Available online at www.sciencedirect.com

SciVerse ScienceDirect

www.elsevier.com/locate/scr

Mesenchymal stem cell-derived exosomes increase ATP levels, decrease oxidative stress and activate PI3K/Akt pathway to enhance myocardial viability and prevent adverse remodeling after myocardial ischemia/reperfusion injury

Fatih Arslan [a,b,c,*], Ruenn Chai Lai [d,e], Mirjam B. Smeets [a,b], Lars Akeroyd [a], Andre Choo [f], Eissa N.E. Aguor [a], Leo Timmers [a,c], Harold V. van Rijen [g], Pieter A. Doevendans [c], Gerard Pasterkamp [a], Sai Kiang Lim [d,h,**], Dominique P. de Kleijn [a,b,h]

[a] *Laboratory of Experimental Cardiology, University Medical Center Utrecht, Utrecht, the Netherlands*
[b] *The Netherlands Heart Institute (ICIN), Utrecht, the Netherlands*
[c] *Department of Cardiology, University Medical Center Utrecht, Utrecht, the Netherlands*
[d] *Institute of Medical Biology, Agency for Science Technology and Research (A*STAR), Singapore*
[e] *NUS Graduate School for Integrative Sciences and Engineering, National University of Singapore (NUS), Singapore*
[f] *Bioprocessing Technology Institute, A*STAR, Singapore*
[g] *Department of Medical Physiology, University Medical Center Utrecht, Utrecht, the Netherlands*
[h] *Department of Surgery, YLL School of Medicine, NUS, Singapore*

Abstract   We have previously identified exosomes as the paracrine factor secreted by mesenchymal stem cells. Recently, we found that the key features of reperfusion injury, namely loss of ATP/NADH, increased oxidative stress and cell death were underpinned by proteomic deficiencies in ischemic/reperfused myocardium, and could be ameliorated by proteins in exosomes. To test this hypothesis *in vivo*, mice (C57Bl6/J) underwent 30 min ischemia, followed by reperfusion (I/R injury). Purified exosomes or saline was administered 5 min before reperfusion. Exosomes reduced infarct size by 45% compared to saline treatment. Langendorff experiments revealed that intact but not lysed exosomes enhanced viability of the ischemic/reperfused myocardium. Exosome treated animals exhibited significant preservation of left ventricular geometry and

 * Correspondence to: F. Arslan, Laboratory of Experimental Cardiology, University Medical Center Utrecht, Heidelberglaan 100, Room G.02.523, 3584 CX Utrecht, the Netherlands. Fax: +31 30252 2693.
** Correspondence to: S.K. Lim, Institute of Medical Biology, Agency for Science Technology and Research (A*STAR), 8A Biomedical Grove, 138648, Singapore.
   E-mail addresses: f.arslan@umcutrecht.nl (F. Arslan), saikiang.lim@imb.a-star.edu.sg (S.K. Lim).

contractile performance during 28 days follow-up. Within an hour after reperfusion, exosome treatment increased levels of ATP and NADH, decreased oxidative stress, increased phosphorylated-Akt and phosphorylated-GSK-3β, and reduced phosphorylated-c-JNK in ischemic/reperfused hearts. Subsequently, both local and systemic inflammation were significantly reduced 24 h after reperfusion. In conclusion, our study shows that intact exosomes restore bioenergetics, reduce oxidative stress and activate pro-survival signaling, thereby enhancing cardiac function and geometry after myocardial I/R injury. Hence, mesenchymal stem cell-derived exosomes are a potential adjuvant to reperfusion therapy for myocardial infarction.

Introduction

Myocardial infarction (MI) and related complications (e.g. heart failure) are a great socio-economic burden to society and healthcare systems. Recent advances in (interventional) cardiology have resulted in timely and optimized coronary flow restoration through the culprit artery. Subsequently, more patients survive the initial infarction, but are susceptible to heart failure or other infarct-related complications (International Cardiovascular Disease Statistics, 2004). The major determinant for heart failure and other complications related to MI is infarct size (Schwartz et al., 2011). The increase in morbidity after MI triggered the search for adjunctive therapeutics to further limit excessive tissue loss and enhance cardiac performance. Pre-clinical studies show a great potential for engineered heart tissue for replacement therapy (Naito et al., 2006; Zimmermann et al., 2006) while stem cell injections may be promising in the treatment of patients with acute MI (Martin-Rendon et al., 2008). Interestingly, there is increasing evidence showing that the observed therapeutic effects are partly mediated by stem cell secretion. This so called 'paracrine hypothesis' has gained much attention and is supported by recent experimental data (Gnecchi et al., 2008). It has been shown that mesenchymal stem cell-conditioned medium (MSC-CM) enhances cardiomyocyte and/or progenitor survival after hypoxia-induced injury (Chimenti et al., 2010; Deuse et al., 2009; Gnecchi et al., 2006; Matsuura et al., 2009; Rogers et al., 2011). Furthermore, MSC-CM induces angiogenesis in the infarcted myocardium (Chimenti et al., 2010; Deuse et al., 2009; Li et al., 2010). We have shown in both murine and porcine models of myocardial ischemia/reperfusion (I/R) injury that MSC-CM reduces infarct size (Timmers et al., 2007). High performance liquid chromatography (HPLC) and dynamic light scatter (DLS) analyses revealed that MSCs secrete cardioprotective microparticles with a hydrodynamic radius ranging from 50 to 65 ηm (Chen et al., 2011; Lai et al., 2010b)). Furthermore, the therapeutic efficacy of MSC-derived exosomes was independent of the tissue source for the MSCs. For example, exosomes from human embryonic stem cell-derived MSCs were similar to those derived from other fetal tissue sources (e.g. limb, kidney). This suggests that secretion of therapeutic exosomes may be a general property of all MSCs (Lai et al., 2010a).

Exosomes are bi-lipid membrane vesicles with a diameter of 50–100 ηm. They are secreted by various cell types through the fusion of multivesicular bodies with the plasma membrane. Exosomes carry a complex cargo load of proteins and RNA with the potential to effect many cellular processes and pathways. They are involved in complex cellular interactions such as immune responses, intercellular communication and antigen presentation (Thery et al., 2009). Multivesicular bodies store the exosomes within the cell and release them upon fusion with the plasma membrane. The identification of exosomes as the cardioprotective factor in MSC secretion makes it a potential therapeutic tool in myocardial infarction. In contrast to cell-based therapy, MSC-derived exosomes provide an 'off-the-shelf' therapeutic. Furthermore, exosomes are potentially safer as they are non-viable and will incur less manufacturing and storage costs. Although we previously described that exosomes reduce infarct size in mice (Lai et al., 2010a) the functional consequence and the molecular alterations in vivo responsible for its cardioprotective actions remain unknown.

We have recently profiled the proteome of ischemic/reperfused mouse hearts (Li et al., 2012) and MSC-derived exosomes (Lai et al., 2013), to elucidate a molecular mechanism for the cardioprotective action of MSC-derived exosomes. Briefly, we found that the most significant proteomic changes in ischemic/reperfused mouse hearts were the depletion of proteins in fatty acid (FA) oxidation, glycolysis, tricarboxylic acid (TCA) cycle, redox homeostasis, gluthatione S-transferase and heat shock proteins. We also observed accumulation of proteins involved in apoptosis and the electron transport chain (ETC) (Li et al., 2012). Taken together, the observed proteomic changes predicted reduced ATP production, increased oxidative stress and apoptosis; all are key features of cell death after myocardial I/R injury (Ovize et al., 2010; Yellon and Hausenloy, 2007). Interestingly, the predicted biochemical changes as a result of the depletion or accumulation could be reversed or circumvented by the >800 proteins found in our MSC-derived exosomes (data available at www.exocarta.org; Lai et al., 2013. MSC exosomes were found to contain all five enzymes in the ATP generating stage of glycolysis namely glyceraldehyde 3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase (PGK), phosphoglucomutase (PGM), enolase (ENO) and pyruvate kinase m2 isoform(PKm2) as well as the phosphorylated PFKFB3 which upregulates phosphofructose kinase, the rate limiting glycolytic enzyme. This glycolytic potential was evident when exposure to MSC exosomes increased ATP production in oligomycin-treated H9C2 cardiomyocytes in which oxidative phosphorylation was inhibited. Furthermore, oxidative stress was reduced via peroxiredoxins and glutathione S-transferases in MSC-derived exosomes. Finally, we found that MSC-derived exosomes have enzymatically active CD73. CD73 is the major enzyme responsible for the formation of extracellular adenosine from released adenine nucleotides (Zimmermann, 2000). We have shown that exosomes activate adenosine receptors and induce adenosine-induced phosphorylation of ERK1/2 and Akt in H9C2 cardiomyocytes (Li et al., 2012).

In the present study, we examined the above-mentioned properties of MSC-derived exosomes to limit cell death after myocardial I/R injury in vivo. We show that intact exosomes

directly target cardiac cells to reduce infarct size. Furthermore, we demonstrate that, in line with our *in vitro* data, exosomes exert a therapeutic effect via increased ATP production, decreased oxidative stress and induced PI3K/Akt signaling.

Materials and methods

Animals and experimental design

Male C57Bl6/J mice (10–12 weeks old, 25–30 g) received standard diet and water *ad libitum*. Saline or exosomes were administered intravenously via the tail vein, 5 min before reperfusion. Myocardial infarction was induced by temporary left coronary artery ligation, just below the left atrial appendage as described previously (Arslan et al., 2010b). Where possible, recommendations from the National Heart Lung and Blood Institute (NHLBI) Working Group on the Translation of Therapies for Protecting the Heart from Ischemia (Bolli et al., 2004) were applied; the surgeon was blinded for the treatment. Digital images of the infarcts were encrypted before being analyzed by the researcher. Heart function and geometry assessments were done by a technician blinded to treatment. All animal experiments were performed in accordance with the Directive 2010/63/EU of the European Parliament, national guidelines on animal care and with prior approval by the Animal Experimentation Committee of Utrecht University.

Myocardial I/R injury *in vivo*

Mice were anesthetized with a mixture of fentanyl (Jansen-Cilag) 0.05 mg/kg, midazolam (Dormicum, Roche) 5 mg/kg and medetomidine 0.5 mg/kg through an intraperitoneal injection. The adequacy of anesthesia was monitored by checking interdigital pain reflexes, righting reflexes, limb/muscle tone and spontaneous breathing frequency. Core body temperature was maintained around 37 °C during surgery by continuous monitoring with a rectal thermometer and automatic heating blanket. Mice were intubated and ventilated (Harvard Apparatus Inc.) with 100% oxygen. The left coronary artery (LCA) was ligated for 30 min using an 8-0 Ethilon (Ethicon) with a section of polyethylene-10 tubing placed over the LCA. Ischemia was confirmed by bleaching of the myocardium and transient tachycardia. In sham operated animals the suture was placed beneath the LCA without ligating. Reperfusion was initiated by releasing the ligature and removing the polyethylene-10 tubing. Reperfusion of the endangered myocardium was characterized by typical hyperemia in the first few minutes. In a subgroup of mice, a piece of the loosened suture was left in place to determine the area-at-risk (AAR) during termination. The chest wall was closed and the animals received subcutaneously atipamezole (Antisedan, Pfizer) 2.5 mg/kg, flumazenil (Anexate, Roche) 0.5 mg/kg and buprenorphine (Temgesic, Schering-Plough) 0.1 mg/kg.

Prior to termination, mice were anesthetized as described above before cervical dislocation. Hereafter, the hearts were rinsed with saline and subsequently explanted for further biochemical and/or histological analysis.

Exosome purification

Exosomes were purified from huES9.E1 derived MSC conditioned media (CM) using HPLC, as described earlier (Lai et al., 2010a; Sze et al., 2007). Briefly, to prepare the CM, 80% confluent HuES9.E1 cell cultures were washed three times with PBS and cultured overnight in a chemically defined medium consisting of DMEM without phenol red (Invitrogen) and supplemented with insulin, transferrin, and selenoprotein (ITS; Invitrogen), 5 ng/ml FGF2 (Invitrogen), 5 ng/ml PDGF AB (Peprotech, Rocky Hill, NJ), glutamine–penicillin–streptomycin, and β-mercaptoethanol. The cultures were then rinsed three times with PBS, and fresh defined medium was added. After 3 days, the medium was collected and centrifuged at 500 *g*, and the supernatant was filtered using a 0.2-μm filter. No serum was used in the preparation of the CM and the cells were not stimulated.

Serum-free CM collected from MSCs culture was concentrated 50× by tangential flow filtration using a membrane with a 100 kDa MWCO (Sartorius, Goettingen, Germany). Hereafter, CM was passed through chromatography columns (TSK Guard column SWXL, 6×40 mm and TSK gel G4000 SWXL, 7.8×300 mm, Tosoh Corp., Tokyo, Japan). Exosomes were collected from the first peak of the elution, concentrated using 100 kDa MWCO filter (Sartorius). Exosomes were filtered with a 0.22 μm filter before storage at −80 °C.

Infarct size

Infarct size (IS) as a percentage of the area-at-risk (AAR) was determined using Evans' blue dye injection and TTC staining, 1 day after reperfusion (n = 6/group).

Mice were anesthetized as described above with a mixture of fentanyl, midazolam and medetomidine. The LCA was ligated once again at the level marked by the suture left in place. Evans' blue dye (4%) was injected via the thoracic aorta in a retrograde fashion. By doing so, one can demarcate non-ischemic region in order to determine the AAR; the extent of myocardial tissue that underwent ischemia (i.e. endangered myocardium). Hearts were rapidly explanted, rinsed in 0.9% saline and put in −20 °C freezer for 1 h. Hereafter, hearts were mechanically sliced into four 1-mm cross sections. Heart sections were incubated in 1% triphenyltetrazolium-chloride (Sigma-Aldrich) at 37 °C for 15 min before placing them in formaldehyde for another 15 min. Viable tissue stains red and infarcted tissue appears white. Heart sections were digitally photographed (Canon EOS 400D) under a microscope (Carl Zeiss®). IS, AAR and total LV area were measured using ImageJ software (version 1.34). Infarct size was corrected for the weight of the corresponding heart slice.

Myocardial I/R injury *ex vivo*

Mice (n = 4/group) were given heparin 50 IE subcutaneously and were operated under general anesthesia as described above with a mixture of fentanyl, midazolam and medetomidine. The suture was placed beneath the LCA *in vivo* without ligating. Hereafter, the heart was excised and aortic root was canulated and perfused in the Langendorff setup. After 10 min recovery, the suture was tightened to

induce ischemia for 30 min. Just 5 min prior to reperfusion, the perfusion buffer was changed for a second buffer containing 0.4 μg/ml MSC-derived exosomes or control. Reperfusion was initiated by releasing the suture. Following 3 h of reperfusion, Evans' blue dye was injected after re-ligating the suture to demarcate the AAR. Subsequently, TTC staining was performed for infarct size assessment.

Cardiac magnetic resonance imaging

Twenty-six mice (n = 10/group in ischemic and n = 6/group in sham operated mice) underwent serial assessment of cardiac dimensions and function by high resolution magnetic resonance imaging (MRI, 9.4 T, Bruker, Rheinstetten, Germany) under isoflurane anesthesia before, 1, 7 and 28 days after MI. Long axis and short axis images with 1.0 mm interval between the slices were obtained and used to compute end-diastolic volume (EDV, largest volume) and end-systolic volume (ESV, smallest volume). The ejection fraction (EF) was calculated as $100 \Box (EDV - ESV)/EDV$. Wall thickness (WT) and systolic wall thickening (SWT) were assessed from both the septum (remote myocardium) and free wall (infarct area). All MRI data are analyzed using Qmass digital imaging software (Medis, Leiden, the Netherlands).

Pressure—volume loop recordings

In a subset of mice, invasive assessment of cardiac performance and LV pressure development was performed 28 days after infarction. Mice were anesthetized as described above with a mixture of fentanyl, midazolam and medetomidine. A Millar 1.4 F pressure catheter (model SPR-839) was inserted in a retrograde fashion via the right common carotid artery. Systolic function was assessed by LV end-systolic pressure and dP/dtmax, whereas diastolic function by LV end-diastolic pressure and dP/dtmin.

Immunohistochemistry

Upon termination (as described above), hearts were rinsed with saline and excised and fixated in 4% formaldehyde and embedded in paraffin. Paraffin sections were stained for Ly-6G (for neutrophils; rat anti-mouse Ly-6G 1:100, Abcam, Cambridge, United Kingdom) and MAC-3 (for macrophages; rat anti-mouse MAC-3 1:30, BD Pharmingen, Breda, the Netherlands).

Sections were stained by overnight incubation with the first antibody at 4 °C for MAC-3 or by 1 h incubation at RT for Ly-6G. Before staining, sections were deparaffinized and endogenous peroxidase was blocked by 30 min incubation in methanol containing 1.5% H_2O_2. Antigen retrieval was performed by 20 min boiling in citrate buffer (MAC-3 and Ly-6G).

For MAC-3, sections were pre-incubated with normal goat serum and incubated with the primary antibody (1:30 overnight at 4 °C). Sections were then incubated for 1 h at RT with a biotin labeled secondary antibody, followed by 1 h incubation with streptavidin—horseradish peroxidase at RT and developed with AEC.

For Ly-6G, sections were incubated with the primary antibody (1:100 for 1 h at RT). Sections were then incubated for 30 min with a secondary antibody followed by 30 min incubation with Powervision poly-HRP anti-rabbit IgG

(ImmunoVision Technologies, Daily City, USA). The staining was immediately visualized with Vector NovaRED™ substrate kit following the manufacturer's instructions (Vector Laboratories Inc., Burlingame, USA).

Macrophage and neutrophil counts were assessed per heart section within the infarcted area using Olympus Cell^P software. The AEC (for MAC-3) and NovaRED™ (for Ly-6G) staining on cells were digitalized (i.e. RGB-HSI color codes) for each heart section. Neutrophils and macrophages were defined as "particles" with i) staining-positivity and ii) had an adjacent minimum pixel count of 5 for neutrophils and 20 for macrophages. Initial manual identification of neutrophils and macrophages revealed that a minimum pixel count of 5 and 20 correlates with the actual cell size. Each particle containing 5 or 20 adjacent pixels positive for, respectively, Ly-6G or MAC-3 staining was counted as 1 cell by the software.

Peripheral white blood cell (WBC) count was determined using a Cell-Dyn® 1800 (Abbott Laboratories) differential analyzer.

Protein isolation

Total protein was isolated from snap frozen infarcted heart sections using 40 mM Tris pH 7.4.

Flow cytometry

Phosphorylated target protein for Akt (Ser473), glucogen synthase kinase-3α/β (GSK-3α/β; Ser21/Ser9), extracellular signal-regulated kinases-1/2 (ERK1/2; Thr202/Tyr204), c-Jun N-terminal kinase (c-JNK; Thr183/Tyr185) were measured using the Bio-Plex Multiplex Assay (Bio-Rad Laboratories) according to the instructions of the manufacturer, after 1:5 dilution in assay buffer.

ATP/ADP and NADH/NAD+

ATP/ADP and NADH/NAD+ ratios were measured in the ischemic area of mice. MI was induced as described earlier. After 0 min, 5 min and 30 min of reperfusion, the ischemic area was excised and snap frozen using liquid nitrogen. Frozen tissue samples were homogenized with 1 ml extraction buffer and supernatant was collected after spinning at 14,000 g for 5 min. Hereafter, the supernatant was passed through 10 kDa MWCO filter (Biovision) before performing the ATP/ADP and NADH/NAD+ assays according to the manufacturer's protocol (Biovision, Mountain view, CA).

Oxidative stress assay

Oxidative stress was measured on 10 μg Tris-isolated protein using the Oxyblot protein oxidation detection kit (S7150; Millipore, Billerica MA, USA). Measurements were performed according to the manufacturer's instructions.

Statistical analysis

Data are represented as mean ± SEM. One-way ANOVA with post-hoc 2-sided Dunnett t-test adjustment (saline was set as control) was used for infarct size comparison between the

groups. Non-parametric t-test was used for 2 group comparisons. All statistical analyses were performed using SPSS 15.1.1. and $p < 0.05$ was considered significant. The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agreed to the manuscript as written.

Results

Intact exosomes reduce myocardial I/R injury through direct interaction with myocardial cells

Thirty minutes ischemia followed by 24 h reperfusion in saline treated animals resulted in $39 \pm 1.8\%$ infarction within the AAR (IS/AAR; Fig. 1A). A single intravenous bolus of exosomes 5 min prior to reperfusion reduced infarct size by 45% ($21 \pm 2.2\%$ IS/AAR; $p < 0.001$), in a dose dependent manner. Infarct size reduction in myocardial I/R injury is achieved by either enhanced cardiomyocyte viability, reduced detrimental activation of blood components or both Arslan et al., 2008, 2010b; Yellon and Hausenloy, 2007). We performed *ex vivo* I/R experiments in the Langendorff setup, to determine the relative contribution of parenchymal and circulating cells to the cardioprotection mediated by exosomes. Three hours reperfusion with normal buffer after 30 min ischemia resulted in $49 \pm 5.3\%$ IS/AAR. Exosome-containing buffer perfusion just prior to reperfusion significantly reduced infarct size to the same extent as in the *in vivo* situation ($23 \pm 1.5\%$ IS/AAR; $p = 0.002$). This suggests that exosomes interact directly with cardiac cells to reduce infarct size. It is postulated that exosomes are internalized by target cells by endocytosis or phagocytosis in order to release their content (Feng et al., 2010; Tian et al., 2010). For this reason, we tested if the cardioprotection was dependent on the integrity of the exosomes as lipid
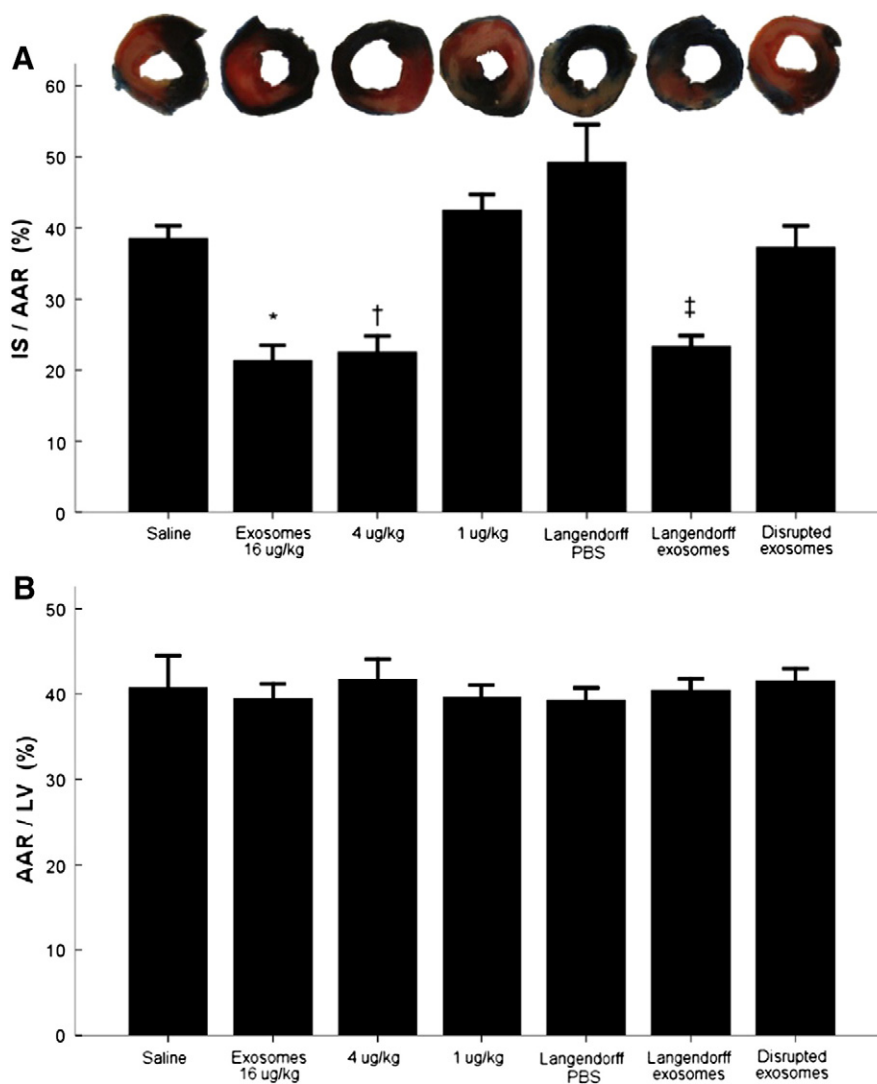

Figure 1 MSC-derived exosomes reduce myocardial I/R injury *in vivo* and *ex vivo*. A, Infarct size (IS) as a percentage of the area-at-risk (AAR) 1 day after I/R injury; *$p < 0.001$, †$p = 0.001$ and ‡$p = 0.001$ compared to saline. B, AAR as a percentage of the left ventricle (LV). Each bar represents mean±SEM, n=6/group for *in vivo* and n=4/group for *ex vivo* experiments.

microvesicles and not just their cargo. Exosomes were physically disrupted by vigorous agitation in a homogenizer before administration. The homogenized exosomes failed to reduce infarct size *in vivo* (37 ± 3.0% IS/AAR; *p* = .994). In all experiments, the extent of myocardium at risk was similar in all groups (mean AAR/LV = 40 ± 1.1%; Fig. 1B).

Exosome treatment prevents left ventricular dilatation and improves cardiac performance

We next examined the functional consequences of exosome treatment during 28 days follow-up. We performed serial cardiac MRI measurements to assess both left ventricular (LV) function and geometry after I/R injury. There were no differences in LV function and dimensions at baseline. The

infarct size reduction in exosome treated animals translated into significant preservation of both end-diastolic and -systolic volume (EDV, ESV) during follow-up. In addition, ejection fraction was significantly improved after exosome treatment at all time points (Figs. 2A−C). Wall thickness (WT) of the infarcted area (free wall) was equally increased in saline and exosome treated animals 1 day after reperfusion (Fig. 2D), presumably caused by tissue edema. Although infarct size was reduced, exosome treatment did not reduce reperfusion-induced edema as shown by equal increase in thickness of the free wall (infarct area) 1 day after reperfusion. The similar extent of tissue edema at day 1 in the MRI studies indicated that the amount of endangered myocardium did not differ between the groups. However, exosome treatment resulted in decreased thinning of the infarct area during scar maturation. This finding was in line
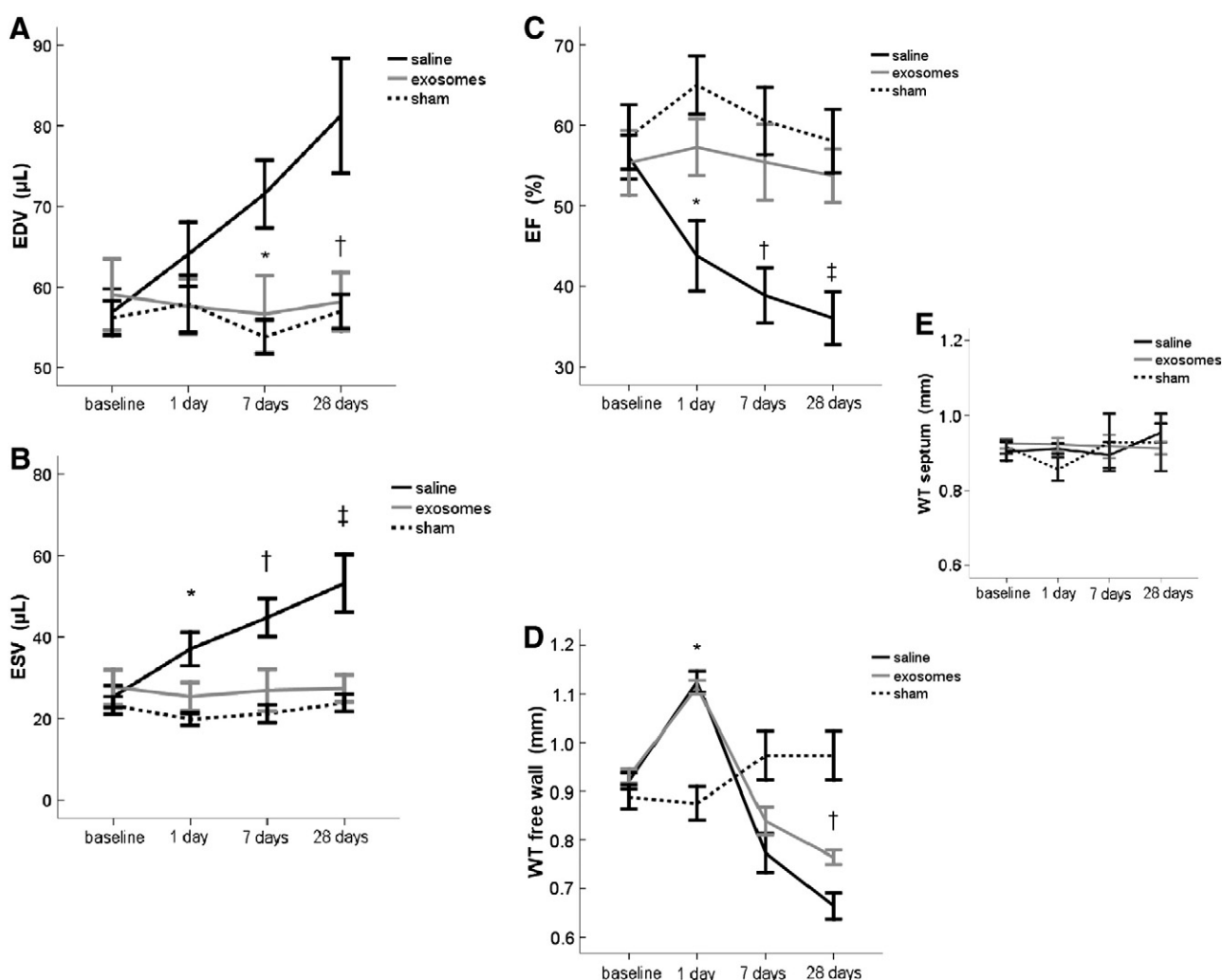

Figure 2 MSC-derived exosomes prevent LV dilation and improve systolic function after myocardial I/R injury. A, End-diastolic volume (EDV, μl); *p = 0.029 and †p = 0.006 compared to saline. B, End-systolic volume (ESV); *p = 0.04, †p = 0.017 and ‡p = 0.002 compared to saline. C, Ejection fraction (EF, %); *p = 0.035, †p = 0.015 and ‡p = 0.002 compared to saline. D, Wall thickness of the infarct area (WT, mm); *p < 0.001 compared to baseline and †p = 0.04 compared to saline. E, WT of the remote myocardium. F, systolic wall thickening (SWT, %); *p = 0.011, †p = 0.042 and ‡p = 0.040 compared to saline. G, SWT of the remote myocardium; *p = 0.012 compared to saline. Representative MRI images are shown. Note the increased LV dimensions at systole and diastole in saline treated animals. Each bar represents mean ± SEM, n = 10/group for ischemic/reperfused animals n = 6/group for sham operated mice.
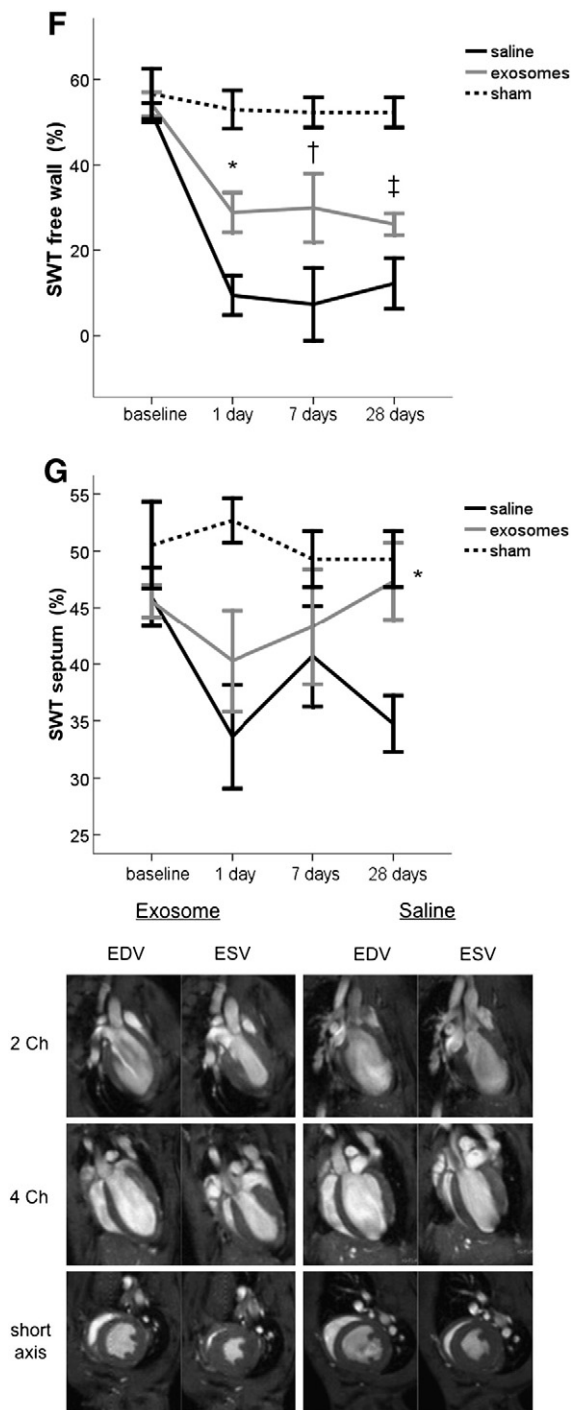
Figure 2 (contnued).

Table 1 Invasive left ventricular pressure measurements 28 days after infarction.

	Saline	Exosomes	p
BPM	480 ± 7	476 ± 3	.60
ESP	110 ± 2	137 ± 10	.12
EDP	15 ± 1	7 ± 1	.009
dP/dT$_{max}$	7496 ± 152	8951 ± 246	.009
dP/dT$_{min}$	-6680 ± 75	-8241 ± 396	.009

Data are represented as mean $\pm$ SEM, $n = 5$/group. BPM = beats per minute, ESP = end-systolic pressure, EDP = end-diastolic pressure.

with the infarct size reduction seen after exosome treatment. There were no differences in wall thickness of the remote myocardium between the groups (Fig. 2E). The higher extent of viable tissue after exosome treatment also significantly improved systolic thickening of the infarcted area at all time points (Fig. 2F), and in the remote myocardium 28 days after infarction (Fig. 2G).

At day 28, we also assessed the hemodynamic consequences using invasive LV pressure−volume loop recordings after exosome treatment. Consistent with improved LV function and geometry, we observed higher contractility and relaxation and reduced end-diastolic pressure (EDP) in exosome treated animals (Table 1).

Exosome treatment restores energy depletion and redox state within 30 min after I/R

Mitochondrial dysfunction is one of the most important determinants of viability loss after I/R injury (Murphy and Steenbergen, 2008). Mitochondrial dysfunction aggravates the loss of ATP and NADH during ischemia where Kreb's cycle and oxidative phosphorylation are inhibited by the lack of oxygen. During reperfusion, ATP production is again disrupted by depletion of enzymes essential for ATP production. Restoring ATP and NADH levels will therefore be critical for the recovery of the ischemic/reperfused myocardium. We observed that within 30 min after reperfusion, the ATP/ADP and NADH/NAD+ ratios in exosome-treated animals were significantly increased (Figs. 3A and B). In addition, the extent of oxidative stress, as a hallmark feature of myocardial I/R injury, was also reduced within the first hour of reperfusion (Fig. 3C). These data demonstrate that exosome treatment is highly effective in eliciting a rapid biochemical response to restore ATP and NADH levels and to reduce oxidative stress.

Exosome treatment phosphorylates Akt/GSK3 pathway and inhibits c-JNK signaling after I/R

Paracrine action of MSC secretion has been shown to be mediated via enhanced phosphorylation of survival pathways, especially of PI3K/Akt pathway (Chimenti et al., 2010; Deuse et al., 2009; Gnecchi et al., 2006; Gnecchi et al., 2008; Matsuura et al., 2009). Recently, we have shown that exosomes can activate adenosine receptors and subsequently phosphorylate PI3K/Akt pathway *in vitro* using H9C2 cardiomyocytes (Li et al., 2012). In the present study, exosome treatment elicited an immediate and significant increase in Akt and GSK3 phosphorylation within an hour after reperfusion, and this increase was maintained over the next 24 h (Figs. 4A and B). ERK1/2 phosphorylation has also shown to be protective in myocardial I/R injury (Murphy and Steenbergen, 2008), however, was not altered by exosome treatment (Fig. 4C). Phosphorylation of c-JNK, a known activator of pro-apoptotic signaling, was rapidly and significantly reduced in exosome treated animals within 1 h reperfusion (Fig. 4D). Total protein levels did not differ between saline and exosome treated animals (Fig. S1).
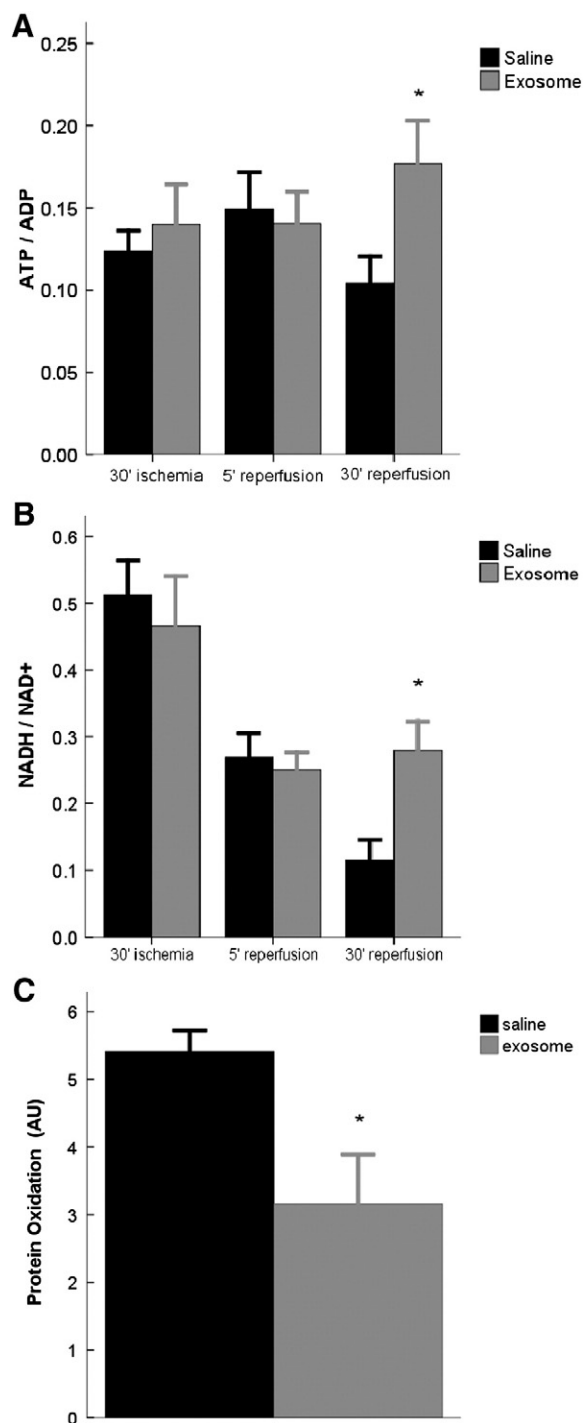
Figure 3 MSC-derived exosomes restore ATP/ADP, NADH/NAD+ levels and reduce oxidative stress. Tissue levels of A, ATP/ADP; *p=0.047 compared to saline. B, NADH/NAD+; *p=0.027 compared to saline. C, Mean oxidized protein levels at 1 h reperfusion; *p=0.043 compared to saline. Each bar represents mean±SEM, n=6/group/time point.

Exosome treatment reduces inflammation after I/R

Myocardial I/R is characterized by accentuated cardiac and systemic inflammation (Arslan et al., 2008, 2010a, 2011).

Cardiac inflammation was assessed by neutrophil and macrophage infiltration. Exosome treatment reduced neutrophil and macrophage infiltration in the hearts at days 1 and 3 after reperfusion (Figs. 5A and B). High peripheral white blood cell (WBC) count is associated with larger infarct size, worse cardiac performance and poor clinical outcome (Barron et al., 2001; Bauters et al., 2007; Chia et al., 2009; Prasad et al., 2007; Smit et al., 2008). In line with reduced infarct size and improved cardiac performance, exosome treated animals exhibited significantly reduced WBC count compared to saline treated mice after I/R injury (Fig. 5C).

Discussion

Previous studies have shown that MSC transplantation improves cardiac function after infarction. The initial hypothesis that this efficacy was mediated by the engraftment and differentiation of transplanted MSCs to replace injured tissues is increasingly untenable. Most studies suggest that MSCs mediate their therapeutic efficacy through the secretion of paracrine factors (Chimenti et al., 2010; Gnecchi et al., 2005, 2006, 2008). Like others (Deuse et al., 2009; Matsuura et al., 2009; Rogers et al., 2011), we have demonstrated that culture medium conditioned by MSCs reduces I/R injury in a pig and mouse model (Timmers et al., 2007). We identified the active component in this CM as an exosome, a 50−100 ηm bi-lipid membrane secreted microvesicle (Lai et al., 2010a, 2010b).

In this study, we demonstrated that exosomes not only reduced infarct size, but also resulted in a long-term preservation of cardiac function and reduced adverse remodeling. Interestingly, the therapeutic effect of MSC-derived exosomes was dependent on their physical integrity such that vigorous agitation and homogenization, likely to disrupt the bi-lipid membrane, prevented cardioprotection. These observations are consistent with the reported uptake of exosomes by cells via endocytosis or phagocytosis (Feng et al., 2010; Tian et al., 2010). More specifically, we have also demonstrated that MSC-derived exosomes are taken up by H9C2 cardiomyocytes (Chen et al., 2010). Furthermore, *ex vivo* Langendorff experiments revealed that exosomes are able to reduce infarct size to the same extent as in the *in vivo* situation. These findings strongly indicate that exosomes exert their therapeutic effect via viability enhancement of cardiac tissue and do not require the presence of circulating blood cells. Unfortunately, we were not able to follow exosomes *in vivo* after administration. Both fluorescent (GFP[pos]-MSC secreted GFP[pos]-exosomes and *ex vivo* protein labeling) and radioactive (^{111}In-oxinate) labeling proved to be too insensitive for detection (data not shown). The main issue is that the efficacious dosage is relatively low at 0.1−0.4 μg per mouse. More sensitive labeling techniques are needed to explore the dynamics of exosomes after *in vivo* administration.

Our recent proteomic profiling of the ischemic/reperfused heart (Li et al., 2012) and MSC-derived exosome (Li et al., 2012; www.exocarta.org) demonstrated that many of the hallmark biochemical features of reperfusion injury, namely ATP deficit, oxidative stress and cell death are underpinned by either depletion or accumulation of proteins in the reperfused ischemic heart tissues. MSC-derived
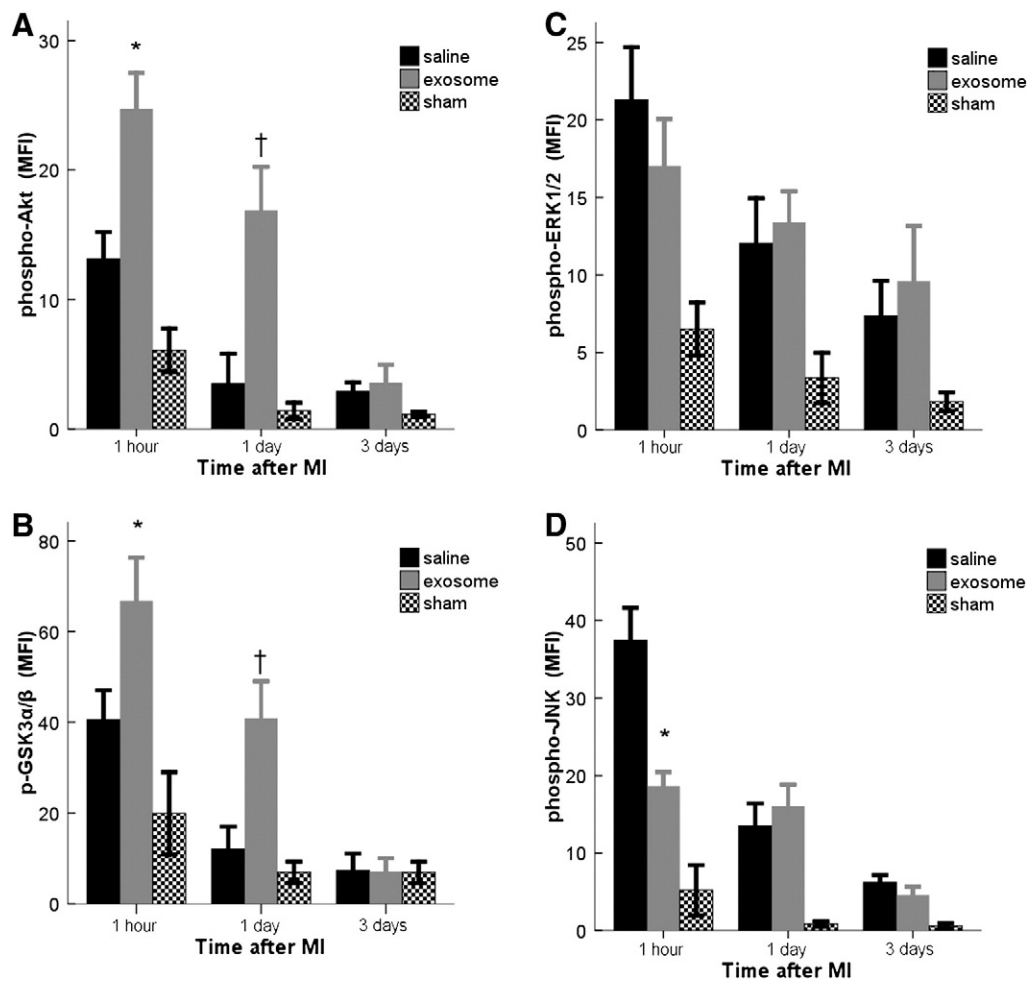
Figure 4 MSC-derived exosomes induce phosphorylation of Akt and GSK3, and reduce c-JNK phosphorylation after myocardial I/R injury. Tissue levels of A, phospho-Akt (MFI); *$p = 0.025$ and †$p = 0.025$ compared to saline. B, phospho-GSK3α/ß; *$p = 0.025$ and †$p = 0.016$ compared to saline. C, phospho-ERK1/2. D, phospho-c-JNK; *$p = 0.045$ compared to saline. Each bar represents mean ± SEM, n = 6/group/time point.

exosomes have either complementary or compensating proteins to revert or circumvent these biochemical features. Based on the proteomic composition of the ischemic heart and exosomes, we hypothesized that MSC-derived exosomes elicited protection against myocardial I/R injury. The mode of action may be by replenishing depleted glycolytic enzymes to increase ATP production and supplementing the reperfused cardiomyocytes with additional protein components of the cellular antioxidant system (such as the peroxiredoxins and glutathione S-transferases) to reduce oxidative stress and activating adenosine-mediated RISK pathway to reduce cell death (Li et al., 2012).

Consistent with our hypothesis, we observed that within an hour of reperfusion, exosome treatment significantly increased tissue level of ATP and NADH in the heart. We presumed that this increase resulted from increased glycolysis through replenishment by glycolytic enzymes from exosomes. It should be noted that in ischemic/reperfused myocardium, only glycolytic but not the ETC proteins were depleted (Li et al., 2012). In fact, the level of several ETC proteins was increased. Therefore increasing glycolytic flux in the reperfused ischemic myocardium would increase ATP production not only by

glycolysis but also by oxidative phosphorylation. Similarly, we hypothesized that anti-oxidants such as peroxiredoxins and glutathione S-transferases in exosomes could supplement depleted cellular antioxidants in ischemic/reperfused myocardium. In line with our hypothesis, oxidative stress was reduced in exosome-treated mice. Finally, we also predicted that the presence of enzymatically active CD73 on exosomes would produce adenosine from extracellular ATP to activate RISK and enhance myocardial viability (Hausenloy and Yellon, 2004). The importance of RISK such as PI3 kinase—Akt in limiting reperfusion injury was recently highlighted in a position paper from the European Society of Cardiology (Ovize et al., 2010). Adenosine has been shown to be efficacious in limiting infarct size in animal models and human trials (Headrick and Lasley, 2009). More importantly, CD73 has been shown to be the major enzyme responsible for the formation of extracellular adenosine from released adenine nucleotides (Zimmermann, 2000). Consistent with the observed infarct size reduction, exosome treatment significantly increased Akt and GSK3 phosphorylation. Incidentally, pro-apoptotic phosphorylation of c-JNK was reduced. The cause of this reduction remains to be determined.
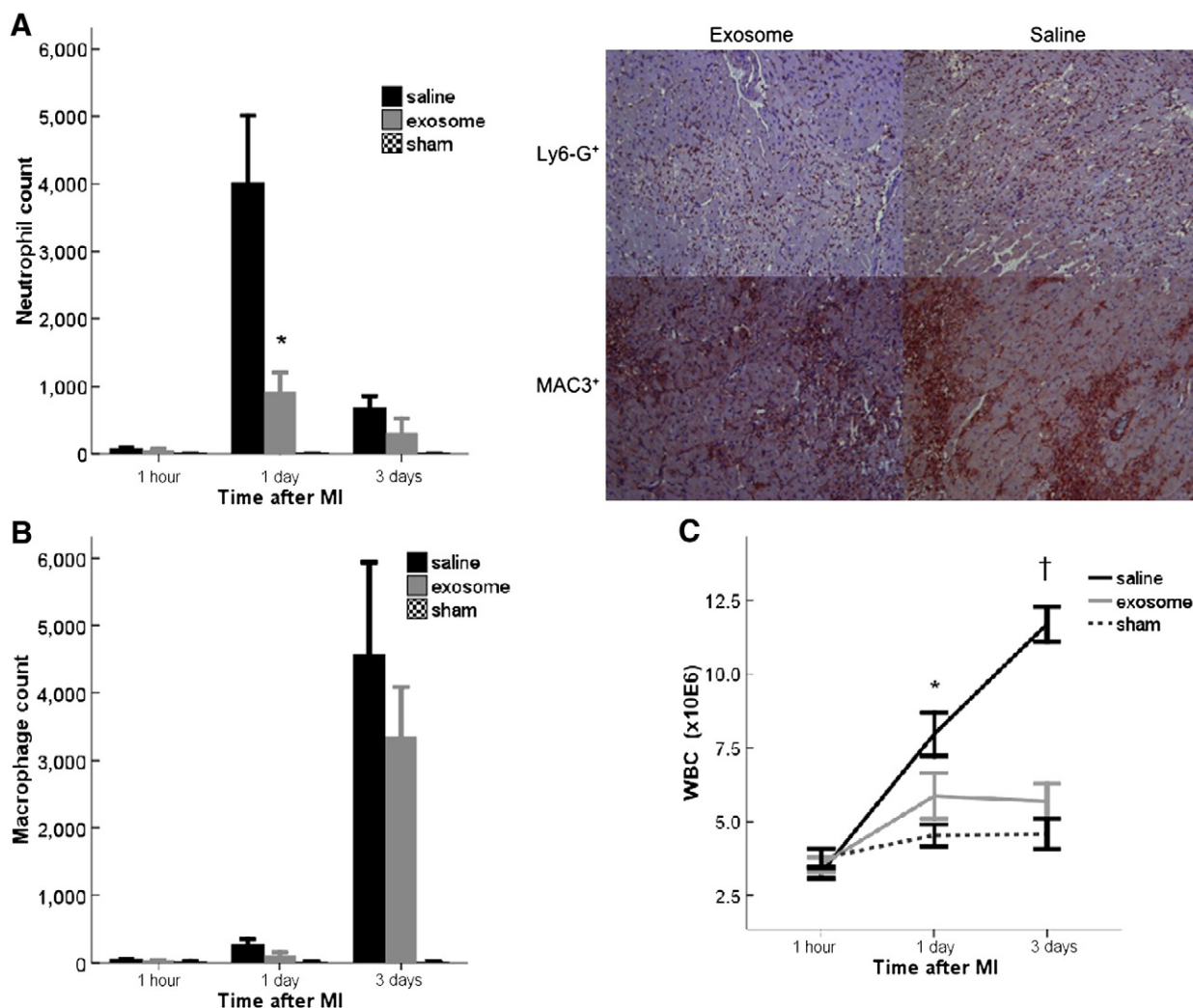
Figure 5 MSC-derived exosomes reduce inflammation after myocardial I/R injury. A, Neutrophil influx (Ly6-G^{pos} cells per heart section); *p=0.016 compared to saline. B, Macrophage influx (MAC3^{pos} cells per heart section). Representative staining are shown. C, Peripheral white blood cell counts after myocardial I/R injury; *p=0.045 and †p=0.009 compared to saline. Each bar represents mean±SEM, n=6/group/time point.

Finally, we observed decreased leukocyte influx in exosome treated animals. It is likely that the reduced influx is secondary to the reduced cardiac injury after exosome treatment, since leukocyte influx did not differ between the groups at 1 h reperfusion. In addition, we found that the reduced infarct size and improved cardiac performance were associated with low WBC counts in exosome treated animals. This finding is in line with clinical studies in which infarct size and clinical outcome is directly proportional to WBC count (Barron et al., 2001; Bauters et al., 2007; Chia et al., 2009; Prasad et al., 2007).

The identification of exosomes as the cardioprotective factor in MSC secretion provides an attractive safer and less costly 'off-the-shelf' therapeutic alternative to cell therapy in the treatment of myocardial infarction. Exosomes will become more attractive if we are able to replicate their efficacy in an ongoing study involving intracoronary injection of exosomes during the reperfusion phase in a porcine model of myocardial I/R injury (Arslan et al., 2012).

Conclusions

We have shown that MSC-derived exosomes rapidly activate multiple cardioprotective pathways to reduce infarct size and prevent heart function deterioration after myocardial I/R injury. Our study demonstrates that a single intravenous administration of MSC-derived exosomes is effective when administered in the late ischemic period, just prior to reperfusion. For these reasons, MSC-derived exosomes are a potential candidate for adjunctive therapy for patients suffering from acute myocardial infarction.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2013.01.002.

Funding

Acknowledgments

Disclosures

None declared.

References

Arslan, F., de Kleijn, D.P., Timmers, L., Doevendans, P.A., Pasterkamp, G., 2008. Bridging innate immunity and myocardial ischemia/reperfusion injury: the search for therapeutic targets. Curr. Pharm. Des. 14, 1205–1216.

Arslan, F., Keogh, B., McGuirk, P., Parker, A.E., 2010a. TLR2 and TLR4 in ischemia reperfusion injury. Mediat. Inflamm. 2010, 704202.

Arslan, F., Smeets, M.B., O'Neill, L.A., Keogh, B., McGuirk, P., Timmers, L., Tersteeg, C., Hoefer, I.E., Doevendans, P.A., Pasterkamp, G., de Kleijn, D.P., 2010b. Myocardial ischemia/reperfusion injury is mediated by leukocytic toll-like receptor-2 and reduced by systemic administration of a novel anti-toll-like receptor-2 antibody. Circulation 121, 80–90.

Arslan, F., de Kleijn, D.P., Pasterkamp, G., 2011. Innate immune signaling in cardiac ischemia. Nat. Rev. Cardiol. 8, 292–300.

Arslan, F., Houtgraaf, J.H., Keogh, B., Kazemi, K., de, J.R., McCormack, W.J., O'Neill, L.A., McGuirk, P., Timmers, L., Smeets, M.B., Akeroyd, L., Reilly, M., Pasterkamp, G., de Kleijn, D.P., 2012. Treatment with OPN-305, a humanized anti-Toll-like receptor-2 antibody, reduces myocardial ischemia/reperfusion injury in pigs. Circ. Cardiovasc. Interv. 5, 279–287.

Barron, H.V., Harr, S.D., Radford, M.J., Wang, Y., Krumholz, H.M., 2001. The association between white blood cell count and acute myocardial infarction mortality in patients > or =65 years of age: findings from the cooperative cardiovascular project. J. Am. Coll. Cardiol. 38, 1654–1661.

Bauters, A., Ennezat, P.V., Tricot, O., Lallemant, R., Aumegeat, V., Segrestin, B., Quandalle, P., Lamblin, N., Bauters, C., 2007. Relation of admission white blood cell count to left ventricular remodeling after anterior wall acute myocardial infarction. Am. J. Cardiol. 100, 182–184.

Bolli, R., Becker, L., Gross, G., Mentzer Jr., R., Balshaw, D., Lathrop, D.A., 2004. Myocardial protection at a crossroads: the need for translation into clinical therapy. Circ. Res. 95, 125–134.

Chen, T.S., Lai, R.C., Lee, M.M., Choo, A.B., Lee, C.N., Lim, S.K., 2010. Mesenchymal stem cell secretes microparticles enriched in pre-microRNAs. Nucleic Acids Res. 38, 215–224.

Chen, T.S., Arslan, F., Yin, Y., Tan, S.S., Lai, R.C., Choo, A.B., Padmanabhan, J., Lee, C.N., de Kleijn, D.P., Lim, S.K., 2011. Enabling a robust scalable manufacturing process for therapeutic exosomes through oncogenic immortalization of human ESC-derived MSCs. J. Transl. Med. 9, 47.

Chia, S., Nagurney, J.T., Brown, D.F., Raffel, O.C., Bamberg, F., Senatore, F., Wackers, F.J., Jang, I.K., 2009. Association of leukocyte and neutrophil counts with infarct size, left ventricular function and outcomes after percutaneous coronary intervention for ST-elevation myocardial infarction. Am. J. Cardiol. 103, 333–337.

Chimenti, I., Smith, R.R., Li, T.S., Gerstenblith, G., Messina, E., Giacomello, A., Marban, E., 2010. Relative roles of direct regeneration versus paracrine effects of human cardiosphere-derived cells transplanted into infarcted mice. Circ. Res. 106, 971–980.

Deuse, T., Peter, C., Fedak, P.W., Doyle, T., Reichenspurner, H., Zimmermann, W.H., Eschenhagen, T., Stein, W., Wu, J.C., Robbins, R.C., Schrepfer, S., 2009. Hepatocyte growth factor or vascular endothelial growth factor gene transfer maximizes mesenchymal stem cell-based myocardial salvage after acute myocardial infarction. Circulation 120, S247–S254.

Feng, D., Zhao, W.L., Ye, Y.Y., Bai, X.C., Liu, R.Q., Chang, L.F., Zhou, Q., Sui, S.F., 2010. Cellular internalization of exosomes occurs through phagocytosis. Traffic 11, 675–687.

Gnecchi, M., He, H., Liang, O.D., Melo, L.G., Morello, F., Mu, H., Noiseux, N., Zhang, L., Pratt, R.E., Ingwall, J.S., Dzau, V.J., 2005. Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. Nat. Med. 11, 367–368.

Gnecchi, M., He, H., Noiseux, N., Liang, O.D., Zhang, L., Morello, F., Mu, H., Melo, L.G., Pratt, R.E., Ingwall, J.S., Dzau, V.J., 2006. Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. FASEB J. 20, 661–669.

Gnecchi, M., Zhang, Z., Ni, A., Dzau, V.J., 2008. Paracrine mechanisms in adult stem cell signaling and therapy. Circ. Res. 103, 1204–1219.

Hausenloy, D.J., Yellon, D.M., 2004. New directions for protecting the heart against ischaemia–reperfusion injury: targeting the reperfusion injury salvage kinase (RISK)-pathway. Cardiovasc. Res. 61, 448–460.

Headrick, J.P., Lasley, R.D., 2009. Adenosine receptors and reperfusion injury of the heart. Handb. Exp. Pharmacol. 189–214.

International Cardiovascular Disease Statistics, 2004. American Heart Association.

Lai, R.C., Arslan, F., Lee, M.M., Sze, N.S., Choo, A., Chen, T.S., Salto-Tellez, M., Timmers, L., Lee, C.N., El Oakley, R.M., Pasterkamp, G., de Kleijn, D.P., Lim, S.K., 2010a. Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. Stem Cell Res. 4, 214–222.

Lai, R.C., Arslan, F., Tan, S.S., Tan, B., Choo, A., Lee, M.M., Chen, T.S., Teh, B.J., Eng, J.K., Sidik, H., Tanavde, V., Hwang, W.S., Lee, C.N., El Oakley, R.M., Pasterkamp, G., de Kleijn, D.P., Tan, K.H., Lim, S.K., 2010b. Derivation and characterization of human fetal MSCs: an alternative cell source for large-scale production of cardioprotective microparticles. J. Mol. Cell. Cardiol. 48, 1215–1224.

Lai, R.C., Yeo, R.W.Y., Tan, S.S., Zhang, B., Yin, Y., Sze, N.S.K., Choo, A., Lim, S.K., 2013. Mesenchymal Stem Cell Exosomes: The Future MSC-Based Therapy? Mesenchymal Stem Cell Therapy vol. 39.

Li, H., Zuo, S., He, Z., Yang, Y., Pasha, Z., Wang, Y., Xu, M., 2010. Paracrine factors released by GATA-4 overexpressed mesenchymal stem cells increase angiogenesis and cell survival. Am. J. Physiol. Heart Circ. Physiol. 299, H1772–H1781.

Li, X., Arslan, F., Ren, Y., Adav, S.S., Poh, K.K., Sorokin, V., Lee, C.N., de, K.D., Lim, S.K., Sze, S.K., 2012. Metabolic adaptation to a disruption in oxygen supply during myocardial ischemia and reperfusion is underpinned by temporal and quantitative changes in the cardiac proteome. J. Proteome Res. 11, 2331–2346.

Martin-Rendon, E., Brunskill, S., Doree, C., Hyde, C., Watt, S., Mathur, A., Stanworth, S., 2008. Stem cell treatment for acute myocardial infarction. Cochrane Database Syst. Rev. CD006536.

Matsuura, K., Honda, A., Nagai, T., Fukushima, N., Iwanaga, K., Tokunaga, M., Shimizu, T., Okano, T., Kasanuki, H., Hagiwara, N., Komuro, I., 2009. Transplantation of cardiac progenitor cells ameliorates cardiac dysfunction after myocardial infarction in mice. J. Clin. Invest. 119, 2204–2217.

Murphy, E., Steenbergen, C., 2008. Mechanisms underlying acute protection from cardiac ischemia–reperfusion injury. Physiol. Rev. 88, 581–609.

Naito, H., Melnychenko, I., Didie, M., Schneiderbanger, K., Schubert, P., Rosenkranz, S., Eschenhagen, T., Zimmermann, W.H., 2006. Optimizing engineered heart tissue for therapeutic applications as surrogate heart muscle. Circulation 114, I72–I78.

Ovize, M., Baxter, G.F., Di, L.F., Ferdinandy, P., Garcia-Dorado, D., Hausenloy, D.J., Heusch, G., Vinten-Johansen, J., Yellon, D.M., Schulz, R., 2010. Postconditioning and protection from reperfusion injury: where do we stand? Position paper from the Working Group of Cellular Biology of the Heart of the European Society of Cardiology. Cardiovasc. Res. 87, 406–423.

Prasad, A., Stone, G.W., Stuckey, T.D., Costantini, C.O., Mehran, R., Garcia, E., Tcheng, J.E., Cox, D.A., Grines, C.L., Lansky, A.J., Gersh, B.J., 2007. Relation between leucocyte count, myonecrosis, myocardial perfusion, and outcomes following primary angioplasty. Am. J. Cardiol. 99, 1067–1071.

Rogers, T.B., Pati, S., Gaa, S., Riley, D., Khakoo, A.Y., Patel, S., Wardlow, R.D., Frederick, C.A., Hall, G., He, L.P., Lederer, W.J., 2011. Mesenchymal stem cells stimulate protective genetic reprogramming of injured cardiac ventricular myocytes. J. Mol. Cell Cardiol. 50, 346–356.

Schwartz, L.L., Kloner, R.A., Arai, A.E., Baines, C.P., Bolli, R., Braunwald, E., Downey, J., Gibbons, R.J., Gottlieb, R.A., Heusch, G., Jennings, R.B., Lefer, D.J., Mentzer, R.M., Murphy, E., Ovize, M., Ping, P., Przyklenk, K., Sack, M.N., Vander Heide, R.S., Vinten-Johansen, J., Yellon, D.M., 2011. New horizons in cardioprotection: recommendations from the 2010 National Heart, Lung, and Blood Institute Workshop. Circulation 124, 1172–1179.

Smit, J.J., Ottervanger, J.P., Slingerland, R.J., Kolkman, J.J., Suryapranata, H., Hoorntje, J.C., Dambrink, J.H., Gosselink, A.T., de Boer, M.J., Zijlstra, F., van 't Hof, A.W., 2008. Comparison of usefulness of C-reactive protein versus white blood cell count to predict outcome after primary percutaneous coronary intervention for ST elevation myocardial infarction. Am. J. Cardiol. 101, 446–451.

Sze, S.K., de Kleijn, D.P., Lai, R.C., Khia Way Tan, E., Zhao, H., Yeo, K.S., Low, T.Y., Lian, Q., Lee, C.N., Mitchell, W., El Oakley, R.M., Lim, S.K., 2007. Elucidating the secretion proteome of human embryonic stem cell-derived mesenchymal stem cells. Mol. Cell. Proteomics 6, 1680–1689.

Thery, C., Ostrowski, M., Segura, E., 2009. Membrane vesicles as conveyors of immune responses. Nat. Rev. Immunol. 9, 581–593.

Tian, T., Wang, Y., Wang, H., Zhu, Z., Xiao, Z., 2010. Visualizing of the cellular uptake and intracellular trafficking of exosomes by live-cell microscopy. J. Cell. Biochem. 111, 488–496.

Timmers, L., Lim, S.K., Arslan, F., Armstrong, J.S., Hoefer, I.E., Doevendans, P.A., Piek, J.J., El Oakley, R.M., Choo, A., Lee, C.N., Pasterkamp, G., de Kleijn, D.P., 2007. Reduction of myocardial infarct size by human mesenchymal stem cell conditioned medium. Stem Cell Res. 1, 129–137.

Yellon, D.M., Hausenloy, D.J., 2007. Myocardial reperfusion injury. N. Engl. J. Med. 357, 1121–1135.

Zimmermann, H., 2000. Extracellular metabolism of ATP and other nucleotides. Naunyn Schmiedeberg's Arch. Pharmacol. 362, 299–309.

Zimmermann, W.H., Melnychenko, I., Wasmeier, G., Didie, M., Naito, H., Nixdorff, U., Hess, A., Budinsky, L., Brune, K., Michaelis, B., Dhein, S., Schwoerer, A., Ehmke, H., Eschenhagen, T., 2006. Engineered heart tissue grafts improve systolic and diastolic function in infarcted rat hearts. Nat. Med. 12, 452–458.