clinical use.

Methods: In the meta-analysis, randomized controlled clinical trials (RCTs) of stem cell treatments for heart failure were retrieved. Primary outcomes were set as all-cause mortality and left ventricular ejection fraction (LVEF) change from baseline. Mean differences and standard deviations between cell therapies and controls were calculated and separated into subgroups based on methodological and clinical differences. In the laboratory, stem cells were expanded with different medium sera and further brought to a severe condition that simulated ischemia-reperfusion injury (AcS). Cell counts and necrotic, apoptotic cells were assessed, and reverse transcription-polymerase chain reaction (RT-PCRs) were performed to measure cell proliferation, cell survival, and gene regulations under normal and stressful conditions.

Results: 42 RCTs were retrieved, and 1984 participants were enrolled. Overall, stem cells were proven to reduce mortality and increase ∆LVEF by 2.88% and 2.33% at short- and long-term follow-ups. Results from trial quality assessments and sensitivity analyses indicated existence of bias risks in randomization and performance that significantly affect clinical outcomes lead to false-positive results. Among various cell origins, perinatal stem cells showed 6.48% and 5.22% of LVEF increase. Cord blood-derived mesenchymal stromal cells were chosen for *in vitro* study. Under human sera, they presented a higher proliferative capacity and shorter expansion time. A higher survival rate was also confirmed against oxidative stress, but apoptosis- and cell cycle-related genes were not regulated in a similar pattern under different medium sera.

Conclusions: Overall, stem cell therapy did not show clinical values for improving cardiac contractility in patients with impaired heart functions. The outcomes were significantly influenced by poor blinding methods leading to false-positive results. Optimizing cord blood-derived mesenchymal stem cells (CB-MSCs) through human sera applications seems to be promising due to better performance in proliferation and resistance against acute pro-apoptotic stress.

Abstrakt (Deutsch)

Ziele: Dieser Arbeit liegt das Ziel zu Grunde die Wirksamkeit von Stammzellen bei Herzinsuffizienz zu evaluieren, um sie für den klinischen Einsatz zu optimieren.

Methoden: In einer Metaanalyse wurden randomisierte kontrollierte klinische Studien zu Stammzellbehandlungen für HFrEF abgerufen. Die primären Ergebnisse wurden als Gesamtmortalität und LVEF-Änderung gegenüber dem Ausgangswert festgelegt. Die mittleren Unterschiede und ihre Standardabweichungen zwischen Zelltherapien und Kontrollen wurden berechnet und auf der Grundlage methodischer und klinischer Unterschiede in Untergruppen unterteilt. Im Labor wurden Stammzellen mit dem Zusatz von humanem Serum von Herzinsuffizienzpatienten vermehrt. Diese Stammzellen wurden dann einer modellierten Ischämie-Reperfusionsverletzung (AcS) ausgesetzt. Zellzahlen und nekrotische sowie apoptotische Zellen wurden quantifiziert und RT-PCRs wurden durchgeführt, um die Zellproliferation, das Zellüberleben und die Genregulation unter regulären und AsC-Bedingungen zu messen.

Ergebnisse: 42 RCTs wurden abgerufen und 1984 Patienten wurden eingeschlossen. Insgesamt konnte gezeigt werden, dass Stammzellen die Mortalität senken und den ∆LVEF bei kurz- und langfristigen Nachuntersuchungen um 2,88% bzw. 2,33% erhöhen. Studienqualitätsbewertungen und Sensitivitätsanalysen ergaben, dass Verzerrungsrisiken bei der Randomisierung und Leistung einen signifikanten Einfluss auf die klinischen Ergebnisse haben können, die zu falsch positiven Ergebnissen führen. Von allen untersuchten Zelltyoen zeigten perinatale Stammzellen einen Anstieg von 6,48% und 5,22% der LVEF. Aus Nabelschnurblut stammende mesenchymale Stromazellen wurden für eine in-vitro-Studie ausgewählt. Unter humanen Seren von Herzinsuffizienzpatienten zeigten sie eine höhere Proliferationskapazität und eine kürzere Expansionszeit. Eine höhere Überlebensrate wurde auch unter oxidativen Stress bestätigt, aber Apoptose- und Zellzyklus-bezogene Gene wurden unter verschiedenen mittleren Seren nicht in einem ähnlichen Muster reguliert.

Schlussfolgerungen: Insgesamt zeigte die Stammzelltherapie zur Verbesserung der Herzkontraktibilität bei Patienten mit Herzfunktionsstörungen bislang keine klinischen Werte. Darüber hinaus wurden die Ergebnisse signifikant durch schlechte Blindheit beeinflusst, die zu falsch positiven Ergebnissen führte. Die Optimierung von CB-MSCs durch die Anwendung von Humanseren scheint vielversprechend zu sein, da die

Proliferationsleistung und die Resistenz gegen akuten proapoptotischen Stress verbessert werden.

Preface

Methods in Part I have been registered in PROSPERO.

Zhiyi Xu, Fengquan Dong, Zihou Liu. Discrepancies of meta-analysis shown in stem cell therapies for heart failure with impaired cardiac function (HFrEF and HFmrEF). PROSPERO 2019 CRD42019118872 is available from https://www.crd.york.ac.uk/prospero/display_record.php?ID=CRD42019118872

Contents in Part II have been previously published.

Timo Z. Nazari-Shafti*, Zhiyi Xu*, Andreas Matthäus Bader, Georg Henke, Kristin Klose, Volkmar Falk, Christof Stamm, “Mesenchymal Stromal Cells Cultured in Serum from Heart Failure Patients Are More Resistant to Simulated Chronic and Acute Stress,” Stem Cells International, vol. 2018, Article ID 5832460, 15 pages, 2018 (*co-first author).

Part I Meta-analyses of stem cell therapies for heart failure patients with reduced ejection fraction

Introduction

Heart failure with reduced ejection fraction (HFrEF) refers to cardiac pump failure that presents a series of symptoms such as shortness of breath, insufficient exercise capacity, swelling in the lower legs and feet, fatigue and weakness, etc., which is mostly caused by coronary artery disease. Approximately 40 million people in the world are impaired by heart failure[1], a condition associated with a 5-year mortality rate of nearly 50%[2] which causes a tremendous burden to health care and leads to loss of many human lives. In contrast to other organs like the liver and epithelium that have significant capability for structure repair, the human heart is known to have very limited regenerative capacity. This is mainly due to the fact that its own pools of cardiac progenitor cells are rapidly depleted after myocardial infarction[3]. However, mobilized peripheral stem cells are insufficient to compensate adequately for the gap between rapid loss of cardiomyocytes after infarction and successive loss during the post-infarct inflammatory response. This lead to the idea of stem cell transplantation with the goal to fill that specific gap.

After infarction, three phases of inflammatory responses are considered to be critical to myocardial repair: clearance of dead cells in the inflammatory phase, secretion of extracellular matrix proteins by myofibroblasts in the proliferative phase, and apoptosis of the majority of reparative cells and scar formation in the maturation phase[4]. The inflammatory response dominats gene expressions in the heart tissues after MI such that pro-inflammatory genes (such as IL-6, MCP-1, G-CSF, GM-CSF) were up-regulated while gene expressions of stem cell attractants (SDF-1, SCF) or angiogenesis (VEGF-a, VEGF-R2) showed a slight decrease[5]. Although the inflammatory response is part of the healing process, prolonged and overshooting inflammation induces sustained loss of cardiomyocytes and matrix degradation, which causes ventricular chamber dilation and finally leads to heart failure. In contrast to myocardial infarction (MI), the main features during the progression of heart failure are fibrosis and ventricular remodeling. The etiologies of heart failure are not restricted to ischemic heart disease, but also rheumatism and idiopathic cardiomyopathy. Thus, the purpose of stem cell therapies for heart failure is to both reverse ventricular dilation and to improve systolic function.

In early 1995, the achievement of producing high yield and pure skeletal myoblasts by Yoon[6] elicited significant enthusiasm for stem cell-related heart failure treatment. In the author's study, the harvested myoblasts were injected into dog hearts and later differentiated into cardiac muscle cell-like cells. In the following twenty years, extensive

preclinical and clinical experiments using stem cells for heart failure were completed and a variety of therapeutic approaches were exploited.

An introduction to stem cell research for heart failure treatment based on data obtained mainly from preclinical trials is given below.

Cell Type

Myoblasts were the earliest stem cells to be investigated for HF treatment. At first, they were favored for their resistance to oxidative stress existing during heart failure and their myogenic capacity but were later proven to be more vulnerable compared to mesenchymal stem cells[7,8]. Even more, they were found to be pro-arrhythmia and tumorigenic[9,10] (Details of advantages and disadvantages are listed in **Table 1** and **Figure 1**). Another type of cardiomyogenic skeletal muscle-derived stem cell that elicited the researchers' interest were muscle-derived stem cells (MDSCs)[7]. Their differentiation potential is more similar to mesenchymal stem cells and has been proven to be osteogenic, chondrogenic, cardiomyogenic, and even hematopoietic[11,12]. Moreover, MDSCs are immune evasive due to their lack of major histocompatibility complex (MHC) expression[13]. They aree reported to be more efficient in cardiac regeneration compared to myoblasts, due to more active paracrine secretion[9]. The above-mentioned advantages are encouraging, however, induced arrhythmias are a prevalent problem that has to be solved urgently [14].

Mesenchymal stromal cells (MSCs) are multipotent stem cells that can be isolated from a variety of tissues, including bone marrow, adipose tissue, heart, perinatal tissues, and other sources. MSCs have following characteristics associated with them in the context of myocardial repair:

> Low immunogenicity

> They are not known to be tumerogenic

> Resistance to stressful microenvironments in ischemic region

> They do not cause ventricular arrhythmias

> They facilitate an immunomodulatory response vial paracrine signaling[15,16].

Adipose tissue-derived mesenchymal stem cells (ATMSCs) with easier accessibility and higher VEGF and HGF secretion are reported to be more efficient in HF treatment compared to bone marrow-derived mesenchymal stem cells (BMMSCs)[17,18]. Perinatal MSCs (such as umbilical cord-derived mesenchymal stromal cells [UCMSCs], cord blood-derived mesenchymal stromal cells [CBMSCs] and others) were shown to be even less

immunogenic[19,20] and more advantageous in anti-inflammation and neovascularization[21,22]. The biggest advantages of UCMSCs over the other two origins of MSCs are their accessibility and the fact that there are no associated ethical issues. MSCs are believed to improve impaired cardiac functions through a paracrine mechanism instead of cardiomyogenesis. In contrast to skeletal muscle-derived stem cells, there is a lack of evidence that MSCs differentiate into cardiomyocyte-like cells *in vivo*. *In vitro* cardiomyocyte differentiation is proven to be feasible by 5-Azacytidine; nevertheless, it has no value for clinical application because of its toxicity[23,24].

Bone marrow mononuclear cells (BMMNCs) are the unfractionated stem cells isolated from bone marrow aspirates that have been used primarily in clinical application. Another similar product is the adipose-derived stromal vascular fraction (AT-SVF). Both can be quickly infused into target areas after collection instead of long-term *in vitro* expansion. Furthermore, immunoselected purified-constituent subsets, for instance $CD34^+$, were explored and demonstrated to lead to improved cardiac functions after transplantations compared to BMMNCs[25]. Likewise, $STRO\text{-}3^+$ mesenchymal progenitor cells (MPCs), directly immunoselected from BMMNCs by STRO-3 antibody and expanded during cultivation, are considered to be subsets of BMMSCs with an even, purified constituent of regenerative cells. Another cell product from BMMNCs is Ixmyelocel-Ts. Through specific expansion techniques, MSCs and macrophages are expanded over 50 to 200-fold, while the amounts of other cell components shrink. Ixmyelocel-Ts have advantages over other cell types in anti-inflammation, so they are promising for suppressing immunological reactions after MI or during HF.

Endogenous cardiac stem/progenitor cells (CSCs/CPCs) are believed to have large potential in heart regeneration. They are more effective than other cell types in improving cardiac function, without inducing ventricular arrhythmias and tumorigenesis[26,27]. Contrarily, they are found to decrease the incidence of arrhythmias[28,29]. However, they need long-term *in-vitro* expansion before cell infusion due to the tiny amount of isolated primary cells from the collection and immunosuppressive therapy is needed during allogeneic transplantation, which limits their widespread application.

To date, pluripotent stem cells, such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), do not show any promise for widespread use in humans, owing to their tumorigenic effects[30,31]. In contrast, induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) are considered to be a stem cell option despite the induction of arrhythmias and tumorigenesis[32,33]. Long-term laboratory preparation is still required

due to reprogramming and differentiation. Counterintuitively, immune rejection still happens during autologous transplantation because genetic modifications could cause immunogenicity of autologous iPSC-derived cells[34]**.** Potential causes include low MHC-I levels, genetic and epigenetic changes, xenogeneic or non-physiological culture reagents, gene corrections for the treatment of specific diseases, and other factors. Although iPSC-CMs are proven to be quite similar to adult cardiomyocytes based on gene expression analysis[35], this advantage could be turned into a disadvantage. iPSC-derived cardiomyocytes could also be vulnerable to the same unfavorable microenvironments that cause successive loss of the original cardiomyocytes. Also, insufficient engraftment of iPSC-CMs makes it very difficult to detect improvements in cardiac function.

In summary, the advantages and disadvantages of stem cells for cardiac repair are listed in **Table 1** and **Figure 1**. From existing results, we can establish that the ideal cells for cardiac repair should satisfy (but not limited to) the following requirements: easy accessibility, being ready-to-use, immune evasion, resistance to stressful conditions, prevention of arrhythmias, being free of tumorigenesis or teratogenesis, immunomodulation in overactive inflammation, cardiac repair such as neovascularization, cardioprotection and anti-apoptosis and cardiomyogenesis. To date, none of the cell types that have already been studied could fulfill all the listed requirements. Therefore, different cell types with separate advantages have been combined in transplantation to complement each other for a better outcome.

Table 1. Advantages and disadvantages of various stem cell origins for heart repair

Cell types	Advantages	Disadvantages
Skeletal muscle-derived cells		
> Myoblasts /satellites	> differentiate into cardiomyocyte-like cells[7] > easy collection through skeletal muscle biopsy > resistant to oxidative stress[9]	> need immunosuppression in allogeneic application[36,37] > poor differentiation potential > tumorigenic[37] > induce arrhythmias[10]
> MDSCs	> lack of MHC II expression[13] > multipotent stem cells[38] > more tolerant to oxidative stress[9] > tend to differentiate into cardiomyocytes[7]	> rare in tissue and need long-term *in vitro* expansion[39] > indicate tumorigenesis[40] > induce arrhythmias[14]
Bone marrow-derived cells		

Table 1. Advantages and disadvantages of various stem cell origins for heart repair

Cell types	Advantages	Disadvantages
> BMMNCs	> quick preparation > beneficial paracrine secretion	> invasive collection > components of different cells types > need immunosuppression if allogeneic application
> $CD34^+$ BMMNCs	> same as BMMNCs	> same as BMMNCs
> $CD133^+$ BMMNCs	> enriched stem cells > stronger capacity in improving cardiac function[25]	> immune-suppression is needed > relative sensitivity to oxidative stress[41] > induced arrhythmias depending on delivery route[42]
> BMMSCs	> immune evasive[43] > free of tumorigenesis[44,45] > prevent arrhythmias[46] > tolerant to oxidative stress[41] > beneficial effects of immunomodulation, neovascularization, cardio-protection through abundant paracrine[21,47,48]	> long term *in vitro* expansion > chemicals of *in vitro* cardiomyocyte differentiation are toxic[23,24] > lack of *in vivo* cardiomyogenic evidence
> STRO3+ MPCs	> purified cell constituents > same as BMMSCs	> same as BMMSCs
> Ixmyelocel-Ts	> same paracrine effects as BMMSCs[49] > enlarged immunomodulatory effects[50]	> specific technique of *in vitro* expansion[51] > same as BMMSCs
Adipose tissue-derived cells		
> AT-SVF	> minimal invasive tissue collection > enriched $CD34^+$ cells > similar advantages to BMMNCs	> mixed population of clear and unclear cell types > immuno-suppressive therapy is needed in allogeneic transplantation
> ATMSCs	> similar as BMMSCs > stronger immunomodulatory capacity[52,53]	> similar as BMMSCs
Perinatal tissue-derived cells		
> UCMSCs	> No conflict in ethics > high concentration MSCs isolated from umbilical cord tissue. > less immunogenicity than BMMSCs > more proliferative > more active paracrine secretion	> same as BMMSCs
Cardiac endogenous cells		

Table 1. Advantages and disadvantages of various stem cell origins for heart repair

Cell types	Advantages	Disadvantages
> CSCs/CPCs	> cardiomyogenesis[54] > prevent arrhythmias in ischemic heart[28,29] > paracrine secretion for cardiac repair[26,27,54] > tolerant to oxidative stress[54] > immune evasive[55]	> cell collection by cardiac tissue biopsy > rare inside heart tissue > long term *in vitro* expansion
>CDCs	> low immunogenicity[56]	> same as CSCs/CPCs
Pluripotent cells		
> ESCs	> pluripotent stem cells[57] > more proliferative	> ethic issues > teratogenic[30,31] > less tolerant to oxidative stress compared to MSCs[58,59] > less reports and evidence of improving cardiac function > clinical practice infeasible so far
> iPSCs	> same as ESCs > various cell origins	> same as ESCs > long-term laboratory cultivation for gene transduction and expansion
Induced cardiomyocytes		
> iPSC-CMs	> various origins	> longer preparation than iPSCs > immune rejection could be seen even in autologous transplantation[34] > tumorigenesis is reported[60] > induced arrhythmias are also reported[32,33] > sensitive to oxidative stress[35]

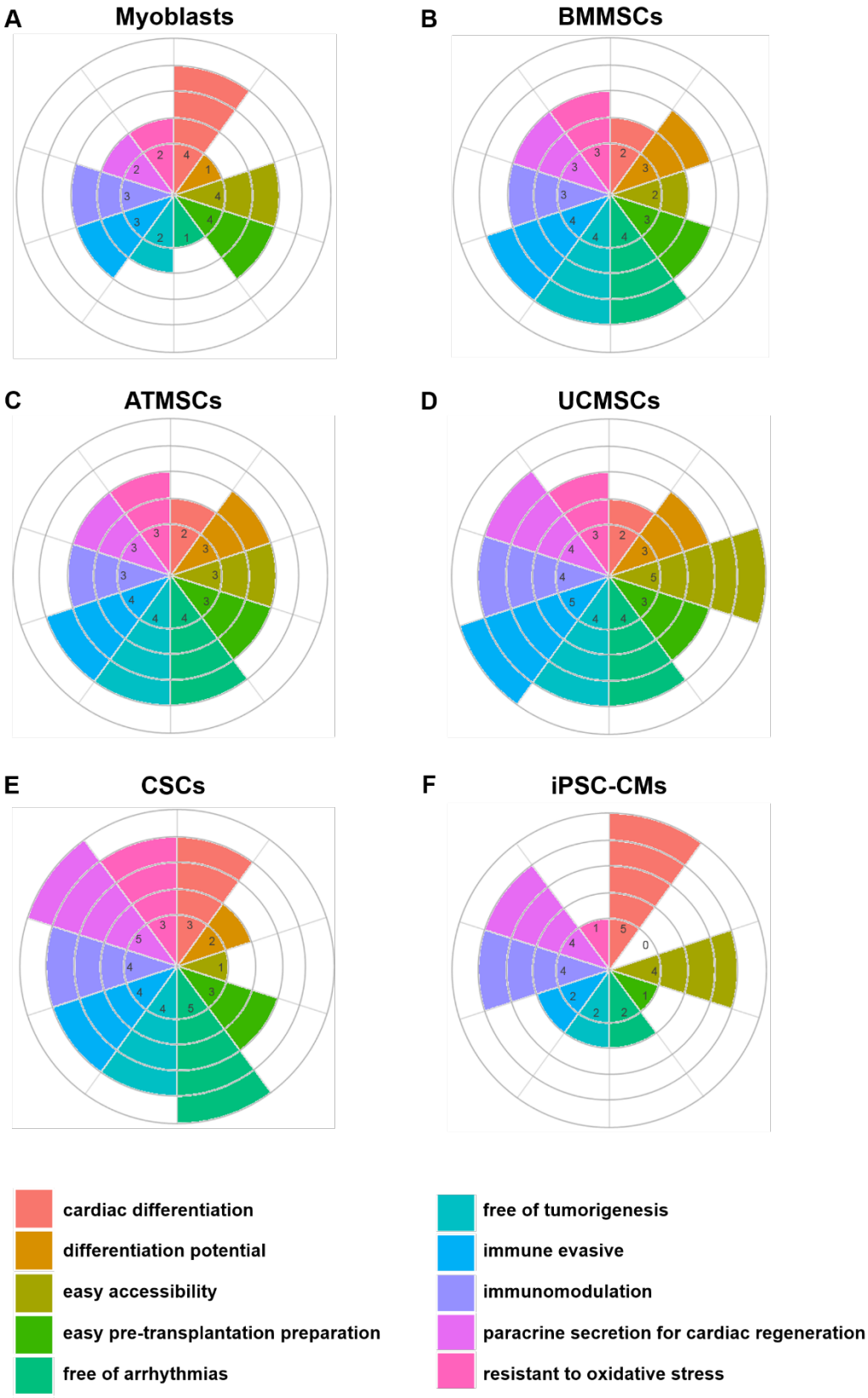


Figure 1. Summary of stem cell grade charts

0-5 grades were given to 10 categories for evaluation of attributes of (A) myoblasts, (B) BMMSCs, (C) ATMSCs, (D) UCMSCs, (E) CSCs, and (F) iPSC-CMs in cardiac repair. The higher grade the stem cells can get, the more advantages they possess in that property. Grades given were based on Table 1.

Cell Sources

Autologous, allogeneic, and xenogeneic stem cells are tested in animal experiments, whilst for human use, only two cell sources are allowed: autologous and allogeneic. To date, most completed clinical studies have used autologous stem cells such as BMMNCs, ATSVFs and myoblasts. Although there is no concern about acute or chronic immune rejection to stem cells from autologous origin, aging, and heart disease could impact the quantity and quality of stem cells[61]. MSCs from aging donors possess impaired proliferative and cardioprotective capacity[62]. The concentration of $CD34^+$ cells in BMMNCs from donors over 55 years old is less than half of that found in cells collected from healthy donors[63,64]. In human CSCs, capacities of proliferation and paracrine secretion are less influenced by aging[65,66], but the concentration of c-kit^+ CSCs drops, and telomere length is shortened in HF patients[66,67].

In order to increase the clinical efficacy of heart failure treatment, allogeneic immunoprivileged stem cells from young and healthy donors have been utilized in cell transplantation[68,69]. The effectiveness of allo-stem cells for improving cardiac function has been confirmed by several clinical trials[68,70]. Similar cardiac functional improvements between syngeneic and allogeneic stem cells were shown in a meta-analysis of large animals studies[71], but no meta-analyses of clinical trials have been published yet. Differences in the cardiac functional improvements between auto- and allo- BMMSCs were compared in two clinical trials, but the results were inconsistent. The earlier one reported insignificant improvements in the allogeneic group[72], while the latter demonstrated significant improvements, including increases in LVEF and 6MW distance, reduction in MACEs at 1-year follow-up, and efficient immune suppression[73]. However, the characteristic details of the healthy donors in these two trials are unclear. The application of allogeneic stem cells has been shown to be feasible and alternative to autologous stem cells in HF treatment, but due to insufficient and contradictory published data, it is still too early to draw any conclusions.

Delivery Route

The delivery strategies of stem cells into the failing heart include direct intramyocardial injection (IMI) or intracoronary infusion (ICI) alone, or concurrent application with revascularization, transendocardial stem cells injection (TESI) guided by an electrical mapping system, intravenous infusion (IVI) or a bio-engineered patch embedded with stem cells. The necessity of revascularization has been shown by

numerous experiments. Clinical patients under coronary artery bypass grafting (CABG) or percutaneous coronary intervention (PCI) have higher survival rates compared to patients who are not[74,75]. Another novel revascularization by transmyocardial laser-made channels also increased stem cell retention and up-regulated the expression of proangiogenic proteins[76]. For cell deliveries without surgical revascularizations, the cell retention rate is positively correlated with LV function restoration. Several studies proved that TESI and direct IMI have better clinical outcomes than ICI and IVI[77–79], but the former two delivery routes were also reported to be proarrhythmic, possibly because of slowing in the conduction area formed by a mass of injected stem cells[42,80].

Cell Dose

There are an estimated 2-7 billion cardiomyocytes in a healthy human heart, and over 50% could be lost after MI and during HF progression[81,82], but the numbers of transplanted stem cells in either preclinical or clinical trials were never able to compensate for the cell loss, especially considering the low cell retention and low survival after injection. In large animal experiments, the dose of transplanted stem cells goes typically over 100 million, and even 1 billion[83]. A meta-analysis divided cell doses into four ranges ($<10^7$, 10^7-10^8, 10^8-10^9, $>10^9$) to explore the correlation between dose escalation and LVEF increase. It showed an 8% increase in LVEF at doses over 10^7 and 6% less than 10^7. Clinical trials studying BMMNCs found a positive correlation between $CD34^+$ cell concentration and cardiac functional improvements[84,85]. The dose-dependent effects have also been seen with BMMSCs[86]. For STRO-3^+ MPCs, cardiac functional improvements increased by dose escalation was confirmed in sheep[87], but data from clinical trials were hard to interpret. In a high-quality double-blinded randomized control clinical trial[70], patients with 150 million MPCs showed reverse LV remodeling and reduced LVEF, but conversely, patients with 25 million cells had accelerated LV dilation and increased LVEF. Patients receiving 75 million MPCs had the highest occurrence of heart failure-related major adverse cardiac events (HF-MACEs) and worst performance in the 6MW test. In the trials of myoblasts, dose escalation does not enhance LVEF improvement or increase 6MW distances, but triggers more ventricular arrhythmias[88,89]. Explorations of the dose-response effect of stem cells are not always satisfactory, and always depend on proper cell types, indicating the necessity for more well-designed studies.

New strategies to supplement stem cell therapy

The application of stem cells for heart failure treatment has been investigated for over 20 years. The safety and feasibility of various stem cell types have been repeatedly verified in laboratory experiments and clinical trials. Obstacles that hinder the widespread application are the following: low retention rate, even less successful engraftments, and subsequent cell loss. The cell retention rate was detected to be nearly 5% two hours after transplantation, and dropped to only 1% after 24 hours[90,91]. To increase retention and survival rate, biomaterial scaffold embedded with stem cells could be dispatched inside the epicardium or injected into target areas[92,93]. Embedding choices are multiple, which could be nearly all cell types[94,95], stem cell cocktails, extracellular matrix, and paracrine exosomes[96]. The migration of transplanted cells from the patch into the cardiac tissue was observed in rats[97]. LV dilation was then seen to be reversed, and the ventricular walls became thicker.

Preconditioning of stem cells is another way of increasing cell survival and regenerative functions[98,99]. In dysfunctional hearts, regardless of the etiologies, chronic inflammation and ischemia inside heart tissue lead to successive loss of myocardium and as well as engrafted stem cells. Preconditioning through hypoxia or repeated ischemia-reperfusion cycles are the two most common treatments that are used to induce cell adaption to severe conditions. Several papers have already reported the increased survival rate and enhanced ability to repair infarcted myocardium with preconditioned stem cells[98,99].

Another way of increasing stem cell efficacy is cardiac unloading. In the progress of heart failure, volume overload caused by weak myocardial contractility causes the development of pathological hypertrophy in non-ischemic zones that aggravate cardiomyocyte oxidative stress, loss of mitochondrial membrane potential, and consequently leads to mitochondrial apoptosis[100]. Loss of cardiomyocytes, in turn, exacerbates volume overload, which perpetuates a vicious circle that eventually leads to eccentric hypertrophy. Mechanical assist devices achieving cardiac unloading, combined with regenerative therapies, could synergistically break this circle[101]. Intrinsic stem cells, including c-kit+ stem cells and side population cells, significantly increase in the myocardium after ventricular unloading[102], and cardiomyocytes even show an increase in mitosis and cytokinesis[103]. To study the cell performance in an unloaded heart, researchers constructed an animal model through heterotopic transplantation of an infarcted heart into a healthy mouse while keeping the orthotopic heart intact[104]. Cardiac stem cells were later injected into this hemodynamically unloading heart. Compared to

the loading control heart, a higher survival rate of stem cells was seen in the unloading heart, and more proliferative (Ki67 stained) cells were detected at 21 days post-transplantation. Therefore, the combination of ventricular assist devices (VADs) and stem cells was proposed to be a novel approach to myocardial recovery in which wearing VADs could decrease ventricular oxygen consumption and facilitate cardiac repair by stem cells[105,106]. However, a series of randomized control clinical trials that combine LVADs with MPCs showed consistently negative results[107,108]. The proportions of successful LVAD weaning at different stages were all similar between the MPC and the sham-control groups. The LVEF data from patients, who tolerated the LVAD weaning, did not shown the difference either. On the other hand, activated myostatin in patients after LVAD implantation raised some concerns that long-term LVAD wearing might cause difficulty in removing[109]. Long-term cardiac disuse causes relative myocardial atrophy that further exacerbates this problem[110]. These findings might partially explain why the combined therapy failed in the clinical trials. Nevertheless, despite the similar likelihood of LVAD weanings, patients receiving additional MPCs could tolerate a longer LVAD turn-off than patients in the control group at the 12-month follow-up[107]. Therefore, it is still too early to confirm the lack of efficacy of stem cells in HF patients with VADs. So far, only trials of MPCs were published, and other cell types are still missing for these trials. In addition, extracorporeal membrane oxygenation (ECMO) was also tried in combination with stem cells but the adherence of stem cells to membrane oxygenator negatively influenced its efficacy[111].

In the end-stage of heart failure, irreversible fibrosis leads to ventricular dilation. In the third phase of cardiac repair after MI, collagen secretion and scar formation replace apoptotic or necrotic cardiomyocytes to keep ventricular integrity; however, they could also act as barriers that hamper stem cells from settling down in the ischemic myocardium. A study proved that interference with fibrogenesis is beneficial for the restoration of heart function after MI[112]. MiR-29b was the microRNA that targeted several mRNAs encoding ECM proteins involved in fibrosis. After transfecting with mimic miR-29b, the fibroblasts showed suppressed collagen I and III gene expressions. In the animal models at four weeks after inducing MI, heart tissue overexpressing miR-29b had the smallest fibrotic area and the thickest ventricular wall in either the infarcted, border or remote area that facilitated the iPSCs penetration to a greater extent. Significant vascularization in the miR-29b pretreated group was confirmed by higher volume and density of newly formed vessels. Two-dimensional echocardiography supported these notable *in vitro* and *in vivo*

data in which rat hearts overexpressing miR-29b had limited LV dilation and restored contractility. In the end, the above-mentioned anti-fibrosis, angiogenesis, and subsequent improvements in cardiac functions all disappeared in the anti-29b pretreated group. Fibrogenesis elimination or collagen digestion seems to be an exciting novel therapeutic modality in cardiac repair, further research to fill the existing gaps of knowledge.

After 20 years of consistent exploration, regenerative therapeutics for heart failure are still stagnating in terms of finding the ideal cell or cell-related candidates for transplantation. Previously, we summarized the evidence regarding different cell types that support cardiac repair in preclinical experiments. Although controversies still exist[113], a meta-analysis of animal experiments had highlighted the positive effects of stem cell therapy[79,83], which were also confirmed by several systematic reviews of clinical trials[114,115]. However, the power of that specific meta-analysis was challenged by negative results from one individual-level data review[116,117]. It raised concern that the “in favor of stem cells”-result from the meta-analysis might be, in fact, a neutral result. Contradictory results from literature-based meta-analyses and individual-based meta-analyses in stem cell treatment for acute MI have an impact on the validity of meta-analysis for HF. However, in that individual-level study[117], most patients received BMMNCs at a dose of 150 million, while in other literature-based meta-analyses, stem cell types were not limited to BMMNCs but also included BMMSCs or other purified cell products. Second, the dose of juvenile stem cells should have been estimated at < 5 million in that paper since the concentration of real stem cells was quite low in BMMNCs (concentration of CD34+ cells in BMMNCs from HF patients versus healthy volunteers: 1.5% versus 3.6%[63]). The heterogeneities within cell types and cell doses suggest that it is still too early to deny the benefits of stem cells for patients who underwent MI or had heart failure. We also noticed that statisticians or reviewers in general blended cultured stem cells with unfractionated cells; therefore, creating insufficient subgrouping. For that reason, in Part I, we will subgroup trials based on cell cultivation by separating expanded cells and freshly isolated primary cells. More information will be obtained from bone marrow cells, and each cell product from this source will be analyzed. Doses of juvenile stem cells will be re-calculated to determine whether a dose-response effect exists. However, the content in Part I mainly focuses on the analyses of stem cells from different origins, and the results of more subgroup analyses are beyond the scope of this dissertation. Therefore, they were finished and listed in **Table 5** but are not shown in the Results and Discussion.

Hypotheses

Combined with the information provided in the introduction, we constructed our hypotheses as follows:

1. Functional cardiac improvements of stem cells might be very limited in cardiac repair.
2. The robustness of overall positive outcomes might be challenged by inappropriate trial designs and execution.
3. The cultured cells that were modified and optimized in the laboratory might bring better cardiac functional improvements compared to isolated primary cells.
4. Different cell types might cause fluctuations in the effectiveness and efficacy of stem cell transplantation.

Methods

This meta-analysis was registered in PROSPERO with the reference of CRD42019118872.

Definition of reduced ejection fraction

Systolic heart failure was defined as weak myocardial contractility and failed to meet the body's demands. Patients presenting with heart failure symptoms, including dyspnea, fatigue, reduced exercise capacity, and edema in the legs, ankles and feet, etc., also supported by ultrasonic and radiological examination with LVEF less than 40%, were diagnosed as HFrEF. A new category of heart failure, Heart Failure with Mid-Range Ejection Fraction (HFmrEF, LVEF 40%-49%), was introduced in 2016[118]. In this meta-analysis, we included heart failure patients with reduced ejection fraction (HFrEF and HFmrEF).

Outcome measures

Primary outcomes include all-cause mortality for safety evaluation and change in LVEF from baseline for efficacy. The definition of all-cause mortality was a composite of all causes of mortality, heart transplantation, and LVAD implantation (Table 2).

Secondary outcomes include heart failure-related major adverse cardiovascular events (HF-MACEs) and serious adverse events (SAEs) for safety, left ventricular end-diastolic diameter/ left ventricular end-diastolic volume/ left ventricular end-diastolic volume index (LVEDD/ LVEDV/ LVEDVI), brain natriuretic peptide/ N-terminal brain natriuretic peptide (BNP/ NT-proBNP), six-minute walk distance (6MW distance)/ walking speed, New York heart association functional status (NYHA functional status), and quality of life for efficacy. HF-MACEs in this meta-analysis are defined as a composite of nonfatal stroke, nonfatal myocardial infarction, cardiac death, decompensated HF, and persistent ventricular arrhythmias. LVEDD, LVEDV, and LVEDVI were measured to quantify the extent of LV remodeling and dilation. BNP and NT-proBNP were put together to assess the severity of cardiac overload, and 6MW distance in addition to walking speed were used to evaluate exercise ability.

Retrieving strategies and study selection

Publications and registered clinical trials were retrieved from Medline, EMBASE, and clinicaltrials.gov in August 2018. The search formula was (heart failure OR myocardial

ischemia OR heart infarction OR ischemic heart disease) AND (stem cell transplantation OR transplantation). In the PubMed database, the filter for article types was applied so that only clinical trials were shown in the results.

The inclusion criteria were as follows:

-- clinical trials

-- adult patients (>18 years old)

-- HF secondary to ischemic or non-ischemic reason: LVEF<50%, NYHA II-IV.

-- studies reporting primary outcomes

-- follow-up >3 months after transplantation

-- publications written in English

Exclusion criteria:

-- review, preclinical experiments, case reports

-- juvenile patients

-- HFrEF due to Chagas disease

-- patients receiving LVAD implantations

-- publications written in non-English languages

-- papers that are not accessible

For further meta-analysis, only randomized controlled trials (RCTs) were included.

Notably, in patients that underwent LVAD implantation, most deaths were attributed to LVAD dysfunctions, pump thrombus, multi-system organ failure, and sepsis that are irrelevant to cell therapy. The wearing of LVAD unloading left ventricles also impacts the accuracy of graphic assessments. Therefore, trials of patients who underwent LVAD implantations were excluded.

Calculation of stem cell numbers

Since subgroup analysis based on cell doses was expected, the numbers of stem cells were then calculated by the expressions of specific biomarkers. For myoblasts, cell numbers were calculated by their expression of CD56 or desmin. For BMMNCs and ATSVFs, stem cell numbers were estimated by the expression of CD34 biomarkers (explained in the Discussion section). As shown in Table 3 and other clinical records[119,120], the concentrations of $CD34^+$ cells in BMMNCs from heart failure patients were reported from 0.7% to 3%. However, not all the BMMNC trials had published detail concentrations or amounts of $CD34^+$ cells. Therefore, we settled those cell concentration that missing in the papers as 1% (The purpose of this rough estimate was only to divide those trials into

four subgroups of ranges of increasing cell doses. In **Table 3**, the estimations of $CD34^+$ cell numbers were shown in digital intervals instead of specific numbers, for instance *<1, 1-10*, *10-100, etc.* Meanwhile, the results based on the cell doses were not reported and discussed but only listed in **Table 5** as supplementary data). In trials with concentrated cell products such as $CD34^+$, $CD133^+$, or $ALDH^+$ cells, cells were immunoselected and highly purified by their biomarkers, so the concentraions or detail numbers of stem cells were all needed and published in the papers that we can use directly. Cultured cells such as BMMSCs or MPCs were already purified during *in vitro* expansion, so no extra calculation was needed. Additionally, stem cells in Ixmyelocel-T cells were calculated by the percentage of CD93+ cells.

Subgroup analyses to detect possible sources of heterogeneities

Heterogeneous values were expected in our meta-analysis due to the diversity of study designs. Patient data were divided into subgroups based on clinical differences to help researchers investigate the origin of heterogeneities:

> Cell cultivation: primary cells after isolation and cultured cells after in-vitro cultivation.

> Cell origin: skeletal muscle-derived stem cells, BM-derived stem cells, AT-derived stem cells, perinatal stem cells, cardiac endogenous stem cells.

> BM-derived cell line: BMMNCs and selected CD34+, CD133+, ALDH+ cells, BMMSCs, STRO-3+ MPCs, BM-derived cardiopoietic stem cells, Ixmyelocel-T cells.

> HF type: heart failure secondary to myocardial ischemia, dilated cardiomyopathy.

> Cell source: autologous stem cells from patients, allogeneic sources from volunteers.

> Delivery route: IVI, ICI, IMI, cell delivery after CABG, TESI, bioengineered cell sheets. The intervention of revascularization referred to surgical CABG or PCI.

> Dose escalation of estimated stem cells: <1 million, 1-10 million, 10-100 million, $\geqslant$ 100 million. Patients were stratified into four groups according to numbers of stem cells: <1 million, 1-10 million, 10-100 million and $\geqslant$100 million.

Data extraction

Data on primary and secondary outcomes were extracted by two reviewers. In the case of data extraction from multi-arm trials with different interventions, to avoid repeating input, the number of participants in the control group was divided and equally distributed

among the different comparisons. This data manipulation was only necessary when multiple comparisons were placed in one single analysis.

By eliminating bias caused by baseline imbalance and diverse imaging techniques, the change from baseline is more accurate; however, many of the investigators reported data as mean at the endpoint, but without change from baseline. However, the mean change from baseline could be easily calculated from the mean at the endpoint, according to the Cochrane handbook, de Jong[121], and Hristov et al[122]. Therefore, in this meta-analysis, the change from baseline of continuous variables such as LVEF, LVEDV was calculated as below:

$$M_{change}=M_{endpoint}-M_{baseline}$$

$$SD_{change}=\sqrt{SD_{baseline}^2+SD_{endpoint}^2-(2\times corr\times SD_{baseline}\times SD_{endpoint})}$$

In the papers which provided all the necessary data, *corr* was calculated according to the following formula:

$$Corr=\frac{SD_{baseline}^2+SD_{endpoint}^2-SD_{change}^2}{2\times SD_{baseline}\times SD_{endpoint}}$$

The mean *corr* values in LVEFs was calculated to be 0.76 and 0.62 in short- and long-term follow-ups, 0.91 and 0.97 in LVEDV, but the calculation was not feasible for other variables due to insufficient data. The estimated mean and SD of change were then filled.

In case that data combination was needed, the data were combined according to the Cochrane handbook (**N**umber of Samples, **M**ean, **S**tandard **D**eviation):

$$N_{combined}=N_1+N_2$$

$$M_{combined}=\frac{N_1M_1+N_2M_2}{N_1+N_2}$$

$$SD_{combined}=\sqrt{\frac{(N_1-1)SD_1^2+(N_2-1)SD_2^2+\frac{N_1N_2}{N_1+N_2}(M_1^2+M_2^2-2M_1M_2)}{N_1+N_2-1}}$$

Statistics

Meta-analysis was performed in Stata15 and RevMan v5.3 for double checking. Characteristic details, including data type, data unit, data source, and effect measures in the meta-analysis are listed in **Table 2**.

Risks of bias were evaluated based on RoB2 (a revised Cochrane risk-of-bias tool for randomized trials[123]). The graph and summary were automatically generated by the tool. Publication biases were assessed by funnel plots and Egger's test. P value >0.05 indicates no evidence of any small-study effect.

Since heterogeneity was expected and found in the pre-study, random effect and inverse variance models were used to control unobserved heterogeneity. Data are shown as mean [95% confidence intervals]. I^2 and Tau^2 were utilized to quantify the extent of heterogeneities, in which I^2>50% stood for substantial heterogeneity, and p<0.05 (from Q test for heterogeneity) indicated statistically significant heterogeneity. Sensitivity and subgroup analyses were conducted based on trial quality, clinical and methodological differences including cell cultivation, cell origins, HF types, cell sources, delivery routes, and cell doses, to identify the sources of heterogeneities and their influence on the effectiveness and efficacy of stem cell therapy for HF.

Table 2. Data characteristics of primary and secondary outcomes in meta-analysis

Outcomes	Data type	Data unit	Data source	Effect measures
Primary outcomes				
LVEF	continuous	%	change from baseline	mean difference
mortality	dichotomous	death/total patient number	incidence at endpoints	risk ratio
Secondary outcomes				
LVEDV LVEDD LVEDVI	continuous	ml mm ml/m^2	change from baseline mean at endpoints	std. mean difference
BNP NT-proBNP	continuous	pg/ml	change from baseline mean at endpoints	std. mean difference
6MW distance walk speed	continuous	meters mph	change from baseline mean at endpoints	std. mean difference
NYHA	continuous	-	change from baseline mean at endpoints	mean difference
quality of life	continuous	-	change from baseline mean at endpoints	mean difference
HF-MACEs	dichotomous	events/total patient number	incidence at endpoints	risk ratio
SAEs	dichotomous	events/total patient number	incidence at endpoints	risk ratio

std. mean difference = standardized mean difference.

Results

The workflow of this meta-analysis is depicted in **Figure 2A**.

In total, 1,810 records were retrieved from Medline and EMBASE, and 84 trials that were registered at clinicaltrial.gov. 42 studies were included according to the inclusion and exclusion criteria (**Figure 2B**). 2,699 patients were included after randomization, 136 excluded before the intervention, and 121 lost during follow-up. Common reasons for exclusion before transplantation were withdrawal of consent, off-specification of cell products, death during waiting, rare machine malfunction, and ineligibility of patients. Loss during follow-up might be due to medical device implantation, undefined withdrawal, and so on.

Characteristics of the 42 included RCTs are described in **Table 3**. **Figure 3A-B** summarize the methodological quality of enrolled studies through the Cochrane risk-of-bias tool. Since high heterogeneities were found in the overall results of LVEF change (high I^2 in **Figure 4**), sensitivity analysis, which separated trials by different qualities in this meta-analysis, was performed to determine whether low trial qualities impacted the outcome robustness. An extra clarification is need here: as seen in **Figure 3A**, selection bias occurs in two ways, inadequate generation of randomized sequence and foreknowledge of treatment assignment. The breaking of blindness in either randomization or allocation would include unrepresentative samples into the trials. Therefore, in the sensitivity analysis based on selection bias, we grouped all the trials together into low risk group if they have low risks in both randomization and allocation, and assign the rest to high/unclear group. In **Figure 3C-F**, trials that did not employ sufficient methods of randomization and blinding during operation (high/unclear risk in selection bias and performance bias, respectively) had significantly better outcomes of LVEF increase than trials that utilized more appropriate randomization and blinding methods. However, the quality of blindness during follow-up examination (risk of detection bias) did not significantly influence the size of mean difference in LVEF change (**Figure 3G-H**).

Overall effects on primary and secondary outcomes

Details of group sizes, p values of effects, I^2 for heterogeneity, effect sizes of comparisons, and corresponding 95% confidence intervals are provided in **Table 4**.

In **Figure 4A-B**, the favorability of cell therapy in reducing mortality of HF patients was not shown within 12-month observation (RR=0.80 [0.45, 1.43], p=0.45, I^2=0%) but

was seen over 12-month follow-up (RR=0.71 [0.54, 0.93], p=0.01, I^2=7%). A 2.88% increase of LVEF among stem cell groups over controls was demonstrated at short-term follow-up ([1.70, 4.06], p<0.00001, I^2=83.7%) (**Figure 4C**), but slightly decreased to 2.33% [0.64, 4.02] after long-term follow-up (p=0.07, I^2=83.8%) (**Figure 4D**). High heterogeneities were shown with LVEF data at both short-term and long-term follow-ups, implying significant clinical differences among those trials. Meanwhile, funnel plots and Egger's tests for small-study effects based on primary outcomes showed no sign of publication bias in the meta-analysis (**Figure 5**).

Detail effect sizes of overall cell therapies on secondary clinical outcomes are shown in **Table 4**. The incidence of MACEs and SAEs were significantly reduced by stem cells at long-term follow-up. In LV remodeling, the standardized mean difference between cell therapy and control suggested benefits of stem cells for ameliorating left ventricular dilation. In the short term after transplantation, LV sizes in cell groups became smaller than controls (-0.20 [-0.36, -0.03], p=0.02), and over a long-term period the extent of LV size reduction went even further (-0.34 [-0.54, -0.13], p=0.001). Moreover, the benefits of stem cells also included improvements in exercise capacity. Patients receiving stem cells could walk further or faster than patients in control. The beneficial effects were also extended over a long-term time period (std. mean difference from mean at endpoint: 0.55 [0.17, 0.94] <12 months and 0.85 [0.07, 1.64] ≥12 months). Applying the New York Heart Association classification to HF symptoms, no favorability of cell therapy was detected in the short term, but over 12 months patients in the cell groups had a significantly decreased NYHA class (-0.46 [-0.74, -0.17], p=0.002) compared to controls indicating better general functional status among them. For other analyses, including life quality (MLHFQ) and biomarkers reflecting HF severity (BNP/NT-proBNP), no statistical difference was observed between cell therapy and control.

Discrepancies in outcomes due to cell cultivation techniques

Within 12 months, no differences in primary outcomes were seen between primary and cultured cells. No advantage of either stem cell types was present in regard to mortality, but increases in LVEF were shown in both categories (**Figure 6A, C**). No sign of LV-size shrinking in either primary or cultured cell group compared to control patients was shown (**Figure 6E**), but results were slightly in favor of primary cells in relation to NYHA class improvement (**Figure 6G**).

Over 12 months, the risk ratio in mortality between patients in primary cell group and control group is 68**%** ([47%, 97%], p=0.03), a 32% decrease in risk of death after primary cells infusion (**Figure 6B**). Meanwhile a 3.05% [1.00, 5.10] higher increase in LVEF with primary cell transplantation (p=0.004) (**Figure 6D**). Freshly isolated primary cells also decreased LV sizes (std. Mean difference-change from baseline: -0.44 [-0.75, -0.13], p=0.005) (**Figure 6F**). Contrarily, *in vitro* expanded cells had a similar effect of increasing LVEF as primary cells at short-term follow-up (2.92% [0.86, 4.99], p=0.005) (**Figure 6C**). But over 12 months this effect became insignificant that from **Figure 6D** their horizontal line of confidential intervals was seen to cross the vertical dotted no-effect line. Moreover, it was also found in the long-term follow-up that cultured cells could also help in relieving HF symptoms. The patients who received cultured stem cells had lower NYHA classes compared to the control groups (Mean difference-mean at the endpoint: -0.73 [-1.25, -0.2], p=0.007) (**Figure 6H**).

Discrepancies in outcomes due to cell origins

42 RCTs were sub-grouped based on diverse cell origins: skeletal muscle-derived stem cells, BM-derived stem cells, AT-derived stem cells, and perinatal stem cells (**Figure 7, Table 4**).

At short-term follow-up, meta-analysis failed to find any beneficial effects from myoblasts on cardiac repair, either in increasing LVEF (-0.03[-2.08, 2.02], p=0.98, I^2=0%, **Figure 7C**), or reducing mortality (2.27 [0.60, 8.62], p=0.23, **Figure 7A**) or by any improvements in the other outcomes (**Figure 7E, G**). Over long-term follow-up, myoblasts were only found to improve NYHA functional status significantly (mean difference: -1.30 [-1.75, -0.85], p<0.00001, **Figure 7H**). Conversely, BM-derived cells proved their effectiveness in cardiac recovery, having a short-term advantage of increasing LVEF by 3.29% (95%CI: [2.03, 4.54], p<0.00001, **Figure 7C**), and also a long-term effect of 2.66% (95%CI: [0.81, 4.51], p=0.005, **Figure 7D**) over controls. Other capacities as slowing down the progression of LV dilation, decreasing mortality, the incidence of SAEs at short-term follow-up, and a long-term decrease in NYHA class, are listed in **Table 4**. Perinatal stem cells, specifically UCMSCs in the meta-analysis, showed the potential to be a better cell choice for heart recovery. Peinatal stem cells increased more LVEF than BM-derived cells. The effect size after the short-term follow-up was 6.48% [2.84, 10.15] (p=0.0005) and remained over 5% after the long-term follow-up (5.22 [0.84, 9.6], p=0.02). Moreover, they seemed to be more efficient in reversing LV dilation (**Figure 7E**). However, as shown,

only two included trials used perinatal stem cells, and the robustness of these results is not strong enough to draw any conclusions. Similarly, the same problem exists with AT-derived cells. Unfortunately, insufficient data from these two different cells is difficult to supplement in a short period because the number of relevant registered or undergoing RCTs is very few.

The influence of other methodological and clinical differences on stem cell efficacy, such as cell types, HF types, cell sources, surgical interventions, and cell doses, was also evaluated. Data on their effect size, heterogeneities, and p values are listed in Table 5.

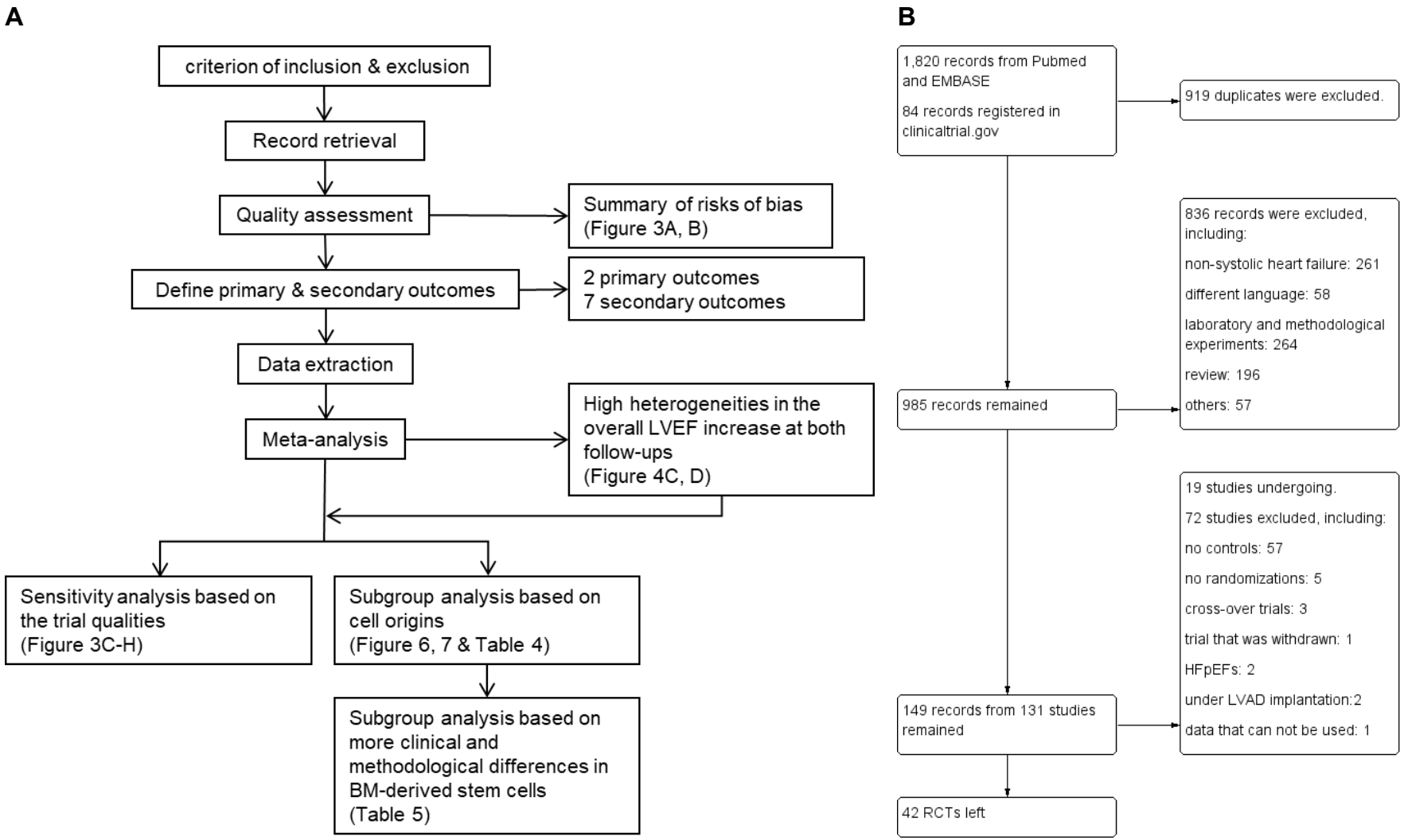


Figure 2. Visualizations of meta-analysis and study retrieval

A) Workflow of meta-analysis

B) Flowchart of systematic retrieval of stem cell therapy for clinical cardiac repair, conducted in August 2018.

Table 3. Characteristics of 42 included randomized controlled trials (RCTs) for meta-analysis

Study identifier	Group size	HF type	Cell source	Cell type	Nucleated cells (Million)	Cell purity	Stem cell numbers (Million)	Ex vivo expansion	Intervention	Follow-up (months)
Skeletal muscle-derived cells										
Brickwedel 2013[124] MAGIC II	7	ischemic	autologous	myoblasts (>50%)	-	-	400 800	cultured but not mentioned	CABG+IMI	12
Dib 2009[125] CAuSMIC	23	ischemic	autologous	myoblasts	-	-	30 100 300 600	15-20% FBS	TESI	3, 6, 12
Duckers 2011[126] SEISMIC	47	ischemic	autologous	myoblasts	596±194	$CD56^+$: 57.2±23.9%	*>100*	15-20% FBS	IMI	3, 6
Menasche 2008[88] MAGIC	120	ischemic	autologous	myoblasts	-	89%	400 800	FBS	CABG+IMI	6
Povsic 2011[89]	23	ischemic	autologous	myoblasts	400 800	-	400 800	FBS	TESI	6
BM-derived cells										
Ang 2008[127]	63	ischemic	autologous	BMMNCs	IMI arm:84±56 ICI arm:115±73	-	$CD34^+CD117^+$ IMI arm: 0.142±0.166 ICI arm: 0.245±0.254		CABG+ICI/IMI	6
Assmus 2013[128] CELLWAVE	103	ischemic	autologous	BMMNCs	low-dose BMMNCs: 150±77 high-dose BMMNCs: 123±69	-	*1-10*		Shockwave+ICI	4
Bartunek 2013[129] C-CURE	47	ischemic	autologous	BM-derived cardiopoietic stem cell	733	-	733	5% human pooled platelet lysate	TESI	6, 2 years
Bartunek 2017[130] CHART-1	348	ischemic	autologous	BM-derived cardiopoietic stem cell	600 before cryopreservation	-	up to 480 (20% lost after thawing)	5% human platelet lysate	retention enhanced TESI	6, 9, 12 months

Table 3. Characteristics of 42 included randomized controlled trials (RCTs) for meta-analysis

Study identifier	Group size	HF type	Cell source	Cell type	Nucleated cells (Million)	Cell purity	Stem cell numbers (Million)	Ex vivo expansion	Intervention	Follow-up (months)
Choudhury 2017[131] REGENERATE-IHD	105	ischemic	autologous	BMMNCs (peripheral after mobilization)	115.1	-	*1-10*		G-CSF+TESI/ICI	6, 12
Frljak 2018[132] REMEDIUM	60	non-ischemic	autologous	BMMNCs (peripheral after mobilization)	-	-	CD34+: 80		TESI	6
Hamshere 2015[133] REGENERATE-DCM	60	non-ischemic	autologous	BMMNCs	216±221.8	-	*CD34+: 4.91±2.75*		G-CSF+ICI	3, 12
Heldman 2014[134] TAC-HFT	65	ischemic	autologous	BMMNCs & BMMSCs	BMMSCs: 200 BMMNCs: 200	-	*BMMSCs: >100* *BMMNCs: 1-10*	20% FBS	TESI	6, 12
Hendrikx 2006[84]	23	ischemic	autologous	BMMNCs	60.25±31.35	$CD34^+$: 1.42±0.99%	*<1*		CABG+IMI	4
Henry 2014[135] IMPACT-DCM	61	both	autologous	Ixmyelocel-T	Catheter-DCM: 13±1.6 IMPACT-DCM: 25±2.6	$CD90^+$: 5-55% $CD45^+CD14^+$: 45-95%	*1-10*	20% FBS	IMI/TESI	12
Hu 2011[136]	60	ischemic	autologous	BMMNCs	131.7±106.6	-	*1-10*		CABG+ICI	6, 12
Martino 2015[137] dilated cardiomyopathy arm of MiHeart	160	non-ischemic	autologous	BMMNCs	236±271	$CD34^+$: 2.26% of mean $CD133^+$: 0.03% of mean	*1-10*		ICI	6, 12
Mathiasen 2015[138] MSC-HF	60	ischemic	autologous	BMMSCs	77.5±67.9	-	77.5±67.9	10% FBS cultured for 50 days	TESI	1, 3, 6
Maureira 2012[139]	14	ischemic	autologous	BMMNCs	342±41	$CD34^+$: 3±1% $CD73^+$: 0.2±0.6%	*10-100*		CABG+IMI	1, 6

Table 3. Characteristics of 42 included randomized controlled trials (RCTs) for meta-analysis

Study identifier	Group size	HF type	Cell source	Cell type	Nucleated cells (Million)	Cell purity	Stem cell numbers (Million)	Ex vivo expansion	Intervention	Follow-up (months)
Nasseri 2014[140] Cardio133	60	ischemic	autologous	BM-derived CD133+ cells	-	$CD133^+$: 73.6%	$CD133^+$: 5.1(3.0-9.1) medium (IQR)		CABG+IMI	6
Noiseux 2016[141] IMPACT-CABG	33	ischemic	autologous	BM-derived CD133+ cells	-	63.3±15.5%	$CD133^+CD34^+CD45^+$: 6.5±3.1		CABG+IMI	6
Patel 2005[142]	20	ischemic	autologous	BM-derived CD34+ enriched mononuclear cells	-	70%	medium of $CD34^+$: 22		CABG+IMI	1, 3, 6
Patel 2015[143] REVIVE	60	both	autologous	concentrated BMMNCs	3700±900	-	$CD34^+$: 39.4		ICI	1, 3, 6, 12
Patel 2016[143] IxCELL-DCM	126	ischemic	autologous	Ixmyelocel-T	40–200	$CD45^+CD90^+$ ≥90% CD90+: ≥3%	*$CD90^+$: 1-10*	20% FBS	TESI	3, 6, 12
Patila 2014[144]	39	ischemic	autologous	BMMNCs	840(medium)	CD34+: 10(medium) CD34+CD133+: 7.5(medium)	*CD34+: 10-100*		CABG+IMI	12
Perin 2011[63] FOCUS-HF	30	ischemic	autologous	BMMNCs	484.1±313	CD34+: 1.5±0.4%	*1-10*		TESI	6
Perin 2012a[145] FOCUS-Br	20	ischemic	autologous	sorted ALDH+ bright BMMNCs	2.94±1.31	-	*$ALDH^+$: 2.37±1.31*		TESI	6
Perin 2012b[85] FOCUS-CCTRN	92	ischemic	autologous	BMMNCs	100	$CD34^+$: 2.6% $CD133^+$: 1.2%	*1-10*		TESI	6
Perin 2015[70]	60	both	allogeneic	STRO-3+ MPCs	25 75 150	-	25 75 150	10% FBS	TESI	3, 6, 12

Table 3. Characteristics of 42 included randomized controlled trials (RCTs) for meta-analysis

Study identifier	Group size	HF type	Cell source	Cell type	Nucleated cells (Million)	Cell purity	Stem cell numbers (Million)	Ex vivo expansion	Intervention	Follow-up (months)
Pokushalov 2010[146] ESCAPE	109	ischemic	autologous	BMMNCs	41±16	$CD34^+$: 2.5±1.6%	*1-10*		TESI	3, 6, 12
Sant'Anna 2014[147] INTRACELL	30	non-ischemic	autologous	BMMNCs	106±43	$CD34^+$: 1.5±0.7%	*1-10*		transthoracic+IMI	3, 6, 12
Santoso 2014[148] END-HF	28	ischemic	autologous	BMMNCs	-	-	$CD34^+$: 2.4±1.2/ml 8-15 injections 0.1ml per injection		TESI	6
Seth 2006[149] ABCD	85	non-ischemic	autologous	BMMNCs	168±96	-	$CD34^+$: 2.7±1.5		ICI	6, 3years
Steinhoff 2017[150] PERFECT	82	ischemic	autologous	BM-derived CD133+ cells	-	-	2.29±1.42		CABG+IMI	6
Trifunovic 2015[151]	30	ischemic	autologous	BMMNCs	70.7±32.4	-	$CD34^+$: 3.96±2.77 $CD133^+$: 2.65±1.71		CABG+IMI	2, 6, 12
Vrtovec 2011[152]	110	non-ischemic	autologous	sorted CD34+ cells (peripheral after mobilization)	-	-	$CD34^+$: 113±26		G-CSF+ICI	1, 5 years
Xiao 2017[153]	53	non-ischemic	autologous	BMMNCs & BMMSCs	BMMNCs: 510±200 BMMSCs: 490±170	-	BMMNCs: 10-100 BMMSCs: >100	BMMSC: 15% autologous serum	ICI	3, 12
Zhao 2008[154]	36	ischemic	autologous	BMMNCs	659±512	-	*10-100*		CABG+IMI	1, 3, 6
Adipose-derived stem cells										
Henry 2017[155] ATHENA	31	ischemic	autologous	AT-SVF	40±9	-	-		TESI	1, 3, 6, 12

Table 3. Characteristics of 42 included randomized controlled trials (RCTs) for meta-analysis

Study identifier	Group size	HF type	Cell source	Cell type	Nucleated cells (Million)	Cell purity	Stem cell numbers (Million)	Ex vivo expansion	Intervention	Follow-up (months)
Perin 2014[156] PRECISE	27	ischemic	autologous	AT-SVF	42	$CD34^+$: 70.4%	*10-100*		TESI	6, 18
Perinatal cells										
Bartolucci 2017[69] RIMECARD	30	non-ischemic	allogeneic	UCMSCs	-	-	1/kg	10% AB plasma	IVI	12
Zhao 2015[68]	59	non-ischemic	allogeneic	UCMSCs	-	-	-	10%FBS	ICI	1, 6

The *italicized* numbers in the column for stem cells represent the estimated amounts of stem cells based on information provided in publications.

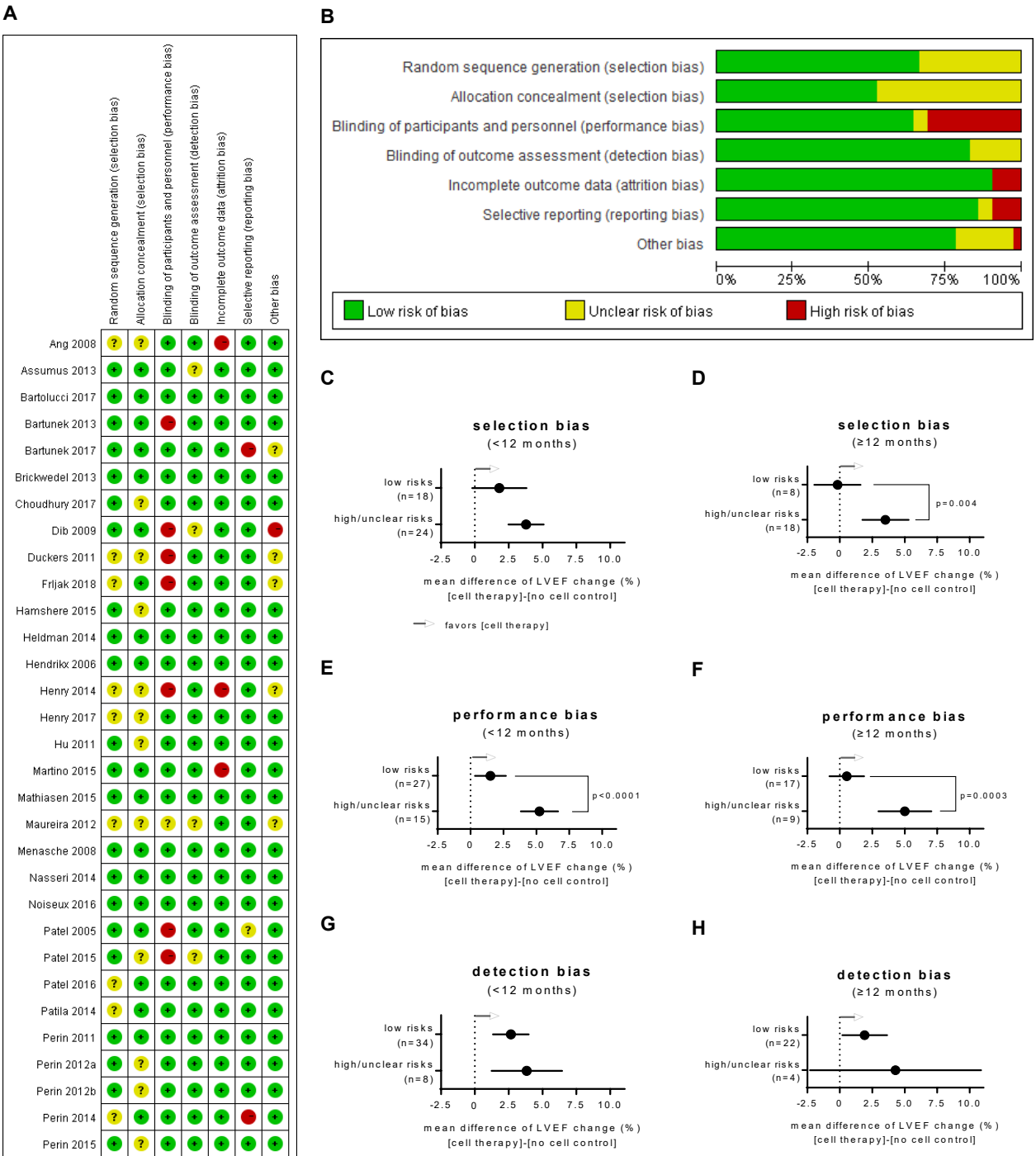


Figure 3. Quality assessments of 42 RCTs

(**A**) Risk of bias graph. (**B**) Risk of bias summary. Influences of (**C&D**) selection bias in LVEF increase at short-term (p=0.1) and long-term follow-up (p=0.004), (**E&F**) performance bias at short-term (p<0.001) and long-term follow-up (p=0.0003), and (**G&F**) detection bias at short-term follow-up (p=0.43) and long-term follow-up (p=0.49).

N beneath the grid name on the y-axis indicates the number of comparisons enrolled in that subgroup, and that more than one comparison could be extracted from one study.

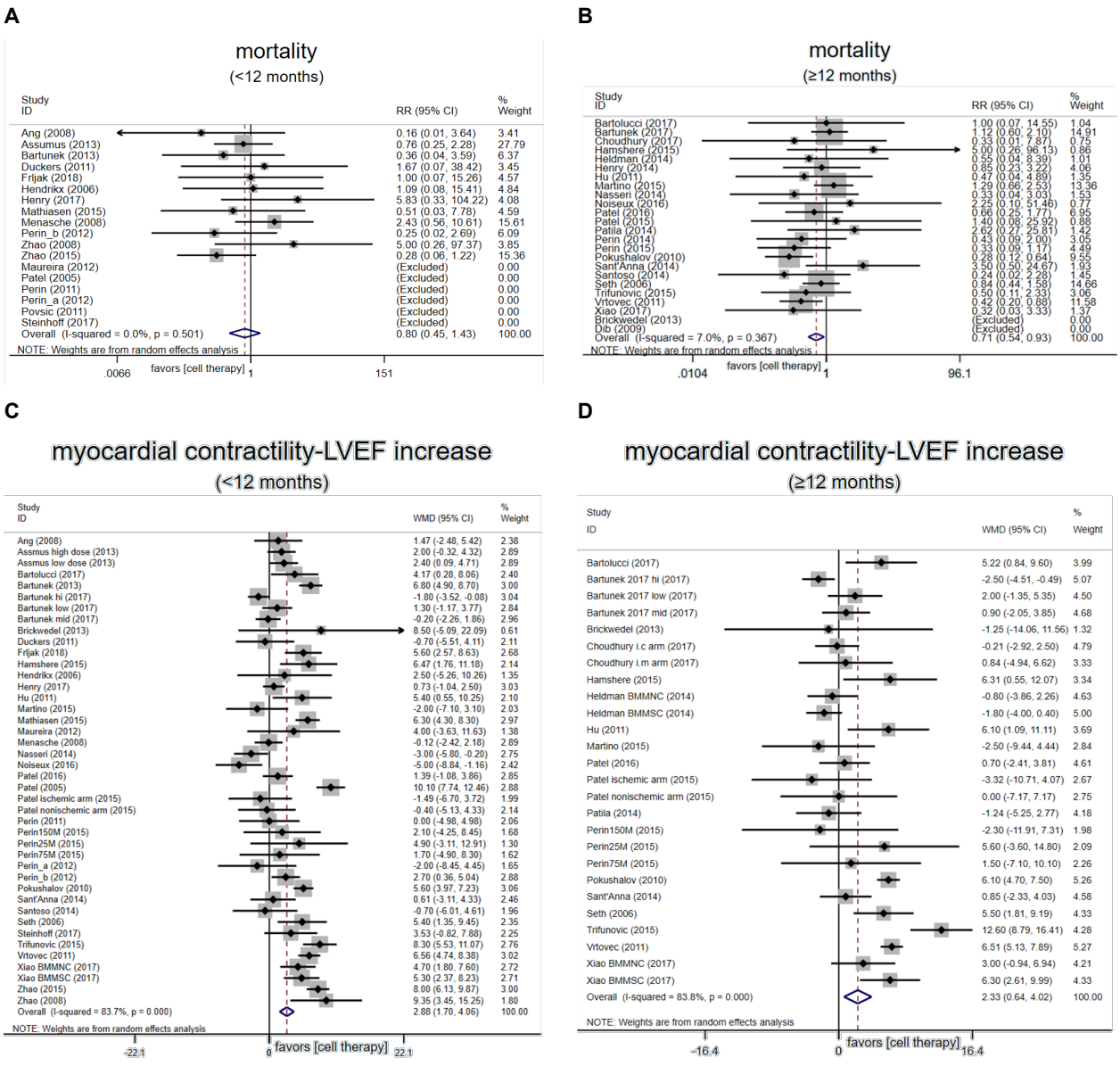
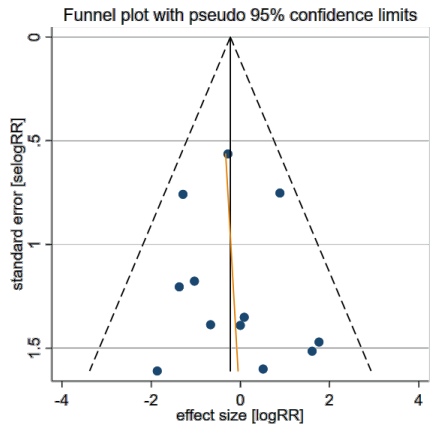


Figure 4. Overview of primary outcomes

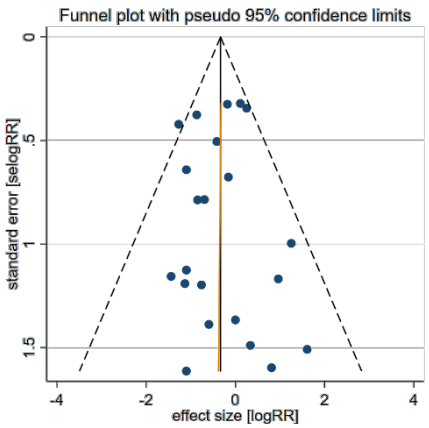
Results of primary outcomes of mortality (**A&B**) and LVEF change from baseline (**C&D**) were shown at both short-term and long-term follow-ups.

The P value in each plot indicates for the heterogeneity.

A. Funnel plot of mortality (<12 months)



B. Funnel plot of mortality (≥12 months)



C. Egger's test for mortality (<12 months)

Number of studies = 12 Root MSE = 1.007

Std_Eff	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
slope	-.479964	.8274875	-0.58	0.575	-2.323721	1.363793
bias	.2663558	.8037818	0.33	0.747	-1.524582	2.057293

Test of H0: no small-study effects P = 0.747

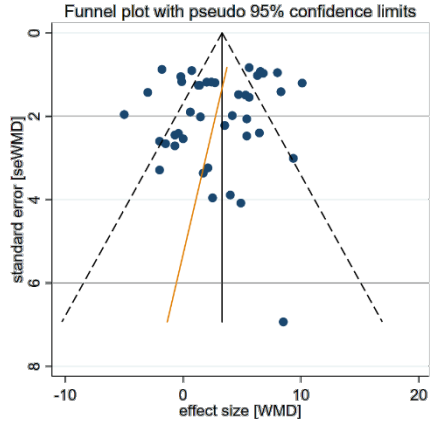
D. Egger's test for mortality (≥12 months)

Number of studies = 22 Root MSE = 1.062

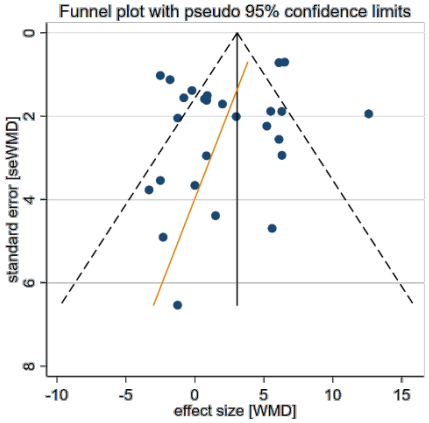
Std_Eff	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
slope	-.3095037	.2673113	-1.16	0.261	-.8671053	.2480979
bias	-.0431423	.4396813	-0.10	0.923	-.9603014	.8740168

Test of H0: no small-study effects P = 0.923

E. Funnel plot of LVEF change (<12 months)



F. Funnel plot of LVEF change (≥12 months)



G. Egger's test for LVEF (<12 months)

Number of studies = 42 Root MSE = 2.481

Std_Eff	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
slope	4.408362	1.32841	3.32	0.002	1.723545	7.09318
bias	-.829983	.9072516	-0.91	0.366	-2.663607	1.003641

Test of H0: no small-study effects P = 0.366

H. Egger's test for LVEF (≥12 months)

Number of studies = 26 Root MSE = 2.457

Std_Eff	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
slope	4.653616	1.494957	3.11	0.005	1.568178	7.739055
bias	-1.169909	.9505656	-1.23	0.230	-3.13178	.7919617

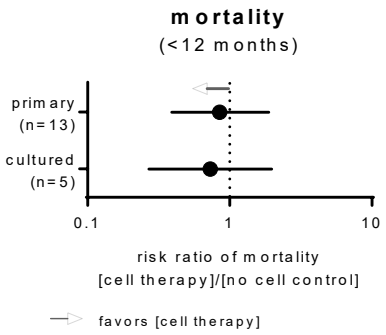
Test of H0: no small-study effects P = 0.230

Figure 5. Publication bias assessments in primary outcomes

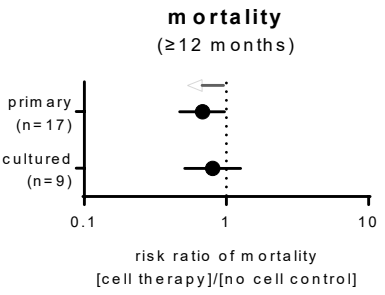
Funnel plots indicate fitted regression lines (orange) and egger's test for small-study effects (**A, C**) of mortality at short-term and (**B, D**) long-term follow-ups. The same assessments were performed on LVEF change at short-term (**E, G**) and long-term (**F, H**) follow-ups. Both oblique regression lines in LVEF change plots (**E&F**) indicate lacking positive results for stem cell therapy, despite no significance in Egger's tests.

WMD: weighted mean difference.

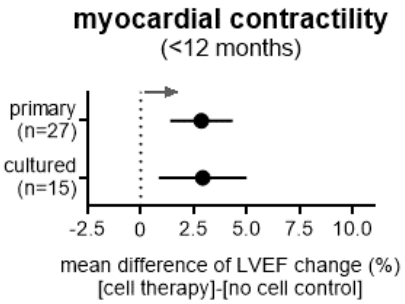
A


mortality
(<12 months)
primary
(n=13)
cultured
(n=5)
0.1
1
10
risk ratio of mortality
[cell therapy]/[no cell control]
favors [cell therapy]

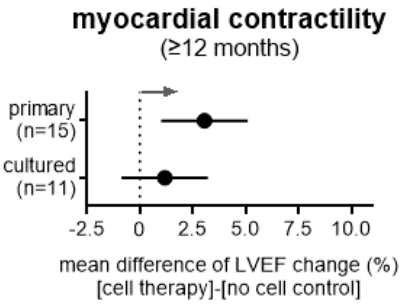
B


mortality
(≥12 months)
primary
(n=17)
cultured
(n=9)
0.1
1
10
risk ratio of mortality
[cell therapy]/[no cell control]

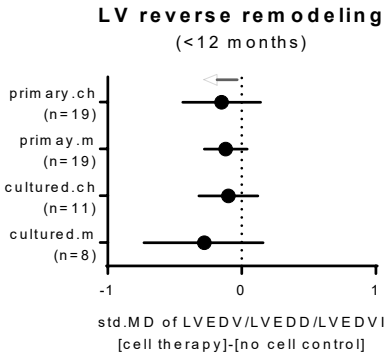
C


myocardial contractility
(<12 months)
primary
(n=27)
cultured
(n=15)
-2.5
0
2.5
5.0
7.5
10.0
mean difference of LVEF change (%)
[cell therapy]-[no cell control]

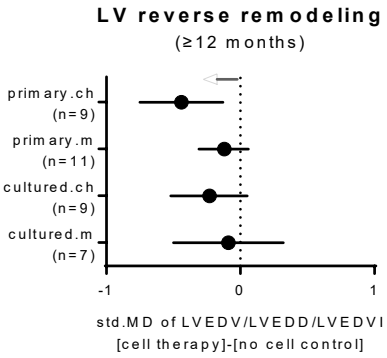
D


myocardial contractility
(≥12 months)
primary
(n=15)
cultured
(n=11)
-2.5
0
2.5
5.0
7.5
10.0
mean difference of LVEF change (%)
[cell therapy]-[no cell control]

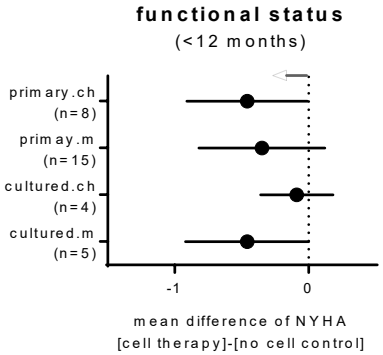
E


LV reverse remodeling
(<12 months)
primary.ch
(n=19)
primay.m
(n=19)
cultured.ch
(n=11)
cultured.m
(n=8)
-1
0
1
std.MD of LVEDV/LVEDD/LVEDVI
[cell therapy]-[no cell control]

F


LV reverse remodeling
(≥12 months)
primary.ch
(n=9)
primary.m
(n=11)
cultured.ch
(n=9)
cultured.m
(n=7)
-1
0
1
std.MD of LVEDV/LVEDD/LVEDVI
[cell therapy]-[no cell control]

G


functional status
(<12 months)
primary.ch
(n=8)
primay.m
(n=15)
cultured.ch
(n=4)
cultured.m
(n=5)
-1
0
mean difference of NYHA
[cell therapy]-[no cell control]

H

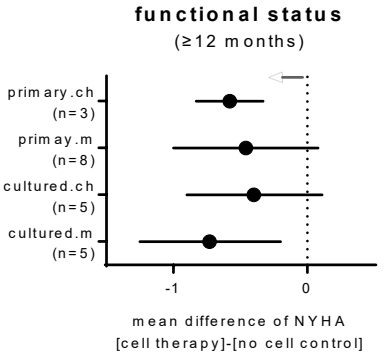

functional status
(≥12 months)
primary.ch
(n=3)
primay.m
(n=8)
cultured.ch
(n=5)
cultured.m
(n=5)
-1
0
mean difference of NYHA
[cell therapy]-[no cell control]

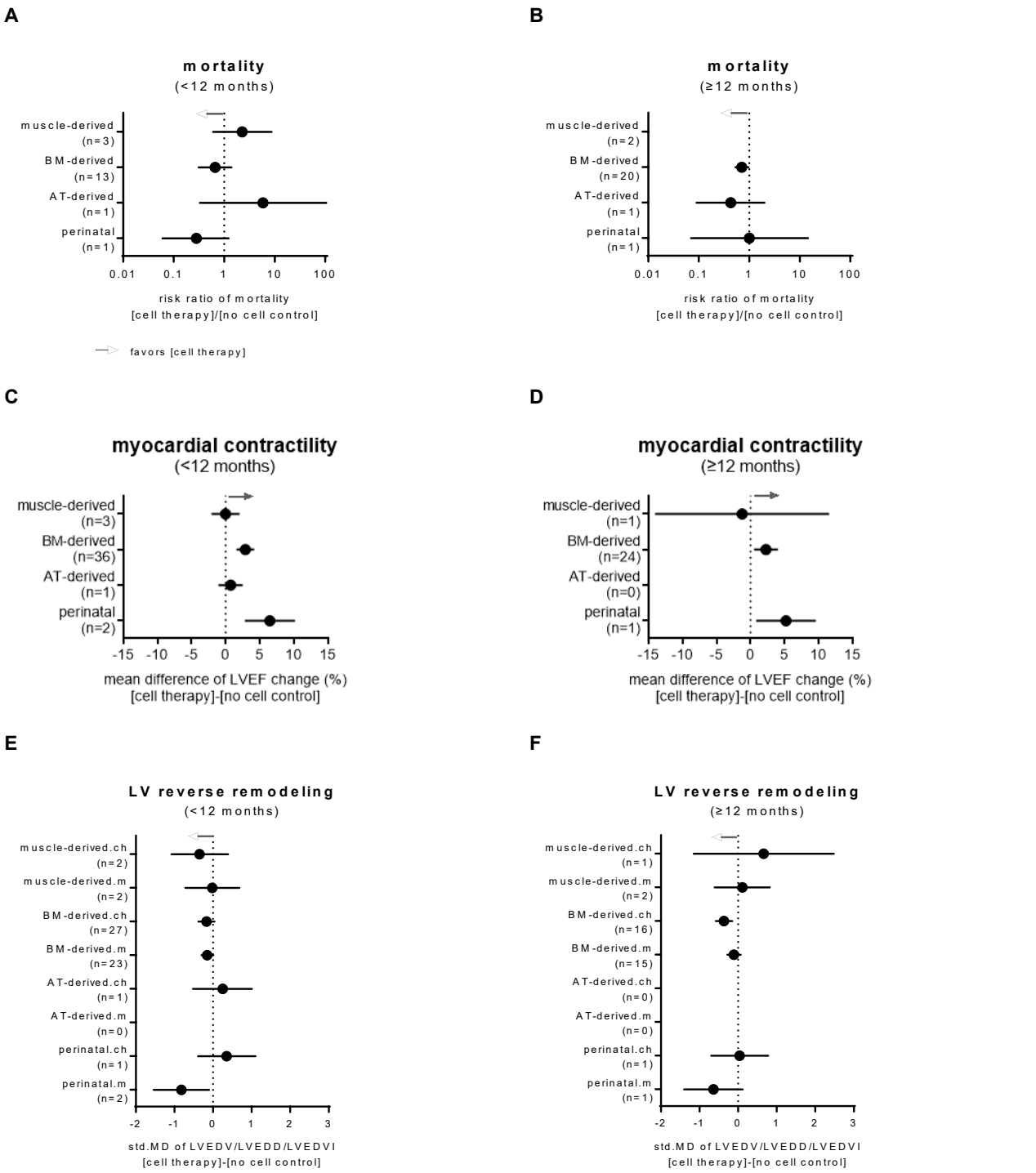
Figure 6. Summary of subgroup analyses in primary and *in vitro* cultured cells.

(**A&B**) Mortality at short- and long-term follow-ups; (**C&D**) LVEF increase from baseline at short- and long-term follow-ups; (**E&F**) left ventricular size; (**G&H**) NYHA functional status.

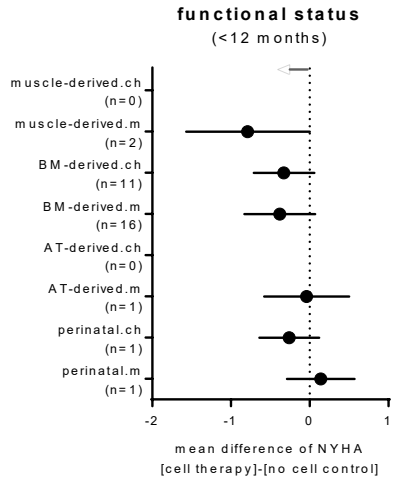
N beneath the grid name on the y-axis indicates the number of comparisons enrolled in that subgroup, and that more than one comparison could be extracted from one study.

.ch: data of change from baseline

.m: data of mean at the endpoint



G



H

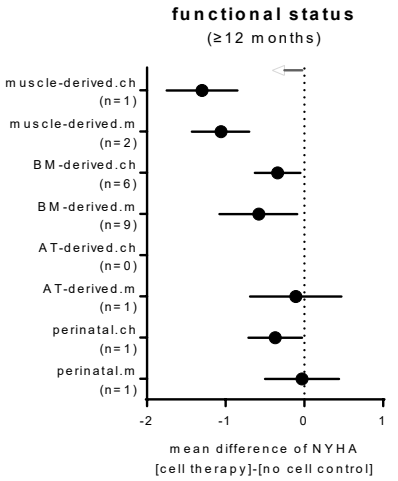


Figure 7. Summary of subgroup analyses in different cell origins

(**A&B**) mortality; (**C&D**) LVEF increase from baseline; (**E&F**) left ventricular size; (**G&H**) NYHA functional status

N beneath the grid name on the y-axis indicates the number of comparisons enrolled in that subgroup, and that more than one comparison could be extracted from one study.

.ch: data of change from baseline

.m: data of mean at the endpoint

Table 4. Summary of results of all clinical outcomes in overall pooled analysis and subgroup analysis by cell origins

Number of comparisons[participants] p value of the effect heterogeneity, I^2 effect size[95%CI]		LVEF	LV remodeling Change from baseline	LV remodeling Mean at endpoint	BNP/NT-proBNP Change from baseline	BNP/NT-proBNP Mean at endpoint	Exercise capacity Change from baseline	Exercise capacity Mean at endpoint	NYHA Change from baseline	NYHA Mean at endpoint	Quality of life Change from baseline	Quality of life Mean at endpoint	Mortality	MACEs	SAEs
Overall	<12 months	42[1773]	30[1306]	[27]930	4[193]	7[232]	11[516]	14[677]	12[477]	20[686]	10[317]	7[334]	18[857]	8[466]	13[870]
		<0.00001	0.20	0.02	0.16	0.79	0.03	0.005	0.07	0.06	0.61	0.34	0.45	0.96	0.31
		84%	64%	30%	95%	0%	85%	81%	87%	97%	39%	88%	0%	0%	7%
		2.88 [1.70, 4.06]	-0.13 [-0.33, 0.07]	-0.20 [-0.36, -0.03]	-1.03 [-2.47, 0.42]	0.04 [-0.23, 0.30]	0.65 [0.08, 1.21]	0.55 [0.17, 0.94]	-0.32 [-0.67, 0.03]	-0.38 [-0.78, 0.01]	1.95 [-5.58, 9.47]	-7.75 [-23.61, 8.11]	0.80 [0.45, 1.43]	1.01 [0.72, 1.42]	1.15 [0.86, 1.56]
	>12 months	26[1023]	18[837]	18[618]	2[71]	5[106]	6[232]	8[361]	8[242]	13[388]	8[301]	5[249]	24[1502]	12[571]	7[364]
		0.007	0.001	0.15	0.29	0.12	0.08	0.03	0.002	0.006	0.54	0.44	0.01	0.04	0.03
		84%	48%	0%	99%	0%	87%	90%	68%	91%	64%	92%	7%	0%	8%
		2.33 [0.64, 4.02]	-0.34 [-0.54, -0.13]	-0.12 [-0.29, 0.04]	-7.90 [-22.63, 6.83]	-0.33 [-0.75, 0.09]	0.75 [-0.08, 1.57]	0.85 [0.07, 1.64]	-0.46 [-0.74, -0.17]	-0.57 [-0.97, -0.16]	-3.44 [-14.39, 7.51]	-8.29 [-29.32, 12.73]	0.71 [0.54, 0.93]	0.72 [0.53, 0.98]	0.75 [0.57, 0.98]
Primary cells	<12 months	27[1071]	19[806]	19[716]	4[193]	4[246]	8[415]	6[347]	8[390]	15[526]	4[176]	4[246]	13[566]	6[329]	8[400]
		0.0001	0.3	0.13	0.16	0.41	0.07	0.1	0.05	0.15	0.41	0.58	0.69	0.6	0.75
		82%	23%	58%	95%	88%	92%	89%	90%	97%	42%	92%	0%	0%	27%
		2.86 [1.39, 4.33]	-0.15 [-0.44, 0.14]	-0.12 [-0.28, 0.04]	-1.03 [-2.47, 0.42]	-0.32 [-1.10, 0.45]	0.70 [-0.06, 1.45]	0.59 [-0.11, 1.28]	-0.46 [-0.91, -0.01]	-0.35 [-0.82, 0.12]	-5.05 [-17.09, 6.99]	-7.22 [-32.83, 18.40]	0.85 [0.39, 1.88]	0.90 [0.61, 1.33]	0.96 [0.75, 1.23]
	≥12 months	15[576]	9[445]	11[481]	2[71]	1[26]	5[207]	4[224]	3[132]	8[299]	4[220]	3[196]	17[898]	7[267]	4[199]
		0.004	0.005	0.19	0.29	0.36	0.1	0.13	<0.00001	0.1	0.16	0.62	0.03	0.97	0.03
		82%	38%	19%	99%	-	90%	95%	21%	93%	70%	94%	14%	0%	0%
		3.05 [1.00, 5.10]	-0.44 [-0.75, -0.13]	-0.12 [-0.31, 0.06]	-7.90 [-22.63, 6.83]	-0.36 [-1.14, 0.41]	0.83 [-0.17, 1.83]	1.16 [-0.33, 2.65]	-0.58 [-0.83, -0.33]	-0.46 [-1.00, 0.08]	-13.50 [-32.50, 5.49]	-9.20 [-45.39, 27.00]	0.68 [0.47, 0.97]	0.99 [0.57, 1.73]	0.58 [0.35, 0.96]
Cultured cells	<12 months	15[702]	11[500]	8[214]	-	5[136]	3[101]	8[330]	4[87]	5[160]	6[141]	3[88]	5[291]	2[137]	5[470]
		0.005	0.37	0.21	-	0.43	0.008	0.02	0.53	0.05	0.05	0.18	0.53	0.28	0.06
		87%	73%	3%	-	91%	0%	69%	0%	76%	6%	24%	18%	0%	58%
		2.92 [0.86, 4.99]	-0.10 [-0.32, 0.12]	-0.28 [-0.73, 0.16]	-	-0.57 [-2.00, 0.85]	0.56 [0.14, 0.97]	0.52 [0.07, 0.97]	-0.09 [-0.36, 0.18]	-0.46 [-0.92, -0.00]	7.58 [-0.10, 15.26]	-8.07 [-19.78, 3.63]	0.73 [0.27, 1.97]	1.48 [0.72, 3.03]	1.72 [0.97, 3.04]
	≥12 months	11[447]	9[392]	7[137]	-	4[80]	1[25]	4[137]	5[110]	5[89]	4[81]	2[53]	9[624]	6[321]	3[165]
		0.26	0.11	0.67	-	0.27	0.41	0.08	0.12	0.007	0.35	0.48	0.34	0.01	0.78
		65%	58%	0%	-	12%	-	0%	78%	67%	15%	69%	0%	0%	45%
		1.17 [-0.87, 3.21]	-0.23 [-0.52, 0.05]	-0.09 [-0.50, 0.32]	-	-0.30 [-0.84, 0.23]	0.35 [-0.47, 1.17]	0.32 [-0.04, 0.68]	-0.40 [-0.90, 0.11]	-0.73 [-1.25, -0.20]	4.34 [-4.73, 13.42]	-6.68 [-25.28, 11.92]	0.80 [0.51, 1.26]	0.73 [0.53, 0.99]	0.93 [0.55, 1.56]
Myoblast	<12 months	3[127]	2[32]	2[32]	0[0]	0[0]	1[38]	1[38]	0[0]	2[52]	1[20]	2[60]	3[157]	2[137]	4[180]
		0.98	0.35	0.95	-	-	0.15	0.76	-	0.05	0.05	0.05	0.23	0.28	0.24
		0%	3%	0%	-	-	-	-	-	80%	-	6%	0%	0%	0%
		-0.03 [-2.08, 2.02]	-0.35 [-1.08, 0.39]	-0.35 [-1.08, 0.39]	-	-	0.49 [-0.18, 1.16]	0.10 [-0.56, 0.76]	-	-0.79 [-1.57, -0.00]	11.50 [-0.04, 23.04]	-13.11 [-26.45, 0.22]	2.27 [0.6,8.62]	1.48 [0.72,3.03]	1.28 [0.85, 1.93]
	>12 months	1[9]	1[6]	2[31]	0[0]	0[0]	0[0]	0[0]	1[25]	2[30]	0[0]	1[25]	2[30]	0[0]	1[23]
		0.85	0.47	0.77	-	-	-	-	p<0.00001	p<0.00001	-	0.009	-	-	0.32
		-	-	0%	-	-	-	-	-	0%	-	-	-	-	-

Table 4. Summary of results of all clinical outcomes in overall pooled analysis and subgroup analysis by cell origins

		Number of comparisons[participants] p value of the effect heterogeneity, I^2 effect size[95%CI]	LVEF	LV remodeling Change from baseline	LV remodeling Mean at endpoint	BNP/NT-proBNP Change from baseline	BNP/NT-proBNP Mean at endpoint	Exercise capacity Change from baseline	Exercise capacity Mean at endpoint	NYHA Change from baseline	NYHA Mean at endpoint	Quality of life Change from baseline	Quality of life Mean at endpoint	Mortality	MACEs	SAEs
			-1.25 [-14.06, 11.56]	0.66 [-1.15, 2.48]	0.11 [-0.61, 0.82]	-	-	-	-	-1.30 [-1.75, -0.85]	-1.06 [-1.43, -0.70]	-	-15.00 [-26.18, -3.82]	-	-	1.47 [0.69, 3.14]
BM-derived	<12 months		36[1541]	27[1244]	23[803]	4[193]	7[304]	10[478]	12[589]	11[449]	16[581]	[9]297	4[246]	13[610]	5[298]	7[369]
			<0.00001	0.12	0.07	0.16	0.74	0.04	0.02	0.1	0.09	0.98	0.58	0.27	0.41	0.88
			83%	66%	10%	95%	80%	90%	78%	88%	97%	33%	92%	0%	0%	34%
			2.89 [1.61, 4.17]	-0.17 [-0.38, 0.04]	-0.15 [-0.30, 0.01]	-1.03 [-2.47, 0.42]	-0.10 [-0.68, 0.48]	0.66 [0.04, 1.29]	0.48 [0.09, 0.87]	-0.33 [-0.71, 0.06]	-0.38 [-0.83, 0.07]	0.08 [-7.98, 8.14]	-7.22 [-32.83, 18.40]	0.66 [0.31,1.39]	0.84 [0.56,1.27]	0.98 [0.73, 1.31]
	>12 months		24[986]	16[803]	15[559]	2[71]	4[78]	6[232]	8[361]	6[189]	9[306]	8[301]	3[196]	20[1415]	10[514]	6[341]
			0.01	0.0008	0.22	0.29	0.5	0.29	0.35	0.02	0.02	0.54	0.62	0.03	0.09	0.002
			85%	51%	0%	99%	0%	87%	90%	49%	90%	64%	94%	14%	0%	0%
			2.26 [0.50, 4.01]	-0.37 [-0.58, -0.15]	-0.11 [-0.28, 0.07]	-7.90 [-22.63,6.83]	-0.17 [-0.67, 0.33]	0.75 [-0.08, 1.57]	0.85 [0.07, 1.64]	-0.34 [-0.63, -0.05]	-0.58 [-1.08, -0.09]	-3.44 [-14.39,7.51]	-9.20 [-45.39, 7.00]	0.71 [0.53, 0.96]	0.76 [0.55,1.04]	0.69 [0.54, 0.88]
AT derived	<12 months		1[27]	1[27]	0[0]	0[0]	0[0]	0[0]	0[0]	0[0]	1[25]	0[0]	0[0]	1[31]	1[31]	1[31]
			0.42	0.53	-	-	-	-	-	-	0.88	-	-	0.23	0.41	0.52
			-	-	-	-	-	-	-	-	-	-	-	-	-	-
			0.73 [-1.04, 2.50]	-0.35 [-1.08, 0.39]	-	-	-	-		-	-0.04 [-0.58, 0.50]	-	-	5.83 [0.33, 104.22]	1.65 [0.50,5.42]	0.82 [0.45, 1.49]
	>12 months		0[0]	0[0]	0[0]	0[0]	0[0]	0[0]	0[0]	0[0]	1[24]	0[0]	0[0]	1[27]	1[27]	0[0]
			-	-	-	-	-	-	-	-	0.71	-	-	0.28	0.16	-
			-	-	-	-	-			-	-	-	-	-	-	-
			-	-	-	-	-	-		-	-0.11 [-0.69, 0.47]	-	-	0.43 [0.09, 2.00]	0.29 [0.05,1.62]	-
Perinatal	<12 months		2[78]	1[28]	2[78]	0[0]	2[78]	0[0]	1[50]	1[28]	1[28]	0[0]	1[28]	1[59]	0[0]	0[0]
			0.0005	0.36	0.03	-	0.2	-	<0.00001	0.18	0.52	-	0.98	0.09	-	-
			67%	-	55%	-	96%	-	-	-	-	-	-	-	-	-
			6.48 [2.81, 10.15]	-0.35 [-1.08, 0.39]	-0.82 [-1.54, -0.10]	-	-1.86 [-4.74, 1.01]	-	1.90 [1.22, 2.58]	-0.26 [-0.64, 0.12]	0.14 [-0.29, 0.57]	-	0.21 [-16.27, 16.69]	0.28 [0.06, 1.22]	-	-
	>12 months		1[28]	1[28]	1[28]	0[0]	1[28]	0[0]	0[0]	1[28]	1[28]	0[0]	1[28]	1[30]	1[30]	0[0]
			0.02	0.92	0.1	-	0.07	-	-	0.04	0.9	-	0.64	1	0.19	-
			-	-	-	-	-	-	-	-	-	-	-	-	-	-
			5.22 [0.84, 9.60]	0.04 [-0.70, 0.78]	-0.64 [-1.40, 0.12]	-	-0.71 [-1.48, 0.06]	-	-	-0.37 [-0.71, -0.03]	-0.03 [-0.50, 0.44]	-	4.14 [-13.43, 21.71]	1.00 [0.07, 14.55]	0.25 [0.03,1.98]	-

Summary results of meta-analyses were performed in RevMan v5.3.

Table 5. Summary of results of subgroup analyses in BM-derived stem cells by clinical and methodological differences.

number of comparisons[participants] p value heterogeneity, I2 effect size[95%CI]		LVEF	LV reverse remodeling Change from baseline	LV reverse remodeling Mean at endpoint	BNP/NT-proBNP Change from baseline	BNP/NT-proBNP Mean at endpoint	exercise capacity Change from baseline	exercise capacity Mean at endpoint	NYHA Change from baseline	NYHA Mean at endpoint	quality of life Change from baseline	quality of life Mean at endpoint	mortality	MACEs	SAEs
BMMNCs	<12 months	20[832]	13[588]	14[546]	3[140]	3[192]	7[370]	6[341]	6[316]	11[416]	5[212]	4[246]	7[362]	3[188]	3[183]
		<0.00001	0.64	0.06	0.48	0.3	0.27	0.07	0.007	0.15	0.28	0.58	0.28	0.66	0.22
		61%	72%	21%	94%	88%	87%	88%	65%	97%	25%	92%	0%	21%	56%
		3.33 [2.06, 4.61]	-0.08 [-0.42, 0.26]	-0.20 [-0.40, 0.01]	-0.58 [-2.19, 1.03]	-0.49 [-1.40, 0.42]	0.34 [-0.26, 0.95]	0.64 [-0.04, 1.33]	-0.38 [-0.65, -0.10]	-0.37 [-0.87, 0.13]	-5.31 [-14.94, 4.31]	-7.22 [-32.83, 18.40]	0.62 [0.27, 1.46]	0.87 [0.45, 1.66]	0.58 [0.24, 1.39]
	≥12 months	14[539]	8[400]	11[489]	1[26]	1[26]	4[162]	4[224]	3[132]	7[283]	4[220]	3[196]	13[668]	6[240]	3[89]
		0.02	0.006	0.17	0.23	0.36	0.26	0.13	<0.00001	0.07	0.16	0.62	0.27	0.66	0.45
		81%	14%	0%	-	-	50%	95%	21%	93%	70%	94%	20%	0%	0%
		2.68 [0.39, 4.96]	-0.31 [-0.53, -0.09]	-0.13 [-0.31, 0.06]	-0.48 [-1.26, 0.31]	-0.36 [-1.14, 0.41]	0.28 [-0.21, 0.78]	1.16 [-0.33, 2.65]	-0.58 [-0.83, -0.33]	-0.52 [-1.07, 0.04]	-13.50 [-32.50, 5.49]	-9.20 [-45.39, 27.00]	0.77 [0.49, 1.22]	1.14 [0.63, 2.06]	0.77 [0.38, 1.53]
CD34+ cells	<12 months	2[65]	2[65]	1[20]	1[53]	0[0]	1[45]	0[0]	1[20]	1[20]	0[0]	0[0]	1[20]	0[0]	0[0]
		<0.00001	0.06	0.12	<0.00001	-	<0.00001	-	<0.00001	<0.00001	-	-	-	-	-
		82%	-	83%	-	-	-	-	-	-	-	-	-	-	-
		8.25 [4.78, 11.71]	-1.54 [-3.18, 0.09]	-0.72 [-1.63, 0.19]	-2.42 [-3.13, -1.70]	-	3.45 [2.50, 4.40]	-	-2.10 [-2.59, -1.61]	-2.00 [-2.66, -1.34]	-	-	-	-	-
	≥12 months	1[45]	1[45]	0[0]	1[45]	0[0]	1[45]	0[0]	0[0]	0[0]	0[0]	0[0]	1[110]	0[0]	1[110]
		<0.00001	<0.0001	-	<0.00001	-	<0.00001	-	-	-	-	-	0.02	-	0.02
		-	-	-	-	-	-	-	-	-	-	-	-	-	-
		6.51 [5.13, 7.89]	-1.49 [-2.16, -0.82]	-	-15.51 [-18.91, -12.10]	-	2.76 [1.92, 3.60]	-	-	-	-	-	0.42 [0.20, 0.88]	-	0.42 [0.20, 0.88]
CD133+ cells	<12 months	3[136]	2[106]	3[136]	0[0]	1[54]	0[0]	1[42]	1[54]	1[54]	0[0]	0[0]	1[77]	2[110]	3[166]
		0.46	0.19	0.71	-	0.48	-	0.85	0.04	0.004	-	-	-	0.96	0.21
		78%	0%	0%	-	-	-	-	-	-	-	-	-	-	0%
		-1.66 [-6.11, 2.79]	0.25 [-0.13, 0.64]	0.06 [-0.27, 0.40]	-	0.19 [-0.33, 0.72]	-	0.06 [-0.56, 0.68]	0.48 [0.02, 0.94]	0.54 [0.17, 0.91]	-	-	-	1.08 [0.07, 16.67]	1.31 [0.86, 1.99]
	≥12 months	0[0]	0[0]	0[0]	0[0]	0[0]	0[0]	0[0]	0[0]	0[0]	0[0]	0[0]	2[93]	0[0]	0[0]
		-	-	-	-	-	-	-	-	-	-	-	0.61	-	-
		-	-	-	-	-	-	-	-	-	-	-	0%		-
		-	-	-	-	-	-	-	-	-	-	-	0.63 [0.10, 3.81]	-	-
ALDH+ cells	<12 months	1[20]	1[20]	1[30]	0[0]	0[0]	0[0]	0[0]	0[0]	1[20]	0[0]	0[0]	1[20]	0[0]	1[20]
		0.54	0.47	0.86	-	-	-	-	-	0.28	-	-	-	-	1
		-	-	-	-	-	-	-	-		-	-	-	-	-
		-2.00 [-8.45, 4.45]	-0.32 [-1.21, 0.56]	-0.07 [-0.83, 0.69]	-	-	-	-	-	0.20 [-0.16, 0.56]	-	-	-	-	1.00 [0.83, 1.20]
	≥12 months	0[0]	0[0]	0[0]	0[0]	0[0]	0[0]	0[0]	0[0]	0[0]	0[0]	0[0]	0[0]	0[0]	0[0]
		-	-	-	-	-	-	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 5. Summary of results of subgroup analyses in BM-derived stem cells by clinical and methodological differences.

number of comparisons[participants] p value heterogeneity, I2 effect size[95%CI]		LVEF	LV reverse remodeling Change from baseline	LV reverse remodeling Mean at endpoint	BNP/NT-proBNP Change from baseline	BNP/NT-proBNP Mean at endpoint	exercise capacity Change from baseline	exercise capacity Mean at endpoint	NYHA Change from baseline	NYHA Mean at endpoint	quality of life Change from baseline	quality of life Mean at endpoint	mortality	MACEs	SAEs
BMMSCs	<12 months	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		2[94]	1[55]	1[25]	0[0]	0[0]	1[27]	1[55]	0[0]	2[89]	1[28]	0[0]	2[96]	0[0]	0[0]
		<0.00001	0.26	0.03	-	-	0.67	0.76	-	0.1	0.56	-	0.73	-	-
		0%	-	-	-	-	-	-	-	56%	-	-	21%	-	-
		5.88 [4.35, 7.41]	0.32 [-0.24, 0.89]	-0.94 [-1.80, -0.07]	-	-	0.17 [-0.63, 0.97]	0.09 [-0.48, 0.65]	-	-0.47 [-1.03, 0.09]	-7.00 [-30.76, 16.76]	-	1.48 [0.16, 14.12]	-	-
	≥12 months	2[56]	0[0]	1[31]	0[0]	0[0]	1[25]	0[0]	0[0]	1[31]	1[25]	0[0]	2[65]	2[63]	1[30]
		0.59	-	0.05	-	-	0.41	-	-	0.14	0.4	-	0.36	0.58	0.81
		94%	-	-	-	-	-	-	-	-	-	-	-	0%	-
		2.16 [-5.77, 10.10]	-	-0.72 [-1.45, 0.01]	-	-	0.35 [-0.47, 1.17]	-	-	-0.80 [-1.86, 0.26]	-6.70 [-22.29, 8.89]	-	0.25 [0.01, 4.81]	0.67 [0.16, 2.73]	1.16 [0.36, 3.73]
cardiopoietic cells	<12 months	4[263]	4[255]	0[0]	0[0]	0[0]	1[36]	0[0]	0[0]	0[0]	0[0]	0[0]	1[36]	0[0]	1[290]
		0.45	<0.0001	-	-	-	0.01	-	-	-	-	-	0.38	-	0.0002
		94%	0%	-	-	-	-	-	-	-	-	-	-	-	-
		1.52 [-2.44, 5.48]	-0.52 [-0.77, -0.27]	-	-	-	0.92 [0.22, 1.62]	-	-	-	-	-	0.36 [0.04, 3.59]	-	3.94 [1.91, 8.13]
	≥12 months	3[220]	3[219]	0[0]	0[0]	0[0]	0[0]	0[0]	0[0]	1[7]	0[0]	0[0]	1[271]	0[0]	0[0]
		0.95	0.0006	-	-	-	-	-	-	0.03	-	-	0.73	-	-
		70%	9%	-	-	-	-	-	-	-	-	-	-	-	-
		-0.10 [-2.99, 2.79]	-0.51 [-0.79, -0.22]	-	-	-	-	-	-	-0.92 [-1.74, -0.10]	-	-	1.12 [0.60, 2.10]	-	-
STRO-3$^+$ MPCs	<12 months	3[57]	3[55]	3[55]	0[0]	3[58]	0[0]	3[57]	3[59]	0[0]	3[57]	0[0]	0[0]	0[0]	0[0]
		0.19	0.38	0.58	-	0.31	-	0.09	0.66	-	0.04	-	-	-	-
		0%	31%	0%	-	0%	-	0%	0%	-	0%	-	-	-	-
		2.64 [-1.33, 6.62]	-0.35 [-1.14, 0.44]	0.18 [-0.46, 0.83]	-	0.35 [-0.40, 1.10]	-	0.54 [-0.09, 1.17]	0.09 [-0.30, 0.47]	-	12.46 [0.49, 24.43]	-	-	-	-
	≥12 months	3[56]	3[54]	3[54]	0[0]	3[52]	0[0]	3[55]	3[57]	0[0]	3[56]	0[0]	1[60]	1[60]	0[0]
		0.53	0.64	0.25	-	0.9	-	0.04	0.79	-	0.09	-	0.09	0.38	-
		0%	46%	0%	-	0%	-	0%	0%	-	0%	-	-	-	-
		1.70 [-3.56, 6.96]	0.21 [-0.66, 1.08]	0.38 [-0.26, 1.01]	-	-0.04 [-0.69, 0.61]	-	0.67 [0.03，1.31]	-0.05 [-0.42, 0.32]	-	8.63 [-1.25, 18.50]	-	0.33 [0.09, 1.17]	0.67 [0.27, 1.64]	-
lxmyelocel-T	<12 months	1[92]	1[92]	0[0]	0[0]	0[0]	0[0]	1[94]	0[0]	0[0]	0[0]	0[0]	0[0]	0[0]	0[0]
		0.27	0.67	-	-	-	-	0.36	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-	-	-	-	-	-	-
		1.39 [-1.08, 3.86]	-0.09 [-0.50, 0.32]	-	-	-	-	0.19 [-0.22, 0.60]	-	-	-	-	-	-	-
	≥12 months	1[85]	1[85]	0[0]	0[0]	0[0]	0[0]	1[82]	0[0]	0[0]	0[0]	0[0]	2[168]	2[168]	1[112]
		0.66	0.68	-	-	-	-	0.48	-	-	-	-	0.42	0.04	0.02

Table 5. Summary of results of subgroup analyses in BM-derived stem cells by clinical and methodological differences.

number of comparisons[participants] p value heterogeneity, I2 effect size[95%CI]		LVEF	LV reverse remodeling Change from baseline	LV reverse remodeling Mean at endpoint	BNP/NT-proBNP Change from baseline	BNP/NT-proBNP Mean at endpoint	exercise capacity Change from baseline	exercise capacity Mean at endpoint	NYHA Change from baseline	NYHA Mean at endpoint	quality of life Change from baseline	quality of life Mean at endpoint	mortality	MACEs	SAEs
		-	-	-	-	-	-	-	-	-	-	-	0%	0%	-
		0.70 [-2.41, 3.81]	-0.09 [-0.52, 0.34]	-	-	-	-	0.16 [-0.28，0.59]	-	-	-	-	0.72 [0.33, 1.60]	0.64 [0.42, 0.98]	0.70 [0.53, 0.93]
ischemic heart failure	<12 months	24[1114]	18[908]	14[501]	1[50]	4[244]	5[225]	7[413]	7[366]	12[478]	4[119]	5[235]	12[550]	5[295]	6[570]
		0.003	0.92	0.04	0.51	0.56	0.2	0.01	0.04	0.12	0.08	0.2	0.31	0.28	0.79
		87%	58%	6%	-	89%	52%	83%	91%	98%	0%	94%	0%	32%	82%
		2.54 [0.89, 4.19]	-0.01 [-0.23, 0.21]	-0.20 [-0.39, -0.01]	0.19 [-0.38, 0.76]	-0.25 [-1.09, 0.59]	0.27 [-0.14, 0.68]	0.63 [0.13, 1.14]	-0.51 [-1.00, -0.01]	-0.41 [-0.92, 0.10]	-9.69 [-20.55, 1.17]	-10.36 [-26.29, 5.58]	0.68 [0.31, 1.45]	0.70 [0.36, 1.35]	1.08 [0.62, 1.90]
	≥12 months	13[601]	8[491]	5[272]	0[0]	1[21]	2[52]	3[194]	2[109]	5[176]	3[131]	3[137]	12[879]	7[350]	6[290]
		0.22	<0.0001	0.14	-	0.48	0.4	0.08	0.0004	0.19	0.08	0.22	0.02	0.54	0.03
		89%	0%	0%	-	-	0%	96%	49%	94%	45%	97%	0%	11%	0%
		1.54 [-0.91, 3.99]	-0.43 [-0.61, -0.25]	-0.21 [-0.48, 0.07]	-	0.39 [-0.70, 1.49]	0.25 [-0.32, 0.81]	1.53 [-0.18, 3.24]	-0.58 [-0.91, -0.26]	-0.46 [-1.15, 0.23]	-13.27 [-28.21, 1.68]	-15.45 [-40.06, 9.16]	0.63 [0.43, 0.92]	0.86 [0.53, 1.39]	0.76 [0.59, 0.97]
non-ischemic heart failure	<12 months	9[370]	5[256]	6[230]	3[143]	2[60]	5[253]	2[119]	1[24]	4[103]	3[149]	2[121]	1[60]	1[29]	0[0]
		<0.0001	0.09	0.62	0.09	0.78	0.09	0.33	0.76	0.34	0.98	0.05	1	0.38	-
		62%	83%	29%	94%	0%	95%	0%	-	61%	48%	0%	-	-	-
		4.03 [2.20, 5.85]	-0.57 [-1.22, 0.09]	-0.09 [-0.43, 0.26]	-1.44 [-3.11, 0.24]	0.08 [-0.48, 0.64]	1.13 [-0.18, 2.43]	-0.18 [-0.54, 0.18]	-0.10 [-0.74, 0.54]	-0.24 [-0.74, 0.26]	-0.13 [-8.53, 8.28]	12.29 [-0.12, 24.71]	1.00 [0.07, 15.26]	1.63 [0.55, 4.87]	-
	≥12 months	8[329]	5[258]	7[288]	2[71]	1[26]	4[180]	2[112]	1[23]	5[179]	3[138]	2[114]	7[447]	2[78]	2[140]
		0.0005	0.13	0.39	0.29	0.36	0.12	0.92	0.41	0.007	0.64	0.55	0.68	0.51	0.14
		63%	76%	0%	99%	-	92%	26%	-	0%	76%	55%	34%	0%	26%
		3.92 [1.72, 6.12]	-0.42 [-0.96, 0.12]	-0.10 [-0.34, 0.14]	-7.90 [-22.63, 6.83]	-0.36 [-1.14, 0.41]	1.01 [-0.26, 2.28]	-0.02 [-0.51, 0.46]	-0.33 [-1.11, 0.45]	-0.37 [-0.63, -0.10]	-3.99 [-20.93, 12.96]	6.57 [-15.11, 28.26]	0.89 [0.52, 1.54]	0.70 [0.25, 2.02]	0.55 [0.25, 1.23]
both	<12 months	3[57]	3[55]	0[0]	0[0]	3[58]	0[0]	3[57]	3[59]	0[0]	3[57]	0[0]	1[59]	0[0]	0[0]
		0.19	0.38	-	-	0.29	-	0.09	0.66	-	0.03	-	0.82	-	-
		0%	31%	-	-	0%	-	0%	0%	-	0%	-	-	-	-
		2.64 [-1.33, 6.62]	-0.35 [-1.14, 0.44]	-	-	0.33 [-0.28, 0.93]	-	0.55 [-0.08, 1.18]	0.09 [-0.30, 0.47]	-	12.96 [0.94, 24.98]	-	0.85 [0.23, 3.22]	-	-
	≥12 months	3[56]	3[54]	5[114]	0[0]	3[52]	0[0]	3[55]	3[57]	0[0]	3[56]	0[0]	2[90]	1[60]	0[0]
		0.53	0.64	0.62	-	0.9	-	0.03	0.79	-	0.07	-	0.93	0.38	-
		0%	46%	0%	-	0%	-	0%	0%	-	0%	-	0%	-	-
		1.70 [-3.56, 6.96]	0.21 [-0.66, 1.08]	0.11 [-0.34, 0.56]	-	-0.04 [-0.69, 0.61]	-	0.69 [0.05, 1.33]	-0.05 [-0.42, 0.32]	-	9.23 [-0.61, 19.06]	-	1.05 [0.35, 3.15]	0.67 [0.27, 1.64]	-
autologous	<12 months	32[1445]	23[1153]	20[741]	4[193]	4[246]	9[442]	8[496]	8[390]	16[581]	6[232]	7[356]	13[633]	6[324]	6[570]
		<0.0001	0.23	0.05	0.16	0.41	0.07	0.07	0.05	0.09	0.51	0.55	0.46	0.51	0.79
		84%	69%	13%	95%	88%	91%	86%	90%	97%	23%	93%	0%	35%	82%
		2.75 [1.39, 4.10]	-0.14 [-0.36, 0.09]	-0.16 [-0.33, 0.00]	-1.03 [-2.47, 0.42]	-0.32 [-1.10, 0.45]	0.64 [-0.05, 1.32]	0.47 [-0.04, 0.97]	-0.46 [-0.91, -0.01]	-0.38 [-0.83, 0.07]	-1.92 [-7.58, 3.74]	-4.53 [-19.23, 10.17]	0.78 [0.40, 1.51]	0.82 [0.45, 1.48]	1.08 [0.62, 1.90]

Table 5. Summary of results of subgroup analyses in BM-derived stem cells by clinical and methodological differences.

number of comparisons[participants] p value heterogeneity, I2 effect size[95%CI]		LVEF	LV reverse remodeling Change from baseline	LV reverse remodeling Mean at endpoint	BNP/NT-proBNP Change from baseline	BNP/NT-proBNP Mean at endpoint	exercise capacity Change from baseline	exercise capacity Mean at endpoint	NYHA Change from baseline	NYHA Mean at endpoint	quality of life Change from baseline	quality of life Mean at endpoint	mortality	MACEs	SAEs
		21[930]	13[749]	14[536]	2[71]	1[26]	6[232]	5[306]	3[132]	9[326]	6[269]	5[251]	18[1296]	9[428]	8[430]
	≥12 months	0.01	0.0001	0.11	0.29	0.36	0.08	0.09	<0.00001	0.11	0.14	0.46	0.06	0.23	0.006
		87%	50%	0%	99%	-	87%	94%	21%	92%	70%	95%	17%	0%	0%
		2.30 [0.46, 4.14]	-0.42 [-0.64, -0.21]	-0.15 [-0.33, 0.03]	-7.90 [-22.63, 6.83]	-0.36 [-1.14, 0.41]	0.75 [-0.08, 1.57]	0.95 [-0.14, 2.03]	-0.58 [-0.83, -0.33]	-0.44 [-0.97, 0.10]	-8.14 [-18.87, 2.58]	-7.55 [-27.76, 12.66]	0.73 [0.53, 1.02]	0.79 [0.54, 1.16]	0.72 [0.58, 0.91]
allogeneic		3[57]	3[55]	3[55]	0[0]	3[58]	0[0]	3[57]	3[59]	0[0]	3[57]	0[0]	0[0]	0[0]	0[0]
	<12 months	0.19	0.38	0.58	-	0.31	-	0.09	0.66	-	0.03	-	-	-	-
		0%	31%	0%	-	0%	-	0%	0%	-	0%	-	-	-	-
		2.64 [-1.33, 6.62]	-0.35 [-1.14, 0.44]	0.18 [-0.46, 0.83]	-	0.31 [-0.29, 0.92]	-	-0.55 [-1.18, 0.08]	0.09 [-0.30, 0.47]	-	12.96 [0.94, 24.98]	-	-	-	-
		3[57]	3[54]	3[54]	0[0]	3[52]	0[0]	3[55]	3[57]	0[0]	3[56]	0[0]	1[60]	1[60]	0[0]
	≥12 months	0.53	0.64	0.25	-	0.9	-	0.03	0.79	-	0.07	-	0.09	0.38	-
		0%	46%	0%	-	0%	-	0%	0%	-	0%	-	-	-	-
		1.70 [-3.56, 6.96]	0.21 [-0.66, 1.08]	0.38 [-0.26, 1.01]	-	-0.04 [-0.69, 0.61]	-	0.69 [0.05, 1.33]	-0.05 [-0.42, 0.32]	-	9.23 [-0.61, 19.06]	-	0.33 [0.09, 1.17]	0.67 [0.27, 1.64]	-
revascularization		11[345]	7[192]	7[219]	0[0]	1[54]	1[57]	3[129]	2[71]	4[138]	0[0]	0[0]	7[249]	2[91]	3[157]
	<12 months	0.06	0.38	0.13	-	0.48	0.11	0.05	0.53	0.18	-	-	0.86	0.32	0.34
		89%	76%	31%	-	-	-	70%	98%	94%	-	-	0%	0%	76%
		3.39 [-0.19, 6.97]	-0.29 [-0.94, 0.36]	-0.27 [-0.61, 0.08]	-	0.19 [-0.34, 0.73]	0.43 [-0.10, 0.96]	0.67 [-0.01, 1.34]	-0.81 [-3.34, 1.72]	-0.60 [-1.49, 0.28]	-	-	1.14 [0.28, 4.73]	2.69 [0.38, 19.05]	0.37 [0.05, 2.88]
		3[89]	1[50]	1[50]	0[0]	0[0]	0[0]	1[30]	0[0]	1[30]	0[0]	0[0]	5[213]	3[123]	2[89]
	≥12 months	0.18	0.03	0.18	-	-	-	<0.0001	-	0.0001	-	-	0.47	0.38	0.66
		92%	-	-	-	-	-	-	-	-	-	-	0%	0%	0%
		5.83 [-2.66, 14.33]	-0.63 [-1.20, -0.06]	-0.38 [-0.94, 0.18]	-	-	-	1.76 [0.90, 2.62]	-	-0.73 [-1.10, -0.36]	-	-	0.70 [0.27, 1.82]	1.55 [0.66, 3.65]	1.18 [0.56, 2.49]
no revascularization		26[1203]	21[897]	16[581]	4[193]	8[308]	9[421]	9[460]	9[375]	12[443]	10[325]	7[356]	9[482]	4[233]	4[432]
	<12 months	<0.0001	0.13	0.32	0.16	0.86	0.06	0.1	0.05	0.24	0.98	0.55	0.25	0.32	0.62
		79%	63%	0%	95%	77%	91%	81%	65%	97%	24%	93%	0%	42%	91%
		2.70 [1.42, 3.97]	-0.17 [-0.38, 0.05]	-0.09 [-0.26, 0.09]	-1.03 [-2.47, 0.42]	-0.05 [-0.61, 0.51]	0.70 [-0.02, 1.41]	0.41 [-0.07, 0.89]	-0.25 [-0.51, 0.00]	-0.31 [-0.83, 0.21]	0.05 [-5.21, 5.32]	-4.53 [-19.23, 10.17]	0.68 [0.35, 1.31]	0.73 [0.40, 1.35]	1.23 [0.50, 3.02]
		21[897]	15[753]	14[509]	2[71]	6[149]	6[232]	7[331]	6[189]	9[325]	9[325]	5[251]	16[1173]	7[365]	7[361]
	≥12 months	0.05	0.003	0.39	0.29	0.83	0.08	0.09	0.02	0.19	0.56	0.46	0.04	0.04	0.005
		84%	53%	0%	99%	99%	87%%	90%	49%	93%	61%	95%	29%	0%	0%
		1.74 [-0.03, 3.51]	-0.35 [-0.58, -0.12]	0.08 [-0.26, 0.10]	-7.90 [-22.63, 6.83]	-0.04 [-0.38, 0.31]	0.75 [-0.08, 1.57]	0.73 [-0.11, 1.57]	-0.34 [-0.63, -0.05]	-0.38 [-0.96, 0.19]	-2.28 [-9.87, 5.31]	-7.55 [-27.76, 12.66]	0.68 [0.48, 0.98]	0.67 [0.46, 0.99]	0.72 [0.58, 0.91]
		2[45]	2[45]	2[25]	0[0]	0[0]	0[0]	0[0]	0[0]	0[1]	0[0]	0[0]	2[84]	0[0]	1[61]
<1 Mio	<12 months	0.35	0.22	0.18	-	-	-	-	-	-	-	-	0.72	-	0.02
		0%	25%	0%	-	-	-	-	-	-	-	-	0%	-	-

Table 5. Summary of results of subgroup analyses in BM-derived stem cells by clinical and methodological differences.

number of comparisons[participants] p value heterogeneity, I2 effect size[95%CI]		LVEF	LV reverse remodeling Change from baseline	LV reverse remodeling Mean at endpoint	BNP/NT-proBNP Change from baseline	BNP/NT-proBNP Mean at endpoint	exercise capacity Change from baseline	exercise capacity Mean at endpoint	NYHA Change from baseline	NYHA Mean at endpoint	quality of life Change from baseline	quality of life Mean at endpoint	mortality	MACEs	SAEs
		1.68 [-1.84, 5.20]	-0.45 [-1.18, 0.27]	-0.62 [-1.52, 0.28]	-	-	-	-	-	-	-	-	0.71 [0.11, 4.55]	-	0.09 [0.01, 0.72]
	≥12 months	0[0]	0[0]	0[0]	0[0]	0[0]	0[0]	0[0]	0[0]	0[0]	0[0]	0[0]	0[0]	0[0]	0[0]
		-	-	-	-	-	-	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-	-	-	-	-	-	-
1-10 Mio	<12 months	18[878]	13[687]	13[587]	2[80]	4[246]	6[319]	7[441]	6[346]	12[47]	5[204]	6[301]	7[365]	4[284]	4[219]
		0.02	0.48	0.91	0.3	0.41	0.84	0.07	0.18	0.57	0.54	0.39	0.15	0.29	0.96
		79%	27%	0%	0%	88%	37%	87%	83%	98%	35%	93%	0%	0%	0%
		2.31 [0.60, 4.01]	0.07 [-0.12, 0.25]	-0.06 [-0.24, 0.11]	0.23 [-0.21, 0.68]	-0.32 [-1.10, 0.45]	0.03 [-0.26, 0.33]	0.52 [-0.05, 1.10]	-0.26 [-0.63, 0.11]	-0.22 [-0.75, 0.31]	-2.15 [-9.03, 4.73]	-6.71 [-22.12, 8.70]	0.71 [0.32, 1.57]	0.79 [0.52, 1.21]	1.00 [0.85, 1.18]
	≥12 months	11[513]	9[485]	10[460]	1[26]	1[26]	4[162]	5[306]	3[132]	7[280]	5[244]	5[251]	13[805]	7[346]	4[230]
		0.01	0.01	0.21	0.23	0.36	0.26	0.09	<0.00001	0.05	0.19	0.53	0.08	0.07	0.02
		84%	10%	0%	-	-	50%	94%	21%	94%	75%	95%	20%	17%	0%
		3.31 [0.78, 5.85]	-0.24 [-0.44, -0.05]	-0.12 [-0.31, 0.07]	-0.48 [-1.26, 0.31]	-0.36 [-1.14, 0.41]	0.28 [-0.21, 0.78]	0.95 [-0.14, 2.03]	-0.58 [-0.83, -0.33]	-0.54 [-1.10, 0.01]	-9.07 [-22.50, 4.35]	-6.76 [-27.95, 14.44]	0.76 [0.55, 1.04]	0.71 [0.49, 1.03]	0.74 [0.57, 0.96]
10-100 Mio	<12 months	9[279]	6[189]	6[137]	1[60]	2[44]	1[60]	3[97]	3[64]	3[108]	2[42]	1[55]	4[153]	1[14]	0[0]
		<0.0001	0.38	0.06	<0.00001	0.38	<0.00001	0.16	0.44	0.006	0.01	0.21	0.6	0.17	-
		72%	87%	21%	-	0%	-	0%	96%	85%	0%	-	0%	-	-
		4.46 [2.11, 6.81]	-0.42 [-1.34, 0.51]	-0.24 [-0.61, 0.13]	-2.20 [-2.85, -1.55]	0.29 [-0.35, 0.92]	2.27 [1.61, 2.93]	0.31 [-0.13, 0.74]	-0.62 [-2.18, 0.94]	-0.96 [-2.02, 0.10]	16.19 [3.58, 28.80]	7.92 [-4.40, 20.24]	0.72 [0.11, 4.83]	7.00 [0.43, 114.70]	-
	≥12 months	6[153]	2[40]	5[129]	0[0]	2[40]	0[0]	2[41]	2[42]	2[29]	2[41]	0[0]	6[170]	5[155]	2[60]
		0.34	0.12	0.65	-	0.7	-	0.02	0.97	0.94	0.03	-	0.34	0.73	1
		4%	22%	30%	-	43%	-	0%	0%	0%	0%	-	0%	0%	0%
		1.07 [-1.12, 3.26]	0.61 [-0.16, 1.39]	0.11 [-0.36, 0.58]	-	-0.18 [-1.09, 0.73]	-	0.79 [0.11, 1.48]	-0.01 [-0.40, 0.38]	0.04 [-0.90, 0.97]	12.04 [1.38, 22.69]	-	0.68 [0.31, 1.49]	0.90 [0.50, 1.62]	1.00 [0.34, 2.91]
>100 Mio	<12 months	7[370]	6[335]	2[61]	1[53]	1[28]	3[108]	2[64]	1[29]	1[34]	3[92]	0[0]	0[0]	0[0]	1[290]
		0.05	0.02	0.12	<0.00001	0.18	0.1	0.14	1	0.01	0.94	-	-	-	0.0002
		92%	54%	48%	-	-	93%	0%	-	-	0%	-	-	-	-
		2.89 [-0.04, 5.82]	-0.42 [-0.75, -0.08]	-0.58 [-1.31, 0.14]	-2.41 [-3.13, -1.69]	0.51 [-0.24, 1.27]	1.49 [-0.27, 3.26]	0.38 [-0.12, 0.88]	0.00 [-0.46, 0.46]	-0.80 [-1.41, -0.19]	0.41 [-9.90, 10.73]	-	-	-	3.94 [1.91, 8.13]
	≥12 months	7[349]	5[291]	2[59]	1[45]	1[24]	2[70]	1[27]	1[29]	1[34]	2[53]	0[0]	6[513]	3[93]	2[140]
		0.42	0.0003	0.2	<0.00001	0.45	0.2	0.23	0.6	0.11	0.87	-	<0.05	0.7	0.35
		93%	57%	33%	-	-	94%	-	-	-	0%	-	12%	0%	52%
		1.43 [-2.04, 4.91]	-0.69 [-1.07, -0.31]	-0.42 [-1.05, 0.22]	-15.51[-18.91, -12.10]	0.31 [-0.49, 1.12]	1.55 [-0.81, 3.91]	0.47 [-0.30, 1.23]	-0.13 [-0.61, 0.35]	-0.80 [-1.79, 0.19]	-0.85 [-10.76, 9.05]	-	0.65 [0.42, 0.99]	0.85 [0.37, 1.94]	0.63 [0.24, 1.66]

Summary results of subgroups meta-analysis by clinical and methodological differences performed in RevMan v5.3

Discussion

Bias assessments

A crossover clinical study is a prospective study where patients are assigned to a crossed sequence of treatments and hence is considered a valid and important type of trial design[157]. Each patient serves as their own control which can minimize confounding covariates. However, its noted disadvantage involves confounding treatment effects caused by carry-over effects. Stem cells injected after transplantation that successfully engraft in the target areas have been proven to survive for long durations and function in their specific “intended” ways. Therefore, the carry-over effects of these stem cells can last as long as they exist. The methods of delivering stem cells into the heart are commonly invasive. This means a high-quality crossover trial designed as a double blinded RCT is nearly impossible, because in such trials each patient has to receive thoracotomy and invasive cell transplantation twice, or else blinding is easily violated. During retrieval and inclusion, three crossover trials were excluded. One was a single-arm crossover trial, and one was still ongoing (or completed but no published yet). Another cross-over trial was reported, and in that trial[158] the ischemic tolerant mesenchymal stem cells (itMSC) were given to patients in the treatment group, and crossover was performed only after three months. It was therefore excluded due to too short follow-up and likely carry-over effects.

Among 42 enrolled RCTs for meta-analysis, 13 trials did not perform well in blinding, and two did not report details. Most of them were open-label trials using autologous stem cells that originated from bone marrow or skeletal muscles. In cases of invasive stem cell harvest, but with a possible future sham injection, patients were less willing to participate in randomized control trials. In CAuSMIC[125] and SEISMIC[126] trials, muscle biopsies were only performed in patients in the treatment group, and control patients would only receive medical therapy. The same open-label design was also seen in autologous BM-derived stem cell transplantation. For example, in Patel 2005[142] investigators performed off-pump coronary artery bypass grafting in patients from treatment and control groups, but the patients in the treatment group would tolerate bone marrow aspiration. In Zhao 2015[68], only patients in the cell therapy group were treated with allogeneic UCMSCs through catheter-based intracoronary delivery, but patients in the control group only received conventional medical treatments. In these 15 trials (either did not perform well in blinding or did not report in details), risk of performance bias existed, since investigators would

devote more attention to them, causing the difference in medical care levels. Besides that, the possibility of a conflict of interest leading to false-positive results was highly possible. Investigators who were aware of group assignments tended to modulate medical treatments to get positive outcomes. In unblinded trials, if blocked randomization was used, especially with a fixed size, the bias would happen due to a predictable future. Therefore, in sensitivity analyses, trials with high risks of performance bias were isolated to reduce the heterogeneity and verify the robustness of the results.

Attrition bias happens in a situation of incomplete outcome data. Less than 5% of loss during follow-up could be accepted, but more than 20% attrition can severely threaten validity[159]. In the *MiHeart trial*[137], 45 patients out of a total of 160 were lost during follow-up, causing a high risk of attrition bias. Twenty-one patients were lost in the treatment group (n=82) and 24 lost in the control group. The details were not discussed in that paper, but common reasons for abandonment in heart failure might be a severe illness, or dissatisfaction with poor efficacy. In their primary outcome as LVEF change from baseline, no significant difference was shown between the two groups. If we assume the worst-case scenario, which is that lost patients in the treatment group all had increased LVEF, and all lost patients in the control group had decreased LVEF or vice versa, the outcomes could changed from statistically insignificant to significant or vice versa.

Patients who felt dissatisfied with their treatments during clinical trials would declare withdrawal of consent in order to obtain better medical services, which would cause an excessive lack of negative results. To avoid this situation, investigators designed a single crossover from controls to the experimental group. For example, in FOCUS-HF[63] and FOCUS-Br[145] trials, patients who were randomized to control groups would be given the experimental treatments after a certain period of follow-up. Transendocardial injections were applied in these studies, which is practicable to be performed in trial-crossed control patients twice, thereby keeping blindness and avoid post-operative trauma at the same time.

Another type of incomplete data happened in trial *Ang 2008*[127] and *Steinhoff 2017*[150] when patients were still reachable but rejected participation in imaging assessment of cardiac functions. In *Ang 2008*[127], 60 patients were randomized and treated, but at the follow-ups, only 33 patients participated in MRI assessments. Thus, the validity of primary outcomes was threatened by their rejection.

Three trials did not report all the outcomes mentioned in their methods. In these trials, outcomes with insignificant results were mentioned, but the details of the data were missing, which caused a risk of reporting bias in meta-analysis.

Clinical outcomes and overall effects

In stem cell trials, cardiac functional improvement generally manifests as an increase of LVEF, which is mostly set as the primary outcome for evaluating the effectiveness and efficacy of stem cells in cardiac repair. The increase in LVEF should not be attributed to a single beneficial function from stem cells but be viewed as a comprehensive result due to different effects, including anti-apoptosis, immunomodulation, cardiomyogenesis, and angiogenesis. Therefore, it is considered as one of the most suitable clinical indicators of whether stem cells could truly function well in cardiac repair. For example, myoblasts were once thought to be quite promising in heart regeneration for their myogenic property and tolerance to ischemic environments but failed to show any improvement in LVEF in RCTs. Conversely, BM-derived cells that mainly reported with specific paracrine effects demonstrated an increase of over 2%.

In the overall effect of stem cells in LVEF, a mean 3.2% increase over the control group was obtained in this meta-analysis; however, responders to stem cell therapies in clinical tirals are commonly defined by an increase over 5%[160,161]. Data from a pilot study[160] showed that 48% of patients responding well to heart failure therapy had LVEF increases of ≥ 5% and 57% had 6MWD > 50 meters, and a strong association between the clinical relevant increase in LVEF and the improvement in 6MW-test was also revealed. Moreover, responders also had a better outcome with respect to all-cause mortality and hospitalization. Therefore, the increase in LVEF at 5% was set as a predictor in the prognosis of stem cell therapy for heart failure[162].

Meta-analyses of bone marrow-derived mesenchymal stem cells in heart repair reported improvements of LVEF in all animal models. Nevertheless, different efficacies of BMMSCs exist among different animal models[79]. In small animals, BMMSCs were demonstrated to increase LVEF by ≈12% over controls. In large animal models, LVEF improvement kept at ≈12% in chronic ischemic cardiomyopathy but dropped to ≈7% in the models of acute myocardial infarction. A similar discrepancy in clinical efficacy between small and large animals was also seen with cardiac stem cells[163]. Cardiac stem cells were associated with an 11.51% increase of LVEF in small animal models, but a 5.22% increase in large ones. The pooled effect of all stem cell types on LVEF was ≈7%

in preclinical trials[71,83]; however, it was proven to be around 2-4% in clinical trials[164–167]. For patients with heart failure, a 2.06% LVEF increase was found after less than 1 year and only 1.26% over 1 year[114], which is consistent with the results from our meta-analysis where we found an overall effect of a 2.88% increase in LVEF with stem cells in less than 12 months and 2.33% at the 12-month or longer follow-up. The disparity between a 5-13% increase in animals and nearly 2-4% in patients represents a dissatisfying transition from bench to bedside in stem cell therapy for heart failure. Tentative explanations for different aspects will be described below.

Patients with stable heart failure included in clinical trials were several months after MI, or had chronic dilated cardiomyopathy with diverse etiologies. Successive loss of cardiomyocytes and the progression of ventricular dilation in heart failure, as mentioned in the background, is different from an active inflammatory reaction to massive cell death after MI. Animal models that were studied for cardiac repair were commonly conducted in two ways, with coronary artery permanent ligation and ischemia-reperfusion cycles, which could not be used to simulate the microenvironments of chronic decompensating hearts.

The efficacy gap between animal experiments and clinical trials could also be attributed to inequivalent injected cell doses. The cell doses injected into small animals like mice and rats were mostly applied in a range of 0.1-10 million, while in large animals with similar heart size as human beings, cell doses were used from 10 to >100 million, which was relatively lower than those used in small animals[79,163]. If we assume the same percentage of cardiomyocytes loss after MI, and the same survival rate of stem cells after transplantation, in addition to a similar heart-to-body mass ratio in mammals in mice and swine[168], the equivalent dose of 0.1 million stem cells into mice (≈20g) would be over 1 trillion into swine (≈200kg). At least 300 - 400 million stem cells would be needed if the investigators wanted to successfully repeat the positive results in adult patients (60-80kg). Obviously, stem cell injection at these doses was hard to be performed in clinics.

Moreover, assessments of cardiac functions in animal experiments were usually performed less than 12 weeks after injection, while the measuring time in clinical trials was mostly at 6 months. However, improvement in LVEF due to stem cells began to fade as early as 9 weeks after injection[83]. In clinical trials, patients receiving bone marrow cells had a 5.58% increase in LVEF at 4 months after transplantation, but only 3.76% at 6 months[169].

Influence of cell cultivation

An extra explanation for applying random effect models in meta-analysis is needed here: Since clinical trials differ reasonably in regard to treatment regimens, clinical heterogeneity caused by patient selection, trial methodologies, or duration of follow-up, etc., and they also differ in statistical heterogeneity. Simple, direct conclusions drawn from the overall results would be too imprecise and less convincing for validating the effectiveness of stem cells in cardiac repair[170]. In case of high heterogeneities, random effect analyses were applied, and subgroup analyses in terms of cell cultivation and cell origins, etc. were executed in order to better understand the clinical relevance from the results.

In 2000, the first stem cells used in clinical trials for HF treatment were myoblasts[171]. Although the application of cultured stem cells began earlier than primary cells, primary cells were preferred in clinical use for their convenient isolation and short preparation time. But over years of development, cultured cells gradually became more adopted, due to higher injected cell density after cell cultivation. However, the maintenance of stem cell quality during passaging became a severe challenge[172]. CD34 biomarkers lost during subsequent cultivation[173,174]. Properties of stem cells were modulated by medium components like basal medium and medium serum[175,176]. In practice, FBS was reported to cause cessation of cell cycling, suppression of proliferation-related and cell cycle-related genes, and other types of genes such as growth factors and metabolism-related genes. Combined with different growth factors, FBS could increase MSC proliferation but also negatively affect their immune-phenotypes[177]. Human-derived serum products like pooled human platelet lysate and autologous or allogeneic human serum seemed to be the best candidates for medium serum but were identified to compromise their immuno-suppressive properties of MSCs, and were suspicious of changing gene expression by undefined proteins[178–180]. Thus, the alterations of large amounts of gene expressions in *in-vitro* expanded stem cells compared to purified uncultured stromal stem cells[181] and remarkably impaired stemness and pluripotency indicate that cultured cells might not be ideal for cell therapies[182].

Influence of cell origins

No increase in LVEF was discovered with myoblasts. No other improvement, except an increase in NYHA functional status at long-term follow-up, was achieved. As pointed out in the Introduction, the effectiveness of myoblasts in heart repair was expected

through transdifferentiating into cardiomyocyte-like cells; however, retention and survival rates were measured as quite low. To date, 17 clinical studies are on record, 16 have been published and only one that began in 2007 is still ongoing or already suspended. 5 out of 16 studies were RCTs that began between 2002 and 2007, and the latest myoblast study was initiated in 2010. It is not surprising that the negative results from the completed RCTs and the evidence of inducing ventricular arrhythmias impeded further clinical trials of myoblasts. In contrast, positive results from bone marrow-derived cells raised more interest in widespread applications.

Perinatal stem cells increased LVEF after short- and long-term follow-ups; the mean effect sizes in LVEF increase were both over the 5% line. As described in the introduction, perinatal stem cells (UCMSCs in **Figure 1**) are more advantageous in immune evasion, paracrine secretion, and survivability under unfavorable conditions. Therefore, the superiority of perinatal stem cells for heart recovery from ischemic injury is suggested in this meta-analysis, and further experiments are needed.

Ongoing trials

Although the Egger's test has proven no publication bias in the overall primary outcomes at both short- and long-term follow-ups, the effectiveness and efficacy of some specific cell types should be carefully affirmed due to limited published trials. For instance, umbilical cord mesenchymal stem cells were shown to cause the highest increase in LVEF and strongly induce LV remodeling reversion; however, only two trials were included in this meta-analysis. Hence, the results of ongoing UCMSC trials are highly anticipated so that more data will be pooled and analyzed to see whether they are consistent with published ones. Furthermore, two trials of allogeneic ATMSCs are awaiting completion. Among ongoing trials of BM-derived cells, 4 are studying BMMSCs, 2 are studying BM-derived mesenchymal precursor cells, and 1 is studying $CD34^+$ cells.

Limitation

This analysis is a literature-based meta-analysis with 42 included RCTs. Efforts have been made to obtain missing but necessary data. For these missing data, which were still unavailable after data requests, a calculation using formulas, based on the Cochrane Guide and other publications, was employed to maximize dataset. Meanwhile, we are aware of the associated disadvantage, whereas the calculated data could impact the robustness of the results.

Insufficient data represents very weak evidence to be used for drawing any conclusions about specific cells. As discussed in the section of ongoing trials, more RCTs are expected to be added in the future.

Conclusion

Despite decades of research in stem cell therapy for heart failure treatment, clincal science is still at any early stage of finding the ideal cell type that provides optimal cardiac functional improvements and reduced mortality. Benefits from stem cell therapy should be clinically significant; the benefits should also maintain stable after a long-term follow-up. From this meta-analysis of 42 RCTs, we learned that stem cells could reduce all-cause mortality in HF patients, but their improvement in LVEF showed no clinical significance. Perinatal stem cells might have the potential to be efficient in HF treatment, but definitive confirmation still requires more RCTs. Inappropriate trial design and execution causing high risks of selection and performance biases could produce false-positive results that significantly impact the robustness of the results and should be avoided in RCTs.

Part II. Application of human sera in the cultivation of cord blood-derived mesenchymal stromal cells to increase their resistance to oxidative stress

Introduction

In the introduction of Part I, we described the relevant properties of MSCs in clinical trials that focosed on cardiac repair. In recent years, the application of MSCs for cardiac repair has been questioned as a result of unsatisfactory pooled clinical outcomes[183]. Their cardiomyogenic capacity has been observed in vitro but no *in vivo* data has supported this claim [23,24], while their angiogenic properties have been confirmed by many studies[184,185]. MSCs transdifferentiate into endothelial cells and also secrete beneficial growth factors and microRNAs. It is clear that MSCs are regenerative cells and are also worthy of clinical application; however, evidence as differentiation into cardiomyocytes is missing. In contrast, their anti-apoptotic and immunomodulative capabilities have been well studied.

Cell rescue from ischemia-reperfusion injury

Under oxidative stress, especially severe ischemia-reperfusion (I/R) injury, reactive oxygen species formation (ROS) and calcium overload are the two main pathological consequences that lead to mitochondrial damage and ultimately to cell death[186]. Autophagy is an intrinsic self-rescuing mechanism in cells that is activated by the same stimuli and helps cells survive in adverse conditions. Exosomes from MSCs were reported to prevent cardiomyocyte deaths by inducing autophagy potentially via the activation of AMPK and Akt pathways[187]. Similarly, co-culturing with MSCs can restore mitochondrial respiratory function and ATP production in hibernating myocardium[188]. The proliferator-activated receptor gamma coactivator 1-alpha-driven mitochondrial biogenesis was also observed to increase after co-cultivation. Since the mitochondrial transfer was discovered during co-cultivation, another explanation for the mechanism of their anti-apoptotic function was introduced. There are several schematic patterns of mitochondrial transfer, with tunneling nanotubes being the most well studied in the cardiovascular field[189]. The transfer was demonstrated via stained donor MSCs mitochondria that were seen inside recipient myoblasts with TNT-like structures connecting each other. TNT formation inhibitor restrained the anti-apoptotic potential in the co-culture system[190]. Animal cardiomyocytes were later reported to receive mitochondria from MSCs of different origins, including BMMSCs, ADMSCs, dental pulp MSCs and Wharton's jelly MSCs (WJMSCs), and WJMSCs performed best in reducing mtROS in cardiomyocytes[191].

Immunomodulation to prevent overactive inflammation after ischemia-reperfusion injury

The rupture of cell membrane releases pro-inflammatory substances and leads to an unbalanced inflammatory reaction that plays a critical role in sustainable cell loss and tissue damage after I/R injury[186]. Mesenchymal stem cells regulate immune cells in regard to multiple aspects. They not only promote macrophage and dendritic cell polarization toward an anti-inflammatory phenotype but also suppress both T and NK cell proliferation[192]. Additionally, they are known to promote the induction of regulatory B-cells, which leads to immunological tolerance. Counterintuitively, apoptotic MSCs were reported to be even more effective than living MSCs in tissue protection against inflammation, oxidative stress, and apoptosis[193,194]. This “dying stem cell hypothesis” could be traced back to 2005[195] in which apoptotic MSCs were proposed to suppress immunoactivity in local regions. Further studies suggested that this might be due to the release of intracellular membrane particles[196]. MSCs were damaged in 4°C cold water, and the separated released membrane particles were found to negatively regulate the activity of pro-inflammatory monocytes. Even more, when MSCs were stimulated by IFN-γ that is only secreted by both activated T and NK cells, the isolated membrane particles could additionally cause an increase in the percentage of anti-inflammatory monocytes. In a publication from the same research group, heat-inactivated MSCs, which were not capable of secreting extracellular particles or actively participating in cross-talk with immune cells, were still immunomodulatory, probably through the cognition of membrane biomarkers by immune cells[197].

MSCs transplantation for post-operative complications

Post-operative complications after cardiac surgery, such as stroke, renal failure, and prolonged intubation, are recognized to be the major contributors to operative death[198]. The invention of the cardiopulmonary bypass (CPB) created the opportunity for the development of cardiac surgeries. However, in many retrospective studies the prolonged CPB running time and combined heart procedures, such as CABG combined with valvular surgeries, have been shown to be independent factors of early stroke, acute renal failure, delayed extubation, and post-operative low cardiac output syndrome (LCOS)[199–203]. Hemolysis, hemodilution, and unstable hemodynamics during CPB would cause local inflammation, hypoxemia, and cell loss in multiple organs. Thus, considering MSCs

properties of anti-apoptosis and immunomodulation, it is reasonable to investigate whether they are effective or efficient in preventing incidence of severe post-operative complications after a long, complicated cardiac surgery.

In stroke patients, MSCs were demonstrated to attenuate those pathophysiological processes, showing that the lesion volume was reduced by nearly 20%[204]. Despite insignificant outcomes from several animal experiments to restore kidney function[205,206], preventative post-operative applications of MSCs are still of great interest (undergoing clinical trials: registered trials of NCT04194671 & NCT03552848 in clinicaltrials.gov). Similarly, MSCs were proven to attenuate pulmonary vascular permeability and ameliorate acute lung injury in animal models[207,208], yet no specific data from clinical trials in this area has been published.

Cardiac injury after long-term CPB often manifests as cardiac stunning, post-operative low cardiac output syndrome, and even cardiogenic shock. An increasing release of cardiac biomarkers was detected after long-term CPB[209]. Mitochondrial dysfunction, microvascular endothelial dysfunction, and unrestricted inflammation are thought to be the main pathological changes in I/R injury[210]. The application of MSCs could lower the cellular stress response to I/R injury. In a swine model of CABG surgery, mitochondrial morphology and function were improved by MSC transplantation[211]. MSC transplantation, combined with hyperbaric oxygenation, was demonstrated to cause significant recovery in tissue oxygenation two weeks after ischemia simulation[212]. The oxygenation of cardiac tissue was assessed by a new technique termed electron paramagnetic resonance (EPR), which has been utilized in several studies to evaluate mitochondrial function preservation via different therapies after ischemic cardiac injury[213]. Although MSC-induced anti-apoptotic and anti-inflammatory effects on injured cardiomyocytes have been explored abundantly in animal models, MSC clinical trials for post-operative cardiac complications are still scarce. No record of any registered clinical trials of stem cells for post-operative cardiac complications was shown in clinicaltrials.gov, and there are also no relevant data from trials studying other cardiac diseases. Most relevant trials were registered for ischemic heart disease and cardiomyopathy, and the outcomes are mainly mortality, SAEs, LVEF, LVEDV, NYHA, and others instead of angiogenesis or immune-relevant clinical indicators.

Arrhythmia is the most common post-operative complication indicating cardiac injury, a prognosis with a higher risk in morbidity, increased length of hospital stay, and an increase in economic costs[214]. Unstable hemodynamics could also aggravate other

comorbidities; abnormal perfusion aggravates post-operative cerebral dysfunction, renal insufficiency, and other complications. It is still controversial whether MSCs could be applied to treat arrhythmias. Animal experiment studies showed MSCs to be pro-arrhythmic, but others also found that they could ameliorate the inducibility of ventricular arrhythmias by enhancing electrical viability[214–216]. Results from any relevant clinical trials are not yet available either.

Application of perinatal stem cells

Perinatal mesenchymal stem cells seem to be promising for prevention of post-operative complications after cardiac surgery. Compared to other sources of MSCs, as discussed in the introduction of Part I, perinatal stem cells are more proliferative, less senescent, and have higher immunomodulative properties. Age-related damage such as DNA-damage and telomere dysfunction are not present in perinatal MSCs[217–219]. In contrast to aging MSCs, for example, aging autologous MSCs from old patients, MSCs from younger donors are more resistant to ROS and can preserve more therapeutic potency under unfavorable conditions during transplantation[220]. Therefore, perinatal MSCs could be a good alternative to cells that have been mostly applied in clinical trials, such as autologous BMMSCs.

Compared to other perinatal stem cells, such as WJMSCs and UCMSCs, CBMSCs are more easily isolated in the lab. Many public and private cord blood banks exist worldwide, which is a huge advantage over the other cell types. Nevertheless, the disadvantage is also clear in which a low yield of primary cells has been seen. Only a very limited amount of cord blood can be obtained at the time of birth, and the primary plastic adherent cell yield is often not satisfactory. Thus, long-term *in vitro* expansion is needed to reach the minimum injection dose.

Issues during preparation for transplantation

The choice of media serum becomes an issue and needs much deliberation. In **Figure 8**, we summarized the application of different medium sera from 42 clinical trials that used the cultured stem cells for heart failure treatment. Thirty trials used FBS in their expansion medium. Human serum was only applied in a total of eight clinical trials, of which five were autologous heart failure serum, two were allogeneic platelet lysate, and another one was allogeneic donated serum. The application of FBS introduces xenogeneic substances to human stem cells during *in vitro* cultivation and results in

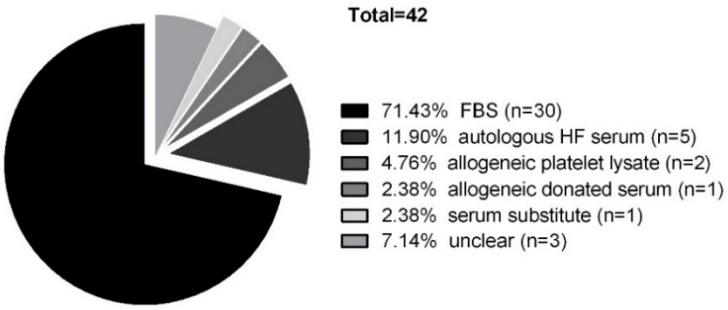


Figure 8. Applications of different medium sera during *in vitro* expansion.
42 trials were identified using cultured stem cells for heart failure treatment.

inferior proliferation profiles and a wide range of gene down-regulation[175]. Autologous HF serum was also found to impact the clonality and proliferation profiles of CB-MSCs[221]. Therefore in Part II, different proliferation profiles among FBS, healthy control serum, and heart failure serum will be compared.

On the other side, a critical factor for successful stem cell engraftment is cell survival in the target area. We noticed that the procedure of cell transplantation, for instance, syringe production, transportation, and final injection, would bring massive stress, especially oxidative stress to the transplanted cells. Thus, in this study we investigated the responses of CB-MSCs in different medium sera to the programmed ischemia-reperfusion injury simulating the conditions during cell transplantation. Hypoxic preconditioning was also given to those cells to test if this additional method could be used to increase their survivability.

Hypotheses

The *in vitro* research on CB-MSCs was based on the following hypotheses:

1. The proliferation capacity of CB-MSCs would be more preserved when they are cultured with human serum.
2. The resistance of CB-MSCs against ischemia-reperfusion injury, which mimics the situation during clinical transplantation, is better under human serum cultivation.
3. Hypoxic preconditioning could enhance stem cell resistance against oxidative stress.

Methods

Donor recruitment

In accordance with the Declaration of Helsinki, this study was approved by the ethics committee of Charité-University Medicine Berlin. Potential patient donors were those who had been confirmed heart failure with reduced ejection fraction (HFrEF) and had received maximal medical treatments during hospitalization. Those who had reduced LVEF (<40%) confirmed by echocardiography, with class III or IV heart failure functional status (NYHA III or IV), were further enrolled. Meanwhile, patients who had malignant tumors, infectious disease, and diabetes and were excluded. In parallel, older healthy volunteers without a history of myocardial infarction (MI) or any sign of cardiovascular diseases (CVDs) were also enrolled and assigned to the control serum group. Each donor was informed of the scientific application of their serum and every participant signed the informed consent form.

Serum extraction

Blood was drawn using a BD Vacutainer Safety-Lok blood collection set. Whole blood samples were left undisturbed at room temperature for 30 minutes, and then centrifuged at 3500 g for 15 mins at 4°C to remove the clotting, and all remaining cellular particles. Serum supernatants were then sterilely filtered, aliquoted, flash-frozen in liquid nitrogen and then stored at -80°C for later use. Samples with hemolytic serum present were excluded. In order to minimize systematic errors, serum concentrations were normalized to the lowest concentration within our samples by dilution with DPBS containing Ca^{2+} and Mg^{2+}. The total protein concentration of each sample was quantified in all human serums using the Pierce BCA Protein Assay Kit. Details of experimental materials were listed in **Table 6**.

In vitro cultivation of human CB-MSCs

Human cord blood mesenchymal stromal cells were made available by courtesy of Dr. K. Bieback, who isolated them from umbilical cord blood, and expanded them based on an optimized, previously published protocol[222]. Prior to experimentation, CB-MSCs were expanded in 1g/L glucose DMEM with 10% FBS under antibiotic protection with 1% Streptomycin/Penicillin. Cells were seeded at 800-1,000 cells/cm² in T175 flasks, and cultured under 21% O_2, 5% CO_2, and at 37°C. Partial media changes were performed every 3 days. All cells cultured were screened for the presence of mycoplasma enzymes

on a regular basis. All experiments were performed on cells between passages 4 and 6. The phenotypes of the used CB-MSCs, as well as their ability to differentiate into nonhematopoietic cell types, were repeatedly confirmed in previous experiments by our group[221].

In vitro models for acute stress on CB-MSCs: acute Stress (AcS)

For a model of acute stress, we chose an *in vitro* model of "ischemia-reperfusion injury", where cells are deprived of glucose, serum, and oxygen (0.2% O_2) for 4 hours, and then transferred back into normal culture conditions. All oxygen deprivation studies were performed in a hypoxic chamber. Cells were first seeded at 8-10×10^3/cm² and cultivated with human serum and 1g/L glucose for 24 hours at normal cultivation. Then, cells were exposed to hypoxia (0.2% O_2), as well as serum and glucose deprivation for 4 hours, followed by 4 hours of modeled reperfusion under normal cultivation settings. To assess their recovery, another 5 days of cultivation under the respective medium at a normal condition was continued after programmed acute stress. Hypoxia (0.2% O_2) was also given to the cells with standard FBS-containing medium, before exploring the protection of hypoxic preconditioning against AcS.

Metabolic activity, cell counts and proliferation assays

A 20% MTS Tetrazolium and PMS solution was added to the cells and incubated for 4 hours at 37°C. Absorbance (OD) was measured at 490 nm and 650nm as a reference wavelength. Subsequently, nuclei were stained with Hoechst 33342 in the dark for 20 mins at room temperature and washed with PBS. Cell counts were operated with a high content screener using the Hoechst 33342 channel (excitation/emission: 380/445 nm). The cell survival rate is depicted as the percentage of cells counted after I/R, compared to cells plated. BrdU incorporation was used to quantify cell proliferation. Cells cultured in 96-well plates were first incubated with BrdU labeling solution at 37°C for 4 hours and finished the remaining steps according to the manual. No stop solution was used at the end of the assay, so absorbance was measured in the ELISA reader at 370 nm with the reference wavelength set at 492 nm.

Apoptosis detections assays

In these experiments, two different assays to quantify apoptosis were performed. Fluorochrome-labeled inhibitors of caspase assays were used to detect caspase activity in CB-MSCs. The poly-caspase probe (SR-VAD-FMK) recognizes all different types of

activated poly-caspases. The cells were incubated with the poly-caspase probe for 45 minutes at 37°C, with gentle agitation every 10 minutes. Then, nuclear counterstain with Hoechst 33342 was performed. Cells were then scanned with the high content screener using the Alexa 568 (excitation/emission: 570/615 nm) and Hoechst 33342 channels. Data were analyzed using the Columbus software.

Additionally, a differentiated detection for the late and early stages of apoptosis was performed by Annexin V and Ethidium Homodimer III staining. CB-MSCs were harvested after I/R and stained according to the provided protocol. Cells under normal cultivation were used as the negative control, cells treated with 24 hours of 200uM H_2O_2 as Annexin V positive control, and cells incubated on ice after 10 minutes of 65°C warm water bath as Ethidium homodimer III positive control. Fluorescence was measured using a MacsQuant VYB and analyzed by FlowJo 10.

Real-time RT-PCR

Cells were harvested after the programmed procedures and stored at -20°C. Total RNA was isolated using the NucleoSpin RNA isolation kit. The purity and integrity of isolated RNA were assessed by spectrophotometry and gel electrophoresis. RNA samples from biological replicates were pooled, before cDNA synthesis to account for biological variation[223]. cDNA was synthesized by reverse transcription using the qScript SuperMix. Expression of 96 gene associated apoptosis and cell cycle pathways were tested using qPCR array kits from Realtimeprimers.com, using their recommended amplification protocol.

ΔCT=CT(genes of interest)-CT(housekeeping genes HPRT1)

For apoptosis-related gene regulations:

$\Delta\Delta CT = \Delta CT_{\text{after AcS}} - \Delta CT_{\text{before AcS}}$

For cell-related gene regulations:

$\Delta\Delta CT_{\text{compared to before AcS}} = \Delta CT_{\text{3d after AcS}} - \Delta CT_{\text{before AcS}}$

$\Delta\Delta CT_{\text{compared to FBS}} = \Delta CT_{\text{human sera}} - \Delta CT_{\text{FBS}}$

Fold gene expression=$2^{-\Delta\Delta CT}$

Statistics

Data were obtained from results comparing groups of at least 3 separate biological samples. With a normal distribution, data are shown as mean ± SD. Levene test was performed to test the homogeneity of variances. One-way ANOVA was then executed to

analyze differences between groups. If the assumption of equal population variance was accepted, Tukey's post hoc analysis was carried out between equal amounts of samples, and Games-Howell tests among inequal numbers of samples. If the null assumption was rejected, Welch ANOVA and Games-Howell post hoc tests were performed. In the case of skewed distribution, data are shown as medium [25%, 75% percentile]. Kruskal-Wallis tests were then performed. Differences were considered statistically significant at $P<0.05$. Statistical analyses were performed using the IBM SPSS Statistics software Version 22.

Table 6. Project experimental materials

	Company	**Country**
Instruments & Tools		
Microscopy	Carl Zeiss MicroImaging GmbH	Göttingen, Germany
Balance, new classic MF	Mettler Toledo	Columbus, Ohio, USA
Eppendorf pipettes	Eppendorf AG	Hamburg, Germany
Centrifuge	Beckman Coulter GmbH	Krefeld, Germany
Cell culture flow hood; SAFE 2020	Thermo Fisher Scientific Corporation	Waltham, Massachusetts, USA
Freezing container; Mr. Frosty	Thermo Fisher Scientific Corporation	Waltham, Massachusetts, USA
Incubator	Binder GmbH	Tuttlingen, Germany
Hypoxic incubator	Binder GmbH	Tuttlingen, Germany
Microplate reader: SpectraMax	Molecular Devices, LLC	San Jose, California, USA
High content screen: Operetta®	PerkinElmer, Inc	Waltham, Massachusetts, USA
Spectrophotometer: Nanodrop™ 2000/2000c	Thermo Fisher Scientific	Wilmington, Delaware USA
Real time PCR machine: QuantStudio 6 Flex	Applied Biosystems	Foster City, California, USA
Flow cytometry: MACSQuant® VYB	Miltenyi Biotec GmbH	Bergisch Gladbach, Germany
Vacutainer Safety-Lok blood collection set	BD Medical	Hamburg, Germany
Reagents & Kits		
DMEM Glutamax+, Gibco, 1g/L glucose; no glucose	Life Technologies Corporation	Carlsbad, California, USA
DMSO	AppliChem GmbH	Darmstadt, Germany
Gelatin	Life Technologies Corporation	Carlsbad, California, USA

Table 6. Project experimental materials

	Company	Country
DPBS, invitrogen: -/-; +/+	Thermo Fisher Scientific Corporation	Carlsbad, California, USA
Trypan blue solution 0.4%	Sigma-Aldrich	St. Louis, Missouri, USA
Trypleexpress Enzyme, Gibco, phenol red	Life Technologies Corporation	Carlsbad, California, USA
Penicillin/streptomycin, Gibco	Life Technologies Corporation	Carlsbad, California, USA
FBS/FCS, Gibco	Life Technologies Corporation	Carlsbad, California, USA
Paraformaldehyde	Carl Roth GmbH&Co.Kg	Karlsruhe, Germany
Ethanol 100%	Carl Roth GmbH&Co.Kg	Karlsruhe, Germany
RNase/DNase free water, ultrapure™, Invitrogen	Life Technologies Corporation	Carlsbad, California, USA
Agarose molecular grade	Bioline GmbH	Luckenwalde, Germany
Ethidium bromide	Carl Roth GmbH&Co.Kg	Karlsruhe, Germany
RNase Zap	Ambion, Inc	Foster City, California, USA
NAN3	Carl Roth GmbH&Co.Kg	Karlsruhe, Germany
Hoechst 33342, molecular probes™	Thermo Fisher Scientific	Wilmington, Delaware USA
CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay	Promega GmbH	Madison, Wisconsin, USA
Cell proliferation ELISA BrdU, colorimetric	Roche Holding AG	Basel, Switzerland
Apoptosis/necrosis detection kits	PromoCell GmbH	Heidelberg, Germany
Mycoalert kits	Lonza Group	Basel, Switzerland
RNA isolation kits, Nucleospin	MACHEREY-NAGEL GmbH & Co. KG	Düren, Germany
Qscript cDNA Supermix	Quantabio Reagent Technologies	Beverly, Massachusetts, USA
Power sybr green master mix	Applied Biosystems	Foster City, California, USA
Pierce BCA protein assay kit	Thermo Fisher Scientific Corporation	Waltham, MA, USA
Hyperladder 1kb	Bioline GmbH	Luckenwalde, Germany
Apoptosis & cell cycle primer sets	RealTimePrimers.com	Elkins Park, Philadelphia, USA
Consumables		
Cell culture flasks	Greiner Bio-One GmbH	Kremsmünster, Austria

Table 6. Project experimental materials

	Company	**Country**
96-well culture plate, transparent, CELLSTAR	Greiner Bio-One GmbH	Kremsmünster, Austria
Falcon tubes	Corning Inc.	Corning, New York, USA
Eppendorf tubes	Eppendorf AG	Hamburg, Germany
Software		
Columbus software	PerkinElmer, Inc	Waltham, Massachusetts, USA
FlowJo 10	FlowJo LLC	Ashland, OR, USA
Spss statistics® 22	IBM Corporation	Somers, New York, USA
Graphpad prism	GraphPad Software	San Diego, California, USA

Results

Characteristics of serum donors

From June to December 2014, sera were collected from 12 patients and 12 healthy volunteers. As presented in **Table 7**, healthy volunteers were significantly younger, but with a narrower age distribution (54±5.8, 46-61 years old) compared to patient donors (64±11.8, 44-89 years old). 5 out of 12 healthy donors were female, but among patient donors, the ratio of female to male was 1:3. Only eight patients had a history of myocardial infarction, and all 12 were diagnosed with ischemic heart diseases during hospitalization, 2 of whom had two-vessel coronary artery diseases, and the other 10 had three-vessel diseases. In terms of NYHA functional status, five patients were classified to be at level III, and the remaining seven at level IV; additionally, reduced left ventricular ejection fraction was distributed from 15% to 36% on an average of 22 ± 7.1% among the patients. Other characteristics concerning left ventricular end-diastolic diameter (LVEDD) and drug usage are described in **Table 7**. Additionally, no significance for total protein concentrations was found between the two groups. The lowest protein concentration was 46.6 g/L in an HF2 patient. Therefore, the subsequent medium sera were neutralized by this lowest concentration.

Table 7. Demographics and patient characteristics

	Healthy control (n=12)	**HF patients (n=12)**
History		
Age	54±5.8*	64±11.8
Gender [male/female]	7/5	9/3
Smoker	2	2
Ex-smoker	3	10
Previous MI	-	8
Time since most recent MI (years)	-	8±9.6 (0-25)
Extend of CAD		
Two vessels	-	2
Three vessels	-	10
NYHA functional class		
III	-	5
IV	-	7
Left ventricular end diastolic diameter (LVEDD)[mm]	-	65±9.5
Left ventricular ejection fraction (LVEF)[%]	-	22±7.1

Table 7. Demographics and patient characteristics

	Healthy control (n=12)	**HF patients (n=12)**
Non-cardiac disease		
Reynaud syndrome	1	-
Pulmonary hypertension	-	3
Drug usage		
Anticoagulant	-	9
Anti-platelet agent	-	4
Anti-arrhythmic agent	-	7
Cardiac glycoside	-	5
Sympathomimetic drug	-	5
Adrenergic receptors blocker	-	8
ACE inhibitors	-	10
Xanthine oxidase inhibitor	-	1
Acetylsalicylic acid	-	4
Proton-pump inhibitor	-	4
Diuretics	-	11
Hypolipidemics	-	4
Pain reliever	-	6
Insulin	-	3
Vasodilator	-	7
Calcium channel blocker	-	1
H2 receptor antagonist	-	3
Aromatic L-amino acid decarboxylase	-	1
Bronchial spasms reliever	-	1
Nausea and vomiting	-	4
Antibiotics	-	5
PAH treat	-	1
Synthetic thyroid hormone	1	1
Lower uric acid levels	-	1
Schizophrenia	-	1
Protein concentration	59.07±5.42	55.28±4.68

*p=0.013 between the ages of HFS and CS donors

CB-MSCs become more proliferative under human serum cultivation

As shown by previous publications, the overall morphology or immunophenotype of the cells was unaffected by the cultivation of CB-MSCs with human sera from either healthy individuals or HF patients[221]. As expected, human sera resulted in a higher variation in cell accrual than FBS, a standardized, pooled tissue culture supplement

(**Figure 9A**). The BrdU incorporation assay confirmed more active cell division in cells cultured under human sera (**Figure 9B**). Since day 3, the OD value, reflecting BrdU incorporation activity in cells with FBS, remained at only 0.033 [0.016, 0.077], an impressive drop compared to the human sera group. BrdU incorporation at day 5 also dropped in human sera groups compared to day 3. But this phenomenon could be partially explained by their high percentage of confluence on day 5 (cell numbers: 19414 [18703, 20660] in the CS group and 16261 [14924, 22266] in HFS group). In addition, cells from three groups all presented as thin, spindle-shaped, and fibroblast-like (**Figure 9D- F**). Cells with HF sera were at higher levels of metabolic activity than those with

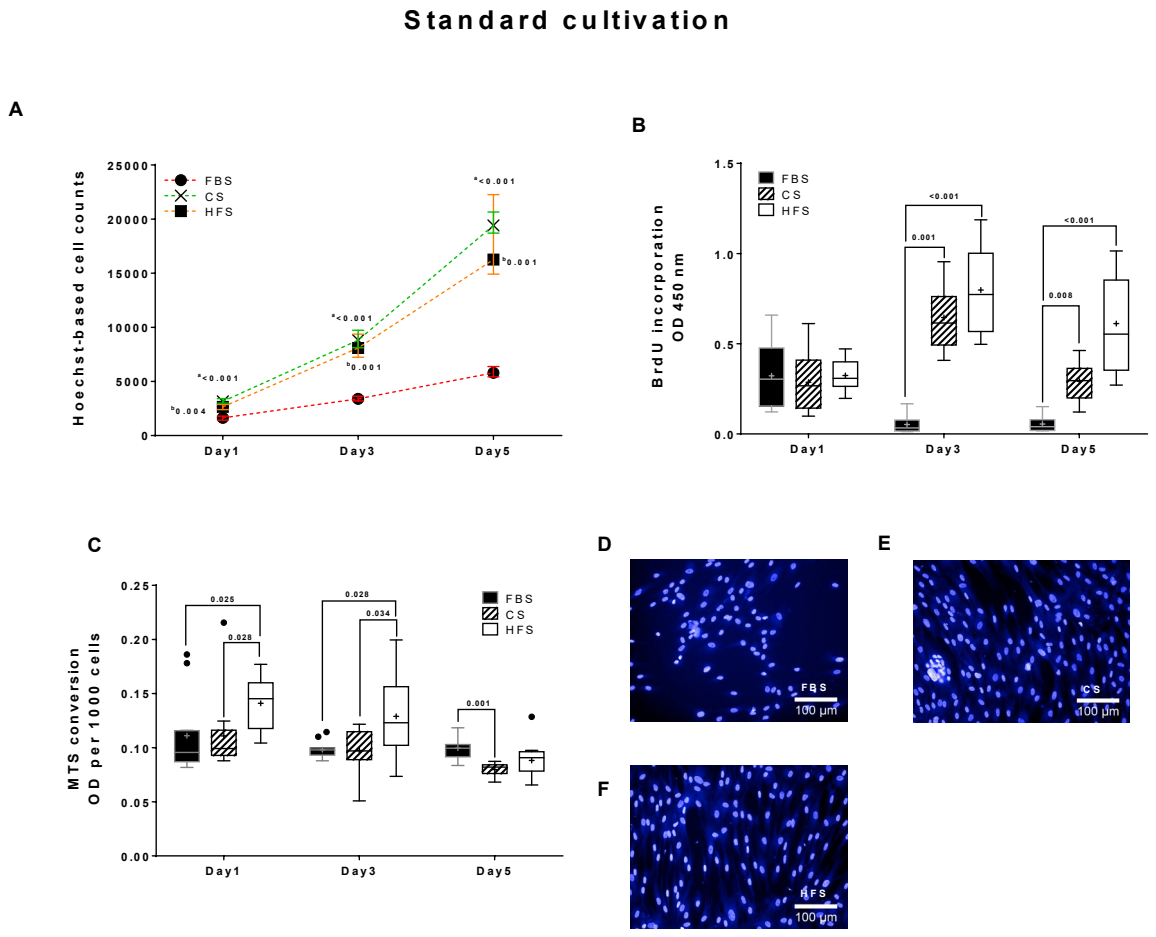


Figure 9. Proliferation and metabolism profiles of CB-MSCs under standard cultivation

Cell accrual (A), DNA synthesis (B), and metabolic activity (C) of CB-MSCs were evaluated with different medium serum under normal conditions (glucose /L, 21% O_2). After 5 days of expansion in two different conditions, cells with three types of medium sera were captured under microscopy (20X) (D, E, F).

In Figure A, data are shown as medium [25%, 75% percentile]. In Figures B and C, box plots are shown with Tukey Whiskers, and black dots are seen as outliers.

[a]p stands for p value between CS and FBS groups. [b]p stands for p value between HFS and FBS groups.

control sera during 5 days. At day 3, CB-MSCs under HFS demonstrated themselves with a significantly more active cellular metabolic rate (0.13 ± 0.03 compared to 0.1 ± 0.02 with CS, p=0.034). These effects were undetectable at day 5 (p=0.201) (**Figure 9C**).

Human serum protects CB-MSCs against acute oxidative stress

Next, we designed a model of acute stress (AcS) imitating an oxidative stressful condition during the process of cell engraftments, which is based on previously published models of ischemia/reperfusion injury[224,225]. In preliminary experiments, we established the combination of 4 hours of hypoxia at 0.2% O_2, and glucose and serum deprivation followed by standard culture conditions that were sufficient for reproducibly induced significant cell loss during AcS. Immediately after acute stress, the salvage of cells cultured with HFS (60.02% ± 13.65%) and CS (65.79% ± 16.03%) was significantly higher than cells treated with FBS (45.08% ± 7.42%) (p=0.035 vs. p=0.006, respectively) (**Figure 10 A**), but there were only mild non-significant differences in cell survival between HFS and CS (p=0.376). Interestingly when we performed Annexin V and EthD-III stainings after AcS, we uncovered that in the HFS group, the presence of apoptotic cells (Annexin V^+) was significantly lower than FBS group (9.02% [8.62%, 11.59%] vs. 13.15% [12.09%, 16.60%], p=0.025), and that of live cells (Annexin V^-/ EthD-III^-) was higher (**Figure 10B**). Comparatively, control sera were shown to protect stem cells against acute stress, additionally brought a faster recovery in cell proliferation (**Figure 10C - 1day after AcS**).

Hypoxic preconditioning increases resistance against AcS of CB-MSCs with FBS

High cell survival is one of the critical factors for successful cell transplantation and the consequent stem cell-induced cardiac repair. In the previous paragraph, we demonstrated the superiority of human serum-cultured cells against oxidative stress during cell transplantation. On the other hand, the disadvantage of human serum application is also clear: limited supply, especially from HF donors. To replace human sera, but maintain the anti-oxidative property, we repeated AcS in all groups of CB-MSCs preconditioned with hypoxia. Consistent with our preliminary data, FBS cultured cells had an increased cell survival after AcS with 81.44% [43.25%, 93.94%] cells left (**Figure 10D**). Through flow cytometry, no significance was found in either dead cells, apoptotic cells, or live cells (**Figure 10E**).

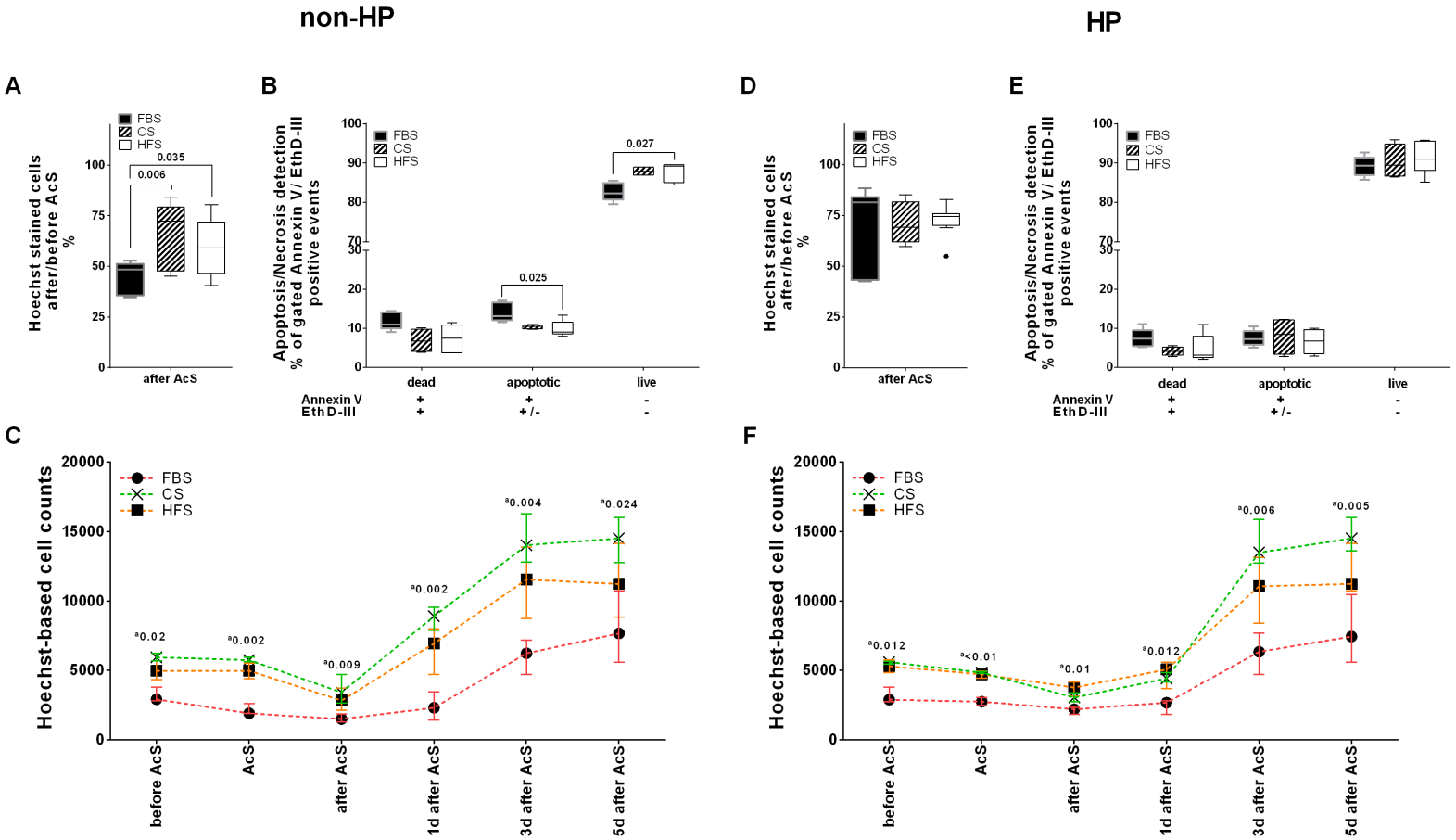


Figure 10. Cell survival profiles of CB-MSCs after AcS

After 4 hours of simulated acute stress without any preconditioning, the numbers of survival CB-MSCs were counted (**A**), and apoptotic, dead, or live cells were stained and assessed through flow cytometry (**B**). Subsequently, the recoveries of cell proliferation were continuously tracked until 5 days after AcS (**C**). Hypoxic preconditioning was also applied to test the benefits of this pretreatment on CB-MSCs against AcS (**D-F**).

In Figures AB and DE, box plots are shown with Tukey Whiskers, and black dots are seen as outliers. In Figure C, F, data were shown as medium [25%, 75% percentile]

[a]p stands for p value between CS and FBS groups.

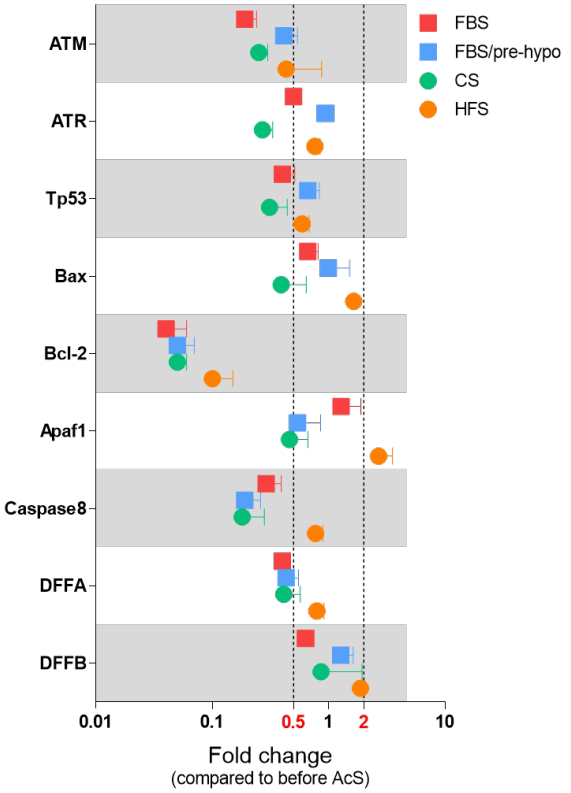


Figure 11. Apoptosis-related gene expression profiles of CB-MSCs under different situations.
Samples were taken after programmed ischemia-reperfusion injury, and their gene expressions were compared to before I/R.

Potential genes involved in cell survival and recovery after AcS

As described previously, human sera in CB-MSCs cultivation presented an anti-oxidative stress property; meanwhile, the gap of cell survival between FBS and human sera was successfully covered by hypoxic preconditioning. To figure out how genes modulated during this process, genes of interest from cells treated with different sera and conditions were measured for intracellular expressions immediately after AcS compared to before. Nine genes were chosen for gene expression analysis based on the key roles of their expressed products during apoptosis. Oxidative stress leading to DNA damage, mitochondrial dysfunction, and activation of the Fas/FADD pathway finally induce cellular apoptosis.

According to **Figure 11**, during activated apoptosis, gene expressions of caspase 8, DFFA, tp53, ATM, and Bcl-2 were downregulated in cells cultured with FBS. A similar pattern of gene modulations was shown in cells under control serum additionally with reduced BAX, ATR, and APAF1 expressions. ATR was additionally suppressed in cells treated with control sera, and the downstream transcription Tp53 was also suppressed, which blocks Bcl-2 and activates BAX so as to modulate the stability of the mitochondrial membrane. APAF1, the critical proteins that combine with cytochrome C, leaked from swallowed and dysfunctional mitochondria and then triggering caspase cascade, were also downregulated, while the expression profiles were different in cells with HF sera. Only ATM and Bcl-2 were downregulated by over 2-fold compared to before AcS.

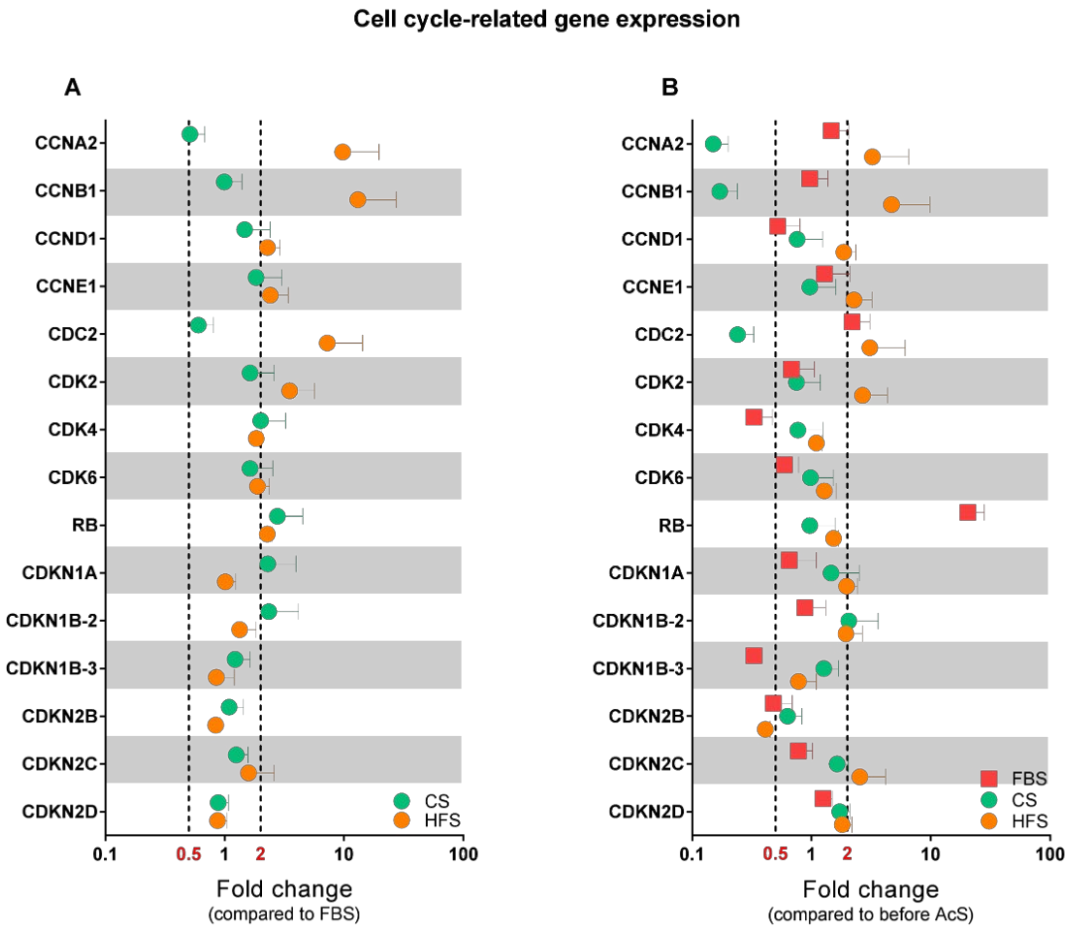


Figure 12. Cell cycle-related gene expression profiles of CB-MSCs under different situations

Samples were taken 3 days after programmed cell injury.

(**A**) Related gene expressions of cells with human sera compared to FBS

(**B**) Related gene expressions of cells with FBS and human sera 3 days after AcS compared to before.

Compared to FBS cultivation, additional hypoxia preconditioning showed unchanged Tp53 and ATR expressions.

Three days after giving AcS, cells cultured with human sera had doubled cell counts compared to FBS as previously shown. Gene expression levels (**Figure 12A-B**) reveal a more active cell cycle-related gene expression in cells with HF sera, and both were compared to FBS and to before AcS. Expression of cyclin encoding genes as CCNA2, CCNB1, CCND1, and CCNE1 in addition to cycle-dependent kinases, CDC2 and CDK2, were up-regulated. Those gene expressions did not increase in cells with control sera compared to FBS and even decreased compared to before AcS. In contrast, we saw an increase in RB, CDKN1A, and CDKN1B-2 expressions in comparison to FBS, which all negatively modulate cell cycles (**Figure 12A**). Limited alterations in gene expression in FBS are shown in **Figure 12B** except for a highly elevated RB level.

Discussion

Impact of aging on cells and the microenvironment

Since regenerative capacity was discovered to be impaired in aged stem cells, concern about the age threshold in the clinical application of stem cells has been raised[226]. As we know, to reach sufficient cell doses for transplantation, harvested primary cells are confronted with replicative stress. After repeated cell division in a short period of time, telomere length would be rapidly shortened[227]. Consequently, damaged DNAs accumulate and eventually undermine intrinsic maintenance[228]. Those aging stem cells with shorter telomere length exhibit weaker proliferative capacity and impaired regenerative functions[229]. It also means that stem cells from old donors have impaired proliferation, and affects potential for cardiac repair, while this is likely to be less of an issue in cells from young donors[63,230].

When cells are successfully engrafted into an old and dysfunctional heart, the aging microenvironment also influences stem cell properties. As the long history of parabiosis and anti-aging research has shown us, aging serum could harm the rejuvenation capacity of progenitor cells, e.g. by prolonging population doubling time, and by increasing the number of senescent cells[231,232]. However, other research discovered that young progenitor cells were more resistant to this negative effect. Young stem cells still maintained more proliferative and juvenile surface markers, while in old cells, they were rapidly lost after overnight culturing with old sera[233].

Applications of different sera

As the most applied media serum in the current clinical application, FBS has been shown to downregulate a wide range of gene expressions in regard to several aspects including cell proliferation, cell differentiation, paracrine secretion and metabolism[175]. In our results, cells cultured with FBS showed less proliferative capacity in standard cultivation compared to cells with human sera. Alternations in gene expression level is discussed in detail in the next section. Therefore, to reach minimum dose for cell infusion in clinical use, human sera is a better choice since they could spare a lot of incubation time.

Choosing either autologous patient sera or allogeneic healthy sera is another issue. In our experiment, we saw that the cell counts in the HF serum group all fell behind the control serum groups, but there was no statistical significance regarding this observed

difference. At the molecular level, higher activities in mitochondrial metabolism were found in the HF serum group under standard cultivation, with insignificantly faster DNA duplication. Moreover, several cell cycle-related genes were also seen upregulated during cell recovery. However, we failed to match the ages of human sera during sera donations where HF sera donors were 9 years older than controls. Thus, we could not eliminate the possibility entirely that donor age affected our proliferation profiles. On the other hand, it is more likely that the different performance between control and HF serum groups and their inconsistencies are due to drug usage, not age. In total, up to 25 different types of drugs were used on HF serum donors during hospitalization. For instance, heparin was used in 6 out of 12 patient donors at the time of blood withdrawal, and heparin was reported to promote the growth of human stem cells through activating FGF signaling, but could also be deleterious at a higher concentration[234]. Another conventional medication that was used in HF patients is aspirin, which prevents platelet accumulation, and is proven to inhibit MSC cell growth during *in vitro* cultivation[235]. Simvastatin, HMG-CoA reductase inhibitor, was even found to induce apoptosis by activating Bax and downregulating Bcl-2. Conversely, only synthetic thyroid hormone was used in healthy volunteers.

Metabolism of stem cells and the model setup of acute stress (AcS)

Under physiological conditions, aerobic metabolism is the most efficient way of utilizing nutrients and providing energy in normal human cells in which the glycolytic metabolism, which is an oxygen-independent metabolic pathway, is suppressed. However, it is highly activated under hypoxia or anoxia. Stem cells are thought to be more resistant to oxidative stress as they have been found to have a higher level of glycolytic metabolism; the switch from oxidative phosphorylation to glycolysis has been observed during iPSC reprogramming[236,237]. Therefore, compared to other cell types, stem cells could more quickly respond and adjust to a change of microenvironment.

However, after cell retention in the injured myocardium, stem cells are exposed to a microenvironment not only characterized by low tissue oxygenation but also by insufficient nutrition supply and an accumulation of metabolic wastes. We set up a simulated condition with 0.2% O_2 and glucose starvation to mimic a similar condition in infarcted myocardium. 0.2% O_2 is the minimum hypoxic condition that we could give for *in vitro* treatments but is not sufficient to create enough oxidative stress on CB-MSCs due

to active glycolysis. Therefore, we also used basal media without glucose to enhance pro-apoptotic stress.

Alterations in gene expressions after programmed AcS

The stem cells in control serum showed a superior anti-oxidative capacity. As depicted and described in **Figure 13A**, several critical pro-apoptotic proteins, such as ATR, Tp53, Bax, Apaf1, caspase 8, and DFFA were downregulated after AcS. The suppression of their expression explained the upraised cell survival in the cells treated with human sera. We expected an up-regulated expression of Bcl-2; however, downregulation actually appears to have occurred. Its intracellular level was reported to be inversely correlated with ROS[240,241], which could explain the negatively modulated expression of BCL-2 under programmed AcS. Different from control serum, HF serum did not suppress the expressions of most pro-apoptotic genes of interest except ATM and Apaf1. Of note, the expression of Apaf1 was elevated, indicating an increase in activation of the downstream caspase cascade. Considering that the survival rate of cells after AcS with HF serum is similar to those with control serum, another anti-apoptotic mechanism and pattern gene modulations are implied in cells in HF sera.

Hypoxic preconditioning is a popular method of drug-free pretreatment against ischemia-reperfusion injury[242]. The preconditioning could decrease the formation of intracellular reactive oxygen species and consequently decrease cell death during ischemic injury[243]. Pretreated stem cells have enhanced survivability, and pro-angiogenic capacity, which was confirmed both *in vitro* and *in vivo*; hence, they have been widely used in the regeneration of multiple organs[225,244,245]. The enhanced survivability of stem cells after hypoxic pretreatment was proven in our experiment after raising stem cell survival rates from 45.08% to 81.44%. Interestingly, the expressions of ATR and Tp53 were slightly suppressed after AcS in the group without hypoxic pretreatment while they were unchanged in the hypoxia pretreated group. These two encoded proteins play essential roles in the cell response to DNA damage and cell adaptation to apoptotic stress (**Figure 13A**). Under severe hypoxia (0.2% O2 or even less), ATR was reported to be activated through the regulation of hypoxia-inducible factor 1(HIF-1), and positively regulate this factor's expression in the feedback. This finding implies that the maintenance of ATR expression might be helpful for cellular adaptation to severe hypoxia[246]. Besides that, the translation of Tp53 was also reported to be enhanced by HIF-1α[247,248]. Thus, the mRNA expressions of these two genes remain stable after hypoxic pre-treatment and

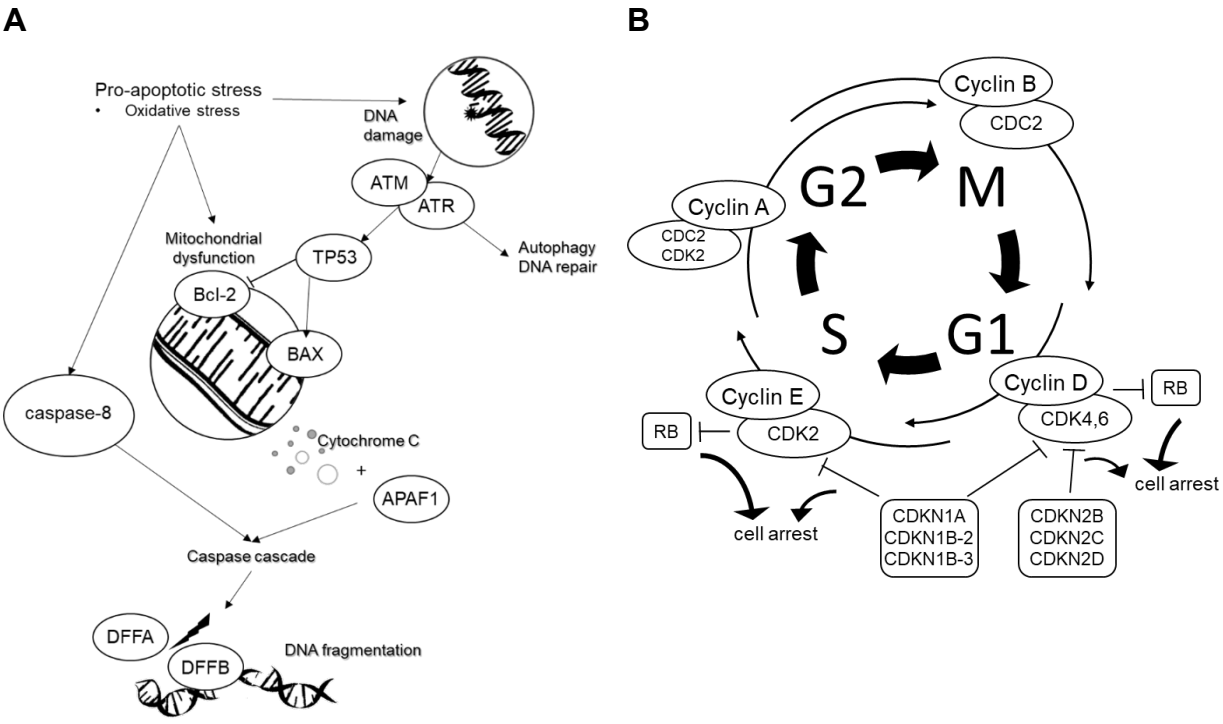


Figure 13. Modulations of apoptosis-related genes in response to AcS and cell cycle-related genes in recovery. The two pathways were drawn based on the information provided by the KEGG pathway database.
(**A**) This plot used ko04210 and ko04115 from the KEGG pathway database for references. ATM is the protein kinase that is recruited when DNA double-strand breaks in response to stimuli and mediates the activation of Tp53. Activated Tp53 leads to the inhibition of Bcl-2 and induction of Bax and promotes apoptosis[238]. Bax and Bcl-2 are both localized to the mitochondrial membrane. Bax opens the mitochondrial voltage-dependent anion channel (VDAC), subsequently leading to the release of cytochrome C, which then binds to APAF. Another pathway to apoptosis is mediated through caspase-8. The stimuli come from the membrane by reactive oxygen species (ROC), generated during ischemia. Pro-caspase 8 is then cleaved and then generates mature caspase 8. Both pathways trigger caspase cascade and lead to the cleavage of DFFA. Subsequently, DFFB activated by DFFA cleavage triggers DNA fragmentation and chromatin condensation[239].
(**B**) This plot used ko04110 from the KEGG pathway database for references. To duplicate, stem cells must go through 4 different cell cycle phases: G1, S, G2 and M. Cyclins, such as Cyclin D, E, A, B, combined with their dependent kinases (CDKs), such as cdk2,4,6 and cdc2, regulate the transition from one cell cycle phase into the next. Cyclin-dependent kinase inhibitors (CDKNs), such as CDKN1A, 1B-2, 1B-3, 2B, 2C, and 2D, arrest the cell cycle through binding and inactivating cyclin-CDK complexes. Retinoblastoma protein (RB) is another protein that keeps cells in the G1 phase by restricting DNA synthesis.
The URL address of ko04210 is:
https://www.kegg.jp/kegg-bin/show_pathway?ko04210+K04451
The URL address of ko04115 is:
https://www.kegg.jp/kegg-bin/show_pathway?ko04115+K04451
The URL address of ko04110 is:
https://www.kegg.jp/kegg-bin/show_pathway?ko04110+K04503

The element picture of DNA breakdown was modified. Its original URL address is: http://www.merckmillipore.com/INTERSHOP/static/WFS/Merck-Site/-/Merck/en_US/Freestyle/BI-Bioscience/Antibodies-Assays/cancer-images/double-helix-break.gif
The element picture of mitochondrial was also modified. Its original URL address is: http://www.alsresearchforum.org/wp-content/uploads/03.03_Nature-Li-Mitochondria_clear_proteins_NV-1.jpg
Both were downloaded and then modified in December 2018.
The element picture of DNA fragmentation was modified. Its original URL address is: https://www.ferty9.com/wp-content/uploads/2019/02/Fragmented-DNA-in-sperm.png

probably maintain cell pro-survival capabilities after severe oxidative stress.

Research related to stem cell recovery after severe ischemia, both *in vitro* and *in vivo*, is still rare. We observed an impaired proliferative potential of cord blood-derived mesenchymal stromal cells after AcS. Cell counts in human serum groups were up to 20,000 per well under standard conditions but only reached between 10,000 and 15,000 at the end of recovery. Three days after programmed AcS, cell expansion seemed to have ceased or become much slower. RT-PCR results from cells with control sera revealed similar cell cycle-related gene modulations as those in FBS. Expressions of CCNA2, CCNB1, and CDC2 were suppressed after AcS compared to the normal condition. These gene expression profiles showed a consistent performance with its halted expansion in cell culture plates at three days after AcS. In this study, we assumed that AcS might impair the proliferative potential of CB-MSCs. However, the expansion of cells with HF sera also ceased, but cell duplication appeared quite active in gene expression levels. Other than RB, the cell-cycle-related gene modulations in FBS were quite limited. RB is a critical tumor suppressor protein that puts the cell cycle into the G0 phase. The high expression of RB with FBS implies a different mechanism of cell cycle suppression from cells with human serum.

Conclusion

In this study, we were able to confirm that CB-MSCs expanded in human sera had better proliferative profiles than those expanded in FBS; higher survival rate after AcS indicated that they were more resistant to severe oxidative stress. However, differences between both heart failure and control sera were not found related to their proliferation capacity or survivability under ischemia-reperfusion injury. Relevant gene modulations in response to AcS were significantly different among sera. Hypoxic preconditioning improved the survivability of CBMSCs under FBS cultivation. Suppression of cell cycle

progression after AcS was detected in cells in all three different medium sera, but it worked in different ways.

Expansion of stem cells by using either human sera or programmed hypoxic pretreatment is a good alternative to FBS in normal conditions. In the preventative therapy of stem cells infusion for post-operative complications after long cardiac surgeries, especially for preventing myocardial injury related to CPB or surgery trauma, stem cells with human sera or hypoxic pretreatment could have a better resistance against oxidative stress, which is the ischemia-reperfusion injury that occurs in heart tissues when removing from CPB. By using the above-mentioned strategies, we optimized CB-MSCs to increase their survivability in the oxidative stressful condition after transplantation into hearts, therefore, to increase their retention rate in order to provide better cardioprotection from operative myocardial injury.

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Statutory Declaration

"I, Zhiyi Xu, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic the study of stem cells in cardiac surgery: from cell type selection to optimization for transplantation, independently and without the support of third parties, and that I used no other sources and aids than those stated.
All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts, and tables) are exclusively my responsibility.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I am aware of the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice and that I commit to comply with these regulations.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me."

Date Signature

Declaration of your own contribution to any publications

Zhiyi Xu contributed the following to the below-listed publications:

Publication 1: Timo Z. Nazari-Shafti*, Zhiyi Xu*, Andreas Matthäus Bader, Georg Henke, Kristin Klose, Volkmar Falk, Christof Stamm, "Mesenchymal Stromal Cells Cultured in Serum from Heart Failure Patients Are More Resistant to Simulated Chronic and Acute Stress," Stem Cells International, vol. 2018, Article ID 5832460, 15 pages, 2018 (*Co-first author)

including experimental designs (establishment of AcS model, IL-6 experiments), executions, data analyses, scientific graphs drawing and primary manuscript writing (abstracts, methods, conclusions, tables, and plots)

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Signature, date and stamp of supervising university professor / lecturer

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Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht

Publication

Timo Z. Nazari-Shafti*, **Zhiyi Xu***, Andreas Matthäus Bader, et al., “Mesenchymal Stromal Cells Cultured in Serum from Heart Failure Patients Are More Resistant to Simulated Chronic and Acute Stress,” Stem Cells International, vol. 2018, Article ID 5832460, 15 pages, 2018. https://doi.org/10.1155/2018/5832460. *Both authors contributed equally

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