PEER REVIEW HISTORY

ARTICLE DETAILS

TITLE (PROVISIONAL)	Behavioral effects of methylphenidate in an animal model of attention-deficit/hyperactivity disorder, the spontaneously hypertensive rats: a systematic review and meta-analysis protocol
AUTHORS	**Douglas Teixeira Leffa (Corresponding Author)** **Alana Castro Panzenhagen** **Diego Luiz Rovaris** **Claiton Henrique Dotto Bau** **Luis Augusto Rohde** **Eugenio Horacio Grevet** **Gabriel Natan Pires**

VERSION 1 - REVIEW

REVIEWER 1	***Sudhish Sharma*** ***University of Maryland***
REVIEW RETURNED	16-04-18

GENERAL COMMENTS	Abstract: Succinct abstract. Introduction: Excellent context for the current study and the value in testing RCVI for cell retention and safety. Line 63: Please correct “proof” to “prove” Methods: Line 124: Please correct “was” to “were” Results: Concisely and effectively describes all salient points related to outcome measurements. Discussion: Excellent discussion which effectively describes implications of results and possible reasons for lower cell retention/adverse safety of RCVI.

REVIEWER 1	***Ramasamy Kannappan*** ***University of Alabama at Birmingham***
REVIEW RETURNED	16-04-18

GENERAL COMMENTS	Reviewers' comments on the article titled " Lower Retention after Retrograde Coronary Venous Infusion Compared to Intracoronary Infusion of Mesenchymal Stromal Cells in the Infarcted Porcine Myocardium". In this study the authors compared two methods, Retrograde Coronary Venous Infusion (RCVI) and Intracoronary Infusion (IC), for delivering stem cells to the heart for the purpose of regenaration. The article was well written and easy to understand. Based on their findings, the authors conclude that RCVI is significantly less efficient in delivering cells to the heart compared to IC infusion. The major part of the conclusion comes from the nuclear imaging and analysis. As the authors have already pointed out in the limitation of the study section, over projection of lungs and heart could lead to overestimation of the retained cells. Moreover, in figure 2, in the heart region individual spots (radioactive signal) could not be seen, they all are overlapped. Quantifying these overlapped spots would lead to either overestimation or underestimation of retained cells. How did the authors decided each radioactive signal count represent a cell? They have not analyzed the amount of radiolabel per cell.

REVIEWER 3	***Wayne Balkan*** ***University of Miami***
REVIEW RETURNED	15-06-18

GENERAL COMMENTS	The manuscript by Gathier et al. compares cell retention in intracoronary (IC) infusion to retrograde coronary venous infusion (RCVI) in a pig model of myocardial infarction (MI). Four weeks post-MI, mesenchymal stromal cells labeled with Indium-111were infused by one of the two methods (6 pigs each) and 4 hours later cell retention was assessed. The authors show that IC infusion results in ~4-fold more cell retention in the heart. While determining the best route of infusion is an important aspect of cell therapy, this manuscript only quantifies the cells after 4 hours rather than directly comparing the cardiac repair associated with the two methods. Referring to Table 2 the authors note that "No significant differences in cell retention were seen in lungs, kidneys, liver, spleen, and bladder between RCVI and IC infusion" but they then state, "although a numeric difference was seen in cell retention in the lungs between both groups". Based on the lack of significance, please remove this latter statement from the Results, and in the Discussion where the authors state, "This could explain the higher retention of cells in the lungs of RCVI treated animals" (page 12). In the Discussion the authors state, "One can imagine that higher

	cell retention in the heart equals a greater effect of cell therapy, making IC infusion preferable over RCVI". This study seems to have provided the opportunity to assess this statement. Furthermore, since it is thought that much of the benefit of mesenchymal stromal cell therapy is due to paracrine effects, retention in the heart may not necessarily correlate with, or be the limiting factor for, reparative ability.

VERSION 1 – AUTHOR RESPONSE

We want to thank the editor and external reviewers for their valuable comments. We have revised the manuscript and added new data. Changes in the revised manuscript are shown with track changes Below we provide answers to the questions raised by the associate editor and reviewers.

Associate Editor Comments:
The study by Gathier et al aims to investigate retention of mesenchymal stromal cells by two delivery methods into the porcine myocardium following cardiac ischaemia. While the reviewers found merit in this study, there were a number of concerns raised that preclude the publication in its current form. Concerns included difficulties associated with the nuclear imaging and assessment of cell retention which could lead to over- or under-estimation of cell number, and associating cell retention data with cardiac repair post-ischaemia to show that actually retaining more cells in the heart can improve outcome. In addition, we felt that a histological investigation that would enable counting the number of cells that were retained in the heart would more definitively show cell retention.
A revised manuscript will need to show accuracy of the counting method that ensures that cell retention in the heart is accurately being measured with the nuclear imaging and analysis technique, any histological cell counts in the heart to show corroboration with the nuclear imaging data, and an assessment of cardiac repair to show whether improved retention is associated with improved cardiac infarct.

Many thanks for the suggested adjustments of our manuscript. We feel that the reviewers addressed valuable points to strengthen our manuscript, and thank them for that. We have addressed their concerns in our latest version of the manuscript. We identify three major points that need to be addressed.
The first being accuracy of the nuclear imaging and analysis. We understand that the reviewers are concerned that cardiac cell retention is not accurately measured due to over projection of heart and lungs. Besides total body imaging, we performed additional ex vivo imaging of major individual organs (heart, lungs, kidneys, liver, spleen). In this way, the issue with organ over projection can be circumvented. We have added an insightful image and accompanying table depicting results from ex vivo imaging. Furthermore, we made textual changes to the manuscript. We are confident that nuclear analysis of cell retention is accurately performed in this study and that our additional data support this.
The second remark is that additional information on histological cell counts and corroboration with the nuclear imaging data is needed to show that we are actually measuring cell retention. We have added images showing retained cells in the myocardium in pigs from both groups. Tissue samples were selected from areas of the heart that showed activity during nuclear imaging. We would like emphasize that the method used to determine cell retention was already described in previous work (van der Spoel TI et al., J Cell Mol Med. 2012). Here we also showed that cells were found in the areas that were selected based on nuclear imaging. However, we believe that using radiolabeled cells and nuclear imaging actually is a superior method to determine the percentage of retention, as with this method we are able to quantify the distribution of cells. Furthermore, histological quantification of cell retention requires very precise selection of all representative tissue samples. This is very difficult in an organ as big as the porcine heart and could easily lead to inadequate quantification of retained cells.
The third remark is that assessment of cardiac repair would strengthen the manuscript. We agree with the reviewers on this point. However, within the design of this confirmatory study we chose to solely assess cell retention after retrograde coronary sinus infusion and intracoronary infusion. To strengthen the evidence for this endpoint we have decided to perform an ex-vivo scan to be able to

analyze retention in individual organs, which made a functional follow-up impossible. However, we do realize that the assumption that a greater retention will advance function is not proven within this study.

Reviewer(s)' Comments to Author:
Comments
Please leave your comments for the authors below

#1 Submitted by : Sudhish Sharma
Abstract: Succinct abstract.

Introduction: Excellent context for the current study and the value in testing RCVI for cell retention and safety.
Line 63: Please correct “proof” to “prove”

Methods:
Line 124: Please correct “was” to “were”

Results: Concisely and effectively describes all salient points related to outcome measurements.

Discussion: Excellent discussion which effectively describes implications of results and possible reasons for lower cell retention/adverse safety of RCVI.

Proposed changes have been made to the manuscript.

#2 Submitted by : Ramaswamy Kannappan
Reviewers' comments on the article titled " Lower Retention after Retrograde Coronary Venous Infusion Compared to Intracoronary Infusion of Mesenchymal Stromal Cells in the Infarcted Porcine Myocardium”. In this study the authors compared two methods, Retrograde Coronary Venous Infusion (RCVI) and Intracoronary Infusion (IC), for delivering stem cells to the heart for the purpose of regenaration. The article was well written and easy to understand. Based on their findings, the authors conclude that RCVI is significantly less efficient in delivering cells to the heart compared to IC infusion. The major part of the conclusion comes from the nuclear imaging and analysis.
As the authors have already pointed out in the limitation of the study section, over projection of lungs and heart could lead to overestimation of the retained cells. Moreover, in figure 2, in the heart region individual spots (radioactive signal) could not be seen, they all are overlapped. Quantifying these overlapped spots would lead to either overestimation or underestimation of retained cells.

We thank the reviewer for this comment. As we pointed out, over projection of lungs and heart will lead to overestimation of the number of retained cells in the heart. This makes it difficult to identify the exact percentage of administered cells retained in the heart. The goal of this study is to compare retention of cells administered either through RCVI or IC infusion. Because we compare the retention of cells in the heart after RCVI and IC infusion in the same way, we have roughly the same overestimation of retention for both techniques, making the results comparable. Therefore, this study was performed in a randomized matter and assessment of the nuclear imaging was performed by blinded experts. In addition, we have determined the number of counts of the individual organs ex-vivo, directly after the t=4 hours total body scan was completed. These data show similar results and are now added to the manuscript. Over projection is circumvented by assessment of individual organs.
Over projection of individual spots in the heart (as seen in figure 2) is not an issue with total body scintigraphy in order to estimate the percentage of retained cells in the heart. Each and every radioactive signal coming from the heart is counted by the gamma camera. The number of counts corresponds with the activity in the heart and thus with cell retention. Although in figure 2 it looks like all counts are merged and undiscernible from each other, all counts are individually recognized by the gamma camera. In this way there can be no over- or underestimation from overlapping spots in one organ.

How did the authors decided each radioactive signal count represent a cell? They have not analyzed the amount of radiolabel per cell.

We thank the reviewer for this comment. You are correct that we did not analyze the amount of radiolabel per cell. As stated in the methods section (2.3 experimental outcomes), we looked at the percentage of total radioactive signal (counts) coming from the heart divided by total radioactive counts coming from the total body of the pig. In this way, we investigate the percentage of total administered cells that are retained in the heart, and not the exact number of cells in the heart. We assume that during the labeling process, every cell binds roughly the same amount of Indium. This would mean that each radioactive signal count or each number of radioactive signal counts represents the same number of cells whether this signal comes from the heart of any other part of the body. Therefore, we are convinced that we can accurately measure the percentage of retained cells in the heart with this technique and compare this percentage in the heart after RCVI and IC infusion in an accurate, reproducible way.

#3 Submitted by: Wayne Balkan
The manuscript by Gathier et al. compares cell retention in intracoronary (IC) infusion to retrograde coronary venous infusion (RCVI) in a pig model of myocardial infarction (MI). Four weeks post-MI, mesenchymal stromal cells labeled with Indium-111were infused by one of the two methods (6 pigs each) and 4 hours later cell retention was assessed. The authors show that IC infusion results in ~4-fold more cell retention in the heart. While determining the best route of infusion is an important aspect of cell therapy, this manuscript only quantifies the cells after 4 hours rather than directly comparing the cardiac repair associated with the two methods.
Referring to Table 2 the authors note that “No significant differences in cell retention were seen in lungs, kidneys, liver, spleen, and bladder between RCVI and IC infusion” but they then state, “although a numeric difference was seen in cell retention in the lungs between both groups”. Based on the lack of significance, please remove this latter statement from the Results, and in the Discussion where the authors state, “This could explain the higher retention of cells in the lungs of RCVI treated animals” (page 12).

We thank the reviewer for his comments.

Regarding pulmonary cell retention: we agree that these contradictive statements may lead to confusion. We meant to say that a trend is seen towards a higher retention of cells in the lungs after RCVI in case of total body imaging. Therefore, we changed this section to:

“No significant differences in cell retention were seen in lungs, kidneys, liver, spleen, and bladder between RCVI and IC infusion, although a trend was seen towards higher retention of cells in the lungs after RCVI.”

We have added additional data on ex vivo analysis of cell retention to overcome the issue with over projection of heart and lungs on the total body scan. This data was produced by performing ex vivo scans of major individual organs (heart, lungs, kidneys, liver and spleen) directly after the t=4 hours in vivo total body scan was completed. We found significant differences in pulmonary cell retention between RCVI and IC infusion if the lungs were analyzed ex vivo. Because of this, we decided to leave the sentence “This could explain the higher retention of cells in the lungs of RCVI treated animals” in the discussion part in place.
We provided additional insight in the manuscript regarding the differences between in vivo and ex vivo results (line 378 – 408). Another remark of the reviewer is that we have only looked at cell retention and not at cardiac repair. This point will be addressed below.

In the Discussion the authors state, “One can imagine that higher cell retention in the heart equals a greater effect of cell therapy, making IC infusion preferable over RCVI”. This study seems to have provided the opportunity to assess this statement. Furthermore, since it is thought that much of the benefit of mesenchymal stromal cell therapy is due to paracrine effects, retention in the heart may not necessarily correlate with, or be the limiting factor for, reparative ability.

Thank you for this comment. The sole purpose of this study was to compare cell retention in the heart after RCVI and IC infusion. Our primary endpoint was cardiac cell retention after 4 hours. We gathered data to answer this question, including ex-vivo scans of the organs. Unfortunately, this decision led to a study design in which it is impossible to collect functional data. The assumption that we make is that a higher retention might lead to a higher efficacy. However, we do agree that this is not proven and neither does this particular study attribute to that discussion. In order to assess benefit from cell therapy, e.g. a longer follow up period, and a larger number of pigs would have been necessary. A longer follow-up period is an issue when working with radioactivity, because pigs would have to be contained in specialized chambers to collect and process all radioactive excrements. To assess differences in cardiac repair between animals that received cells through either RCVI or IC infusion, a different study design is preferred. We are familiar with the hypothesis that functional benefit of mesenchymal stromal cell therapy is caused by paracrine signaling. We believe that more cells in the heart could lead to an increased paracrine effect on the cell's surroundings which would lead to an increase in cardiac repair. To test whether this is true, additional experiments with longer follow up time would be required.
To clarify this, we added the following to the introduction (line 77-78): "We did not aim to provide data on cardiac repair because animals were terminated four hours after cell infusion to enable ex-vivo scintigraphy of different organs.", and also specified this in the discussion (line 345-347).

VERSION 2 - Review

REVIEWER 1	***Ramasamy Kannappan*** ***University of Alabama at Birmingham***
REVIEW RETURNED	03-11-18

GENERAL COMMENTS	I'm satisfied with the authors response to my comments

REVIEWER 3	***Wayne Balkan*** ***University of Miami***
REVIEW RETURNED	23-10-18

GENERAL COMMENTS	The authors addressed my comments.

Lower Retention after Retrograde Coronary Venous Infusion Compared to Intracoronary Infusion of Mesenchymal Stromal Cells in the Infarcted Porcine Myocardium

Gathier WA[1]	wouter_gathier@hotmail.com	ORCID: 0000-0002-0270-8542
van der Naald M[1]	m.vandernaald@umcutrecht.nl	ORCID: 0000-0002-4949-625X
van Klarenbosch BR[1]	b.r.vanklarenbosch@umcutrecht.nl	ORCID: 0000-0001-7064-2891
Tuinenburg AE[1]	a.e.tuinenburg@umcutrecht.nl	ORCID: 0000-0002-3038-715X
Bemelmans JLM[2]	j.bemelmans@umcutrecht.nl	ORCID: no ORCID
Neef K[1,4]	k.neef@umcutrecht.nl	ORCID: 0000-0002-3261-5769
Sluijter JPG[3,4,6]	j.sluijter@umcutrecht.nl	ORCID: 0000-0003-2088-9102
van Slochteren FJ[1]	f.j.vanslochteren@umcutrecht.nl	ORCID: 0000-0003-2657-7409
Doevendans PA[1,4,5,6]	p.doevendans@umcutrecht.nl	ORCID: 0000-0002-6257-7169
Chamuleau SAJ[1,4]	s.a.j.chamuleau@umcutrecht.nl	ORCID: 0000-0002-9952-6701

[1] Department of Cardiology, UMC Utrecht, Heidelberglaan 100, 3584 CX, Utrecht, The Netherlands

[2] Department of Nuclear Medicine, UMC Utrecht, Heidelberglaan 100, 3584 CX, Utrecht, The Netherlands

[3] Department of Experimental Cardiology, UMC Utrecht, UMC Utrecht, Heidelberglaan 100, 3584 CX, Utrecht, The Netherlands

[4] Regenerative Medicine Center Utrecht, Utrecht University, Uppsalalaan 8, 3584 CT, Utrecht, The Netherlands

[5] Central Military Hospital, Lundlaan 1, 3584 EZ, Utrecht, The Netherlands

[6] NL-HI (Dutch Heart Institute), Moreelsepark 1, 3511 EP, Utrecht, The Netherlands

Abstract

Background: Commonly used cell delivery strategies to the heart are intramyocardial injection and intracoronary (IC) infusion, both having their advantages and disadvantages. Therefore, alternative strategies are explored, such as retrograde coronary venous infusion (RCVI). The aim of this confirmatory study was to compare cardiac cell retention between RCVI and IC infusion. As secondary endpoint, the procedural safety of RCVI is assessed.

Methods: Four weeks after myocardial infarction, twelve pigs were randomized to receive mesenchymal stromal cells, labeled with Indium-111, via RCVI (n=6) or IC infusion (n=6). Four hours after cell administration, nuclear imaging was performed to determine the number of cells retained in the heart as a percentage of whole body signal of the pig. Procedural related safety measures were reported.

Results: Cardiac cell retention is significantly lower after RCVI compared to IC infusion (RCVI: median 2.89% vs IC: median 13.74%, p=0.002). Retention of cells in other organs did not significantly differ between RCVI and IC infusion. RCVI led to development of pericardial fluid and hematomas on the frontal wall of the heart in 3 cases. Coronary venous dissection after RCVI was seen in 3 pigs, of which one also developed pericardial fluid and a hematoma. IC infusion led to no-flow in one pig.

Conclusion: RCVI is significantly less efficient in delivering cells to the heart compared to IC infusion. RCVI led to more procedural related safety issues than IC infusion, with multiple cases of venous dissection and development of hematomas and pericardial fluid collections.

1. Introduction

Cell therapy is a suggested as a potential treatment option for ischemic heart disease, yet only moderate improvement in cardiac function is achieved.[1, 2] The delivery of cells to the myocardium is an important limitation of current cell injection methodologies.[3] The ideal strategy is safe, easy to perform and efficient in cell delivery. Intracoronary (IC) infusion and intramyocardial (IM) injection have been thoroughly tested.[4-7] Both techniques present with disadvantages such as the need for patent coronary arteries and the risk of embolization leading to decreased blood flow in case of intracoronary infusion.[8-10] The IM injection procedure is time consuming and requires specialized equipment in the catheterization laboratory. Furthermore, rapid loss of cells via venous drainage is seen after IM injection.[11]. Alternative delivery strategies could possibly overcome these drawbacks. Retrograde coronary venous infusion (RCVI) is less commonly applied, but could be a good alternative to IC infusion and IM injection. However, the available data on technical and safety aspects of RCVI are insufficient and incomplete. At present, there are not enough arguments to proceed with this technique in the clinical arena because well-designed confirmatory studies on retention rates and safety data are required to proof its value.[12]

With RCVI, cells are retrogradely infused in the coronary venous system, which is typically free of atherosclerotic disease, and therefore could potentially improve delivery to the target area compared to IC infusion. An important limitation of cardiac cell therapy is the retention of cells in the heart after delivery. IM injection and IC infusion show comparable retention rates of 10-15%.[4, 13, 14] However, there is only limited data available on safety and the retention of cells in the heart after RCVI in large animal models and in the clinical setting. Currently, no direct comparison is available on cardiac cell retention after RCVI versus IC infusion in the setting of chronic myocardial ischemia. In view of future clinical trials it is important to determine whether RCVI is a good alternative to IC infusion. Therefore, the aim of this confirmatory study is to compare the retention rates of mesenchymal stromal cells (MSCs) in the heart after RCVI and IC infusion

2. Methods

2.1 Ethical statement

All animals received care in compliance with the "Guide for the Care and Use of Laboratory Animals", published by the National Institutes of Health (National Institutes of Health publication 85-23, revised 1985). The study protocol was approved by the Animal Experiment Committee of the University of Utrecht and the governing national Central Animal Experiment Committee (AVD115002015257, 105119-2). It was not possible to perform this experiment without animals due to the fact that the hemodynamics and biologic nature of the heart and the whole body cannot be replicated in such a way that the results of this study would be translatable to the real situation. We minimized the number of animals used by performing a sample size calculation beforehand. Refinement was done by using proven techniques, performed by trained personnel. Furthermore, maximum effort was put into ensuring the best conditions for the animals in terms of housing, enrichment, and analgesia.

2.2 Study design

Myocardial infarction (MI) was induced in sixteen female Dutch Topigs pigs (Van Beek SPF varkensfokkerij B.V., Lelystad, The Netherlands). Pigs were selected as the preferred animal for this experiment because of the resemblance of the pig and human heart in terms of anatomy and hemodynamics. Animals that survived four weeks after MI (n=12) were randomized (1:1) to receive MSCs labeled with Indium-111 (In^{111}) via RCVI (n=6) or IC infusion (n=6). Randomization was performed using a closed envelope system. Nuclear imaging was carried out four hours after MSC delivery, after which the anesthetized animals were euthanized by potassium chloride overdose. Nuclear imaging data was analyzed by lab technicians blinded to the infusion procedure.

The protocol of this study was registered on https://www.preclinicaltrials.eu/ (PCTE0000104) and the ARRIVE guidelines were followed for reporting. Heart rate, mean arterial pressure, left ventricular

internal diameter at diastole and systole (LVIDd, LVIDs) were determined prior to MI (baseline) and directly prior to cell infusion.

2.3 Experimental outcomes

The primary endpoint of this study is retention of cells in the heart four hours after delivery, defined as the percentage of total radioactive signal (counts) coming from the heart divided by total radioactive counts coming from the total body of the pig, including the bladder catheterThe secondary endpoint is safety in terms of procedural related complications such as occurrence of vessel dissections, flow obstruction during or after cell administration, development of pericardial effusion, and development of hematomas on the left ventricular wall. Experimental setup is shown in figure 1.

Fig. 1 *Experimental setup.*

[INSERT FIGURE 1]

MI = myocardial infarction, IC = intracoronary infusion, RCVI = retrograde coronary venous infusion, t = timepoint, n = number of animals

2.4 Experimental procedures

2.4.1 Anesthesia and Analgesia

Prior to MI induction, all animals received a Butrans patch 5 µg/h. Animals were pretreated with Amiodarone (1200 mg/day, 7 days), Clopidogrel (75 mg/day, 3 days) and Carbasalate Calcium (loaded with 320 mg, 1 day), which was continued until the end of the experiment (daily dose 80 mg). Premedication (ketamine 10-15 mg/kg, midazolam 0.7 mg/kg, and atropine 0.5 mg) was delivered intramuscularly. Anesthesia was induced with thiopental sodium 4 mg/kg delivered through the ear vein. General anesthesia and analgesia was maintained with a bolus of midazolam 10 mg and sufentanil 0.25 mg followed by intravenous delivery of midazolam 1 mg/kg/h, sufentanil 10 µg/kg/h, and pancuronium bromide 0.1 mg/kg/h. Animals received 300 mg amiadarone in 500 ml venofundin 6% infused in 30

minutes. Mechanical ventilation was performed using a mixture of O2 and air (1:2) with a tidal volume of 10 ml/kg with 12 breaths per minute. Animals received 5000 IU of heparin every two hours during the procedure.

2.4.2 Myocardial infarction

Myocardial infarction was induced percutaneously by a temporal (90 minute) occlusion of the left anterior descending artery (LAD) using an angioplasty balloon. The preferred occlusion site was after diagonal branch two, but the infarct site was determined per pig based on the anatomy of the coronary arteries (thickness and tract). In case of ventricular fibrillation or ventricular tachycardia without output, 200-joule shocks were delivered using an external defibrillator in order to restore sinus rhythm. Chest compressions were given between shocks to maintain circulation. In addition, amiodarone (maximum of 3 times 150 mg), adrenalin (0.1 mg) and/or atropine (0.5 mg) were administered. Arterial blood pressure, ECG and capnogram were monitored during the entire procedure.

2.4.3 MSC culture and In^{111}-labeling

Allogeneic MSCs were isolated and cultured in αMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 0.2 ng/ml vitamin C (Sigma-Aldrich, St. Louis, MO, USA), 1 ng/ml basic fibroblast growth factor (Sigma-Aldrich, St. Louis, MO, USA) and 1% Penicillin/Streptomycin. The cells were incubated at 37°C and medium was changed every 3 days. Cells were cultured in $75cm^2$ flask and passaged when they reached confluence, until passage 2–3. MSCs were frozen in 10% dimethylsulfoxide and 90% culture medium. Characterization of MSCs was performed as previously described.[15, 16]. Seven days prior to transplantation, MSCs were thawed, plated in flasks, and grown to confluence, until passage 5–7. At the day of cell delivery, cells were trypsinized and 10^7 MSCs were labeled with a median of 36.3 [interquartile range (IQR) 33.5 – 40.5] megabecquerel (MBq) of In^{111} at 37°C for 20 minutes. After incubation, cells were washed up to three times with Hank's Balanced Salt Solution $CaCl_2$+ $MgCl_2$+ (Life Technologies Corp, Grand Island, NY, USA) to remove unbound label. Radiolabel uptake

efficiency was measured with a dose calibrator. After labeling, cell viability was assessed via trypan-blue (Sigma-Aldrich, St. Louis, MO, USA) counting. Before injection, MSCs were resuspended in 10 ml phosphate buffered saline pH 7.4 (Life Technologies Corp, Grand Island, NY, USA).

The protocol on labeling of MSCs with In^{111} can be found at: *https://www.protocols.io/view/labeling-of-porcine-mesenchymal-stromal-cells-mscs-mr9c596*

2.4.4 Retrograde coronary venous infusion

Two different infusion catheters were used for RCVI. In case the coronary sinus (CS) was ≥ 5 mm in diameter a dedicated CS infusion catheter was used (Advance® CS Coronary Sinus Infusion Catheter, Cook Medical, Bloomington, IN, USA). In case the diameter of the CS was < 5 mm, an over the wire balloon catheter (Advance® 35LP Low-Profile PTA Balloon Dilatation Catheter, Cook Medical, Bloomington, IN, USA) was used. Balloons were inflated at low pressure (maximum of 2 atmosphere) in the CS after which a venogram was made to ensure total occlusion of the CS. When total occlusion was observed, 2 ml of cell suspension followed by 8 ml of sodium chloride 0.9% was infused during 60 seconds. This procedure was performed a total of five times in order to infuse a total of 10 ml of cell suspension flushed with 40 ml of sodium chloride 0.9% in five minutes. Occlusion of the CS was maintained for ten minutes after infusion to prevent washout of cells.

2.4.5 Intracoronary infusion

Intracoronary infusion was performed by placing an over-the-wire balloon (Emerge™ over-the-wire PTCA dilatation catheter, Boston Scientific Corp, Natrick, MA, USA) of equivalent size to the LAD at the same site where occlusion was created during MI induction. After inflation of the balloon at low pressure, 3.3 ml of cell suspension was infused in 30 – 45 seconds. The balloon was deflated after three minutes to reinstate flow. After three minutes of flow, the procedure was repeated another two times to infuse a total of 10 ml of cell suspension.

2.4.6 Nuclear imaging and analysis

In vivo total body scintigraphy was performed four hours after MSC administration using a dual head gamma camera (Phillips Skylight). A whole-body scan was acquired at both 174 and 247 kiloelectronvolt energy windows using the following imaging parameters: medium-energy general-purpose collimator and 512 x 1024 projection matrix. The retained activity in syringes was measured with a dose calibrator (Azbil Telstar Benelux). Both anterior and posterior images were captured for each he number of counts used for analysis was based on the geometric mean of the anterior and posterior counts. egions of interest were placed over the major visceral organs and body (figure 2), using manufacturer's software (JETStream workspace; Philips, Best, The Netherlands). The retention of In^{111}-labeled cells in the heart was calculated as a ratio of the total radioactive signal (counts) coming from the heart divided by the total counts coming from the total body of the pig (including bladder catheter), after correction for anatomy. Data analysis was performed by two to three laboratory analysts per animal coming from a pool of four analysts, supervised by an expert analyst, all blinded for treatment allocation.

Fig. 2 *Total body scintigraphy with regions of interest*

[INSERT FIGURE 2]

A: regions of interest placed over visceral organs (heart in red, lungs in blue, kidneys in green, liver in brown, spleen in pink, bladder in light blue), and catheter bag in yellow. B: region of interest placed over total body of pig including catheter bag. Of note: both image A and image B are anterior captures. Both anterior and posterior images were captured for each animal and the number of counts used for analysis was based on the geometric mean of the anterior and posterior counts.

2.4.7 Echocardiography

Transthoracic echocardiography (X5-1 probe, IE-33, Philips, Best, The Netherlands) was performed directly before MI induction and four weeks later, directly before MSC infusion. Chamber dimensions (LVIDd and LVIDs) were obtained in short-axis view at mid-papillary level. Analysis was performed in a blinded fashion by a trained physician.

2.5 Experimental animals

2.5.1 Sample size

A total number of twelve animals (median age and weight at time of MI: 20 weeks [IQR: 18 - 22], and 72 kilograms [IQR: 68 - 76] respectively) was allocated to receive MSCs via either RCVI (n=6) or IC (n=6) infusion. This sample size was predefined, and calculated for an α of 0.05, power of 80%, maximum standard deviation of 4%, and an expected maximum absolute difference in cell retention of 7.5%. Because four animals died during or after MI induction, a total of sixteen animals had to be used to include twelve animals in the analysis.

2.5.2 Housing

Animals were housed in stables with up to two pigs in the same stable before MI. After MI, animals were housed in separate stables to minimize stress. Animals were still able to see, smell, and hear each other through the grates that divide the stables. Straw was used for bedding and environmental enrichment was provided in the form of special rods that the animals could nibble on and play with. Welfare was assessed daily by animal caretakers.

2.6 Statistical analysis

Statistical analysis was performed using IBM SPSS statistics 21 (IBM, Armonk, New York, USA). Baseline characteristics and cell retention are presented as median with interquartile ranges. Comparison of data between two groups was performed using Mann-Whitney U test. A p-value of <0.05 was considered significant.

3. Results

3.1 Procedural data

Ventricular fibrillation (VF) during MI induction occurred in thirteen out of sixteen pigs, of which two died due to refractory VF. Another two pigs died in the stables due to acute heart failure or a heart rhythm disorder (day four and day nineteen) as a result of the MI. The remaining twelve pigs were randomized to RCVI (n=6) or IC infusion (n=6). No significant differences in heart rate, mean arterial pressure, LVIDd, and LVIDs were seen between groups as seen in table 1a, although a trend was seen towards a larger LVIDs in pigs that were allocated to IC infusion both at baseline and at follow up.

[INSERT TABLE 1]

3.2 Cell viability and numbers

The median viability of MSCs after labeling with In^{111} was 66.8% [IQR: 62.1 – 72.4] in the IC group versus 53.6% [IQR: 49.8 – 73.8] in the RCVI group (p=0.418). The median total administered cells was 3.2M [IQR: 3.2 – 3.7] in the IC group versus 2.8M [IQR: 2.1 – 3.1] in the RCVI group (p=0.180) The median number of administered live cells was 2.4M [IQR: 1.6 – 2.4] in the IC group versus 1.6M [IQR: 1.3 – 1.7] in the RCVI group (p=0.167). Results are shown in table 1b.

3.3 Cell retention

A significant difference in MSC retention in the heart was seen between the RCVI and IC infusion group with a median retention of 2.89% [IQR: 2.14 – 3.86] in the RCVI group versus 13.74% [IQR: 10.20 – 15.41] in the IC infusion group (p=0.002).

No significant differences in cell retention were seen in lungs, kidneys, liver, spleen, and bladder between RCVI and IC infusion, although a numeric difference was seen in cell retention in the lungs between both groups. Data are presented in table 2 and figure 3a and 3b.

[INSERT TABLE 2]

Fig. 3 *Retention of cells in major organs presented as a percentage of total body activity*

[INSERT FIGURE 3]

A: Activity in heart, lungs, kidneys, liver, spleen, and bladder presented as a percentage of total body activity: RCVI versus IC infusion. Only activity in the heart differed significantly between RCVI and IC infusion (= p=0.002). B: Magnification of fig 3.A. Retention of MSCs in the heart is significantly worse after RCVI compared to IC infusion.*

3.4 Safety aspects

3.4.1 RCVI group

Dissection of the CS occurred in three out of six pigs at the site of the balloon catheter tip. Two animals with the largest dissection later showed a radioactive hotspot in the heart instead of a more disseminated activity pattern as would be expected in case of cell infusion. Cardiac cell retention in these two pigs was the highest of all RCVI pigs and well above the median of 2.89% with 3.86% and 4.59%, respectively. Three animals presented with a small to moderate, clear pericardial effusion and a hematoma of approximately four cm^2 on the atrioventricular groove of the left ventricle (LV) at termination. Only one animal was free of dissection and development of hematoma and pericardial fluid. In this one animal, the occlusion of the CS was found to be compromised after the infusion was completed, possibly leading to direct drainage of cells into the right atrium. Nevertheless, the retention in this pig was 2.97%.

3.4.2 IC group

One animal in the IC group showed no-flow directly after cell infusion, probably due to thrombus formation. Flow was restored after five minutes of angioplasty.

4. Discussion

Cell retention

To our knowledge, this is the first confirmatory study that directly compared retention between RCVI and IC infusion in a chronic MI pig model. We showed that RCVI of MSCs is inferior to IC infusion in terms of cardiac cell retention with RCVI showing a mean retention of 2.89% versus 13.74% with IC infusion. One can imagine that higher cell retention in the heart equals a greater effect of cell therapy, making IC infusion preferable over RCVI. We chose to infuse the same amount of cells in both the IC infusion group and the RCVI group in order to make sure that the results are comparable. Because retention of cells in the heart is calculated as a percentage of total administered cell dose, it is probable that a higher cell dose would not result in a higher retention rate. However, it is known that infusion of larger volumes of cells ($30 \times 10^6 – 50 \times 10^6$) via the IC route can result in a higher index of microcirculatory resistance.[17, 18] Currently, there is no evidence that larger cell volumes infused via the retrograde route would impair venous flow. This could implicate that more cells can be infused with RCVI, making up for the lower retention. The retention rates that we observed for IC infusion are comparable to results of other studies.[4, 13, 14] Three pig studies with small sample sizes (n=5, n=6 and n=7) and only 1 clinical trial (n=9) reported retention rates after RCVI in a model of acute myocardial infarction. However, no data on cell retention in a chronic ischemia model in large animals are available. Retention of cells, measured as radioactive positive signals coming from the heart, was low in these four trials, ranging from 3% - 8% of total injected activity, corresponding with our results.[13, 14, 17, 19] A possible explanation for the low retention of cells with RCVI is that cells are maintained in the CS after infusion but are directly flushed into the right atrium after abrogation of the CS occlusion and reinstitution of flow. This could explain the higher retention of cells in the lungs of RCVI treated animals. It is also possible that the cells are not adequately pushed though the microvascular bed as is the case with IC infusion, possibly effecting cell retention. Additionally, a low retention with RCVI could occur due to the existence of aberrant (and/or collateral) veins draining directly in the right atrium, effectively negating the blockade of the CS. An experienced cardiologist analyzed the fluoroscopy images made during cell infusion in this study and found anatomical variations of the coronary veins strongly suggesting the presence of aberrant venous drainage in three out of six RCVI treated animals. We could not find a relation between possible aberrant

venous drainage and cell retention in these pigs, possibly due to the small number of pigs and other factors present such as coronary sinus dissection and pericardial effusion.

Safety aspects of RCVI

RCVI was associated with multiple safety issues in this study. We found pericardial fluid and hematoma development on the atrioventricular groove of the LV in three pigs and occurrence of CS dissection in three pigs, of which one also showed a hematoma and pericardial fluid at termination. Only one animal in the RCVI group was free of adverse events. It is striking that in this specific animal, the occlusion of the CS appeared to be incomplete after infusion. We do not know at which time point during the infusion procedure the occlusion was compromised.

In one animal, overinflation of balloon of the Advance® CS Coronary Sinus Infusion Catheter (>2 atmosphere) could have been the cause of development of a CS dissection, hematoma on the atrioventricular groove, and pericardial fluid collection.

The most likely explanation for the development of pericardial fluid and hematoma is a sudden rise in pressure in the coronary venous system during infusion even though we infused cells slowly at 10 milliliters per minute. Significant contrast blushing was seen on the fluoroscopy images made after infusion, supporting this hypothesis. We identified ten studies that used RCVI for cell delivery in pigs.[14, 17, 19-26] The median infused volume in these studies was 15 ml [IQR: 10 – 25 ml], with two studies infusing a higher volume of 40 ml [26], and 250 ml.[19]. The study that infused 40 ml did so during 4 hours, making it likely that no pressure or volume overload could develop.[26] However, the study that infused 250 ml did so during 10 minutes, making both the infused volume and infusion rate higher than in our study.[19] Unfortunately, it is unclear if the CS was occluded during infusion in these two trials, so it is not possible to make a statement on pressure or volume overload in these cases. Three other trials infused cells at a much higher rate and did not report development of pericardial fluid and hematomas.[14, 17, 22] However, the infused volume was only 10 ml in these three trials.

It is unfortunate that the majority of the RCVI pig studies reported did not state anything on procedural safeties. The studies that do, mention absence of arrhythmias and microvascular obstruction, but nothing on occurrence of dissection of the CS or development of hematomas or pericardial fluid. It is also possible that pericardial fluid collection and hematoma formation were related to CS injury in some of the cases.Contrary to RCVI studies, development of hematomas on the atrioventricular groove, pericardial fluid collections, and damage to the CS have been reported in the field of cardiac surgery and have been related to traumatic catheter insertion, overinflation of the balloon in the CS, and elevated CS infusion pressure during retrograde cardioplegia.[27-30] With retrograde cardioplegia, the CS is accessed with a balloon-catheter to occlude the CS and subsequently infuse fluid to arrest the heart and protect the myocardium. This procedure is in a way comparable to RCVI. Injury to the CS was reported to occur in 0.6 to 0.06% of the patients that underwent retrograde cardioplegia, essentially proving safety of this technique.[27, 31] A possible explanation for the high number of adverse events in the RCVI group in this study compared to an event rate of only 0.6 to 0.06% in human cases could be the difference in anatomy of the coronary sinus between humans and pigs. Contrary to humans, the hemiazygos vein drains in the coronary sinus in pigs. This leaves less room for balloon positioning in pigs, increasing the chance to perforate the CS with the catheter tip due to the small operating area. Clinical trials that have used RCVI did not report safety issues beside a rise in cardiac enzymes in some cases.[13, 32-36]

The occurrence of CS dissections did not appear to have a negative effect on cell retention in the heart. On the contrary, the two pigs with the largest dissection showed the highest retention rates of all six RCVI pigs. It is likely that the infused cells collected between the wall layers of the dissected area, effectively trapping the cells and preventing them from washing out. IC infusion was associated with less safety issues with one animal showing no-reflow directly after cell infusion, which could be restored within 5 minutes. Decreased blood flow after IC infusion is a known drawback and has been attributed to coronary embolisms leading to microvascular plugging in the past.[8-10]

Future implications

Here, we found that retention rates with both RCVI and IC infusion are low (<14%), which may hamper the effectiveness of cell therapy. Therefore, alternative approaches to increase cell retention and survival are being investigated. These include the use of carrier materials such as nanomatrix gels, microspheres and cell sheets or patches[37-39], but also pretreatment of grafted cells or target tissues, for instance by overexpressing pro-survival genes to increase survival of grafted cells in a hostile environment.[40, 41]

Conclusion

Cell retention after RCVI is significantly lower compared to IC infusion. Our results confirm previous research comparing retention of cells after RCVI with IC infusion in the setting of acute MI. Furthermore, RCVI presented with more safety issues than IC infusion. Taking both efficiency and safety into account, IC infusion is the preferred method of cell delivery between the two.

5. Strengths and limitations of the study

- To our knowledge, this is the first confirmatory study performed on cell retention after RCVI versus IC infusion in a porcine model of chronic MI.
- Adequate steps were taken to limit the risk of bias: the primary endpoint was prespecified, sample size was calculated beforehand to ensure adequate power of the study and prevent unnecessary use of animals.
- The study was performed in a randomized matter and outcome assessment was performed by blinded investigators.
- Radiolabeling with In^{111} made it possible to quantify cell retention in a very precise way.
- Precise determination of cell retention in the heart on total body images of pigs is challenging due to over projection of lungs and heart. This means counts coming from areas of the lungs that are positioned over the heart are attributed to the heart, leading to a slightly higher cell retention in the heart than was actually the case.

6. Data availability

The dataset generated and/or analyzed during the current study is available in the Open Science Framework (http://osf.io) repository:

https://osf.io/n8wg4/?view_only=55de4fda913d450899e95c3052dcf79b [42]

7. Authors' contributions - CRediT

Gathier WA:	Data curation (lead), Formal analysis (lead), Investigation (lead), Methodology (supporting), Project administration (equal), Validation (equal), Writing – original draft (lead)
van der Naald M:	Data curation (supporting), Investigation (supporting), Methodology (supporting), Validation (equal), Writing – original draft (supporting)
van Klarenbosch BR:	Data curation (supporting), Formal analysis (supporting), Investigation (supporting), Writing – review & editing (equal)
Tuinenburg AE:	Investigation (supporting), Supervision (supporting), Validation (supporting), Writing – review & editing (equal)
Bemelmans JLM:	Data curation (supporting), Investigation (supporting), Formal analysis (supporting), Writing – review & editing (equal)
Neef K:	Methodology (supporting)
Sluijter JPG:	Resources (supporting), Methodology (supporting), Writing – review & editing (equal)
van Slochteren FJ:	Supervision (supporting), Methodology (supporting)
Doevendans PA:	Funding acquisition (equal), Resources (equal), Writing – review & editing (equal)

Chamuleau SAJ:	Conceptualization (Lead), Funding acquisition (equal), Methodology (lead), Resources (equal), Supervision (lead), Validation (equal), Writing – review & editing (lead), Project administration (equal)

8. Sources of funding

This research is part of Cardiovasculair Onderzoek Nederland (grant number: CVON2011-12), an initiative of the Dutch Heart Foundation, Netherlands Federation of University Medical Centres (NFU), Royal Netherlands Academy of Arts and Science (KNAW) and NWO/ZonMW.

9. Conflict of interest

WAG, MvdN, KN, JPGS, PAD, and SAJC report grants from the Netherlands CardioVascular Research Initiative (CVON): the Dutch Heart Foundation, Dutch Federations of University Medical Centers, the Netherlands Organization for Health Research and Development, and the Royal Netherlands Academy of Sciences, during the conduct of the study.

JPGS reports grants from Horizon2020 ERC-2016-COG EVICARE, grants from Technobeat, grants from the Project SMARTCARE-II of the BioMedicalMaterials institute, co-funded by the ZonMw-TAS program, grants from the Dutch Ministry of Economic Affairs, Agriculture and Innovation, during the conduct of the study.

BRvK, AET, and JLMB have nothing to disclose.

WAG, FvS, and SAJC report non-financial support from Cook Regentec, 1102 Indiana Avenue Indianapolis, IN 46202, during the conduct of the study. Catheters for RCVI were provided by Cook Regentec, 1102 Indiana Avenue Indianapolis, IN 46202. On-site training with these catheters was facilitated by Cook Regentec. The authors did not receive payment from Cook Regentec to perform this study. Neither do the authors have stock options in Cook Regentec. Cook Regentec reviewed the manuscript prior to submission.

10. Acknowledgements

We gratefully acknowledge Marlijn Jansen, Joyce Visser, Martijn van Nieuwburg, Helma Avezaat, and Jeroen van Ark for excellent technical assistance and animal care. In addition, the authors acknowledge the excellent technical assistance provided by Esther van Eeuwijk regarding MSC culturing. Chantal Dekker, Monique Jacobs, Ingrid Boots, and Joost Holthof are gratefully acknowledged for their work on the pig in vivo scintigraphy and blinded analysis of the data. We thank Jeannette Wolfswinkel, Anke Wassink, Kees Vos, Andre Dales, and Koen Vaessen for their assistance regarding the health physics aspects of this study and Evelyn Velema and Joris de Brouwer regarding all planning and logistics affairs. We thank Tycho van der Spoel for his assistance in setting up the study protocol, and Rob van Rooij for assisting with nuclear image data analysis.

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547