



Bone marrow mononuclear cells reduce myocardial reperfusion injury by activating the PI3K/Akt survival pathway

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ARTICLE INFO

Article history:

Received 13 January 2010

Received in revised form 22 July 2010

Accepted 23 July 2010

Available online 4 August 2010

Keywords:

Bone marrow
Mononuclear cells
Ischaemia-reperfusion
Stem cell
Myocardial infarction
Proteomics

ABSTRACT

Objective: Adult bone marrow mononuclear cells (BMMNCs) can restore cardiac function following myocardial necrosis. Protocols used to date have administered cells relatively late after ischaemia/reperfusion injury, but there is the opportunity with elective procedures to infuse cells shortly after restoration of blood flow, for example after angioplasty. Our aim was therefore to try and quantify protection from myocardial injury by early infusion of BMMNCs in a rat ischaemia reperfusion (I/R) model.

Methods and results: Male Wistar rats underwent 25 min of ischaemia followed by 2 h reperfusion of the left anterior descending coronary artery. Ten million BMMNCs were injected i.v. at reperfusion. We found BMMNCs caused a significant reduction in infarct size at 2 h when assessed by staining the area at risk with p-nitro blue tetrazolium (42% reduction, $P < 0.01$). Apoptosis and necrosis of isolated cardiomyocytes was significantly reduced in the area at risk. Functional assessment at 7 days using echocardiography and left ventricular catheterisation showed improved systolic and diastolic function in the BMMNC treatment group (LVEF: BMMNC $71 \pm 3\%$ vs. PBS $48 \pm 4\%$, $P < 0.0001$). In functional studies BMMNC injected animals showed increased activation of Akt, inhibition of GSK-3 β , amelioration of p38 MAP kinase phosphorylation and NF- κ B activity compared to control myocardium. Inhibition of PI3K with LY294002 abolished all beneficial effects of BMMNC treatment. Proteomic analysis also demonstrated that BMMNC treatment induced alterations in proteins within known cardioprotective pathways, e.g., heat shock proteins, stress-70 protein as well as the chaperone protein 14-3-3 epsilon.

Conclusions: Early BMMNC injection during reperfusion preserves the myocardium, with evidence of reduced apoptosis, necrosis, and activation of survival pathways.

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1. Introduction

Bone marrow mononuclear cells (BMMNCs) are emerging as a therapeutic modality for the treatment of ischaemic heart disease and its sequelae. The majority of animal experiments, forming the foundation for clinical translation of cell therapy, have been conducted after myocardial infarction has been established, i.e., cells were injected more than 1 h after reperfusion or whilst the coronary artery remained occluded [1]. The possibility that cell therapy

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may have a beneficial outcome if administered near the time of reperfusion, in a model many ways analogous to patients undergoing primary angioplasty (i.e. ischaemia-reperfusion injury – IRI) for acute myocardial infarction, has not been investigated.

BMMNCs have been demonstrated to improve cardiac function and a variety of explanations have been promoted as the cause of induced benefit. A key suggested mechanism of action is transdifferentiation or plasticity, these terms describe the observation that BMMNCs can engraft within ischaemic and non-ischaemic myocardium and modify their cell phenotype to that of cardiac muscle or vascular cells thus improving cardiac function by direct contribution to contractile function and increasing perfusion of cardiac tissue [2].

Others have failed to observe transdifferentiation [3] and subsequent investigators have suggested local release of paracrine factors as the key mechanism of action of transplanted cells. BMMNCs are capable of secreting a wide range of cytokines [4]. The paracrine factors themselves have been shown to induce a number of beneficial effects including induction of angiogenesis in host tissues [1], reducing apoptosis [4], immunomodulation of injury, e.g., benefits of transplantation following myocardial ischaemia have been shown to be dependent on cytokines such as IL-10 [5], a further documented paracrine benefit is the stimulation of, the recently described, resident cardiac stem cells [6].

The optimum timing of BMMNC cell injection in acute myocardial infarction has been identified as an unanswered question [7], and currently there is little published data to answer this question.

We speculated that an early time point of injection could optimise benefits: the earlier the offending coronary artery is treated during myocardial infarction the more cardiac tissue is preserved, and the better the outcome [8]. If the stem cells evoke their beneficial effect through paracrine actions, rather than ‘de-novo’ myogenesis, in acute myocardial infarction, then the biggest opportunity to save muscle would be before completion of infarction. Thus we suggest that the stem cell delivery would ideally be in less than 3 h after ischaemia onset, an important threshold observed in thrombolysis trials [8]. This viewpoint is underlined by observations in a rat stroke model that earlier intervention led to greater brain tissue survival and improved functional outcome [9]. Early intervention, ideally in minutes, is also needed to ameliorate ischaemia-reperfusion injury, an important modifiable determinant of infarct size, and an obvious potential target for paracrine factors released by BMMNCs [10].

Our hypothesis was that early injection of BMMNCs would induce a significant benefit after ischaemia-reperfusion injury and that this benefit would be induced by paracrine factors affecting the ischaemia-reperfusion injury pathway. Thus, we designed a series of experiments to test whether there is an early window of opportunity during which injection of bone marrow derived cells leads to a significant decrease in infarct size, in an animal model of IRI.

2. Materials and methods

All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All studies were performed using male Wistar rats (Charles River, UK) weighing 250–350 g receiving a standard diet and water ad libitum.

2.1. Experiment outline

We performed five experimental protocols in parallel, due to the mutually exclusive nature of the outcome measures used in our study (see [Supplementary Fig. 1](#)). The aim of each study was to

assess the effect of intravenous delivery of 10^7 BMMNCs in a rat IRI model.

The first two studies were designed to assess changes in infarct size, and effects on apoptosis and necrosis. The third study evaluated cardiac function and myocardial fibrosis after seven days reperfusion. The fourth study evaluated activation of the PI3K/Akt survival pathway including downstream mediators, finally we attempted to quantify proteomic changes (see [online Supplementary Fig. 1](#) for the experimental designs). We chose 10^7 BMMNCs as our therapeutic dose since several studies have shown functional benefit at this dose [11,12], in addition a dose–response effect has been documented so we tried to take advantage of this [13].

2.2. Donor bone marrow mononuclear cell preparation

Whole bone marrow was harvested from femurs and tibiae of male Wistar rats, BMMNCs were isolated by Percoll (Amersham Biosciences, UK) density gradient centrifugation, as previously described [14]. BMMNCs were characterised by flow cytometry using monoclonal antibodies for c-kit (Santa Cruz, sc-5535, USA), CD34 (Santa Cruz, sc-9095, USA), CD45 (BD, 554875, USA) and CD133 (Santa Cruz, sc-30219, USA). BMMNCs were c-kit+ ($7 \pm 1\%$, $n = 10$), CD34+ ($7 \pm 1\%$, $n = 10$), CD45+ ($54 \pm 6\%$, $n = 10$), and CD133+ ($15 \pm 1\%$, $n = 10$).

2.3. Measurement of infarct size

Rats were anaesthetised with thiopentone, tracheotomised and ventilated with air–oxygen mix (30%), and subjected to LAD-occlusion (25 min) [15] and reperfusion (for 30 min, 2 h or 7 days). Area at risk (AAR) of the left ventricle was demarcated by perfusion with Evans Blue and infarct size was measured by staining the AAR with nitro blue tetrazolium, as previously described [16].

2.4. Detection of apoptosis and necrosis in cardiac myocytes

Cardiomyocytes (CMCs) from the AAR of the previously ischaemic and reperfused (2 h) myocardium were isolated and analysed for CMC apoptosis and necrosis by flow cytometry as previously described [17]. Gating of the CMC population was determined by the degree of binding of troponin-T monoclonal antibody (SC-20025, Santa Cruz, USA). Troponin-T binding in the gated population was $70 \pm 6\%$ ($n = 5$).

Annexin 5-FITC/propidium iodide apoptosis detection kit (BD Biosciences Pharmingen, UK) was used to dual stain live AAR CMC isolates. All stained AAR CMC isolate samples were analysed within 1 h using a flow cytometer (FACScan, Becton Dickinson, UK) with Cell-Quest software (BD, UK). Cardiomyocyte apoptosis was also confirmed using a caspase-9 FLICA assay kit (Sigma–Aldrich, UK).

2.5. Determination of cardiac function after 7 days reperfusion

Cardiac function was analysed using echocardiography (Vevo-770 imaging system and 23.5 MHz probe, Visual Sonics, USA) under anaesthetic (1.5% isoflurane) on the day before and 7 days post I/R injury. The percentage fractional area of contraction (%FAC) was assessed with 2D images at the papillary muscle level. Left ventricle ejection fraction (LVEF) was measured by M-mode.

Haemodynamic catheter analysis of LV function was performed (on the same days as above) by inserting a 2 Fr micro-tipped pressure transducer (Millar Instruments; SPR-320) through the right carotid artery and advanced into the LV for measurement of ventricular pressure. Left ventricular peak systolic pressure (LVPS), end diastolic pressure (LVEDP), maximal slope of systolic pressure increment ($+dP/dt$), diastolic decrement ($-dP/dt$), the relaxation

time constant (τ), and heart rate were all analysed using ChartPro software.

2.6. Determination of LV fibrosis after 7 days reperfusion

Excised hearts were immediately fixed by perfusion (via aortic cannulation) with 4% paraformaldehyde, followed by immersion in 4% paraformaldehyde, on ice for 30 min. The hearts were then washed with PBS and incubated in PBS containing 30% sucrose (w/v) at 4 °C overnight.

The fixed hearts were then cut transversely into three pieces, each of which was embedded in OCT compound (BDH, UK), frozen in liquid nitrogen-cooled isopentane and stored at –80 °C. Subsequently, cryosections were stained with 0.1% picosirius red F3B (BDH, UK) for 10 min at room temperature. The sections were rinsed 5 times in deionised water and then rinsed for 1 min in picric alcohol (20 ml absolute alcohol; 70 ml H₂O; 10 ml saturated aqueous picric acid). The sections were then dehydrated through a methanol series and mounted in DPX (VWR, UK). The degree of picosirius red staining was visualized by an all-in-one microscope (Keyence BZ8000, UK).

2.7. Determination of activation of the PI3K/Akt-signaling pathways by Western blot analysis

Animals were subjected to 25 min ischaemia followed by 2 h reperfusion and ex vivo hearts were frozen in liquid nitrogen. Cytosolic and nuclear protein homogenates were prepared from the frozen myocardium (AAR only) as described [18]. Protein extracts from the cytosol were analysed for phosphorylation of Akt, GSK-3 β , and p38 MAPK by western blots as previously described [19]. Protein extracts from both the cytosol and nucleus were analysed for nuclear translocation of p65 NF- κ B, as described [19]. Immunodetection was performed using primary antibodies against mouse anti-phosphorylated AktSer473 mouse, anti-phosphorylated p38 MAPK (both from Cell Signaling Biotechnology), rabbit anti-total Akt, rabbit anti-total GSK-3 β , goat anti-phosphorylated GSK-3 β Ser9, and mouse anti-NF- κ B p65 (all from Santa Cruz). Blots were then incubated with specific secondary antibodies conjugated with horseradish peroxidase and developed with an ECL detection system (Amersham).

Immunoreactive bands were visualised by autoradiography and band density was evaluated using the Gel Pro[®] Analyzer 4.5, 2000 software (Media Cybernetics). The proportion of phosphorylated to total protein was normalised to the vehicle treated group. Membranes were then stripped and incubated with β -actin monoclonal antibody and subsequently with anti-mouse antibody to assess gel-loading homogeneity.

2.8. Analysis of the cardiac proteome after LAD-occlusion and reperfusion (6 h)

We assessed changes in the cardiac proteome by two-dimensional gel electrophoresis (2-DE) coupled with electro-spray ionization (ESI) mass spectrometry (MS) to identify differentially expressed protein spots in myocardial samples from sham operated animals plus PBS, ischaemia/reperfusion (25 min/6 h) plus PBS, and from ischaemia/reperfusion (25 min/6 h) plus BMMNC ($n = 3$, for all groups).

Protein homogenates were individually prepared for each rat from 80 to 140 mg of ground frozen myocardium (AAR only) using 1 ml lysis buffer per 100 μ g tissue (9.5 M Urea, 4% CHAPS, 1% DTT, complete protease inhibitor [Roche, UK], and phosphatase inhibitor cocktail [Sigma]). Protein concentration of homogenates was quantified by Bio-Rad protein assay (Bio-Rad). Duplicate 2D gels were produced for each sample using 200 μ g total protein separated on

18 cm non-linear (NL) pH4-7 immobilised pH gradient (IPG) strips (GE-Healthcare Lifesciences, UK). Large format, homogenous, 12% SDS PAGE gels were used for second dimension separation and then stained using the plus one silver staining kit (GE-Healthcare Lifesciences, UK). Images were scanned and analyzed using PDQuest software (BioRad, UK). Spot intensity values were 'normalised' using 'total density in gel' method. 2DE using immobilized pH gradients (IPGs) is a well established method capable of resolving thousands of different proteins with excellent reproducibility [20,21]. This technique enables comparative analyses of high and medium abundant proteins; it also enables intact proteins to be visualized in a way that makes post-translational modifications evident.

Fold changes and unpaired Mann–Whitney P -values were calculated for 'SHAM vs. PBS' and 'PBS vs. BMMNC' comparisons. Over a hundred spots had significant >2.5-fold differences ($P \leq 0.05$) in either or both of the comparisons, and were selected for trypsin digestion, peptide extraction, and protein identification using LC–MS/MS, Q-ToF Micro (Micromass, UK). The resultant peak list data (PKL file) was then used to search the SwissProt database using the MASCOT (Matrix Science) online server for a match. Only peptides with scores over 45 were considered to be significant, the MASCOT scores were calculated from these.

2.9. Western blot methods

Twenty μ g of protein homogenate was electrophoresed in duplicate on 10% Bis-tris gels (Invitrogen, UK) and transferred to PVDF membranes (GE-Healthcare Lifesciences, UK), and subsequently blocked in Tris buffered saline containing 0.05% Tween-20 (TBS-Tween) with 3% non-fat dry milk at 4 °C overnight. Blots were probed with either anti-14-3-3 ϵ or anti-SDHA antibodies (Abcam, UK). Loading was verified by probing the duplicate gel with anti-GAPDH antibodies (Abcam, UK) for each experiment. Blots were incubated with the appropriate secondary antibody (Santa Cruz Biotechnology, USA). Chemiluminescence was detected using ECL reagents (GE-Healthcare, UK). Bands were detected and quantified using TotalLab v1.1 (Phoretix, UK), and volumes were normalised to the GAPDH volumes for the duplicate gels.

2.10. Statistical analyses

GraphPad Prism 5 statistics package was used to analyse the results. Data for physiological variables are expressed as means \pm standard error, and analysed by one-way ANOVA followed by Dunnett's post hoc test for multiple comparisons. For western blots, a one-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons was used.

For 2-DE data a two-tailed unpaired Mann–Whitney P -value <0.05 was considered to be statistically significant.

3. Results

3.1. Intravenous administration of BMMNCs upon reperfusion reduces infarct size

Intravenous jugular administration of 10 million BMMNCs, in 0.5 ml PBS, immediately upon reperfusion to animals subjected to ischaemia then reperfusion for 2 h resulted in a 42% reduction in infarct size (Fig. 1). There was no significant difference in the percentage of left ventricle at risk for the BMMNC group compared to the PBS control group (50.85% vs. 49.65%, respectively, $P = 0.68$). From experiments not shown here, we quantified donor cells within the rat hearts using a cell tracker dye, CFDA SE, we estimate that <1% of the injected cells remained in the heart 2 h following injection.

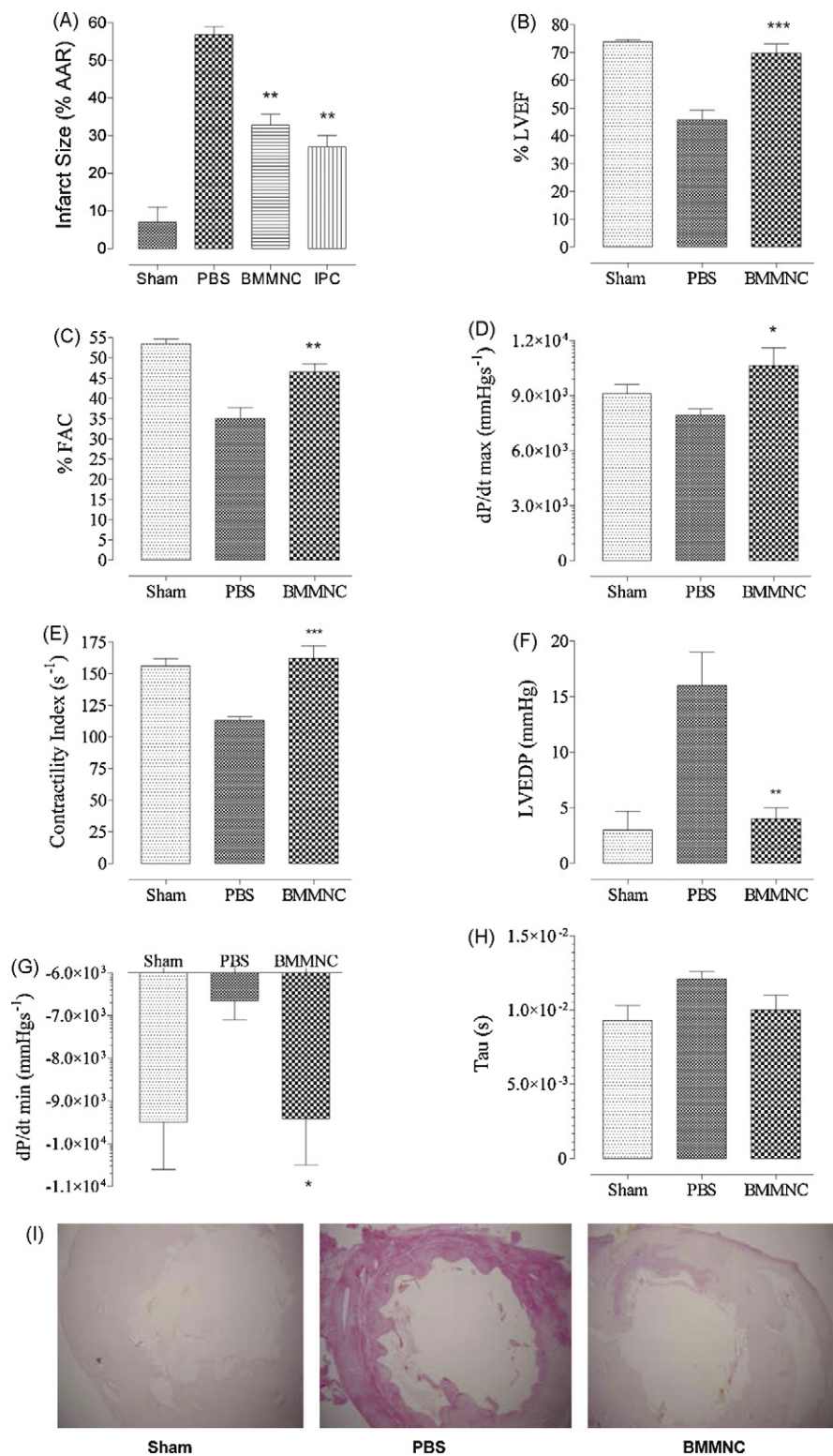


Fig. 1. (A) Infarct size was measured in animals subjected to 25 min regional myocardial ischaemia followed by 2 h reperfusion (I/R2h) treated with phosphate buffered saline (PBS), bone marrow mononuclear cells (BMMNCs) or ischaemic preconditioning (IPC), sham animals received a thoracotomy only and were not rendered ischaemic. The area at risk (AAR) was expressed as a % of the left ventricle (LV) and the infarct size was expressed as a % of the AAR. (B–H) Cardiac function was analysed by echocardiography and LV hemodynamic catheterisation in animals subjected to 25 min LAD-occlusion followed by 7 days reperfusion (I/R7D). When compared to vehicle, administration of BMMNCs upon reperfusion prevented systolic and diastolic dysfunction. (I) Myocardial fibrosis in hearts subjected to (I/R7D) was assessed by picosirius red staining of myocardial cryosections. When compared with PBS, administration of BMMNC dramatically attenuated the degree of post-I/R fibrosis. Statistical significance was determined by ANOVA followed by Dunnett's post hoc test * $P < 0.05$, ** $P < 0.01$.

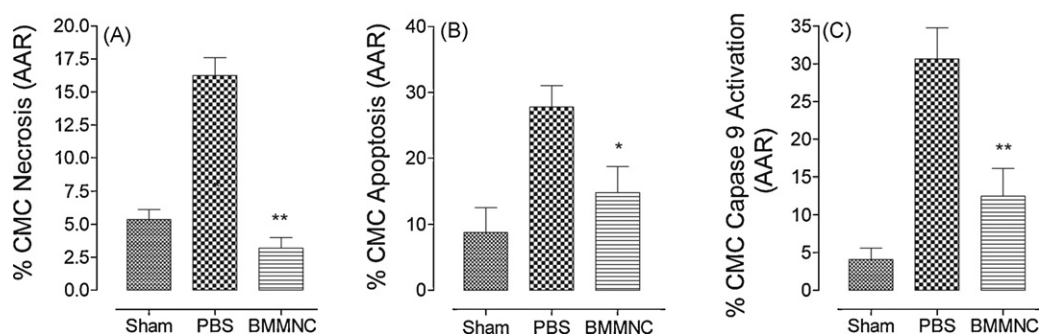


Fig. 2. BMMNC treatment significantly reduced apoptosis, necrosis and caspase 9 activation compared to controls. The area at risk (AAR) of rat myocardium, following LAD ischaemia for 25 min then reperfusion for 2 h, was isolated from animals in sham, vehicle, and BMMNC treated groups. The cardiomyocyte (CMC) fraction, for all groups, was then assessed for: (A) necrosis (B) apoptosis (C) caspase 9 activation. Statistical significance was determined by ANOVA followed by Dunnett's post hoc test * $P < 0.05$, ** $P < 0.01$.

We next demonstrated that administration of BMMNCs, at the beginning of a 7 days reperfusion period, reduced IRI-induced infarct size and fibrosis, i.e., cardiac scar formation. These findings were associated with prevention of impairment in systolic and diastolic LV function measured by echocardiography and haemodynamic catheterization. Improvements in systolic function by BMMNC therapy were demonstrated by significantly higher values for LVEF (BMMNC $71 \pm 3\%$ vs. PBS $48 \pm 4\%$, $n = 11$, $P < 0.0001$); FAC (BMMNC $47 \pm 2\%$ vs. PBS $36 \pm 3\%$, $n = 11$, $P < 0.01$); dP/dt_{max} (BMMNC $10 \times 10^3 \text{ mmHg s}^{-1} \pm 0.4\%$ vs. PBS $8 \times 10^3 \text{ mmHg s}^{-1} \pm 1\%$, $n = 7$, $P < 0.05$); and Contractility Index (BMMNC 162 ± 10 vs. PBS 113 ± 3 , $n = 7$, $P < 0.001$). Similarly, improvements in diastolic function by BMMNC-therapy were demonstrated by lower values for LVEDP (BMMNC $4 \pm 1 \text{ mmHg}$ vs. PBS $16 \pm 3 \text{ mmHg}$, $n = 7$, $P < 0.01$); dP/dt_{min} (BMMNC $-9 \times 10^3 \pm 0.5 \text{ mmHg s}^{-1}$ vs. PBS $-7 \times 10^3 \pm 1 \text{ mmHg s}^{-1}$, $n = 7$, $P < 0.01$); and Tau (BMMNC $12 \times 10^{-3} \pm 0.5$ vs. PBS $10 \times 10^{-3} \pm 1$, $n = 7$, $P = 0.075$) (Fig. 1b–i).

3.2. Apoptosis and necrosis

A significant decrease in the number of necrotic myocytes was seen when BMMNCs were injected immediately upon reperfusion ($n = 5$ all groups, $6.1 \pm 1.1\%$ in shams vs. $16.3 \pm 1.3\%$ myocyte necrosis in control vs. $4.9 \pm 1\%$ myocyte necrosis in BMMNC treated animals, $P < 0.01$).

Similarly, injection of BMMNCs immediately after ischaemia significantly reduced the proportion of cells undergoing apoptosis ($n = 5$ all groups, $7.8 \pm 0.5\%$ in shams, $27.7 \pm 3.2\%$ in vehicle controls, and $13.9 \pm 3.4\%$ in BMMNC group, $P < 0.05$, Fig. 2). This observation was confirmed by measurement of caspase 9 expression, the percentage of cells isolated from the area at risk expressing caspase 9 was measured and found to be significantly reduced in cell treated compared to vehicle treated animals (BMMNCs $12.7 \pm 2.6\%$ vs. PBS $30.6 \pm 4.2\%$, $n = 5$, $P < 0.01$).

3.3. The reduction in infarct size caused by BMMNC is associated with activation of PI3K/Akt survival pathway

The cardioprotective effect of BMMNCs was associated with the increased expression of the pro-survival PI3K/Akt-signalling pathway. BMMNCs resulted in a significant increase, in phosphorylation of serine-473Akt and serine-9GSK-3 β (Fig. 3) which was abolished by the pre-treatment of animals with the PI3-K inhibitor LY294002 (0.3 mg/kg i.v.), indicating that the activation of Akt (and the subsequent inhibition of GSK-3 β) were secondary to the activation of PI3K. Furthermore, BMMNCs resulted in a significant reduction in the phospho-

rylation of p38-MAPK and nuclear translocation of NF- κ B, and both effects were attenuated by the PI3K inhibitor (0.3 mg/kg i.v.).

3.4. The cardioprotection afforded by BMMNCs is associated with a reversal in metabolism and mitochondrial protein disturbances

We next investigated changes in the cardiac proteome of hearts that had been subjected to IRI by comparing the Sham operated group to PBS, and then we investigated the proteomic alterations attributable to BMMNC application by comparing the PBS group to the BMMNC group in the hope this would elucidate pro-survival mechanisms afforded by the treatment. In this analysis we were able to match ~ 1100 different proteins across 18 different 2D gels. The proteins identified from spots found to be significantly altered are detailed in Table 1. Fold changes and P -values of these proteins are shown in Table 2 along with the observed and theoretical Mr and pI (Table 1). An image of a 2DE gel produced in this analysis labeled with the positions of the identified proteins is available (Supplementary Fig. 2).

The proteins identified as being altered by IRI were involved in mitochondrial respiration, stress, cellular energy metabolism, and sarcomeric/cytoskeletal structure and function. Disturbances in mitochondrial oxidative phosphorylation components (complex I and complex III and ATP synthase) were observed in IRI suggesting mitochondrial dysfunction. Several stress responsive proteins were also altered in IRI: stress-70 protein (Hspa9), and the cytoplasmic antioxidant Peroxiredoxin-6 were both affected by IRI. The expression levels of several energy metabolism enzymes were also disturbed, possibly reflecting a switch in substrate preference [22]. Galectin-5, a beta-galactoside-binding lectin, was up-regulated from undetectable levels in the PBS myocardial samples compared to Sham.

Many of these alterations were largely reversed when the BMMNC group was compared to PBS, such that levels were similar to that seen in the Sham group. For example, the spots identified as NADH-ubiquinone oxidoreductase 75 kDa subunit, and cytochrome b-c1 subunit 6 which had -10 - and 6 -fold changes respectively in the Sham vs. PBS comparison were seen to have 7 - and -10 -fold changes respectively in PBS vs. BMMNC. Fragments of ATP synthase subunit beta, identified in 16 different spots, all showed down-regulation in Sham vs. PBS, possibly reflecting enhanced protein degradation in IRI, and up-regulation in PBS vs. BMMNC, four of these had significant alteration in both comparisons showing similar degree of expression change (-13 -, -6 -, -3 - and -2 -fold decrease in Sham vs. PBS; 13 -, 9 -, 3 - and 3 -fold increase in PBS vs. BMMNC). IRI related expression changes were also 'corrected' for Enoyl CoA hydratase and lactate dehydrogenase, hinting at a reversal of the IRI related substrate switch. We also observed

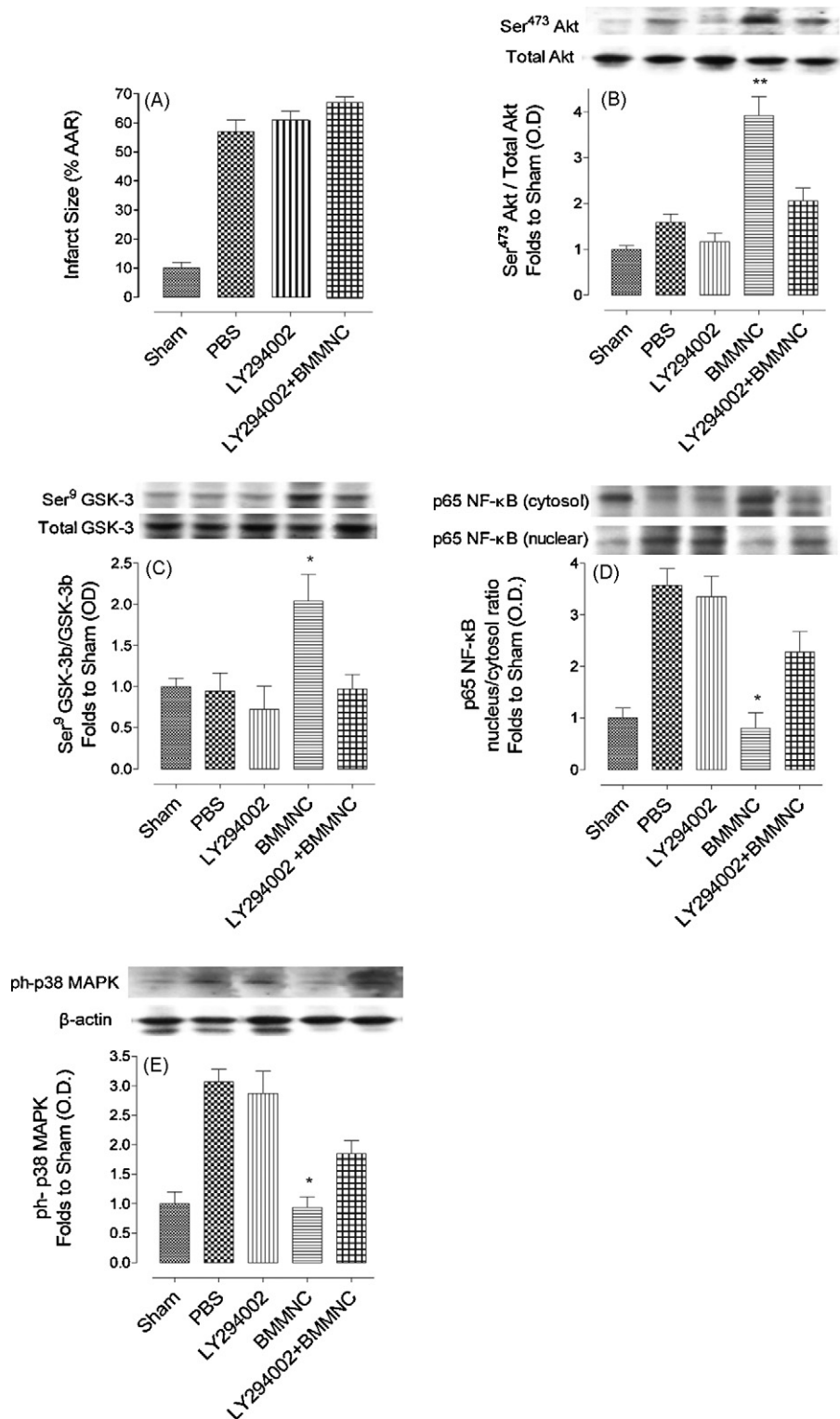


Fig. 3. (A) Infarct size was measured in animals subjected to 25 min regional myocardial ischemia followed by 2 h reperfusion (I/R2h) or sham animals. The area at risk (AAR) was expressed as a % of the left ventricle (LV) and the infarct size was expressed as a % of the AAR. When compared with PBS, pre-treatment with the LY294002 (0.3 mg/kg IV) abolished the attenuation of infarct size caused by the systemic intravenous injection of 10 million BMMNC upon reperfusion in I/R2h (B–E). Western blots demonstrating that systemic intravenous injection of 10 million BMMNC upon reperfusion significantly augmented the phosphorylation of Aktserine-473 and GSK-3 β serine-9, reduced the phosphorylation of p38-mitogen activated protein kinase (MAPK) and the nuclear translocation of NF- κ B. The PI3-K inhibitor LY294002 abolished all of these effects of BMMNCs. Statistical significance was determined by ANOVA followed by Dunnett's post hoc test * $P < 0.05$, ** $P < 0.01$.

a 12-fold up-regulation in the chaperone protein 14-3-3 epsilon in the BMMNC group compared to PBS. The expression of two proteins (Shda and 14-3-3 epsilon) was validated by Western blot (see Fig. 4).

4. Discussion

We have discovered that the intravenous systemic administration of BMMNCs (10 million: c-Kit+, CD34+, CD45+, CD133) at the

Table 1

Spots with significant fold changes for which a single protein ID was found. SSP are spot identification numbers assigned by PDQuest software. Mascot details are as follows; mascot score (the sum of the peptide scores), the number of peptides found and the sequence coverage of these peptides (only including peptides with individual scores over 35). Theoretical Mr and PI values for the protein identified are shown as well as the observed Mr and PI of the spot as estimated from its position in the gel.

SSP	Observed in gel		Theoretical		Protein name	UniProtKB/Swiss-Prot accession	Mascot		
	Mr	PI	Mr	PI			Score	Pep's	% sc
1430	3,4500	4.40	29,155	4.63	14-3-3 protein epsilon	P62260	164	3	20
2616	47,500	4.80	32,803	4.80	40S ribosomal protein SA	P38983	50	1	4
2546	45,000	4.80	51,556	7.16	Adenylyl cyclase-associated protein 1	Q08163	55	1	4
3209	20,000	5.00	56,318	5.19	ATP synthase subunit beta	P10719	144	2	5
1225	21,000	4.50	56,318	5.19	ATP synthase subunit beta	P10719	192	2	7
3208	22,000	5.00	56,318	5.19	ATP synthase subunit beta	P10719	51	1	4
4235	24,000	5.40	56,318	5.19	ATP synthase subunit beta	P10719	110	2	4
4329	29,000	5.40	56,318	5.19	ATP synthase subunit beta	P10719	69	1	2
1424	34,000	4.60	56,318	5.19	ATP synthase subunit beta	P10719	131	2	8
3433	34,000	5.00	56,318	5.19	ATP synthase subunit beta	P10719	436	6	18
2443	35,000	4.80	56,318	3.00	ATP synthase subunit beta	P10719	91	1	3
3523	40,000	5.20	56,318	5.19	ATP synthase subunit beta	P10719	280	4	14
3632	47,000	5.20	56,318	5.19	ATP synthase subunit beta	P10719	314	5	16
2609	47,500	4.80	56,318	5.19	ATP synthase subunit beta	P10719	457	6	12
2626	52,500	4.90	56,318	5.19	ATP synthase subunit beta	P10719	167	3	8
3626	54,500	5.20	56,318	5.19	ATP synthase subunit beta	P10719	711	9	29
3636	57,000	5.10	56,318	5.19	ATP synthase subunit beta	P10719	226	5	15
3706	57,000	5.10	56,318	5.19	ATP synthase subunit beta	P10719	215	3	9
2712	57,500	4.90	56,318	5.19	ATP synthase subunit beta	P10719	707	8	26
2119	14,500	4.80	10,417	4.90	Cytochrome b-c1 complex subunit 6	Q5M9I5	131	1	20
3627	57,000	5.20	53,424	5.21	Desmin	P48675	463	7	20
7722	62,000	6.60	67,123	8.76	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex	P08461	228	4	9
7323	31,500	6.60	31,496	8.39	Enoyl-CoA hydratase	P14604	71	1	3
7110	16,000	6.30	16,186	6.17	Galectin-5	P47967	157	3	21
7702	70,000	6.40	58,307	5.85	Hydroxysteroid dehydrogenase-like protein 2	Q4V8F9	75	2	4
6425	37,000	6.20	36,589	5.70	L-lactate dehydrogenase B chain	P42123	77	1	6
4521	37,000	5.40	36,589	5.70	L-lactate dehydrogenase B chain (or LDHA)	P42123	72	1	3
8853	67,500	6.70	79,293	7.70	Methylcrotonoyl-CoA carboxylase subunit alpha, mitochondrial (mouse)	Q5I0C3	166	3	6
3305	25,000	5.00	22,142	5.03	Myosin light chain 3	P16409	334	5	33
1206	17,500	4.40	18,868	4.86	Myosin regulatory light chain 2	P08733	57	1	8
1213	17,500	4.40	18,868	4.86	Myosin regulatory light chain 2	P08733	108	2	16
3228	15,000	5.20	18,868	4.86	Myosin regulatory light chain 2	P08733	135	3	20
4207	19,000	5.40	223,370	5.59	Myosin-6	P02563	279	4	3
1207	19,000	4.40	113,780	8.4	NAD(P) transhydrogenase	Q61941	58	1	2
7112	17,000	6.50	79,362	5.65	NADH-ubiquinone oxidoreductase 75 kDa subunit	Q66HF1	102	2	2
5408	32,000	5.80	24,803	5.64	Peroxisomal oxidoreductin-6	O35244	388	7	44
3518	42,500	5.10	73,812	5.97	Stress-70 protein (aka HSPA9)	P48721	85	1	2
4702	60,000	5.20	73,812	5.97	Stress-70 protein (aka HSPA9)	P48721	739	10	21
7723	67,500	6.60	71,570	6.75	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	Q920L2	73	1	2
1320	29,000	4.50	32,661	4.69	Tropomyosin alpha-1 chain (or Tropomyosin beta chain)	P04692	58	1	4
1248	25,000	4.50	35,709	4.95	Troponin T, cardiac muscle	P50753	59	1	3
4523	43,000	5.60	49,892	4.95	Tubulin alpha-4A chain	Q5XIF6	191	3	11
8710	67,000	6.60	66,140	6.15	WD repeat-containing protein 1	Q5RKI0	157	4	11

beginning of reperfusion, in a myocardial ischaemia-reperfusion injury model (25 min ischaemia, 2 h reperfusion), results in a significant reduction in myocardial infarct size, and reduces the number of cardiomyocytes undergoing apoptosis within the area subjected to IRI.

The cardioprotection induced by BMMNCs was not transient, but remained evident when hearts were subjected to regional myocardial ischaemia and treated with BMMNCs upon reperfusion, followed by 7 days of reperfusion. Under these experimental conditions, BMMNCs reduced the development of a scar tissue (reduction of necrosis and intra-mural fibrosis assessed by histology) and

prevented the development of a significant systolic and diastolic cardiac dysfunction, which was seen in control animals.

Notably, the cardioprotection afforded by BMMNCs was similar to that afforded by ischaemic preconditioning, a known gold standard for the experimental reduction of myocardial infarct size [23]. The cardioprotective effect of preconditioning is induced by pro-survival kinases such as PI3K-Akt and MEK1/Erk1/2, these protective kinases are collectively termed the reperfusion injury salvage kinase pathway (RISK).

Activation of the RISK pathway at the time of myocardial reperfusion can confer powerful infarct size reduction. A diverse range

Table 2
Proteins identified from spots that had significant fold changes in either the Sham vs. PBS or the PBS vs. BMMNC comparisons. Average spot volumes and standard deviations for each group are shown alongside fold changes and *P*-values (unpaired, 2 tailed, Mann–Whitney). ‘On’ and ‘Off’ in the FC column indicate changes to presence or absence in spots, e.g., in spot 1206 ‘On’ indicates the spot was absent in Sham but present in PBS.

SSP	Protein name	Sham		PBS		BMMNC		6 h reperfusion			
		Average	StDev	Average	StDev	Average	StDev	SHAM vs. PBS		PBS vs. BMMNC	
								FC	<i>P</i> -value	FC	<i>P</i> -value
<i>Mitochondrial/oxidative phosphorylation</i>											
4329	ATP synthase subunit beta	110.16	35.61	19.83	23.57	106.70	92.44	−5.6	0.0095	5.4	ns
1225	ATP synthase subunit beta	291.53	151.54	22.25	23.86	283.33	231.65	−13.10	0.0238	12.7	0.0476
3636	ATP synthase subunit beta	271.00	120.04	47.25	52.81	411.01	114.47	−5.7	0.0190	8.7	0.0095
3433	ATP synthase subunit beta	924.75	333.91	357.85	251.86	1052.96	350.05	−2.6	0.0022	2.9	0.0087
2609	ATP synthase subunit beta	621.15	297.10	311.42	168.21	781.41	136.07	−2.0	ns	2.5	0.0022
3523	ATP synthase subunit beta	468.32	318.28	244.35	136.88	670.89	119.11	−1.9	ns	2.7	0.0022
1424	ATP synthase subunit beta	394.95	298.27	272.46	165.49	776.50	227.39	−1.5	ns	2.8	0.0022
3632	ATP synthase subunit beta	130.97	81.35	76.08	59.83	218.44	101.06	−1.7	ns	2.9	0.0152
3626	ATP synthase subunit beta	846.73	155.91	386.66	214.61	1178.68	396.37	−2.2	0.0043	3.0	0.0022
2712	ATP synthase subunit beta	708.74	441.93	292.35	251.10	955.57	597.67	−2.4	ns	3.3	0.0260
2443	ATP synthase subunit beta	652.00	498.23	177.28	268.43	639.85	170.64	−3.7	ns	3.6	0.0152
2626	ATP synthase subunit beta	91.75	60.28	90.81	52.64	334.31	223.37	−1.0	ns	3.7	0.0303
3208	ATP synthase subunit beta	248.58	168.64	133.85	129.33	511.89	119.33	−1.9	ns	3.8	0.0043
3706	ATP synthase subunit beta	368.77	159.79	172.42	95.57	776.99	334.44	−2.1	ns	4.5	0.0095
3209	ATP synthase subunit beta	261.67	224.65	101.93	124.18	470.32	252.87	−2.6	ns	4.6	0.0260
4235	ATP synthase subunit beta	70.55	65.79	21.61	12.18	176.25	102.63	−3.3	ns	8.2	0.0238
2119	Cytochrome b-c1 complex subunit 6	330.30	406.92	1901.07	1217.20	185.77	128.67	5.8	0.0303	−10.2	0.0087
7112	NADH-ubiquinone oxidoreductase 75 kDa subunit	156.83	102.34	16.00	10.00	113.15	111.86	−9.8	0.0022	7.1	0.0260
1207	NAD(P) transhydrogenase	384.6	636.9	1474.3	808.7	1325.4	851.1	3.83	0.026	−1.1	ns
7723	Succinate dehydrogenase [ubiquinone] flavoprotein subunit	314.78	202.61	115.62	63.81	282.54	143.14	−2.7	ns	2.4	0.0260
<i>Energy metabolism</i>											
7722	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex	39.01	24.22	225.82	164.74	45.42	22.77	5.8	0.0411	−5.0	ns
7323	Enoyl-CoA hydratase	696.98	400.62	1634.53	371.06	639.89	608.94	2.4	0.0022	−2.6	0.0152
6425	L-lactate dehydrogenase B chain	426.25	148.40	69.00	82.66	385.72	480.03	−6.2	0.0087	5.6	0.0303
4521	L-lactate dehydrogenase B chain (or LDHA)	700.40	749.67	252.34	315.77	676.73	160.45	−2.8	ns	2.7	0.0317
8853	Methylcrotonoyl-CoA carboxylase subunit alpha (mouse)	320.41	221.74	120.29	80.60	403.40	239.65	−2.7	ns	3.4	0.0173
<i>Antioxidants</i>											
5408	Peroxiredoxin-6	410.63	200.37	162.61	156.60	393.45	123.55	−2.5	ns	2.4	0.0260
<i>Heat shock proteins</i>											
3518	Stress-70 protein (aka HSPA9)	369.67	142.60	112.02	144.99	323.75	223.68	−3.3	0.0260	2.9	0.0411
4702	Stress-70 protein (aka HSPA9)	508.89	402.11	1035.09	584.46	360.54	271.60	−1.9	ns	2.7	0.0152
<i>Cytoskeletal/sarcomeric</i>											
3627	Desmin	491.87	264.82	206.72	46.34	545.80	182.40	−2.4	0.0159	2.6	0.0043
3305	Myosin light chain 3	1691.27	957.03	545.68	498.17	1472.06	904.54	−3.1	0.0411	2.7	ns
1206	Myosin regulatory light chain 2			490.18	236.51	305.05	331.21	On		−1.6	ns
1213	Myosin regulatory light chain 2	147.58	126.06	1049.19	385.02	626.65	440.62	7.1	0.0095	−1.7	ns
3228	Myosin regulatory light chain 2	204.57	219.47	1850.40	1241.85	1218.78	1357.75	9.1	0.0022	−1.5	ns
4207	Myosin-6	508.89	402.11	1035.09	584.46	360.54	271.60	2.0	ns	−2.9	0.0152
1320	Tropomyosin alpha-1 chain (or Tropomyosin beta chain)	172.31	68.46	17.32	9.72	195.82	115.85	−9.95	0.0238	11.30	0.0238
1248	Troponin T, cardiac muscle	133.87	228.64			173.79	243.95	Off		On	
4523	Tubulin alpha-4A chain	705.21	218.21	157.23	193.67	404.76	319.42	−4.5	0.0087	2.6	ns
<i>Others</i>											
1430	14-3-3 protein epsilon	225.70	167.56	24.77	15.25	309.80	180.01	−9.1	ns	12.5	0.0043
2616	40S ribosomal protein SA	98.56	104.48	83.32	54.58	278.01	167.07	−1.2	ns	3.3	0.0173
2546	Adenylyl cyclase-associated protein 1	89.10	44.41	21.66	13.56	134.59	57.71	−4.1	0.0303	6.2	0.0043
7110	Galectin-5			654.97	1246.68	66.30	101.51	On		−9.9	ns
7702	Hydroxysteroid dehydrogenase-like protein 2	150.13	98.79	54.77	48.38	200.66	103.56	−2.7	ns	3.7	0.0087
8710	WD repeat-containing protein 1	358.36	214.12	169.16	88.09	454.56	216.81	−2.1	ns	2.7	0.0043

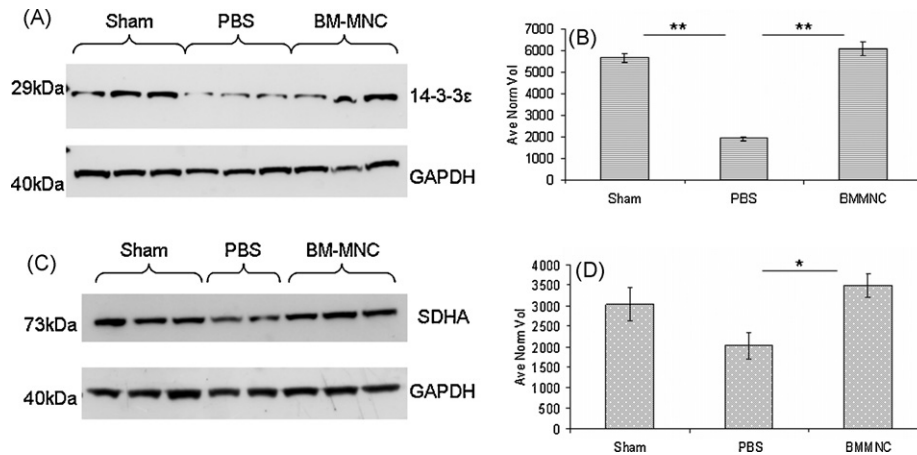


Fig. 4. (A) Western blot of expression levels of 14-3-3 epsilon with the normalised volumes shown in panel B. (C) Western blot of expression levels of succinate dehydrogenase with the normalised volumes shown in D. (B and D) Average normalised volumes and standard deviations, lines with stars indicate significance as calculated by one-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons.

of activating mechanisms have been demonstrated, these include growth factors [24], many of which have been shown to be released by stem cells, for example VEGF and FGF [25].

The cardioprotective actions of RISK pathway activation remain to be completely resolved, but leading suggestions with experimental evidence include inhibition of the mitochondrial permeability transition pore (mPTP), activation of anti-apoptotic mechanisms including inhibition of pro-apoptotic factors, e.g., BAD and BAX, and facilitation of calcium uptake into the sarcoplasmic reticulum ameliorating calcium triggered mPTP opening [26].

We next investigated possible mechanisms for our observations. The small quantity of cell engraftment 2 h after injection, <1% (measured in experiments not shown here), combined with the speed of the cardioprotective effect of BMMNC administration, coupled to the finding that BMMNCs can secrete mediators of the RISK pathway suggested to us that the observed cardioprotective effects could be due to activation of the RISK pathway. Various mediators of reperfusion injury – oxidative stress, changes in pH, inflammation, intracellular calcium levels, and the mitochondrial permeability transition pore are all possible targets for interaction with BMMNCs injected at the time of reperfusion. As BMMNCs have already been shown to exhibit release of paracrine factors and expression of cardioprotective genes that may act beneficially on the RISK pathway we chose to investigate this possibility preferentially over other hypotheses [4]. Specific growth factors released by BMMNCs that have been shown to be cardioprotective include vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), fibroblast growth factor (FGF), insulin-like growth factor 1 (IGF-1), and transforming growth factor (TGF) [25].

A number of our observations support the hypothesis that paracrine growth factors secreted by BMMNCs contribute to the cardioprotective effects of BMMNCs observed here. This hypothesis is supported by the following findings, administration of BMMNCs upon reperfusion was associated with activation of the PI3K/Akt phosphorylation survival kinase pathway, and when PI3K activity was inhibited, with LY294002, the cardioprotective effect seen with injection of BMMNCs was negated, highlighting the key importance of the activation of the PI3K/Akt pro-survival pathway in the observed beneficial effects. The increase in Ser-473Akt phosphorylation also resulted in the inhibition of GSK3β, suppression of which has been documented to reduce infarct size and to cause pronounced anti-inflammatory effects [27], which are, at least in part, due to prevention of the activation of NF-κB24. The findings that the inhibition of activation of PI3K with LY294002 abolished all of the above effects of BMMNCs on cell signaling as well as their

cardioprotective effects supports the view that the cardioprotective effects of BMMNC may be secondary to activation of the PI3K/Akt survival pathway.

In order to further investigate the down stream molecular mechanisms associated with PI3K/Akt-signaling we analysed changes in the global proteome of hearts subjected to IRI and treated upon reperfusion with BMMNCs. Within the proteomic dataset, many of the protein alterations measured in the BMMNC group compared to PBS control seem likely to be secondary to the reduced cell death associated with treatment, however, several proteins with a possible mechanistic role in cardioprotection were identified in the BMMNC treated group. Two significant proteins were the heat shock protein stress-70 protein (aka HSPA9 or HSP70), which has also been shown to be up-regulated in cardioprotection as afforded by hydrogen sulphide [28], and the chaperone protein 14-3-3 epsilon which is known to have pro-survival functions including its ability to bind and inhibit the Bcl-2 related protein Bad in its phosphorylated conformation [29]. Bad phosphorylation is thought to be one of the many downstream actions of the PI3K/Akt pathway [30].

In conclusion, a single dose of systemic intravenous BMMNCs upon reperfusion reduces infarct size and cardiac dysfunction caused by regional myocardial ischaemia and reperfusion. This reduction in infarct size was associated with activation of the PI3K/Akt survival pathway. Furthermore, BMMNC treatment induced alterations in protein expression consistent with known cardioprotective pathways. Results from clinical BMMNC trials suggest positive effects on cardiac function when cells are administered 5 days after successful reperfusion of myocardial infarction [31]. However, in another study when cells were injected at a time closer to the point of successful reperfusion (24 h) no beneficial effect was seen [32]. The data presented in this paper suggests that there may be a very early window of opportunity (i.e., less than 24 h following reperfusion) that exists for bone marrow derived cell therapy to prevent myocardial necrosis, and that this time point should be considered when designing future clinical trials. In addition, it appears that the cardioprotective benefit shown here is too rapid to be related to stem cell plasticity and may be attributable to activation of the RISK pathway. This raises the possibility of unearthing one or more molecules that could be administered 'in lieu' of BMMNCs to achieve similar protection, thus avoiding the need for cell collection and any potential harmful effects that may manifest as a consequence of cell injection.

Disclosures

The authors acknowledge the support of the Medical Research Council (K.L. Lee and M. Yasin), William Harvey Research Foundation (M. Yasin), the British Heart Foundation (M. Lythgoe and M. Lovell), and the Engineering and Physical Sciences Research Council (K.C.). No conflicts of interest are declared. This work originates in part from the Barts and the London Cardiovascular Biomedical Research Unit. The grant number is: FS/04/018 PhD studentship.

Contributions

Study concept and design: MJL, MY, MMY, KS, MFL, CT, AM; Phenotype studies (infarct size, effects on apoptosis and necrosis, cardiac function and myocardial fibrosis: MJL, MY, KC, YS, AS, KT, AK, KS, MFL, JM, AM; 2-DE and mass spectrometry: KLL; PI3/Akt survival analyses: MY, MC, AK; Data analyses and interpretation: MJL, MY, KLL, MC, KYL, PBM, CT, AM; Writing of the paper: MJL, MY, KLL, CT, AM; Critical review of the manuscript: MJL, KLL, MFL, JM, PBM, CT, AM.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.atherosclerosis.2010.07.045](https://doi.org/10.1016/j.atherosclerosis.2010.07.045).

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