Randomized controlled trial on the cardioprotective effect of bone marrow cells in patients undergoing coronary bypass graft surgery

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Aims
This randomized study investigates whether bone marrow cells (BMCs) can reduce ischaemic injury during cardiac surgery.

Methods and results
Forty-four elective coronary artery bypass grafting patients were randomized to control group or BMCs group (whereby autologous BMCs were administered with each dose of cardioplegia antegradely into the coronaries). Troponin I and CK-MB were measured during the first 48 h after surgery and were not significantly different between the control and BMCs groups. The role of cardiopulmonary bypass (CPB) on the cardioprotective effects of BMCs was also studied using an in vitro model of stimulated ischaemia and reoxygenation on right atrial appendages obtained from controls either before or 10 min after the initiation of CPB. Bone marrow cells significantly reduced myocardial injury in muscles obtained prior to CPB. This effect was comparable with ischaemic preconditioning (IP), although their combination did not afford additional benefit. However, when muscles were harvested after CPB, myocardial injury in the ischaemic group alone was less, and BMCs or IP did not exert further protection.

Conclusion
Bone marrow cells did not afford additional benefit when used as an additive to cardioplegia during CPB. However, BMCs offer cardioprotection as potent as IP, when the heart is not subjected to stress, such as CPB, that per se can precondition the myocardium.

Keywords
Bone marrow cells • Cardioprotection • Coronary artery bypass grafting • Cardioplegia • Ischaemic preconditioning

Introduction
There has been considerable interest on the use of bone marrow cells (BMCs) for myocardial repair following initial encouraging results from animal studies.1,2 However, the mechanisms of the beneficial effects of BMCs remain unclear; and clinical trials on its applications following acute3–6 and chronic myocardial ischaemia7–10 have produced mixed results.

Recently, our laboratory has demonstrated that BMCs have a potent effect against ischaemic injury of the human myocardium in an in vitro model of acute ischaemia, reducing creatinine kinase (CK) release, apoptosis, and necrosis.11 This was in agreement with other studies in animal models whereby BMCs have been shown to decrease the expression of pro-apoptotic proteins and reduce apoptosis.12–14 To further exploit this potential cardioprotective effect in the clinical setting, we have conducted the first randomized controlled trial to investigate whether the administration of autologous BMCs as an additive to cardioplegia solution during cardiac surgery can reduce myocardial ischaemic injury, which remains a main cause of complications and morbidity after cardiac surgery.15

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Methods

Study population
Patients aged between 20 and 80 years, undergoing first-time elective coronary artery bypass grafting (CABG) by a single surgeon (M.G.), were considered for the study. For inclusion in the study, the patients must have three graftable diseased coronary vessels and a left ventricular ejection fraction greater than 40%. The exclusion criteria were: unstable angina, cardiogenic shock (systolic blood pressure less than 80 mmHg, requiring intravenous inotropes or an intra-aortic balloon pump), percutaneous coronary intervention during the preceding 3 months, pre-existing bone marrow conditions, bleeding disorders, hepatic or renal failure, diabetics, chronic inflammatory disease, previous neoplasm, chronic treatment with oral antibiotic agents, or the K$_{ATP}$ channel opener, nicorandil.

The study was approved by the local Ethics Committee and was conducted in accordance with Medicine for Human Use (Clinical Trials) Regulations 2004 and EU Clinical Trials Directive in UK. The clinical trial is also registered on the International Standard Randomized Control Number Register (ISRCTN 22639836).

Study design
Patients were randomly assigned by computer-generated block randomization to receive BMCs during each dose of cardioplegia (BMCs group) or to be control. Physicians treating the patients as well as the investigators analyzing the results were blinded to the randomization.

The primary end-point was a reduction in myocardial injury during the first 48 h after the release of aortic cross-clamp. The secondary end-point was an improvement in cardiac function during the first 24 h after aortic cross-clamp release. Information was also collected regarding any post-operative complications prior to the patient’s discharge.

Operative procedure
All patients underwent CABG under standard anaesthetic protocol, operative techniques, and post-operative care. Briefly, patients were given Temazepam 20 mg and ranitidine 150 mg as pre-medication 2 h before their scheduled operation. Intravenous access was established in the induction room, before the patients were pre-oxygenated and monitored with ECG, pulse oximetry, and arterial line pressure tracing. Anaesthesia was then induced with fentanyl 5–10 µg/kg, midazolam 0.05–0.1 mg/kg, and rocuronium 1 mg/kg and maintained with O$_2$/air mixture and isoflurane to achieve a Bispectral Index System reading of less than 50. Patients were then intubated and a central venous catheter as well as Swan Ganz pulmonary artery flow catheter were inserted. All operations were performed through a median sternotomy using standard techniques with cardiopulmonary bypass (CPB) under full heparinization (3–4 mg/kg intravenously), and regular doses of cold blood cardioplegia (ratio of blood to St Thomas’ cardioplegic solution No 1 of 1:4–1:1000 mL was given during the first dose, subsequently 500 mL was given at 15–20 min interval, coinciding with the completion of each bypass graft). Bone marrow was aspirated from all patients before the start of CABG. In patients assigned to the BMCs group, BMCs were given together with the cardioplegia at the end of each dose of cardioplegia (see below). After their operation, patients were transferred to the Cardiac Intensive Care Unit for further care, keeping their cardiac filling pressures (central venous pressure between 8 and 12 mmHg and pulmonary capillary wedge pressure between 12 and 16 mmHg with appropriate transfusion), heart rate (between 70 and 90 b.p.m. with atrial pacing if required), and systemic vascular resistance index (between 1200 and 1800 U using vasodilators such as GTN and vasoconstrictors as norepinephrine and vasopressin if required) within the physiological range. Patients were extubated and cared for post-operatively as per unit standard routine procedures until discharge.

Bone marrow cells preparation and administration
After anaesthesia but before CABG, 120 mL of bone marrow were aspirated from the patient’s iliac crest into preservative-free heparin (10 U/mL) and diluted with normal saline. The BMCs were separated by density centrifugation with Lymphoprep® (AXIS-SHIELD PoC AS, Oslo, Norway) and diluted in 30 mL of cardioplegia solution. The viability of BMCs (assessed by trypan blue exclusion) after processing and immediately before administration was more than 94%. Ten millilitre of diluted BMCs was delivered at the end of each cardioplegia infusion antragradely into the coronary circulation via a side-arm of the administration set close to the aortic root.

Assessment of myocardial injury
Blood samples were taken before surgery and at 4, 12, 24, and 48 h after the cross-clamp release for the determination of plasma levels of Troponin I and CK-MB. Troponin I and CK-MB were measured using ADVIA Centaur CP system (Siemens, Deerfield, USA) based on direct chemiluminescence technology. The minimal detection limits of the assay were 0.04 and 3.03 ng/mL for Troponin I and CK-MB, respectively.

Assessments of cardiac function
Cardiac index was measured by thermodilution using a Swan-Ganz pulmonary artery flow catheter at different time points: before surgery and 1, 2, 4, 8, 12, and 18–24 h after aortic cross-clamp release.

In vitro study
To study the role of CPB on the cardioprotective effect of BMCs, in vitro studies were performed and the effects of BMCs were compared with that of ischaemic preconditioning (IP). To do this, the right atrial appendage from 12 control patients was collected either immediately before or 10 min after the initiation of CPB and then subjected to 90 min of ischaemia followed by 120 min of reoxygenation. Briefly, samples were collected in cold (4°C) aerobic Krebs/Henseleit/HEPES (KHH) buffered medium (containing in mM: NaCl 118, KCl 4.8, NaHCO$_3$ 27.2, KH$_2$PO$_4$ 1.0, MgCl$_2$ 1.0, CaCl$_2$ 1.25, glucose 10, and HEPES 20, at pH 7.4 and pre-bubbled with 95% O$_2$/5% CO$_2$). Tissue was sectioned with a skin-graft blade (Swann-Morton Ltd, Sheffield, UK) to slices of 300–500 µm thick and weight of 30–50 mg each, under constant cold aerobic KHH irrigation. All the sliced muscles were first equilibrated in normothermic aerobic condition (aerobic KHH of pH 7.4, bubbled continuously with 95% O$_2$/5% CO$_2$) for 50–60 min before they were allocated to one of the following groups: aerobic control, ischaemia/reoxygenation alone (IR), and IP, BMCs, or IP + BMCs followed by IR. For the IR group, ischaemia was induced by incubating the muscles in glucose-free KHH bubbled with 95% N$_2$/5% CO$_2$ (pH 6.8) at 37°C for 90 min. Muscles were then reoxygenated under normothermic aerobic condition for 120 min. For IP group, IP was induced using a protocol that has been shown to induce maximal protection in an identical experimental model and this involved 5 min of ischaemia followed by 5 min of reoxygenation. In the BMCs group, BMCs isolated by density centrifugation, as described above, were co-incubated with muscles (5 x 10$^6$)
BMCs (per flask) throughout IR. Muscles subjected to aerobic condition for identical experimental duration served as time-matched controls.

Myocardial injury was assessed by total CK release into the incubation media during the 120 min of reoxygenation. Creatinine kinase was measured by ELISA method with a plate reader (Benchmark, Bio-Rad Laboratories, CA, USA) using a commercial CK assay kit (30-3060/R2; Abbott Laboratories, Kent, UK) and expressed as IU/mg wet tissue.

Cell death by necrosis and apoptosis was assessed on frozen sections of muscles, using propidium iodide (Sigma Aldrich, Dorset, UK) and TUNEL (Roche Diagnostics, Penzberg, Germany) staining, respectively, and with 4′, 6-Diamidino-2-phenylindole (DAPI, Molecular Probes, OR, USA) as nuclear counterstain. At least ten fields at ×40 magnification per section were examined in each experimental condition using Axio 200M Zeiss fluorescent microscope (Carl Zeiss Inc., Göttingen, Germany). The images were acquired with OpenLab Version 5 software (Improvement Ltd, Coventry, UK) and analysed using the Scion NIH Image software (Scion Corporation, MD, USA). Only necrotic or apoptotic signals coinciding with DAPI were considered as true events. Fluorescent signals with areas less than 16 µm² were also excluded to avoid the inclusion of artefacts.

Statistics and expression of results
As this was a novel study, we did not know the variability associated with the primary end-point at the start of the study. Therefore, to determine the sample size, we specified that we wish to be able to detect whether the treated group was as much as one standard deviation better off than the control group. Assuming 80% power, an α = 0.05 and an estimated 20% drop-out rate, 22 subjects per group were required. Continuous variables that were normally distributed were presented as mean ± standard deviation (SD), and differences between two groups were compared using independent t-tests. For non-parametric data, the χ² test or the approximate non-parametric Mann–Whitney test was used. For the analysis of myocardial injury and cardiac function between groups, area under the curve (AUC) was calculated for Troponin I, CK-MB, and cardiac index over their, and values were almost identical in both study groups in the first 48 h after surgery, and the elevations had a similar profile for the first post-operative day in the BMCs group (Figure 1A and B), the plasma Troponin I and CK-MB were not significantly different from those seen in the control group (Troponin I: 165.6 ± 153.2 vs. 188.2 ± 213.8 µg/L in control and CK-MB 639.6 ± 714.9 vs. 567.1 ± 454.2 µg/L in control; P = NS in both instances).

Cardiac enzymes
As shown in Figure 1A and B, the plasma Troponin I and CK-MB level were equally raised after surgery, and the elevations had a similar profile for the first post-operative 48 h in the BMCs treated and control groups. Analysis of the AUC, also for the first 48 h after surgery confirmed that the plasma levels for Troponin I and CK-MB in the BMCs treated group were similar to those in the control group before surgery. As this was a novel study, we did not know the variability associated with the primary end-point at the start of the study. Therefore, to determine the sample size, we specified that we wish to be able to detect whether the treated group was as much as one standard deviation better off than the control group. Assuming 80% power, an α = 0.05 and an estimated 20% drop-out rate, 22 subjects per group were required. Continuous variables that were normally distributed were presented as mean ± standard deviation (SD), and differences between two groups were compared using independent t-tests. For non-parametric data, the χ² test or the approximate non-parametric Mann–Whitney test was used. For the analysis of myocardial injury and cardiac function between groups, area under the curve (AUC) was calculated for Troponin I, CK-MB, and cardiac index over their, and values were almost identical in both study groups in the first 24 h after surgery.

Cardiac function
As seen in Table 2, the mean cardiac index in the BMCs treated group was similar to those in the control group before surgery and values were almost identical in both study groups in the first 24 h after surgery.

In vitro study
In contrast to the clinical in vivo results, Figure 2A–C shows that BMCs significantly reduced CK release and the degree of cell administration. A patient in the control group developed post-operative atrial fibrillation requiring medical treatment. One other patient in the BMCs group had low-systemic resistance after surgery and required vasoconstrictor therapy (norepinephrine) for 48 h. Another patient from the BMCs group also was re-intubated the first post-operative day a further 24 h of mechanical ventilation due to poor gas exchange after initial successful extubation.

Results
Baseline characteristics and operative and post-operative data
A total of 44 patients were consented for the study. Two patients, one from each group, withdrew from the study before the surgery. A mean of 155 ± 78 × 10⁶ BMCs were administered in the treated group, comprising of a mean of 24.9 ± 30.0 × 10⁶ CD34/117 positive cells, 6.8 ± 9.4 × 10⁶ CD45 positive cells, 78.4 ± 130.8 × 10⁶ CD34 positive cells, and 20.3 ± 22.3 × 10⁶ CD133 positive cells. As shown in Table 1, the baseline characteristics and operative date were similar in both study groups. There was no identifiable complication associated with the BMCs application during CPB. The myocardiectomy wound was closed with continuous 2-0 nylon suture. A patient in the control group developed post-operative atrial fibrillation requiring medical treatment. One other patient in the BMCs group had low-systemic resistance after surgery and required vasoconstrictor therapy (norepinephrine) for 48 h. Another patient from the BMCs group also was re-intubated the first post-operative day a further 24 h of mechanical ventilation due to poor gas exchange after initial successful extubation.

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necrosis and apoptosis when compared with control when muscles were obtained prior to the initiation of CPB. The benefit induced by BMCs was similar to the one that obtained with IP, however their use in combination did not result in further improvement to the one seen with each intervention. Importantly, as shown in Figure 3A–C, when muscles were harvested 10 min after the initiation of CPB, cell necrosis and apoptosis seen in the control group were less than in the muscles obtained prior to CPB. In this instance, the mean values for the BMCs- and IP-treated groups alone and in combination were not significantly different from the control values, all suggesting that the muscles were already protected by the initiation of CPB.

Discussion

This randomized study is the first to investigate the potential cardioprotective effect of BMCs in the clinical setting. The results have shown that the use of BMCs as an adjunct to the cardioplegic solution does not afford additional cardioprotection, as assessed by the plasma values of Troponin I and CK-MB. In contrast, the in vitro studies demonstrated that although cardioprotection can be obtained when the muscles are harvested prior to CPB, the effect is dissipated when they were harvested 10 min after the initiation of CPB. The absence of additional cardioprotection in muscles obtained after the initiation of CPB was due to a reduction of ischaemic injury in the control muscles rather than to a loss of cardioprotective effect of BMCs or IP; this suggesting that CPB preconditions the heart and that the additional use of BMCs and IP do not induce further protection. These results are of relevance for the understanding of the mechanism underlying the beneficial effect of BMCs and warrant further discussion.

There are still controversies as to whether BMCs contribute to myocardial repair and if so whether is by myocyte regeneration, neovascularization and other paracrine effects. Having previously demonstrated the cardioprotective effect of bone cells against ischaemic injury in an in vitro model, here we set off to explore the potential beneficial effect of BMCs in limiting myocardial ischaemic injury during cardiac surgery. Thus, unlike previous clinical trials to-date that were not specifically designed to address the mechanism of action, we have attempted to test a specific mechanistic property of BMCs. This clinical set-up was chosen because the initiation of myocardial ischaemia and the timing of reperfusion are controlled. Furthermore, to reduce study variability, patients with good LV function and those requiring only three bypass grafting were selected. Hence, all patients received the same dose of cardioplegia and the bypass and cross-clamp time were also similar in all study patients. Although, BMCs did not confer additional cardioprotective benefits when used as an adjunct to cardioplegia and during CPB, the findings are of particular importance for explaining the reported controversial results from clinical studies.

The apparent dichotomy of the presence of cardioprotection by BMCs in the in vitro studies but absence of a significant anti-ischaemic effect in the clinical study may be explained by the activation of cardioprotection by CPB, which is supported by the abolition of the benefit on CK release and cell necrosis and apoptosis in control muscles exposed to CPB prior to their harvesting rather than due to a loss of protection by BMCs or IP. The findings are further supported by previous results from our laboratory showing that the reduction in the plasma leakage of cardiac enzyme induced by IP in patients undergoing CABG without the

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<th>Intervention</th>
<th>Time after cross clamp release (h)</th>
<th>P-value</th>
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<tr>
<td></td>
<td>Baseline</td>
<td>1</td>
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<tr>
<td>Control</td>
<td>2.51 ± 0.59</td>
<td>3.63 ± 0.54</td>
</tr>
<tr>
<td>BMCs</td>
<td>2.31 ± 0.60</td>
<td>3.29 ± 0.86</td>
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use of CPB was absent in patient operated on CPB. Indeed, preconditioning of the myocardium can be induced by a large number of stress conditions including ischaemia, hypoxia, heat, stretch, etc, and it is possible to conclude that, in the presence of CPB, the use of BMCs does not induce further protection against ischaemic injury.

The finding that BMCs and IP induce similar degree of cardio-protection when used alone and that their use in combination does not result in additional benefit support the thesis that both interventions share similar mechanism of action. This view is supported by previous study from our laboratory demonstrating that the cardioprotection induced by BMCs can be abrogated by blocking the activation of the kinases PKC and p38 MAPK, which are also essential to elicit protection by IP. Although there are still work to be done to fully elucidate the cardioprotective mechanisms of BMCs and IP, our findings are of great clinical relevance since to obtain maximal cardioprotection by of BMCs, they should be administered at a time when the myocardium is not preconditioned by other means. This could provide explanation to the variable results of clinical trial where BMCs were
Cardioprotective effect of BMC

injected at different times ranging from 3 h to 7 days after an acute myocardial infarction. It is worth noting that the study was performed under clinical conditions where double blinded randomization could not be achieved. Furthermore, as the inter-subject variability in cardiac enzymes was large, the power of the study was limited for moderate or smaller treatment effects. In addition, the assessment of endpoints only up to 48 h, and the absence of additional evaluations of left ventricular and regional function may represent limitations of the study.

In conclusion, we have shown that the use of BMCs as an additive to cardioplegia during CPB did not confer additional cardioprotection above and beyond that of cardioplegia. However, the in vitro study has demonstrated that BMCs can induce cardioprotection in the absence of stress conditions such as CPB that per se, may have already preconditioned the heart. These results are of clinical relevance and need to be taken into consideration for the planning of future clinical trials.

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