CHARACTERISATION AND MECHANISM OF BONE MARROW CELL-INDUCED MYOCARDIAL PROTECTION

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ABSTRACT – Characterisation and Mechanism of Bone Marrow Cell-Induced Myocardial Protection – Vien Khach Lai

It is controversial whether the administration of bone marrow cells (BMCs) can repair the heart after an ischaemic insult and whether the mechanism of the putative beneficial effect is due to a paracrine action. By using an experimental model of simulated ischaemia of the atrial myocardium, I set up this thesis to understand the protective properties of BMCs on the human myocardium with different clinical conditions and to investigate the underlying mechanism. The results provide clear data that myocardial protection of BMCs is dose-dependent with a maximal protection, comparable to that of ischemic preconditioning, at a dose of ~$1.5x10^6$ cells/mg wet myocardium and that the similar effect can be achieved when BMCs were present before, during or after ischaemia. Allogeneic BMCs are as protective as the autologous cells and their capacity is unaffected by frozen storage or culture, but lost by magnetic isolation. In addition, the conditioned media, obtained after 30min of incubation of BMCs, can induce a protection similar to that of BMCs, an effect that is abolished by selective blockade of the insulin-like growth factor-1 receptor. Furthermore, disease states such as diabetes and poor left ventricular function or the long-term administration of the mitochondrial K_{ATP} channel opener nicorandil prevents the protective effect of BMCs, a consequence that appears to be due to an alteration in the myocardium for diabetes and nicorandil and mainly due to a defect in BMCs and also to a less responsive myocardium in heart failure. A final clinical study aimed to elucidate the effect of BMCs during cardiac surgery showed that BMCs have no additive protection to conventional cardioplegia during coronary artery bypass grafting. In this thesis I have characterised the cardioprotective potential of BMCs and identified some of the underlying mechanisms. The results are of clinical interest and they should generate novel hypotheses.

Dedication

To my parents for their academic inspiration and to my wife and son for their love and support.

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List of abbreviation

ADP	: Adenosine diphosphate
Akt/PKB	: Protein kinase B
AMP	: Adenosine monophosphate
ANOVA	: Analysis of variance
ASTAMI	: Autologous Stem Cell Transplantation in Acute Myocardial Infarction
ATP	: Adenosine triphosphate
AUC	: Area under the curve
BMCs	: Bone marrow cells
BOOST	: BOne marrOw transfer to enhance ST-elevation infarct regeneration
CABG	: Coronary artery bypass grafting
CAD	: Coronary artery disease
CK	: Creatine kinase
CK-MB	: Creatine kinase MB isoenzyme
CPB	: Cardiopulmonary bypass
CXCR	: Alpha chemokine receptor
Cy	: Cyanine
DAPI	: 4', 6-Diamidino-2-phenylindole
ECG	: Electrocardiography
EF	: Ejection fraction
EGFP	: Enhanced green fluorescent protein
EPCs	: Endothelial progenitor cells
FITC	: Fluorescein isothiocyanate
FGF	: Fibroblast growth factor
GFP	: Green fluorescent protein
GTN	: Glyceryl trinitrate
HGF	: Hepatocyte growth factor
HSCs	: Haematopoietic stem cells
IGF	: Insulin-like growth factor
IGF-R	: Insulin-like growth factor receptor
Is	: Ischaemia

IP	: Ischaemic preconditioning
I/R	: Ischaemia/reoxygenation
K_{ATP}	: Adenosine triphosphate dependent potassium
LV	: Left ventricular
MAPCs	: Multipotent adult progenitor cells
MAPK	: Mitochondrial activated protein kinase
MPTP	: Mitochondrial permeability transition pore
MSCs	: Mesenchymal stem cells
NHS	: National Health Service
NIH	: National Institute of Health
PBS	: Phosphate buffered saline
Pi	: Inorganic Phosphate
PI	: Propidium iodide
PKC	: Protein kinase C
Re	: Reoxygenation
REPAIR-AMI	: Reinfusion of Enriched Progenitor Cells and Infarct Remodelling in Acute Myocardial Infarction
ROS	: Reactive Oxygen Species
SD	: Standard deviation
SDF	: Stromal cell-derived factor
SEM	: Standard error of the mean
SP	: Side population
STEMI	: ST-Elevation acute Myocardial Infarction
SWOP	: Second window of protection
TOPCARE-CHD	: Transplantation Of Progenitor Cells And Recovery of left ventricular function in patients with Chronic ischaemic Heart Disease
TUNEL	: Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling
VEGF	: Vascular endothelial growth factor

Publication arising from this thesis

Abstracts

1. **Vien Khach Lai**; José Linares-Palomino; Bernardo Nadal-Ginard; Manuel Galiñanes. Potency and Mechanism of Bone Marrow Cells-Induced Cardioprotection. Circulation, Oct 2008; 118: S_537. (Oral presentation at the annual meeting of the American Heart Association, New Orleans, Louisiana, USA. 8-12 November 2008)

2. **Vien Khach Lai**, José Linares-Palomino, Manuel Galiñanes. Is the Cardioprotective Paracrine Action of Bone Marrow Cells Influenced by Clinical Conditions? J. Am. Coll. Cardiol., March 10, 2009; 53: A306. (Supplement A). (Poster presentation at the 58th Annual Scientific Session of American College of Cardiology, Orlando, Florida, USA. 29-31 March 2009)

Articles

1. **Vien Khach Lai,** Manuel Galiñanes. Protection of Human Myocardium by Bone Marrow Cells: Role of Long-term Administration of the Mitochondrial K_{ATP} Channel Opener Nicorandil. Journal of Surgical Research. 2010 (accepted).
2. **Vien Khach Lai,** José Linares-Palomino, Bernardo Nadal-Ginard, Manuel Galiñanes. Bone Marrow Cell-Induced Protection of the Human Myocardium: Characterization and Mechanism of Action. Journal of Thoracic Cardiovascular Surgery. 2009 Dec;138(6):1400-08.
3. **Vien Khach Lai**, Keng-Leong Ang, Wendy Rathbone, Nicholas James Harvey, Manuel Galiñanes. Randomized controlled trial on the cardioprotective effect of bone marrow cells in patients undergoing coronary bypass graft surgery. European Heart Journal, 2009 Oct;30(19):2354-9.

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CHAPTER 1: INTRODUCTION

According to the 2006 report of the World Health Organization, coronary heart disease is the leading cause of death worldwide and its incidence is continuously rising and becoming a true pandemic. Despite improvements in survival rates, in the USA, 1 in 4 men and 1 in 3 women still die within a year of a recognised first heart attack[1].

The reduction or complete cut-off of blood to the myocardium leads to the loss of cardiomyocytes by necrosis and apoptosis, which in turn is replaced by fibrous tissue. This results in progressive remodelling of the left ventricle that can ultimately cause irreversible heart failure.

A large portfolio of therapies to combat ischaemia/reperfusion injury has been investigated for the last few decades. Among these, ischaemic preconditioning (IP), a powerful natural mechanism of protection in which short episodes of ischaemia followed by reperfusion prevent the damage from prolonged subsequent ischaemia and reperfusion injury, was discovered in 1986 by Murry and his colleagues[2], has been thoroughly studied experimentally and clinically. However, IP still has limitations when applied in the clinical setup and because of this, investigators have tried to activate the cellular mechanisms responsible for the cardioprotection by pharmacological means to achieve a similar outcome as that of IP.

The discovery that haematopoietic and mesenchymal stem cells (MSCs) from bone marrow cells (BMCs) can acquire mature cell lineages different from their tissue of origin has started a new intriguing scientific revolution[3, 4]. The belief is that the administration of BMCs can help to repair the injured myocardium following a heart attack[5] although the capacity of BMCs to trans-differentiate into heart tissue has been

recently challenged[6-10], other investigators, including ourselves, have demonstrated that these cells may reduce myocardial ischaemic injury and improve cell survival, an effect that is possibly medicated by a paracrine factor(s)[11-13].

1.1 Myocardial ischaemia/reperfusion injury

1.1.1 Ischaemic injury

Both hypoxia and ischaemia represent a phenomenon in which the oxygen supply to the tissue is interrupted, but in ischaemia the blood flow is also discontinued which in turn causes the accumulation of lactic acid and other waste products from the anaerobic metabolism of cells[14]. A lack of oxygen results in the inhibition of oxidative phosphorylation and in a dramatic loss of ATP with a concomitant rise in ADP, AMP and Pi. This metabolic process is incapable of producing sufficient ATP and as a result normal cardiac contraction can not be maintained. In addition, CO_2 and lactic acid accumulate[15] causing tissue and cellular acidosis, leading ultimately to cell death[16]. Alterations in the ion transport systems of the sarcolemma and organellar membranes develop[17, 18], which are responsible for the development of ventricular arrhythmias. An increased osmotic load caused by the accumulation of metabolites and Pi leads to an increase in K^+ efflux and an increase in free Mg^{2+} which is then followed by a decrease in total Mg^{2+}. With a substantial decline in ATP, the Na^+/K^+-ATPase pump is inhibited, resulting in a further decline in intracellular K^+ and an increase in Na^+. The influx of Na^+, Cl^- and water leads to cell swelling. An early increase in cytosolic Ca^{2+} also develops due to multifactorial changes in transport systems in the sarcolemma and sarcoplasmic reticulum. The rise in cytosolic Ca^{2+} induces activation of proteases causing alterations in contractile proteins that, together with a decreased sensitivity to

Ca^{2+}, are responsible for sustained impairment of the contractility despite the elevated cytosolic Ca^{2+}. If ischaemia is prolonged, myocytes undergo structural changes including swelling of mitochondria and sarcoplasmic reticulum, with enlargement of the cytoplasm and physical defects in the sarcolemma, and also clumping of nuclear chromatin and linear densities in the mitochondria. The progression from reversible to irreversible myocardial cell injury is accompanied by changes in the myocardial interstitium and microvasculature and, al together, the necrosis of myocytes and nonmyocytes triggers an inflammatory reaction with subsequent tissue organization and healing[19].

Global ischaemia is an essential feature of cardiac surgery whereas regional ischaemia is a more common clinical circumstance caused by partial or complete coronary occlusion with drastic reduction in coronary blood supply. Ischaemia can also be seen in cases of increasing myocardial oxygen demand, of reducing oxygen supply or a combination of the two.

1.1.2 Reperfusion injury

The concept of reperfusion injury has been a topic of debate for many years and whether cardiomyocytes are injured primarily during ischaemia, which may be revealed at the start of reperfusion, or whether additional injury occurs during reperfusion remains unclear. This point has important clinical implications, because if additional injury occurs on reperfusion, this would allow an opportunity to intervene with cardioprotective drugs on reperfusion[20, 21]. The potential mechanisms of reperfusion induced injury are complex and include the release of oxygen-derived free radicals, dysregulation of intracellular and mitochondrial calcium, microvascular dysfunction

leading to incomplete return of blood flow to areas of the microcirculation (the non-reflow phenomenon), and an inflammatory reaction involving influx of various populations of immune cells.

Below I expand on each of these mechanisms that contribute to cell death during reperfusion.

Reactive oxygen species (ROS)

During reperfusion with the return of oxygen and nutrients there is a large formation of ROS. These radicals are generated mainly during the early reperfusion period leading to extensive oxidative tissue damage and the loss of cell viability[22-24]. Vanden Hoek et al[25] found in isolated cardiomyocytes that irreversible cell membrane injury, as measured by propidium iodide uptake, increased from 4% of cells at the end of ischaemia to 73% after reperfusion and implied that ROS participated in the injury. A potential mechanism through which ROS leads to cellular toxicity may be complement activation and an increasing binding ability of neutrophils[26]. More recently, the production of ROS during early reperfusion has been linked to the opening of mitochondrial permeability transition pore (MPTP) which in turn leads to cell death[27].

Evidence for a role of ROS in myocardial stunning in human beings, myocardial stunning: mechanical dysfunction that persists after reperfusion despite the absence of irreversible damage and despite restoration of normal or near-normal coronary flow[28], has also been provided[29], and treatment with antioxidants *in vivo* and *in vitro* studies reduced ROS production and stunning[30]. However, despite these findings, there is

still controversy on the role of antioxidants with clinical trials failing to prove a reduction in infarct size[31-33] while experimental studies showing benefits[34, 35].

Calcium loading

During ischaemia, ATP declines and pH decreases due to anaerobic glycolysis. The increase in proton stimulates Na^+/H^+ exchange and in turn the Na^+/Ca^{2+} exchange, results in an increase in cytosolic Ca^{2+}[36, 37]. Much of the ATP generated by glycolysis is consumed by the reverse mode of the mitochondrial F_1F_0-ATPase, which uses the energy to generate mitochondrial membrane potential and then can be used to take up Ca^{2+} into the mitochondria[38]. The increase in mitochondrial Ca^{2+} can activate the MPTP, but the existing low pH inhibits the pore during ischaemia so that this is not activated until reperfusion, when the pH is restored to normal[20]. During reperfusion, extracellular pH rapidly returns to normal. However, initially, the intracellular pH is still acidic, and this pH gradient facilitates extrusion of H^+ from the cell in exchange for Na^+ via the Na^+/H^+ exchange. The increased cytosolic Na^+ can be extruded by Na^+/K^+-ATPase pump and also by the Na^+/Ca^{2+} exchanger, thereby raising, at least transiently, cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$). Arrhythmias can be triggered by altered Ca^{2+} homeostasis and that is a major cause of death during ischaemia/reperfusion. In the absence of arrhythmias, $[Ca^{2+}]_i$ returns quickly to near-normal levels in myocytes that survive[20]. However, in some cases, $[Ca^{2+}]_i$ remains high during reperfusion, accompanied with the cell re-energization, can lead to hypercontracture that *per se* will contribute to further decline in ATP and deterioration of the myocyte resulting in irreversible injury[39, 40]. The dysfunction of the sarcoplasmic reticulum contributes to the Ca^{2+} oscillations leading to further ATP depletion and exacerbation of arrhythmias[20]. Therefore, targeting the sarcoplasmic reticulum during the initial of

reperfusion can regulate Ca^{2+} cytosolic levels and reduce ischaemic reperfusion injury[41, 42]. The administration of Na^{+}/H^{+} exchange inhibitors at the beginning of reperfusion may also improve $[Ca^{2+}]_i$ regulation and elicit protection[43]; however, initial clinical trials have failed to show a benefit with the administration of these agents[44, 45].

Neutrophils

Artery occlusion critically reduces blood flow to the portion of the myocardium subserved, markedly impairing the energy metabolism. In occlusions of the coronary arteries as short as 5min, functional abnormalities of the reperfused myocardium are observed for as long as 24–48 hrs. These abnormalities are not followed by lethal injury and the ischaemic myocardium is ultimately recovered. Ischaemia of significant duration to induce infarction results in an inflammatory response, which is both accelerated and augmented if the ischaemic tissue is reperfused. Evidence that inflammation can extend myocardial injury has come from the administration of anti-inflammatory strategies in animal models of myocardial ischaemia and reperfusion[26]. The systemic administration of corticosteroids was shown to decrease infarct size in a canine model of experimental myocardial infarction, but it may also delay healing and collagen deposition that, in turn, may cause catastrophic results by increasing the incidence of ventricular arrhythmias and extending infarct size[26]. By employing neutrophil depletion in an animal model undergoing 90min ischaemia followed by reperfusion, a marked reduction in infarct size was found, leading to the belief that neutrophils may contribute to the mediation of reperfusion injury by releasing oxidants and proteases and possibly the expression of factors capable of increasing chemotaxis[46, 47]. Indeed, the release of chemo-attractants after an acute myocardial

infarction draws neutrophils into the infarct zone during the first 6 hours of reperfusion and then during the next 24 hours they migrate into the myocardial tissue, a process that is facilitated by cellular adhesive molecules.

Neutrophils have been implicated as primary and secondary mediators of lethal injury after reperfusion to coronary vascular endothelium and cardiomyocytes. However, whether neutrophils are directly involved in the pathogenesis of lethal reperfusion injury in the myocardium, are just the first responders to inflammatory signals released after the onset of reperfusion, or are important to an early but not clinically important phase of pathology are still points of controversy[48]. Experimental studies have shown reductions in infarct size of up to 50% with several interventions aimed at neutrophils during myocardial reperfusion. These interventions include leukocyte-depleted blood, antibodies against the cell-adhesion molecules such as P-selectin, CD11 and CD18, and the intercellular adhesion molecule-1 and also pharmacological inhibitors of complement activation. Yet again, despite the positive animal studies, clinical trials have failed to demonstrate meaningful cardioprotective effects from such interventions[28].

1.1.3 Consequences of Myocardial Ischaemia: Cell Death by Necrosis, Apoptosis and Autophagy

Classically there are two recognized patterns of cell death: necrosis and apoptosis. A third cellular process known as autophagy has been recently described as another potential cellular mechanism. The precise proportion of cell death following ischaemic reperfusion may be varied and much depends on the model of experimental designs. Below I describe the three forms of cell death. Other consequences of myocardial

ischaemia such as arrhythmias and myocardial stunning derived from mild, non-lethal ischaemia are beyond the scope of my thesis and are not discussed.

Necrosis

The term "necrosis" is derived from the Greek "nekros" for corpse. Necrosis came to be used to describe an uncontrolled process or accidental/pathologic cell death. Necrotic cell death was believed to occur as a result of an irreversible bioenergetic compromise. The cell dying by necrosis has been viewed as a victim of extrinsic events beyond its control. The fundamental feature that distinguishes most forms of necrosis is the rapid loss of cellular membrane potentials. The inability to maintain the electrochemical potentials results in cytoplasmic and mitochondrial swelling, rupture of the plasma membrane, and cytolysis without severe damage to nuclei[49]. This loss of membrane potential may be the consequence of cellular energy depletion, damage to membrane lipids, and/or loss of function of homeostatic ion pumps/channels. It is difficult to determine whether the loss of ATP production or the disruption of channel function occurs first, but these defects can amplify in the induction of necrotic cell death.

The necrosis-induced release of cytosolic components results in an inflammatory response which is, as discussed before, a critical event of ischaemia/reperfusion injury. The release of cardiac enzymes such as troponins and creatine kinases seen after ischaemia/reperfusion is likely to be the product of necrosis of intracellular components. Until recently, necrosis was thought to be an unregulated process. However, recent evidence suggests that a cell can initiate its own deterioration by necrosis in a programmed manner. The genetic components of this programmed cell necrosis may involve (1) gene products that function in the dying cell to induce an irreversible

bioenergetic compromise that results in cell death, and (2) gene products that are selectively released into the extracellular environment to trigger a host response[50]. Therefore, programmed cell necrosis can be the consequence of extracellular signalling or can be initiated as a form of cellular suicide in response to intracellular perturbations. Programmed cell death by necrosis is not only a cardiac phenomenon but also plays a role in a number of diseases including neurodegenerative diseases, infection, inflammatory diseases, exposures to toxins, and cancer[50].

Apoptosis

Apoptosis , a term derived from the Greek word describing the falling off of petals from a flower or leaves from a tree, is a form of programmed cell death originally described by Kerr et al in 1972[51]. Apoptosis can happen under physiological or pathological conditions as a result of endogenous or exogenous stimuli. The morphological features of apoptosis are characterized by nuclear and cytoplasmic condensation with blebbing of the plasma membrane, cell shrinkage and eventually break up into particles, which contain apoptotic bodies including intact organelles as well as portions of the fragmented nucleus. These apoptotic bodies are then rapidly phagocytised by other cells. Apoptosis is an important process to maintain normal cell turnover during embryogenesis and in adult tissues that typically does not release intracellular components and then it does not induce inflammation or tissue scarring.

The mechanism of apoptosis may involve a series of molecular, biochemical, and morphological events including (1) gene activation (programmed cell death); (2) activation of a cascade of cytosolic aspartate specific cysteine proteases (caspases); (3) mitochondrial alterations such as loss of membrane potential, initiation of the MPTP,

and cytochrome C release; (4) endonuclease activation leading to double-stranded DNA fragmentation; and (5) selective alteration of cell membranes and their permeability with an increased expression of phosphatidylserine in the outside of cells[19, 51, 52]. The inhibition of these mechanisms can reduce the occurrence of apoptosis. Thus, for example, the inhibition of caspases, that target a number of mitochondrial proteins can result in limitation of infarct size after a lethal ischaemia[53-55], although some investigators have argued that the inhibition of caspases while decreasing apoptosis may switch on other pathways of cell death such as necrosis or autophagy[56, 57]. Certainly, the role of caspases in ischaemia/reperfusion injury still remains the subject of debate[58, 59].

Autophagy

Besides apoptotic and necrotic cell death, another important cellular pathway that has been recently implicated in cardiac cell death is autophagy. Autophagy is generally defined as a cellular self-process involved in the breakdown of intracellular proteins and organelles via lysosomal degradation. There are three pathways for autophagy that have been detected in mammalian cells: (1) microautophagy involves the local uptake of protein by the lysosome itself; (2) chaperone-mediated autophagy relies on the delivery of proteins to the lysosome; (3) macroautophagy is the most common and active form of autophagy, and the term autophagy in the literature generally refers to macroautophagy[60].

The process of autophagy begins with the processing of an intracellular membrane fragment called a phagophore that may have been synthesized *de novo*, or may have originated in the sarcoplasmic/endoplasmic reticulum[60]. It is believed that autophagy

is a homeostatic cellular process required for the recycling of proteins and damaged organelles, and to promote cell survival. At present, there is accumulating evidence that autophagy can also trigger and mediate programmed cell death under certain pathological situations and that over-activation of autophagy can lead to a complete self digestion of the autophagic cell particularly when mitochondria is also damaged[61].

Experimental *in vitro* studies have reported that autophagic activity during hypoxia confers protection[62, 63] and, similarly, the induction of autophagy *in vivo* experimental animal models has been shown to promote functional recovery of the heart after ischaemia/reperfusion[64], supporting the hypothesis that up-regulation of autophagy provides protection against ischaemia/reperfusion injury[65]. Therefore, The role of autophagy seems to be protective but the molecular events involved in this process during ischaemia/reperfusion still remains unclear[60].

There are a number of connections between autophagy and other forms of cell death and death following ischaemia/reperfusion injury appears to be a mixture of apoptosis, autophagy, and necrosis. It has been speculated that the distinction between the modes of death may not be important, since recent data suggest that all three forms of cell death are interrelated[20]. Obviously, the importance is that cell death during ischaemia/reperfusion appears to be a regulated active process, which can be inhibited with appropriate interventions.

1.2 Cardioprotection by ischaemic preconditioning

1.2.1 Preconditioning the non-pathological myocardium

In 1986, Murry and colleagues observed in the dog heart that four sublethal ischaemic periods of 5min each, induced by occlusion of the coronary artery, interspaced by short reperfusion periods of also 5min duration prior to prolonged ischaemia resulted in a 75% infarct size reduction[2]. This phenomenon, known as IP, has been proposed as "the strongest form of *in vivo* protection against myocardial ischaemic injury other than early reperfusion"[66]. The infarct size reduction by IP occurred when hearts were subjected to approximately 60-90min of ischaemia with lost of benefit when the coronary occlusion was prolonged to two to three hours or more, this showing the critical importance of early reperfusion even after lethal ischaemia[2]. The cardioprotection by IP is bimodal with a first phrase of protection (first window or classical preconditioning) lasting 1-2 hours after the preconditioning trigger, followed by an unresponsive period of between 1-4 hours according to models and species[67, 68], and finally by a second window of protection (SWOP) that is less effective but longer than the first window lasting approximately between 24 and 72 hours after the IP stimulus[69]. (See figure 1).

Figure 1 has been removed due to copyright issue. Please refer to Figure 2 of reference 69.

Figure 1: Diagrammatic representation of the temporal nature of the two windows of preconditioning[69].

The mechanism of IP has been thoroughly investigated although the precise signal transduction sequence and the potential end-effector(s) remain unclear. Initially, it was proposed that protection was induced by slowing the consumption of high energy phosphates in the dog heart[2, 70]. However, other investigators demonstrated in the isolated rat heart that in fact ATP consumption was accelerated in preconditioned hearts that also went into ischaemic contracture earlier and this was translated in a better recovery of contractility during reperfusion in the preconditioned hearts than in the non-preconditioned hearts[71]. It is accepted that the phenomenon is mediated by the activation of A1 and A3 adenosine receptors and other surface receptors such as opioid, muscarinic and adrenoreceptors leading to the activation of protein kinase C coupled with G proteins and the opening of K_{ATP} channels in the mitochondria rather than sarcolemma, that in turn and via activation of various protein kinases would result in inhibition of the MPTP, which has been suggested as the potential end-effector[20, 69, 72].

In 2003, it was demonstrated that the myocardium can also be salvaged at the time of reperfusion, with the same potency as that of IP, by applying short periods of ischaemia and reperfusion following prolonged ischaemia, a phenomenon that was termed postconditioning[73]. It was reported in an *in vivo* rabbit model that postconditioning is

only effective if applied within the first 10min of reperfusion[74], and if coronary occlusion duration is not extended more than 45min in the conscious rat model[75]. Interestingly, it has been shown that postconditioning confers protection through activation of similar mediators involved in IP, including the activation of several protein kinases and the inhibition of the MPTP[76-79].

1.2.2 Preconditioning the pathological myocardium: role of diabetes, K_{ATP} channel openers, and heart failure

There is conflicting evidence on whether the diabetic myocardium can be protected by IP as some studies have reported a beneficial effect[80, 81] and others have shown an absence of protection[82, 83]. It has been proposed that the loss of protection by IP in the diabetic myocardium is due to dysfunction of mitochondrial K_{ATP} channels[84, 85] that have been altered in ventricular myocytes from diabetic rats[86]. However, by increasing the cycles of IP from 1 to 3, a manoeuvre that induced a greater Akt phosphorylation[87], there is suggestion that sufficient intensity of the trigger ischaemia is needed to elicit protection. It is clear that more research is required to fully elucidate the role of IP in the diabetic heart.

Mitochondrial K_{ATP} channels are central to the signal transduction mechanism of IP and their opening has been demonstrated to induce cardioprotection[88]. Furthermore, there are clinical trials showing that the intravenous administration of nicorandil, a specific K_{ATP} channel opener, during coronary angioplasty induces cardioprotection[89, 90]. It could be argued that the regular administration of nicorandil could result in a state of permanent protection, however the myocardium of patients receiving nicorandil as an anti-anginal treatment was unresponsive to protection by IP[91]. Nonetheless,

protection could still be obtained when the protein kinases PKC and p38 MAPK, placed downstream of the mitochondrial K_{ATP} channels in the signalling transduction pathway of IP, were activated[91]. These results are of clinical importance by opening a window of therapeutic potential.

The severity of heart failure corresponds with the degree of oxidative stress which was lead to the notion that free radicals may play an important role in the progression of heart failure. Indeed, mitochondria isolated from failing cardiac myocytes produce 2.8 folds more superoxide than normal and this may alter the response of the myocardium to ischaemic stress[92, 93]. Our laboratory has demonstrated that, using an *in vitro* model of simulated ischaemia, the myocardium from patients with poor heart function (EF <30%) can not be protected by IP while exhibiting a degree of injury similar to that of patients with a greater EF[94]. The study also showed that the mitochondrial K_{ATP} channel opener diazoxide is capable of eliciting protection of the muscle from hearts with poor EF, this suggesting that the deficit of cardioprotection in patients with heart failure is due to a defect in the signal transduction pathway upstream of mitochondrial K_{ATP} channels and, importantly that the therapeutic interventions targeted to components of the signal transduction pathway beyond the malfunctioning point can be used with identical potency to that of IP[94].

1.3 Myocardial self-renewal and repair

A stem cell is defined by three main criteria: self-renewal, ability to differentiate into multiple cell types, and ability to achieve the phenotype of tissue where is seeded. A fertilized egg is capable of not only forming cells of the mesoderm, endoderm and ectoderm layer, and germ cells, but also supporting the trophoblast that is required for

the survival of the developing embryo. These cells that are at the top of the hierarchy of stem cells are termed ‘totipotent’. Embryonic stem cells and embryonic germ cells, isolated from the inner cell mass of the blastocyst or from primordial germ cells of an early embryo, give rise to mesoderm, endoderm, ectoderm and germ cells but not extra-embryonic tissues, and are therefore termed ‘pluripotent’. Stem cells isolated from various adult organs that self-renew and differentiate into multiple organ-specific cell types are termed ‘multipotent stem cells’. Committed cells that have limited or no self-renewal ability and differentiate into only one defined cell type are termed ‘progenitor cells’ or ‘precursor cells’[95].

For decades it has been widely accepted that the adult mammalian heart is a post-mitotic organ, based on the belief that cardiomyocytes can not re-enter the cell cycle and the mature heart possesses a relatively constant number of cardiomyocytes from shortly after birth and throughout adulthood and senescence and that the heart lacks stem/progenitor cells to re-constitute dying cells with new myocytes. According to this concept, the response of the heart to stresses such as pressure and volume overload would consist in myocardial hypertrophy and nuclear hyperplasia. However, in 2001, Orlic et al[5] reported that cardiac muscles can be regenerated in mice and the infarcted area reduced after ligature of the left anterior descending coronary artery when BMCs were injected in the damaged myocardium. These results contradicted the above dogma and opened the possibility that the heart may be a self-renewable organ with capacity for repair. Other investigators were unable to replicate these findings using an identical species and experimental model[7, 8], creating an intense debate in the field.

1.3.1 Cardiac progenitors, stem cells and cardiac regeneration

The concept that the heart possesses a pool of stem/progenitor cells which can contribute to myocardial regeneration is an exciting and promising prospect, which challenges the belief that the heart is a terminally differentiated organ. Up to date, adult hearts have been reported to contain cells such as c-kit$^+$, sca-1$^+$, isl1$^+$, and side population cells considered to be cardiac stem/progenitor cells. These cells have the ability to proliferate and differentiate into myocardial cells both *in vitro* and *in vivo*, and therefore they might have the potential to regenerate the injured heart. Stem cells have been delivered to the infarcted areas through different routes (direct intramuscular, intracoronary, intravenous injection), however, myocardial proliferation appears to be restricted to the viable myocardium in the peri-infarcted zone or in remote areas from the infarction rather than in the ischaemic region[12, 96].

Cardiac c-kit positive cells

C-kit$^+$ stem cells were first isolated from adult rat hearts using an antibody against the surface marker c-kit[97]. The isolated c-kit positive cells were negative for blood linage makers (Lin$^-$/c-kit$^+$ cells), did not possess specific protein markers for the myeloid, lymphoid, and erythroid lineages (CD34, CD45, CD20, CD45RO, CD8, and TER-119), but partially expressed cardiac transcription factors Nkx2.5, α-sarcomeric actin, GATA4, and MEF2. C-kit$^+$ cardiac stem cells are self-renewing, clonogenic, and multipotent and also have the ability to differentiate into three minimum cell types of the heart such as myogenic, endothelial and smooth muscle cell lineages *in vitro*. When implanted into the border zone of a myocardial infarcted heart, these cells or a mixture of cells including c-kit$^+$ cells differentiate into myocardium, forming new vessels and myocytes with the characteristics of young cells, in the absence of cell fusion, and

resulting in significant improvement of the heart performance[97]. It has also been suggested that injection of c-kit$^+$ cells from the bone marrow favourably influence the myofibroblast response in infarcted hearts, preventing infarct expansion and improving the recovery of cardiac function[98].

Cardiac sca-1 positive cells

The adult mammalian heart also has cells expressing the stem cell antigen-1 (sca-1$^+$) that has also been depicted as another cardiac progenitor population. Sca-1$^+$ cells expressed CD31 but not c-kit, CD45, CD34, Flt-1 or Flk-1. They possess the properties of side population cells able to extrude the Hoechst 33342 dye but differ from haematopoietic stem cells (HSCs), endothelial progenitor cells (EPCs), and muscular progenitor sca-1$^+$ cells. Treatment with the cytosine analog 5'-azacytidine allows these cells to differentiate into cells expressing the cardiac specific marker Nkx2.5. When cardiac sca-1$^+$ cells were given intravenously in a mouse model of coronary occlusion, they were able to home to injured myocardium and generate cardiomyocytes both with and without cell fusion in a roughly equal distribution[99]. In addition, sca-1$^+$ cells treated with oxytocin improve the gene expression for cardiac specific transcription factors and contractile proteins and cells develop sarcomeric structures and spontaneous beating. Treatment of these cells with isoproterenol increases the beating rate with characteristic intracellular Ca^{2+} transients[100].

Cardiac isl1-positive cells

The isl1$^+$ cells identified in the postnatal rat, mouse and human myocardium have also been considered as cardiac progenitors. These cells have the ability to self-renew and are capable of adopting a fully differentiated cardiomyocyte phenotype. Isl1-positive

cells do not express the Hoechst 33342 dye and do not express c-kit or sca-1, demonstrating that isl1^{+} cardiac stem cells are distinct from other populations. When co-cultured with neonatal rat myocytes, isl1^{+} cells can adopt the phenotype of authentic endogenous cardiac progenitors (cardioblasts) that displayed highly efficient conversion to mature cardiomyocytes with stable expression of myocytic markers in the absence of cell fusion, intact Ca^{2+} cycling, and the generation of action potentials[101]. Recently, it has been reported that the embryonic isl1^{+} cardiac cells of 1–5 days old mice are able to differentiate to all three lineages[102], however it is not clear if the same population exists in adult mammalian hearts.

Cardiac side population cells

The side population (SP) cells, first identified in bone marrow by their ability to extrude to Hoechst 33342, can be found not only in the bone marrow but also in non-haematopoietic tissues serving as progenitors for haematopoietic cells, skeletal muscle, and endothelium[103-105]. Recent studies have demonstrated that the adult heart contains SP cells, which are high positive for sca-1, but low positive for c-kit, CD34, and CD45, and are capable of proliferating and differentiating into myocytes[106, 107], and participating in myocardial repair when injected intravenously after cold burned injury was induced to the mouse heart[108]. SP cells appear as a mixture of vascular endothelial cells, smooth muscle cells, and mesenchymal stem/progenitor cells including potential cardiomyogenic cells[109]. Interestingly, it has been reported that among cardiac SP cells, the greatest potential for cardiomyogenic differentiation is restricted to CD31^{-}/sca1^{+} cells[110].

Bone marrow stem cells

Bone marrow, the soft tissue found in the hollow interior of bones, constitutes around 2-5% of total body weight. In adults, bone marrow produces new blood cells, and contains a pool of MSCs, EPCs[111], side population cells[112] as well as an undefined population termed as multipotent progenitor cells[113] which are capable of generating all three germ layers. There is strong evidence that circulating mobilised BMCs can home into the myocardium as transsexual allogeneic human cardiac transplants have shown that female hearts in a male host had a significant number of y-chromosome positive cardiomyocytes, vascular endothelial and smooth muscle cells[3]. A number of studies have suggested that adult bone marrow stem cells retained the capacity to produce cells of unrelated tissues, including cardiomyocytes and vascular endothelium[5, 114, 115]. Also, it has been warned that transplantation of unselected bone marrow cells into acutely infarcted myocardium can cause intramyocardial calcification, an effect that might be due to the differentiation potential of MSCs into bone, cartilage, fat, and a connective tissue[116, 117].

In 2001, Orlic et al reported that $lin^-/c\text{-}kit^+$ HSCs isolated from bone marrow can differentiate into cardiomyocytes, vascular endothelial and smooth muscle cells when injected in the border zone of infarcted myocardium in mice, causing an extraordinary regeneration of more than 60% of the infarcted area and improving cardiac function[5]. The same investigators have reported that HSCs mobilization from BM by administration of growth factors such as stem cell factor and granulocyte colony-stimulating factor may result in reduced mortality and improved cardiac function in mice with ligation of the left coronary artery[118]. They also showed substantial tissue repair with proliferating myocytes and newly formed vessels in mice receiving growth factors than in the control animals[118]. As mentioned earlier in the chapter, other

investigators have not been able to reproduced the above results with injection of BMCs using an identical experimental model[7, 8] and this has fuelled the controversy on the regenerative potential of BMCs. It is important to clarify that these experiments were performed using the whole unfractionated BMCs and that the various cell fractions might have different degrees of regenerative potential. Thus, for example, the SP population ($CD34^{-/low}$, c-kit^{+}, sca-1^{+} cells) isolated from BMCs have been reported to migrate and trans-differentiate into cardiomyocytes and endothelial cells, contributing to the formation of functional myocardium in ischaemic mice hearts[98, 115, 119]

Similarly, MSCs have been shown to differentiate *in vitro* into cardiac myocytes, as recognized by the formation of myotube-like structures and typical sarcomeres, spontaneous beating, and expression of muscle-specific proteins when treated with 5-azacytidine[120]. Several *in vivo* animal studies have also shown that MSCs from the bone marrow regenerate cardiac tissue and improve heart function[121-123]. Human MSCs, labelled with lacZ and transplanted into myocardial infracted areas in mice, have been shown to trans-differentiate, in spite of low frequency, into cells with *de novo* expression of desmin, ß-myosin heavy chain, α-actinin, cardiac troponin T, sarcomeric contractile proteins and phospholamban at levels comparable to those of the host cardiomyocytes, suggesting *in situ* differentiation of MSCs into myocytes[121]. Other studies in rats[124] and pigs[125] have also demonstrated the feasibility and functional effect of MSCs when injected into infarcted myocardium, although different levels of MSCs engraftment were reported.

EPCs have been derived from human umbilical cord blood, as well as from bone marrow and peripheral blood. Mature EPCs express a variety of endothelial markers

such as CD34, VE-cadherin, CD31, von Willebrand factor, and a vascular endothelial growth factor receptor called fetal liver kinase[105, 126]. It is recognised that EPCs could be mobilised from bone marrow into the peripheral circulation and incorporated into foci of neovascularisation in adult animals, particularly in the presence of ischaemia in responding to stimuli[127, 128]. *Ex vivo* expanded EPCs have been proved to form significant greater capillary density and reduce scarring formation than controls in an *in vivo* animal model of myocardial infarction[129, 130]. Also, in *in vitro* studies, it has been shown that human EPCs can trans-differentiate into functionally active cardiomyocytes when co-cultured with neonatal rat cardiomyocytes and in the presence of E-cadherin[131].

One population of multipotent adult progenitor cells (MAPCs) expresses minimal levels (less than 3%) of CD90, CD105, and CD117, and is capable of differentiating into cells forming all germ layers including those expressing cardiac-specific genes. Another population of MAPCs expresses CD34, CD45, c-kit, sca-1, Flk-1, Thy-1, CD13, and stage-specific antigen 1; however, compared with the former population they do not show a similar range of trans-differentiation[126]. The potential of MAPCs is not fully defined and other investigators have reported that MAPCs although having the potential to differentiate into liver, lung, gut and haematopoietic lineages, do not seem to differentiate into skeletal or cardiac muscle[113].

Recently, a novel human bone marrow stem cell has been described to be able to expand *in vitro* with multipotent activity and characteristics that do not appear to belong to any previously described bone marrow stem cell population[132]. The cells have been tested *in vivo* for therapeutic and differentiation potential and shown to have the capacity to

induce neovascularisation and cardiomyogenesis by both differentiation and fusion[132]. Certainly, a greater definition of this cell type is required.

Inspired by experimental results, even the underlying mechanisms are not clearly understood, several clinical trials have been carried out using autologous BMC transplantation to repair the damaged heart in acute and chronic ischaemia. The admission of cells has been proved to be safe[133, 134] but the beneficial outcome has been controversial with modest improvement of the left ventricular (LV) ejection fraction (EF)[135, 136] or no evident benefit in the others[137-139]. For example, the REPAIR-AMI (Reinfusion of Enriched Progenitor Cells and Infarct Remodelling in Acute Myocardial Infarction) and the TOPCARE-CHD (Transplantation Of Progenitor Cells And Recovery of left ventricular function in patients with Chronic ischaemic Heart Disease) trials showed small but significant improvement in EF of 3-5% as compared to controls, and concluded that patients with more severe reduction of ventricular function obtained the greatest benefit[135, 136, 140]. By contrast, the STEMI (ST-Elevation acute Myocardial Infarction) and the ASTAMI (Autologous Stem Cell Transplantation in Acute Myocardial Infarction) trials performed after an acute myocardial infarction and a recent trial from our group in which BMCs were injected into scarred myocardium did not show improvement in global cardiac function[137-139, 141]. The BOOST II (BOne marrOw transfer to enhance ST-elevation infarct regeneration) trial also performed in the acute phase of a myocardial infarction revealed significant improved EF after 4 months followed-up, a benefit that was dissipated after 18 months[142]. It has been argued that the different results between trials could be due to the handling and preparation of BMCs[143]. Moreover, meta-analyses of clinical trials on BMC administration following an acute myocardial

infarction have also suggested the existence of a dose-response association between injected cell number and LV EF change[144-146]. Certainly, the standardisation of the preparation of BMCs as well as the number of cells injected and their quality need to be defined.

Other adult stem/progenitor cells

Adipose stroma stem cells have been recently mentioned to contain pluripotent stemness and could acquire the cardiomyocyte phenotype, including beating state, after culture without addition of 5-azacytidine[147].

Spermatogonial stem cells derived from mice have been reported to have raised all three germ layers. Using the hanging drop method, spermatogonial stem cells have been able to differentiate into beating cardiomyocytes with expression of cardiac specific L-type Ca^{2+} channels and responding to Ca^{2+} channel modulating drugs[148]. Differentiated cells showed the presence of functional gap junctions among cardiomyocytes and action potentials demonstrated the presence of pacemaker-, ventricular-, atrial-, and Purkinje-like cardiomyocytes[148].

Skeletal myoblasts have also been described as a potential source of cells for cardiac regeneration. Myoblast cells, the precursors of the skeletal muscle, are considered as satellite cells and normally lie in a quiescent state under the basal membrane of skeletal muscle fibres and proliferate to mend the injured muscle. Following successful experimental studies[149-151], the clinical transplantation of the myoblasts showed no improvement in the global contractile function and raised the serious concern of development of ventricular arrhythmias[152]. Indeed, myoblasts differentiate into

multinuclear myotubes with a lack of electrophysiological coupling with the neighbouring host cardiomyocytes, that could be the reaction for the greater susceptibility to induce arrhythmias[153-155]. The utility of myoblasts to repair the damaged heart however has not yet been fully investigated and the possibility of over-expression of gap junction proteins for a better integration with the host tissue and the elimination of ventricular arrhythmias may appear promising.

1.3.2 Myocardial regeneration and cell fusion

The concept of cell fusion refers to the joining of two fully differentiated cells or the coalescence of a stem cell and a differentiated cell, the growth of which is regulated by the nucleus of the differentiated cell. The observation that BMCs or cardiac progenitors can spontaneously fuse with other cells and subsequently adopt the phenotype of the recipient cells including cardiac cells[99, 156-158] could be an alternative mechanism of tissue repair although this is a rare event that may happen only occasionally[158]. Furthermore, some investigators have reported that fusion of a primitive cell with a terminally differentiated myocyte would not be able to activate the cell cycle of the fused cell[97, 101, 159]. Therefore, it is unlikely that cell fusion is an important mechanism of cardiac repair as the required volume of the formed cells to be regenerated is of at least 20,000 μm^3 or larger[160].

1.3.3 Paracrine action in cardiac repair

While the concept of differentiation has been questioned, other mechanisms of action such as paracrine effects and immune modulation have been suggested. The paracrine action of transplanted stem cells is not fully understood, although activation of

angiogenesis, mobilisation of both circulating and bone marrow stem cells, activation of cardiac resident stem cells and protection of myocardium from cell death have been suggested.

Neovascularisation

Transplanted stem cells may secrete angiogenic growth factors that may be considered as a pro-survival response to diseased states. BMCs can express vascular endothelial growth factor (VEGF) which may enhance the survival of the host cells and also promote angiogenesis that would result in improvement of the regional blood flow in the ischaemic myocardium[161-163]. Transplantation of c-kit^{+} cells[119] and MSCs[164] induces an increase of the angiogenic factors fibroblast growth factor (FGF)-2, VEGF, and the stem cell homing factor stromal cell-derived factor (SDF)-1 in treated hearts compared with control hearts, this leading to an increase in capillary density of approximately 40%. The action was also accompanied by a reduction of proapoptotic protein in ischaemic myocardium[164], suggesting an additional cytoprotective benefit. Furthermore, the combination of growth factors such as VEGF-1 and FGF-2 has been reported to induce mural cell recruitment and formation of functional neovasculature[165] and the combination of VEGF to the injection of marrow-derived stromal cells might facilitate angiogenesis and functional improvement[166].

Mobilisation of circulating and bone marrow stem cells

Circulating EPCs can be mobilised from the peripheral circulation, and MSCs mobilised from the bone marrow to the injured myocardium[167]. EPCs play an important role in the physiological maintenance of the endothelium, assisting in both reendothelialisation

and neovascularisation. Recently it has been reported that the alpha chemokine receptor (CXCR)-4 signalling axis plays an essential role in the recruitment of stem cells to the heart. CXCR-4 is the cell surface receptor for SDF-1 and is expressed in EPCs and HSCs[168, 169]. It has also been suggested that monocyte chemotactic protein-3 is another regulator for the homing of stem cells[170]. These findings suggest that recruitment of stem cells through paracrine signalling may be one of the potential mechanisms facilitating the migration of stem cells from the circulation or the bone marrow to the ischaemic myocardium.

Activation of cardiac resident stem cells

The ability of stem/progenitor cells to secrete growth factors and cytokines may cause not only myocardial survival but also myocardial proliferation in the injured cardiac tissue[171-173]. Experimental studies have shown that the combined treatment of insulin-like growth factor (IGF)-1/hepatocyte growth factor (HGF) can activate cardiac progenitor cells that have the properties of stemness[174, 175], however, this potential action needs to be further investigated.

Cardioprotection

Our group has been the first to report that BMCs have a potent anti-ischaemic effect in human myocardium using an *in vitro* model. It was shown that BMCs co-incubated with cardiac muscle during 90min ischaemia and 120min reoxygenation significantly reduce tissue injury and cell necrosis and apoptosis, an action that was abrogated by the blockage of the protein kinases PKC and p38 MAPK, both of which are essential components of the signal transduction pathway of IP[11]. The results suggest that the

beneficial action of the injection of BMCs may be due, at least in part, to cardioprotection by diminishing ischaemic injury.

The above findings are supported by experimental animal studies. Thus, it has been reported that the injection of BMCs and also the administration of the culture media into ischaemic myocardium can induce angiogenesis and improve cardiac function after myocardial infarction[13, 176, 177]. The intramyocardial injection of bone marrow MSCs overexpressing Akt has been proved to inhibit ventricular remodelling and restore cardiac function, an action that was suggested to be mediated by the secretion of several growth factors such as VEGF, FGF-2, HGF, IGF-1, and thymosin β-4 that posses anti-apoptotic properties[178]. Also in a rodent model, BMCs enhanced cardiomyocyte survival under hypoxic conditions, probably by the secretion of cytokines like VEGF, FGF-2, SDF-1 and IGF-1[161], and preconditioning of injected BMCs further enhanced this benefit[13].

The influences of stem cell-derived cytokines and growth factors in the cardioprotective effect have been mentioned for years. Distinct from other secreted factors, IGF-1 has been observed to prevent apoptosis and promote survival of cardiomyocytes, to improve cardiac function and preserve structural damage during I/R[179-182]. *Ex vivo* administration of IGF-1 has been shown to regulate the recovery of cardiac function, limit myocardial infarction and reduce apoptosis of animal hearts subjected to ischaemia[183-185], an action has also been seen in IGF-1 overexpression heart and *in vivo* pretreatment of IGF-1[182, 186]. The downstream cascade pathway of IGF-1 stimulation in myocardial protection appears to involve activation of the Akt/PKB, PKC and MAPK molecules[181, 186-188]. IGF-1 treatment has also shown to up-regulate

Bcl-xL, suppress pro-apoptotic Bax protein, decrease mitochondrial cytochrome *c* level, inhibit calcium sensitive mitochondrial swelling, prevent mitochondrial damage and reduce ROS production from I/R leading to protection[179, 183, 189, 190]. In addition, IGF-1 deficiency is known to associate with cardiac atrophy, impaired cardiac function and increased cardiovascular mortality[187], and cardiovascular disease risk may also be elevated among healthy individuals who have IGF-1 serum levels below the normal range[191].

Immune modulation

Another potential mechanism by which BMCs could exert benefit is the modulation of the inflammatory response that follows a myocardial infarction. Transplantation of MSCs overexpressing Akt into ischaemic myocardium can inhibit cardiac remodelling by reducing intramyocardial inflammation, collagen deposition and cardiac myocyte hypertrophy[192]. Indeed, the inflammatory response seen in patients after an acute MI, as measured by the plasma level of tumor necrosis factor alpha and monocyte chemoattractant protein 1, were slightly but significantly reduced after 3 months follow-up in individuals receiving intracoronary autologous BMCs compared to control subjects[193], further supporting the role of immuno-modulation in the benefit obtained from BMCs transplantation.

1.4 Aims of the thesis

Based on the knowledge obtained from the previous works of our laboratory and the capability of BMC-derived factors, the hypothesis of my thesis was that BMCs possess

potent cardioprotective properties against ischaemic injuries. To further understand the mechanisms of BMC-induced benefit, the aims of my thesis were as followed:

- To characterise the most effective dose of BMCs to induce cardioprotection
- To elucidate whether the cardioprotective effect of BMCs is as potent as that of IP
- To investigate whether the manipulation of BMCs affects their protective properties
- To characterise the best time of administration
- To investigate the mechanism underlying the cardioprotection by BMCs
- To identify the cell types within the BMCs responsible for cardioprotection
- To elucidate whether disease states (such as diabetes, heart failure) and medical treatments such as K_{ATP} channel openers (nicorandil) alter the cardioprotective effect of BMCs
- To investigate whether the administration of BMCs as an additive to cardioplegia solution during cardiac surgery can reduce myocardial ischaemic injury.

CHAPTER 2: METHODS

In this chapter I will describe in detail the methods used in my thesis

2.1 Donor patients of atrial myocardial tissue and bone marrow

The right atrial appendage was obtained from patients undergoing elective heart surgery such as coronary bypass surgery or aortic valve surgery prior to the initiation of cardiopulmonary bypass. All the studies were conducted according to the Declaration of Helsinki principles and approval was obtained from the Local Ethics Committee. In addition, all participants provided written consent. Patients with atrial fibrillation, cancer, diabetes, infection, poor LV function (EF<30%) or with additional surgical procedures or those being treated with opioid, catecholamines, K_{ATP} channel opener nicorandil were excluded from the study. However, in Chapter 10 samples from patients with diabetes or poor LV function (EF<30%) were also used and in Chapter 11 patients treated with K_{ATP} opener nicorandil were also recruited.

2.2 Experimental preparation for the induction of ischaemia/reoxygenation-induced injury

The experimental preparation has been previously described and fully characterized by our laboratory[194]. Upon harvesting, samples were immediately immersed in cold (4 °C) Krebs/Henseleit/Hepes (KHH) buffered medium containing (in mM) NaCl 118, KCl 4.8, $NaHCO_3$ 27.2, KH_2PO_4 1, $MgCl_2$ 1, $CaCl_2$ 1.25, glucose 10, and Hepes 20 pre-bubbled with 95% O_2/5% CO_2 and a pH of 7.4. The tissue was immediately laid with epicardium faced down between a filter paper fixed to a rectangular glass plate (5x25cm) and a slide and sectioned manually with a skin-graft blade (Swann-Morton

Ltd, Sheffield, U.K.) to slices of 300-500μm thickness and 30-50mg weight. The tissue and the apparatus were kept wet during sectioning with cold and oxygenated KHH medium (4-10^{0}C).

After preparation, the muscles were immersed in 25ml Erlenmeyer flasks containing 15ml KHH medium pre-bubbled with O_2. Then the flasks were placed in a shaking water bath with 80 cycles per minute under aerobic conditions (95% O_2/5% CO_2) and at 37^{0}C for 50-60min equilibration. After this, the muscles were washed with KHH medium (without glucose and at pH 6.8 for the muscles allocated to ischaemia/reoxygenation (I/R) groups and aerobic condition for aerobic control groups) and then immersed into new flasks containing 10ml of media and receiving different treatments according to the study protocol.

For the induction of simulated ischaemia, the slices were subjected to 90min of anaerobic condition at 37°C, obtained by continuously bubbling the media with 95% N_2/5% CO_2 in the absence of glucose and at a pH of 6.8. The used media was pre-bubbled with 95% N_2/5% CO_2 for at least 20min to obtain complete hypoxic conditions before the immersion of the tissues. At the end of ischaemic period, slides were transferred to flasks containing 10ml oxygenated media with glucose, at a pH of 7.4 and a temperature of 37^{o}C for further 120min of reoxygenation.

For the induction of IP, at the end of the equilibration period, the atrial muscles were transferred to another flask containing 10ml of anaerobic solution with 95% N_2/5% CO_2 for 5min, followed by an additional 5min of aerobic conditions prior to induction of ischaemia[195].

The time-matched aerobic controls incubated for the entire experimental time were used in every set of experiments.

2.3 Aspiration of bone marrow and preparation of bone marrow cells

Under general anaesthesia and before the initiation of surgery, 40-120ml (10ml in 1000 units of heparin) of bone marrow was aspirated from the iliac crests of patients using a special harvesting needle (Medical Device Technologies Inc., Gainesville, Florida, USA). The bone marrow was placed in 50ml Falcon tubes (BD Biosciences, New Jersey, USA) and immediately immersed in ice for transportation to the laboratory.

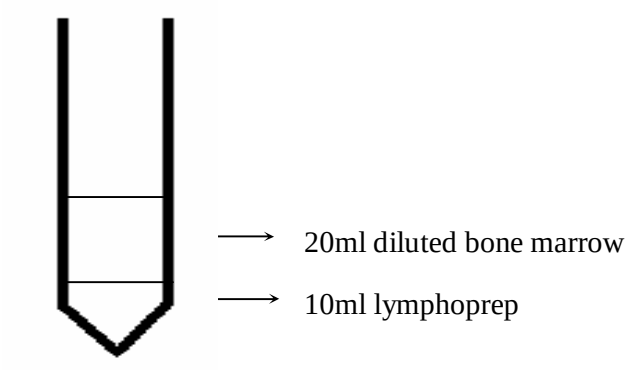


Figure 2: Illustration of bone marrow and lymphoprep layers.

For the separation of cells, the bone marrow was diluted at a ratio of 1:1 with normal saline, every of 20ml diluted bone marrow solution was gently pipetted onto a 50ml conical centrifuge Falcon tube containing 10 ml Lymphoprep (Axis-Shield PoC AS, Oslo, Norway) in such a way that the bone marrow laid over the Lymphoprep, and the two layers were clearly demarcated by a line. Then the tubes were centrifuged at 800G for 20min at 10^0C without braking. Fat and clots were removed from the tubes and the

separated mononuclear cell layer was collected without shaking the tubes. The mononuclear fraction was spun for further 10min at 500G and 10^0C condition. Following this, supernatant was discarded and the cells were washed using 10ml KHH medium and re-suspension and centrifugation at 500G and 10^0C. The number of cells was counted using the Coulter Counter (Beckmancoulter, Fullerton, California, USA). All the procedures were performed under aseptic conditions.

For the experiments using fresh bone marrow cells, the cells were suspended in KHH solution at the concentration of $5x10^6$ cells per 100 µl medium.

For culturing, fresh cells were re-suspended in DMEM F12 (1:1) GlutaMAX media (Gibco, Invitrogen corporation, California, USA) containing 10% FBS (Hyclone, Utah, USA) and penicillin/streptomycin at the concentration of 100 IU/ml and 100µg/ml, respectively (Lonza, Basel, Switzerland). Then cells were seeded to 6 wells plates (Nunc Brand Products, Roskilde, Denmark) at the concentration of $15\text{-}20x10^6$ cells per well in 4.5 ml cultured medium and incubated for 7 days in normothemic conditions and 5% CO_2 / 95% O_2 without changing the media. Initial studies from our laboratory have determined that under this condition, GlutaMax media allows BMCs to maintain their viability and number during 7 days of incubation. After this, cells were re-suspended and the adherent cells were de-attached in 4-5min with 0.05% Trypsine EDTA (Lonza, Basel Switzerland) at 37^{o}C. Trypsine was then neutralised by FBS. After this, cells were placed into a 50ml Falcon tube and centrifuged at 100G and 10^0C for 5min, the supernatant was discarded and the cells were washed and centrifuged with 10ml KHH solution. The cells were then counted using the Coulter Counter and re-suspended in KHH solution at the concentration of $5x10^6$ cells per 100 µl medium.

For freezing, the fresh cells were re-suspended in FBS containing 10% dimethyl sulfoxide (DMSO, Sigma-Aldrich, Dorset, UK), placed into cryovials and stored at -80^0C in a jacket over night and then transferred to liquid nitrogen until cells were used. When they were required for experimentation, cells were quickly thawed at 37^0C for 3-4min and then centrifuged at 100G and 10^0C for 5min. The supernatant was removed and cells were re-suspended and transferred into a 50ml Falcon tube and washed with 10ml KHH. The cells were then counted using the Coulter Counter and re-suspended in KHH medium at the concentration of $5x10^6$ cells per 100 µl solution.

To assess the BMCs viability, 40 µl of medium containing cells was incubated with the same amount of Trypan blue (Sigma-Aldrich, Dorset, UK) for 5min at room temperature, and then cells were counted under an ordinary microscope. The viability of BMCs, assessed by Trypan blue exclusion, after processing and immediately before administration was ≥ 95%. Cell viability was calculated as follows:

$$\text{Percentage of BMC viability} = \frac{\text{Living cells}}{\text{Living cells} + \text{Dead cells}} \times 100$$

The bone marrow cells from each individual were used a maximum of twice, one with the atrium from the same donor and another time with the atrium from a different subject.

2.4 Study groups

After equilibration, the sliced myocardial muscles from a single donor were not used twice for the same groups and were randomly allocated to one of the various groups as described in each Chapter. For all the studies, myocardial slices were aerobically incubated for the entire duration of the protocol (aerobic control) and the remaining muscles were subjected to the 90min ischaemia/120min reoxygenation alone (ischaemic control) or in co-incubation with different treatments according to the protocol. IP was induced by 5min of ischaemia followed by 5min of reoxygenation before the I/R protocol.

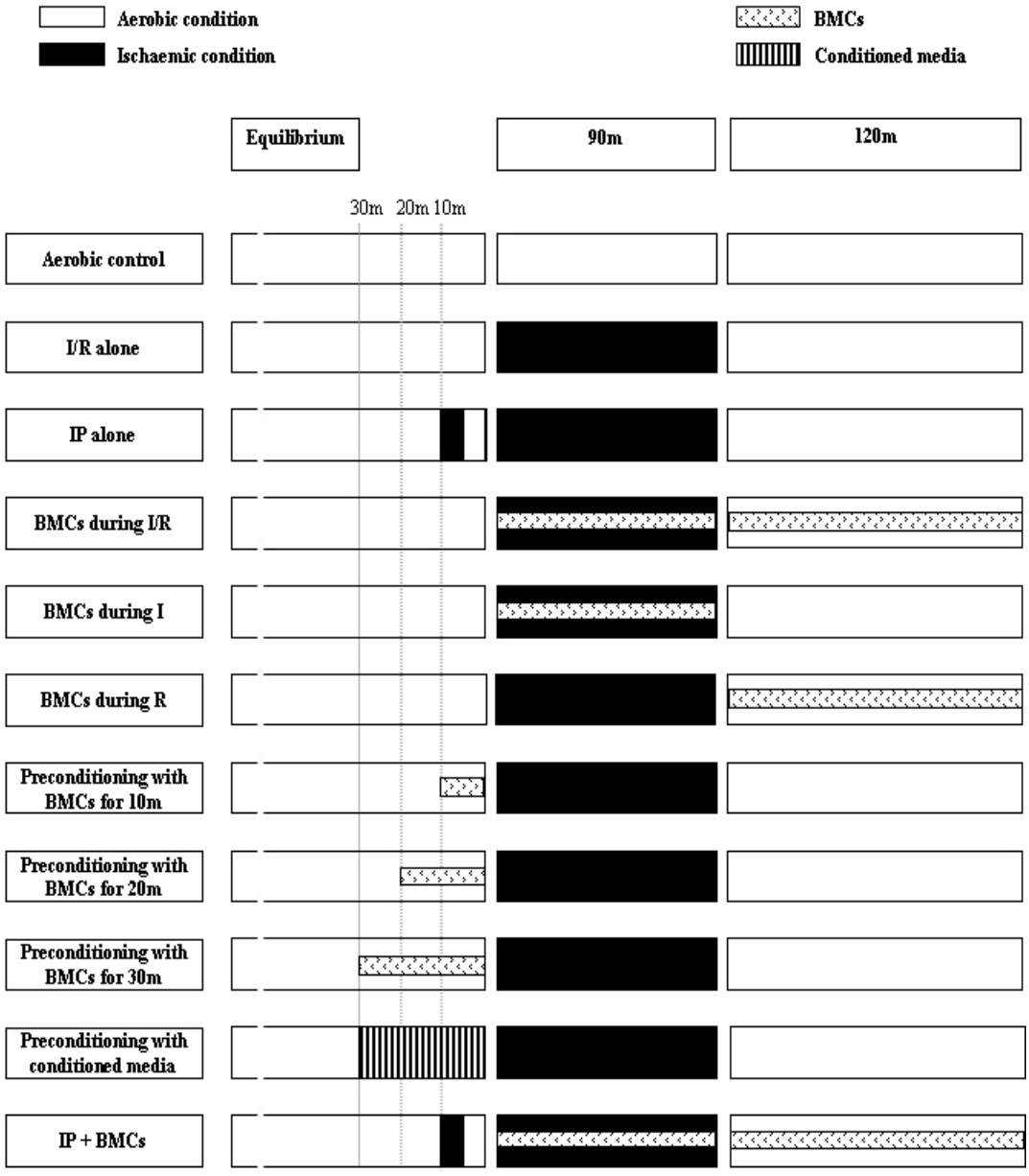


Figure 3: Study protocols to characterise the cardioprotective potential of BMCs. Various groups of experiments were used depending on the aims of the studies.

2.5 Assessment of tissue injury

Tissue injury was assessed by measurement of creatine kinase (CK) release into the media during the 120min reoxygenation period. The enzyme activity was measured by

an ultraviolet method based on the formation of NADP employing a commercial assay kit (30-3060/R2: Abbott Laboratories, Diagnostic Division, Kent, UK). The enzymatic reactions involved in the assay are as follows:

Creatine phosphate + ADP $\xleftrightarrow{CK}$ Creatine + ATP

ATP + Glucose $\xleftrightarrow{HK}$ ADP + G-6-P

G-6-P + NADP $\xleftrightarrow{G6PDH}$ 6-PG + NADPH

During this oxidisation, the amount of NADP reduced to NADPH is read at an absorbance of 340nm. The rate of change in absorbance (ΔA/min) represents the CK activity.

For this assay, the two reagents used were mixed together and then incubated with the media at 37^0C for 1min. After that, the incubation medium was dispensed into a 96 well flat bottom micro plate (Costar, Corning Life Sciences, Lowell, Massachussetts, USA) and measured at 0,1,2,3,4min using a spectrophotometer (Benchmark, Bio-Rad Laboratories, California, USA). The CK activity expressed, in units per litre, was calculated using the formula:

$$\text{CK activity (IU)} = \frac{(\Delta A/\text{min} \times TV \times 1000)}{(\varepsilon \times LP \times SV)}$$

TV: Total reaction volume (ml)

ε = 6.22: Millimolar absorptivity of NADPH at 340nm

LP: Light patch = 1

SV: Sample volume (ml)

1000: conversion of units per millilitre to units per litre

For each experiment, single samples were performed in triplicate with a variation of values less than 10%. The mean of calculated CK activity was then divided by the weight of the muscle, measured at the end of the experiments, so that the results were expressed as IU/mg wet weight.

2.6 Assessment of cell death

At the end of the experimental protocol, tissues were incubated for 15min at room temperature on the rocker with 20μg/ml propidium iodide (PI, Sigma-Aldrich, Dorset, UK) in phosphate buffered saline (PBS) at a pH of 7.4 to identify the necrotic nuclei. Muscles were then washed with PBS twice for 5min each time before fixation in 4% paraformaldehyde. They were kept overnight at 4-10^{o}C and then transferred to sucrose 30% until the tissue sunk. All the above steps were performed in the dark to avoid exposure to light. Following this, the muscles were embedded with Optical Cutting Temperature embedding matrix (Tissue-Tek®, Agar Scientific Ltd, Essex, UK). Frozen sections were then cut at 7μm thickness in a Bright cryotome (model OTF) at circa -25^{o}C, and sections were collected on SuperFrost Plus slides (Menzel Glasser, Braunschweig, Germany). The slides were then kept at -80^{o}C.

To assess apoptosis, the slides were brought from -80^{o}C to room temperature, washed with 20mM PBS, and then permeabilised for 1min in a microwave oven at 850 watts in 200ml of 0.1% Triton X-100 in 0.1M Tri-sodium citrate buffer at a pH of 6.0. After this, the slides were rapidly cooled by adding 80ml distilled water and transferred to 20mM PBS solution. In addition, they were immersed into 3% bovine serum albumin (Sigma Aldrich, Dorset, UK) and 20% foetal bovine serum (Hyclone, Utah, USA) in 0.1M Tris-HCl buffer at a pH of 7.5 for 30min to block unspecific labelled activity. The terminal

deoxynucleotidyl transferase (TdT) was used to incorporate fluorescein (FITC) labelled dUTP oligonucleotides to DNA strand breaks at the 3'-OH termini in a template dependent manner (TUNEL technique) for 90min at 37^{o}C in a humidity chamber using a commercially available kit (Roche Diagnostics, Penzberg, Germany). A negative control, obtained by removing the enzymes, was performed every time.

To distinguish the total number of nuclei, sections were counter-stained with 1µg/ml 4', 6-Diamidino-2-phenylindole (DAPI) (Molecular Probes, Eugene, Oregon, USA) in PBS for 1min. Then the slides were washed in PBS 3 times for 5min each. To reduce photobleaching, the sections were mounted with anti-fade solution (Prolong Antifade kit, Molecular Probes, Eugene, Oregon, USA) and covered with coverslips (Menzel Glasser, Braunschweig, Germany).

A fluorescent microscope (Axiovert 200M, Zeiss fluorescent microscope, Göttingen, Germany) at 40x magnification was used to assess necrosis and apoptosis and pictures were taken using OpenLab 5 software (Improvision ltd, Coventry, UK). At least ten fields per section and one section per staining were examined for each experiment. PI and FITC labelled nuclei were detected by the Cy3 and EGFP channels respectively, whilst DAPI labelling was detected by the DAPI channel. Only the necrotic or apoptotic signals coinciding with DAPI were considered true events. The NIH Image software (Scion Corp, Frederick, Maryland, USA) was used to determine the total events for each field. To avoid the inclusion of artefacts only fluorescent signals with areas greater than 16µm^2 were counted.

2.7 Determination of blood Troponin I and CK-MB

For the clinical study on the cardioprotective effect of BMCs in patients undergoing coronary bypass graft surgery, blood samples were taken before surgery and at 4, 12, 24 and 48 hours 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 and were performed independently by the Dept. of Biochemistry, University Hospitals of Leicester, NHS trust.

2.8 Assessment of cardiac function

For the clinical study, cardiac output was determined by thermodilution using Swan-Ganz catheters placed in the pulmonary artery at different time points: before surgery and 1, 2, 4, 8, 12, and 18-24 hours after aortic cross-clamp release. Three measurements were made at each time point through the injection of 10 ml 5% glucose solution at ambient temperature into the catheters. An injection was replaced if its value differed from the others greater than 10%. Cardiac output was calculated from the measured temperature changes over a given distance recording by the same catheter and cardiac index was then adjusted to body surface area. All the values were computed by clinical devices in the Cardiac Theatre and Intensive Care Unit, Glenfield hospital, UK.

2.9 Statistical analyses

- In all the *in vitro* studies, the data presented were obtained by subtracting the aerobic control values. Results from all experiments are expressed as mean ± SEM except for the results from the *in vitro* part of the clinical study which are expressed

as mean ± SD. One way ANOVA followed by Bonferroni's test were used to determine any effect of the calculation (SPSS 14.0 statistical analysis software).

- For the *in vivo* study, 22 subjects per group were determined as a result of the specification of 1SD better off than the control group with 80% power, an alpha=0.05 and an estimated 20% drop-out rate[196]. Continuous variables that were normally distributed were presented as mean ± SD, and differences between 2 groups were compared using independent t-tests. For non-parametric data, the Chi-Square 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 respective time points, and compared using the appropriate tests.
- Differences were considered to be statistically significant if $p<0.05$.

CHAPTER 3: THE ROLE OF CELL DOSE AND THE POTENCY OF BONE MARROW CELL-INDUCED MYOCARDIAL PROTECTION

3.1 Introduction

Experimental data have demonstrated that cell therapy has the potential to repair the damaged myocardium. This expectation and the clinical need for an effective treatment to reduce the injury sustained during an acute myocardial infarction and to repair the failing heart have accelerated the performance of clinical trials mainly using autologous BMCs even before the underlying mechanisms of their potential effects have been elucidated. However, the promising results of the initial non-randomized, feasibility phase I trials[133, 134, 197] has been followed by controversial outcomes in the randomized, efficacy phase II trials[136-140, 198]. It has been argued that differences in the cell composition and manipulation of BMCs may be responsible for the observed differences in some of these studies[143]. All this has prompted investigators to go back to the laboratory to clarify what BMCs can do and the best modalities of application.

Until recently, BMCs have been used based on the assumption that they induce therapeutic angiogenesis[199], myocardial regeneration by differentiation of the administrated BMCs[5, 159] or by stimulation of the putative cardiac stem/progenitor cells[174, 175] or cytoprotection via a paracrine action[200]. It has been suggested the existence of a dose-response between the injected BMC number and LV EF change in some clinical trials[144, 145], but so far no study has been performed to elucidate what

is the degree of the BMC-induced cardioprotection and whether the beneficial effect is dose-dependent.

In this study, my aims were to investigate the myocardial protective dose-response of BMCs and to determine the degree of this benefit as compared to that of IP, which has been considered the most powerful cardioprotective intervention in nature[66].

3.2 Methods

Experimental preparation

Experiments were performed on the myocardium and BMCs obtained from patients undergoing elective heart surgery such as coronary bypass surgery or aortic valve replacement prior to the initiation of cardiopulmonary bypass. The selection of patients, the bone marrow aspiration and separation, and also the experimental preparation were described in Chapter 2.

Assessment of tissue injury and cell death

CK release into the media during the 120min of reoxygenation and cell death by necrosis and apoptosis were determined as described in Chapter 2.

Study protocols

After equilibration for 50-60min, the atrial myocardial slices (n=6/group) were randomly allocated to different groups and BMCs from one single donor were not utilized more than twice. In each experiment, some myocardial slices were aerobically incubated for the whole experimental time serving as time-matched aerobic controls and

others subjected to 90min ischaemia/120min reoxygenation alone served as ischaemic controls. The BMCs studied groups were sequentially carried out as followed:

Study 1: To study the most effective dose of BMC-induced myocardial protection

To find out the number of BMCs required to obtain maximal protection, different doses of fresh BMCs (0.5, 1, 5 and $10x10^6$cells/preparation) were co-incubated with the atrial myocardial slices during the 90min ischaemia and 120min reoxygenation

Study 2: To elucidate the potency of protective effect of BMCs

This was achieved by comparing the protection obtained by the most effective lower dose of fresh cells ($5x10^6$ cells/preparation) co-cultured with atrial myocardial slices during the ischaemic and reoxygenation periods with the effect of IP induced by 5min ischaemia/5min reoxygenation prior to the 90min of ischaemia that previously has been demonstrated to afford maximal protection in this model[195].

Statistical analyses

Data are expressed as mean ± SEM. Each reported value was obtained after subtracting the corresponding time-matched aerobic control value. One way ANOVA followed by post hoc Bonferroni's test was used on the SPSS program. Differences were considered to be statistically significant if $p<0.05$.

3.3 Results

Study 1: The dose- response of BMC-induced myocardial protection

Figures 4A-4C show that the CK release and the percent of cell necrosis and apoptosis seen in the myocardial slices subjected to I/R alone were not significantly affected by

doses $\leq 1x10^6$ BMCs. However, significant reduction on the three indexes of injury were observed and plateaued at dose of $5x10^6$ cells, equivalent to $0.142\pm0.006x10^6$ cells/mg wet wt myocardium. *$p<0.05$ vs. I/R alone.

Study 2: The potency of cardioprotective effect of BMCs

Figures 5A-5C show that myocardial slices when used with BMCs at a dose of $5x10^6$ during I/R periods resulted lesser CK release as well as necrosis and apoptosis as compared to I/R alone, confirming the results in study 1. Importantly, they also demonstrate that the protection afforded by BMCs is of similar degree to the one obtained with IP. *$p<0.05$ vs. I/R alone.

3.4 Discussion

The results provide clear evidence on the capacity of BMCs to induce myocardial protection of the human myocardium, which potency is similar to that of IP, by significantly decreasing the death of cells at risk, which is in agreement with previous results from our laboratory[11]. Other studies have also suggested that ischaemic changes in the murine, rat and pig heart can be prevented or decreased by BMCs via a paracrine effect[166, 172, 201]. Therefore, it is possible that the modest improvement in cardiac function seen in some clinical trials using BMCs[135, 136, 140, 198, 202] is due, at least in part, to an early effect which reduces the loss of cells destined to die in the area at risk. Of course, such a mechanism would not exclude the possibility that BMCs may ameliorate cardiac function by late tissue regeneration either via differentiation of the transplanted BMCs or through the stimulation of resident stem cells, although these are two topics that remains the source of intense debate.

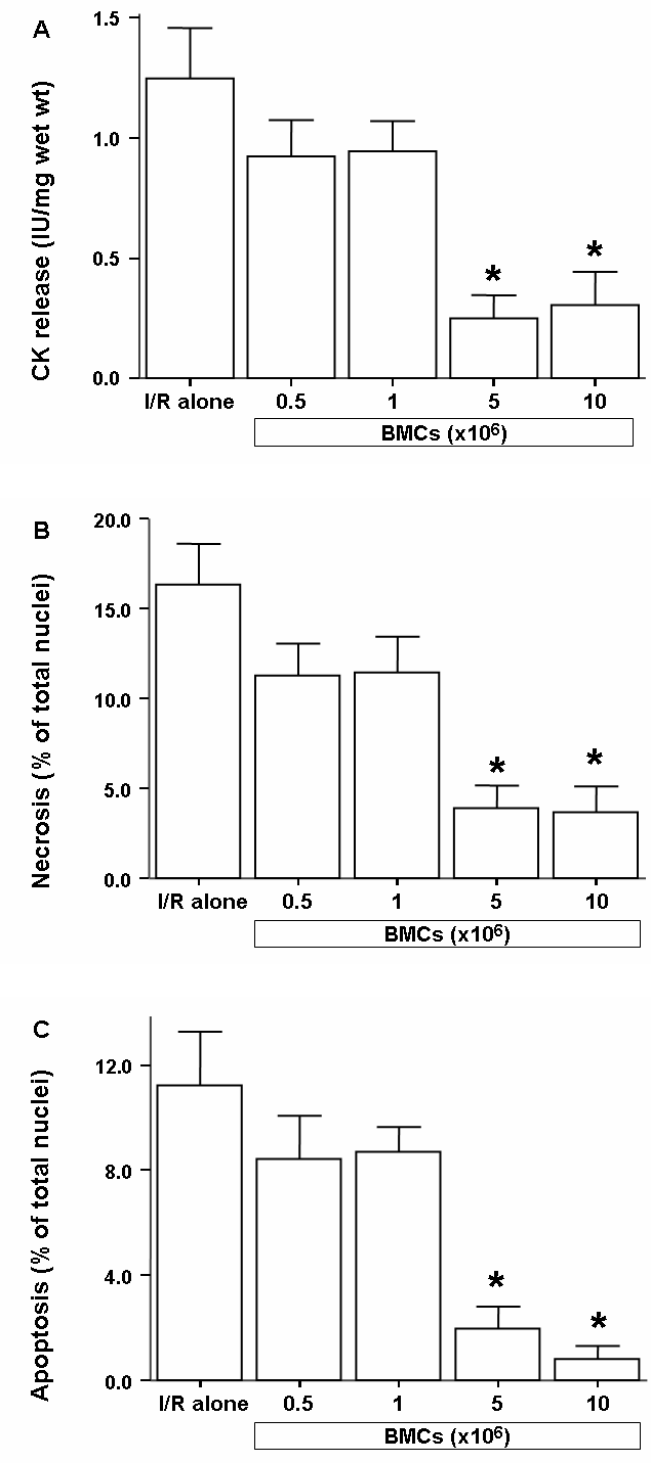


Figure 4: Dose-response effect of BMCs.

The effect of BMC doses on CK release (A), cell necrosis (B) and apoptosis (C) of the myocardium, n=6. *$p<0.05$ versus I/R alone.

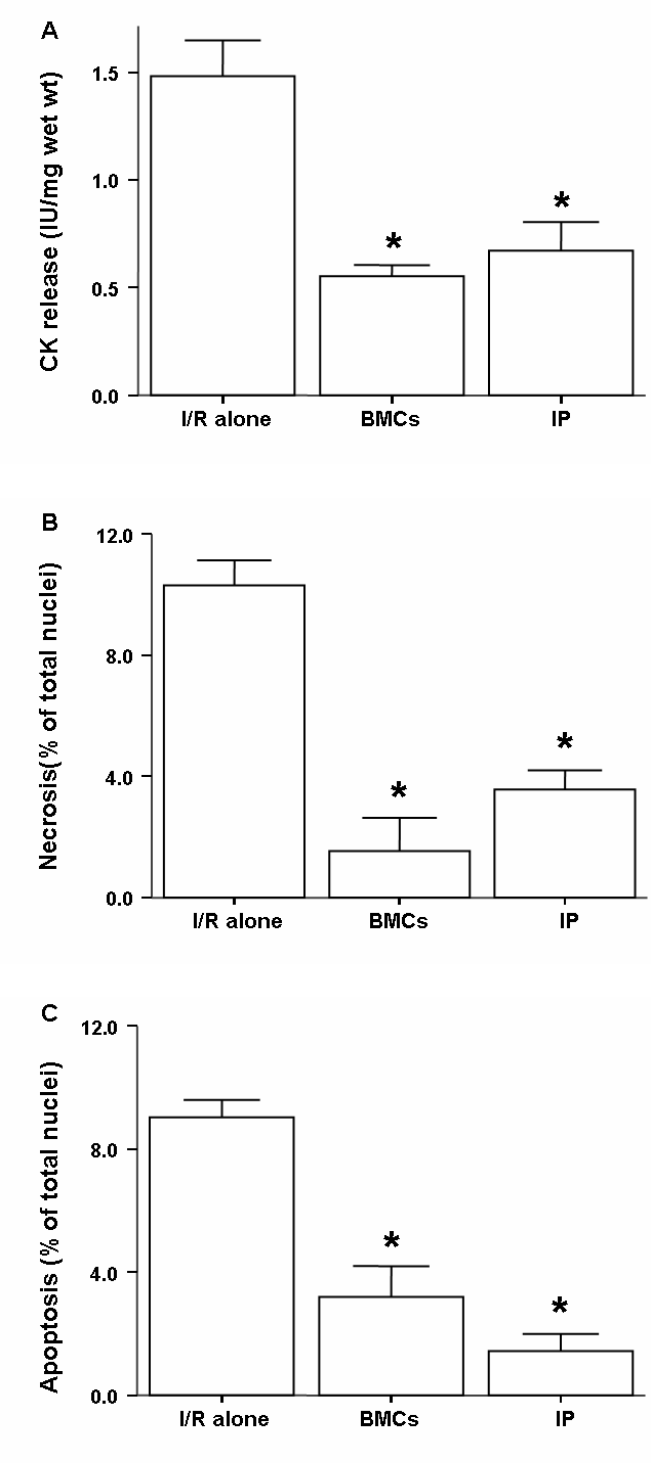


Figure 5: The cardioprotective potency of BMCs as compared to IP.

Potency of BMCs as compared to ischaemic preconditioning to reduce CK release (A), cell necrosis (B) and apoptosis (C), n=6. *$p<0.05$ versus I/R alone

Here I have also shown that the protective effect of BMCs is dose-dependent and that doses of $0.142\pm0.006x10^6$ cells/mg wet wt myocardium are required to obtain maximal benefit with no additional protection with higher doses. This implies that $71x10^9$ (or $\sim10^{11}$) BMCs could be required to obtain optimal protection of a heart weighting 500g. This is an important finding that may explain the variable results reported by recent clinical trials. Thus, for example the REPAIR-AMI[140] and BOOST[198] trials showed an improvement in cardiac function when a medium of $198x10^6$ or a mean of $246x10^7$ BMCs, respectively, were administrated, whereas the ASTAMI[139] and Leuven[138] trials reported no benefit when a median of $68x10^6$ BMCs or $172x10^6$, respectively, were given. It is worth noting that the doses of cells used in the former two trials, even if all the administrated cells had gone to the myocardium and not been lost into the systemic circulation, were well below the optimal minimal dose seen in my study and it is possible that the reported benefit was only partial. The argument that the cardioprotective action of BMCs is dose-dependent is also reinforced by the finding that the infusion of a high number of BMCs in patients with established postinfarction heart failure was associated with reduced mortality[203]. Moreover, meta-analyses of clinical trials on intracoronary BMCs therapy after acute myocardial infarction have also suggested the existence of a dose-response association between injected cell number and LV EF change[144, 145]. Therefore the design of future clinical studies must take into consideration the importance of selecting an appropriate dose of BMCs. Furthermore, the demonstration that <5% of BMCs administrated via the coronary arteries remain in the myocardium is an additional concern that would account for the critical issue of cell dosage[204].

3.5 Conclusions

The finding that the myocardial protection by BMCs is dose-dependent, which potency is similar to that of IP, may lead to the clinical exploitation of these cells to reduce the injury sustained during an ischaemic insult (eg, acute myocardial infarction, cardiac surgery) and should be taken into account when planning new trials. According to the present results, maximal protection was obtained and plateaued at dose of $5x10^6$ autologous cells (~$1.5x10^5$ cells/mg wet myocardium). However, it is difficult to maintain this optimal number of cells in the beating heart as data showed that after an intracoronary injection, the retention of transplanted cells in the myocardium is less than 5%[204]. On the other hand, a suboptimal number of cells have been used in the majority of reported clinical studies adding to the existing controversy on the therapeutic efficacy of BMCs. It is worth noting that the protective potency of BMCs can be enhanced by subjecting cells to hypoxic conditions[13], and it is possible to speculate that stimulation of BMCs will produce and release more cell survival factors that could compensate for a lower number of cells.

CHAPTER 4: THE RELEVANCE OF THE SOURCE AND THE EFFECT OF MANIPULATION OF BONE MARROW CELLS

4.1 Introduction

Having demonstrated the cardioprotective potency of BMCs and characterised the dose-response of such an effect, I embarked to investigate whether the manipulation of cells could influence this beneficial action. It has been postulated that variations in cell processing and storage have a major impact on the functional activity of cells, affecting their capacity to migrate[143], an effect that could explain the controversial results of some clinical trials . The elucidation of this issue is of great clinical importance and, therefore, in the present series of experiments I investigated whether the manipulation (culture, freezing) of BMCs affects their cardioprotective properties. Because in these experiments I need to use allogeneic BMCs, I also aimed to determined whether the cardioprotective efficacy of cells is influenced by the source (autologous versus allogeneic).

4.2 Methods

Experimental preparation

Experiments were performed on the right atrial myocardium and BMCs obtained from patients undergoing elective heart surgery prior to the initiation of cardiopulmonary bypass. The selection of patients, the bone marrow aspiration and separation, and also

the experimental preparation were described in Chapter 2. The methods of freezing and culture of BMCs can also be found in the same chapter.

Assessment of tissue injury and cell death

CK release into the media during the 120min of reoxygenation and cell death by necrosis and apoptosis were determined as described in Chapter 2.

Study protocols:

After the equilibrium period, the atrial myocardial slices (n=6/group) were randomly allocated to different protocols as described below:

Study 1: To investigate the protective efficacy of allogeneic BMCs

As explained earlier, because the study on the manipulation of BMCs would require the use of allogeneic cells, it was necessary to identify whether the source of cells has an influence on the degree of myocardial protection from I/R injury. To this end, atrial myocardial slices were incubated during ischaemia and during reoxygenation with fresh autologous and fresh allogeneic BMCs ($5x10^6$cells/preparation).

Study 2: To investigate whether the manipulation (culture, freezing) of cells affects the myocardial protection of BMCs

For this study, atrial myocardial slices were incubated during ischaemia and during reoxygenation ($5x10^6$ cells/preparation) with fresh autologous BMCs and with allogeneic cells cultured for 7 days or frozen for 1-9 weeks.

Statistical analyses

Means of data were analysed using one way ANOVA followed by post hoc Bonferroni's test (SPSS software) as described in Chapter 2. Significant differences were considered if $p<0.05$.

4.3 Results

Study 1: The protective efficacy of allogeneic BMCs

Figures 6A-6C show that allogeneic BMCs reduce the CK release and cell death by necrosis and apoptosis seen in the I/R alone group to an identical degree to that obtained with autologous BMCs. *p<0.05 vs. I/R alone.

Study 2: The effect of manipulation (culture, freezing) to the myocardial protection of BMCs

Figure 7 shows that the culture (7A-7C) or the freezing (7D-7F) of allogeneic BMCs significantly reduce CK release and cell death when compared to I/R alone, an effect that again was comparable to the one observed with autologous fresh BMCs. *p<0.05 vs. I/R alone.

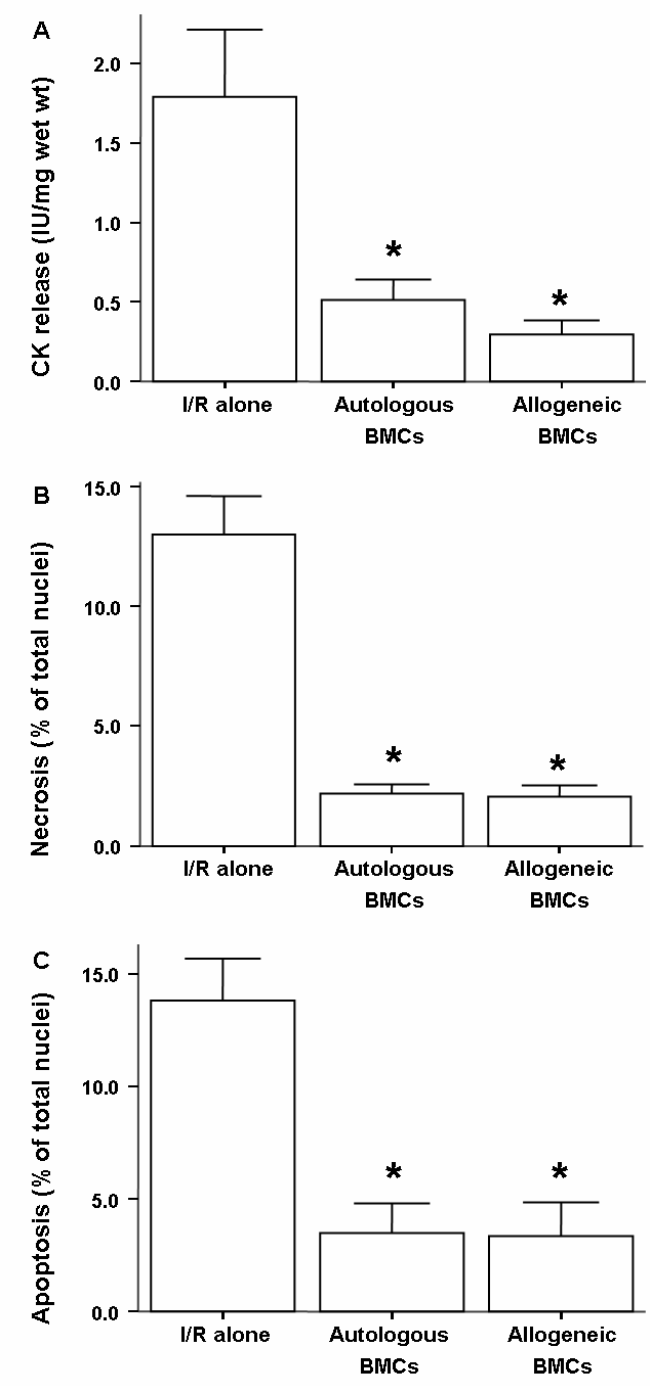
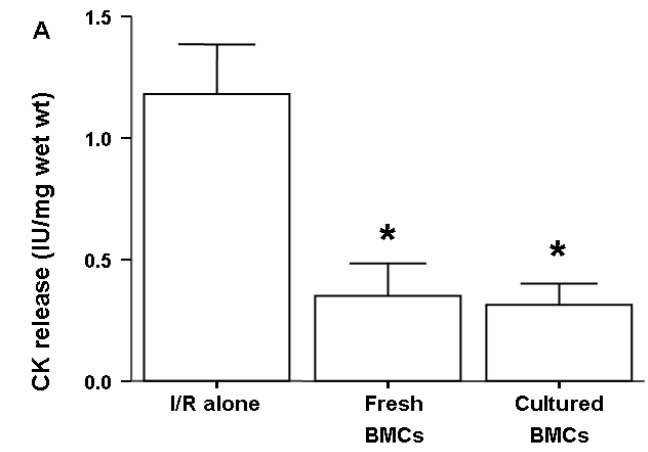
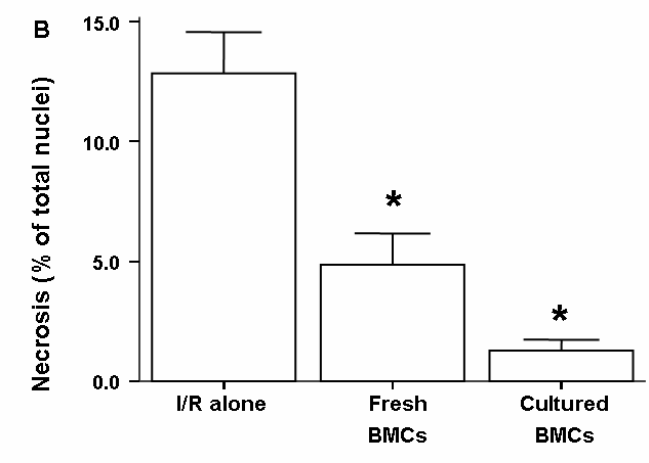
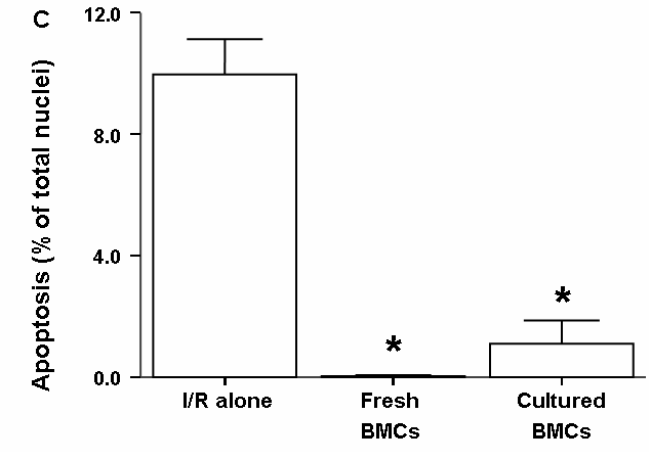


Figure 6: The effect of allogeneic BMCs.

The cardioprotective effect of allogeneic BMCs as compared to autologous BMCs on CK release (A), cell necrosis (B) and apoptosis (C), n=6. *$p<0.05$ versus I/R alone.


A
CK release (IU/mg wet wt)
1.5
1.0
0.5
0.0
*
*
I/R alone
Fresh BMCs
Cultured BMCs


B
Necrosis (% of total nuclei)
15.0
10.0
5.0
0.0
*
*
I/R alone
Fresh BMCs
Cultured BMCs


C
Apoptosis (% of total nuclei)
12.0
8.0
4.0
0.0
*
*
I/R alone
Fresh BMCs
Cultured BMCs

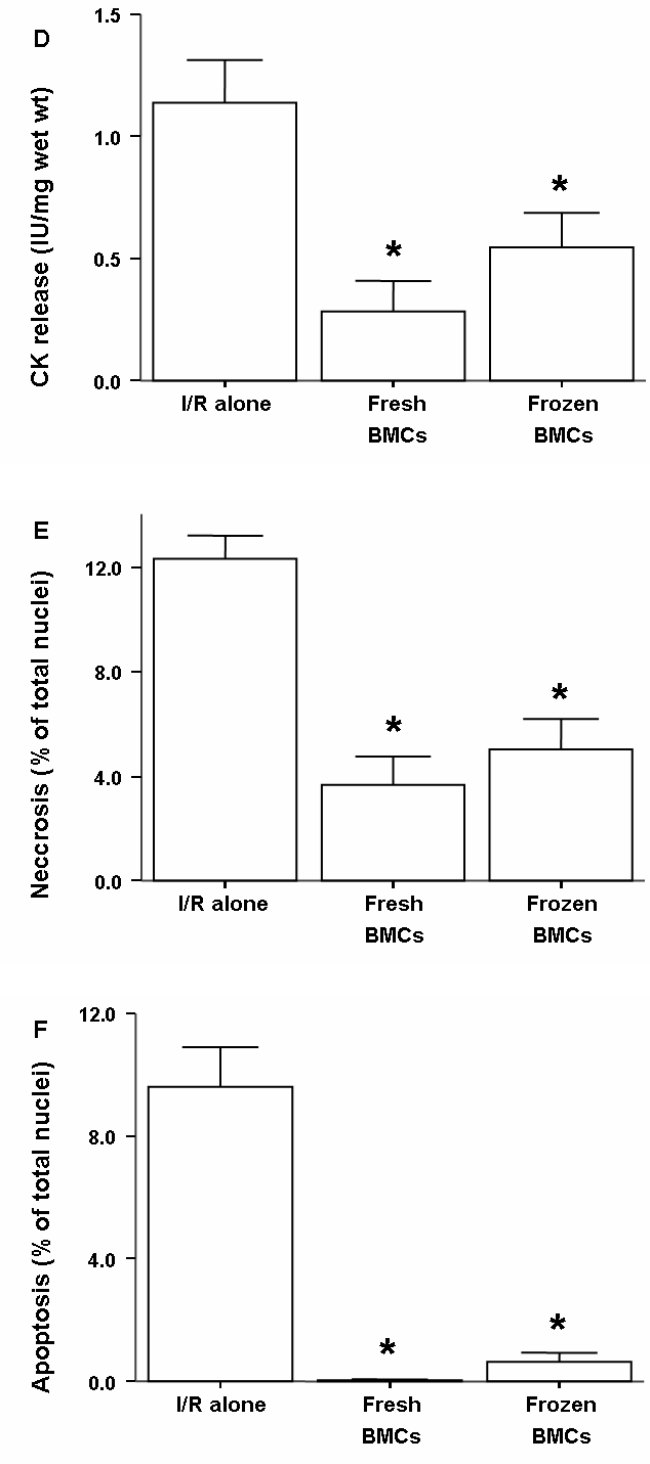


Figure 7: The effect of culturing and freezing of BMCs.

The effect of culture (A,B,C) and freezing (D,E,F) on the capacity of BMCs to reduce CK release, cell necrosis and apoptosis, n=6. *$p<0.05$ versus I/R alone.

4.4 Discussion

Transplantation of allogeneic BMCs for haematopoietic diseases has been reported to be feasible and effective in animal studies[205, 206] and human beings[207, 208]. Here I have demonstrated for the first time that allogeneic BMCs protect the myocardium against ischaemic injury to a degree similar to that of autologous BMCs. The results also suggest that allogeneic BMCs, being readily available, could be successfully applied in clinical conditions where autologous cells cannot be timely prepared or are defective. It should be conceded that immune reaction remains a considerable problem following allogeneic cell transplantation[209]. However, allogeneic MSCs, a subset of BMCs, have been reported to engraft without rejection and contribute to heal necrotic tissue[122].

Previously, it has also been suggested that subtle differences in cell processing and storage may affect the number and functionality of infused progenitor cells that, in turn, could explain the discordance between clinical trials[143]. By contrast, my studies have also clearly demonstrated that freezing or culturing of BMCs do not affect their protective property. It is also interesting to note that culturing of BMCs under hypoxic conditions enhances their protective potential after transplantation probably by releasing more survival factors[13]. However, the finding that BMCs and other progenitor cells can lose their differentiation potential and protective ability with increasing time in culture and high number of passages should be taken into consideration[210, 211]. I cannot fully explain the discrepancy between the claim that therapeutic potential of BMCs can be affected by their manipulation[143] and the contrary results reported here. However, it should be noted that whilst BMC-induced protection was the property investigated in my studies, the capacity to graft was assessed in the other study[143].

4.5 Conclusions

The observation that allogeneic BMCs can afford similar myocardial protection to that of autologous BMCs adds flexibility to their clinical application since they will be readily available at the point of need. The protective properties of BMCs may not be affected by the possible immune reaction that could follow the injection of allogeneic cells since the former action would precede the latter. The findings that the handlings of BMCs such as culturing or freezing do not alter the protective properties of the cells may lead to the clinical exploitation of these cells to reduce the injury sustained during an ischaemic insult. However, it is not clear whether the time of administration might have an effect on the protective action of BMCs.

CHAPTER 5: THE ROLE OF THE TIME OF ADMINISTRATION ON THE PROTECTIVE EFFECT OF BONE MARROW CELLS

5.1 Introduction

In the previous chapters, I have shown that BMCs administered during I/R have a potent protective effect against ischaemic injury. Other experimental and clinical investigations using BMCs have also obtained satisfactory results by reducing infarct size, salvaging dying myocardium and ameliorating cardiac contractility after the onset of an acute myocardial infarction[5, 136]. However it is not clear when BMCs should be applied to achieve maximal benefit and it remains the subject of debate in clinical studies[212]. Therefore, in the present chapter I aimed to determine whether the time of administration of BMCs influences their myocardial protection.

5.2 Methods

Experimental preparation

As in previous chapters, the experimental presented in this chapter were performed using the myocardium from the right atrial appendage of patients undergoing elective cardiac surgery obtained prior to the initiation of cardiopulmonary bypass. The selection of patients, the bone marrow aspiration and separation, and also the experimental preparation were described in Chapter 2.

Assessment of tissue injury and cell death

The CK release into media during the 120min of reoxygenation and cell death by necrosis and apoptosis were determined as described in Chapter 2.

Study protocol

For this study, the most effective lower dose of fresh autologous BMCs ($5x10^6$ cells/preparation) seen in Chapter 3 was used to co-culture with atrial myocardial slices (n=6/group) during ischaemia or during reoxygenation alone or during both times (throughout).

Statistical analyses

One way ANOVA followed by Bonferroni's test was used to determine the significance between means ± SEM of groups as described in Chapter 2. Statistical differences were considered if $p<0.05$.

5.3 Results

Figures 8A-8C show that the incubation of atrial myocardial slices with BMCs during the ischaemic or during the reoxygenation period alone was equally effective in reducing myocardial CK release (A), cell necrosis (B) and apoptosis (C) seen in I/R alone and that the protection observed in each of these two groups was similar to that of BMCs when present during both periods of ischaemia and reoxygenation. *$p<0.05$ vs. I/R alone.

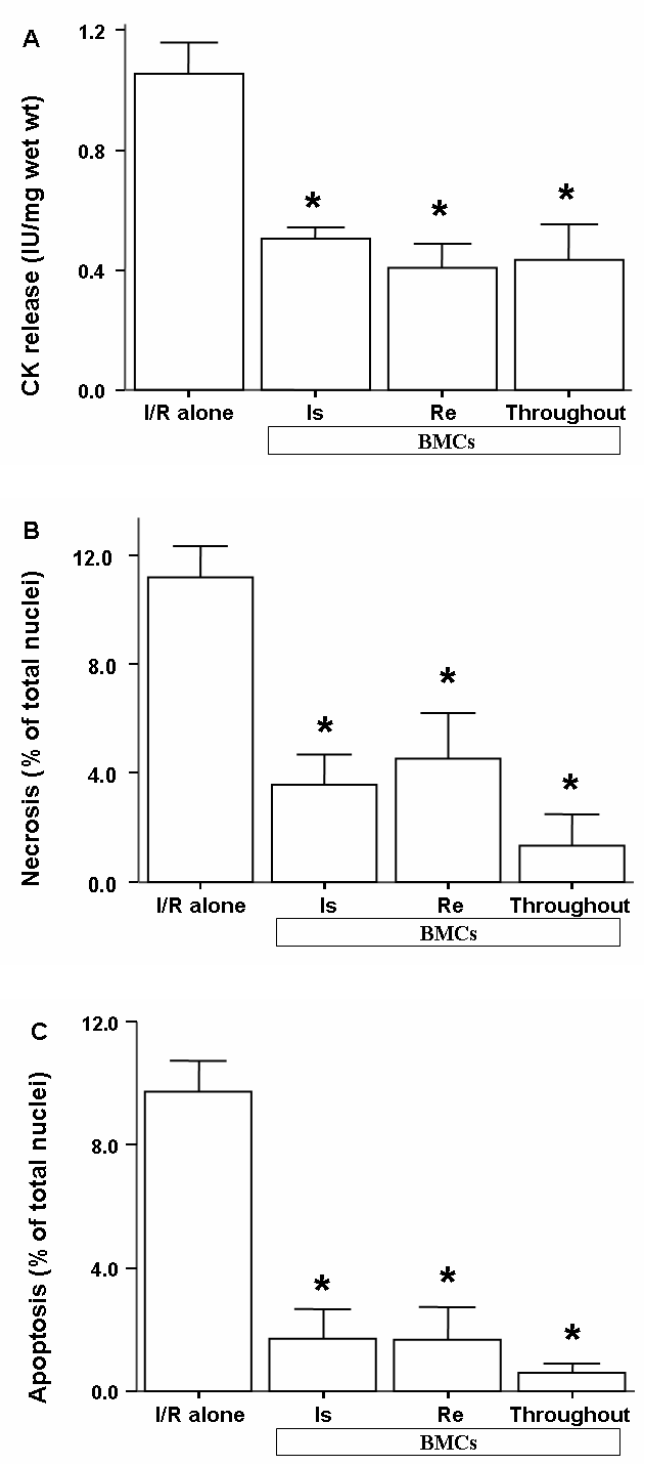


Figure 8: The role of the time of administration.

The effect of the time of administration of BMCs on CK release (A), cell necrosis (B) and apoptosis(C), n=6. *$p<0.05$ versus I/R alone (Is: ischaemia, Re: reoxygenation).

5.4 Discussion

The finding reported in this Chapter that myocardial protection by BMCs can be obtained when cells are applied during ischaemia or during reoxygenation and that the degree of protection is similar in all instances without further improvement when they are administrated at more than one time point, it is also of clinical relevance since maximal cardioprotection can be achieved at anytime, particularly after the ischaemic insult; an invaluable property that adds flexibility to the therapeutic potential of BMCs. However, it should be noted that in my studies BMCs were administrated for the first 2 hours of reoxygenation and that is not known whether they may be as effective when given at later stages of reperfusion. Indeed, the time of BMCs administration following a myocardial infarction has been argued as a key factor to improve cardiac function[212, 213] and a clear improvement was claimed in a preliminary report when the BMCs were administrated in the first few hours after the myocardial infarction[214]. It is clear that further studies will be required to characterize the time-course of the benefit of BMcs when applied clinically.

5.5 Conclusions

Here I have demonstrated that the degree of myocardial protection afford by BMCs can be obtained when the cells are applied during ischaemia or during reoxygenation or during both time periods, adding flexibility to their potential clinical utility. However it remains unknown whether the application of BMCs prior to ischaemia can induce a myocardial protective response and how it is compared to IP.

CHAPTER 6: PRECONDITIONING WITH BONE MARROW CELLS

6.1 Introduction

In the previous Chapter I showed that BMCs afforded myocardial protection to a similar degree when they were administrated during ischaemia or during the reoxygenation period when given during both ischaemia and reoxygenation. However, whether BMCs can precondition the myocardium when administrated before the induction of ischaemia remains unclear, although the equipotency exhibited by BMCs with IP and the ability to block the BMC-induced protection by PKC and p38 MAPK inhibitors[11], essential factors to elicit protection by IP[215], would suggest that BMCs also can precondition the myocardium. Therefore, in this Chapter my aim was to investigate whether BMCs can precondition the human myocardium and if so, to determine the degree of the protection.

6.2 Methods

Experimental preparation

Again, experiments were performed using the myocardium from the right atrial appendage of patients undergoing elective cardiac surgery obtained prior to the initiation of cardiopulmonary bypass. The selection of patients, the bone marrow aspiration and separation, and also the experimental preparation were described in Chapter 2.

Assessment of tissue injury and cell death

The CK release into media during the 120min of reoxygenation and cell death by necrosis and apoptosis were determined as described in Chapter 2.

Study protocol

After equilibration, the atrial myocardial slices (n=6/group) were randomly allocated to different groups on which fresh autologous BMCs were co-incubated with the myocardial slices for various time periods (10, 20 and 30min) prior to the 90min ischaemia/120min reoxygenation and their anti-ischaemic effect was compared to the one obtained with IP. To reduce the possibility that the concentrations of BMCs could play a role, particularly with the shorter periods of co-incubation, a maximum dose of $10x10^6$cells/preparation was selected for this study.

Statistical analyses

The data were reported as mean ± SEM and analysed as described in Chapter 2. Statistical significance is considered if $p<0.05$.

6.3 Results

The results shown in Figure 9 demonstrates that the incubation of the myocardial slices with BMCs for 10, 20 and 30min prior to I/R resulted in progressive protection as compared to I/R alone with maximal reduction in CK release (A), cell necrosis (B) and apoptosis (C) in the 30min incubation group, values that were almost identical to those obtained with IP. *p<0.05 vs. I/R alone.

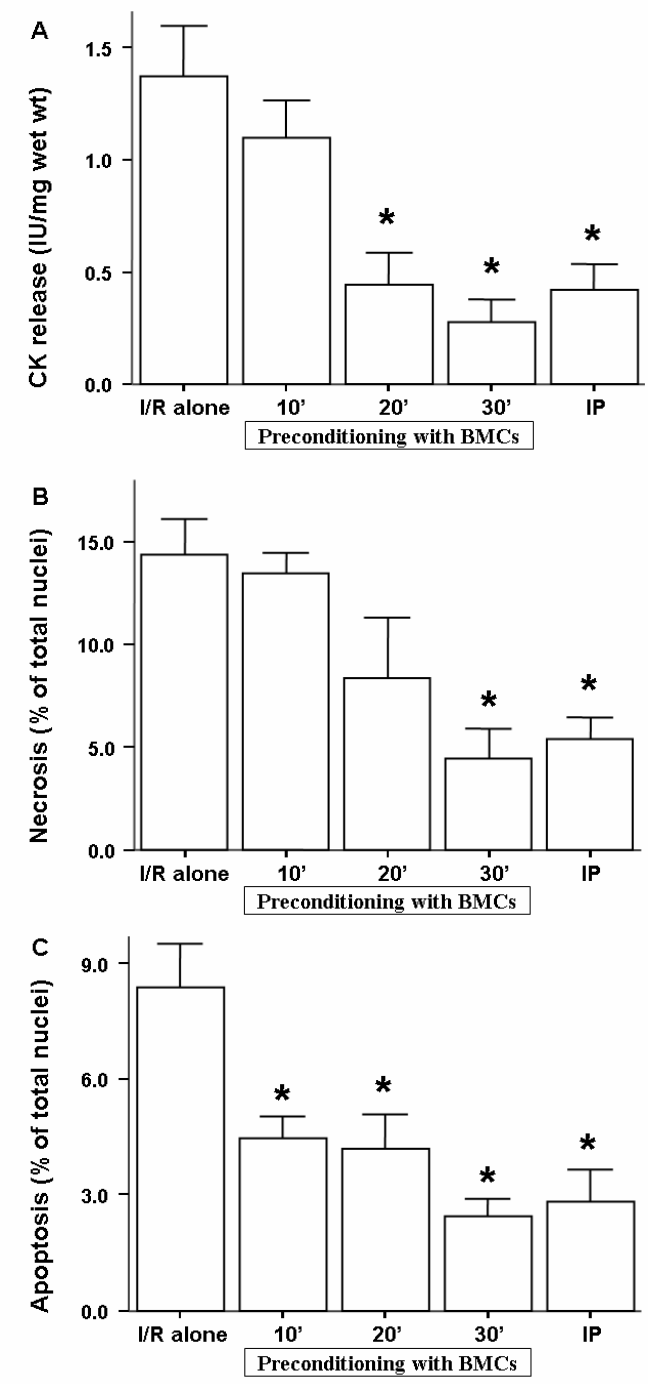


Figure 9: Preconditioning with BMCs.

The effect of preconditioning with BMCs, induced by co-incubation of BMCs with atrial muscles for various time periods prior to ischaemia, on CK release (A), cell necrosis (B) and apoptosis (C), n=6. Some atrial muscles were also subjected to IP. *$p<0.05$ versus I/R alone.

6.4 Discussion

In this chapter I have demonstrated for the first time that BMCs can precondition the human myocardium and that the degree of the myocardial protection is similar to the one obtained with IP. I also have shown that the BMC-induced myocardial protection is achieved gradually and is not an all-or-none phenomenon with some protection seen after 10min of co-incubation with the myocardial slices and maximal benefit obtained after 30min of exposure.

If both forms of preconditioning, with BMCs and with ischaemia, are equipotent and sharing cellular mechanisms, then it is possible that the preconditioning with BMCs is subjected to the therapeutic windows exhibited by IP. If this is the case, it is possible that after the protective trigger of an ischaemic insult, no additional benefit may be obtained from the administration of BMCs. The experimental model used in my thesis is not appropriate to investigate this important point but it is an issue that would require full clarification if BMCs are going to be used clinically to combat ischaemic injury.

6.5 Conclusions

Here I have demonstrated that BMCs confer precondition to the human myocardium by 30min exposure, an action that was as potent as that of IP. However, this study did not show whether the benefit was induced by a direct effect of the BMCs on the myocardium or it was the consequence of a factor(s) secreted by the cells.

CHAPTER 7: THE ROLE OF SECRETED FACTORS ON THE BONE MARROW CELL-INDUCED MYOCARDIAL PROTECTION

7.1 Introduction

Having demonstrated in previous chapters that BMCs possess potent protective properties, I turn my attention to the question of whether this action is due to a direct effect of the cells or caused by secretion of a factor(s). In deed, the original hypothesis underlying the putative beneficial effect of BMC transplantation has been questioned and a paracrine action has been put forward as a more likely mechanism[200]. Early studies suggested that the implanted cells respond to the new microenvironment by secreting cytokines and growth factors, which may act both in an autocrine fashion on the donor cells and in a paracrine fashion on nearby host cells[178, 216]. Several strategies using different cell types to improve the survival of the myocardium have demonstrated significant improvement in cardiac function[192, 217-219], a benefit that could be associated with increasing release of secreted factors from the cells. Hence, in this chapter my aim was to elucidate whether the myocardial protection is induced by factor(s) secreted by the BMCs.

7.2 Methods

Experimental preparation

As in previous chapters, experiments were performed using the myocardium from the right atrial appendage of patients undergoing elective cardiac surgery harvested prior to

the initiation of cardiopulmonary bypass. The selection of patients, the bone marrow aspiration and separation, and also the experimental preparation were described in Chapter 2.

Assessment of tissue injury and cell death

The CK release into media during the 120min of reoxygenation and cell death by necrosis and apoptosis were determined as described in Chapter 2.

Study protocol

For this experiment, fresh autologous BMCs ($10x10^6$cells/preparation) were incubated in 10ml KHH solution under aerobic conditions for 30min. The KHH supernatant (conditioned media) was then obtained and co-incubated with atrial myocardial slices for another 30min prior to 90min ischaemia/120min reoxygenation. For comparison, the effect of fresh autologous BMCs ($10x10^6$cells/preparation), also co-incubated with the myocardial slices in KHH solution for the same time period before ischaemia, and that of IP were studied (n=6/group). KHH solution was always used to maintain BMCs, therefore no other controls were needed.

Statistical analyses

The results were analysed with ANOVA followed by Bonferroni's test as described in Chapter 2. Statistical significance is considered if $p<0.05$.

7.3 Results

As shown in Figure 10, the conditioned media (supernatant) significantly reduced CK release and cell necrosis and apoptosis, as compared to I/R alone, when co-incubated with the myocardial slices for 30min prior to 90min ischaemia/120min reoxygenation. The results were almost identical to those obtained by preconditioning with BMCs (30min) or by IP, this suggesting that the observed protective effect is mediated by a factor(s) produced by BMCs and contained in the media.

7.4 Discussion

Here I have shown that the incubation of BMCs-conditioned cell-free media with human myocardium before ischaemia confers protection similar to that afforded by BMCs themselves and by IP. Taken together, the results of this and the previous chapter suggest that the beneficial action of BMCs is mediated by a secreted factor(s) that require at least 30min of cells incubation so that s sufficient concentration can be accumulated in the media to achieve maximal therapeutic efficacy. These results in man are supported by an animal study showing that the use of conditioned media from BMCs significantly increases microvessel density, decrease fibrosis and prevents apoptosis in the infarcted myocardium, this leading to improvement in cardiac function[176]. However, in this experimental study the conditioned media was obtained after culturing BMCs in normoxic and hypoxic conditions and then administrated directly into the myocardium immediately after coronary ligation and subsequently intraperitoneally at several time points (2, 4 and 6 days) after the infarction[176].

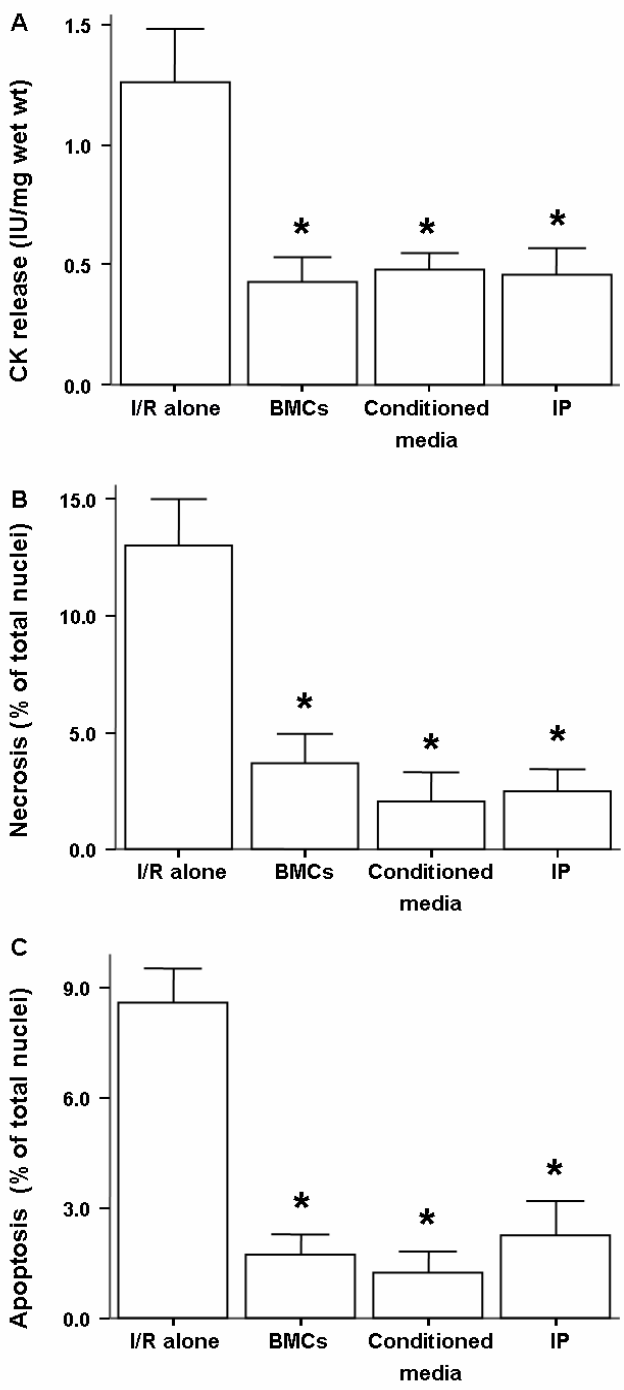


Figure 10: The role of conditioned media in the BMC-induced myocardial protection. The effect of preconditioning with conditioned media, as compared to BMCs and IP, on CK release (A), cell necrosis (B) and apoptosis (C), n=6. *$p<0.05$ versus I/R alone.

The composition of BMCs-derived media appears to be a complex cocktail of cytokines and growth factors[13, 176, 177], and it is possible to speculate that some of these factors individually or in combination may be responsible for the cardioprotective effect of BMCs. The clinical value of the present results should not be underestimated because the desired cardioprotective effect could be achieved without the need of administration of BMCs, saving time and resources and adding flexibility and applicability.

7.5 Conclusions

The finding that full myocardial protection against ischaemic injury can be achieved by BMCs-conditioned media without the need for the administration of cells is of great clinical relevance since the conditioned media can be stored and be readily available when needed. However, further refinement of this anti-ischaemic intervention can be attended by a better understanding of the protective mechanism that I will explore in the next chapter.

CHAPTER 8: THE MECHANISM OF BONE MARROW CELL -INDUCED MYOCARDIAL PROTECTION

8.1 Introduction

Stem cell transplantation into the injured heart has been considered to be a promising therapy for the treatment of ischaemic cardiac diseases, yet the potential mechanism is not clearly understood. Beyond the putative regenerative capacity, the use of BMCs has been demonstrated to express paracrine signalling factors including cytokines and growth factors that increase angiogenesis and reduce cell death[200, 220]. Among these factors, IGF-1 acts predominately as a growth, survival, and differentiation factor for many cell types including myocytes[179, 180, 221]. IGF-1 is produced by BMCs and both IGFs (IGF-1 and IGF-2), whose biological activity is mediated mainly through IGF-1 receptors (IGF-1R), have been shown in animal experimentation to induce cardioprotection by reducing cell death and limiting infarct size[13, 161, 176, 182, 222]. Therefore, IGF-1R can be a potential mediator of the BMC- and conditioned media-induced myocardial protection seen in the previous chapter. Due to the cardioprotection being induced by both cells and conditioned media within 30min of exposure and because they mimicked the benefit of IP, my hypothesis was that extracellular adenosine produced by ecto-5'-nucleotidase/CD73-CD39 could be the trigger for protection; however, preliminary experiments using a universal adenosine receptor inhibitor (theophylline) did not support the thesis of an involvement of adenosine. Hence, the aim of this study was to investigate the role of IGF-1/IGF-1R in the cardioprotection induced by BMC-conditioned media.

8.2 Methods

Experimental preparation

The experimental preparation using the right atrial myocardium and BMCs from patients undergoing elective cardiac surgery is described in Chapter 2.

Assessment of tissue injury and cell death

The CK release into media during the 120min of reoxygenation and cell death by necrosis and apoptosis were determined as described in Chapter 2.

Study protocols

In this chapter, the following studies were carried out:

Study 1: To elucidate whether IGF-1R mediates the cardioprotection induced by BMCs

To do this, atrial myocardial slices (n=6/group) were incubated for 30min with conditioned media, previously obtained from the culture of $10x10^6$ fresh autologous BMCs for 30min, prior to 90min ischaemia/120min reoxygenation. Experiments were performed in the presence of various concentrations (0, 1.5, 15, 150μM) of the specific IGF-1R blocker PQ401 (Sigma-Aldrich, Dorset, UK) that directly inhibits autophosphorylation of the kinase domain of the receptor[223]. Some atrial muscles were concomitantly co-incubated with the highest dose of PQ401 prior to I/R and served as the control for the influence of the blocker in this experimental model.

Study 2: To define the concentration of IGFs in the conditioned media

In this study, the concentration of IGFs secreted into the media by $10x10^6$ fresh autologous BMCs (conditioned media) was determined after 10, 20 and 30min of incubation (n=6 determinations at each time point) by ELISA assays.

The concentrations of IGF-1 and IGF-2 in the conditioned media were analysed using the ELISA method (plate reader Benchmark, California, USA), in which IGF-1 was measured by using human IGF-1 detection kit (R&D, Minneapolis, Minnesota, USA) and IGF-2 by using human IGF-2 detection kit (Bio supply, Norwich, Norfolk, UK) as described by the manufacturers. The sensitivity of the measurement is 62.5ng/ml and 40ng/ml for IGF-1 and IGF-2 respectively.

Study 3: To investigate whether IGFs at a concentration above the detection levels can confer protection

In this additional study, to examine whether the observed benefit from BMCs is mediated by the IGFs present in the media, myocardial slices (n=6/group) were incubated with IGF-1 at the concentration of 62.5ng/ml and IGF-2 at the concentration of 40ng/ml (both from Sigma-Aldrich, Dorset, UK) alone and in combination for 30min prior to 90min ischaemia/120min reoxygenation. These concentrations were higher than those detected in the conditioned media and represented the minimal detected levels by the used ELISA assays.

Statistical analyses

The reported values were analysed with ANOVA followed by Bonferroni's test and were described in Chapter 2. Statistical significance is considered if $p<0.05$.

8.3 Results

Study 1: IGF-1R mediates the myocardial protection induced by BMCs

Figures 11A-11C show that the specific IGF-1R blocker PQ401 at the highest concentration used (150μM) did not affect the CK release and cell necrosis and

apoptosis caused by I/R. They also demonstrate that the favourable effect of the conditioned media in reducing CK release and cell death was abolished by PQ401 at concentration ≥15μM, suggesting that the myocardial protection induced by BMCs is mediated by IGF-1R.

Study 2: The concentration of IGFs in the conditioned media

IGF-1 and IGF-2 could not be detected in the conditioned media at the end of 10, 20 and 30min incubation with BMCs. Because of that, the minimum detectable concentrations for the assays of the two factors (62.5ng/ml for IGF-1 and 40ng/ml for IGF-2) were used alone and in combination for the next study to determine whether IGFs at a known concentration can cause any effect.

Study 3: The role of IGFs in BMC-conditioned media induced myocardial protection

The results shown in Figures 12A-12C demonstrate that IGF-1 and IGF-2 alone or in combination at concentration above the level of detection did not significantly affect the CK release and cell death caused by I/R.

This suggests that although IGFs acting through the IGF-1R could be necessary, they are not sufficient for the myocardial protection afforded by BMCs and that other factor(s) are required to achieve the benefit.

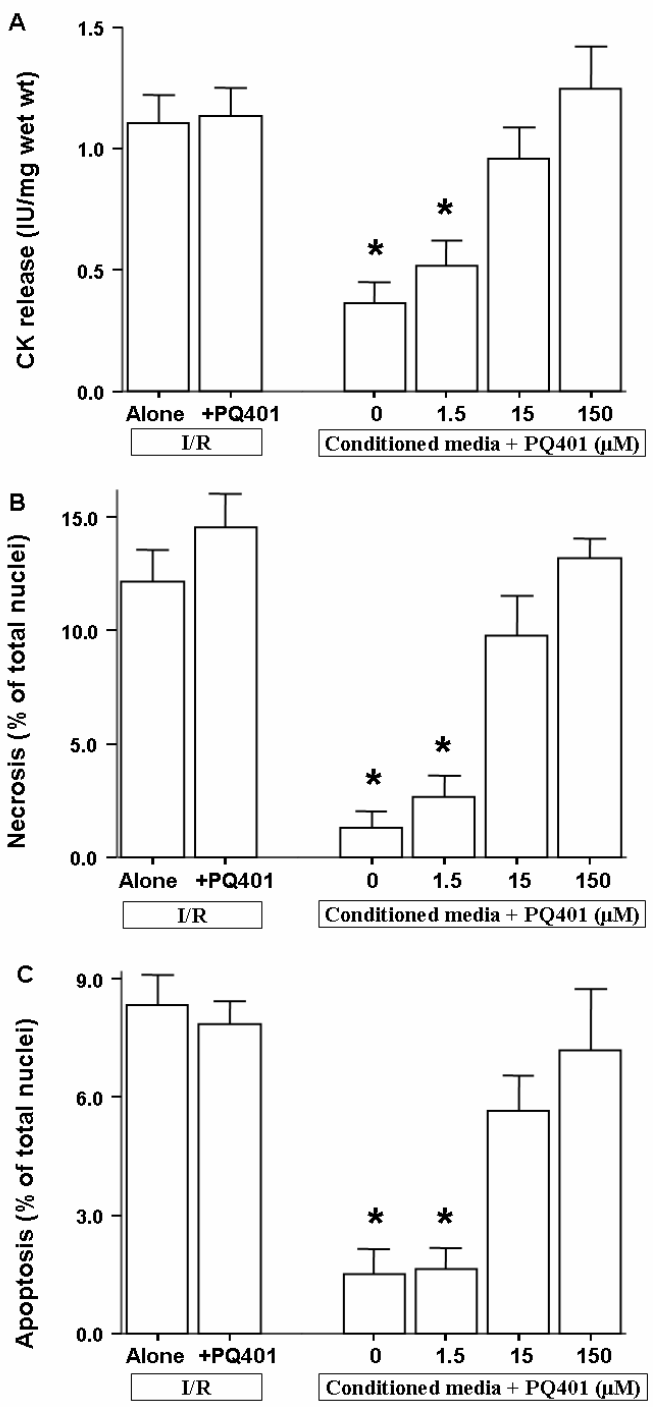


Figure 11: The effect of the blockade of IGF-1R on myocardial protection.

The effect of IGF-1R blocker PQ401 at different concentrations in the conditioned media, when co-incubated with the muscles for 30min prior to ischaemia, on CK release (A), cell necrosis (B) and apoptosis (C), n=6. *$p<0.05$ versus I/R alone.

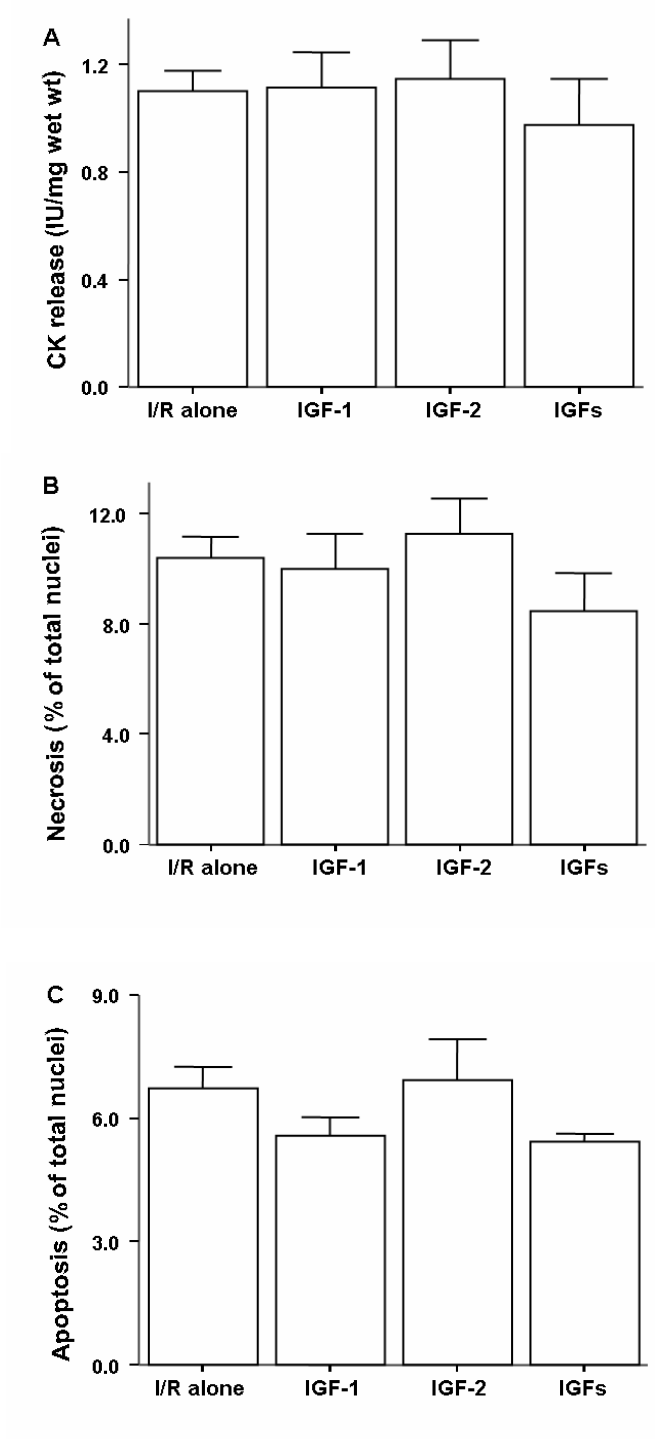


Figure 12: The effect of IGF-1 and IGF-2.

IGF-1 (62.5ng/ml) and IGF-2 (40ng/ml) alone and in combination did not significantly affect the CK release (A), cell necrosis (B) and apoptosis (C) caused by I/R, n=6.

8.4 Discussion

In the present studies, the IGF-1R was identified as a necessary mediator of the myocardial protection induced by BMCs-conditioned media since the benefit was completely abolished by the IGF-1R specific antagonist PQ401 in a dose-dependent manner. This finding would suggest that a factor responsible for the beneficial action of the BMCs-conditioned media is IGF, a thesis that would be supported by previous experimental studies on the protective properties of this growth factor[179, 180, 182, 221, 224]. However, the two IGF isoforms, IGF-1 and IGF-2, were below the level of detection of commercial ELISA kit in the BMCs-conditioned media and when both IGFs were used at higher concentrations to those present in the media they were not sufficient to reduce ischaemic injury. These results suggest that factor(s) other than IGF-1/IGF-2 acting through IGF-1R are necessary to induce protection or another mechanism is also involved. Given the complex biological actions triggered by the BMCs or their conditioned medium, it seems reasonable that other factors alone or in combination with IGF are needed to produce the protective effect. Although IGF-1 and IGF-2 could not be measured in the conditioned medium, it would be interesting to see whether antibodies against either of these two factors or both of them could block the effect. Actually, in agreement with my findings, several studies have shown that blocking IGF-1R is associated with promotion of apoptosis, an important factor responsible for ventricular remodelling after myocardial infarction[225, 226].

As mentioned in the Introduction of this chapter, a preliminary study using theophylline, a non-selective adenosine receptor blocker, did not abolish the myocardial protection induced by the BMCs-conditioned media, thus excluding a role for adenosine

and adenosine receptors. Certainly, a full elucidation of the factor(s) responsible for cardioprotection would require further investigation.

The myocardial protection obtained with the BMC-conditioned media was as potent as that of IP. A previous study from our laboratory[11] demonstrated that the BMC-induced cardioprotection can be abolished by blocking the activation of kinases such as PKC and p38MAPK, both of which have been shown to be integral parts of the signalling transduction mechanism of IP[215] and these kinases are also downstream effectors of the IGF-1R[227, 228]. Therefore, it is likely that both interventions, BMCs and IP, are sharing some of the mechanism required to elicit their protective action.

8.5 Conclusions

The results shown in this chapter suggest that the myocardial protection induced by BMCs-conditioned media is mediated, at least in part, through the IGF-1R, that although IGF-1 and IGF-2 could be necessary, they are not sufficient for the protection afforded by BMCs and that other factor(s) are required to achieve the benefit. Following this, I turn my attention to the identification of the BMCs type that may be responsible for the protective effect.

CHAPTER 9: THE IDENTITY OF THE CELL TYPE RESPONSIBLE FOR THE BONE MARROW CELL-INDUCED MYOCARDIAL PROTECTION

9.1 Introduction

Unselected BMCs represent a mixed population of various stem cells, progenitor cells, and haematopoietic cells at various maturation stages. It is not yet clear which cell population(s) promote functional recovery in the subjects after some of the intracoronary transfer. In experimental models of acute myocardial infarction, functional improvements have been reported after transplantation of unselected or highly selected BMC populations, including HSCs, EPCs[5, 129, 199] and MSCs[121]. Importantly, the use of $CD133^+$, a subset of BMCs with lots of stemness, has been reported to enhance tissue repair after an acute ischaemic insult[197, 229, 230] or when used clinically during coronary bypass graft surgery in the context of chronic ischaemia[231]. While the differentiation of BMCs to cardiac tissue is not clear from these studies, my previous data have demonstrated a strong myocardial protective effect of the unfractionated BMCs, and because of this my aim in this chapter was to investigate the role of $CD133^+$ cells in mediating myocardial protection.

9.2 Methods

Experimental preparation

The experimental preparation using the right atrial myocardium and BMCs from patients undergoing elective cardiac surgery is described in Chapter 2. In addition,

$CD133^+$ cells were isolated by the use of antibody-magnetic beads (CD133 indirect kits, Miltenyi Biotec, Bergisch Gladbach, Germany) following the company's protocol. After that, the percentage of $CD133^+$ in the selected cell population was determined.

Assessment of tissue injury and cell death

The CK release into media during the 120min of reoxygenation and cell death by necrosis and apoptosis were determined as described in Chapter 2.

Study protocol

To identify whether $CD133^+$ cells, a subset of BMCs that have been used in clinical trials for cardiac repair[197, 229, 230, 232], myocardial slices (n=5/group) were incubated with fresh autologous $CD133^+$ and $CD133^-$ cells for 30min prior to 90min ischaemia/120min reoxygenation. The $CD133^+$ and $CD133^-$ cells used in each preparation were those obtained after their separation from $10x10^6$ fresh BMCs. In addition, myocardial slices (n=5/group) from the same donors were subjected to an identical protocol using unfractionated fresh BMCs ($10x10^6$ cells). This study was restricted to the cardioprotective effect of CD133 cells and no further exploration on the composition of the CD133 cells was established.

Statistical analyses

Data were reported as mean ± SEM and compared as described in Chapter 2. Statistical significance is considered if $p<0.05$.

9.3 Results

Figures 13A-13C demonstrate that while the reduction in CK release and in cell necrosis and apoptosis can be replicated by the incubation of the myocardial slices with BMCs for 30min prior to I/R, the $CD133^+$ (0.3±0.04% BMCs) and $CD133^-$ population failed to significantly modify the mean values for the three indexes of injury when compared to the I/R group. The unexpected results suggest that the immunobeads isolation procedure eliminate the protective properties of BMCs, possibly by suppression of the production/secretion of the putative beneficial factor(s).

9.4 Discussion

The stemness of $CD133^+$ cells has made them a likely candidate responsible for the putative benefit of BMCs and as a result they have been used in several clinical trials[197, 229, 230, 232]. Here I have shown that neither the $CD133^+$ nor the remaining BMCs population were able to reduce ischaemic injury, which suggests that cell separation by magnetic immunobeads impairs the capacity of cells to secrete the factor(s) required to withstand an ischaemic insult. Therefore, the question of whether $CD133^+$ cells are responsible for the BMC-induced myocardial protection still remains unanswered.

In Chapter 4, I demonstrated that the culturing and freezing of BMCs did not affect their protective potential; however, as shown here, the use of other manipulations, such as the use of immunobeads, can reduce or abolish this property. This finding emphasises the importance of standardisation of the protocols used for the preparation of cells and the need for detailed analysis of their capabilities before use.

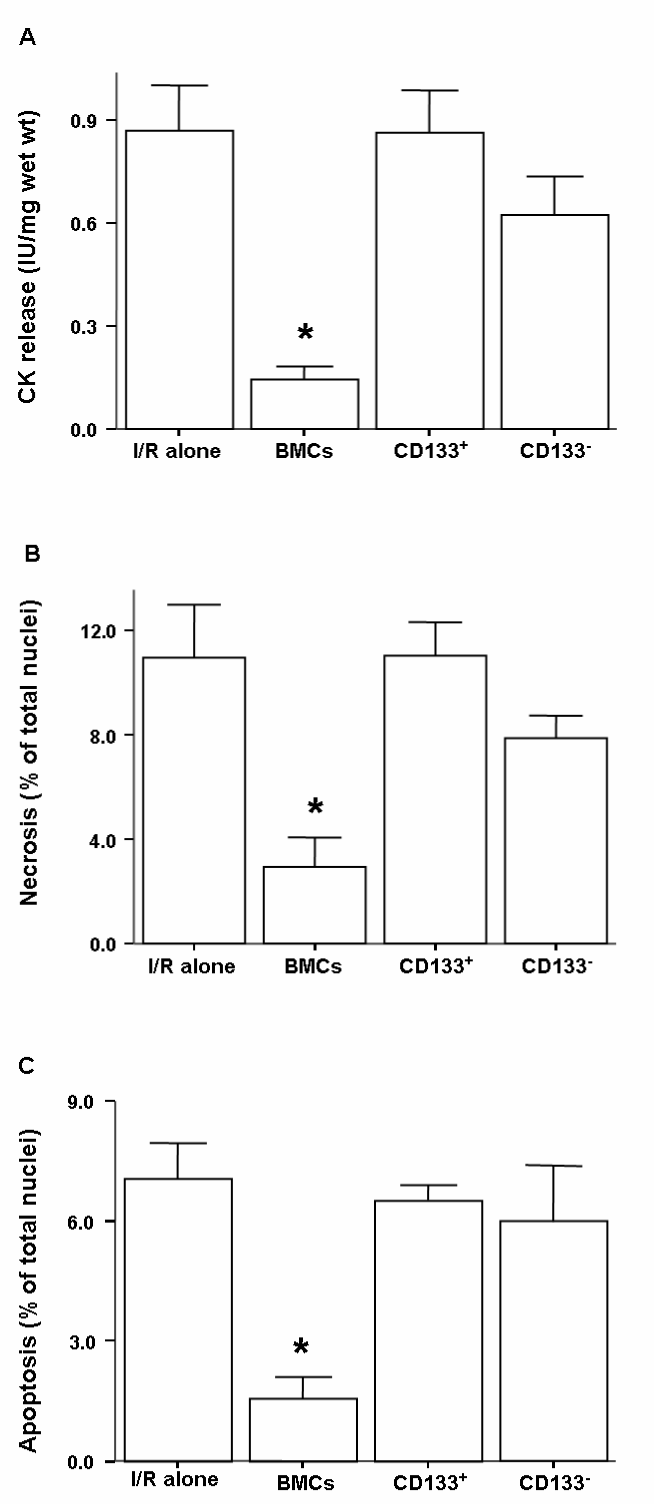


Figure 13: The role of $CD133^+$ cells in the BMC-induced myocardial protection.

The role of CD133+ cells, as compared to $CD133^-$ cells and unfractioned BMCs, on CK release (A), cell necrosis (B) and apoptosis (C), n=5. *$p<0.05$ versus I/R alone.

9.5 Conclusions

The effect of the immunobeads on the isolation of BMCs has prevented me from elucidating whether CD 133+ cells are responsible for the myocardial protection of BMCs. This unexpected result may suggest that to obtain the cardioprotection of BMCs, it would be more efficacious to use the unfractionated, whole BMCs population, rather than a specific cell subset. However, it should be conceded that the culturing of separated cells could allow them to regain their capabilities, an issue that will need to be clarified in future studies.

CHAPTER 10: THE EFFECT OF DIABETES AND POOR LEFT VENTRICULAR FUNCTION ON BONE MARROW CELL-INDUCED MYOCARDIAL PROTECTION

10.1 Introduction

Diabetes mellitus is a major risk factor for cardiovascular diseases and complications[233]. In addition, diabetes reduces the tolerance to ischaemia[234] and patients with this condition are more susceptible to develop heart failure[235], that constitutes a growing health problem further exacerbated by the increase in the aging population. Indeed, mortality is 4-8 times greater in people with heart failure than in the general population of the same age[236]. To investigate and overcome this clinical problem, it is necessary to have a better understanding of how these conditions are affecting the cardiovascular system.

We have demonstrated that the myocardium of patients with diabetes or very poor LV function (EF<30%) cannot be protected by interventions such as IP[85, 94] but that this deficit can be reversed by the manipulation of the intracellular signalling pathway of cardioprotection[85]. As described in Chapter 3, I have also found that BMCs confer protection of the myocardium from subjects without diabetes or poor LV function, an action that was as potent as that obtained with IP. The aims of the present study were to investigate whether such clinical conditions influence the myocardial protection elicited by BMCs and whether the cause for any loss in protection resides in the BMCs or in the myocardium.

10.2 Methods

Experimental preparation

Experiments were performed on the right atrial myocardium and BMCs obtained from patients undergoing elective heart surgery prior to the initiation of cardiopulmonary bypass. The selection of patients, the bone marrow aspiration and separation, and also the experimental preparation were described in Chapter 2.

Assessment of tissue injury and cell death

CK release into the media during the 120min of reoxygenation and cell death by necrosis and apoptosis were determined as described in Chapter 2.

Study protocols:

In this chapter, the most effective lower dose of BMCs ($5x10^6$ cells/preparation) seen in Chapter 3 was used to co-culture with atrial myocardial slices (n=6/group) during 90min ischaemia/120min reoxygenation in the following studies:

Study 1: To investigate the myocardial protective potential of BMCs from subjects with diabetes and to elucidate whether the response depends on the myocardium or the BMCs

This was achieved by co-incubating the myocardium from subjects with diabetes with autologous BMCs and with allogeneic BMCs from subjects without diabetes during the entire ischaemia and reoxygenation periods and compared to the efficacy of IP. In addition, the myocardial slices from subjects without diabetes were co-incubated with autologous BMCs and with allogeneic BMCs from subjects with diabetes.

Study 2: To investigate the myocardial protective potential of BMCs from subjects with poor LV function and to determine whether the response depends on the myocardium or the BMCs

For this study, the myocardium from subjects with failing hearts (LV EF <30%) were co-cultured with autologous BMCs and with allogeneic BMCs from subjects with normal function hearts during entire ischaemia and reoxygenation periods and compared to the efficacy of IP. In further experiments, the myocardium from hearts with normal LV function was co-incubated with autologous BMCs and with allogeneic BMCs from subjects with failing hearts.

Statistical analyses

The results were expressed as mean ± SEM. One way ANOVA followed by Bonferroni's test was used as described in Chapter 2. Statistical significance is considered if $p<0.05$.

10.3 Results

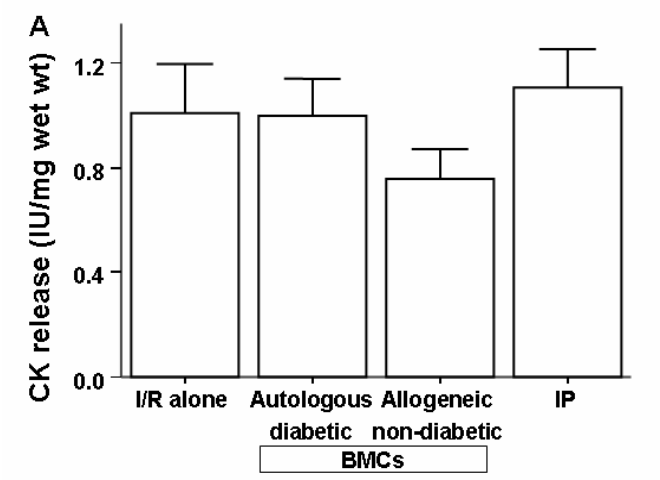
Study 1: Protection of the myocardium from subjects with diabetes mellitus: role of BMCs and myocardium

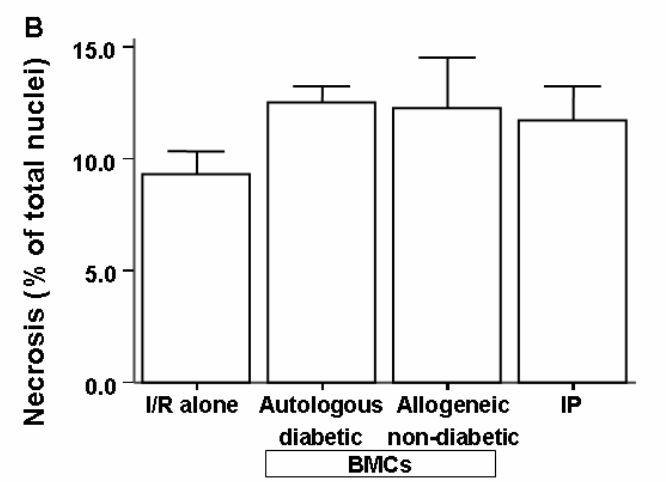
Figures 14A-14C show that I/R alone of the diabetic myocardium induced substantial myocardial injury as measured by CK release, and cell necrosis and apoptosis. They also demonstrated that the diabetic myocardium cannot be protected by IP or by the co-incubation with autologous BMCs or allogeneic BMCs from subjects without diabetes. By contrast, Figures 14D-14F show that the CK release, and the cell necrosis and apoptosis induced by I/R of the non-diabetic myocardium are significantly and similarly reduced by the autologous BMCs or by allogeneic BMCs from subjects with diabetes.

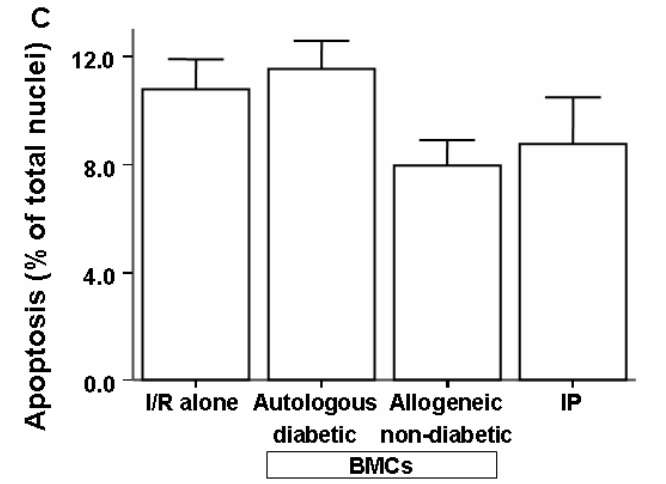
These results suggest that the failure to protect the diabetic myocardium is due to a deficit in the myocardium and that the protective properties of BMCs from diabetic subjects are preserved.

Study 2: Protection of the myocardium from subjects with poor LV function: role of BMCs and myocardium

Figures 15A-15C demonstrate that, as expected[94], IP did not reduce the CK release and cell death by necrosis and apoptosis of the myocardium from failing hearts subjected to I/R. They also show that, as IP, autologous BMCs did not afford protection, however the co-incubation of myocardium from failing hearts with allogeneic BMCs from subjects with normal LV function resulted in significant reduction in CK release and in a modest, but still statistically significant, decrease in cell necrosis without a detectable benefit in apoptosis. Importantly, Figures 15D-15F show that whilst the co-incubation of the myocardium from subjects with normal LV function effectively reduced CK release and cell necrosis and apoptosis as compared to I/R alone, the use of BMCs from subjects with poor LV function failed to do so. These results suggest that the BMCs from subjects with poor LV function have lost the protective potential although the failing myocardium also exhibits a deficit in the protection brought about by IP and BMCs.


A
CK release (IU/mg wet wt)
1.2
0.8
0.4
0.0
I/R alone
Autologous diabetic
Allogeneic non-diabetic
IP
BMCs


B
Necrosis (% of total nuclei)
15.0
10.0
5.0
0.0
I/R alone
Autologous diabetic
Allogeneic non-diabetic
IP
BMCs


C
Apoptosis (% of total nuclei)
12.0
8.0
4.0
0.0
I/R alone
Autologous diabetic
Allogeneic non-diabetic
IP
BMCs

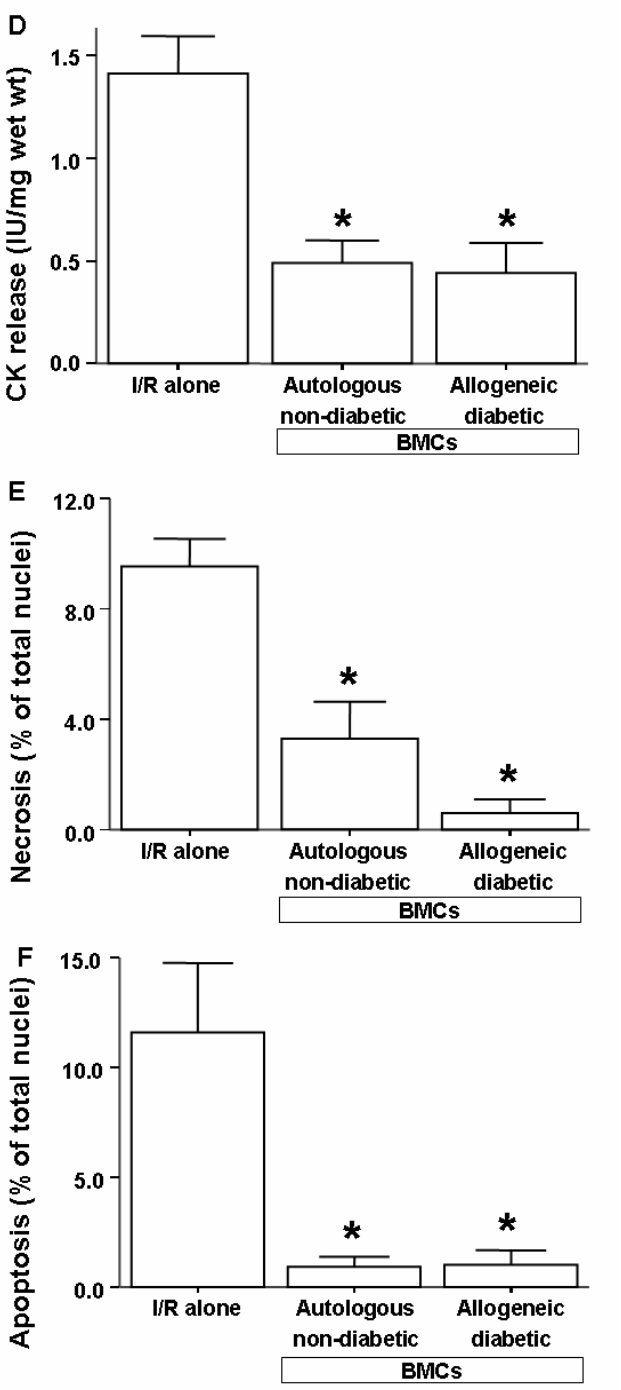
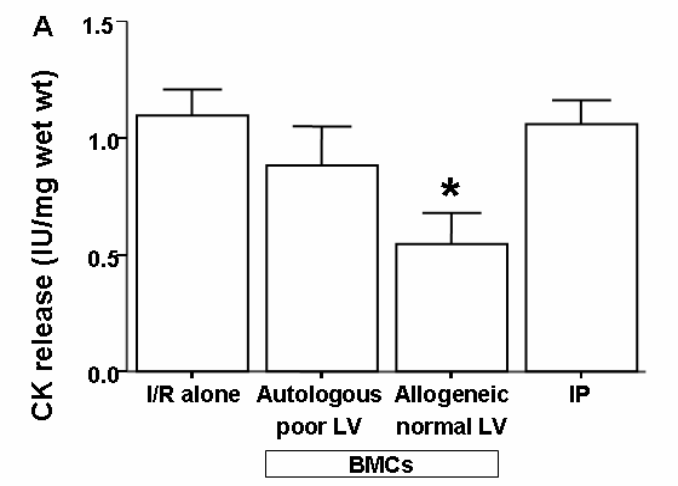
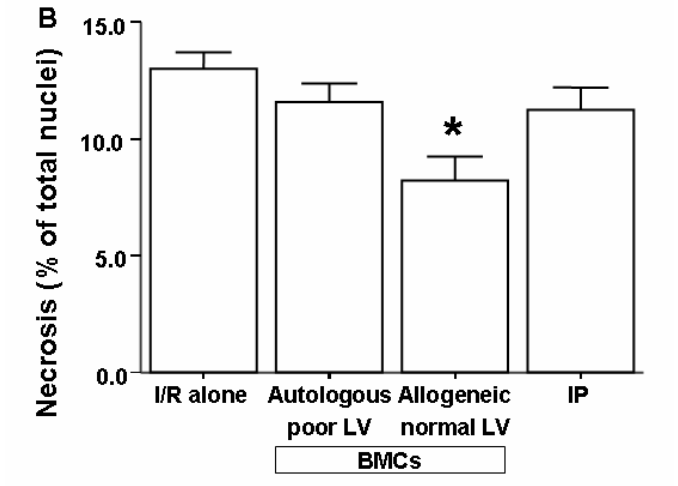
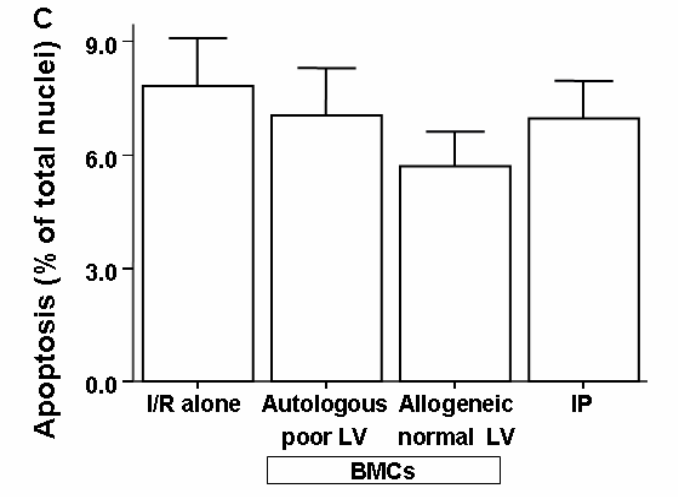


Figure 14: The effect of BMCs and myocardium from subjects with diabetes.

CK release (A), cell necrosis (B) and apoptosis (C) in myocardium from subjects with diabetes co-cultured with diabetic and non-diabetic BMCs; and CK release (D), cell necrosis (E) and apoptosis (F) in myocardium from subjects without diabetes co-cultured with non-diabetic and diabetic BMCs, n=6. * $p<0.05$ versus I/R alone.


A
CK release (IU/mg wet wt)
1.5
1.0
0.5
0.0
*
I/R alone
Autologous poor LV
Allogeneic normal LV
IP
BMCs


B
Necrosis (% of total nuclei)
15.0
10.0
5.0
0.0
*
I/R alone
Autologous poor LV
Allogeneic normal LV
IP
BMCs


C
Apoptosis (% of total nuclei)
9.0
6.0
3.0
0.0
I/R alone
Autologous poor LV
Allogeneic normal LV
IP
BMCs

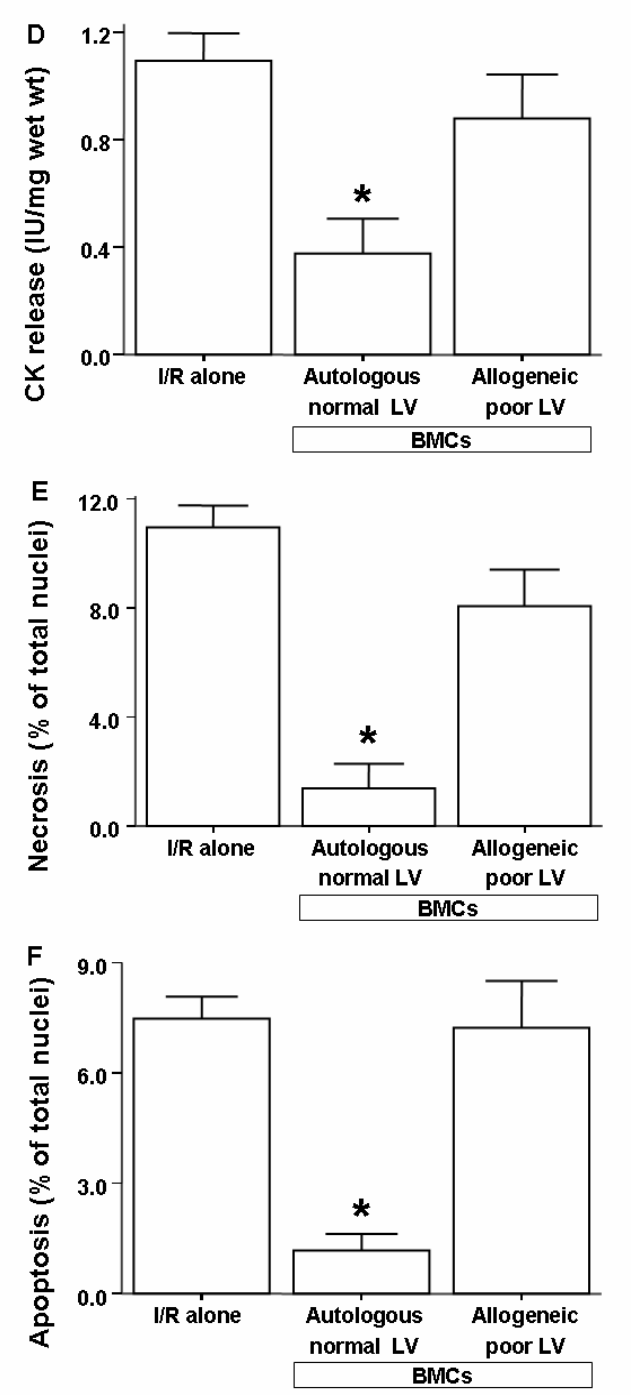


Figure 15: The effect of BMCs and myocardium from subjects with poor LV function. CK release (A), cell necrosis (B) and apoptosis (C) in myocardium from failing hearts co-cultured with poor and normal LV function BMCs; and CK release (D), cell necrosis (E) and apoptosis (F) in myocardium from subjects with normal LV function co-cultured with normal and poor LV function BMCs, n=6. *$p<0.05$ versus I/R alone.

10.4 Discussion

Diabetes and myocardial protection

Diabetes is a systemic disease that also causes cardiomyopathy[237-239]. Patients with diabetes are at an increased risk of ischaemic events[240-242] and diabetes also represents an important risk factor for poor outcomes after coronary revascularisation[243, 244]. Despite the overwhelming clinical evidence that the diabetic heart is more sensitive to ischaemia/reperfusion injury[245], experimental studies are divided, reporting more[246, 247], less[248] or similar[249] susceptibility. Our laboratory was the first in demonstrating that the myocardium from subjects with diabetes cannot be preconditioned by ischaemia[94], that the reason is dysfunctional mitochondria[85] but that the deficit can be overcome by activation of the signalling transduction pathway beyond the mitochondria[215]. In the present studies, it was demonstrated that the BMCs of patients with diabetes posses similar protective potential to the BMCs of those without diabetes. Since neither autologous BMCs from diabetic nor allogeneic BMCs from non-diabetic subjects were able to afford protection of the diabetic myocardium, it can be hypothesised that the protection of BMCs is mediated by a mechanism operating upstream of the mitochondria.

The finding that the protective potential of BMCs is preserved in diabetes does not necessary mean that the other properties of the BMCs from these subjects are not affected by the disease. Thus, it has been reported that the diabetic bone marrow mononuclear cells have an impaired potency to induce therapeutic angiogenesis[250, 251]. Certainly, a full elucidation of how diabetes may affect BMCs will be required for a safe and efficacious exploitation of their therapeutic potential in this group of patients.

Heart failure and myocardial protection

It is believed that the failing heart is more sensitive to ischaemia/reperfusion injury probably due, at least in part, to a greater oxidative stress[92, 252, 253] and increased production of cytokines, such as tumour necrosis factor alpha[254], in congestive heart failure. We have also demonstrated that the myocardium from subjects with poor LV function cannot be protected by IP although the mitochondrial K_{ATP} channel opener diazoxide elicited protection[94], this suggesting that the defect in cardioprotection was located upstream of the mitochondria. Here I have confirmed that IP does not protect the failing myocardium and demonstrated for the first time that allogeneic BMCs from subjects with normal cardiac function can afford some, but not full, protection. Importantly, I also have shown for the first time that the BMCs from patients with poor LV function have lost the protective properties on the myocardium from subjects with normal LV function. Previously, I have reported that the myocardial protection induced by BMCs is triggered by secreted factors and mediated by IGF-1R, an effect that can be blocked by inhibition of the activity of PKC and p38 MAPK[11]. Overall, these results support the thesis that BMCs induce myocardial protection by a mechanism similar to that of IP and that the failure to induce protection by BMCs from subjects with poor LV function is probably due to a deficit in the secretion of some, as yet unknown, growth factors and/or chemokines. The elucidation of such factors may be of clinical importance for increasing the tolerance to ischaemic insults and ameliorating the survival of cardiomyocytes of failing hearts.

10.5 Conclusions

The finding that autologous BMCs cannot protect the myocardium of patients with diabetes and poor LV function has important clinical implications and should be taken into consideration for the design of clinical trials on the use of BMCs. It is worth emphasising that the use of allogeneic BMCs from normal donors will not evoke protection of the diabetic myocardium since the defect is located in the cardiac tissue and not in the BMCs. However, the use of allogeneic BMCs from normal donors could still elicit some degree of protection in the failing heart.

It will be valuable to reveal the factors produced by BMCs whose absence could be responsible for the protective deficit in patients with heart failure in order to avoid the complexity of the use of allogeneic cells. None the less, a more direct and effective approach could be the activation of intracellular survival mechanisms, particularly the ones downstream of the mitochondria that have been shown to be operative in diabetes. But the use of such a therapy would still require the availability of selective and non-toxic pharmacological agents with appropriate tissue diffusion and cellular penetrability.

CHAPTER 11: THE EFFECT OF LONG-TERM ADMINSTRATION OF THE MITOCHONDRIAL K_{ATP} CHANNEL OPENER NICORANDIL ON MYOCARDIAL PROTECTION OF BONE MARROW CELLS

11.1 Introduction

The mitochondria play an important role in ischaemia/reperfusion-induced injury of the mammalian heart[255, 256] and mitochondrial K_{ATP} channels are central to the protection against this damage[257-259]. Previously our laboratory and other investigators have demonstrated that the acute opening of mitochondrial K_{ATP} channels with diazoxide can elicit an identical protection to that of IP in the human myocardium[85] and in the other myocardium of animal species[257, 260, 261]. However, our group also has observed that the permanent opening of mitochondrial K_{ATP} channels with long-term administration of the anti-anginal agent nicorandil rather than to afford a lasting states of protection it renders the myocardium unresponsive to protection by IP or by pharmacological preconditioning with diazoxide[91]. Despite this, the loss in protection of the myocardium from subject on long-term treatment with nicorandil can be regained by activation of PKC and p38 MAPK[91], kinases that are located beyond the mitochondria in the cellular signalling pathway of IP[215].

Initially, it was speculated that BMCs differentiate into cardiac tissue restoring the lost myocardium. However, this thesis has been questioned and there is a growing belief that BMCs induce benefit by a paracrine mechanism via stimulation of resident

progenitor/stem cells and/or improvement in tissue survival[11, 200]. Indeed, if the benefit of BMCs is due, at least in part, to a greater protection against myocardial ischemic injury it is possible that such an effect may be influenced by clinical conditions (eg, diabetes, heart failure) or medical treatments (eg, mitochondrial K_{ATP} channel openers). Hence, the aims of this study were to investigate whether the long-term administration of the mitochondrial K_{ATP} channel opener nicorandil influences the protection elicited by BMCs and whether they can overcome the unresponsiveness of the myocardium to cardioprotection.

11.2 Methods

Experimental preparation

Experiments were performed on the right atrial myocardium and BMCs obtained from patients undergoing elective heart surgery prior to the initiation of cardiopulmonary bypass. The selection of patients, the bone marrow aspiration and separation, and also the experimental preparation were described in Chapter 2.

Assessment of tissue injury and cell death

CK release into the media during the 120min of reoxygenation and cell death by necrosis and apoptosis were determined as described in Chapter 2.

Study protocol:

For this study, the most effective lower dose of BMCs ($5x10^6$ cells/preparation) seen in Chapter 3 was used to co-culture with atrial myocardial slices during 90min ischaemia/120min reoxygenation. The aims were achieved by co-incubating the myocardium (n=6/group) from subjects on long-term treatment with nicorandil with

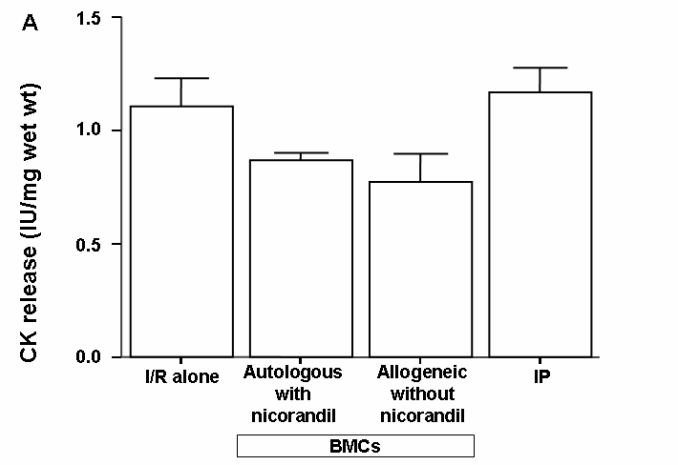
autologous BMCs and with allogeneic BMCs from subjects non-treated with nicorandil during the entire ischemic and reoxygenation periods and compared to the efficacy of IP. In addition, the myocardial slices from subjects non-treated with nicorandil were co-incubated with autologous BMCs and with allogeneic BMCs from subjects on long-term treatment with nicorandil during I/R.

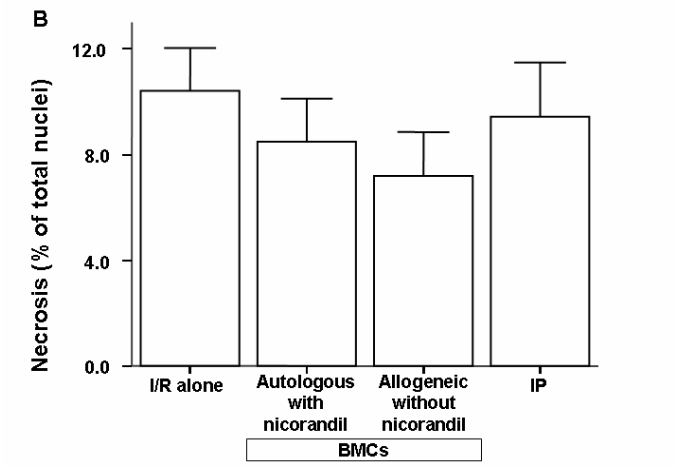
Statistical analyses

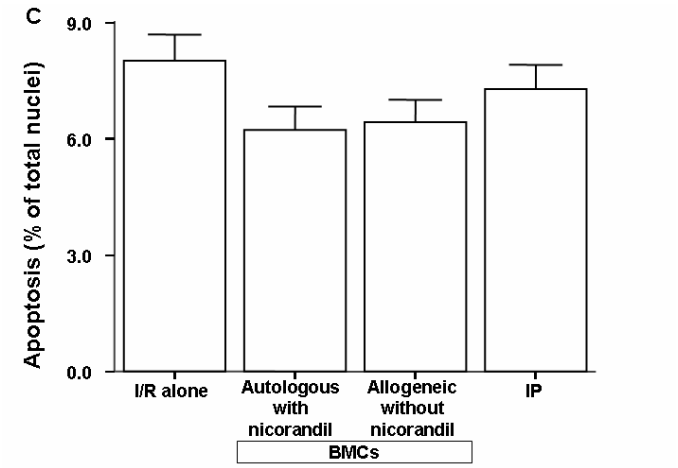
The results were expressed as mean ± SEM. One way ANOVA followed by Bonferroni's test was used as described in Chapter 2. Statistical significance is considered if $p<0.05$.

11.3 Results

Figures 16A-16C show that I/R alone induced substantial damage of the myocardium from subjects on long-term treatment with nicorandil as measured by CK release, cell necrosis and apoptosis. They also demonstrated that, as expected, this myocardium cannot be protected by IP. Furthermore, the incubation of myocardium from subjects on nicorandil with autologous BMCs and with allogeneic BMCs from subjects non-treated with nicorandil did not significantly reduce the mean values of any of the indexes of injury. However, Figures 16D-16F show that the CK release and cell necrosis and apoptosis induced by I/R of the myocardium from subjects non-treated with nicorandil were significantly and similarly reduced by autologous BMCs and allogeneic BMCs obtained from individuals on long-term treatment with nicorandil. These results suggest that the protective properties of BMCs are unaffected by nicorandil treatment.


A
CK release (IU/mg wet wt)
1.5
1.0
0.5
0.0
I/R alone
Autologous with nicorandil
Allogeneic without nicorandil
IP
BMCs


B
Necrosis (% of total nuclei)
12.0
8.0
4.0
0.0
I/R alone
Autologous with nicorandil
Allogeneic without nicorandil
IP
BMCs


C
Apoptosis (% of total nuclei)
9.0
6.0
3.0
0.0
I/R alone
Autologous with nicorandil
Allogeneic without nicorandil
IP
BMCs

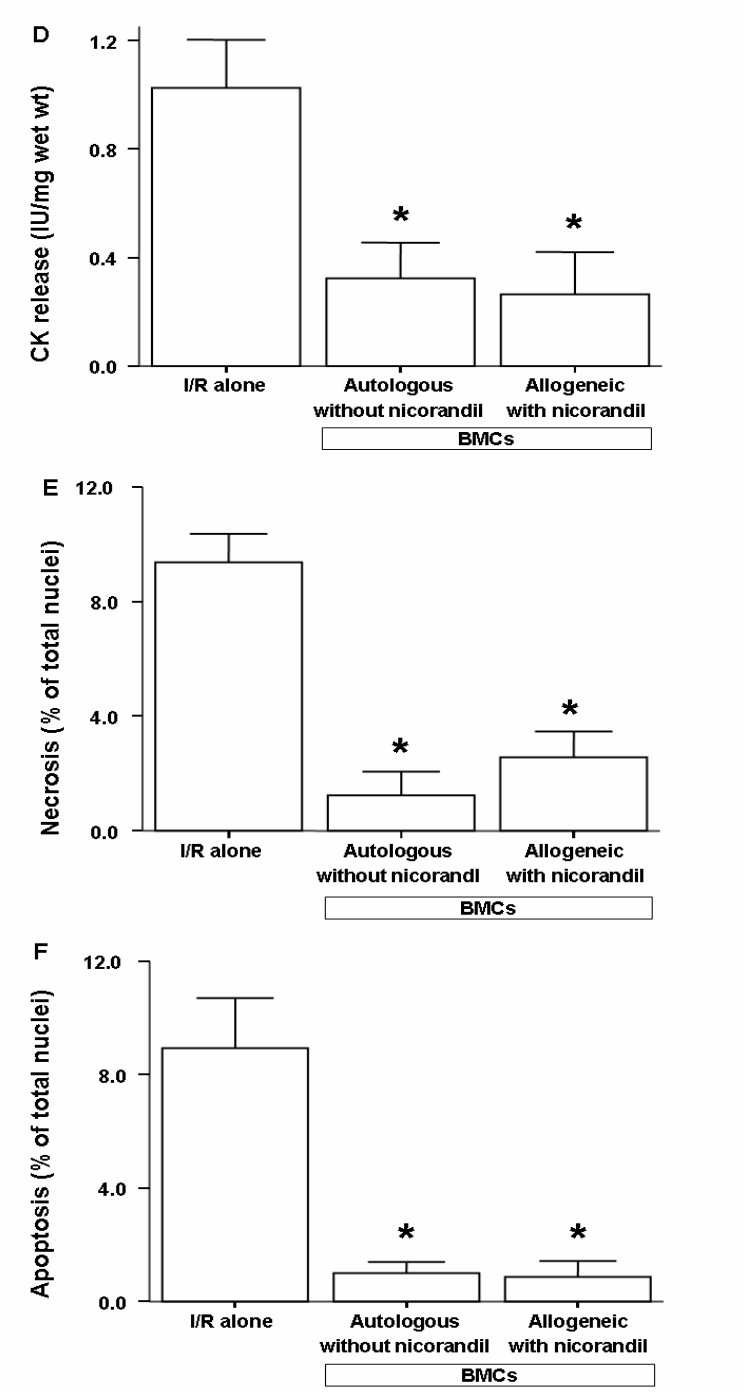


Figure 16: The effect of nicorandil on BMC-induced myocardial protection.

CK release (A), and cell necrosis (B) and apoptosis (C) in nicorandil myocardium co-cultured with autologous BMCs and allogeneic non-nicorandil BMCs; and CK release (D), cell necrosis (E) and apoptosis (F) in non-nicorandil myocardium co-cultured with autologous BMCs and allogeneic nicorandil BMCs, n=6. *$p<0.05$ versus I/R alone.

11.4 Discussion

The failure to protect the myocardium from subjects on long-term treatment with nicorandil is probably due to permanent opening of the mitochondrial K_{ATP} channels that as a result would prevent the activation of the downstream intracellular signalling. Previously, we have demonstrated that activation of PKC and p38 MAPK can elicit protection of a similar magnitude to that of ischemic and pharmacological preconditioning in the myocardium from subjects on long-term nicorandil treatment[91], this suggesting that the signalling pathway beyond the mitochondrial K_{ATP} channels remains unaffected. Therefore, the observation in the present studies that autologous and allogeneic BMCs cannot reverse the deficit in cardioprotection caused by the long-term administration of nicorandil supports the view that opening of mitochondrial K_{ATP} channels is necessary for the BMC-induced myocardial protection. This finding is of critical importance for the planning and patients' selection of clinical trials investigating the therapeutic potential of BMCs. It is also worth noting that whilst the long-term administration of nicorandil affects the innate protective mechanism of the myocardium, the capacity of BMCs to induce myocardial protection was unaffected. Certainly, this is another point of clinical relevance if allogeneic BMCs may be considered for therapeutic purpose.

In contrast with the results reported here, the IONA randomised trial has demonstrated a reduction of major coronary events in patients with stable angina[262]. The reason for these apparent opposite results is not clear but it is possible to speculate that the clinical benefit obtained by the long-term administration of the drug is exerted by mechanisms different from the activation of the cell signalling responsible for cardioprotection[263, 264]. Indeed, mechanisms such as vasodilation, upregulation of nitric oxide,

improvement of endothelial function and anti-inflammation could explain the discrepancy[262, 263]. It should be mentioned that, in addition to the effect on the mitoK_{ATP} channels, nicorandil acts as a nitric oxide donor and therefore it could be speculated that the effect of the long-term administration of this agent is due, at least in part, to a nitric oxide-derived action. However, previous findings from our laboratory (M Galiñanes, unreported results) have shown that the long-term administration of nitrates does not render the myocardium unresponsive to IP. Nonetheless, it is clear that further studies should be performed to fully elucidate the exact mechanism of the action, the therapeutic potential and the potential side effects of long-term administration of nicorandil.

11.5 Conclusions

The present study has shown for the first time that the opening of the mitochondrial K_{ATP} channels by long-term administration of nicorandil does not eliminate or reduce the cardioprotective potential of BMCs but they are unable to rescue the unresponsiveness of the myocardium from these subjects to protection. It is also important to note that the present findings relate to the protective properties of BMCs and that other potential beneficial actions of the cells may not be influenced in the same way by long-term therapy with nicorandil.

CHAPTER 12: RANDOMISED CONTROLLED TRIAL ON THE CARDIOPROTECTIVE EFFECT OF BONE MARROW CELLS IN PATIENTS UNDERGOING CORONARY BYPASS GRAFT SURGERY

12.1 Introduction

There has been considerable interest on the use of BMCs for myocardial repair following initial encouraging results from animal studies[265, 266]. However, the mechanisms of the beneficial effects of BMCs remain unclear; and clinical trials on its applications following acute[144-146, 267] and chronic myocardial ischaemia[137, 231, 268, 269] 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 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[13, 176, 200]. 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[270].

12.2 Methods

Study population

Patients, aged between 20 to 80 years, having 3 graftable diseased coronary vessels and a left ventricular EF > 40%, undergoing first-time elective coronary artery bypass grafting (CABG) by a single surgeon (Manuel Galiñanes) were considered for the study. Patients with unstable angina, cardiogenic shock (systolic blood pressure<80mmHg, 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, infection, previous neoplasm, chronic treatment with oral antibiotic agents or the K_{ATP} channel opener nicorandil were excluded from the study.

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 the UK. The clinical trial is also registered on the International Standard Radomised Control Number Register (ISRCTN 22639386).

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 hours after the release of aortic cross-clamp. The secondary end-point was an improvement in

cardiac function during the first 24 hours after aortic cross-clamp release. Information was also collected regarding any postoperative 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 150mg as premedication 2 hours 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 5-10μg/kg, midazolam 0.05-0.1mg/kg and rocuronium 1mg/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 No1 of 1:4 – 1000ml was given during the first dose, subsequently 500ml was given at 15-20min 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 12mmHg and pulmonary capillary wedge pressure between 12 and 16mmHg with appropriate transfusion), heart rate (between 70

and 90 beats/minute with atrial pacing if required) and systemic vascular resistance index (between 1200 and 1800 units using vasodilators such as GTN and vasoconstrictors as norepinephrine and vasopressin if required) within the physiological range. Patients were extubated and cared for postoperatively as per unit standard routine procedures until discharge.

BMC preparation and administration

After anaesthesia but before CABG, 120ml of bone marrow were aspirated from the patient's iliac crest and BMCs were separated by density centrifugation as described in Chapter 2. BMCs were then diluted in 30ml of cardioplegia solution and each 10ml of diluted BMCs was delivered at the end of each cardioplegia infusion antegradely 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 hours after the cross-clamp release for the determination of plasma levels of Troponin I and CK-MB as described in Chapter 2.

Assessments of cardiac function

Cardiac index was measured by thermodilution using a Swan-Ganz pulmonary artery as described in Chapter 2.

***In vitro* study**

To study the role of CPB on the cardioprotective effect of BMCs *in vitro,* the right atrial appendage from 12 control patients was collected either before or 10min after the

initiation of CPB (n=6/group) and then subjected to 90min ischaemia/120min reoxygenation. The preparation and experimental procedures were described in Chapter 2.

In addition, BMCs were co-incubated with myocardial slices (5 $x10^6$ cells/preparation) during I/R and the slices were allocated to different groups: controls, IP, BMCs, and IP+BMCs (BMCs after the induction of IP).

Myocardial injury assessed by CK release, and cell death by necrosis and apoptosis assessed by PI and TUNEL were determined as described in Chapter 2.

Statistics and expression of results

As this was a novel study, 22 subjects per group were required as described in chapter 2. Continuous variables that were normally distributed were presented as mean ± SD, differences between 2 groups were compared using independent t-tests. For non-parametric data, the Chi-Square test or the approximate non-parametric Mann-Whitney test was used.

12.3 Results

Baseline characteristics and operative and postoperative 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 x 10^6 BMCs were administered in the treated group, comprising of a mean of 24.9 ± 30.0 x 10^3 CD34/117 positive cells, 6.8 ± 9.4 x 10^6 CD45 positive cells, 78.4 ± 130.8 x 10^3 CD34 positive cells and 20.3 ± 22.3 x 10^3 CD133 positive cells. As shown on Table 1, the baseline characteristics and operative date were similar in both study groups. There was no

identifiable complication associated with the BMCs 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 (norepinerphine) for 48 hours. Another patient from the BMCs group also was re-intubated the first postoperative day a further 24 hours of mechanical ventilation due to poor gas exchange after initial successful extubation.

Cardiac enzymes

As shown in Figures 17A–17B, the plasma Troponin I and CK-MB level were equally raised after surgery and the elevations had a similar profile for the first postoperative 48 hours in the BMCs treated and control groups. Analysis of the AUC, also for the first 48 hours after surgery confirmed that the plasma levels for Troponin I and CK-MB in the BMCs treated group were not significantly different from those seen in the control group (Troponin I: 165.6 ± 153.2 vs 188.2 ± 213.8µg/L in control and CK-MB 639.6 ± 714.9 vs 567.1 ± 454.2µg/L in control; p=NS in both instances).

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 hours after surgery.

In vitro study

Figure 18A–18C show that, in contrast to the clinical *in vivo* results, BMCs significantly reduced CK release and the degree of cell necrosis and apoptosis as compared to 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 Figures 18D–18F, when muscles were harvested 10min 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 BMC- 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.

Table 1: Patients' characteristics and operative data

Characteristics	Control	BMCs	p value
Demographics:			
Age (years)	62.2 ± 11.2	64.4 ± 8.8	0.49
Male sex – (%)	76.2	76.2	1.00
Risk Factors:			
Hypertension – (%)	71.4	61.9	0.51
Hyperlipidemia – (%)	61.9	57.1	0.75
Smoking history – (%)	61.9	57.1	0.75
Family history of CAD– (%)	57.1	66.7	0.53
Pre-op medication:			
Aspirin – (%)	85.7	95.2	0.61
Clopidogrel – (%)	33.3	9.5	0.13
Beta-blockers – (%)	57.1	76.2	0.19
ACE inhibitors – (%)	52.4	23.8	0.11
Statins – (%)	81.0	90.5	0.66
Diuretics – (%)	19.0	19.0	1.00
Operative details:			
Bypass time (minutes)	108.5 ± 19.3	108.6 ± 14.5	0.99
Cross-clamp time (minutes)	62.3 ± 12.0	64.3 ± 11.6	0.57

Abbreviation: CAD- coronary artery disease

Table 2: Cardiac index before and after coronary bypass grafting

Intervention	Time after cross clamp release (hrs)							p value
	Baseline	1	2	4	8	12	24	
Control	2.51 ± 0.59	3.63 ± 0.54	3.54 ± 0.68	2.86 ± 0.74	3.26 ± 0.81	3.42 ± 0.73	3.08 ± 0.62	0.47
BMCs	2.31 ± 0.60	3.29 ± 0.86	3.24 ± 0.79	2.94 ± 0.87	3.26 ± 0.63	3.31 ± 0.56	3.11 ± 0.39	

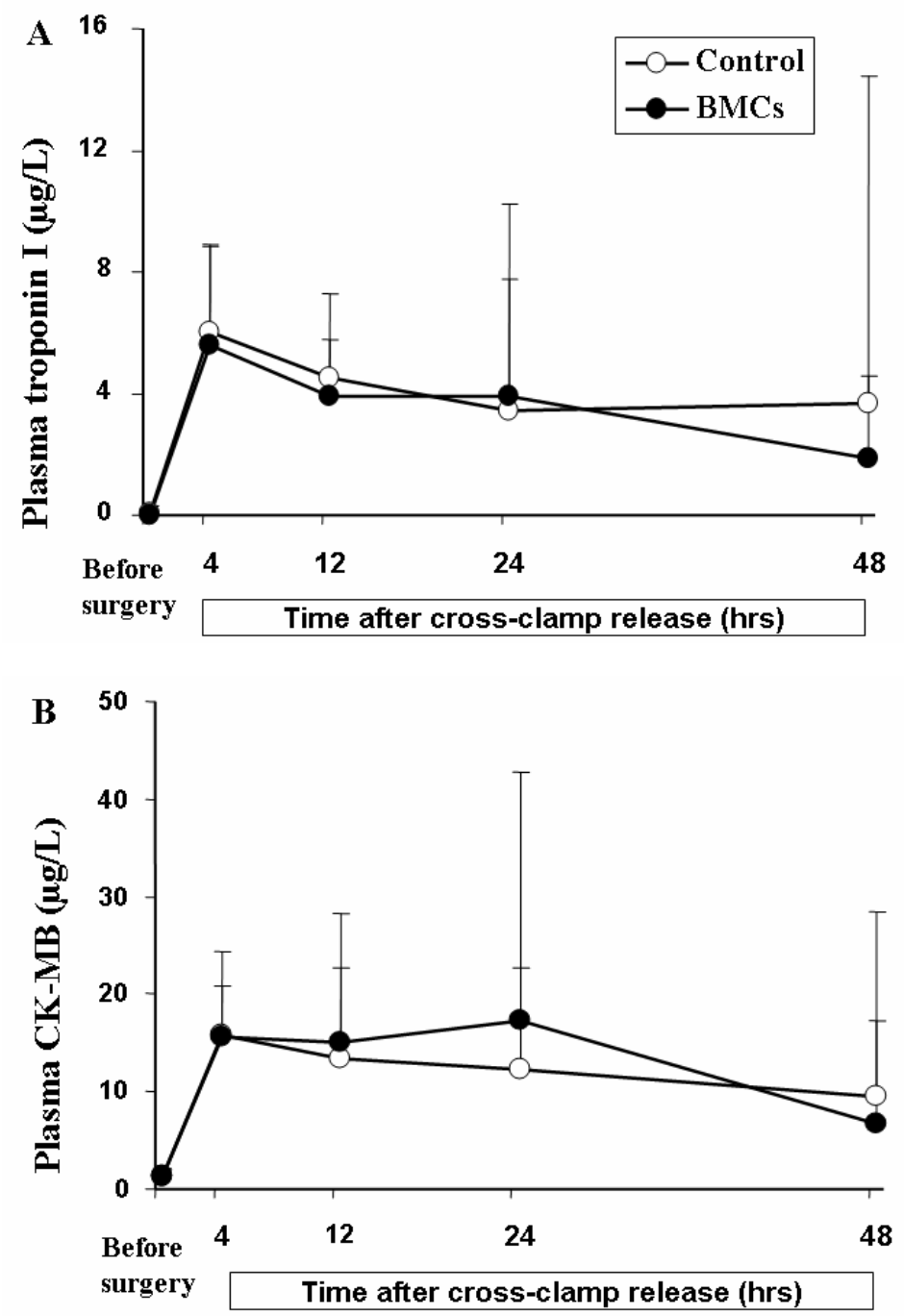
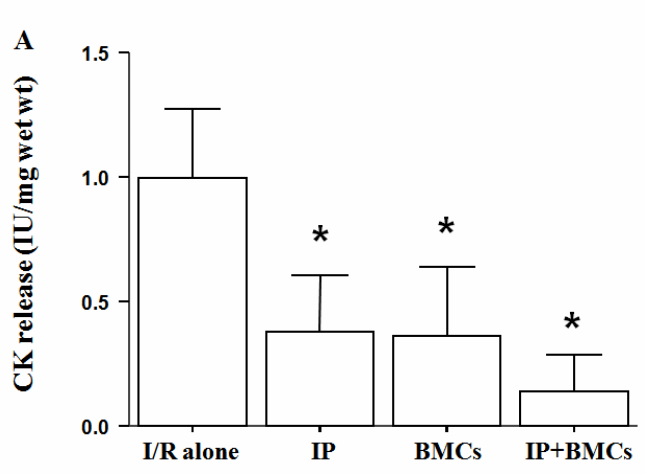
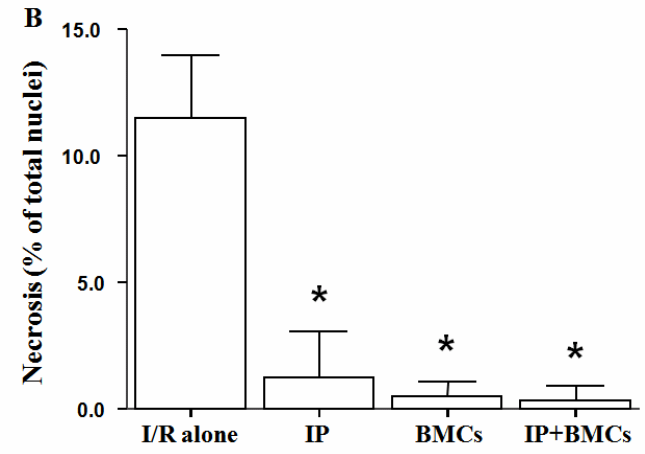
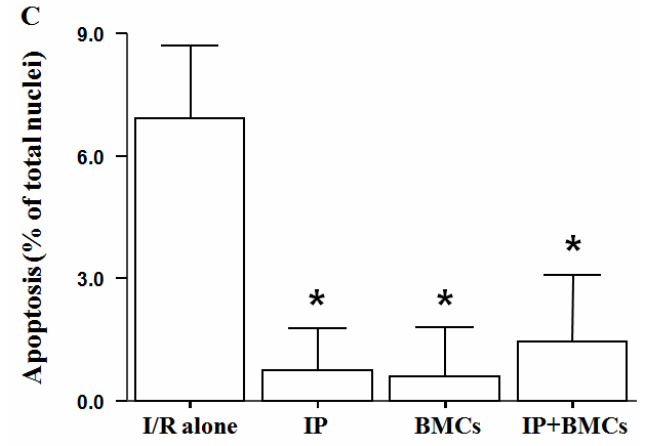


Figure 17: Cardiac enzymes before and during the first 48 hours following aortic cross-clamp release.

Plasma Troponin I (A) and CK-MB (B) levels in patients from the control and treated groups at baseline and during 48 hours after aortic cross-clamp release were not significantly different.


A
CK release (IU/mg wet wt)
1.5
1.0
0.5
0.0
*
*
*
I/R alone
IP
BMCs
IP+BMCs


B
Necrosis (% of total nuclei)
15.0
10.0
5.0
0.0
*
*
*
I/R alone
IP
BMCs
IP+BMCs


C
Apoptosis (% of total nuclei)
9.0
6.0
3.0
0.0
*
*
*
I/R alone
IP
BMCs
IP+BMCs

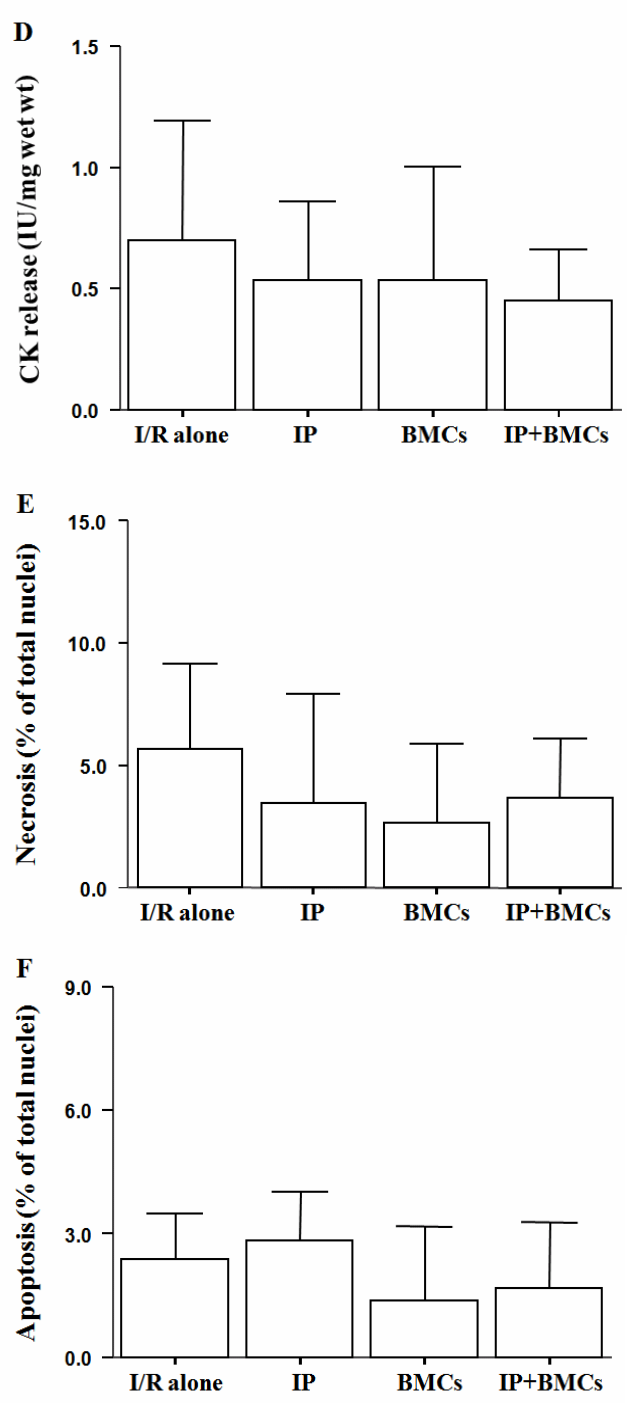


Figure 18: Protective effect of BMCs on myocardial slices obtained before and after cardiopulmonary bypass.

The effect of BMCs to reduce CK release, cell necrosis and apoptosis in myocardium obtained before (A,B,C) and 10min after (D,E,F) the initiation of cardiopulmonary bypass, n=6. *$p<0.05$ versus I/R alone.

12.4 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. By 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 10min 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, neovascularisation and other paracrine effects. Having previously demonstrated the cardioprotective effect of bone cells against ischaemic injury in an *in vitro* model[11], 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 setup 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 3 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[137, 144-146, 231, 267-269].

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 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 coronary artery bypass grafting without the use of CPB was absent in patient operated on CPB[271]. 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 cardioprotection when used alone and that their use in combination does not results in additional benefit support the thesis that both interventions share similar mechanism of action. This view is supported by previous study from our laboratory[11] demonstrating that the cardioprotection induced by BMCs can be abrogated by blocking the activation of the kinases PKC and p38 MAPK, that are also essential to elicit protection by IP[215]. While there are still work to be done to fully elucidate the cardioprotective mechanisms of BMCs & IP, our

findings are of great clinical relevance since to obtain maximal cardioprotection by 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 injected at different times ranging from 3 hours to 7 days after an acute myocardial infarction.

Limitations

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 end-points only up to 48 hours, the complexity and potential sources of inaccuracy in haemodynamic assessment[272] and the absence of additional evaluations of left ventricular and regional function may represent limitations of the study.

12.5 Conclusions

I and my colleagues 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* studies have 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.

CHAPTER 13: CONCLUSIONS

13.1 Conclusions

Controversy still remains whether the administration of BMCs to the heart can cause myocardial regeneration or preservation[139, 140, 273]. Thus, beside the putative capacity of BMCs to transdifferentiate, the work presented in this thesis has demonstrated that BMCs possess potent cardioprotection against ischaemia/reoxygenation-induced injury, similar to that of IP. This property may lead to the clinical exploitation of these cells to reduce the myocardial injury sustained during an acute ischaemic insult. The fact that maximal benefit can be obtained when cells are administrated either prior to or during ischaemia, or during reoxygenation, provides enormous potential and flexibility for an effective clinical application. However, it is necessary to note that certain manipulations, like cell fractionation using immunobeads, can abolish the BMC-induced myocardial protection, although others such as culturing or freezing do not.

The finding that allogeneic BMCs can afford identical myocardial protection to that of autologous BMCs also adds flexibility to their clinical application since they will be readily available when needed. The argument that allogeneic cells may cause immunoreaction could be overcome by the use of BMC-conditioned media. Furthermore, the identification of the secreted factor(s) and its (their) utilization, or the direct activation of the IGF-1R, shown to be the mediator of this cardioprotection in my studies, may simplify clinical application even more by making redundant the need for BMCs.

It should be pointed out that, because BMC-induced cardioprotection may share some elements of the intracellular signalling pathway with IP, it may be possible to argue that both interventions are similarly regulated and that, if so, it could explain why BMCs lost their protective benefit when applied after induction of IP. Of course, this may be the reason why BMCs used as an adjunct to cardioplegic solution during cardiac surgery could not be protective in the presence of cardiopulmonary bypass that *per se* induces preconditioning of the heart. These findings may allow to speculate that BMCs may also be subjected to therapeutic windows as IP does (eg, early or first window and delayed or second window). If this is the case, then it may have important clinical implications for choosing the most appropriate time of administration following an acute myocardial infarction. One is tempted to argue that this could be an explanation for the conflicting clinical results on the administration of BMCs following an acute myocardial infarction.

The finding of my thesis that the myocardial protection by BMCs is dose-dependent is also of clinical relevance and should be taken into account when planning new trials. According to the present results, it is possible that a suboptimal number of cells have been used in the majority of reported clinical studies adding to the existing controversy on the therapeutic efficacy of BMCs.

The findings that BMCs failed to induce protection of the diabetic myocardium or of the myocardium being treated with the K_{ATP} channel opener nicorandil is due to a defect in the myocardium but not to the cells may have therapeutic implication for the design of future clinical studies as a large part of the population suffering from coronary artery disease have diabetes and nicorandil is a common anti-anginal agent. The results may

also help to explain some of the negative results from clinical trials and are particularly meaningful if cardioprotection is the dominating mechanism in cardiac repair rather than regeneration. It was also important that although autologous BMCs could not protect the failing heart, allogeneic BMCs were able to partly induce protection of this poorly contractile myocardium, suggesting that the BMCs from subjects with failing heart do not release some of the factors essential for cardioprotection. Certainly, it would be worthwhile to elucidate the factors responsible for cardioprotection so that their therapeutic use could eliminate the complexity of using BMCs.

13.2 Limitations

A limitation of my *in vitro* studies is the use of atrial tissue, as it may not fully represent the response of ventricular myocardium. Nonetheless, recent studies in our laboratory (unpublished data) have shown that the degree of injury induced by I/R of the human atrial myocardium is similar to the one observed in muscles from the left ventricle of mice and rats suggesting that the response of the atrial and ventricular myocardium to I/R is comparable. In addition, we[274] and other investigators[275] have shown that preconditioning induces similar degree of protection of the atrial and ventricular myocardium. Another potential limitation may be that most of my studies were performed in an *in vitro* preparation that is devoid of blood components. However, the latter shortcoming could also be advantageous to examine the direct relationship between the intervention and the myocardium, removing the potential confounding effects of blood and vasculature. Despite the above considerations, caution must be taken when extrapolating the present results to clinical conditions.

13.3. Future directions

In defining the future directions that could follow the studies reported in my thesis, I believe that the most urgent and burning issue to be investigated is the identification of secreted factors by the BMCs responsible for the observed potent cardioprotection. It is clear that a full definition of these factors would simplify the clinical applicability by avoiding the aspiration, preparation and administration of BMCs. The use of these factors would also mean that larger proportion of the population could receive the treatment to reduce the lost of muscle after an acute ischaemic insult with important societal and economic implications.

In my view, the elucidation of cell type(s) producing the cardioprotective factors should also be performed. My failure to show whether $CD133^+$ cells may play a role was probably due to the use of magnetic beads for their separation, a technique that may have an effect on the function of cells since both $CD133^+$ cells and the remaining BMCs fraction were unable to elicit the protective effect. Therefore, it would be necessary to use other alternative cell separation methods when investigating which cell type(s) is the producer of the protective factors.

A full clarification of the cell signalling pathway mediating the cardioprotective response should also be investigated. This knowledge could allow further simplification of the therapeutic approach used and also make possible to bypass the defective part of the signalling cascade present in clinical conditions such as diabetes and heart failure.

While there is a growing belief that BMCs do not differentiate into heart tissue it still remains unclear whether these cells have the potential to induce proliferation of resident

progenitor cells. In my thesis I did not investigate this issue but it may be possible to speculate that if such an effect exists it may be induced by the same factor(s) causing cardioprotection. Indeed, a full elucidation of these properties will be required to refine and advance the therapeutic potential of BMCs or their products.

BIBLIOGRAPHY

[1] Mackay J, Mensah GA. The Atlas of Heart Disease and Stroke. The World Health Organization 2006:48-49.

[2] Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation 1986;74:1124-1136.

[3] Quaini F, Urbanek K, Beltrami AP, Finato N, Beltrami CA, Nadal-Ginard B, *et al.* Chimerism of the transplanted heart. N Engl J Med 2002;346:5-15.

[4] Grove JE, Bruscia E, Krause DS. Plasticity of bone marrow-derived stem cells. Stem Cells 2004;22:487-500.

[5] Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, *et al.* Bone marrow cells regenerate infarcted myocardium. Nature 2001;410:701-705.

[6] Vieyra DS, Jackson KA, Goodell MA. Plasticity and tissue regenerative potential of bone marrow-derived cells. Stem Cell Rev 2005;1:65-69.

[7] Murry CE, Soonpaa MH, Reinecke H, Nakajima H, Nakajima HO, Rubart M, *et al.* Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. Nature 2004;428:664-668.

[8] Balsam LB, Wagers AJ, Christensen JL, Kofidis T, Weissman IL, Robbins RC. Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. Nature 2004;428:668-673.

[9] Kucia M, Ratajczak J, Ratajczak MZ. Are bone marrow stem cells plastic or heterogenous--that is the question. Exp Hematol 2005;33:613-623.

[10] Wagers AJ, Sherwood RI, Christensen JL, Weissman IL. Little evidence for developmental plasticity of adult hematopoietic stem cells. Science 2002;297:2256-2259.

[11] Kubal C, Sheth K, Nadal-Ginard B, Galiñanes M. Bone marrow cells have a potent anti-ischemic effect against myocardial cell death in humans. J Thorac Cardiovasc Surg 2006;132:1112-1118.

[12] Lehrke S, Mazhari R, Durand DJ, Zheng M, Bedja D, Zimmet JM, *et al.* Aging impairs the beneficial effect of granulocyte colony-stimulating factor and stem cell factor on post-myocardial infarction remodeling. Circ Res 2006;99:553-560.

[13] Uemura R, Xu M, Ahmad N, Ashraf M. Bone marrow stem cells prevent left ventricular remodeling of ischemic heart through paracrine signaling. Circ Res 2006;98:1414-1421.

[14] Sperelakis N. Defining ischaemia. Bmj 1995;311:890-891.

[15] Yan GX, Kleber AG. Changes in extracellular and intracellular pH in ischemic rabbit papillary muscle. Circ Res 1992;71:460-470.

[16] Anversa P, Kajstura J. Myocyte cell death in the diseased heart. Circ Res 1998;82:1231-1233.

[17] Buja LM, Hagler HK, Willerson JT. Altered calcium homeostasis in the pathogenesis of myocardial ischemic and hypoxic injury. Cell Calcium 1988;9:205-217.

[18] Thandroyen FT, Bellotto D, Katayama A, Hagler HK, Willerson JT, Buja LM. Subcellular electrolyte alterations during progressive hypoxia and following reoxygenation in isolated neonatal rat ventricular myocytes. Circ Res 1992;71:106-119.

[19] Buja LM. Myocardial ischemia and reperfusion injury. Cardiovasc Pathol 2005;14:170-175.

[20] Murphy E, Steenbergen C. Mechanisms underlying acute protection from cardiac ischemia-reperfusion injury. Physiol Rev 2008;88:581-609.

[21] Gross GJ, Auchampach JA. Reperfusion injury: does it exist? Journal of Molecular & Cellular Cardiology 2007;42:12-18.

[22] Zweier JL, Flaherty JT, Weisfeldt ML. Direct measurement of free radical generation following reperfusion of ischemic myocardium. Proc Natl Acad Sci U S A 1987;84:1404-1407.

[23] Zweier JL. Measurement of superoxide-derived free radicals in the reperfused heart. Evidence for a free radical mechanism of reperfusion injury. J Biol Chem 1988;263:1353-1357.

[24] Kevin LG, Camara AK, Riess ML, Novalija E, Stowe DF. Ischemic preconditioning alters real-time measure of O2 radicals in intact hearts with ischemia and reperfusion. Am J Physiol Heart Circ Physiol 2003;284:H566-574.

[25] Vanden Hoek TL, Shao Z, Li C, Zak R, Schumacker PT, Becker LB. Reperfusion injury on cardiac myocytes after simulated ischemia. Am J Physiol 1996;270:H1334-1341.

[26] Frangogiannis NG, Smith CW, Entman ML. The inflammatory response in myocardial infarction. Cardiovasc Res 2002;53:31-47.

[27] Kim JS, Jin Y, Lemasters JJ. Reactive oxygen species, but not Ca2+ overloading, trigger pH- and mitochondrial permeability transition-dependent death of adult rat myocytes after ischemia-reperfusion. Am J Physiol Heart Circ Physiol 2006;290:H2024-2034.

[28] Yellon DM, Hausenloy DJ. Myocardial reperfusion injury. N Engl J Med 2007;357:1121-1135.

[29] Bolli R, Marban E. Molecular and cellular mechanisms of myocardial stunning. Physiol Rev 1999;79:609-634.

[30] Bolli R. Causative role of oxyradicals in myocardial stunning: a proven hypothesis. A brief review of the evidence demonstrating a major role of reactive

oxygen species in several forms of postischemic dysfunction. Basic Res Cardiol 1998;93:156-162.

[31] Murohara Y, Yui Y, Hattori R, Kawai C. Effects of superoxide dismutase on reperfusion arrhythmias and left ventricular function in patients undergoing thrombolysis for anterior wall acute myocardial infarction. Am J Cardiol 1991;67:765-767.

[32] Ooiwa H, Stanley A, Felaneous-Bylund AC, Wilborn W, Downey JM. Superoxide dismutase conjugated to polyethylene glycol fails to limit myocardial infarct size after 30 min ischemia followed by 72 h of reperfusion in the rabbit. J Mol Cell Cardiol 1991;23:119-125.

[33] Vanhaecke J, Van de Werf F, Ronaszeki A, Flameng W, Lesaffre E, De Geest H. Effect of superoxide dismutase on infarct size and postischemic recovery of myocardial contractility and metabolism in dogs. J Am Coll Cardiol 1991;18:224-230.

[34] Chi LG, Tamura Y, Hoff PT, Macha M, Gallagher KP, Schork MA, *et al.* Effect of superoxide dismutase on myocardial infarct size in the canine heart after 6 hours of regional ischemia and reperfusion: a demonstration of myocardial salvage. Circ Res 1989;64:665-675.

[35] Kilgore KS, Friedrichs GS, Johnson CR, Schasteen CS, Riley DP, Weiss RH, *et al.* Protective effects of the SOD-mimetic SC-52608 against ischemia/reperfusion damage in the rabbit isolated heart. J Mol Cell Cardiol 1994;26:995-1006.

[36] Murphy E, Perlman M, London RE, Steenbergen C. Amiloride delays the ischemia-induced rise in cytosolic free calcium. Circulation research 1991;68:1250-1258.

[37] Imahashi K, Pott C, Goldhaber JI, Steenbergen C, Philipson KD, Murphy E. Cardiac-specific ablation of the Na+-Ca2+ exchanger confers protection against ischemia/reperfusion injury. Circulation research 2005;97:916-921.

[38] Grover GJ, Atwal KS, Sleph PG, Wang FL, Monshizadegan H, Monticello T, *et al.* Excessive ATP hydrolysis in ischemic myocardium by mitochondrial F1F0-ATPase: effect of selective pharmacological inhibition of mitochondrial ATPase hydrolase activity. American journal of physiology Heart and circulatory physiology 2004;287:H1747-1755.

[39] du Toit EF, Opie LH. Inhibitors of Ca2+ ATPase pump of sarcoplasmic reticulum attenuate reperfusion stunning in isolated rat heart. J Cardiovasc Pharmacol 1994;24:678-684.

[40] Piper HM, Meuter K, Schafer C. Cellular mechanisms of ischemia-reperfusion injury. Ann Thorac Surg 2003;75:S644-648.

[41] Piper HM, Kasseckert S, Abdallah Y. The sarcoplasmic reticulum as the primary target of reperfusion protection. Cardiovasc Res 2006;70:170-173.

[42] Zucchi R, Ronca F, Ronca-Testoni S. Modulation of sarcoplasmic reticulum function: a new strategy in cardioprotection? Pharmacol Ther 2001;89:47-65.

[43] Murphy E, Cross H, Steenbergen C. Sodium regulation during ischemia versus reperfusion and its role in injury. Circ Res 1999;84:1469-1470.

[44] Theroux P, Chaitman BR, Danchin N, Erhardt L, Meinertz T, Schroeder JS, *et al.* Inhibition of the sodium-hydrogen exchanger with cariporide to prevent myocardial infarction in high-risk ischemic situations. Main results of the GUARDIAN trial. Guard during ischemia against necrosis (GUARDIAN) Investigators. Circulation 2000;102:3032-3038.

[45] Zeymer U, Suryapranata H, Monassier JP, Opolski G, Davies J, Rasmanis G, *et al.* The Na(+)/H(+) exchange inhibitor eniporide as an adjunct to early reperfusion therapy for acute myocardial infarction. Results of the evaluation of the safety and cardioprotective effects of eniporide in acute myocardial infarction (ESCAMI) trial. J Am Coll Cardiol 2001;38:1644-1650.

[46] Litt MR, Jeremy RW, Weisman HF, Winkelstein JA, Becker LC. Neutrophil depletion limited to reperfusion reduces myocardial infarct size after 90 minutes of ischemia. Evidence for neutrophil-mediated reperfusion injury. Circulation 1989;80:1816-1827.

[47] Jordan JE, Zhao ZQ, Vinten-Johansen J. The role of neutrophils in myocardial ischemia-reperfusion injury. Cardiovasc Res 1999;43:860-878.

[48] Vinten-Johansen J. Involvement of neutrophils in the pathogenesis of lethal myocardial reperfusion injury. Cardiovasc Res 2004;61:481-497.

[49] Eguchi Y, Shimizu S, Tsujimoto Y. Intracellular ATP levels determine cell death fate by apoptosis or necrosis. Cancer Res 1997;57:1835-1840.

[50] Zong WX, Thompson CB. Necrotic death as a cell fate. Genes Dev 2006;20:1-15.

[51] Fadeel B, Orrenius S. Apoptosis: a basic biological phenomenon with wide-ranging implications in human disease. J Intern Med 2005;258:479-517.

[52] Bialik S, Cryns VL, Drincic A, Miyata S, Wollowick AL, Srinivasan A, *et al.* The mitochondrial apoptotic pathway is activated by serum and glucose deprivation in cardiac myocytes. Circ Res 1999;85:403-414.

[53] Mocanu MM, Baxter GF, Yellon DM. Caspase inhibition and limitation of myocardial infarct size: protection against lethal reperfusion injury. Br J Pharmacol 2000;130:197-200.

[54] Yaoita H, Ogawa K, Maehara K, Maruyama Y. Attenuation of ischemia/reperfusion injury in rats by a caspase inhibitor. Circulation 1998;97:276-281.

[55] Chandrashekhar Y, Sen S, Anway R, Shuros A, Anand I. Long-term caspase inhibition ameliorates apoptosis, reduces myocardial troponin-I cleavage, protects left ventricular function, and attenuates remodeling in rats with myocardial infarction. J Am Coll Cardiol 2004;43:295-301.

[56] Vandenabeele P, Vanden Berghe T, Festjens N. Caspase inhibitors promote alternative cell death pathways. Sci STKE 2006;2006:pe44.

[57] Yu L, Wan F, Dutta S, Welsh S, Liu Z, Freundt E, *et al.* Autophagic programmed cell death by selective catalase degradation. Proc Natl Acad Sci U S A 2006;103:4952-4957.

[58] Gottlieb R. ICE-ing the heart. Circ Res 2005;96:1036-1038.

[59] Czerski L, Nunez G. Apoptosome formation and caspase activation: is it different in the heart? J Mol Cell Cardiol 2004;37:643-652.

[60] Shaw J, Kirshenbaum LA. Molecular regulation of autophagy and apoptosis during ischemic and non-ischemic cardiomyopathy. Autophagy 2008;4:427-434.

[61] Rami A, Kogel D. Apoptosis meets autophagy-like cell death in the ischemic penumbra: Two sides of the same coin? Autophagy 2008;4:422-426.

[62] Dosenko VE, Nagibin VS, Tumanovska LV, Moibenko AA. Protective effect of autophagy in anoxia-reoxygenation of isolated cardiomyocyte? Autophagy 2006;2:305-306.

[63] Hamacher-Brady A, Brady NR, Gottlieb RA. Enhancing macroautophagy protects against ischemia/reperfusion injury in cardiac myocytes. J Biol Chem 2006;281:29776-29787.

[64] Decker RS, Poole AR, Crie JS, Dingle JT, Wildenthal K. Lysosomal alterations in hypoxic and reoxygenated hearts. II. Immunohistochemical and biochemical changes in cathepsin D. Am J Pathol 1980;98:445-456.

[65] Gustafsson AB, Gottlieb RA. Eat your heart out: Role of autophagy in myocardial ischemia/reperfusion. Autophagy 2008;4:416-421.

[66] Kloner RA, Bolli R, Marban E, Reinlib L, Braunwald E. Medical and cellular implications of stunning, hibernation, and preconditioning: an NHLBI workshop. Circulation 1998;97:1848-1867.

[67] Burckhartt B, Yang XM, Tsuchida A, Mullane KM, Downey JM, Cohen MV. Acadesine extends the window of protection afforded by ischaemic preconditioning in conscious rabbits. Cardiovasc Res 1995;29:653-657.

[68] Sack S, Mohri M, Arras M, Schwarz ER, Schaper W. Ischaemic preconditioning--time course of renewal in the pig. Cardiovasc Res 1993;27:551-555.

[69] Yellon DM, Downey JM. Preconditioning the myocardium: from cellular physiology to clinical cardiology. Physiol Rev 2003;83:1113-1151.

[70] Murry CE, Richard VJ, Reimer KA, Jennings RB. Ischemic preconditioning slows energy metabolism and delays ultrastructural damage during a sustained ischemic episode. Circ Res 1990;66:913-931.

[71] Kolocassides KG, Seymour AM, Galinanes M, Hearse DJ. Paradoxical effect of ischemic preconditioning on ischemic contracture? NMR studies of energy metabolism and intracellular pH in the rat heart. J Mol Cell Cardiol 1996;28:1045-1057.

[72] Baines CP. The mitochondrial permeability transition pore and ischemia-reperfusion injury. Basic Res Cardiol 2009;104:181-188.

[73] Zhao ZQ, Corvera JS, Halkos ME, Kerendi F, Wang NP, Guyton RA, *et al.* Inhibition of myocardial injury by ischemic postconditioning during reperfusion:

comparison with ischemic preconditioning. Am J Physiol Heart Circ Physiol 2003;285:H579-588.

[74] Yang XM, Proctor JB, Cui L, Krieg T, Downey JM, Cohen MV. Multiple, brief coronary occlusions during early reperfusion protect rabbit hearts by targeting cell signaling pathways. J Am Coll Cardiol 2004;44:1103-1110.

[75] Tang XL, Sato H, Tiwari S, Dawn B, Bi Q, Li Q, *et al.* Cardioprotection by postconditioning in conscious rats is limited to coronary occlusions <45 min. Am J Physiol Heart Circ Physiol 2006;291:H2308-2317.

[76] Hausenloy D, Yellon D. Reperfusion injury salvage kinase signalling: taking a RISK for cardioprotection. Heart Fail Rev 2007;12:217-234.

[77] Hausenloy D, Yellon D. Preconditioning and postconditioning: united at reperfusion. Pharmacol Ther 2007;116:173-191.

[78] Heusch G. Postconditioning: old wine in a new bottle? J Am Coll Cardiol 2004;44:1111-1112.

[79] Liem DA, Honda HM, Zhang J, Woo D, Ping P. Past and present course of cardioprotection against ischemia-reperfusion injury. J Appl Physiol 2007;103:2129-2136.

[80] Ravingerová T, Neckár J, Kolár F. Ischemic tolerance of rat hearts in acute and chronic phases of experimental diabetes. Mol Cell Biochem 2003;249:167-174.

[81] Tatsumi T, Matoba S, Kobara M, al e. Energy metabolism after ischemic preconditioning in streptozotocin-induced diabetic rat hearts. J Am Coll Cardiol 1998;31:707-715.

[82] Hausenloy DJ, Yellon DM. Preconditioning and postconditioning: new strategies for cardioprotection. Diabetes Obes Metab 2008;10:451-459.

[83] del Valle H, Lascano E, Negroni J, Crottogini A. Absence of ischemic preconditioning protection in diabetic sheep hearts: Role of sarcolemmal KATP channel dysfunction. Mol Cell Biochem 2003;249:21-30.

[84] Cleveland JC, Jr., Meldrum DR, Cain BS, Banerjee A, Harken AH. Oral sulfonylurea hypoglycemic agents prevent ischemic preconditioning in human myocardium. Two paradoxes revisited. Circulation 1997;96:29-32.

[85] Hassouna A, Loubani M, Matata B, Fowler A, Standen N, Galiñanes M. Mitochondrial dysfunction as the cause of the failure to precondition the diabetic human myocardium. Cardiovasc Res 2006;69:450-458.

[86] Smith J, Wahler G. ATP-sensitive potassium channels are altered in ventricular myocytes from diabetic rats. Mol Cell Biochem 1996;158:43-51.

[87] Tsang A, Hausenloy D, Mocanu M, Carr R, Yellon D. Preconditioning the diabetic heart: the importance of Akt phosphorylation. Diabetes 2005;54:2360-2364.

[88] Pomerantz BJ, Robinson TN, Morrell TD, Heimbach JK, Banerjee A, Harken AH. Selective mitochondrial adenosine triphosphate-sensitive potassium channel activation is sufficient to precondition human myocardium. J Thorac Cardiovasc Surg 2000;120:387-392.

[89] Ishii H, Ichimiya S, Kanashiro M, Amano T, Imai K, Murohara T, *et al.* Impact of a single intravenous administration of nicorandil before reperfusion in patients with ST-segment-elevation myocardial infarction. Circulation 2005;112:1284-1288.

[90] Matsuo H, Watanabe S, Segawa T, Yasuda S, Hirose T, Iwama M, *et al.* Evidence of pharmacologic preconditioning during PTCA by intravenous pretreatment with ATP-sensitive K+ channel opener nicorandil. Eur Heart J 2003;24:1296-1303.

[91] Loubani M, Galiñanes M. Long-term administration of nicorandil abolishes ischemic and pharmacologic preconditioning of the human myocardium: role of

mitochondrial adenosine triphosphate-dependent potassium channels. J Thorac Cardiovasc Surg 2002;124:750-757.

[92] Ide T, Tsutsui H, Kinugawa S, Suematsu N, Hayashidani S, Ichikawa K, *et al*. Direct evidence for increased hydroxyl radicals originating from superoxide in the failing myocardium. Circ Res 2000;86:152-157.

[93] Ide T, Tsutsui H, Kinugawa S, Utsumi H, Kang D, Hattori N, *et al*. Mitochondrial electron transport complex I is a potential source of oxygen free radicals in the failing myocardium. Circ Res 1999;85:357-363.

[94] Ghosh S, Standen N, Galiñanes M. Failure to precondition pathological human myocardium. J Am Coll Cardiol 2001;37:711-718.

[95] Lakshmipathy U, Verfaillie C. Stem cell plasticity. Blood Rev 2005;19:29-38.

[96] Beltrami AP, Urbanek K, Kajstura J, Yan SM, Finato N, Bussani R, *et al*. Evidence that human cardiac myocytes divide after myocardial infarction. N Engl J Med 2001;344:1750-1757.

[97] Beltrami AP, Barlucchi L, Torella D, Baker M, Limana F, Chimenti S, *et al*. Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell 2003;114:763-776.

[98] Cimini M, Fazel S, Zhuo S, Xaymardan M, Fujii H, Weisel RD, *et al.* c-kit dysfunction impairs myocardial healing after infarction. Circulation 2007;116:I77-82.

[99] Oh H, Bradfute SB, Gallardo TD, Nakamura T, Gaussin V, Mishina Y, *et al.* Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. Proc Natl Acad Sci U S A 2003;100:12313-12318.

[100] Matsuura K, Nagai T, Nishigaki N, Oyama T, Nishi J, Wada H, *et al.* Adult cardiac Sca-1-positive cells differentiate into beating cardiomyocytes. J Biol Chem 2004;279:11384-11391.

[101] Laugwitz KL, Moretti A, Lam J, Gruber P, Chen Y, Woodard S, *et al.* Postnatal isl1+ cardioblasts enter fully differentiated cardiomyocyte lineages. Nature 2005;433:647-653.

[102] Moretti A, Caron L, Nakano A, Lam JT, Bernshausen A, Chen Y, *et al.* Multipotent embryonic isl1+ progenitor cells lead to cardiac, smooth muscle, and endothelial cell diversification. Cell 2006;127:1151-1165.

[103] Asakura A, Rudnicki MA. Side population cells from diverse adult tissues are capable of in vitro hematopoietic differentiation. Exp Hematol 2002;30:1339-1345.

[104] Asakura A, Seale P, Girgis-Gabardo A, Rudnicki MA. Myogenic specification of side population cells in skeletal muscle. J Cell Biol 2002;159:123-134.

[105] Guan K, Hasenfuss G. Do stem cells in the heart truly differentiate into cardiomyocytes? J Mol Cell Cardiol 2007;43:377-387.

[106] Martin CM, Meeson AP, Robertson SM, Hawke TJ, Richardson JA, Bates S, *et al.* Persistent expression of the ATP-binding cassette transporter, Abcg2, identifies cardiac SP cells in the developing and adult heart. Dev Biol 2004;265:262-275.

[107] Yoon J, Choi SC, Park CY, Shim WJ, Lim DS. Cardiac side population cells exhibit endothelial differentiation potential. Exp Mol Med 2007;39:653-662.

[108] Oyama T, Nagai T, Wada H, Naito AT, Matsuura K, Iwanaga K, *et al.* Cardiac side population cells have a potential to migrate and differentiate into cardiomyocytes in vitro and in vivo. Journal of Cell Biology 2007;176:329-341.

[109] Yamahara K, Fukushima S, Coppen SR, Felkin LE, Varela-Carver A, Barton PJ, *et al.* Heterogeneic nature of adult cardiac side population cells. Biochem Biophys Res Commun 2008;371:615-620.

[110] Pfister O, Mouquet F, Jain M, Summer R, Helmes M, Fine A, *et al.* CD31- but Not CD31+ cardiac side population cells exhibit functional cardiomyogenic differentiation. Circulation Research 2005;97:52-61.

[111] Iwami Y, Masuda H, Asahara T. Endothelial progenitor cells: past, state of the art, and future. J Cell Mol Med 2004;8:488-497.

[112] Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med 1996;183:1797-1806.

[113] Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, *et al.* Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 2002;418:41-49.

[114] Badorff C, Brandes RP, Popp R, Rupp S, Urbich C, Aicher A, *et al.* Transdifferentiation of blood-derived human adult endothelial progenitor cells into functionally active cardiomyocytes. Circulation 2003;107:1024-1032.

[115] Jackson KA, Majka SM, Wang H, Pocius J, Hartley CJ, Majesky MW, *et al.* Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. J Clin Invest 2001;107:1395-1402.

[116] Yoon YS, Park JS, Tkebuchava T, Luedeman C, Losordo DW. Unexpected severe calcification after transplantation of bone marrow cells in acute myocardial infarction. Circulation 2004;109:3154-3157.

[117] Breitbach M, Bostani T, Roell W, Xia Y, Dewald O, Nygren JM, *et al.* Potential risks of bone marrow cell transplantation into infarcted hearts. Blood 2007;110:1362-1369.

[118] Orlic D, Kajstura J, Chimenti S, Limana F, Jakoniuk I, Quaini F, *et al*. Mobilized bone marrow cells repair the infarcted heart, improving function and survival. Proc Natl Acad Sci U S A 2001;98:10344-10349.

[119] Fazel S, Cimini M, Chen L, Li S, Angoulvant D, Fedak P, *et al*. Cardioprotective c-kit+ cells are from the bone marrow and regulate the myocardial balance of angiogenic cytokines. J Clin Invest 2006;116:1865-1877.

[120] Makino S, Fukuda K, Miyoshi S, Konishi F, Kodama H, Pan J, *et al*. Cardiomyocytes can be generated from marrow stromal cells in vitro. J Clin Invest 1999;103:697-705.

[121] Toma C, Pittenger MF, Cahill KS, Byrne BJ, Kessler PD. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. Circulation 2002;105:93-98.

[122] Amado LC, Saliaris AP, Schuleri KH, St John M, Xie JS, Cattaneo S, *et al*. Cardiac repair with intramyocardial injection of allogeneic mesenchymal stem cells after myocardial infarction. Proc Natl Acad Sci U S A 2005;102:11474-11479.

[123] Martens TP, See F, Schuster MD, Sondermeijer HP, Hefti MM, Zannettino A, *et al.* Mesenchymal lineage precursor cells induce vascular network formation in ischemic myocardium. Nat Clin Pract Cardiovasc Med 2006;3 Suppl 1:S18-22.

[124] Wang JS, Shum-Tim D, Galipeau J, Chedrawy E, Eliopoulos N, Chiu RC. Marrow stromal cells for cellular cardiomyoplasty: feasibility and potential clinical advantages. J Thorac Cardiovasc Surg 2000;120:999-1005.

[125] Shake JG, Gruber PJ, Baumgartner WA, Senechal G, Meyers J, Redmond JM, *et al.* Mesenchymal stem cell implantation in a swine myocardial infarct model: engraftment and functional effects. Ann Thorac Surg 2002;73:1919-1925; discussion 1926.

[126] Ang KL, Shenje LT, Srinivasan L, Galinanes M. Repair of the damaged heart by bone marrow cells: from experimental evidence to clinical hope. Ann Thorac Surg 2006;82:1549-1558.

[127] Takahashi T, Kalka C, Masuda H, Chen D, Silver M, Kearney M, *et al.* Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. Nat Med 1999;5:434-438.

[128] Asahara T, Masuda H, Takahashi T, Kalka C, Pastore C, Silver M, *et al.* Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. Circ Res 1999;85:221-228.

[129] Kawamoto A, Tkebuchava T, Yamaguchi J, Nishimura H, Yoon YS, Milliken C, *et al.* Intramyocardial transplantation of autologous endothelial progenitor cells for therapeutic neovascularization of myocardial ischemia. Circulation 2003;107:461-468.

[130] Kawamoto A, Gwon HC, Iwaguro H, Yamaguchi JI, Uchida S, Masuda H, *et al.* Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia. Circulation 2001;103:634-637.

[131] Koyanagi M, Urbich C, Chavakis E, Hoffmann J, Rupp S, Badorff C, *et al.* Differentiation of circulating endothelial progenitor cells to a cardiomyogenic phenotype depends on E-cadherin. FEBS Lett 2005;579:6060-6066.

[132] Yoon YS, Wecker A, Heyd L, Park JS, Tkebuchava T, Kusano K, *et al.* Clonally expanded novel multipotent stem cells from human bone marrow regenerate myocardium after myocardial infarction. J Clin Invest 2005;115:326-338.

[133] Galiñanes M, Loubani M, Davies J, Chin D, Pasi J, Bell PR. Autotransplantation of unmanipulated bone marrow into scarred myocardium is safe and enhances cardiac function in humans. Cell Transplant 2004;13:7-13.

[134] Strauer BE, Brehm M, Zeus T, Kostering M, Hernandez A, Sorg RV, *et al.* Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans. Circulation 2002;106:1913-1918.

[135] Assmus B, Honold J, Schachinger V, Britten MB, Fischer-Rasokat U, Lehmann R, *et al.* Transcoronary transplantation of progenitor cells after myocardial infarction. N Engl J Med 2006;355:1222-1232.

[136] Schachinger V, Erbs S, Elsasser A, Haberbosch W, Hambrecht R, Holschermann H, *et al.* Improved clinical outcome after intracoronary administration of bone-marrow-derived progenitor cells in acute myocardial infarction: final 1-year results of the REPAIR-AMI trial. Eur Heart J 2006;27:2775-2783.

[137] Ang KL, Chin D, Leyva F, Foley P, Kubal C, Chalil S, *et al.* Randomized, controlled trial of intramuscular or intracoronary injection of autologous bone marrow cells into scarred myocardium during CABG versus CABG alone. Nat Clin Pract Cardiovasc Med 2008;5:663-670.

[138] Janssens S, Dubois C, Bogaert J, Theunissen K, Deroose C, Desmet W, *et al.* Autologous bone marrow-derived stem-cell transfer in patients with ST-segment elevation myocardial infarction: double-blind, randomised controlled trial. Lancet 2006;367:113-121.

[139] Lunde K, Solheim S, Aakhus S, Arnesen H, Abdelnoor M, Egeland T, *et al.* Intracoronary injection of mononuclear bone marrow cells in acute myocardial infarction. N Engl J Med 2006;355:1199-1209.

[140] Schachinger V, Erbs S, Elsasser A, Haberbosch W, Hambrecht R, Holschermann H, *et al.* Intracoronary bone marrow-derived progenitor cells in acute myocardial infarction. N Engl J Med 2006;355:1210-1221.

[141] Lunde K, Solheim S, Aakhus S, Arnesen H, Abdelnoor M, Forfang K. Autologous stem cell transplantation in acute myocardial infarction: The ASTAMI randomized controlled trial. Intracoronary transplantation of autologous mononuclear bone marrow cells, study design and safety aspects. Scand Cardiovasc J 2005;39:150-158.

[142] Meyer GP, Wollert KC, Lotz J, Steffens J, Lippolt P, Fichtner S, *et al.* Intracoronary bone marrow cell transfer after myocardial infarction: eighteen months' follow-up data from the randomized, controlled BOOST (BOne marrOw transfer to enhance ST-elevation infarct regeneration) trial. Circulation 2006;113:1287-1294.

[143] Seeger FH, Tonn T, Krzossok N, Zeiher AM, Dimmeler S. Cell isolation procedures matter: a comparison of different isolation protocols of bone marrow mononuclear cells used for cell therapy in patients with acute myocardial infarction. Eur Heart J 2007;28:766-772.

[144] Lipinski MJ, Biondi-Zoccai GG, Abbate A, Khianey R, Sheiban I, Bartunek J, *et al.* Impact of intracoronary cell therapy on left ventricular function in the setting of acute myocardial infarction: a collaborative systematic review and meta-analysis of controlled clinical trials. J Am Coll Cardiol 2007;50:1761-1767.

[145] Martin-Rendon E, Brunskill SJ, Hyde CJ, Stanworth SJ, Mathur A, Watt SM. Autologous bone marrow stem cells to treat acute myocardial infarction: a systematic review. Eur Heart J 2008;29:1807-1818.

[146] Abdel-Latif A, Bolli R, Tleyjeh IM, Montori VM, Perin EC, Hornung CA, *et al.* Adult bone marrow-derived cells for cardiac repair: a systematic review and meta-analysis. Arch Intern Med 2007;167:989-997.

[147] Planat-Benard V, Menard C, Andre M, Puceat M, Perez A, Garcia-Verdugo JM, *et al.* Spontaneous cardiomyocyte differentiation from adipose tissue stroma cells. Circ Res 2004;94:223-229.

[148] Guan K, Wagner S, Unsold B, Maier LS, Kaiser D, Hemmerlein B, *et al.* Generation of functional cardiomyocytes from adult mouse spermatogonial stem cells. Circ Res 2007;100:1615-1625.

[149] Winitsky SO, Gopal TV, Hassanzadeh S, Takahashi H, Gryder D, Rogawski MA, *et al.* Adult murine skeletal muscle contains cells that can differentiate into beating cardiomyocytes in vitro. PLoS Biol 2005;3:e87.

[150] Murry CE, Wiseman RW, Schwartz SM, Hauschka SD. Skeletal myoblast transplantation for repair of myocardial necrosis. J Clin Invest 1996;98:2512-2523.

[151] Taylor DA, Atkins BZ, Hungspreugs P, Jones TR, Reedy MC, Hutcheson KA, *et al.* Regenerating functional myocardium: improved performance after skeletal myoblast transplantation. Nat Med 1998;4:929-933.

[152] Menasche P, Alfieri O, Janssens S, McKenna W, Reichenspurner H, Trinquart L, *et al.* The Myoblast Autologous Grafting in Ischemic Cardiomyopathy (MAGIC) trial: first randomized placebo-controlled study of myoblast transplantation. Circulation 2008;117:1189-1200.

[153] Fernandes S, Amirault JC, Lande G, Nguyen JM, Forest V, Bignolais O, *et al.* Autologous myoblast transplantation after myocardial infarction increases the inducibility of ventricular arrhythmias. Cardiovasc Res 2006;69:348-358.

[154] Reinecke H, MacDonald GH, Hauschka SD, Murry CE. Electromechanical coupling between skeletal and cardiac muscle. Implications for infarct repair. Journal of Cell Biology 2000;149:731-740.

[155] Menasche P. Skeletal myoblast for cell therapy. Coronary Artery Disease 2005;16:105-110.

[156] Alvarez-Dolado M, Pardal R, Garcia-Verdugo JM, Fike JR, Lee HO, Pfeffer K, *et al.* Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. Nature 2003;425:968-973.

[157] Nygren JM, Jovinge S, Breitbach M, Sawen P, Roll W, Hescheler J, *et al.* Bone marrow-derived hematopoietic cells generate cardiomyocytes at a low frequency through cell fusion, but not transdifferentiation. Nat Med 2004;10:494-501.

[158] Terada N, Hamazaki T, Oka M, Hoki M, Mastalerz DM, Nakano Y, *et al.* Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. Nature 2002;416:542-545.

[159] Kajstura J, Rota M, Whang B, Cascapera S, Hosoda T, Bearzi C, *et al.* Bone marrow cells differentiate in cardiac cell lineages after infarction independently of cell fusion. Circ Res 2005;96:127-137.

[160] Leri A, Kajstura J, Anversa P. Cardiac stem cells and mechanisms of myocardial regeneration. Physiol Rev 2005;85:1373-1416.

[161] Xu M, Uemura R, Dai Y, Wang Y, Pasha Z, Ashraf M. In vitro and in vivo effects of bone marrow stem cells on cardiac structure and function. J Mol Cell Cardiol 2007;42:441-448.

[162] Tse HF, Siu CW, Zhu SG, Songyan L, Zhang QY, Lai WH, *et al.* Paracrine effects of direct intramyocardial implantation of bone marrow derived cells to enhance neovascularization in chronic ischaemic myocardium. Eur J Heart Fail 2007;9:747-753.

[163] Zeng L, Hu Q, Wang X, Mansoor A, Lee J, Feygin J, *et al.* Bioenergetic and functional consequences of bone marrow-derived multipotent progenitor cell

transplantation in hearts with postinfarction left ventricular remodeling. Circulation 2007;115:1866-1875.

[164] Tang YL, Zhao Q, Qin X, Shen L, Cheng L, Ge J, *et al.* Paracrine action enhances the effects of autologous mesenchymal stem cell transplantation on vascular regeneration in rat model of myocardial infarction. Ann Thorac Surg 2005;80:229-236; discussion 236-227.

[165] Kano MR, Morishita Y, Iwata C, Iwasaka S, Watabe T, Ouchi Y, *et al.* VEGF-A and FGF-2 synergistically promote neoangiogenesis through enhancement of endogenous PDGF-B-PDGFRbeta signaling. J Cell Sci 2005;118:3759-3768.

[166] Kinnaird T, Stabile E, Burnett MS, Shou M, Lee CW, Barr S, *et al.* Local delivery of marrow-derived stromal cells augments collateral perfusion through paracrine mechanisms. Circulation 2004;109:1543-1549.

[167] Cheng AS, Yau TM. Paracrine effects of cell transplantation: strategies to augment the efficacy of cell therapies. Semin Thorac Cardiovasc Surg 2008;20:94-101.

[168] Abbott JD, Huang Y, Liu D, Hickey R, Krause DS, Giordano FJ. Stromal cell-derived factor-1alpha plays a critical role in stem cell recruitment to the heart after myocardial infarction but is not sufficient to induce homing in the absence of injury. Circulation 2004;110:3300-3305.

[169] Yamaguchi J, Kusano KF, Masuo O, Kawamoto A, Silver M, Murasawa S, *et al.* Stromal cell-derived factor-1 effects on ex vivo expanded endothelial progenitor cell recruitment for ischemic neovascularization. Circulation 2003;107:1322-1328.

[170] Schenk S, Mal N, Finan A, Zhang M, Kiedrowski M, Popovic Z, *et al.* Monocyte chemotactic protein-3 is a myocardial mesenchymal stem cell homing factor. Stem Cells 2007;25:245-251.

[171] Linke A, Muller P, Nurzynska D, Casarsa C, Torella D, Nascimbene A, *et al.* Stem cells in the dog heart are self-renewing, clonogenic, and multipotent and regenerate infarcted myocardium, improving cardiac function. Proc Natl Acad Sci U S A 2005;102:8966-8971.

[172] Wang X, Hu Q, Nakamura Y, Lee J, Zhang G, From AH, *et al.* The role of the sca-1+/CD31- cardiac progenitor cell population in postinfarction left ventricular remodeling. Stem Cells 2006;24:1779-1788.

[173] Wang M, Crisostomo PR, Herring C, Meldrum KK, Meldrum DR. Human progenitor cells from bone marrow or adipose tissue produce VEGF, HGF, and IGF-I in response to TNF by a p38 MAPK-dependent mechanism. Am J Physiol Regul Integr Comp Physiol 2006;291:R880-884.

[174] Gonzalez A, Rota M, Nurzynska D, Misao Y, Tillmanns J, Ojaimi C, *et al.* Activation of cardiac progenitor cells reverses the failing heart senescent phenotype and prolongs lifespan. Circ Res 2008;102:597-606.

[175] Rota M, Padin-Iruegas ME, Misao Y, De Angelis A, Maestroni S, Ferreira-Martins J, *et al.* Local activation or implantation of cardiac progenitor cells rescues scarred infarcted myocardium improving cardiac function. Circ Res 2008;103:107-116.

[176] Takahashi M, Li TS, Suzuki R, Kobayashi T, Ito H, Ikeda Y, *et al.* Cytokines produced by bone marrow cells can contribute to functional improvement of the infarcted heart by protecting cardiomyocytes from ischemic injury. Am J Physiol Heart Circ Physiol 2006;291:H886-893.

[177] Korf-Klingebiel M, Kempf T, Sauer T, Brinkmann E, Fischer P, Meyer GP, *et al.* Bone marrow cells are a rich source of growth factors and cytokines: implications for cell therapy trials after myocardial infarction. Eur Heart J 2008;29:2851-2858.

[178] Gnecchi M, He H, Noiseux N, Liang OD, Zhang L, Morello F, *et al.* Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. Faseb J 2006;20:661-669.

[179] Yamamura T, Otani H, Nakao Y, Hattori R, Osako M, Imamura H. IGF-I differentially regulates Bcl-xL and Bax and confers myocardial protection in the rat heart. Am J Physiol Heart Circ Physiol 2001;280:H1191-1200.

[180] Li Q, Li B, Wang X, Leri A, Jana KP, Liu Y, *et al.* Overexpression of insulin-like growth factor-1 in mice protects from myocyte death after infarction, attenuating ventricular dilation, wall stress, and cardiac hypertrophy. J Clin Invest 1997;100:1991-1999.

[181] Fujio Y, Nguyen T, Wencker D, Kitsis RN, Walsh K. Akt promotes survival of cardiomyocytes in vitro and protects against ischemia-reperfusion injury in mouse heart. Circulation 2000;101:660-667.

[182] Buerke M, Murohara T, Skurk C, Nuss C, Tomaselli K, Lefer AM. Cardioprotective effect of insulin-like growth factor I in myocardial ischemia followed by reperfusion. Proc Natl Acad Sci U S A 1995;92:8031-8035.

[183] Davani EY, Brumme Z, Singhera GK, Cote HC, Harrigan PR, Dorscheid DR. Insulin-like growth factor-1 protects ischemic murine myocardium from ischemia/reperfusion associated injury. Crit Care 2003;7:R176-183.

[184] Otani H, Yamamura T, Nakao Y, Hattori R, Kawaguchi H, Osako M, *et al.* Insulin-like growth factor-I improves recovery of cardiac performance during reperfusion in isolated rat heart by a wortmannin-sensitive mechanism. J Cardiovasc Pharmacol 2000;35:275-281.

[185] Friehs I, Stamm C, Cao-Danh H, McGowan FX, del Nido PJ. Insulin-like growth factor-1 improves postischemic recovery in hypertrophied hearts. Ann Thorac Surg 2001;72:1650-1656.

[186] Yamashita K, Kajstura J, Discher DJ, Wasserlauf BJ, Bishopric NH, Anversa P, *et al.* Reperfusion-activated Akt kinase prevents apoptosis in transgenic mouse hearts overexpressing insulin-like growth factor-1. Circ Res 2001;88:609-614.

[187] Ren J, Samson WK, Sowers JR. Insulin-like growth factor I as a cardiac hormone: physiological and pathophysiological implications in heart disease. J Mol Cell Cardiol 1999;31:2049-2061.

[188] Foncea R, Andersson M, Ketterman A, Blakesley V, Sapag-Hagar M, Sugden PH, *et al.* Insulin-like growth factor-I rapidly activates multiple signal transduction pathways in cultured rat cardiac myocytes. J Biol Chem 1997;272:19115-19124.

[189] Gustafsson H, Soderdahl T, Jonsson G, Bratteng JO, Forsby A. Insulin-like growth factor type 1 prevents hyperglycemia-induced uncoupling protein 3 down-regulation and oxidative stress. J Neurosci Res 2004;77:285-291.

[190] Pi Y, Goldenthal MJ, Marin-Garcia J. Mitochondrial involvement in IGF-1 induced protection of cardiomyocytes against hypoxia/reoxygenation injury. Mol Cell Biochem 2007;301:181-189.

[191] Vasan RS, Sullivan LM, D'Agostino RB, Roubenoff R, Harris T, Sawyer DB, *et al.* Serum insulin-like growth factor I and risk for heart failure in elderly individuals without a previous myocardial infarction: the Framingham Heart Study. Ann Intern Med 2003;139:642-648.

[192] Mangi AA, Noiseux N, Kong D, He H, Rezvani M, Ingwall JS, *et al.* Mesenchymal stem cells modified with Akt prevent remodeling and restore performance of infarcted hearts. Nat Med 2003;9:1195-1201.

[193] Solheim S, Seljeflot I, Lunde K, Aukrust P, Yndestad A, Grogaard HK, *et al*. Inflammatory responses after intracoronary injection of autologous mononuclear bone marrow cells in patients with acute myocardial infarction. Am Heart J 2008;155:55 e51-59.

[194] Zhang JG, Ghosh S, Ockleford CD, Galiñanes M. Characterization of an in vitro model for the study of the short and prolonged effects of myocardial ischaemia and reperfusion in man. Clin Sci (Lond) 2000;99:443-453.

[195] Ghosh S, Standen N, Galiñanes M. Preconditioning the human myocardium by simulated ischemia: studies on the early and delayed protection. Cardiovasc Res 2000;45:339-350.

[196] Guyatt GH, Townsend M, Kazim F, Newhouse MT. A controlled trial of ambroxol in chronic bronchitis. Chest 1987;92:618-620.

[197] Stamm C, Westphal B, Kleine HD, Petzsch M, Kittner C, Klinge H, *et al*. Autologous bone-marrow stem-cell transplantation for myocardial regeneration. Lancet 2003;361:45-46.

[198] Wollert KC, Meyer GP, Lotz J, Ringes-Lichtenberg S, Lippolt P, Breidenbach C, *et al.* Intracoronary autologous bone-marrow cell transfer after myocardial infarction: the BOOST randomised controlled clinical trial. Lancet 2004;364:141-148.

[199] Kocher AA, Schuster MD, Szabolcs MJ, Takuma S, Burkhoff D, Wang J, *et al.* Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. Nat Med 2001;7:430-436.

[200] Gnecchi M, Zhang Z, Ni A, Dzau VJ. Paracrine mechanisms in adult stem cell signaling and therapy. Circ Res 2008;103:1204-1219.

[201] Hinkel R, El-Aouni C, Olson T, Horstkotte J, Mayer S, Muller S, *et al.* Thymosin beta4 is an essential paracrine factor of embryonic endothelial progenitor cell-mediated cardioprotection. Circulation 2008;117:2232-2240.

[202] Tse HF, Thambar S, Kwong YL, Rowlings P, Bellamy G, McCrohon J, *et al.* Prospective randomized trial of direct endomyocardial implantation of bone marrow cells for treatment of severe coronary artery diseases (PROTECT-CAD trial). Eur Heart J 2007;28:2998-3005.

[203] Assmus B, Fischer-Rasokat U, Honold J, Seeger FH, Fichtlscherer S, Tonn T, *et al.* Transcoronary transplantation of functionally competent BMCs is associated with a decrease in natriuretic peptide serum levels and improved survival of patients with chronic postinfarction heart failure: results of the TOPCARE-CHD Registry. Circ Res 2007;100:1234-1241.

[204] Hofmann M, Wollert KC, Meyer GP, Menke A, Arseniev L, Hertenstein B, *et al.* Monitoring of bone marrow cell homing into the infarcted human myocardium. Circulation 2005;111:2198-2202.

[205] Koh CY, Welniak LA, Murphy WJ. Lack of correlation between an assay used to determine early marrow allograft rejection and long-term chimerism after murine allogeneic bone marrow transplantation: effects of marrow dose. Biol Blood Marrow Transplant 2005;11:252-259.

[206] Suzuki Y, Adachi Y, Zhang Y, Minamino K, Iwasaki M, Mukaide H, *et al.* Administration of granulocyte colony-stimulating factor to recipients followed by intra-bone marrow-bone marrow transplantation accelerates acceptance of allogeneic bone marrow cells in mice. Immunobiology 2008;213:427-435.

[207] Cahn JY, Labopin M, Schattenberg A, Reiffers J, Willemze R, Zittoun R, *et al.* Allogeneic bone marrow transplantation for acute leukemia in patients over the age of

40 years. Acute Leukemia Working Party of the European Group for Bone Marrow Transplantation (EBMT). Leukemia 1997;11:416-419.

[208] Robin M, Guardiola P, Dombret H, Baruchel A, Esperou H, Ribaud P, *et al.* Allogeneic bone marrow transplantation for acute myeloblastic leukaemia in remission: risk factors for long-term morbidity and mortality. Bone Marrow Transplant 2003;31:877-887.

[209] Bacigalupo A. Management of acute graft-versus-host disease. Br J Haematol 2007;137:87-98.

[210] Crisostomo PR, Wang M, Wairiuko GM, Morrell ED, Terrell AM, Seshadri P, *et al.* High passage number of stem cells adversely affects stem cell activation and myocardial protection. Shock 2006;26:575-580.

[211] Derubeis AR, Cancedda R. Bone marrow stromal cells (BMSCs) in bone engineering: limitations and recent advances. Ann Biomed Eng 2004;32:160-165.

[212] Bartunek J, Wijns W, Heyndrickx GR, Vanderheyden M. Timing of intracoronary bone-marrow-derived stem cell transplantation after ST-elevation myocardial infarction. Nat Clin Pract Cardiovasc Med 2006;3 Suppl 1:S52-56.

[213] Charwat S, Gyöngyösi M, Lang I, Graf S, Beran G, Hemetsberger R, *et al.* Role of adult bone marrow stem cells in the repair of ischemic myocardium: current state of the art. Experimental hematology 2008;36:672-680.

[214] Huang R, Yao K, Qian J, Ge L, Li Y, Niu Y, *et al.* Emergency Intracoronary Stem Cell Transplantation in Patients with Acute Myocardial Infarction (TCT-STAMI-2): Long-Term Outcome. Circulation 2008;118:S_791 "Abstract".

[215] Loubani M, Galiñanes M. Pharmacological and ischemic preconditioning of the human myocardium: mitoKATP channels are upstream and p38MAPK is downstream of PKC. BMC Physiol 2002;2:10-23.

[216] Gerber HP, Malik AK, Solar GP, Sherman D, Liang XH, Meng G, *et al.* VEGF regulates haematopoietic stem cell survival by an internal autocrine loop mechanism. Nature 2002;417:954-958.

[217] Askari A, Unzek S, Goldman CK, Ellis SG, Thomas JD, DiCorleto PE, *et al.* Cellular, but not direct, adenoviral delivery of vascular endothelial growth factor results in improved left ventricular function and neovascularization in dilated ischemic cardiomyopathy. J Am Coll Cardiol 2004;43:1908-1914.

[218] Noiseux N, Gnecchi M, Lopez-Ilasaca M, Zhang L, Solomon SD, Deb A, *et al.* Mesenchymal stem cells overexpressing Akt dramatically repair infarcted myocardium and improve cardiac function despite infrequent cellular fusion or differentiation. Mol Ther 2006;14:840-850.

[219] Wang Y, Haider HK, Ahmad N, Xu M, Ge R, Ashraf M. Combining pharmacological mobilization with intramyocardial delivery of bone marrow cells over-expressing VEGF is more effective for cardiac repair. J Mol Cell Cardiol 2006;40:736-745.

[220] Burchfield JS, Dimmeler S. Role of paracrine factors in stem and progenitor cell mediated cardiac repair and tissue fibrosis. Fibrogenesis Tissue Repair 2008;1:4.

[221] Kooijman R. Regulation of apoptosis by insulin-like growth factor (IGF)-I. Cytokine Growth Factor Rev 2006;17:305-323.

[222] Vogt AM, Htun P, Kluge A, Zimmermann R, Schaper W. Insulin-like growth factor-II delays myocardial infarction in experimental coronary artery occlusion. Cardiovasc Res 1997;33:469-477.

[223] Gable KL, Maddux BA, Penaranda C, Zavodovskaya M, Campbell MJ, Lobo M, *et al.* Diarylureas are small-molecule inhibitors of insulin-like growth factor I receptor signaling and breast cancer cell growth. Mol Cancer Ther 2006;5:1079-1086.

[224] Santini MP, Tsao L, Monassier L, Theodoropoulos C, Carter J, Lara-Pezzi E, *et al.* Enhancing repair of the mammalian heart. Circ Res 2007;100:1732-1740.

[225] Olivetti G, Quaini F, Sala R, Lagrasta C, Corradi D, Bonacina E, *et al.* Acute myocardial infarction in humans is associated with activation of programmed myocyte cell death in the surviving portion of the heart. J Mol Cell Cardiol 1996;28:2005-2016.

[226] Saraste A, Pulkki K, Kallajoki M, Henriksen K, Parvinen M, Voipio-Pulkki LM. Apoptosis in human acute myocardial infarction. Circulation 1997;95:320-323.

[227] Vincent AM, Feldman EL. Control of cell survival by IGF signaling pathways. Growth Horm IGF Res 2002;12:193-197.

[228] Pecherskaya A, Solem M. IGF1 activates PKC alpha-dependent protein synthesis in adult rat cardiomyocytes. Mol Cell Biol Res Commun 2000;4:166-171.

[229] Bartunek J, Vanderheyden M, Vandekerckhove B, Mansour S, De Bruyne B, De Bondt P, *et al.* Intracoronary injection of CD133-positive enriched bone marrow progenitor cells promotes cardiac recovery after recent myocardial infarction: feasibility and safety. Circulation 2005;112:I178-183.

[230] Stamm C, Kleine HD, Westphal B, Petzsch M, Kittner C, Nienaber CA, *et al.* CABG and bone marrow stem cell transplantation after myocardial infarction. J Thorac Cardiovasc Surg 2004;52:152-158.

[231] Stamm C, Kleine HD, Choi YH, Dunkelmann S, Lauffs JA, Lorenzen B, *et al.* Intramyocardial delivery of CD133+ bone marrow cells and coronary artery bypass grafting for chronic ischemic heart disease: safety and efficacy studies. J Thorac Cardiovasc Surg 2007;133:717-725.

[232] Goussetis E, Manginas A, Koutelou M, Peristeri I, Theodosaki M, Kollaros N, *et al.* Intracoronary infusion of CD133+ and CD133-CD34+ selected autologous bone marrow progenitor cells in patients with chronic ischemic cardiomyopathy: cell

isolation, adherence to the infarcted area, and body distribution. Stem Cells 2006;24:2279-2283.

[233] Preis SR, Hwang SJ, Coady S, Pencina MJ, D'Agostino RB, Sr., Savage PJ, *et al.* Trends in all-cause and cardiovascular disease mortality among women and men with and without diabetes mellitus in the Framingham Heart Study, 1950 to 2005. Circulation 2009;119:1728-1735.

[234] Paulson DJ. The diabetic heart is more sensitive to ischemic injury. Cardiovasc Res 1997;34:104-112.

[235] Bauters C, Lamblin N, Mc Fadden EP, Van Belle E, Millaire A, de Groote P. Influence of diabetes mellitus on heart failure risk and outcome. Cardiovasc Diabetol 2003;2:1.

[236] Kannel WB. Incidence and epidemiology of heart failure. Heart Fail Rev 2000;5:167-173.

[237] Bell DS. Diabetic cardiomyopathy. Diabetes Care 2003;26:2949-2951.

[238] Mildenberger RR, Bar-Shlomo B, Druck MN, Jablonsky G, Morch JE, Hilton JD, *et al.* Clinically unrecognized ventricular dysfunction in young diabetic patients. J Am Coll Cardiol 1984;4:234-238.

[239] Borow KM, Jaspan JB, Williams KA, Neumann A, Wolinski-Walley P, Lang RM. Myocardial mechanics in young adult patients with diabetes mellitus: effects of altered load, inotropic state and dynamic exercise. J Am Coll Cardiol 1990;15:1508-1517.

[240] Kannel WB. Lipids, diabetes, and coronary heart disease: insights from the Framingham Study. Am Heart J 1985;110:1100-1107.

[241] Woodfield SL, Lundergan CF, Reiner JS, Greenhouse SW, Thompson MA, Rohrbeck SC, *et al.* Angiographic findings and outcome in diabetic patients treated with

thrombolytic therapy for acute myocardial infarction: the GUSTO-I experience. J Am Coll Cardiol 1996;28:1661-1669.

[242] Zuanetti G, Latini R, Maggioni AP, Santoro L, Franzosi MG. Influence of diabetes on mortality in acute myocardial infarction: data from the GISSI-2 study. J Am Coll Cardiol 1993;22:1788-1794.

[243] Barsness GW, Peterson ED, Ohman EM, Nelson CL, DeLong ER, Reves JG, *et al.* Relationship between diabetes mellitus and long-term survival after coronary bypass and angioplasty. Circulation 1997;96:2551-2556.

[244] Herlitz J, Wognsen GB, Emanuelsson H, Haglid M, Karlson BW, Karlsson T, *et al.* Mortality and morbidity in diabetic and nondiabetic patients during a 2-year period after coronary artery bypass grafting. Diabetes Care 1996;19:698-703.

[245] Galiñanes M, Fowler AG. Role of clinical pathologies in myocardial injury following ischaemia and reperfusion. Cardiovasc Res 2004;61:512-521.

[246] Hearse DJ, Stewart DA, Chain EB. Diabetes and the survival and recovery of the anoxic myocardium. J Mol Cell Cardiol 1975;7:397-415.

[247] Haider B, Ahmed SS, Moschos CB, Oldewurtel HA, Regan TJ. Myocardial function and coronary blood flow response to acute ischemia in chronic canine diabetes. Circ Res 1977;40:577-583.

[248] Tani M, Neely JR. Hearts from diabetic rats are more resistant to in vitro ischemia: possible role of altered Ca2+ metabolism. Circ Res 1988;62:931-940.

[249] Broderick TL, Quinney HA, Lopaschuk GD. L-carnitine increases glucose metabolism and mechanical function following ischaemia in diabetic rat heart. Cardiovasc Res 1995;29:373-378.

[250] Li TS, Furutani A, Takahashi M, Ohshima M, Qin SL, Kobayashi T, *et al.* Impaired potency of bone marrow mononuclear cells for inducing therapeutic

angiogenesis in obese diabetic rats. Am J Physiol Heart Circ Physiol 2006;290:H1362-1369.

[251] Tamarat R, Silvestre JS, Le Ricousse-Roussanne S, Barateau V, Lecomte-Raclet L, Clergue M, *et al.* Impairment in ischemia-induced neovascularization in diabetes: bone marrow mononuclear cell dysfunction and therapeutic potential of placenta growth factor treatment. Am J Pathol 2004;164:457-466.

[252] Belch JJ, Bridges AB, Scott N, Chopra M. Oxygen free radicals and congestive heart failure. Br Heart J 1991;65:245-248.

[253] McMurray J, Chopra M, Abdullah I, Smith WE, Dargie HJ. Evidence of oxidative stress in chronic heart failure in humans. Eur Heart J 1993;14:1493-1498.

[254] McTiernan CF, Feldman AM. The role of tumor necrosis factor alpha in the pathophysiology of congestive heart failure. Curr Cardiol Rep 2000;2:189-197.

[255] Schwartz D, Sack M. Targeting the mitochondria to augment myocardial protection. Current Opinion in Pharmacology 2008;8:160–165.

[256] Burwell LS, Brookes PS. Mitochondria as a Target for the Cardioprotective Effects of Nitric Oxide in Ischemia–Reperfusion Injury. Antioxidants & Redox Signaling 2008;10:579-599.

[257] Garlid K, Paucek P, Yarov-Yarovoy V, Murray H, Darbenzio R, D'Alonzo A, *et al.* Cardioprotective effect of diazoxide and its interaction with mitochondrial ATP-sensitive K+ channels. Possible mechanism of cardioprotection. Circ Res 1997;81:1072-1082.

[258] O'Rourke B. Evidence for mitochondrial K+ channels and their role in cardioprotection. Circ Res 2004;94:420–432.

[259] Zingman LV, Alekseev AE, Hodgson-Zingman DE, Terzic A. ATP-sensitive potassium channels: metabolic sensing and cardioprotection. J Appl Physiol 2007;103:1888-1893.

[260] Matejikova J, Kucharska J, Pinterova M, Pancza D, Ravingerova T. Protection Against Ischemia-Induced Ventricular Arrhythmias and Myocardial Dysfunction Conferred by Preconditioning in the Rat Heart: Involvement of Mitochondrial KATP Channels and Reactive Oxygen Species. Physiol Res 2009;58:9-19.

[261] Schwartz LM, Reimer KA, Crago MS, Jennings RB. Pharmacological preconditioning with diazoxide slows energy metabolism during sustained ischemia. Exp Clin Cardiol 2007;12:139-147.

[262] IONA. Effect of nicorandil on coronary events in patients with stable angina: the Impact Of Nicorandil in Angina (IONA) randomised trial. Lancet 2002;359:1269-1275.

[263] Galinanes M, James M, Codd V, Baxi A, Hadjinikolaou L. TNF-alpha gene promoter polymorphism at nucleotide -308 and the inflammatory response and oxidative stress induced by cardiac surgery: role of heart failure and medical treatment. Eur J Cardiothorac Surg 2008;34:332-337.

[264] Ishibashi Y, Takahashi N, Tokumaru A, Karino K, Sugamori T, Sakane T, *et al*. Effects of long-term nicorandil administration on endothelial function, inflammation, and oxidative stress in patients without coronary artery disease. J Cardiovasc Pharmacol 2008;51:311-316.

[265] Orlic D, Kajstura J, Chimenti S, Bodine DM, Leri A, Anversa P. Transplanted adult bone marrow cells repair myocardial infarcts in mice. Ann N Y Acad Sci 2001;938:221-229; discussion 229-230.

[266] Tomita S, Li RK, Weisel RD, Mickle DA, Kim EJ, Sakai T, *et al.* Autologous transplantation of bone marrow cells improves damaged heart function. Circulation 1999;100:II247-256.

[267] Kang S, Yang YJ, Li CJ, Gao RL. Effects of intracoronary autologous bone marrow cells on left ventricular function in acute myocardial infarction: a systematic review and meta-analysis for randomized controlled trials. Coron Artery Dis 2008;19:327-335.

[268] Hendrikx M, Hensen K, Clijsters C, Jongen H, Koninckx R, Bijnens E, *et al.* Recovery of regional but not global contractile function by the direct intramyocardial autologous bone marrow transplantation: results from a randomized controlled clinical trial. Circulation 2006;114:I101-107.

[269] Patel AN, Geffner L, Vina RF, Saslavsky J, Urschel HC, Jr., Kormos R, *et al.* Surgical treatment for congestive heart failure with autologous adult stem cell transplantation: a prospective randomized study. J Thorac Cardiovasc Surg 2005;130:1631-1638.

[270] Breisblatt WM, Stein KL, Wolfe CJ, Follansbee WP, Capozzi J, Armitage JM, *et al.* Acute myocardial dysfunction and recovery: a common occurrence after coronary bypass surgery. J Am Coll Cardiol 1990;15:1261-1269.

[271] Ghosh S, Galiñanes M. Protection of the human heart with ischemic preconditioning during cardiac surgery: role of cardiopulmonary bypass. J Thorac Cardiovasc Surg 2003;126:133-142.

[272] Nishikawa T, Dohi S. Errors in the measurement of cardiac output by thermodilution. Can J Anaesth 1993;40:142-153.

[273] Anversa P, Leri A, Rota M, Hosoda T, Bearzi C, Urbanek K, *et al.* Concise review: stem cells, myocardial regeneration, and methodological artifacts. Stem Cells 2007;25:589-601.

[274] Linares-Palomino J, Husainy MA, Dickenson J, Lai VK, Standen N, Galiñanes M. Specific Inhibition of Protein Kinase B Protects Against Ischemic Myocardial Injury in Rat and Man. Biophysical Journal 2009;96:682a-683a. "Abstr".

[275] Speechly-Dick ME, Grover GJ, Yellon DM. Does ischemic preconditioning in the human involve protein kinase C and the ATP-dependent K+ channel? Studies of contractile function after simulated ischemia in an atrial in vitro model. Circ Res 1995;77:1030-1035.