The inflammatory response in myocarditis and acute myocardial infarction

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Intravenous adipose-derived stem cell therapy 24 hours after acute myocardial infarction results in fast recovery of cardiac function in rats


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SUMMARY

**Background:** Adipose-derived stem cells (ASCs) are a promising new therapeutic option for acute myocardial infarction (AMI) patients. Previously, we found that ASCs coupled to antibody targeted gas-filled microbubbles (StemBells; StB) improved cardiac function when administered intravenously 7 days post-AMI in rats. In this study, we compared the efficacy of intravenous StB administration at different administration time points following AMI in rats.

**Methods.** AMI, followed by reperfusion, was induced in four groups of male Wistar rats, which subsequently received an intravenous 1x10⁶ StB bolus 1 day post AMI (StB1, n=8), 7 days post-AMI (StB7, n=9), at both time points (StB1+7, n=7) or neither (Control, n=7). The effect on cardiac function was determined using echocardiography prior to AMI and at 7 days and 42 days post-AMI. The effect on infarct size and macrophages in the infarct core were determined (immuno)histochemically 42 days post-AMI.

**Results.** At 42 days post-AMI, all three StB groups had a significantly improved fractional shortening compared to the control group. Between the StB-treated groups the effects did not differ significantly at 42 days post-AMI. At 7 days post-AMI, the StB1 group had a significantly improved fractional shortening compared to the control and StB7 groups. No significant changes in infarct size or macrophage numbers were found compared to the control group for any StB group.

**Conclusion.** StB administration 1 day post-AMI resulted in an improved cardiac function compared to controls, as measured at both 7 days post-AMI and 42 days post-AMI. This improvement was not related to a reduction in infarct size.
INTRODUCTION

Acute myocardial infarction (AMI) is a major cause of death and morbidity worldwide. A potential treatment option for AMI is mesenchymal stem cell administration. In 2002, it was first described that adipose tissue also contains mesenchymal stem cell populations. Adipose tissue forms an attractive source to obtain mesenchymal stem cells (adipose-derived stem cells, ASCs), as it is easily accessible and stem cell-rich. ASCs have been demonstrated to differentiate into several intracardiac cell types, and secrete paracrine factors which can stimulate cardiac regeneration. Therapeutic application of ASCs for AMI has been investigated thoroughly in pre-clinical animal studies. However, there is limited clinical experience, although thus far with positive results. In general, an important limitation of stem cell therapy for AMI is poor stem cell retention and survival in the infarcted myocardium. We recently developed a technique to increase ASC engraftment into the post-AMI myocardium. We coupled gas-filled microbubbles to ASCs, forming a complex referred to as ‘StemBells’ (StB). The microbubbles are coated with antibodies targeting CD90 (ASC surface marker) and ICAM-1 (adhesion molecule present on activated endothelial cells during inflammation). This facilitates a binding of the StBs to the activated endothelial cells in intramyocardial blood vessels following AMI. Furthermore, StBs can be pushed against vessel walls using ultrasound, as previously demonstrated intravitaly in chicken embryos.

We demonstrated that administrating StB intravenously in a rat model of AMI at 7 days post-AMI significantly improved cardiac function compared to regular ASCs. However, no increase in the number of stem cells was found in the infarcted myocardium, at least not at 42 days post-AMI. This suggested an important paracrine effect of StB which improved cardiac function independent of an increase of stem cells within the heart. A reduction in infarct size however was not found for StBs or ASCs administrated at day 7 post-AMI however.

Between day 1 and day 7 post-AMI, a strong inflammatory response is induced in the infarcted heart. This inflammatory response clears the wound of necrotic cells and debris and provides signals to initiate reparative pathways. This inflammatory response however also causes further damage to cardiomyocytes, resulting in an increased infarct size and adverse cardiac remodeling. We hypothesized that protective effects of StB can protect against this inflammatory damage when administered 1 day post-AMI, and thus decrease infarct size and restore cardiac function even more compared to StB administration 7 days post-AMI. For this, we compared in a rat AMI model the efficacy of intravenous StB administration at 1 day post-AMI with administration 7 days post-AMI.
MATERIALS & METHODS

Laboratory animals
All animals were treated according to national guidelines and with permission of the Institutional Animal Care and local Animal Ethical Committee of the VU University Medical Center (Amsterdam, The Netherlands), which conforms with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996). All animals were housed under constant temperature (21-22°C), humidity (60-65%) and light-dark periodicity (L:D 12:12). Experimental procedures started after two weeks of acclimatization.

Isolation and expansion of adipose-derived stem cells
Adipose tissue was resected from the inguinal fat pads of 18 male Wistar rats (Harlan, Horst, the Netherlands), and was pooled prior to processing. The tissue was washed in sterile phosphate-buffered saline (PBS), minced manually with a sterile surgical blade and digested with 0.0125% Liberase TM Research Grade medium Thermolysin (Roche Diagnostics, Indianapolis, USA, dissolved in PBS with 1% Bovine Serum Albumin) under intermittent shaking for 25 min at 37°C. The mixture was filtered (100μm; Codan, Deventer, the Netherlands) and centrifuged (10 min, 600g). The supernatant was discarded and the cell pellet was re-suspended in PBS with 1% Bovine Serum Albumin, and washed by centrifugation (5 min, 600g). Finally, the cell pellet (stromal vascular fraction; SVF) was harvested, frozen in Recovery cell culture freezing medium (Gibco, Thermo Fisher Scientific, Waltham, MA), and subsequently stored in liquid nitrogen.

Prior to StB assembly, SVF from liquid nitrogen storage was thawed and seeded at 100,000 cells/cm² in low glucose Dulbecco’s modified Eagle’s medium (DMEM, Gibco) containing 100 U/ml penicillin, 100 μg/ml streptomycin (all Gibco) and 10% foetal bovine serum (FBS; Hyclone, GE Healthcare, Logan, UT), and culturing in a humidified atmosphere of 5% CO2 at 37°C. When the ASCs reached 90% confluency, cells were detached with 0.5 mM EDTA/0.05% trypsin (Gibco).

StemBell assembly
Biotinylated microbubbles with a C4F10 gas core were made by sonication, as described by Klibanov et al. The shell was composed of DSPC (59.4 mol %; Sigma-Aldrich, St Louis, MO); PEG-stearate (35.7 mol %; Sigma-Aldrich); DSPE-PEG (2000) (4.1 mol %; Avanti Polar Lipids, Alabaster, AL) and DSPE-PEG (2000)-biotin (0.8 mol %; Avanti Polar Lipids). Microbubbles (10⁰ per ml) had an average diameter of 3.5 μm and were stored in sealed glass vials with a C4F10 gas head-space to prevent deflation.

Dual-targeted microbubbles were made according to protocols as described previously by Woudstra et al. Briefly, microbubbles (100 μl) were first washed
twice with PBS/C$_4$F$_{10}$ by centrifugation (1500 rpm, 1 min, 4°C) to remove superfluous biotin and resolved in PBS/ C$_4$F$_{10}$. Next, streptavidin (1 mg/ml; Sigma-Aldrich) was added and the mixture was incubated at 4°C for 25 min. Microbubbles were again washed to remove superfluous streptavidin and resolved in PBS/ C$_4$F$_{10}$. Next, biotinylated mouse-anti-rat-CD90 (1 μg; BD Biosciences, Franklin Lakes, NJ) and biotinylated mouse-anti-rat-ICAM-1 (1 μg; Acris antibodies, Herford, Germany) were added and the mixture was incubated at 4°C for 25 min. These dual-targeted microbubbles were again washed and resolved in DMEM. To assemble the StB, ASCs were incubated with dual-targeted microbubbles in a 1:100 ratio under continuous rotation at room temperature for 25 min. A minimum of 10 microbubbles were coupled to each ASC.

**Induction of AMI in rats**

Rats (n=31) were anaesthetized using subcutaneous hypnorm/dormicum (fentanyl and fluanison 0.5 ml/kg, midazolam 5mg/kg) injection, and were ventilated at 75 breaths/min, 10-0.4 mbar (Zovent ventilator, Instruvet, Amerongen, The Netherlands). Heart rate was monitored using Einthoven I ECG. A left thoracotomy in the fourth intercostal space was made, and the left anterior descending coronary artery (LAD) was ligated using a 6.0 prolene suture (Ethicon, Somerville, NJ). Ischemia was maintained for 40 min, followed by reperfusion and chest closure. This procedure results in relatively small non-aneurysmatic infarcts, comparable to what occurs in the majority of patients suffering from AMI.

**Experimental procedures**

After induction of AMI, the rats were subdivided into four groups: Control (n=7), StB1 (n=8), StB7 (n=9) and StB1+7 (n=7). For ethical reasons concerning animal use, we compared the StB1 and StB1+7 groups to the same control and StB7 groups used previously. One day post-AMI, the rats in the StB1 and StB1+7 group received a bolus of 1x10$^6$ StB in 600 μl DMEM in the tail vein, while the rats in the StB7 and control group received a control injection of 600 μl DMEM. Seven days post-AMI, the rats in the StB7 and StB1+7 group received a bolus of 1x10$^6$ StB in 600 μl DMEM in the tail vein, while the rats in the StB1 and control group received antrol injection of 600 μl DMEM. At both time points, all injections were performed under 3% isoflurane anaesthesia and rats received ultrasound exposure following injection. Ultrasound was applied using a 1-MHz unfocused transducer (V303-SU, Panametrics Inc, Waltham, MA) or a 500kHz PZT transducer (V318, Panametrics Inc) coupled to an arbitrary waveform generator (33220A, Agilent, Palto Alto, CA) and a linear 60-dB power amplifier (150A100B, Amplifier Research, Bothell, WA). The transducer was placed parasternal at the anterior wall and location of the infarcted area and rats were exposed to 1 kHz pulse repetition frequency for one minute. The ultrasound
signal was monitored by a synchronized digital oscilloscope (GOULD DSO 465, Valley View, OH). Peak negative acoustic pressure was 100 kPa as verified with a calibrated hydrophone (PA076; Precision Acoustics, Dorchester, United Kingdom). Rats were sacrificed 42 days post-AMI, where after the hearts were isolated and fixed in 4% formaldehyde, sliced transversally and embedded in paraffin. For infarct size determination and macrophage quantification, two sections were examined per heart, one section approximately 2mm above the tip of the apex and the other approximately 2mm below the site where the LAD was ligated.

**Analysis of cardiac function**

For analysis of cardiac function, 2D-echocardiography was performed immediately prior to AMI, immediately prior to the second injection (7 days post-AMI) and immediately prior to sacrifice (42 days post-AMI), using a 13 MHz linear-array transducer (ProSound SSD-4000 PureHD, Aloka, Tokyo, Japan). Echocardiographic images were analysed using Image-Arena 2.9.1 (TomTec Imaging Systems, Unterschleissheim, Germany) To ensure that the (repeated) measurements in the rats were taken at the same mid-ventricular location, the echocardiographic images were recorded using the attachment site of the papillary muscle for orientation. For each rat and time point, lumen diameter and wall thickness was measured in three separate images, and the average values of the three images were used for the analysis. The fractional shortening (FS) was determined by calculating the degree of shortening of left ventricular diameter between end-systole and end-diastole. Contraction of posterior and anterior walls was determined by calculating the difference between end-systolic and end-diastolic wall thickness.

**Infarct size determination**

To determine the infarct size an Elastica von Giesson (EvG) staining was performed on 4 μm thick paraffin-embedded heart slides. Slides were placed in ethanol and washed in regular tap water. Next, the slides were placed in Lawson’s solution (Klinipath, Duiven, the Netherlands) for 30 minutes, followed a brief (several seconds) immersion in 100% ethanol. The slides were washed, stained with haematoxylin, washed again, and placed in Van Giesson solution (saturated picric acid solution with 0,075% fuchsine acid) for 5 minutes. Surplus fluid was drained after which the slides were air dried and covered. The slides were scanned using a Pannoramic DESK digital slide scanner (3DHistech Ltd., Budapest, Hungary), and surface areas of the magenta-stained infarct areas, as well as the total cross-sectional surface areas of the hearts, were measured using Pannoramic Viewer v1.15.3 (3DHistech Ltd.). The infarct area relative to the total cross-sectional surface was determined at two heights: ±2mm below the ligation and ±2mm above the apex. The infarct size was determined as the mean relative infarct size of both sections.
Immunohistochemical staining of macrophages
The number and the subtype of macrophages in the infarcted area was determined by immunohistochemistry on serial 4 μm thick paraffin-embedded heart slides. A mouse-anti-rat CD68 antibody was used to identify macrophages (1:100, RT, 60 min; AbD Serotec, Puchheim, Germany) after antigen retrieval with 0.1% pepsin (in 0.02M HCl, 37°C, 30 min). A mouse-anti-rat ED2 antibody was used to identify the anti-inflammatory subtype of macrophages (1:200, RT, 60 min; a gift from prof. C.D. Dijkstra, VUmc, Amsterdam, the Netherlands) after antigen retrieval through boiling the slides in 10mM sodium citrate buffer, pH 6.0 for 10 min. As secondary antibody Envision-HRP (1:200, 30 min, Dako, Glostrup, Denmark) was used. Staining was visualized using Envision-diaminobenzidin (Dako Cytomation). Control slides incubated with PBS instead of primary antibody yielded no staining (not shown). All CD68 and ED2 positive cells in the infarcted area were scored microscopically using a 20x objective (Zeiss, Oberkochen, Germany). For each rat, the CD68- and ED2-stainings were done on serial tissue slides. For the number of cells per mm² infarct area, the infarct area was measured again on the CD68-stained sections using Pannoramic Viewer v1.15.3, and these infarct sizes were used specifically for both CD68- and ED2-stainings.

Statistical analysis
Statistical analysis was performed with Prism 6.0 (Graphpad software, La Jolla, CA). Normality of each data set was analysed using the Shapiro-Wilk normality test. Infarct sizes, macrophage numbers and echocardiographic changes in anterior wall contraction and posterior wall thickness were analysed with the Mann-Whitney U-test, as not all data sets were normally distributed. Echocardiographic data compared between different time-points within the same experimental group were analysed with a paired T-test. Echocardiographic data compared between different experimental groups within the same time point were analysed with an independent T-test. A p-value below 0.05 was considered statistically significant for all analyses. Data-values in text are displayed as mean ± standard error of the mean.

RESULTS
The effect of different StemBell administration time points on fractional shortening
The effect of the StB administration on the fractional shortening (FS) was determined at baseline (day 0, immediately before induction of AMI), 7 days post-AMI and 42 days post-AMI (figure 1A). At baseline, the FS did not differ.
Figure 1. Fractional shortening
A. Time-lapsed echocardiographic image (left ventricular short-axis view) of a control rat created at day 0 and day 42, displaying the lumen diameter (yellow line) during systole (S) and diastole (D). AW: anterior wall. L: lumen. PW: posterior wall. B. Mean fractional shortening (systolic lumen diameter relative to diastolic lumen diameter) compared between different time points within each experimental group. C. Mean fractional shortening compared between different experimental groups within each time point. Error bar: Standard error of the means. *: p<0.05 compared to control group. **: p<0.01 compared to control group. ***: p<0.001 compared to day 0. #: p<0.05 compared to StB 7 group. The star colour above the data points in figure B indicates the group for which a significant difference was found. Of one control rat (all time points) and one rat from the StB1 group (day 0), echocardiographic images were of insufficient quality for analysis. Of one control rat (all time points) and two rats from the StB1 group (one rat: all time points, one rat: day 7), echocardiographic images were of insufficient quality for determination of fractional shortening.

between the groups (control: 0.50±0.02, StB1: 0.50±0.03, StB7: 0.50±0.02, StB1+7: 0.49±0.02).

At 7 days, the FS in the control group and StB7 group (0.31±0.03 and 0.33±0.02 respectively) was significantly decreased compared to baseline (figure 1B) as result of AMI. Interestingly, at 7 days the StB1 and StB1+7 groups showed an improvement in FS (0.45±0.02 and 0.42±0.04 respectively) compared to the control and StB7 groups, which was significant for the StB1 group (figure 1C).

At 42 days, the FS of the control group (0.28±0.02) did not differ significantly compared to day 7 and remained significantly lower compared to baseline (figure 1B). Compared to the control group, at 42 days the StB1 group (0.46±0.04), the StB7 (0.41±0.04) and the StB1+7 group (0.44±0.03) all showed a significantly improved FS, but did not differ significantly between each other (figure 1C).

Summarized, StB administered at 1 day post-AMI resulted in a higher FS on the short-term compared to the control group (7 days post-AMI), while on the long term (42 days post-AMI) StB administration at 1 day post-AMI, 7 days post-
AMI or at both time points all similarly counteracted the AMI-induced effects on FS.

The effect of different StemBell administration time points on cardiac muscle contraction

Next, we investigated the effects of StB administration on the contraction of the anterior wall (containing the infarction) and the (non-infarcted) posterior wall (figure 2A), expressed as the end-systolic wall thickness relative to the end-diastolic wall thickness. At baseline, the anterior wall contraction did not differ between groups.

Compared to baseline, at 7 days post-AMI a significant decrease in anterior wall contraction was observed for the control (from 1.73±0.05 to 1.24±0.14) and StB7 groups (from 1.78±0.09 to 1.26±0.08) (figure 2B). For the StB1 (from 1.68±0.04 to 1.52±0.04) and StB1+7 (from 1.71±0.05 to 1.51±0.05) the anterior wall contraction was significantly decreased as well compared to baseline, although it remained significantly higher compared to the StB7 group (figure 2C).

At 42 days, the anterior wall contraction was similar for all three StB groups (StB1: 1.46±0.09, StB7: 1.30±0.09, StB1+7: 1.37±0.04). Importantly, these values were all increased compared to the anterior wall contraction of the control group (1.09±0.04), although only significantly for the StB1 and StB 1+7 groups (figure 2C). Contraction of the posterior wall did not significantly differ between the different time points, nor between different groups (figure 2D).

Summarized, StB administration at 1 day post-AMI resulted in a significantly improved anterior wall contraction at 7 days post-AMI. In addition, at 42 days post-AMI all StB groups had a significantly improved anterior wall contraction compared to the control group.

The effect of different StemBell administration time points on ventricular remodelling

We were also interested whether the StB therapy had an effect on the end-diastolic ventricular wall thickness. In the (infarcted) anterior wall, the control group had a slight decline in wall thickness from baseline to 42 days post-AMI. In the StB1 and StB1+7 groups the wall thickness showed a slight increase from baseline to 42 days post-AMI. However, no significant differences were found between the groups compared per time point, nor between the different time points (figure 2E).

The posterior wall thickness was similar for all groups at baseline (control: 0.19±0.01 cm, StB1: 0.20±0.01 cm, StB7: 0.19±0.01 cm, StB1+7: 0.20±0.01 cm) and at 7 days post-AMI (control: 0.16±0.01 cm. StB1: 0.18±0.01 cm, StB7: 0.17±0.01 cm, StB1+7: 0.17±0.01 cm). At 42 days post-AMI, the control group had a significantly higher
Figure 2. Wall contraction and thickness
A. Time-lapsed echocardiographic image (left ventricular short-axis view) of a control rat created at day 0 and day 42, displaying the wall thickness (yellow line) during systole (S) and diastole (D). AW: anterior wall. L: lumen. PW: posterior wall. B+C. Mean contraction (systolic wall thickness relative to diastolic wall thickness) of the anterior wall, compared between different time points within each experimental group (B) and compared between different experimental groups within each time point (C). D. Mean contraction (systolic wall thickness relative to diastolic wall thickness) of the posterior wall, compared between different time points within each experimental group. E+F. Diastolic wall thickness of the anterior wall (E) and posterior wall (F) compared between different time points within each experimental group. G. Diastolic wall thickness of the posterior wall compared between different experimental groups within each time point. Error bar: Standard error of the means. *: p<0.05 compared to control group. **: p<0.01 compared to day 0. ***: p<0.001 compared to day 0. #: p<0.05 compared to 7 days post-AMI. The symbol colour above the data points in figure B indicates the group for which a significant difference was found. Of one control rat (all time points) and one rat from the StB1 group (day 0), echocardiographic images were of insufficient quality for wall analysis.

Posterior wall thickness compared to earlier time points (0.24±0.02 cm) (figure 2F). Remarkably, at day 42 all three StB groups did not display this increase in posterior wall thickness compared to earlier time points (all 0.19±0.01 cm). For the StB1 and StB7 group, the posterior wall thickness at 42 days post-AMI was significantly decreased compared to the control group (figure 2G).

Summarized, StB treatment prevented long-term (42 days post-AMI) posterior wall thickening independent of administration time point.
The effect of StemBell administration on infarct size

To determine whether the long-term beneficial functional effects of StB therapy were related to a decrease in infarct size, the infarct areas were quantitatively analysed using an EvG-staining at 42 days post-AMI (figure 3A). Compared to the control group (5.83±0.85%), in the StB1 group (3.63±1.25%), the StB7 group (7.72±1.56%) and the StB1+7 group (4.24±1.50%), the infarct size did not differ significantly. However, for the StB1 group the infarct size was significantly decreased compared to the StB7 group (figure 3B). Taken together, the long-term improvement of cardiac function does not correspond to a significant reduction in infarct size.

The effect of different StemBell administration time points on macrophages

Finally, we analysed whether the long-term beneficial effects of StB therapy were related to effects on cardiac inflammation. For this, we quantified the density of macrophages present in the heart 42 days post-AMI, as macrophages are known to remain present in increased numbers for an extended period of time in the infarcted myocardium while other inflammatory cells do not.19 Virtually no macrophages were observed outside of the infarct area in any of the groups (not shown). In the infarct area, the total number of macrophages in the control group (225±46 cells/mm²) did not significantly differ compared to the StB1 group (220±41 cells/mm²), the StB7 group (132±27 cells/mm²) or the StB1+7 group (182±31 cells/mm², figure 4A). Compared to StB1 however, the total number of macrophages were significantly decreased in the StB7 group. Similar to the total
Figure 4. Macrophages
A. Mean macrophage numbers per mm² of infarcted tissue, compared between experimental groups at day 42. B. Mean ED2-positive macrophage numbers in the infarct area compared between experimental groups at day 42. C. ED2-positive macrophage numbers, relative to total macrophage numbers, compared between experimental groups at day 42. Error bar: Standard error of the means. *: p<0.05. Of two control rats and one rat of the StB1+7 group, cardiac tissue was insufficient for macrophage analysis.

number of macrophages, the number of ED2-positive macrophages (the anti-inflammatory M2 macrophage subset) did not differ significantly between the control group (193±35 cells/mm²), the StB1 group (161±21 cells/mm²), the StB7 group (115±29 cells/mm²) and the StB1+7 group (134±23 cells/mm²)(figure 4B). Also, the percentage of ED2-positive macrophages relative to the total number of macrophages was comparable between groups (87±6%, 82±9%, 81±6% and 75±9% respectively for control, StB1, StB7 and StB1+7, figure 4C).

Summarized, the long-term functional improvement of the heart does not relate to the state of cardiac inflammation (macrophages) at day 42.

DISCUSSION

The aim of this study was to compare in rats the therapeutic efficacy of StB administration 1 day after post-AMI with administration 7 days post-AMI. At 42 days post-AMI, both time points of administration resulted in a similar
improvement of cardiac function. Additionally, StB administration prevented long-term posterior wall hypertrophy independent of administration time-point. However, despite that these long term effects were equal, StB administration 1 day post-AMI resulted in a faster recovery of cardiac function, measured at 7 days post-AMI. A second StB bolus administered at 7 days post-AMI provided no additional long-term functional benefits. Interestingly, the observed functional benefits did not correlate with a reduction in infarct size, nor a reduction of residual cardiac inflammation at 42 days post-AMI.

Theoretically, the fast recovery of cardiac function observed after StB administration 1 day post-AMI could have been caused by an attenuated infarct expansion during the first week following AMI. However, we did not find significant differences in infarct size between the groups at 42 days post-AMI. Also, the improvement of cardiac function appears to be primarily attributed to the non-infarcted areas of the myocardium. Despite the absence of a significant infarct size reduction, the contraction of the (infarcted) anterior wall was significantly improved at day 42 for the StB1 and StB1+7 groups compared to the control group. In line with this, cardiac hypertrophy in the non-infarcted myocardium is a well-known compensatory response to the reduced contractile function of the infarcted myocardium.\textsuperscript{20} The observed reduction in posterior wall hypertrophy for all StB groups is therefore possibly an indirect effect caused by the StB-induced improvement of anterior wall contraction.

It has been demonstrated before that AMI also results in a decrease in cardiomyocyte contractility outside of the infarct area.\textsuperscript{21} Paracrine factors secreted by the StBs may have improved cardiac function by improving cardiomyocyte contractility. Indeed, Takahashi \textit{et al.} previously demonstrated that bone marrow-derived stem cell (BMSC)-treated culture medium increased \textit{in vitro} contraction of individual cardiomyocytes.\textsuperscript{22} Which secreted factors caused this increased contractility was not determined however, nor has such an effect been demonstrated yet for ASCs.

In several animal studies, ASC administration at 1 day post-AMI has been studied before. Danoviz \textit{et al.} injected 1x10\textsuperscript{6} rat ASCs directly in the cardiac muscle 1 day post-AMI in rats, and observed an increased capillary density in the infarcted myocardium and decreased susceptibility to afterload stress 28 days post-AMI.\textsuperscript{23} In contrast, Zhu \textit{et al.} administrated a large number of human ASCs (1x10\textsuperscript{6}) intravenously 1 day post-AMI in rats and observed improvements in cardiac function (FS), increased capillary density in the infarcted myocardium, as well as a reduction in infarct size 28 days post-AMI.\textsuperscript{24} Our data show that using the StB technique, it is possible to improve cardiac function after ASC administration 1 day post-AMI, using a low cell quantity (1x10\textsuperscript{6}) and a low-invasive administration route (intravenously).

We previously compared intravenous administration of uncultured adipose tissue cell isolates (the so-called 'stromal vascular fraction' or 'SVF', 5x10\textsuperscript{6} cells) also between 1 day and 7 days post-AMI in rats. Here we found no change in
infarct size for SVF administered at day 1, while SVF administration at day 7 did result in a significant infarct size reduction. At day 1, the inflammation in the infarcted rat myocardium starts, while at day 7, inflammation is subsiding and reparative mechanisms have been activated. We previously hypothesized that these differences in cellular environment caused SVF administration to be less effective at day 1 compared to day 7. However, for StBs we found no indication in our current study that this inflammatory environment reduces the efficacy of administration at day 1 post-AMI. In addition, it is known that AMI causes inflammation in remote areas of the myocardium, and this is hypothesized to induce adverse remodelling outside of the infarct area. The paracrine effects of the StBs may attenuate this remote inflammation, and hereby improving contraction of the anterior wall. Further research is needed to confirm this however, as at 42 days post-AMI we saw no signs of inflammation outside the infarcted areas in any group.

Between day 3 and day 5 following AMI (in mice), a macrophage phenotype switch occurs in the infarcted myocardium, where pro-inflammatory M1 macrophages are replaced by M2 macrophages, which suppresses inflammation and stimulates repair. In AMI patients, higher levels of M2 monocytes in blood are associated with decreased infarct size and increased left ventricular ejection fraction. Importantly, BMSCs are known to influence this switch in favour of the M2 macrophage. StB administration 1 day post-AMI precedes this switch, and may therefore influence this process. In our study, StB administration did not appear to influence macrophage numbers or M1/M2 subtype composition compared to control at day 42 post-AMI, independent of administration time point. We cannot exclude however that ASCs did accelerate the macrophage transition towards a M2 phenotype in our StB-treated groups, although cardiac tissue obtained at earlier time points is needed to study this further.

Compared to StB administration at 1 day post-AMI alone, a second administration at 7 days post-AMI did not result in additional benefits. We were unable to find a reason why no cumulative improvement was observed for the StB1+7 group, as to the best of our knowledge we are the first to study this 'dual treatment' in an AMI animal model.

In our study, we did not measure cardiac function at the first time point of StB administration (1 day post-AMI). Therefore, based on our data alone we cannot rule out that the near-baseline FS measured at 7 days post-AMI after StB administration on day 1 is an inhibited decline of FS rather than a 'fast recovery'. However, from other studies using rat AMI models we do know that FS declines significantly already in the first day following AMI induction. Therefore, we consider a fast recovery a more likely scenario than a reduced decline. A second limitation is the fact that we chose to obtain cardiac tissue only at 42 days post-AMI, as the primary objective was to determine the effects of StB administration on cardiac function. However, because of this we could not study putative effects of StB administration on cardiac inflammation at earlier time points. Finally, the
low number of rats and large variance in some datasets may have contributed to an underestimation of differences between groups and time points.

The fast recovery of cardiac function after StB administration at 1 day post-AMI may be beneficial for patients. A large population-based study on AMI patients determined that mortality can be as high as 6.5% 7 days post-AMI, and more than half of these deaths occur after day 1. Therefore, our data indicate that StB administration 1 day post-AMI is preferable over 7 days. An additional practical advantage of StB administration 1 day post-AMI is that this allows the administration during the initial hospitalization period of the AMI patients, which in modern times is generally less than 4 days following the ischemic event.

References

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