CERTIFICATE

I, Deepthi R.S hereby certify that I had personally carried out the work depicted in the thesis entitled, **Response of human cardiosphere derived cells to hypoxia-reoxygenation injury and its implications for myocardial regeneration.** No part of the thesis has been submitted for the award of any other degree or diploma prior to this date.

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Clearance was obtained from the Institutional Ethics Committee/Institutional Animal Ethics for carrying out the study.

Date Dr R.Renuka Nair

The thesis entitled

RESPONSE OF HUMAN CARDIOSPHERE DERIVED CELLS TO HYPOXIA-REOXYGENATION INJURY AND ITS IMPLICATIONS FOR MYOCARDIAL REGENERATION

Submitted by

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LIST OF ABBREVIATIONS

ACEIs	Angiotensin- converting-enzyme inhibitors
AMI	Acute myocardial infarction
BMMNCs	Bone marrow mononuclear cells
CABG	Coronary artery bypasses graft
CAD	Coronary artery disease
CDC	Cardiosphere Derived Cells
CEM	Complete explant medium
CRT	Cardiac resynchronization therapy
CSs	Cardiospheres
cTnI	Cardiac troponin I
DCFDA	2, 7-dichlorodihydrofluorescein diacetate
EF	Ejection fraction
ELISA	Enzyme Linked Immunosorbant Assay
EPCs	Endothelial Progenitor Cells
ESCs	Embryonic stem cells
FACS	Fluorescence- activated cell sorting
HGF	Hepatocyte growth factor
HLA	human leukocyte antigen
HR injury	Hypoxia reoxygenation injury
ICD	Implantable cardioverter-defibrillator
IHD	Ischemic heart disease
IR injury	Ischemia reperfusion injury
MI	Myocardial infarction
MSCs	Mesenchymal stem cells
PGF	Primary graft failure
PI	Propidium iodide
PTCA	Percutaneous transluminal coronary angioplasty
ROS	Reactive oxygen species
Sca-1	stem cell antigen-1
SDF-1	Stromal derived growth factor
SP	Side population
SSEA	Stage-specific embryonic antigens
TBS	Tris Buffered Saline
UCBCs	Umbilical Cord Blood Cells
VEGF	Vascular endothelial growth factor

SYNOPSIS

Background

Ischemic injury of the myocardium leading to progressive ventricular failure accounts for a large proportion of cardiac deaths. Myocardial infarction (MI) is associated with necrosis of heart muscle secondary to prolonged ischemia, due to blockade of coronary artery. Reoxygenation of the ischemic myocardium upon restoration of blood flow may further damage the heart muscle (Ischemia- reperfusion injury) due to oxidative stress and result in augmentation of the infarct size. Cardiac remodeling consequent to ischemia-reperfusion injury can lead to the development of congestive cardiac failure. The adult human myocardium has limited regenerative capacity and the cardiomyocytes lost due to ischemia- reperfusion injury, is replaced by noncontractile scar tissue leading to cardiac dysfunction. Although a broad array of treatment options are available none of the conventional management strategies successfully replace lost cardiomyocyte mass or myocardial scar with new contractile cells. Pre-clinical and clinical trials indicate the possibility of stem cell-based myo cardial regeneration. A variety of stem cell types has been experimented for their myocardial regeneration potential. Of these, bone marrow derived cells and cardiac stem cells have been found to be promising for successful myocardial regeneration. Cardiac stem cells have the advantage that they are tissue specific and are suitable for autologous transplantation. Among the different forms in which cardiac stem cells can be transplanted, cardiosphere derived cells (CDCs) were found to be having the maximum regenerative potential as observed in preclinical and clinical trials. Their expansion capacity in culture makes CDCs the cells of choice for

transplantation, as this property is helpful in obtaining the desired number of cells for therapy. However, there is a lacuna in information on the response of cardiosphere derived cells to hypoxic injury, and the effect of cardiomyocyte injury on proliferation and migration of cardiac stem cells. This study is aimed at understanding the response of cardiosphere derived cells to hypoxia-reoxygenation injury, and identifying the ideal conditions for ex-vivo expansion of these cells, so as to facilitate stem cell based myocardial regeneration. This study is based on the **assumption** that**,** Cardiosphere Derived Cells are resistant to hypoxia- reoxygenation injury and that cells expanded in culture can be transplanted for myocardial regeneration.

Atrial appendage obtained at the time of coronary artery bypass graft surgery will be the source of cardiosphere derived cells for the experimental studies.

The main Objectives of the study are:

- Understand the response of human cardiosphere derived cells (CDCs) to hypoxia-reoxygenation injury
- Evaluate the effect of hypoxically injured myocardium on CDCs.
- Assess the effect of hypoxically-injured CDCs on cardiomyocytes
- Compare the response of CDCs and bone marrow mesenchymal stem cells to hypoxia-reoxygenation injury
- Identify the optimal culture condition for the expansion of CDCs prior to transplantation

Methods

The study was carried out in Cardiosphere derived cells isolated from the right atrial appendage of patients undergoing coronary artery bypass graft (CABG) surgery. Mesenchymal stem cells for comparison were isolated from sternal bone marrow of the patients. The cells isolated from the tissue samples were expanded in culture and used for experimental studies. Cells from passages 2-4 were used for the studies. Ischemia-reperfusion injury was simulated in culture by incubating the cells in serum free medium for 2hrs in a hypoxia incubator with 0.5% O_2 followed by incubation in 10% serum containing medium, in ambient (20%) O_2 for 6hr (Hypoxia reoxygenation injury- HR injury). Cardiomyocyte cell lines (H9c2) for the experiments were obtained from National Centre for Cell Science, Pune. In vivo cells are exposed to tissue oxygen levels that generally range from 2-7%. Assuming that the oxygen levels can affect the properties of cells, CDCs cultured in 3% O_2 was compared with those cultured in ambient O_2 for proliferation, survival and differentiation. Cells were assessed for survival using Annexin/ PI cell survival assay, β galactosidase cell senescence assay and Tunnel assay. Proliferation was assessed by Cell cycle analysis, Ki 67 nuclear antigen and population doubling time. Cardiomyocyte differentiation was evaluated from analysis of gene expression by Real time PCR and immunocytochemistry. Angiogenesis was assessed by endothelial tube formation assay. Migration was assessed by scratch wound assay and transwell migration assay. ROS generation was evaluated by DCFDA-FACS, Flourimetry, release of growth factors was quantified by ELISA and secretion of

prosurvival factors by western blot analysis.Statistical significance was assessed by one way ANOVA followed by Student's t test.A value of $P \leq 0.05$ was considered significant.

Significant Findings

- **Response of human cardiosphere derived cells to hypoxic injury**

A small percentage of CDCs were found to undergo apoptosis (2.2%) and senescence (11%) after Hypoxia- reoxygenation injury. The basal level of apoptosis was 3.5± 0.2, soon after HR injury it increased to 6.7±0.3. There was a two fold increase in the expression of cell survival factors pAkt and BCl2. No cell cycle arrest was observed after HR injury. Cell proliferation increased along with the improved secretion of hepatocyte growth factor ($p<0.01$), a mediator of growth and proliferation. Reduced expression of cardiac troponin I and connexin 43 (1.5 fold reduction) and reduced number of branching points respectively ($p<0.01$) suggest that differentiation of cells to cardiac lineage are affected by HR injury. Migratory capacity of CDC was also reduced following HR injury ($p<0.05$). The observations indicate that Hypoxia- reoxygenation injury affects the behavior of CDCs

- **Effect of hypoxically injured stem cells on cardiomyocytes**

CDC conditioned medium was found to have a protective effect on injured cardiomyocytes, Conditioned medium from both normal CDCs ($p<0.01$) and injured CDCs ($p<0.05$) reduced apoptosis of HR injured cardiomyocytes. Both normal CDC

conditioned medium and injured CDC conditioned medium positively influenced G1-S progression of injured H9c2 cells.

- **Effect of hypoxically injured myocardium on stem cell behavior**

Myocyte injury promotes migration and survival of cardiac stem cells. Both normal H9c2 conditioned medium ($p<0.05$) and injured H9c2 conditioned medium ($p<0.05$) positively influenced G1-S progression of CDCs irrespective of hypoxic injury. Injured myocardial tissue and injured H9c2 conditioned medium promoted transwell migration of normal CDCs. CDCs were more resistant to hypoxic injury compared to MSCs.

- **Comparison of the response of CDCs and MSCs to hypoxia- reoxygenation injury.**

MSCs were found to be affected more by HR injury than CDCs. This was well reflected by the 2-fold increase in survival rate and 2.5-fold increase in differentiation rates of CDCs even after HR injury. Basel cell proliferation rate was greater for CDCs ($p<0.05$). Release of essential cytokines, cell survival factors (2 fold increase) and migratory rate (3 fold increase) in response to HR injury was higher for CDCs. MSCs also have the disadvantage that their efficiency to undergo osteogenic (1.75 fold) and adipogenic(4 fold) differentiation increases after HR injury in comparison with HR CDCs. Therefore, CDCs appear to be the appropriate cell type for transplantation for regeneration of injured myocardium

- **Exvivo expansion of cardiosphere derived cells in niche levels of O_2 (3% O_2)**

The physiological oxygen level of heart approximates 5% O_2. In the normal practice the cells are cultured in the atmospheric levels of oxygen- 20% O_2. In the cardiac stem cell niches, the cells are maintained in much lower oxygen levels. Since cells have to be transplanted in to reduced oxygen conditions, cardiosphere derived cells were expanded in 3% O_2. Ex-vivo expansion of cardiac stem cells in 3% oxygen, increased proliferation rate and the yield of viable stem cells by 2 fold along with a 3 fold reduction in ROS generation compared to culture in ambient O_2. The angiogenic property was found to be enhanced, >1.5 fold with increased number of endothelial tubes. Survival and proliferation was better when cultures maintained at 3% O_2 were shifted to physiological levels of O_2 (5% O_2). Thus, expansion of cells in 3% O_2 culture is advantageous when used for transplantion for myocardial regeneration.

Implications of the findings

Hypoxia-reoxygenation injury to stem cells reduces the survival, proliferation and migration. This can be the underlying cause for inefficient endogenous repair of injured myocardium. Transplantation of stem cells therefore appears to be the most viable option to induce myocardial regeneration. Cardiosphere derived cells expanded in physiological O_2 were found to have better survival. Hence, expansion of cells in 3% O_2 appears to be appropriate for expansion of stem cells intended for transplantation for myocardial regeneration.

I. INTRODUCTION

Myocardial infarction (MI) and the subsequent development of congestive heart failure are the leading cause of mortality worldwide. MI occurs when blood flow to the myocardium is restricted (ischemia) by narrowing or obstruction of a coronary artery causing damage to the heart muscle. Reperfusion of the coronary artery is necessary to resuscitate the ischemic or hypoxic myocardium. Timely reperfusion facilitates cardiomyocyte salvage and decreases cardiac morbidity and mortality. Reperfusion of an ischemic area may result, however, in paradoxical cardiomyocyte dysfunction, a phenomenon termed "reperfusion injury," possibly mediated by excessive generation of reactive oxygen species. Cardiac remodeling consequent to ischemia reperfusion injury can lead to the development of congestive cardiac failure. Current treatment modalities, including pharmacological, interventional and surgical approaches help to relieve symptoms, but does not arrest disease progression. Endogenous repair mechanisms are triggered soon after ischemia/reperfusion (I/R) injury. However, the inherent repair mechanisms appear to be inadequate to salvage the infracted muscle, possibly due to the insufficient number of stem cells, compounded with the hostile, proapoptotic environment in the ischemically injured area. Cardiomyocytes lost due to ischemia reperfusion injury, is generally replaced by noncontractile scar tissue leading to cardiac dysfunction.

Cell based therapy has been conceptualized as a strategy to repopulate the site of injury and stimulate myocardial regeneration, with consequent improvement of ventricular function. The intention of stem cell therapy for myocardial regeneration is to transplant stem cells that provide a renewable source of proliferating, functional cardiomyocytes as well as a network of newly formed blood vessels, thereby promoting reverse remodeling.

Experimental studies in large and small animals using different stem and progenitor cells have recorded beneficial effect on the heart (Orlic et al., 2001a, Barbash et al., 2003a, Prasad et al., 2009a, Quevedo et al., 2009a, Ji et al., 2010)**.** Two promising cell types of autologous origin popularly identified for myocardial regeneration are the bone marrow derived mesenchymal stem cells due to easy availability and adult resident cardiac stem cells because of cardio specificity. Of the different forms in which cardiac stem cells can be transplanted, cardiosphere derived cells (CDCs) are advocated as the potential cardiac stem cell candidates due to superior paracrine potency and differentiation efficacy (Koninckx et al., 2011a, Li et al., 2012a, Cheng et al., 2014a). This is well supported by the encouraging results obtained in the ongoing clinical trials (Makkar et al., 2012). Mesenchymal stem cells (MSCs) have attracted attention as a source for cell transplantation therapy, because of their safety, multilineage differentiation potential (Pittenger et al., 1999) and immunomodulatory (Boyle et al., 2010) and antifibrotic effects (Ian P. Counihan et al.,2012). With the support of several promising preclinical studies in large and small animals(Quevedo et al., 2009a, Orlic et al., 2001a), clinical translation is in progress with Phase Iand II clinical trials (Hare et al., 2009b).

Cardiac stem cell response to ischemia- reperfusion injury is an unexplored area. The response of resident cardiac stem cells and the fate of transplanted cells in the event of ischemic injury are not known. Cardiac fibroblasts are relatively resistant to ischemic injury (Sangeetha et al, 2011) and they proliferate and populate the regions of myocyte loss resulting in scar tissue formation. Though the optimal timing of cell-based therapy after myocardial infarction is currently not standardized, a recent infarct still in the

healing phase appears to be a more favorable substrate for cell-based repair than the scar tissue. The milieu into which the cells are transplanted, determines the fate of the cells. Whether cardiosphere derived cells survive ischemia reperfusion injury, without losing its innate characteristics is an important unanswered question.

Despite the progress in stem cell therapy, the anticipated outcome is affected by low cell engraftment and poor survival of the stem cells in the hostile environment of the failing heart.Stem cell survival after transplantation is influenced by a number of factors such as ischemic conditions, succeptibility to ischemia reperfusion injury, inflammatory response and quality of donor cells. The choice of the appropriate functionally competent cell types that tolerate ischemia, and differentiate in the infarct area remains a matter of concern. Although CDCs and MSCs have emerged as potential candidates for cell based therapy, their post transplantational response to ischemic injury is not known. The autocrine and paracrine effects on survival, proliferation and migration in the event of ischemic injury can vary with the cell type. Previous studies have reported that MSCs failed to engraft in the ischemic region many weeks after MI (Barbash et al., 2003a), resulting in the accumulation of cells in lungs. MSCs are known to be sensitive to hypoxic injury (Potier et al., 2007). Only around 1–5% of delivered cells engrafted within the infarct zone (Freyman et al., 2006a). The sensitivity of CDCs to ischemic injury and the comparison with MSCs has not been evaluated. This study was therefore taken up with the aim of comparing the migration, survival and proliferation of CDCs and MSCs in response to hypoxia-reoxygenation injury.

Preparation of sufficient number of cells in short duration of time and maintenance of stem cell characteristics during expansion are essential prerequisites for the success of cell-based therapy. In conventional practice, stem cells are expanded in CO_2 incubators with 5% CO_2 in air where the oxygen content is close to 20%, that is about four times higher than the physiological, in situ oxygen levels (Roy et al., 2003, Haque et al., 2013). Further, stem cells are maintained in specialized microenvironments called niches (Li and Neaves, 2006), that regulates stem cell function and protects stem cells from exhaustion (Drummond-Barbosa, 2008). Oxygen tension is an important component of stem cell niches that regulates proliferation, differentiation and plasticity of resident stem cells (Mohyeldin et al., 2010). The high oxygen gradient incidental to transplantation of the ex-vivo expanded cells from ambient oxygen to a relatively hypoxic environment can induce cell death and poor engraftment of transplanted cells (Wion et al., 2009). This has prompted investigators to develop potential cytoprotective strategies for transplantation, including hypoxic preconditioning, (Maslov et al., 2013) culturing in low oxygen tension, pharmacological preconditioning (Wisel et al., 2009, Yao et al., 2009), over expression of anti-apoptotic and antioxidant proteins (Noiseux et al., 2006) etc. Reduction of O_2 levels from 20% to physiological levels has been reported to enhance proliferation, stemness and genomic stability of many stem cell types such as MSCs (Grayson et al., 2007), ESCs (Prasad et al., 2009b), IPScs(Guo et al., 2013) and cancer stem cells (Bar et al., 2010). CDCs when expanded in physiological oxygen levels (5% O_2) of the heart, improved cell production and myocardial regeneration potential (Li et al., 2011a). Human cardiospheres contain a heterogeneous cell population, and are reported to recapitulate the properties of

stem cell niches (Li et al., 2010). Oxygen levels in which they are cultured are important to preserve their innate characteristics. CDCs for transplantation are popularly cultured under ambient oxygen tensions. Exposure of stem cells to hyperoxic (20% O_2) condition increases ROS generation(Li and Marbán, 2010), induces spontaneous differentiation (Prasad et al., 2009b) and affects genomic stability (Li and Marbán, 2010). The regenerative potential of cells expanded in ambient oxygen can therefore be compromised.

The O_2 level in the niches is lower than the physiological levels (5%) prevalent in the cardiac tissue (Ivanovic, 2009). Amirrasouli et al have reported that preconditioning of CDCs in 3% O_2 prior to delivery prepare transplanted stem cells for survival in the ischemic myocardium and improve angiogenesis(Amirrasouli et al., 2013). Therefore it is presumed that, expansion of stem cells in physiological levels of oxygen can provide a more conducive atmosphere for the CDCs to grow and preserve their original characteristics, with increased survival rate and proliferation on transplantation.

There is very little information on the response of cardiosphere derived cells to hypoxic injury, and the effect of cardiomyocyte injury on proliferation and migration of cardiac stem cells. The effect of hypoxically injured myocardium on CDCs and the influence of hypoxically-injured CDCs on cardiomyocytes are two important areas that need investigation. The autocrine and paracrine mechanisms associated with hypoxic injury of cardiomyocytes and cardiosphere derived cells are not known. There is a lacuna in information on the response of cardiosphere derived cells to hypoxic injury, and the effect

of cardiomyocyte injury on proliferation and migration of cardiac stem cells. This study is therefore aimed at examining the response of cardiosphere derived cells to hypoxia-reoxygenation injury, and identifying the ideal conditions for ex-vivo expansion of these cells, so as to facilitate stem cell based myocardial regeneration. Based on the available information, it is hypothesised that**,** cardiosphere derived cells have greater resistance to hypoxia- reoxygenation injury and that cells expanded ex-vivo under appropriate culture conditions can improve the survival properties of cells used for myocardial regeneration. The research was focused on identifying the appropriate stem cell type for cell based therapy and the ideal conditions for ex-vivo expansion, aimed at improving the survival of transplanted stem cells within the host environment to augment myocardial repair.

Therefore, this study was carried out with the following objectives:

- Understand the response of human cardiosphere derived cells (CDCs) to hypoxia-reoxygenation injury
- Evaluate the effect of hypoxically injured myocardium on CDCs.
- Assess the effect of hypoxically-injured CDCs on cardiomyocytes
- Compare the response of CDCs and bone marrow mesenchymal stem cells to hypoxia-reoxygenation injury
- Identify the optimal culture condition for the expansion of CDCs prior to transplantation.

Atrial appendage obtained at the time of coronary artery bypass graft surgery formed the source of cardiosphere derived cells for the experimental studies.

The literature pertaining to different treatment strategies for heart failure, and different stem cell types used for myocardial regeneration, including the clinical trials have been reviewed in Chapter II. .

The design of the study and experimental methodology are given in the third chapter. In the fourth chapter, the results are presented and the findings discussed in the light of available information. Salient observations, major conclusions and scope for further studies are summarized in the fifth chapter.

II. LITERATURE REVIEW

II.1 Ischemic heart disease

Ischemic heart disease (IHD) is a group of closely related disorders consequent to myocardial ischemia—an imbalance between the supply and requirement of the heart for oxygenated blood by a partial or complete blockage of coronary arteries. Ischemia includes, lack of availability of oxygen, nutrients and inadequate removal of metabolites consequent to insufficient blood supply(Libby and Theroux, 2005). Myocardial infarction (MI), the death of cardiac muscle resulting from prolonged severe ischemia is the most important form of IHD. Acute MI (AMI) most often occurs when there is rupture of an atherosclerotic plaque into a coronary artery, which may cause thrombosis and occlusion of the artery, stopping the blood supply to that region of the heart and causing necrosis of the affected area (Falk et al., 1995). Restoration of coronary blood flow after myocardial infarction (MI) often aggravates the extent of damage (Ischemia reperfusion injury-IR injury). IR injury is related to the increased reactive oxygen species (ROS), calcium overloading, and the loss of membrane phospholipids especially during the reperfusion (Buja, 2005). The damaged myocardium experiences an immediate loss of contractility and necrosis starts developing 15-30 minutes after coronary occlusion. During the following 3-6 hours, the necrotic region grows and sometimes even spans a large portion of the ventricular wall. After 2-3 months, the infarct heals, leaving a fibrotic, non-contracting region in the ventricular wall, known as the fibrotic scar. The rest of the myocardium may not be able to compensate for compromised function of the ischemically injured region and the consequent increased work load. Since the heart possesses only a limited regenerative capacity, it activates a number of other compensatory mechanisms

such as the Renin– Angiotensin–Aldosterone pathway. These factors eventually lead to increased wall stress with secondary changes in myocyte size, metabolism, and gene expression, as well as ventricular dilation and ultimately reduced systolic function. Collectively this process is known as myocardial remodeling and is the phenotypic hallmark of HF.

The main cause of myocardial ischemia is reduction in coronary blood flow due to atherosclerotic plaque formation and gradual obstruction of coronary artery (Figure 1). Thus, ischemic heart disease is often termed coronary artery disease (CAD) or coronary heart disease which usually represents a continuum of illnesses ranging from coronary atherosclerosis, myocardial infarction (MI), post infarct heart failure (HF), and ultimately to end-stage heart failure (Mann et al., 2014). Despite dramatic advances in the treatment of coronary heart disease including pharmacological, interventional and surgical therapies, ischemic heart disease remains one of the leading cause of morbidity and mortality world-wide (Roger et al., 2012). Irrespective of the aetiology, most cardiovascular diseases ultimately lead to heart failure (HF), which is progressive and irreversible (Bui et al., 2011).

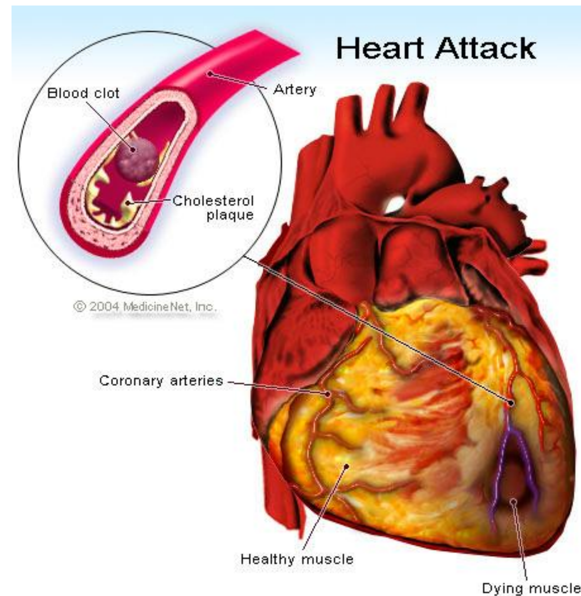


Figure 1: Myocardial infarction due to reduced blood flow to the heart muscle (Adopted from www.medicine net.com)

II.2 Current therapies for Heart Failure

II.2.1 Medical therapy

Conventional pharmacological agents for HF include diuretics, angiotensin-converting-enzyme inhibitors (ACEIs), beta-blockers, angiotensin-receptor blockers (ARBs), and aldosterone antagonists (McMurray, 2010). These drugs act mainly through the modification of ventricular remodeling and the systemic responses, (sympathetic and renin-angiotensin-aldosterone systems) (McMurray, 2010). Diuretics are proven to be helpful in the management of fluid retention by getting rid of excess salt and fluid to alleviate symptoms such as dyspnea and edema (Dickstein et al., 2008, Hunt et al., 2009). As the first-line therapy, ACEIs have been shown to improve symptoms, decrease

ventricular hypertrophy and augment the ejection fraction (EF) moderately (Dickstein et al., 2008, Hunt et al., 2009). Like ACEIs, beta-blockers are also in the list of first-line drugs in patients with HF, which can increase the EF and improve left ventricular function if tolerated. Angiotensin II Receptor Blockers (ARBs) are similar in action to ACEIs in patients with chronic HF (Cohn et al., 2001). Other drugs, such as hydralazine/ isosorbide dinitrate digoxin, dobutaminetc are also given to improve symptoms and quality of life depending on patients' requirements. Medical treatment provided to date can slow down, but not prevent, the progression to end-stage HF

II.2.2 Interventional therapy

Interventional therapy, which is minimally invasive than surgery, includes percutaneous transluminal coronary angioplasty (PTCA), implantable cardioverter-defibrillator (ICD) and biventricular cardiac pacing (cardiac resynchronization therapy; CRT), which could benefit patients with HF under certain circumstances. Elective PTCA improves symptoms and heart function in patients with ischemic HF and viable myocardium by coronary revascularization with or without the use of stents. But there are chances of restenosis to occur in 25% to 30% of the patients(Hurst, 1987). A systematic review of clinical trials using ICDs showed reduction of mortality in HF patients with a high risk of sudden cardiac death (Ezekowitz et al., 2003).

II.2.3 Surgical therapy

Coronary artery bypass graft (CABG) is an effective treatment in patients with chronic ischemic cardiomyopathy, or reversible myocardial ischemia, and leads to better outcomes than medical therapy (McCarthy, 2008). CABG is shown to alleviate symptoms and reduce the risk of death among patients who have limiting angina that persists despite optimal medical treatment and suitable coronary anatomy (Sobolev et al., 2008) .

The gold standard therapy for end-stage HF remains heart transplantation, which improves patients' symptoms (95% symptom-free rate) and extends their life span with about 90% 1-year survival and 60% 10-year survival (Taylor et al., 2007). Nevertheless, patients receiving heart transplantation require lifelong immunosuppression and face the possibilities of severe post-operative complications, such as primary graft failure (PGF), and transplant vasculopathy.

II.2.4 Stem cell based therapy

In an attempt to replace cardiomyocytes lost after ischemia, cellular therapy/ cardiomyoplasty has been conceptualized as a viable option in the last few years.

Over the past decade, several preclinical studies(Orlic et al., 2001b, Barbash et al., 2003b, Quevedo et al., 2009b) and early small and intermediate sized clinical trials(Assmus et al., 2002, Strauer et al., 2002, Fuchs et al., 2003,Makkar et al., 2012, Bolli et al., 2011) have examined the effects of stem cell-based cardiac therapy that aims to replace noncontractile fibrous scar tissue after MI and modulate myocardial remodeling.

II.3 Stem cells

Stem cells are capable of self renewal, meaning that they can divide and give rise to stem cell progeny with similar properties. They are clonogenic ie each cell can form a colony in which all the cells are derived from this single cell with identical genetic makeup. In adult mammalian tissue, stem cells participate in normal tissue homoeostasis through repair and regeneration on injury. A variety of stem cell types have been experimented for their myocardial regeneration potential, which include bone marrow mononuclear cells, embryonic stem cells, mesenchymal stem cells, induced pluripotent stem cells, umbilical cord derived cells and cardiac stem cells. For stem cell therapy to be effective, picking up the proper cell type and delivery options are important. Survival, retention and integration of the transplanted cells in the ischemic myocardium are the challenges of stem cell based therapy

II.4 Ideal stem cell for myocardial regeneration

Identifying the ideal cell type for myocardial repair is one of the important challenges in stem cell based regenerative therapy. Chronically infarcted myocardium lacks functional blood vessels as well as efficient contractile machinary. Hence, there is a need for angiogenesis, myogenesis and electrical integration of the delivered cells. The ideal cell then for postinfarction cardiac repair would be an ischemia resistant electrically compatible cell, capable of directing new vessel growth and myocyte formation (Jameel and Zhang, 2010). This could occur either via a direct differentiation of transplanted cells or by an indirect mechanism whereby the injected cells promote myogenesis and

angiogenesis via stimulation or recruitment of endogenous cells in the heart (Chimenti et al., 2010). Injected cells are exposed to a hostile, ischemic environment. The more cardiospecific and ischemia-resistant a cell is, the higher its engraftment rate will be. For clinical applications, a regenerative cell source should be (i) abundant, (ii) accessible, (iii) easy to deliver within the scope of a single operation, (iv) tested and optimized in appropriate preclinical models, (v) non-tumorigenic and non-immunogenic, and (vi) should stimulate regeneration in a predictable manner with a well characterized dose response (Russo et al., 2014)

II.5 Stem cells used for myocardial regeneration

II.5.1 Skeletal myoblasts

Myoblasts or satellite cells are precursor cells attached to skeletal muscle fibers. In normal muscles, satellite cells are mitotically quiescent, but in response to signals released following damage or in response to increased workload, they will become activated to divide (Danoviz and Yablonka-Reuveni, 2012). After division, satellite cell progeny (myoblasts) undergo terminal differentiation and become incorporated into mature muscle fibers as post-mitotic myonuclei. Early trials delivering skeletal myoblasts to heart failure patients showed long-term functional benefits with evidence for persistence of functional skeletal muscle within transplanted hearts (Menasché et al., 2001). Unfortunately, some patients experienced some form of ventricular tachycardia (Menasché et al., 2003, Smits et al., 2003, Siminiak et al., 2004). This was followed by several clinical trials including

MAGIC (Myoblast Autologous Graft in Ischemic Cardiomyopathy) trial (Menasché et al., 2008). MAGIC trial was a Phase 2 randomized clinical trial. The intracardiac electrophysiological inhomogeneities created by electrically uncoupled skeletal muscle cells gave rise to a high incidence of life-threatening arrhythmias. Investigations in small and large animals with ischemic and nonischemic cardiomyopathies have established that myoblasts injected into damaged myocardium can survive in small numbers, proliferate and differentiate into skeletal multinucleated myotubes. Although these cells may align with cardiomyocytes, they do not express gap-junction proteins, such as connexion-43, and do not form connections with host cardiomyocytes. Lineage restriction of skeletal myoblasts prevents differentiation of myoblasts into cardiomyocytes and also prevents teratoma formation in the heart. In a critical review, Durrani and coauthors conclude that use of skeletal myoblasts for myocardial repair has advantages as well as disadvantages. The Authors suggest that skeletal myoblast transplantation accompanied by appropriate interventions such as use of supplements or genetic reprogramming can promote integration, and improve the survival and therapeutic efficacy in clinical conditions (Durrani et al., 2010).

II.5.2 Bone marrow mononuclear cells (BMMNCs)

Lineage-unselected bone marrow derived mononuclear cells from bone marrow aspirates have been used for a significant number of preclinical and preliminary clinical studies. BMMNCs (CD34+ and CD133+) are the widely used bone marrow cell types in clinical trials for patients with acute MI and ischemic cardiomyopathy. Over the last decade these studies have confirmed that BMMNCs administration is safe and feasible, but clinical

advantages to date have not been impressive due to low cell engraftment and lack of cardiomyocyte differentiation potential. Early small animal studies have shown that intramyocardial injection of BMMNCs improve cardiac contractility, reduce infarct size and fibrosis, and differentiate into cardiomyocytes and endothelial cells (Orlic et al., 2001) (Kudo et al., 2003a). However, later research showed that these cells quite often do not differentiate into cardiomyocytes (Balsam et al., 2004, Murry et al., 2004). The exceptional ability of BMMNCs to undergo cell fusion and thereby achieve the phenotype of the cell with which they have fused can explain the apparent transdifferentiation ability of BMCs to cardiomyocytes (Terada et al., 2002). Clinical trials such as TOPCARE-AMI (Assmus et al., 2002), REPAIR-AMI (Lunde et al., 2006), BOOST (Wollert et al., 2004) and FINCELL (Huikuri et al., 2008)- studies comparing autologous BMMNCs to a placebo control- have shown increases in left ventricular ejection fraction (LVEF) in BMMNC treated patients in follow up studies up to 18 months. Long-term follow up for 5-years demonstrated functional benefits in the TOPCARE-AMI trial (Leistner et al., 2011) but not in the BOOST trial (Meyer et al., 2009). Other clinical trials like ASTAMI (Lunde et al., 2006), BONAMI (Roncalli et al., 2011), Leuven-AMI (Janssens et al., 2006), and HEBE (Hirsch et al., 2011) showed no significant increase in left ventricular ejection fraction and myocardial regeneration over the control group. A Phase I trial (Hare et al., 2009a) with allogeneic BMMNCs by Osiris Therapeutics Inc. showed an enhancement in LVEF at 6 months.This study didnot show any improvement in the patient physical performance, as measured by the six minute walk test, highlighting the need for a consensus on standardized accepted metrics for evaluation of the efficacy of

cardiac cell based therapy. Trials carried out by the Cardiovascular Cell Therapy Research Network- TIME (Traverse et al., 2012) and LateTIME- indicated no clinical benefit of BMMNCs in acute myocardial infarction (AMI),. Similarly, no clinical benefit was noted in a trial investigating transendocardial delivery of BMMNCs for heart failure (FOCUS-CCTRN) (Perin et al., 2012). The current ongoing multicentre studies (REVITALIZE (NCT00874354), REGEN-AMI (NCT00765453), REPAIR-ACS (NCT00711542), SWISS-AMI (NCT00355186) and BAMI (NCT01569178)) are aimed to investigate the benefits of autologous bone marrow cell therapy. The overall negative results of completed trials have encouraged exploration of other cell types or "next-generation" cell therapy, where cells are subjected to screening assays to predict regenerative potential before cell transplantation (Behfar et al., 2010). The moderate improvement obtained by of BMMNC can be explained by the paracrine hypothesis, where angiogenic, anti apoptotic and antifibrotic chemokines and growth factors, are secreted by transplanted cells, that encourage cardiac regeneration (Gnecchi et al., 2006). This hypothesis has been supported experimentally through demonstration that BMMNC conditioned media can replicate the effects of stem cell therapy (Gnecchi et al., 2008).

II.5.3 Embryonic stem cells (ESCs)

Embryonic stem cells (ESCs) are derived from the inner cell mass of the developing embryo during the blastocyst stage. hESCs have a normal karyotype, maintain high telomerase activity, and can differentiate into more than 200 cell types of the adult body (Thomson et al., 1998). They have shown the greatest cardiac differentiation potential among all other stem cells (Bradley et al., 2002). A probable challenge for their clinical

use is immunological incompatibility as a result of their allogenic origin. The cell surface antigens used to distinguish hESCs are stage-specific embryonic antigens (SSEA) 3 and 4, and keratin sulfate antigens Tra-1-60 and Tra-1-81(Draper et al., 2002). Genetically-selected mESC derived cardiomyocytes, formed intra cardiac grafts in the mouse heart, which were found to be stable upto 7 weeks (Klug et al., 1996). In another infarcted rat heart model, the transplantation of differentiated mESC-derived cardiomyocytes resulted in long lasting improvement of myocardial performance (Min et al., 2003). When undifferentiated mESC were injected into non-syngeneic or immunotolerant animals, there was generation of treatomas and rejection of cells posing another (Ménard et al., 2005). Their clinical use has been hindered by significant limitations, including their potential tumorigenic and immunogenic properties as well as ethical issues related to their origin (Rutger-Jan Swijnenburg, 2005).

II.5.4 Hematopoietic Stem Cells

Hematopoietic stem cells are characterized by the markers- $CD150^{+}$, $CD48^{-}$, $CD244^{-}$(Oguro et al., 2013). Additionally, hematopoietic stem cells (HSCs) are normally recognized by the expression of CD34 and CD133 cell surface markers. They are shown to transform into cardiomyocytes under strict laboratory conditions. There is as yet no evidence that they can transdifferentiate into cardiomyocytes when transplanted into infarcted myocardium (Nygren et al., 2004, Balsam et al., 2004).

II.5.5 Endothelial Progenitor Cells (EPCs)

EPCs are bone marrow derived cells that are mobilized into peripheral blood and can differentiate into mature endothelial cells (Calzi et al., 2010). They express endothelial lineage markers (CD34+, Flk-1+, VE-cadherin, PECAM-1, von Willebrand factor, eNOS, and E-selectin) and share many surface marker antigens with hematopoietic stem cells (Flk-1, Tie-2, c-Kit, Sca-1, AC133, and CD34) (Levenberg et al., 2007). It has been shown that EPC cell number increases in vascular circulation soon after a myocardial infarction. EPCs are thought to react to ischemic damage in the heart (and other tissues) and migrate to injured areas to induce angiogenesis. A study by Aicher et al showed that when EPCs were injected into either the tail vein or the left ventricular cavity of rats with ischemic myocardial injury, there was more than twofold enhancement in the accumulation of infused EPCs when compared with animals undergoing sham surgery (Aicher et al., 2003). Rats undergoing intravenous injection of EPCs following ischemic myocardial injury showed improved ventricular function, fractional shortening, and regional wall motion when compared with control animals injected with culture media (Kawamoto et al., 2001, Yamahara and Itoh, 2009). To date, EPCs have not been shown to differentiate into myocytes within injured myocardium, but they act through paracrine mechanisms and neovascularization (Rubart and Field, 2006). The efficacy and feasibility of intracoronary and intramyocardial injection of endothelial progenitor cells were demonstrated in clinical trials, resulting in moderate functional benefits (Stamm et al., 2007, Bartunek et al., 2005).

II.5.6 Umbilical Cord Blood Cells (UCBCs)

The blood cells present in the umbilical cord blood are a rich source of hematopoietic, endothelial and mesenchymal stem cells. They can be easily harvested and expanded invitro. UCBCs can self-renew, proliferate and differentiate into varying lineages including cardiomyocytes. They are less immunogenic, with lower incidence of graft-versus-host disease as compared to other varieties of stem cells. Studies in animal models demonstrate that intramyocardial injection of unfractionated cord blood cells showed the potential to improve LV ejection fraction and anteroseptal wall thickening while decreasing infarct size. hUCBCs have significantly limited the size of myocardial infarctions and reduced left ventricular remodeling, thereby preserving LV ejection fraction and rate of rise of left ventricular pressure (dP/dt) without requirements for host immune suppression (Henning et al., 2004, Henning et al., 2006, Henning et al., 2007, Henning et al., 2012, Ma et al., 2005). No evidence yet proposes that cord blood cells injected into infarcted portions of the myocardium have the potential to differentiate directly into mature cardiomyocytes (Moelker et al., 2007).

II.5.7 Mesenchymal stem cells (MSCs)

MSCs are the nonhemotopoetic population in the bone marrow, representing about 0.001–0.01% of total nucleated cells. Apart from the bone marrow, MSCs have been identified in almost every tissue type such as brain, spleen, liver, kidney, lung, muscle, skin, adipose tissue, thymus, aorta, vena cava and pancreas. The most abundant source is the BM. In culture, they possess a spindle-shaped fibroblast-like appearance and are capable of expanding abundantly, without loosing their plastcity. Human MSCs can differentiate into

a cardiomyocyte-like phenotype in adult mouse hearts, expressing β-myosin heavy chain, α-actinin, cardiac troponin I, and phospholamban. Numerous animal studies have demonstrated that transplantation of bone marrow (BM) mesenchymal stem cells improves cardiac function in settings of acute myocardial infarction and ischemic left ventricular dysfunction (Kwon et al., 2013, Nagaya et al., 2004, Bhattacharya and Stubblefield, 2014). In chronic ischemic heart disease, intramyocardial injections have been performed using electromechanical mapping via catheter based delivery and showed modest improvements in left ventricular function(Perin et al., 2012)

MSCs possess immunomodulatory effects, and they do not evoke any immune response on allogenic transplantation due to the inhibition of T-cell proliferation(Patel et al., 2008). MSCs are shown to express HLA (human leukocyte antigen) class I, but not HLA class II antigens on their cell surface membrane. They do not express Class II antigens or the B7 and CD40 ligand costimulatory molecules. Undifferentiated as well as differentiated MSCs do not show proliferative lymphocytic immune responses after allogenic transplantation (Le Blanc et al., 2003).

MSCs were first identified by, Friedenstein et al in the 1970s (Friedenstein et al., 1970). They were the earliest researchers to demonstrate the potential of MSCs to differentiate into mesoderm-derived tissue. Later, different research groups established that MSCs can differentiate into osteoblasts, chondrocytes, and adipocytes (Caplan, 1986, Piersma et al., 1985). In the 1990s, Wakitani et al. showed that MSCs can differentiate into a myogenic phenotype (Wakitani et al., 1995). The ability of mouse BM-derived

MSCs to form cardiomyocytes was reported for the first time by Makino et al.1999 (Makino et al., 1999), and later on Toma et al. showed the same findings in vivo (Orlic et al., 2001a). In 2001, Orlic et al. first demonstrated in a mouse model of MI that MSCs can form new cardiac tissue with an improvement in cardiac contractility (Ryan et al., 2005). Two years later, Kudo et al. demonstrated that bMSC transplantation decreased fibrosis and infarct size, by differenting into cardiomyocytes and endothelial cells within the injured myocardium (Kudo et al., 2003b). Zhang et al compared the effects of intramyocardial injection of bMSCs versus PBMCs and demonstrated that functional improvement with bMSCs (Schuleri et al., 2007). Subsequently, numerous preclinical studies reported reduction of infarct size, after transplantation of MSCs and improved left ventricular (LV) function in small and large animal models- mice (Kudo et al., 2003c, Fazel et al., 2005), rats (de Macedo Braga et al., 2008, Nagaya et al., 2004), swine (Shake et al., 2002), sheep (Houtgraaf et al., 2013).

II.5.7.1 Animal studies using MSCs

In the ischaemic mouse heart, implantation of $5x10^4$ to $5\ x10^5$ bone marrow stem cells, containing MSCs, reduced infarction size and fibrosis (Kudo et al., 2003a). In the porcine MI model, direct injection of $6x\ 10^7$ MSCs into the infarct resulted in engraftment in all animals with expression of muscle-specific proteins, resulting in attenuation of contractile dysfunction and decreased wall thinning (Shake et al., 2002). Studies by Mangi et al have suggested that injection into ischemic myocardium of $5\ x\ 10^6$ genetically engineered rat

MSCs using retroviral transduction to overexpress the pro survival gene Akt1 resulted in regeneration of 80–90% of lost myocardial volume and normalized left ventricular systolic and diastolic cardiac functions (Mangi et al., 2003). In a subsequent study, a highly proliferative bMSC subpopulation was isolated from human bone marrow by Yoon et al (Yoon et al., 2005). Intramyocardial injection of this subpopulation in a nude rat model resulted in a higher degree of engraftment and differentiation as compared to unselected bMSCs, and also induced endogenous cardiomyogenesis. In the swine model, Shake et al. reported strong engraftment of labeled MSCs along with expression of numerous muscle-specific proteins as early as two weeks after intramyocardial implantation. This study proposed that the differentiation of MSCs into cardiomyocyte like cells occurs two weeks after transplantation, followed by a significant improvement of contractile dysfunction and wall thinning (Shake et al., 2002). A similar study by Schuleri et al. showed that intramyocardial transplantation of MSCs resulted in a significant increase of LV function eight weeks after transplantation (Schuleri et al., 2009). These improvements were preceded by an early enhancement of resting myocardial blood flow after one week, which was confirmed by an increase in vessel size in the MSC group compared to the control groups. These observations suggest that transplantation of MSCs can ameliorate cardiac function by reducing infarct size, triggering neovascularization and cardiomyogenesis.

II.5.7.2 Clinical trials with MSCs for myocardial regeneration

By using large animal models, the majority of MSC transplantation studies have proven to be safe and effective. Based on the promising results obtained in animal models, several

clinical trials investigating bMSCs in the treatment of MI have been initiated (Table 1). Chen et al, 2004 was the first to investigate the outcomes of intracoronary injection of autologous BM-MSCs (8–10 $\times 10^9$ cells) in acute MI patients. They showed that that MSC therapy is safe and devoid of deaths and arrhythmias during the follow-up period of three-months. Improvements in myocardial perfusion, LV ejection fraction, and LV chamber dimensions were apparent in MSC-treated patients in comparison with placebo. In 2005, Katritsis et al. investigated the effect of a combination of intracoronary transplantation of BM derived MSCs and EPCs (2–4 $\times 10^6$ cells) on tissue repair in myocardial scars of patients with an anteroseptal MI (Katritsis et al., 2005). A significant reduction in wall motion score index and significant increases in myocardial regeneration and contractility was observed in stem-cell treated patients compared to untreated controls. The safety and practicability of MSCs therapy was investigated by Mohyeddin-Bonab et al in a pilot study of eight patients with old MI (Mohyeddin-Bonab et al., 2007). After 6–18-month followup, they revealed reduced perfusion defect and enhanced ejection fraction, without any adverse side effects. The first clinical trial of MSC transplantation for cardiac regeneration in the United States was conducted by Osiris Therapeutics. They administered allogenic MSCs by intravenous infusion. MSC-treated patients showed improved heart and lung function along with lesser arrhythmic events compared to placebo group after 6 months. Safety of intravenous allogenic transplantation of MSCs in post-MI patients were confirmed by another double-blind, placebo-controlled, dose-ranging trial by Hare et al (Hare et al., 2009a). MSC administration reduced ventricular arrhythmias, improved pulmonary function and increased LV ejection fraction in patients

after 3 months. In 2011, Williams et al. investigated the functional benefits of transendocardial injection of MSCs in patients with chronic ischemic cardiomyopathy secondary to MI (Williams et al., 2011). At 3 months followup MSC-treated patients showed reduced cardiac remodeling and increased regional contractility, and these effects continued up to one year. There was no evidence of ectopic tissue growth or persistant arrhythmias at one year after transplantation. This data indicates the safety and efficacy of MSC therapy for post-MI transplantation. In another clinical trial (C-CURE) for the treatment of ischemic cardiomyopathy, Bartunek et al guided cardiopoietic MSCs to viable but defective myocardium by electromechanical guidance (Bartunek et al 2005). At 6-month follow up, the results showed significant increase in ejection fraction and functional performance in cardiopoietic MSC therapy group compared to controls. There was no evidence of cardiopoietic MSC-induced arrhythmias or toxicity.

Concomitantly, a number of reports have raised concerns about arrhythmic events and tumor formation as a result of using BM-cultured MSCs. In these reports murine-derived BM-MSCs exhibited chromosomal abnormalities that led to tumor formation in many organs (Jeong et al., 2011, Miura et al., 2006)]. Studies have revealed that both MSCs and BM-derived stem cells are associated with calcification and possibly ossification of the heart in a murine model of MI (Breitbach et al., 2007). Contrary to these observations, numerous small and large-animal preclinical studies have demonstrated the safety of MSCs therapy without any incidence of tumor formation or ectopic tissue growth (Amado et al., 2005, Amado et al., 2006, Schuleri et al., 2008, Schuleri et al., 2008, Silva et al., 2005). Early-phase human clinical trials using MSCs showed no evidence of ectopic

tissue growth (Hare et al., 2009a), Williams et al., 2011). However, the data of tumorigenesis in murine models call attention to the necessity of consistent long-term monitoring of patients treated with MSCs. Furthermore, other reports have shown that intracoronary injection of MSCs in canine and swine models of MI resulted in microinfarctions and slow coronary arterial flow due to microvascular obstruction (Freyman et al., 2006b, Vulliet et al., 2004). MSC therapy has been shown to be secure, feasible and efficient in improving LV function, reducing scar size and increasing myocardial tissue regeneration and angiogenesis post-MI in small and large animal models. But it is hard to assess the impact of these preclinical studies on MI patients. Long term follow-up studies and other comparative studies are needed to reach a conclusion regarding their bedside applications.

Table 1: MSC clinical trials in heart diseases.

Group	Condition	Dose(mode of transplantation)	Follow up (Months)	Results
(Chen et al., 2004)	Acute MI	8-10×10^{9}(IC)	3	↑Myocardial perfusion,↑LVEF, and ↓LV chamber dimensions
(Katritsis et al., 2005)	Anteroseptal MI	2-4×10^{6}(IC)	4	↓Wall motion score index and ↑myocardial viability and contractility
(Mohyeddin-Bonab et al.,	Old MI	2.1-9.1×10^{6}(IC)/(DI)	6-18	↓Perfusion defect and ↑LVEF

2007)				
Osiris therapeutics(Hare et al., 2009a)	Acute MI	(IV)	6	↑Heart function and ↓arrhythmic events
(Williams et al., 2011)	Chronic ischemic cariomyopathy secondary to MI	10 repeated injections of 0.5mL of cell suspension (TESI)	3-12	↓Cardiac remodeling,↓ ESV and EDV, and ↑regional contractility
C-CURE (Bartunek et al., 2005)	Heart failure secondary to ischemic cardiomyopathy	6-12×10^8 (EMG)	6	↑LVEF and ↓ESV and EDV

MI: myocardial infarction; IC: intracoronary infusion; DI: direct intramyocardial injection; IV: intravenous infusion; TESI: transendocardial stem cell injection; EMG: electromechanical guidance; LV, left ventricular; EF: ejection fraction; ESV: end-systolic volume; EDV: end-diastolic volume.

II.5.8 Cardiac stem cells

Heart was believed to be a terminally differentiated organ, incapable of repairing any form of damage. Recent studies have reported evidence that myocardium itself contains a resident progenitor cell population capable of giving rise to new cardiomyocytes. Cardiac stem cells are tissue specific progenitor cells, which are limited in number, but can be isolated and expanded invitro. Different membrane markers (C kit, Sca-1, Abcg-2, and Flk-1) and transcription factors (Isl-1, Nkx2.5, and GATA4) have been employed to identify and characterize these cells. These cells most likely mediate endogenous mechanisms for normal homeostasis of the heart, and contribute for ongoing cell turnover

within the adult heart. These cells are clonogenic and induce both myocardial and vascular regeneration after MI.

II.6 Cardiac c-kit^{+} stem cells

Tyrosine protein kinase Kit (c-Kit) is considered as a potential marker for resident CSCs. The existence of resident cardiac c-Kit-positive (c-Kit+) cells was first described in 2003 when Beltrami and colleagues identified a population of multipotent, self-renewing, and clonogenic cells, in the rat heart, that were positive for c-Kit and negative for hematopoietic lineage markers (CD34 and CD45) (Beltrami et al., 2003). Cardiac c-Kit^{+} CSCs express cardiomyogenic markers, mainly early cardiac development transcription factors including Nkx2.5, myocyte enhancer factor 2C (MEF2C), and GATA4 at the early stage of differentiation indicating its committment to myocardial lineages. C-kit^{+}cells were found to have the capacity to generate millions of myocytes when injected into the ischemic myocardium expressing sarcomeric α-actin, cTnI, and connexin 43, without fusing with host cells. Long term pulse chase experiments showed that once committed to the myocyte lineage, a stem cell can undergo three to four rounds of cell divisions before their permanent withdrawal from the cell cycle. When delivered intra coronarily after IR injury, c-Kit^{+} cardiac stem cells reduced infarct size, attenuated LV remodeling and improved LV function. Thus engraftment of CSC resulted in replacement of dead tissue with viable myocardium (Beltrami et al., 2003).

Two different classes of c-Kit þositive CSCs cells were identified, the myogenic (c-Kit^{+}KDR^{-}kinase insert domain receptor) and vasculogenic (c-Kit^{+}/ KDR^{+}) CSCs. These

cells are found within the interstitial space between cardiomyocytes (myogenic c-Kit^{+} cells) or vasculature (vasculogenic c-Kit^{+} cells) within distinct clusters positioned in areas of low wall tension like the atrial appendages or cardiac apex (Hosoda, 2011).

c-Kit (CD117), the tyrosine kinase receptor for the stem cell factor, was initially reported to be the surface marker of hematopoietic stem cells. Studies in the past decade showed that c-Kit$^{[+]}$ subset of CSCs possesses substantial cardiac repair potential in various disease models of diverse conditions such as acute myocardial infarction (AMI)(Bearzi et al., 2007), ischaemia/reperfusion injury (Oh et al., 2003), myocardial infarction (MI) (Lara-Astiaso et al., 2012, Gorbunov et al., 2012), congenital heart defects (CHDs) (Mishra et al., 2011) diffuse myocardial injury (Ellison et al., 2013), diabetic cardiomyopathy (DCM)(Rota et al., 2006), chronic heart failure (CHF, Song et al., 2013).

The activation of cardiac stem cells in response to physiological or pathological stimuli is mediated by paracrine-autocrine feedback loop between myocytes and cardiac stem cells. Cardiac progenitor cells possess a HGF-c-Met and igf- IR receptor system and synthesize and secrete HGF and IGF. HGF promote cell migration through activation of c-Met. Expression of extracellular matrix metalloproteinases break down the extracellular matrix and favor cell locomotion, homing and tissue reconstitution which is important during myocardial regeneration post infarction (Linke et al., 2005, Urbanek et al., 2005). Insitu activation of c-Kit^{+} cardiac stem cells by growth factors, EGF-1 and IGF-1, assessed by locally administering these factors at different doses to the infarcted pig myocardium showed significant regeneration of the cardiomyocytes, arterioles and vessels in the

ischemic area (Ellison et al., 2011). The rate of regeneration was proportional to the doses of growth factor administered. Human c-Kit^+ cells were found in 1.8% ± 1.7% of the unsorted myocardial cell population and included lineage negative cells (52% ± 12%) as well as early committed cells (48% ± 12%). The number of CSCs (Lineage committed cardiac stem cells) within atrial niches was higher than in other areas and depended on the size of the niches (Urbanek et al., 2006). In a recent report, of a study carried out in the MI model in mini pig, Fanton et al concluded that autologous transplantation of atrial appendage cardiac stem cells preserved cardiac function by extensive engraftment and cardiomyogenic differentiation (Fanton et al., 2015). The study highlights the potential of cardiac atrial appendage stem cells for myocardial regeneration.

Administration of human c-Kit positive cells into the infarcted myocardium of immuno-deficient mice and immuno-suppressed rats, generated a chimeric heart, which contained human myocardium composed of myocytes, coronary resistance arterioles, and capillaries that integrated structurally and functionally with the myocardium of the host, enhancing the performance of the infarcted heart. The differentiated human cardiac cells had only one set of human sex chromosomes excluding cell fusion (Bearzi et al., 2007). This observation suggests that an individual's CSCs when expanded in culture and transplanted, it can promote regeneration of the injured myocardium. Kubo et al showed that c-Kit^+ CPCs can be isolated from nearly every human heart and that their number increases in patients with advanced heart failure requiring transplantation (Kubo et al., 2008). Significantly reduced Akt and p38MAPK activity and increased JNK and ERK activity was found in human senescent c-Kit^+ cells compared to control cells. ERK

inhibitors increased proliferation capabilities but differentiation was unaffected. Thus they concluded that specific inhibition of ERK during ex vivo expansion of human c-kit^{+} cardiac stem cells delays their senescence (Choi et al., 2013). In another study a developmental loss of cardiomyogenic differentiation potential in c-Kit^{+} cells in the transition from neonatal to adult life in mice was reported. A subset of the c-Kit^{+} cells from double transgenic neonatal hearts acquired a cardiomyogenic phenotype when cocultured with fetal cardiomyocytes but rarely when cultured alone or when cocultured with mouse fibroblasts. In contrast, c-Kit^{+} cells from normal adult double transgenic hearts failed to undergo cardiomyogenic differentiation when cocultured with nontransgenic fetal cardiomyocytes or when transplanted into normal or infarcted adult mouse hearts (Zaruba et al., 2010).

Administration of cardiac progenitor cells (CPCs) 4 hours after reperfusion was found to improve left ventricular function in rats with old MI. Here exogenous CPC administration activated endogenous CPCs, suggesting the role of paracrine mechanisms (Tang et al., 2010).When Intramyocardial injection of human fetal CSCs (36,000) was compared with two doses of adult MSCs (36,000 and 1,000,000) in nonobese diabetic/severe combined immune deficiency mice after coronary artery ligation, CSCs improved the myocardial infarction-induced enlargement in left ventricular chamber dimensions and increased ejection fraction compared to MSCs(Oskouei et al., 2012). Fengdi Yan in 2012 showed that hypoxia preconditioning of c-Kit+ CPCs activated antiapoptotic pathways and increased their migratory capacity invitro. The beneficial effects generated from the hypoxia preconditioning of c-Kit+ CPCs were mediated by the SDF-1a/CXCR4 axis

signaling pathway. Because of the promising evidence in pre-clinical animal studies, the first phase I human clinical trial using endogenous c-kit+ stem cells in patients with ischemic heart disease has been carried out. The SCIPIO trial by Bolli et al. was initiated to examine the safety and efficacy of intracoronary delivery of autologous CSCs. CSCs were isolated and expanded from right atrial appendages, of patients with ischemic cardiomyopathy. The initial results of the study are encouraging, confirming the safety and feasibility, of intracoronary infusion of autologous c-kit+ CSCs. At one year follow up, significant improvement in LVsystolic function and a substantial reduction in scar size was reported (Bolli et al., 2011).

II.7 Cardiac Sca-1+ stem cells

Stem cell antigen-1 (Sca-1), a member of the Ly-6 family, was first explaind as one of the cell surface markers expressed on hematopoietic stem cells (van de Rijn et al., 1989). Multipotent stem cells derived from bone marrow and skeletal muscles express Sca-1 (Asakura et al., 2002, Qu-Petersen et al., 2002, Gojo et al., 2003). Later various heterogeneous subpopulations of Sca- 1+ cells were identified based on different subsets of markers co-expressed with Sca-1. In 2003, Oh et al (Oh et al., 2003) for the first time isolated Sca-1+/CD31+ stem cells from the adult mouse heart , which were negative for blood cell lineage markers, c-Kit, Flt-1, Flk-1, CD34, and CD45. They expressed cardiac transcription factors such as Gata4, MEF2C and TEF-1. When stimulated with 5-azacytidine, these cells differentiated into cardiomyocytes with expression of cardiac specific genes Nkx2.5, cTnI, and MHC (Oh et al., 2003). When treated with oxytocin, a

population of Sca-1+, cells gave rise to spontaneously beating cardiomyocytes expressing cardiac transcription factors and contractile proteins (Matsuura et al., 2004). Animal studies demonstrated that administration of Sca-1+/ CD31- cells resulted in improved LVEF following MI by myocardial regeneration through paracrine effects, suggesting their therapeutic potential (Wang et al., 2006). Cardiac Sca-1+ stem cells from mouse heart was found to be self-renewing, clonogenic, and multipotent with the potential to differentiate into cardiomyocytes, both in vitro and in vivo(Matsuura et al., 2004, Oh et al., 2003, Tateishi et al., 2007,Tang et al., 2007, Wang et al., 2006). However, the human equivalent of the murine Sca-1 surface marker has not yet been identified. Zongjin Li in a mouse myocardial infarction model assessed CSC survival and therapeutic efficacy for restoration of cardiac function by multimodality noninvasive imaging techniques. They concluded that, Sca-1–positive CSCs provide no long-term engraftment and benefit to cardiac function. These cells are yet to be studied in large animal models and in clinical trials(Li et al., 2009)

II.8 Cardiac side population cells

The side population (SP) cells are characterized by their ability to efflux Hoechst 33342 (a DNA-binding dye) via the transporter, ATPbinding cassette sub-family G member 2 (ABCG2; CDw338). This Hoechst 33342 effluxing SP cells have been identified in several adult tissues such as bone marrow and skeletal muscle (Zhou et al., 2001). Hierlihy et al reported for the first time, the existence of a cardiac SP cell population with stem cell-like activity with cardiomyogenic differentiation potential in postnatal murine

myocardium (Hierlihy et al., 2002). Cardiac side population cells are heterogeneous in nature, consisting of different subpopulations of cells expressing CD31, CD34, VE-cadherin c-Kit, Sca-1, vascular endothelial cells, mesenchymal progenitors, and cardiomyogenic precursors in varying proportions (Yamahara et al., 2008). There are several reports that cardiac side population cells found in rodents are able to differentiate into cardiomyocytes, endothelial cells and smooth muscle cells in vitro (Martin et al., 2004, Pfister et al., 2005, Oyama et al., 2007). When infused into adult rats and mouse, these cells homed to the damaged myocardium and differentiated into three cardiac lineages (Liang et al., 2010, Oyama et al., 2007). More research is needed to explore their therapeutic effects on cardiac function upon transplantation into a myocardial infarction model.

II.9 Isl1+ cardiac progenitors

Isl1+ cells are cardiovascular progenitors which play a crucial role as developmental precursors in the creation of the right ventricle, atria and outflow tract (Urbanek et al., 2003). Isl1+ cells are multipotent progenitors, that can give rise to cells of all three cardiovascular lineages and are optimal for cardiac regeneration (Moretti et al., 2006). Neonatal and fetal tissues have abundant number of Isl1+ progenitor cells, yet with increasing age, Isl1 population is diminished to low or nonexistent levels (Fuentes et al., 2013). The fact that $Isl1^{+}$ cells are rare in the adult myocardium may make them undesirable candidates to use for cell based therapies. Further studies are needed to

explore the potential of human Isl1+ cardiovascular progenitors to be isolated and expanded for therapeutic use specifically from neonates where the cells are abundant.

II.10 Epicardium-derived stem cells

During the development of heart, cells derived from the proepicardial organ migrate over the myocardium to form the epicardium. Consequently these progenitor cells go through epithelial-to-mesenchymal transition, invade the underlying myocardium, and differentiate into specialized cells of different cardiac lineages. Epicardium-derived myocardial and vascular progenitors have been identified in embryonic mouse and adult human heart (Gittenberger-de Groot et al., 2010, Winter et al., 2007, Zhou et al., 2008). When transplanted into an immunodeficient mouse model of myocardial infarction, adult human EPDCs were found to reduce remodelling and increase ejection fraction (Madonna et al., 2011). The migration and angiogenesis of epicardium derived stem cells were found to get stimulated by thymosin beta 4, a small actin-binding protein that activates integrin-linked kinase (Smart et al., 2007).

II.11 Cardiospheres (CSs)

Cardiospheres are self-assembling multicellular clusters from the cellular outgrowth from cardiac explants cultured in suspension in poly–D Lysine plates. These heterogenous clusters contain a core of primitive, proliferating cells and an outer layer of differentiating cells that express cardiomyocyte proteins (Barile et al., 2013). Messina et al, , reported for the first time that cardiac precursor cells can be clonally expanded from murine and human myocardial biopsy specimens and form “spheres” in vitro (Messina et al., 2004b).

When outgrowth cells from cardiac tissue samples were maintained in suspension culture in growth factor enriched medium, these cardiac explant- derived cells spontaneously formed spherical aggregates resembling colonies of embryoid bodies, which after 24 hours, detached from the culture surface into free-floating 3D cardiospheres. Cardiospheres resemble stem cell niches, with a stem cell-rich core surrounded by supporting cells. After injection into the injured heart, cardiospheres showed multilineage differentiation potential and imparted functional benefits (Davis et al., 2009).

Cardiospheres express c-Kit, connexin 43, Nkx2.5, desmin and proliferative cells (Ki 67) in the core with reduced expression of MDR-1. Many cardiospheres manifest cardiomyocyte specific sarcomeric proteins (αMHC, cTnI) in the peripheral cells, indicative of partial myogenic differentiation. On their periphery, cardiospheres also express mesenchymal marker CD105, CD31and CD133. Cardiospheres are negative for CD34 and CD45 (Davis et al., 2009). These characteristics of cardiospheres resemble those of in vivo stem cell niches with enhanced ‘‘stemness’’(Li et al., 2010). qRT-PCR studies showed upregulation of Nanog and Sox2, two important pluripotent stem cell transcription factors, in cardiospheres. Many extracellular matrix protein and adhesion molecules, including laminin-β1, integrin-α2, and E-selectin were also found to be upregulated in cardiospheres. Cardiospheres are reported to have greater resistance to oxidative stress than monolayer- cultured cells.(Kawaguchi et al., 2013)

Cardiospheres under normal culture conditions express intermediate levels of the major histocompatibility complex I on their outer surface, but no evident MHC II or co-

stimulatory molecules CD 80 and CD 86 (Tseliou et al., 2013). MHC I expression on the outer layer, protect cardiospheres from the cytotoxic NK cells, while the absence of MHC II expression gives them the possibility to evade immune response by alloreactive CD4 lymphocytes. Cardiospheres under normal culture conditions express IL-6, IL-7, IL-8, transforming growth factor α, macrophage colony-stimulating factor, and the growth factors HGF and VEGF, thereby preventing local immune responses by avoiding T lymphocyte proliferation (Nian et al., 2004, Tasso and Pennesi, 2009). They are also reported to express stem cell relevant factors and adhesion/extracellular-matrix molecules (ECM) IGF-1, histone deacetylase 2 (HDAC2), Tert, integrin-α2, laminin-β1 and matrix metalloproteinases (MMPs).

Nuria Gago-Lopez et al divided clones derived from cardiospheres into two distinct populations based on the expression of thymocyte differentiation antigen 1 (THY-1/CD90). The first cell population was enriched for the cardiac transcription factor NKX2.5 and ISL1 but negative for mesenchymal stem cell/fibroblast surface marker CD90 and cardiac stem cell marker c-Kit. These $CD90^{-}$ cells differentiated into smooth muscle cells, endothelial cells, and cardiomyocytes, which form complete sarcomeres and beat spontaneously at a very low frequency. The second cell population was positive for CD90 and Periostin and appeared to be a cardiac mesenchymal/fibroblast cell that is NKX2.5low and ISL1low. This population showed endothelial and SMC differentiation. Their cardiac differentiation potential was incomplete, without sarcomere formation or calcium cycling. Therefore, these two populations of cardiogenic clones displayed distinct cell surface markers and unique transcriptomes.

Elvira Forte reported an up-regulation of the expression of epithelial mesenchymal transition (EMT) related genes during CS formation. They suggested that EMT underlies the formation of CSs and the subsequent differentiation of cardiogenic progenitors. They found that EMT is controlled by TGFβ signaling. Thus, cardiosphere and secondary cardiosphere formation involves the up-regulation of markers of EMT (e.g., SNAI1, TGFBR2, TGFBR1, and TWIST) and cardiac progenitors (e.g., TBX5, MEF2C, CDH5, KDR, NOTCH1, and CTNNB1)(Gago-Lopez et al., 2014a) .

II.11.1 Animal studies using cardiospheres

Implantation of cardiospheres in severe combined immunodeficiency (SCID) mouse hearts with acute myocardial infarction was found to improve cell engraftment and myocardial function, relative to monolayer-cultured cells (Li et al., 2010). But when these spheres were dissociated into single cells, the expression of ECM and adhesion molecules decreased, with reduced functional benefits. Deliang Shen, studied the dose dependent benefits of human cardiospheres, delivered via intramyocardial injection, in SCID mice with acute myocardial infarction. Hearts receiving high doses of CSps showed higher vascular densities than those in the low-dose group or the saline group. A similar dose-dependent improvement was found with the cell cycle re-entry of cardiomyocytes. More Ki67+ cardiomyocytes were apparent in hearts from the three high-dose groups (Shen et al., 2012)

Primary cardiosphere derived cells when grown in cardiosphere forming conditions, formed secondary cardiospheres (Cho et al., 2013). When transplanted into the infarcted

myocardium, secondary CSs showed better functional benefits including faster engraftment, improved left ventricular (LV) function, and reduced infarct sizes compared to primary cardiospheres. Robust vascular endothelial growth factor (VEGF) synthesis and enhanced neovascularization were also evident in the infarcted myocardium after secondary cardiosphere transplantation (Cho et al., 2012).

Eleni Tseliou et al investigated the safety and efficacy of allogeneic cardiosphere administration in a rat model of acute MI. Allogeneic cardiospheres conferred beneficial functional outcomes similar to those of syngeneic ones, without eliciting any harmful immune response. Administration of allogeneic cardiospheres increased tissue expression of the growth factors- vascular endothelial growth factor, hepatocyte growth factor and insulin-like growth factor-1, and stimulated angiogenesis in the peri-infarct region. These beneficial effects were sustained for at least 6 months, far beyond the time when allogeneic cardiospheres had been cleared from the site of administration (by 3 weeks) (Tseliou et al., 2013).

II.12 Cardiosphere Derived Cells (CDC)

Messina and colleagues first reported that cardiac progenitor cells that migrated directly from cardiac tissue can be further enriched within spherical aggregates, termed cardiospheres(Messina et al., 2004b). Smith et al, has advanced this method towards clinical translation by expanding cardiospheres into monolayers, on fibronectin coated dishes to yield cardiosphere-derived cells (CDCs)(Smith et al., 2007a). CDCs displayed antigenic and cytological similarities to cardiospheres, as well as discrete differences.

Cardiosphere-derived cells are clonogenic with multilineage differentiation potential, and exert promising functional benefits in preclinical models of myocardial ischemia (White et al., 2013). Under suitable conditions they are able to differentiate into cardiomyocytes, endothelial cells and vascular smooth muscle cells. The use of the cardiospheres, as an intermediate step provides a stem cell enriched starting material for ex-vivo cell expansion and minimize fibroblast contamination.

In the normal culture conditions CDCs express mesenchymal markers- CD 105, CD 90, myocyte specific proteins- connexin 43, α sarcomeric actin, cardiac stem cell markers- c-Kit, and endothelial markers- CD34, and CD31. These cells are negative for MDR1, CD133, CD45, and a cocktail of blood lineage markers, which implies that CDCs are phenotypically different from other types of cardiac stem cells recognized in vivo (c-Kit$^+$ MDR1$^+$). They are also distinct from bone marrow–derived or endothelial progenitor stem cell populations (CD133$^+$ CD34$^+$ CD45$^+$) (Smith et al., 2007a) that might enter the heart via the circulation. hCDCs were consistently negative for other Lin1 hematopoietic surface markers (CD3, CD14, CD16, CD19, CD20, and CD56).In vitro, CDCs express major histocompatibility complex (MHC) class I and not class II antigens or B7 costimulatory molecules. In mixed-lymphocyte cultures, allogeneic CDCs elicited no considerable lymphocyte proliferation and inflammatory cytokine secretion(Malliaras et al., 2012).

Several groups have demonstrated that CDCs survive, differentiate, secrete essential cytokines and growth factors and improve cardiac function after myocardial

infarction. Several small and large animal studies have shown that CDC administration is safe without any evidence of tumor formation, or arrhythmias. No excess life threatening events or mortality in cell-treated groups compared to placebo controls was reported. Administration of cardiosphere-derived cells enhance both cardiomyocyte proliferation and myocyte replenishment by recruited resident stem cells, which results in the regeneneration of healthy heart muscle (Malliaras et al., 2012). Potential mechanisms that lead to functional improvement after CDC administration include prevention of cardiomyocyte apoptosis, modulation of inflammatory processes, augmented angiogenesis, recruitment of resident stem cells and stimulation of cardiomyocyte proliferation (Malliaras and Marbán, 2011).

Chimenti et al showed that human adult CSs and CDCs release many proangiogenic and antiapoptotic growth factors in culture media, which mediate angiogenesis in human umbilical vein endothelial cells and antiapoptotic effects on neonatal rat ventricular myocytes in vitro. When CDCs were transplanted into the peri-infarct zone of a SCID mouse MI model, they secreted several essential growth factors including vascular endothelial growth factior 1 (VEGF1), hepatocyte growth factor (HGF), and IGF1. There was an upregulation of the prosurvival factor Akt, followed by reduced activation of caspase 3 and apoptosis. CDC administration increased capillary density, and enhanced cardiac function. Direct differentiation of the transplanted cells accounted for 20% to 50% of the observed effects. These findings reveal that the functional benefits obtained after CDC transplantation is mainly due to stimulation of endogenous repair mechanisms induced by paracrine factors while direct

cardiac differentiation of CDCs in situ is also having a contributory role (Chimenti et al., 2010).

CDC functionality was found to be correlated with age. When cocultured with neonatal cardiomyocytes, human neonatal CDCs were found to be more cardiomyogenic than CDCs derived from the adults. Human neonatal CDCs express higher levels of c-Kit, KDR, and Isl1, and are more angiogenic and cardiomyogenic than adult-derived CDCs, when transplanted in vivo into infarcted myocardium of immunodeficient rats (Simpson et al., 2012). A recent study in mouse shows that the regenerative potential of CDCs decreases with age, as demonstrated by decreased cell differentiation, reduced cell mobility and proliferation (Hsiao et al., 2014). In contrast, the proportion of cells expressing CD90 (Thy-1) was found to increase with age. Ke Cheng et al found that CDCs from heart failure patients outperformed CDCs from healthy or post-MI donor heart, in acute cardiac protection, and in attenuating LV remodeling. They demonstrated that the therapeutic potential of CDCs was not compromised by advanced cardiomyopathy; and end-stage heart failure gives superior functional potency to CDCs. HF CDCs also secreted higher levels of SDF-1 , which can promote new cardiomyocyte formation and stem cell recruitment (Cheng et al., 2014a).

The percentage of expression of CDC markers varies according to the tissue site from which they are isolated. Both atrial and ventricular epicardial CDCs contain variable numbers of $CD90^+$ cells and c-Kit^+ cells. There was wide variation in expression of CD90 in CDCs cultured from diabetic patients containing 1.6-fold more $CD90^+$ cells than those

from non-diabetic patients (Chan et al., 2012). White et al have demonstrated, by three independent molecular methods, that CDCs originate within the heart, with no significant extracardiac contribution. All three methods used—short tandem repeat analysis, FISH for sex chromosomes, and qPCR of recipient-specific HLA-A alleles—resulted in a concordant conclusion, thereby unambiguously differentiating CDCs from other non-cardiac cell types like BMMSCs (White et al., 2013).

II.12.1 Animal studies using CDCs

Smith et al (Smith et al., 2007a) did the first animal study to investigate the role of human CDCs injected into the border zone of myocardial infarcts. After injection, CDCs were found to engraft and migrate into the infarct zone. Twenty days after the injection, the left ventricular ejection fraction and the percentage of viable myocardium within the infarct zone was greater in the CDC-treated group than in the fibroblast-treated control group. Another study (Mishra et al., 2011) demonstrated the strong regenerative ability of hCDCs from congenital heart disease patients in immunodeficient rat model of myocardial ischemia. HCDC administration promoted greater myocardial regeneration and functional improvement in infarcted myocardium than cardiac fibroblasts. Cheng et al cotransplanted CDCs with platelet gel and compared that with transplantation of platelet gel alone. They demonstrated that pre-seeding platelet gel with CDCs maximized the therapeutic benefit with a healthier heart morphology (Cheng et al., 2012a). In situ polymerizable hydrogels made of hyaluronan and porcine gelatin (Hystem®-C™) were formulated as a liquid and administered along with CDCs in SCID mouse model of myocardial infarction (Linke et al., 2005). CDCs were injected intramyocardially in the

infarct border zone. Histological analysis suggested cardiovascular differentiation of the CDCs in Hystem-C™ with better engraftment and functional performance relative to CDCs alone.

Malliaras et al. compared between syngeneic, allogeneic, and xenogeneic CDCs for cardiac regeneration in vivo, in a rat model of myocardial infarction. Allogeneic CDCs induced a mild, transient, and local immune reaction in the heart, without any signs of immune rejection or systemic immunogenicity (Malliaras et al., 2012). They concluded that allogeneic CDC therapy is safe and do not require immunosuppression. Improvements in cardiac performance were comparable with syngeneic and allogeneic CDCs for a period upto to 6 months. Allogeneic CDCs stimulated paracrine pathways (HGF1, IGF 1 and VEGF 1 signalling) and activated endogenous repair mechanisms (cell cycling, recruitment of c-Kit+ cells, and angiogenesis) equally with syngeneic CDCs. Allogeneic human CDCs there by signify a potential off-the-shelf material for cell based heart therapy.

CDCs are the most widely studied cardiac progenitors in large-animal models of MI. Intracoronary infusion of CDCs to both healthy and infarcted pigs at 4 weeks after MI resulted in the formation of new cardiac tissue with reduction in the relative infarct size. Here, the engraftment was low and the benefits were modest. Significant differences were found in pressure change (dP/dt) maximum, and dP/dt minimum (Johnston et al., 2009). In an attempt to increase engraftment, cells were injected directly into the myocardium through thoracotomy, in a later study. Cardiospheres or CDCs from endomyocardial

biopsies were injected 4 weeks after MI that included 20 intramyocardial injections of 0.5 million cells. The injection of either cardiospheres or CDCs preserved LVEF than the placebo during the 8-week observation period. However, the cardiospheres were found to have superior advantage (over CDCs) in improving regional function, hemodynamics, and in attenuating ventricular remodeling (Lee et al., 2011). The long-term retention of CDCs at 8 weeks postinjection was confirmed by the presence of lacZ+-labeled cardiomyocytes. They found that highest percentage of engraftment in the border zone was associated with lowest dose of cells (0.5 million cells). But direct injection into the infarct scar didnot promote engraftment of cells in that area. This work provided the basis for the "Intracoronary cardiosphere-derived cells for heart regeneration after myocardial infarction" (CADUCEUS) clinical trial (Makkar et al., 2012).

Naofumi Takehara et al demonstrated that transplantation of hCDCs with controlled delivery of bFGF resulted in cardiomyocyte regeneration and stable angiogenesis resulting in a synergistically enhanced cell therapy approach in a pig model of chronic myocardial infarction. Two sets of randomized, placebo-controlled studies were carried out in pigs with anterior myocardial infarctions. Four weeks after MI, 14 pigs were randomly allocated for an intramyocardial injection of CDCs, with or without bFGF-incorporating hydrogel. In the second set, 26 pigs were randomized to give controlled release of bFGF combined with or without hCDCs or bone marrow derived mesenchymal stem cells. Synergistic effects of bFGF were observed in hCDC-injected animals, but were not in animals delivered with human bone marrow–derived mesenchymal stem cells (Takehara et al., 2008).

II.12.2 Current clinical trials using CDC

Cell-based therapies to regenerate the injured myocardium using cardiac progenitors expanded from the same patients (autologous) have been used in CADUCEUS (Makkar et al., 2012) and SCIPIO("Administration of cardiac stem cells in patients with ischemic cardiomyopathy") (Bolli et al., 2011) clinical trials. Both clinical trials reported two distinct functional results. The CADUCEUS (Makkar et al., 2012) was a phase I clinical trial performed to validate the safety of autologous CDC transplantation. The fully randomized clinical trial, consisted of 25 patients (17 treatment, 8 control), with an impaired EF (< 45%), 2-3 weeks after successful revascularization. CDC cultures were established from respective ventricular biopsies for intracoronary delivery (4-8 weeks after ventricular biopsy). No significant difference in patient deaths, carcinogenesis, or major adverse cardiac events occurred during 6 months observatory period after transplantation. CDC administration reduced scar mass, increased viable myocardial mass and improved regional contractility and systolic wall thickening. Final 1-year results revealed that regenerative efficiency of CDCs is not related to percentage of c-Kit+ cells, but it is related to baseline scar size, and inversely related to the percentage of CD90+ cells in CDCs (Malliaras et al., 2014), SCIPIO trial(Bolli et al., 2011), investigated the safety and practicability of using c-kit^{+} CSCs for the treatment of HF followed by ischemic heart disease. A total of 33 patients (20 treatment, 13 control) were enrolled in the SCIPIO trial. In this study, left atrial appendages were harvested from patients undergoing cardiac surgery (EF < 40%). Isolated c-Kit^{+} cells were expanded in culture to required dose (1 million c-Kit+ CSCs) for intracoronary delivery. Cells were

delivered into the surgical graft during coronary artery bypass grafting surgery at least 4 months after MI. At 2 years after CSC infusion, LVEF increased from a baseline of 29.7% to 41.7%, with simultaneous improvements in left ventricular viable mass (12.2% increase) and infarct size (15.7% decrease).

CADUCEUS laid the groundwork for a phase II trial, Allogeneic Heart Stem Cells to Achieve Myocardial Regeneration (ALLSTAR) (Makkar et al., 2014). This clinical trial was designed on the basis of the work by Malliaras et al., which demonstrated the safety and feasibility of allogeneic CDC administration in rats (Malliaras et al., 2012). Allogeneic CDCs reduces the delay associated with tissue harvest and might provide more appropriate delivery of CDCs immediately after infarction (Fuentes and Kearns-Jonker, 2013). In this on going study, patients will receive an intracoronary infusion of 25 million CDCs or placebo and will be followed for 12 months. This study, is now enrolling participants with LVEF < 45%. Cells will be transplanted, 3–12 months after myocardial infarction to optimize the ideal time interval for cell delivery.

Several promising clinical trials using cardiac progenitors are ongoing, but the results are yet to be published. The clinical trial "Transcoronary infusion of cardiac progenitor cells in patients with single ventricle physiology" or TICAP (NCT01273857),79 led by Hidemasa Oh, is intended to treat hypoplastic left heart syndrome in patients less than 6 years old (Ishigami et al., 2015).

The final phase I clinical trial that has been concluded and presented but not published is entitled, Autologous Human Cardiac-Derived Stem Cell to Treat Ischemic Cardiomyopathy (ALCADIA, NCT00981006), lead by Hiroaki Matsubara in Japan. This trial is designed to test the effectiveness of transplanting a combination of CSCs from endomyocardial biopsies and a hydrogel infused with FGF (200 mg), in patients of age 20-80 years with EF >15% and <35%. Injected cells expanded for this trial were positive for the surface markers- CD105, CD90, CD29, NANOG, OCT4, and GATA4 and negative for CD45. Patients received intramyocardial injections of human CSp (500,000 cells per kg of patient body weight). Transplantation of CSCs with controlled release of bFGF is reported to be safe and effective in patients with ischemic cardiomyopathy although final publication of the results has still not occurred. The outcomes of the clinical trial are consolidated in Table 2.

Table 2: Clinical trials using endogenous cardiac progenitors(Fuentes and Kearns-Jonker, 2013)

Clinical trial	ALCADIA (NCT00981006)	CADUCEUS (Makkar et al., 2012)	SCIPIO (Bolli et al, 2012)
Cell type	Autologous CDC with FGF hydrogel	Autologous CDC	Autologous c-kit^{+} cardiac derived cells
Number of treated patients	6	17	20
Inclusion criteria	LVEF 15%–35%, ischemia cardiomyopathy, past history of HF	LVEF 25%–45%, 30 days post-MI, history of angioplasty, area of regional dysfunction	LVEF , 40%, history of Q-wave MI, scheduled for CABG within 2 weeks
Cells collected	No data	30 days after MI	3–5 months post-MI
Number of injected cells	0.5 million cells/kg	12.5–25 million	0.5–1 million
Time frame for assessment	6 months	1 year	2 years
ΔLVEF	9.1% (3-D echo)12.1% (MRI)	5.4% (MRI), NS	12.0% (3-D echo)12.1% (MRI)
Increase in viable tissue	No data	22.6 g	12.20%
Decrease in scar mass	No data	12 g	15.7 g
Wall motion score	−10.6	No data	−3.92

II.13 Comparison of CDCs with other cell types

Remco Koninckx performed a comparative study between human mesenchymal stem cells (hMSCs), purified c-Kit$^+$ CSCs, and cardiosphere-derived cells (CDCs)(Koninckx et al., 2011a). They found that regardless of the difference in isolation protocol, both c-Kit positive CSCs and CDCs are similar in phenotype and functional characteristics. They might have originated from the same precursor cell or even are the same cells. They found that not only cardiac progenitor but also cells unrelated to heart tissue such as bone marrow–derived MSCs and even primary differentiated cells such as human dermal mesenchymal cells and myofibroblasts from colon are able to form spheres when maintained in cardiosphere-specific medium. They have shown that hMSCs are completely different from CSCs by their differentiation capacity towards adipocytes and osteocytes and the expression of CD140b, CD90 and EGFR on their membrane surface. In another study, Human cardiosphere-derived cells (CDCs), bone marrow–derived mesenchymal stem cells, adipose tissue– derived mesenchymal stem cells, and bone marrow mononuclear cells were compared (Li et al., 2012b). CDCs showed a unique phenotype with consistent expression of CD105, incomplete expression of c-Kit and CD90, and insignificant expression of hematopoietic markers. In the invitro conditions, CDCs showed the greatest cardiomyocyte differentiation potential, highest angiogenic potency, and higher production of various angiogenic and antiapoptotic factors. Injection of CDCs into the infarcted mouse hearts resulted in enhancement of cardiac function, cell engraftment, myogenic differentiation rates, and improvements in heart morphology 3 weeks after treatment. The c-Kit$^+$ subpopulation obtained from the whole CDCs, produced

lower levels of paracrine factors and modest functional benefits when compared with unpurified CDCs.

II.14 Stem cells and hypoxia

Stem cell cultures are conventionally carried out in ambient air, which would represent hyperoxia compared to the physiological O_2 levels of 5-10% O_2. Oxygen levels can have a significant influence on the stem cell characteristics. Stem cells normally reside in niches that have subphysiological O_2 levels. The advantage of culturing cells in lower O_2 levels is gaining importance. Enhanced proliferation of progenitor cells including mesenchymal stem cells (Grayson et al., 2007, Hung et al., 2012, Dos Santos et al., 2010), neural stem cells (Santilli et al., 2010) and embryonic stem cells (Forristal et al., 2010), in hypoxia has been reported. Yoshida et al (Yoshida et al., 2009) have shown that mild hypoxia is the optimal condition for both the generation and proliferation of induced pluripotent stem cells (iPSC) from human somatic cells in comparison with 1% or 21% O_2. Li et al observed that CDCs expanded in 5% O_2 had lower number of aneuploid and senescent cells and increase in cell migration from explants (Li et al., 2011a). Fink et al demonstrate that culture in 1% oxygen induced an adipose phenotype in bone marrow-derived MSC but no increase in adipocyte-specific genes (Fink et al., 2004). Fehrer et al. (2007) reported that bone marrow-derived MSCs (BM-MSCs) cultured in 3% O_2 concentration showed significantly increased in vitro proliferative lifespan, with approximately 10 additional population doublings (PDs) before reaching senescence compared to cells cultured in the ambient O_2 environment (Fehrer et al., 2007). MSCs

cultured in hypoxic conditions in each pasages exhibited increased proliferative lifespan along with significant difference in population doublings (Estrada et al., 2012). Higher in vitro expansion rate in hypoxic conditions has also been reported by several other researchers (Basciano et al., 2011, Grayson et al., 2007, Nekanti et al., 2010, Weijers et al., 2011). However, proliferation of MSCs was reduced significantly in 1% or less O_2 concentration (Holzwarth et al., 2010). Basciano et al. (2011) have reported improved osteoblastic and adipogenic differentiation potential of early passaged (P2) MSCs in 5% O_2 concentration(Basciano et al., 2011). Modulation of in vitro culture environment also allows to maintain a higher proportion of rapidly self-renewing MSCs for a longer period of time (Saller et al., 2012). Several other recent reports support that the multilineage differentiation potential of MSCs can be maintained under hypoxic (1–5% O_2 concentration) environment (Grayson et al., 2007, Nekanti et al., 2010, Holzwarth et al., 2010). Increased adipogenic and osteogenic differentiation potentials of adipose tissue-derived MSCs precultured in hypoxic environment have also been reported (Valorani et al., 2012). Tan et al reported that CDC culture can be optimized by preconditioning the cells under hypoxia (2% oxygen), which may reflect the physiological oxygen level of the stem cell niche. Under hypoxia, the CDC proliferation rate increased by 1.4-fold, generating CDCs with higher expression of cardiac stem cell and pluripotency gene markers compared to normoxia. Furthermore, telomerase (TERT), cytokines/ligands involved in stem cell trafficking (SDF/CXCR 4), erythropoiesis (EPO) and angiogenesis (VEGF) were increased under hypoxia (Tan et al., 2015). In a study conducted by Li et al, culture of cardiosphere derived cells in in physiological low oxygen

(5% O_2) conditions, rather than in traditional culture in a general CO_2 incubator (20% O_2) was found to minimise aneuploidy and improve cell quality. Compared with 20% O_2, 5% O_2 culture doubled the cell production and noticeably reduced the frequency of aneuploidy. Cells expanded in 5% O_2 showed lower intracellular levels of reactive oxygen species, less cell senescence, and superior resistance to oxidative stress than those grown in 20% O_2. The expression of stem cell antigens, adhesion molecules and paracrine secretion of growth factors into conditioned media remained same for both cultures. Transplantation of cells grown in 5% O_2 into mouse heart post MI resulted in better cell engraftment and enhanced functional recovery than with conventionally cultured cells (Li et al., 2011b). Another study suggested that the pro-angiogenic potential of CDCs is likely to be enhanced by preconditioning in sub-physiological O_2 (3%) and this may be useful for pro-angiogenic therapy following myocardial infarction (Amirrasouli et al., 2013). There are very few systematic studies on the response of CDCs to culture in physiological O_2 levels.

III. MATERIALS AND METHODS

III.1 MATERIALS

III.1.1 Fine chemicals

IMDM, DMEM-Hams F12Trypsin, DNase, FBS, anti-mouse IgM antibody, anti-rabbit antibody, SDS, Trizma base, agarose, Glycine, gelatine, Poly –D-lysine, Fibronectin, Amphotericin, coomassie protein assay reagent, DNase 1, endothelial cell growth supplement, propidium iodide,5-Azacytidine, Thrombine, polyclonal anti Desmin antibody, anti rabbit secondary antibody, antimouse secondary antibody, Triton X-100, para formaldehyde, crystal violet solution were purchased from Sigma-Aldrich, MO, USA.

The fine chemicals for cDNA synthesis including RT buffer, RNase inhibitor, oligo dt primers, dNTPs and M-MLV Reverse transcripatase were purchased from Promega corporation, Madison, WI, USA.

Polyclonal cardiac troponin I, Myosin Heavy Chain, Connexin 43, Ki 67, P16INK 4A, monoclonal c Kit antibody, ELISA kits- SDF-1, VEGF-1, HGF-1 were purchased from Abcam.

Osteogenic differentiation medium (HiOsteoXL ™ – Himedia) and Adipogenic differentiation medium (HiAdipoXL ™- HiMedia) were purchased from Himedia, India.

Matrigel, Endothelial growth factor, basic Fibroblast growth factor, DAPI were purchased from BD Biosciences, San Diego, CA

Power SYBR Green was purchased from Applied Biosystems, Penicillin (Alembic, India) B27 serum supplement (Invitrogen).

III.1.2 Routine chemicals

Sodium chloride, Potassium chloride, Sodium bicarbonate, Disodium hydrogen phosphate, Magnesium sulpahate, Phenol red, Potassium dihydrogen phosphate, Routine chemicals were purchased from Sigma, USA and Sisco Research Laboratories, India

Ethanol, Methanol, Isopropanol were purchased from Sisco Research labotories, Nice chemicals, Merk India.

III.1.3 Instruments used

Laminar Flow chamber (Clas, India), CO_2 water –jacketed incubator (Sanyo, USA), Hypoxia incubator (Eppendorf), High speed refrigerated centrifuge (Hitachi, Japan), Incubators (Beston India, Kemi, India), Weighing balance (Sartorius,USA and Ohaus), Homogenizer (IKA- Labortechnik, Germany) Deep freezer -20^0C (Vestfrost), Deepfreezer -80^0c(Sanyo), Steam distillation Unit (Beston), Microwave oven (IFB), Water bath (LKB , Sweden), Ice machine (Hoshizaki, Japan), pH meter (Labindia), Phase contrast microscope (Nikon, Japan), Phase contrast microscope (Olympus KX4, Philippines) with camera (Evolution LC, model no:PL-A662,Media cybernetics), Low speed magnetic stirrer (Remi, India), Hot air oven(Tempo, India), Easy pure UV/UF compact reagent grade water system (Barnstead, USA), ELISA reader (Bio-Tek instruments, USA), UV-visible spectrophotometer (Shimadzu), Electrophoresis unit (Biorad labotaroies, USA), Mini Blot (Biorad Laboratories, USA), Programmable thermal Cycler (MJ Research

Inc,USA), Submarine electrophoresis unit (Banglore Genei, India), UV transilluminator (Bangalore Genei, India), FACS (BD FACSAria™), RealtimePCR (ABI biosystems, Life technologies), Cell counter (Biorad), Fluorescent microscope (Nikon eclipse TE 2000-U), Multivolume spectrophotometer (Nanodrop Bio-Tek), Dissolved Oxygen probe (Lutron,DO-5510), Hypoxia incubator (Eppendorf).

III.1.4 Softwares used

Image-Pro Plus for image analysis (Media Cybernetics), GraphPad prism (San Diego, CA), Zotero, ImageJ (1.43 freeware (NIH), Adobe Photoshop CS3 (Adobe Systems, Inc)

III.2 Composition of media, reagents, and buffers

III.2.1 Phenol red free Phosphate Buffered Saline (pH 7.6)

NaCl- 0.8g

KCl- 0.02 g

Na_2HPO_4- 0.181 g

KH_2PO_4- 0.024g

Dissolve and make up to 100ml using deionised water.

III.2.2 Cell lysis buffer

1M Tris (pH-6.8) - 2.4 ml

20% SDS- 3ml

Glycerol- 3ml

β mercapto ethanol- 1.6 ml

Bromo phenol blue – 0.006g

Add equal volume of sample buffer and protein extract. Place it in boiling water bath for 10’. Centrifuge the sample at 10,000g for 10’ at room temperature. Transfer the supernatant to a fresh tube.

III.2.3 RIPA Buffer

Tris HCl	234 mg
NaCl	261 mg
EDTA	11.6 mg
1% Triton X100	3 ml
1% sodium deoxy cholate	3 ml

Made up to 30 ml with distilled water

III.2.4 Resolving gel buffer- 1.5 M tris (pH 8.8)

Weigh out 18.165g of TRIZMA base and dissolve in about 80ml deionized water. Adjust the pH using HCl and make upto 100ml. Store at room temperature.

III.2.5 Stacking buffer- 1 M Tris (Ph 6.8)

Measure about 30ml of stacking gel buffer, adjust the pH to 6.8 using HCl and make up the volume to 5ml. Store at room temperature.

III.2.6 Electrophoresis buffer

Trizma base (25mM) - 3.027 gm/ml

Glycine (192mM) - 14.4gm/ml

SDS (0.1%) - 1gm/ml

III.2.7 30% acrylamide solution

Weigh out 29.2g of acrylamide and 0.8gms of bis acrylamide. Dissolve in 100ml deionised water. In order to facilitate the dissolution of acrylamide, slow warming or keeping at 37^{0c} for some time is required. This solution may not be stable for very long time as the components may undergo slow conversion to acrylic acid.

III.2.8 10% SDS solution

Dissolve 1gm SDS in 10ml of deionized water. Keep it at 37^{0}c for complete dissolution.

III.2.9 10% Ammonium per sulphate solution

Dissolve 0.1gms in 1ml deionized water

III.2.10 Gel preparation

III.2.10.1 Stacking gel (5%)

5% gel can be used as the stacking gel. Composition is as follows: TEMED should be added only just before the gel is loaded.

H_2O- 1.4ml

30% acrylamide mix- 0.35ml

1.0M Tris (pH 6.8)- 0.25ml

10% SDS- 0.02ml

10% APS- 0.02ml

TEMED- 0.002ml

III.2.10.2 Resolving gel (10%) - 10ml

H_2O- 4ml

30% acrylamide mix- 3.3ml

1.0M Tris (pH 6.8)- 2.5ml

10% SDS- 0.1 ml

10% APS- 0.1 ml

TEMED- 0.004ml

III.2.11 Transfer buffer (Towbin's buffer)

Trizma base- 3.027 gm/ml

Glycine- 14.4 gm/l

Dissolve in deionized water, make up to 800 ml. Add 200ml methanol to make up to 100ml just before use.

III.2.12 TBS stock solution (10x) - 100ml

Trizma base- 2.42 gm

NaCl- 8gms

Dissolve in 80ml deionised water. Adjust the pH to 7.6 using HCL. Make upto 100ml with water.

III.2.13 Blocking solution

Skimmed milk-5%

Tween 20-0.01%

Dissolved in 1X TBS solution

III.2.14 DAB substrate solution

6mg DAB in 10ml tris (pH 7.6) containing 10µl of 30% H_2O_2

DEPC- treated deionized water

1ml of DEPC in one litter of deionised water, stirred overnight at room temperature and autoclaved

III.2.15 DNA/RNA gel- loading dye

Bromophenol blue (0.25%); xylene cyanol FF (0.25%); EDTA (1mM); glycerol (50%) in DEPC- treated deionized water

III.2.16 Ethidium bromide (Stock solution)

1mg ethidium bromide in 1ml deionized water; 5µl of this stock solution was added to 20ml of 1% agarose gel for DNA/RNA electrophoresis

III.2.17 Agarose gel (1%) for electrophoresis of RNA samples

Agarose in 20ml of 1X MOPS buffer.

III.3 Experimental Methods

III.3.1 Source of cardiac and mesenchymal stem cells

The study was carried out in stem cells isolated from the right atrial appendage and sternal bone marrow of patients undergoing coronary artery bypass graft (CABG) surgery. The cells isolated from tissue specimens were expanded in culture and used for experimental studies. Tissue samples were obtained with informed consent and the studies conformed to the guidelines established by the Ethical Committee of the Institute.

III.3.2 Isolation and culture of cardiosphere derived cells

Cardiosphere derived cells (CDCs) were isolated and grown from the right atrial appendage of patients undergoing CABG. The tissue samples were processed following

the procedure standardised in the laboratory (Messina et al., 2004a, Rani et al 2008, Smith et al., 2007b). Tissue samples were minced into fine pieces of $1mm^3$, after removing the adherent fat tissue. The tissue bits were placed in dishes coated with 2% gelatin and incubated in complete explant medium (CEM) (Iscove's Modified Dulbecco's Medium [IMDM] supplemented with 20% fetal bovine serum, 100 U/ml penicillin G, 100 U/ml streptomycin, 2 mmol/ l L-glutamine and 0.1 mmol/L 2-mercaptoethanol) at 37^oC in 5% CO_2 and 99% humidity. After one week in culture, a layer of stromal-like cells migrate from adherent explants over which small, round, phase-bright cells appear. On attaining confluency, the cells surrounding the explants were harvested by gentle enzymatic digestion and seeded at a density of 1×10^5 cells/ml on poly-D-lysine–coated dishes in cardiosphere-growing medium (CGM) (35% complete IMDM/65% DMEM–Ham F-12 mix containing 2% B27, 0.1 mmol/ l 2-mercaptoethanol, 10 ng/ml epidermal growth factor [EGF], 20 ng/ml basic fibroblast growth factor [bFGF], 40 nmol/l cardiotrophin-1, 40 nmol/l thrombin, antibiotics, and L-Glu, as in CEM). After 48 hrs in culture, the cardiospheres formed were isolated and plated on fibronectin-coated dishes, leaving behind the cells that remained adherent to the poly-D-lysine–coated dishes. The cardiospheres adhered on to the culture surface and expanded into monolayers of Cardiosphere derived cells (CDCs) in IMDM medium supplemented with 20% FBS (Gibco), 100 U/ml penicillin G, 100 μg/ml streptomycin, 2 mmol/l L-glutamine. CDCs were subsequently passaged by trypsinization and replated at a ratio of 1:2. Isolation of the cardiac outgrowth cells was repeated twice from the same explants.

On attaining 90% confluence, the cells were detached from the culture surface using trypsin-EDTA solution (Sigma), and sub-cultured at a density of $1x10^5$ live cells/ml. Cell number was assessed by using cell counter (Bio rad) by trypan blue dye exclusion method. Experiments were performed using cells of passages 2 to 4, unless specified.

III.3.3 Isolation and culture of Mesenchymal Stem Cells

Human Mesenchymal stem cells (MSCs) were isolated from bone marrow aspirates obtained during sternotomy for CABG. The popular method is based on the ability of the MSCs to selectively adhere to plastic surfaces. Cells from the aspirate were plated in IMDM medium supplemented with 20% FBS, 100 U/ml penicillin G, and 100 µg/ml streptomycin. On reaching confluency the cells were subcultured in the ratio 1:2. Cells from passages 2-4 were used for experimental studies.

III.3.4 Phenotypic characterization

Phenotypic characterization of CDCs and MSCs was carried out using fluorescence-activated cell sorting (FACS) analysis of cell surface markers. The cells were stained with the following primary antibodies: c-Kit, MDR1, CD105, CD90, CD133, CD34, CD45, CD31 along with corresponding isotype controls. After incubation with appropriate fluorescent-labeled secondary antibodies, cells were washed, fixed with 4% paraformaldehyde and analyzed by flow cytometry. The samples were analyzed using a 4-color multiparameter flow cytometer (FACS Aria; Becton Dickinson).

III.3.5 Population doubling time

Cardiosphere derived cells and mesenchymal stem cells from the primary culture were seeded at a density of 2×10^5 cells in 35 mm culture plates and incubated at 37°C with 5% CO_2 and 99% humidity for 48h. This was marked as the first passage (P1). At 48h cells were trypsinized and the cell count was determined. These cells were then replated at a density of 2×10^5 cells/ml and designated as P2. The cell count was determined after 48h and the process was repeated upto P7. The doubling time for each passage was calculated based on the cell count at plating and after 48hr in culture, using the formula; PDT=(log $2\times t$)/ (log Nh - log N_i) where N_h is the number of cells harvested and Ni is the number of cells plated, t is the time in culture.

III.3.6 Hypoxia- reoxygenation injury (HR injury)

Ischemia-reperfusion injury was simulated in cell culture by subjecting the CDCs and MSCs to hypoxia followed by reincubation in normoxic conditions. Briefly, 48hr old, semi confluent cultures were exposed to hypoxic injury by incubating serum deprived cells in 0.5% O_2, 5% CO_2 and 94.5% N_2 for 2 hrs. This was followed by serum replenishment and reintroduction of atmospheric oxygen for 6hrs termed, hypoxia-reoxygenation injury (HR injury).

III.3.7 Assessment of cell viability by annexin-propidium iodide live-dead assay

The effect of HR injury on cell viability of MSCs and CDCs was determined by using the Annexin V-FITC/propidium iodide (PI) apoptosis detection kit (Becton Dickinson) according the manufacturer's instructions. In brief, cells were rinsed with ice-cold PBS and resuspended in 200 μL of binding buffer. Five microliters of Annexin V and 5 μl of propidium iodide (PI) were added to the cells. After incubation for 15 min (25^0C) in the dark, 200 μl of 1X binding buffer was added to each tube and FCM analysis(FACS Aria, Becton- Dickton) was performed immediately. Ten thousand events were analyzed in each of the samples. Annexin V (+) and PI (-) cells were considered as apoptotic cells**.**

III.3.8 Senescence assay by SA-β-Gal staining

A senescence β-galactosidase staining kit (Abcam) was used according to the manufacturer's instructions for SA-β -Gal staining. The assay was carried out in 6 well plates, and cells were seeded in triplicates at a density of $1x10^5$ cells/ ml and grown until 70% confluent. Twenty four hours after HR injury, wells were rinsed twice with PBS, fixed in 1 ml of 1X fixing solution per well, and then incubated at room temperature for 15 min. Following washing with PBS, cells were incubated overnight in freshly prepared staining SA β-gal detection staining solution at 37^0C. SA-β-Gal-positive cells were observed for development of blue color using 10x objective, and bright field images were captured with a digital camera using Image-Pro plus 6.0 software. The percentage of senescent cells was obtained by counting the number of blue-stained cells and the total

cells per field. The experiment was repeated 3 times and the mean percentage of cells expressing β-galactosidase was calculated.

III.3.9 Measurement of intracellular reactive oxygen species

To measure intracellular reactive oxygen species (ROS), generated after HR injury, MSCs and CDCs were seeded at a density of 4 $x10^4$ cells per well in 96-well dark plates. After 24hrs cells were subject to HR injury. The cells were then incubated with 10 µM 2′, 7′-dichlorodihydrofluorescein diacetate (DCFDA-Invitrogen) for 30 min. Normoxic cells were kept as control. DCFDA was deacetylated intracellularly by nonspecific esterase, which was further oxidized by ROS to the fluorescent compound 2,7-dichlorofluorescein (DCF). The fluorescence of 2′,7′- dichlorodihydrofluorescein (DCF) in cells was directly determined using FLx800™ Multi-Detection Microplate Reader with Gen 5 PC software (Biotek) with an excitation wavelength of 495 nm and an emission wavelength of 520 nm.

III.3.10 Western blot analysis for detection of prosurvival factors

Western blot analysis was carried out for the assay of pro survival factors after HR injury. Immediately after HR injury, cells were trypsinized and lysed using RIPA buffer and the cleared total cell lysate was denatured by boiling 35µg of protein was loaded for electrophoresis and separated through 8% to 15% SDS-PAGE gel. After electrophoresis, the proteins were transferred into the polyvinylidene difluoride membranes. The membranes were blocked with 5% skim milk in Tris-buffered saline (TBS) for 1 h at room temperature and incubated with primary antibodies for Akt, phospho-Akt , HIF-1α (Thermoscientific) , BCl2 (Santa Cruz Biotechnology) and Mef 2c (Abcam), at 4°C

overnight. The cells were washed 3 times in Tris Buffered Saline (TBS) containing 0.1% Tween-20 to remove the excessive antibodies and incubated with the appropriate secondary antibody (Sigma) for 1 h. The proteins of interest were detected by using Supersignal west femto chemiluminescent substrate kit and exposed to radiography film. The membranes were reprobed for β actin. Semi-quantitative analysis of the scanned images was performed using ImageJ software and normalised with β actin.

III.3.11 Analysis of Cell Cycle by Flow-cytometry

Cells were plated at a density of $1x10^5$cells/ ml in a 35mm dish. After 48hrs in culture, cells were subject to HR injury. The cells were then harvested, washed twice with PBS, and fixed in 70% ice-cold ethanol, added drop wise while vortexing. The fixation reaction was allowed to proceed overnight while the cells were kept at -20°C. Cells were then collected by centrifugation and resuspended in phosphate-buffered saline containing 20μg/ml RNase and Propidium iodide at a final concentration of 50μg/ml and incubated for 30 minutes at 37°C. Each phase of the cell cycle was measured by FACS.

III.3.12 Measurement of growth factors released by ELISA

For measurement of growth factors released by CDCs and MSCs, the cells were seeded in 35mm culture plates at a density of 2×10^5 cells and incubated for 3 days. The supernatants were collected and the concentrations of the growth factors- hepatocyte growth factor (HGF), stromal derived growth factor (SDF-1), and vascular endothelial growth factor (VEGF) were measured with human ELISA kits (Abcam), according to the manufacturer's instructions.

III.3.13 Differentiation of stem cells to cardiovascular lineage

Myocyte differentiation-MSCs and CDCs were plated at a density of 1x 10^5 cells per well in 6 well plates and incubated in Iscove's modified Dulbecco's medium supplemented with l-glutamine. After 24 hrs cells were given HR injury. To check the effect of HR injury on myocyte differentiation, the cells were stimulated with 5 µM 5′-azacytidine for 24 h followed by TGF-β1 stimulation (1 ng/ml). The medium was changed every 3 days and cells were maintained in culture for 14 days**.** The cells without HR injury treated in the same differentiation medium served as control. Differentiation was assessed by immunostaining for GATA4, Desmin, connexin 43, MHC and Cardiac Troponin I.

For immunocytochemistry, cells were fixed in 4% paraformaldehyde for 20 min at 4^0C and subsequently permeabilized with 0.3% Triton X (Sigma-Aldrich) at room temperature. Cells were incubated overnight with rabbit, anti-Human antibodies to either Desmin (1:200; Sigma), connexin 43 (1:100; Abcam), Myosin Heavy chain (1:100; Abcam), GATA 4(1:500; Abcam) or cardiac troponin I (cTnI; 1:100; Abcam) at 4^oC. The cells were then incubated with a sheep anti-rabbit phycoerythrin -labelled secondary antibody (1:200; Sigma) for 1 hr at room temperature. Cells were visualized with the fluorescence microscope (Nikon eclipse TE 2000-U) and images were captured with the digital camera using Image-Pro plus 6.0 software

III.3.14 In vitro tube formation assay as an indicator of angiogenic differentiation

Cells were suspended in IMDM medium with endothelial growth supplements (Sigma) and 2% FBS. The cells were plated at a density of $5x10^4$ cells per well in 4-well plates coated with Matrigel (Becton Dickinson). After HR injury, cells on matrigel were again maintained in normoxia (20% O_2) for 10 more hrs, and tube formation was examined and branching points were counted using an inverted microscope (Nikon Eclipse TE2000). The cells maintained in the same condition without HR injury served as the control. Pictures were taken using a Nikon DS-L2 camera.

III.3.15 Differentiation of stem cells to noncardiac lineage

III.3.15.1 Osteogenic differentiation

Third passage of CDCs and MSCs were adjusted to a concentration of $2x10^5$cells /ml and cultured on a 6-well culture plate (1 ml/well). Once the cells had reached 100% confluency, the cells were given HR injury, the cells were incubated in an osteogenic differentiation medium (HiOsteoXL ™ – Himedia) for 2–4 weeks during which time the medium was changed every 2–3 days. The cells were fixed with ice-cold 70% ethanol and differentiation was characterised by the accumulation of calcium (crystalline hydroxyapatite) which was detected by Alizarin red (Sigma-aldrich) staining. The alizarin red positive area was analyzed using NIH image software Image J and expressed as percentage of alizarin red positive area.

III.3.15.2 Adipogenic differentiation

The cells were first grown at a density of $2x10^5$cells/ml and cultured on a 6-well culture plate (1 ml/well) for 48 hrs. After HR injury, the cells were incubated in the induction and maintenance media (HiAdipoXL ™- HiMedia) for 21 days and then fixed with 4% paraformaldehyde for 30 min at room temperature. Presence of intracellular lipid vesicles was assessed for fat deposition by Oil Red O (Sigma) staining. Stained Oil Red O was eluted with 100% isopropanol (v/v) and quantified by measuring the optical absorbance at 500nm

III.3.16 Assessment of stem cell migration

III.3.16.1 Transwell migration assay

Tissue repair following ischemic injury is mediated by the homing in of stem cells to the site of injury. The migration of stem cells to the site of injury is possibly mediated by the transmission of signals from the injured cells. Nevertheless, the migratory property of stem cells can be influenced by the ischemic injury. Transwell migration assay was used to assess the behaviour of stem cells following HR injury. Additionally, the effect of injured myocardial tissue on stem cells was also examined. To check the effect of injured myocardial tissue bit on stem cell migration, atrial bits were grown as explants in the lower chamber of transwell system for 11 days. Stem cells were seeded into the upper chamber of the transwell (8.0 µm, pore size, BD) at a concentration of $2 x10^4$cells/ well. After 24hrs the tissue bit and surrounding outgrowth cells in the lower chamber was subjected to hypoxic injury (0.5% O_2 and serum deprivation, for 2hrs). Soon after HR

injury serum deprived stem cells in the 24 well insert was placed on top of the injured myocardial bit. After 6 h of incubation at 37^0C in normal culture conditions, the upper sides of the filters were carefully washed with PBS, and cells remaining were removed with a cotton wool swab. The cells that migrated to the bottom side of the filter were fix ed with 4% paraformaldehyde and stained using 0.1% crystal violet. The number of migrated cells was manually counted in ten random fields per filter at 10x magnification. To evaluate the effect of HR injury to stem cells on migratory capacity of CDCs and MSCs was evaluated. Stem cells were seeded into the upper chamber of the transwell system at a concentration of 2 $x10^4$cells/ well, in serum containing medium. After 24 hrs the cells were given HR injury. After HR injury, the serum deprived cells in the transwell was placed on top of a 24 well plate with 10% serum containing medium. After 6 h of incubation at 37^0C, under normal culture conditions, the inserts were processed for microscopy as described above.

III.3.16.2 Wound-healing assay/Scratch-wound assay

The effect of HR injury on cell migration was also analyzed by scratch-wound assay. Cells were grown to confluence and a scratch wound was made in the monolayer by dragging a pipette tip across the culture dish. Detached cells were washed away with PBS and fresh serum-free medium was used for culture. Cells were given HR injury. After HR injury, cells were maintained in serum free medium in normal culture conditions for 24hrs. Serum-free medium was used to prevent proliferation of migrated cells as serum

containing medium will promote wound-healing by migration as well as proliferation. The control cells were cultured parallely but not subject to HR injury. Photomicrographs were captured under phase contrast optics after 8 and 24 hrs (Nikon, 2000-U), and migrated cells were counted in five scratched areas per well. The number of migrated cells was calculated for each well using Image Pro Plus 6.0 software, and the mean and SD were then calculated from the averages of replicate wells.

III.3.17 Effect of hypoxically injured myocytes on CDCs.

III.3.17.1 Effect of H9c2 conditioned medium on CDCs

H9c2 cells and CDCs were cultured in a 35mm dish at a density of 1.5 x 10^5 cells per dish. After 48 hrs both the H9c2 cells and CDCs were given HR injury (serum deprived cells in 0.5% O_2, 5% CO_2 and 94.5% N_2 for 2 hrs, followed by serum replenishment and reintroduction of atmospheric oxygen for 6hrs). After HR injury, the conditioned medium from injured H9c2 cells was collected and added to HRCDC culture dish, after removing the injured CDC conditioned medium. CDCs were incubated in injured H9c2 conditioned medium for 48 hrs. In another set HR CDCs were incubated in normal H9c2 conditioned medium. Both sets of cells were processed for cell cycle analysis and cell cycle stages were analysed using FACS. Normal CDCs and HR CDCs served as the control.

III.3.17.2 Migration of CDCs towards injured H9c2 cells

To check the effect of injured H9c2 cells on stem cell migration, H9c2 cells were plated at a density of 8x 10^4 cells/well in the lower chamber of transwell system. CDCs were

seeded into the upper chamber of the transwell (8.0 µm, pore size, BD) at a concentration of 2 x10^4cells/ well. After 24hrs H9c2 cells in the lower chamber and CDCs in the upper chamber were subjected to hypoxic injury (0.5% O_2 and serum deprivation, for 2hrs) separately. Soon after Hypoxic injury serum deprived CDCs in the 24 well insert was placed on top of injured H9c2 cells. Migration of normal serum deprived CDCs in the upper chamber towards injured H9c2 and normal H9c2 were compared. After 6 h of incubation in normal culture conditions, the upper sides of the filters were carefully washed with PBS, and cells remaining were removed with a cotton wool swab. The cells that migrated to the bottom side of the filter were fixed with 4% paraformaldehyde and stained using 0.1% crystal violet. The numbers of migrated cells were manually counted in ten random fields per filter at 10x magnification.

III.3.18 Effects of hypoxically injured stem cells on cardiomyocytes

III.3.18.1 Effect of injured CDC conditioned medium on Injured H9c2- Cell cycle analysis

H9c2 cells and CDCs were cultured in a 35mm dish at a density of 1.5 x 10^5 cells per dish. After 48 hrs both the H9c2 cells and CDCs were given HR injury (serum deprived cells in 0.5% O_2, 5% CO_2 and 94.5% N_2 for 2 hrs, followed by serum replenishment and reintroduction of atmospheric oxygen for 6hrs). After HR injury, the conditioned medium from injured CDCs were collected and added to HR H9c2 culture dish, after removing the injured H9c2 conditioned medium. H9c2 cells were incubated in injured CDC conditioned medium for 48 hrs. In another set HR H9c2 cells were incubated in normal CDC

conditioned medium. Both sets of cells were processed for cell cycle analysis and cell cycle stages were analysed using FACS as described previously. Normal H9c2 cells and HR H9c2 cells served as controls for comparison.

III.3.18.2 Effect of CDC conditioned medium on injured H9c2- Annexin – PI live dead assay

H9c2 cells and CDCs were cultured in a 35mm dish at a density of 1.5 x 10^5 cells per dish. After 48 hrs, both the H9c2 cells and CDCs were given HR injury (serum deprived cells in 0.5% O_2, 5% CO_2 and 94.5% N_2 for 2 hrs, followed by serum replenishment and reintroduction of atmospheric oxygen for 6hrs). After HR injury, the conditioned medium from injured CDCs were collected and added to HR H9c2 culture dish, after removing the injured H9c2 conditioned medium. H9c2 cells were incubated in injured CDC conditioned medium for 48 hrs. In another set HR H9c2 cells were incubated in normal CDC conditioned medium. Both sets of cells were processed for annexin- PI live dead assay. FACS was carried out as described previously. Normal H9c2 cells and HR H9c2 cells were served as controls.

III.3.19 Culture of stem cells in physiological oxygen levels

In the conventional cell culture system cells are maintained in ambient oxygen (≈ 20%), which is higher than the physiological levels of 2-8% O_2 in the tissues. Oxygen levels in the cardiac tissue are usually 5% but 3% in the stem cell niches. Cardiosphere derived cells were cultured in O_2 cells simulating that of stem cell niches (3%) and compared with the behaviour of cells cultured in ambient O_2.

III.3.20 The effect of physiological oxygen levels (3% O_2) on the CDCs

To determine the effect of physiological oxygen levels (3% O_2) on the CDCs, the cells were cultured either in a standard CO_2 incubator with 5% CO_2 in air (20% O_2) or a low-oxygen incubator supplied with 5% CO_2 and 3% oxygen. The O_2 concentration was achieved and maintained using delivery of ultrapure nitrogen gas. Media for the low oxygen cultures were pre-equalized in 3% O_2. The dissolved oxygen content of the medium was measured using a Dissolved oxygen probe (Lutron, DO 5510). CDCs were processed and expanded using the same protocol described previously.

III.3.21 Cardiosphere formation assay in different oxygen levels

The cells that migrated from the explants were cultured as cardiospheres following previously described protocol. After 48 hrs in culture, the cardiospheres formed in both 20% O_2 and 3% O_2 were counted and their size was measured using NIH image software Image J.

III.3.22 Cell viability of CDCs cultured in 20% and 3% O_2

III.3.22.1 Apoptosis

The Apoptosis in CDCs was assessed using the Annexin V-FITC/propidium iodide (PI) apoptosis detection kit (Becton Dickinson) according to the manufacturer's instructions and as describer earlier.

III.3.22.2 Senescence assay by SA-β-Gal staining

Senescence was assessed in higher passages. The proportion of senescent cells in the 8th passage (P8) was checked by using a senescence β-galactosidase staining kit (Abcam).

III.3.22.3 Expression of p16INK4a

The expression of p16INK4a, a marker for cell senescence, was checked by flowcytometry. The single cell suspension of CDCs expanded in 3% O_2 and 20% O_2 were incubated with PE-conjugated mouse anti-human p16INK4a antibody for 60 min. The expressions of p16INK4a were quantitatively measured using FACS.

III.3.23 Expression of c-Kit antigen

The presence of c-Kit is an indicator of stemness. To examine whether the c Kit population is affected by oxygen levels, the proportion of c-Kit positive cells was measured by FACS following the procedure described earlier.

III.3.24 Cell proliferation of CDCs cultured in 20% and 3% O_2

III.3.24.1 Population doubling time

Cardiosphere derived cells maintained in 20% O_2 and 3% O_2 from the initiation of culture were seeded at a density of 2×10^5 cells in 35 mm culture plates and incubated at 37°C with 5% CO_2 and 99% humidity for 48h. This was marked as the first passage (P1). The procedure was repeated and the cell count was determined after 48h up to P7. The doubling time for each passage was calculated as described earlier.

III.3.24.2 Cell cycle analysis

Cells from cultures maintained in 20% O_2 and 3% O_2 were plated at a density of $1\text{x}10^5$ cells/ ml in a 35mm dishes. Cells were fixed treated with PI and analysed using FACS as described earlier.

III.3.24.3 Ki 67-proliferation assay by flow cytometry

Cells from 20% O_2 culture and 3% O_2 culture were plated at a density of $1x10^5$ cells/ ml in 35mm culture plates. After 48hrs in culture, the cells were harvested and washed twice with PBS. Cells were suspended in 70% ice cold ethanol while vortexing. They were then incubated at −20°C for 2 hrs. Fixed cells were then washed twice with staining buffer (PBS with 1% foetal calf serum and 20µl triton x100), centrifuged for 10 min at 200g, and resuspended in 100 µl in the staining buffer. Anti-Ki-67 antibody (Abcam) was added at 1:100 dilution. Cells were incubated for 30 min. Cells were washed twice with the staining buffer, centrifuged at 200g for 10 min, and resuspended in 100 µl of staining buffer; the secondary antibody was added at a dilution of 1:200. Cells were reincubated in the dark for 1 hr before being washed twice in staining buffer. The cells were pelletted by centrifugation and resuspended in 50 µl of staining buffer. Ki-67-positive cells were analysed using FACS.

III.3.1 Generation of reactive oxygen species by CDCs in ambient O_2 and 3% O_2

III.3.2 Differentiation potential of CDCs

III.3.2.1 Spontaneous expression of Pluripotency and cardiomyocyte lineage by Real time PCR assay

Total RNA was isolated using an RNeasy mini plus kit (Qiagen) according to the manufacturer's instructions. Genomic DNA contamination of RNA sample was removed

by RNase free DNase -1 (Sigma) treatment. 2 μg of each RNA sample was reverse transcribed to cDNA with MMLV reverse transcriptase using oligo dT primers. Human specific primers for the genes were supplied by Genex. Real Time RT-PCR was performed using Power SYBR Green technology on Applied Biosystem 7500 real time PCR system. PCR amplification was performed in a total volume of 20 μl, containing 60 ng of the cDNA derived from reverse transcription. The threshold cycle (CT) for fluorescence development was used to calculate the fold change using the formula ''$2^{-\Delta\Delta CT}$.

Table 3: The sequences of oligonucleotide primers used for Real time PCR

Gene name	Primer sequence
Cardiac troponin I Forward primer Reverse primer	 5' GAGGTGGTGGAAGAGTACGAG 3' 5' GACGTCTCTCGATCCTGTCTTT 3'
Nkx2.5 Forward primer Reverse primer	 5' GCAGGTCAAGATCTGGTTCCAGA 3' 5' GAGTGAATGCAAAATCCAGGGGAC 3'
GAPDH Forward primer Reverse primer	 5'AATCCCATCACCATCTTCCA 3' 5' TGGACTCCACGACGTACTCA 3'

CDCs expanded under 3% O_2 or 20% O_2 were seeded on matrigel coated 4-well plates at a density of 4×10^4 cells per well in the presence of 2% fetal bovine serum and 50 ng/ml vascular endothelial growth factor. Spontaneous tube formation assay was done without the addition of endothelial growth supplement. After 8 h incubation, the tube formation was imaged. The total number of branching points per field was then measured with Image-Pro Plus software.

III.3.2.2 Osteogenic differentiation

To examine the effect of oxygen levels on ostogenic differentiation, CDCs were plated at a density of $2x10^5$cells/ well in 6-well culture plates. Confluent cultures were incubated in osteogenic differentiation medium (HiOsteoXL ™ – Himedia) for 2–4 weeks. The medium was changed every 2–3 days. The cells were fixed with ice-cold 70% ethanol and differentiation was characterized by the accumulation of calcium (crystalline hydroxyapatite) which was detected by Alizarin red (Sigma-aldrich) staining. The extent of alizarin red staining area was quantified using NIH image software Image J and expressed aspercentage of alizarin red positive area

III.3.2.3 Adipogenic differentiation

Effect of oxygen levels on adipogenic differentiation was assessed. The cells were plated at a density of $2x10^5$cells/well in 6-well culture plates for 48 hrs. The cells were then incubated in the induction media (HiAdipoXL ™- HiMedia) for 21 days and fixed with 4% paraformaldehyde for 30 min at room temperature. Intracellular lipid vesicles were visible and

fat deposition was assessed by Oil Red O (Sigma) staining. Oil Red O was then eluted with 100% isopropanol (v/v) and quantified spectrophotometrically.

III.3.3 Proliferation and survival of cells in physiologic oxygen following culture in subphysiological and ambient oxygen

To examine whether cell survival is affected when cells cultured in 20% O_2 and 3% O_2, are shifted to a milieu with physiological oxygen levels, confluent cultures maintained in different O_2 levels were shifted to 5% O_2 contemplating the oxygen level in the myocardial tissue. After 72 hrs cells were assessed for viability by Annexin-propidium iodide Live-dead assay and proliferation by Ki-67 FACS assay.

III.3.4 Statistical data analysis

All results are presented as mean± SD. The presence of variation between groups was analyzed using ANOVA. In the event of significant difference by ANOVA, the difference between 2 groups was tested using Students t-test. $p \leq 0.05$ was considered statistically significant.

A minimum of 3 replicates was analysed for each variable.

The study was approved by the Institutional Ethics Committee- (No. IEC/263 Dated 2-3-2010)

IV. RESULTS

IV.1 Culture of cardiosphere derived cells (CDCs) from human atrial appendage

IV.1.1 Isolation and expansion of stem cells from atrial appendage

Human CDCs were isolated from 120±30 mg of atrial biopsy samples. Biopsy samples were obtained from the right atrial appendage that was dissected out for insertion of canula during CABG surgery. The tissue bits from the right atrial appendage were minced into bits and were placed on gelatin coated dishes. Cells migrated out of the explant within 5 days of culture and formed a layer of cells, over which small, loosely adherent, phase bright cells were observed (Fig 2i). After two weeks in culture, the cells were harvested and grown in suspension culture on CGM on poly-D-lysine coated dishes to enable cardiosphere assembly. Free-floating, three dimensional cardiospheres were observed between 24 and 48 hrs (Fig 2iii). The cardiospheres adhered to the culture surface of fibronectin coated dishes (Fig 2iv) and expanded to form a monolayer of CDCs (Fig 2vi). On reaching confluence, The CDCs were passaged every 5 days. The total cell yield was found to be an average of $3 \pm 0.36 \times 10^7$ cells/150mg of tissue (1 month)

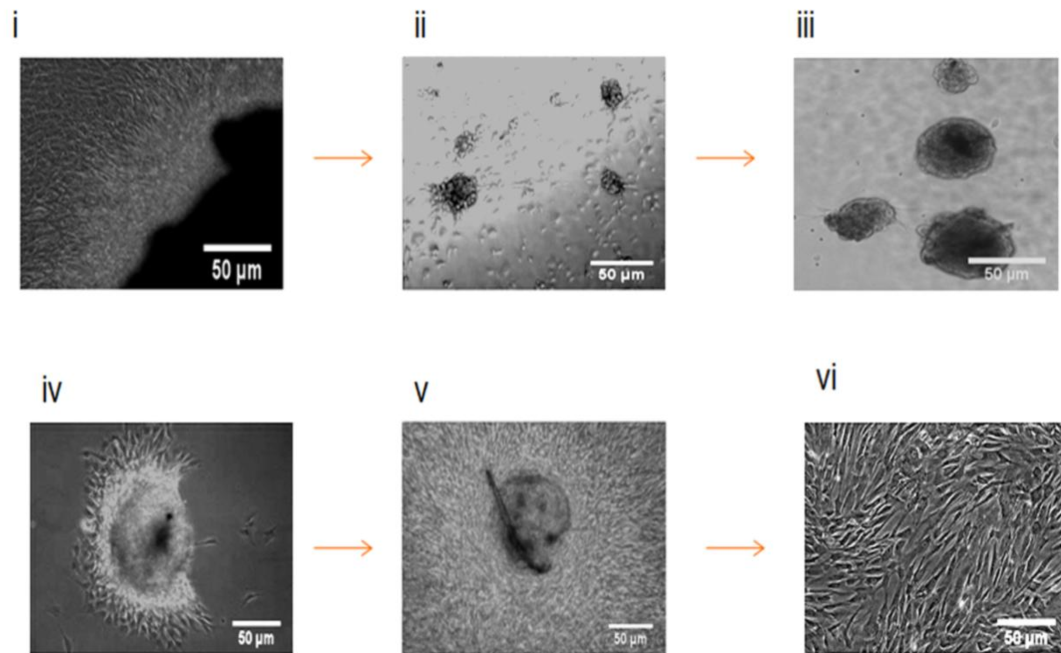


Figure 2: Isolation of Cardiosphere Derived Cells

i) Representative image showing explant culture with cardiac outgrowth after 12 days. ii) Formation of cardiospheres in poly D Lysine coated dishes. iii) Floating cardiospheres- 24-72hrs after culturing the outgrowth cells in cardiosphere growing medium. iv) Cardiosphere attached to culture surface and spreading on fibronectin coated dish V) expanding cardiosphere. vi) Monolayer of cardiosphere derived cells

IV.1.2 Characterization of CDCs

CDCs were characterized for the presence of different cell surface stem cell markers. Flow cytometric analysis revealed that CDCs express cardiac stem cell marker (c Kit) as well as mesenchymal markers, (CD 105, CD 90, and CD 29). CDCs were negative for endothelial and hematopoetic markers (CD 31, CD 34, CD 45, CD 133) (Fig. 3, Table 4).

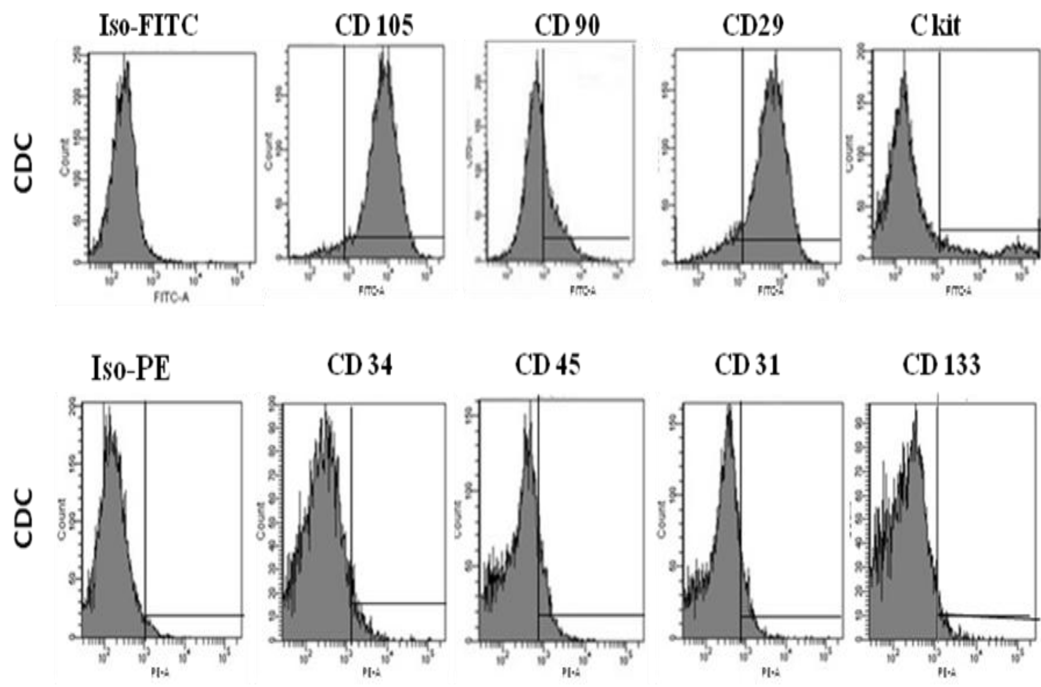


Figure 3: Representative FACS data of expression pattern of cell surface markers of CDCs

*Subconfluent cultures of P4 CDCs were stained with primary antibodies for CD105, CD90,CD29, cKit, CD34, CD45, CD***3***1 and c-Kit, along with corresponding isotype control. After incubation with appropriate fluorescent-labeled secondary antibodies, cells were washed, fixed with 4% paraformaldehyde and analyzed by flow cytometry.*

Table 4 Distribution of stem cell markers in cardiospere derived cells. Data obtained by FACS assay, n=6.

Marker	**Percentage**
CD 105	94±1.5
CD 90	16±1.2
CD 29	99±0.08
C kit	6.3±0.8
CD 34	1.1±0.3
CD 45	0.67±0.21
CD 31	1.06±0.15
CD 133	0.93±0.16

IV.1.3 Expression of myocyte lineage markers by CDCs.

CDCs were immunostained for the expression of myocyte lineage markers GATA 4 and MEF2c by immunocytochemistry. CDCs were found to express both GATA 4 and MEF 2C. (Fig.4)

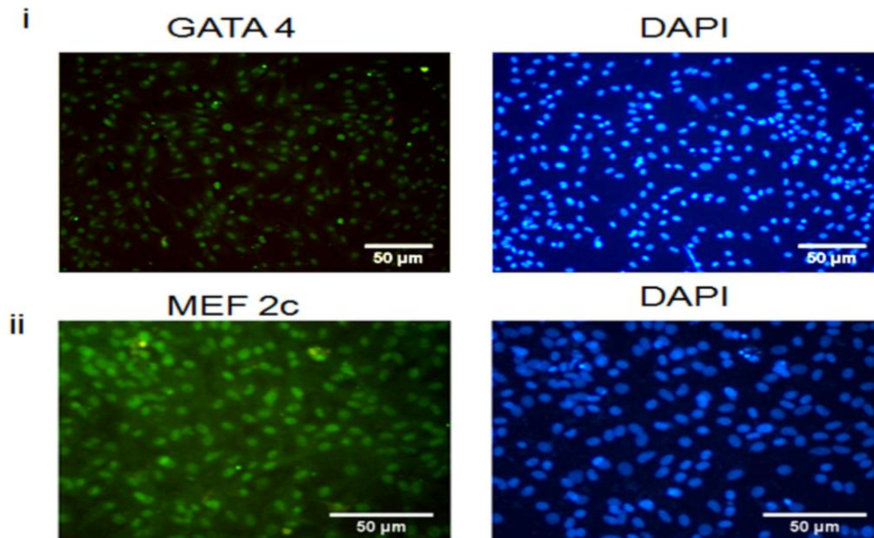


Figure 4: Cardiosphere derived cells expressing myocyte lineage markers (i) GATA 4 and (ii) MEF 2C

P3 CDCs were washed, fixed using paraformaldehyde and immunostained for the expression of myocyte lineage markers GATA 4 and MEF 2c. Nuclei were counter stained using DAPI.

IV.1.4 Population doubling time of CDCs

Population doubling time is an indirect indicator of cell proliferation. Population doubling time increased with increase in passage (Fig 5) after P2. In later passages (P6, P7) CDCs acquired a more senescent morphology

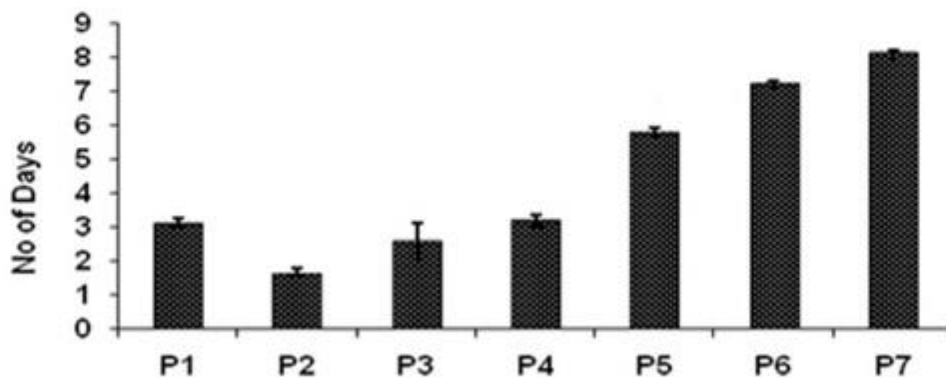


Figure 5: Graphical representation of population doubling time of Cardiosphere derived cells from different passsages

Cardiosphere derived cells from the primary culture were seeded at a density of 2×10^5 *cells in 35 mm culture plates and incubated at 37°C with 5%* CO_2 *and 99% humidity for 48h. This was marked as the first passage (P1). At 48h cells were trypsinized and the cell count was determined. These cells were then replated at a density of* 2×10^5 *cells/ml and designated as P2. The cell count was determined after 48h and the process was repeated upto P7.*

The data confirms the presence of resident cardiac stem cells that can be isolated from atrial biopsies. Isolated cells find application for in depth understanding of stem cell behavior as well as therapeutic use.

IV.2 Effect of hypoxia-reperfusion injury on human cardiosphere derived cells

In the event of myocardial ischemia, stem cells can be affected. Therefore, the effect of ischemia-reperfusion injury on CDCs was analyzed. Ischemia-reperfusion injury was simulated in culture as hypoxia-reoxygenation injury.

To study the effect of hypoxia-reoxygenation HR injury on CDCs, serum deprived cells were placed in a hypoxia incubator (0.5% O_2 for 2 hrs). After hypoxic injury the cells were reoxygenated in the presence of 10% serum containing medium for 6 hrs to simulate ischemia-reperfusion injury. The effect of hypoxia-reoxygenation injury on cell viability, proliferation, migration and differentiation was assessed.

IV.2.1 Effect of HR injury on cell survival

Flow cytometric analysis for Annexin V-FITC/PI live/dead assay was done to evaluate the effect of HR injury on survival of CDCs (Fig 6). A small but significant increase in the percentage of apoptotic cells (6.7± 0.3%) after HR injury was seen compared to 3.5±0.2% in untreated cultures.

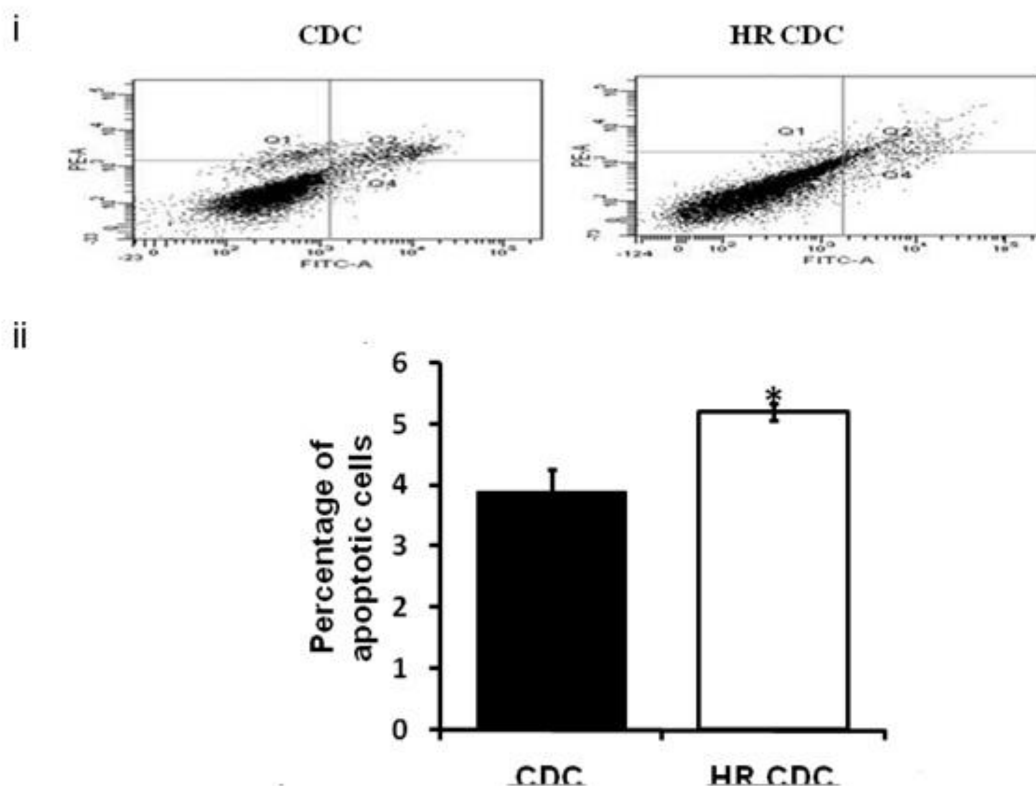


Figure 6: Effect of HR injury on apoptosis of Cardiosphere derived cells assessed by Annexin V /PI live dead assay.

*Cultured cells were subject to HR injury. The level of apoptosis was quantified by FACS analysis after staining with Annexin V and propidium iodide (PI). (i)Representative histograms of Annexin V /PI live dead assay. ii) Quantitative data representing early apoptotic cells by annexin/PI live dead assay. *p<0.05, CDC Vs HR CDC. n=3.*

IV.2.2 β galactosidase cell senescence assay

Cell cultures in the fourth passage were subjected to HR injury and examined for the presence of senescent cells after 24 hrs in standard culture conditions (Fig 7). 24 hr after HR injury, the number of senescent cells increased significantly ($p<0.01$ Vs CDCs). There

were 11± 0.3% senescent cells in treated culture, but senescent cells were rarely seen in untreated cultures.

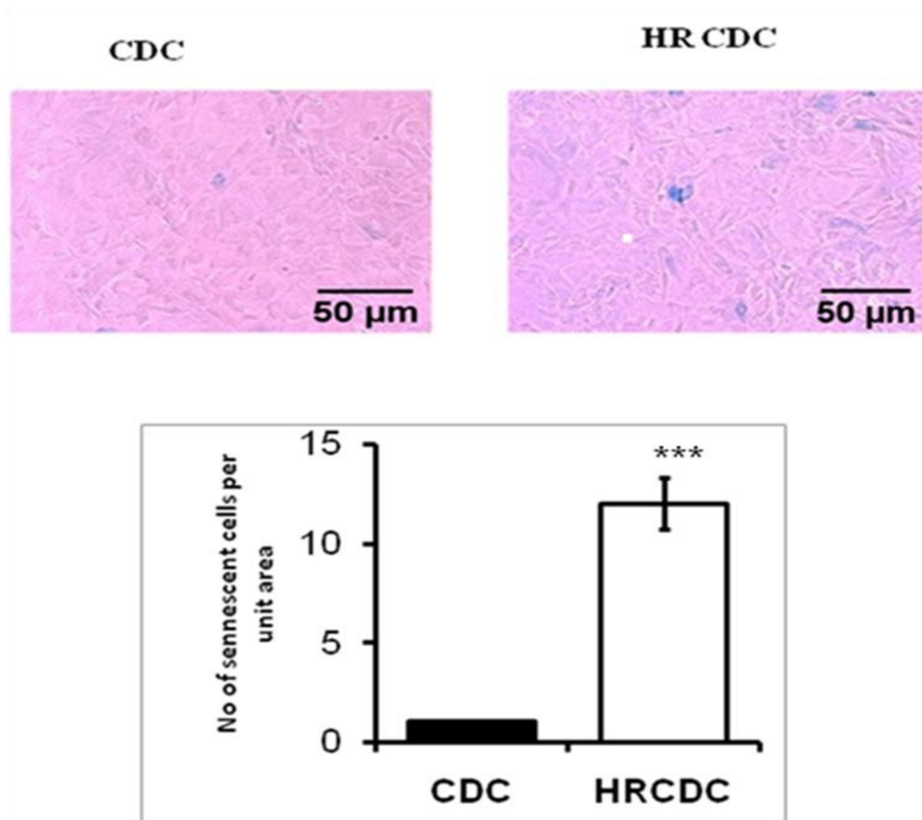


Figure 7: Effect of HR injury on cell senescence. Senescence-associated β-galactosidase (SA-β-gal) positive cells after 24hrs of HR injury.

*P4 CDCs were seeded in triplicates at a density of $1x10^5$ cells/ ml and grown to 70% confluence. Twenty four hours after HR injury, wells were rinsed twice with PBS, fixed in 1 ml fixing solution, and incubated at room temperature for 15 min. Following washing with PBS, cells were reincubated overnight in freshly prepared SA β-gal staining detection solution. Senescent, SA-β-Gal-positive cells developed blue colour. Staining was quantified by positive cell count i) representative images of (SA-β-gal)-positive cells. ii) Graphical representation of SA-β-gal positive cells. *** $p<0.001$ CDC Vs HR CDC, n=3.*

IV.2.3 Stimulation of reactive oxygen species generation after HR injury

Ischemia-reperfusion generally leads to excessive free radical generation. Intracellular ROS generation was assessed by of DCFDA cellular ROS detection assay. In response to hypoxic injury, there was an increase in fluorescence of DCFDA in CDCs (P< 0.05 CDC Vs HR CDC) (Fig. 8) suggesting that HR injury stimulates oxidative stress.

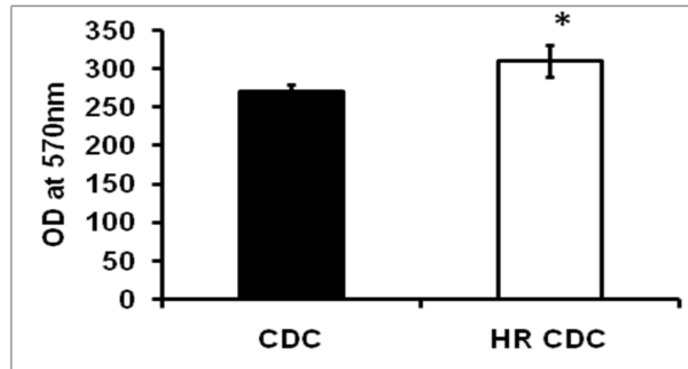


Figure 8: Stimulation of reactive oxygen species (ROS) generation consequent to HR injury

*P4 CDCs were seeded at a density of 4 x10^4 cells per well in 96-well dark plates. After 24hrs cells were subject to HR injury. The cells were then incubated with 10 µM 2', 7'-dichlorodihydrofluorescein diacetate (DCFDA-Invitrogen) for 30 min. Normoxic cells served as control. The fluorescence of 2',7'- dichlorodihydrofluorescein (DCF) in cells was directly determined using FLx800™ Multi-Detection Microplate Reader with Gen 5 PC software (Biotek) at an excitation wavelength of 495 nm and an emission wavelength of 520 nm. Graphical representation of ROS generation after HR injury, quantified by FACS analysis after treating with DCFDA. *p<0.05, CDC Vs HR CDC, n=-3.*

IV.2.4 Expression of prosurvival factors after HR injury

Prosurvival proteins expressed by the cells after HR injury were assessed by Western blot analysis, and compared with those expressed in cells grown under normal culture conditions (Fig 9). HR injury was found to upregulate the expression of prosurvival factors- HIF-1α ($p<0.05$), pAkt ($p<0.01$) and Bcl-2 ($p<0.05$). There was a two fold increase in the expression of cell survival factors pAkt and Bcl-2. The results show that survival of CDCs after HR injury is associated with upregulation of these proteins.

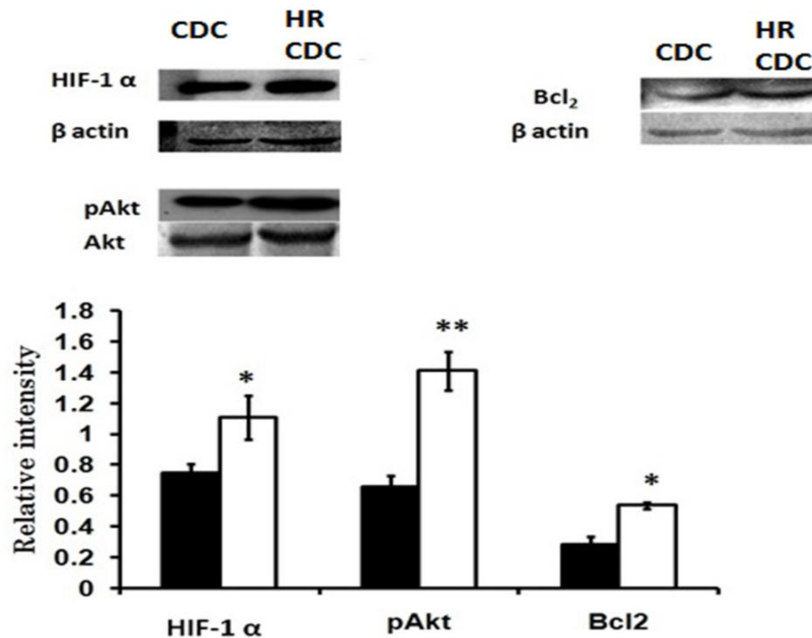


Figure 9: Expression of pro survival factors after HR injury

Proteins expressed by the cells after HR injury was compared with those expressed in cells grown under normal culture conditions by Western blot. i) Representative Western blots for HIF-1α, pAkt, Bcl_2. ii) Quantification of Western blot data.$p<0.05$, ** $p<0.01$ CDC Vs HR CDC, n= 3.*

IV.2.5 Effect of hypoxia- reoxygenation injury on cell cycle progression

Flow cytometry analysis in combination with propidium iodide staining was carried out to determine the proportion of cells in various stages of cell cycle (Fig 10). Cell cycle progression was determined 24 hrs after HR injury. Following HR injury, stimulation of cell proliferation was apparent in CDCs as seen from the increased proportion of cells in the S phase ($p \leq 0.05$).

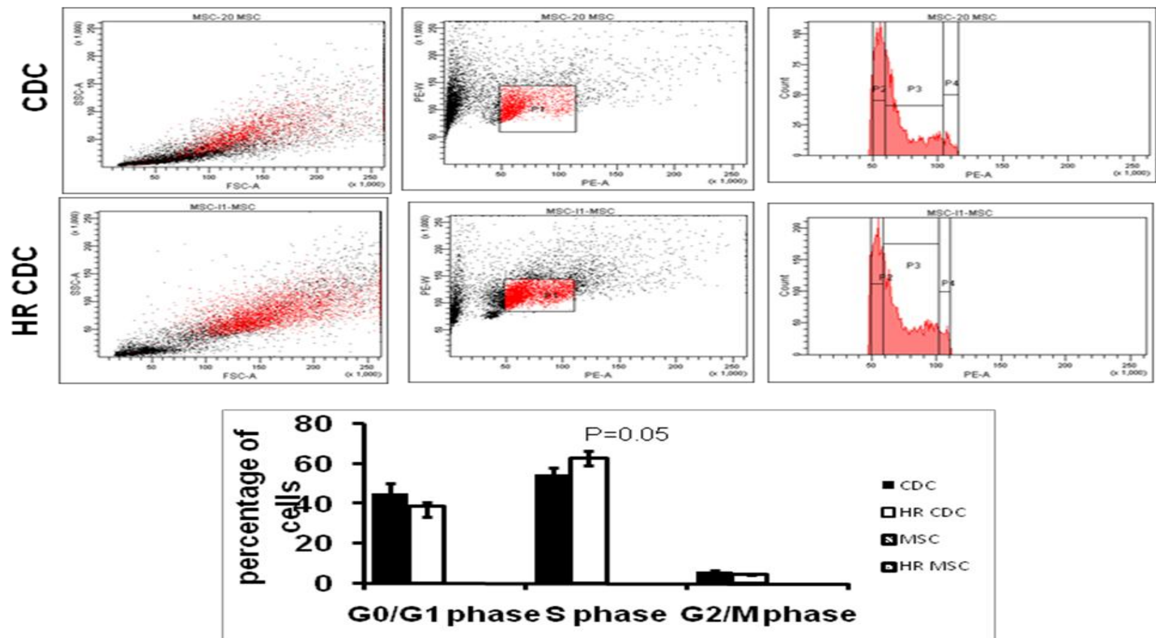


Figure 10: The percentage of Cardiosphere derived cells (CDCs) in each phase of the cell cycle assessed by FACS analysis, 24 hrs after HR injury

*P3 CDCs were plated at a density of $1x10^5$ cells/ml in 35mm dish. After 48hrs in culture, cells were subject to HR injury. The cells were then harvested, washed twice with PBS, and fixed in 70% ice-cold ethanol. After RNase and propidium iodide treatment, each phase of the cell cycle was measured by FACS assay. Normal CDCs served as the controls i) representative histograms of cell cycle analysis ii) graphical representation of percentage of cells in each phase of cell cycle. ANOVA $p < 0.05$, *$p<0.05$ CDC Vs HRCDCs, n= 3.*

IV.2.6 Release of Hepatocyte growth factor after Hypoxia- reoxygenation injury

Hepatocyte growth factor (HGF) is a multifunctional growth factor which regulates DNA synthesis, cell growth and cell motility. HGF levels after HR injury in culture supernatants of CDCs was determined by ELISA (Fig 10). HR injury was found to stimulate HGF release from CDCs ($p<0.01$).

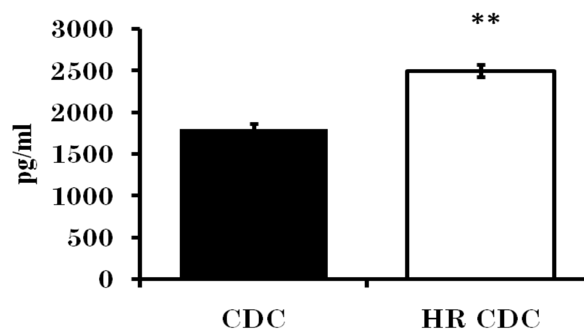


Figure 11: Levels of HGF secreted after HR injury measured by ELISA.

P3 CDCs were seeded in 35mm culture plates at a density of 2×10^5 *cells and incubated for 3 days. The supernatants were collected and the concentration of HGF was* measured using human specific ELISA kits (Abcam). CDCs without HR injury were used as control. ** $p<0.01$ CDC Vs HRCDC, n= 3.

IV.2.7 Effect of HR injury on myogenic differentiation of CDCs

The stem cells were directed to differentiate into cardiomyocytes using 5-azacytidine and TGF β. Differentiation potential was assessed by immunostaining for cardiac-specific markers (Fig 12). Following HR injury and azacytidine induction, the effect of hypoxia and reoxygenation on differentiation of CDCs towards a myocyte lineage was examined. The expression of early myogenesis markers like GATA 4, MHC, and Desmin was unaffected by HR in CDCs but there was an apparent reduction in the expression of connexin 43($p< 0.01$) and cardiac troponin I ($p<0.01$).

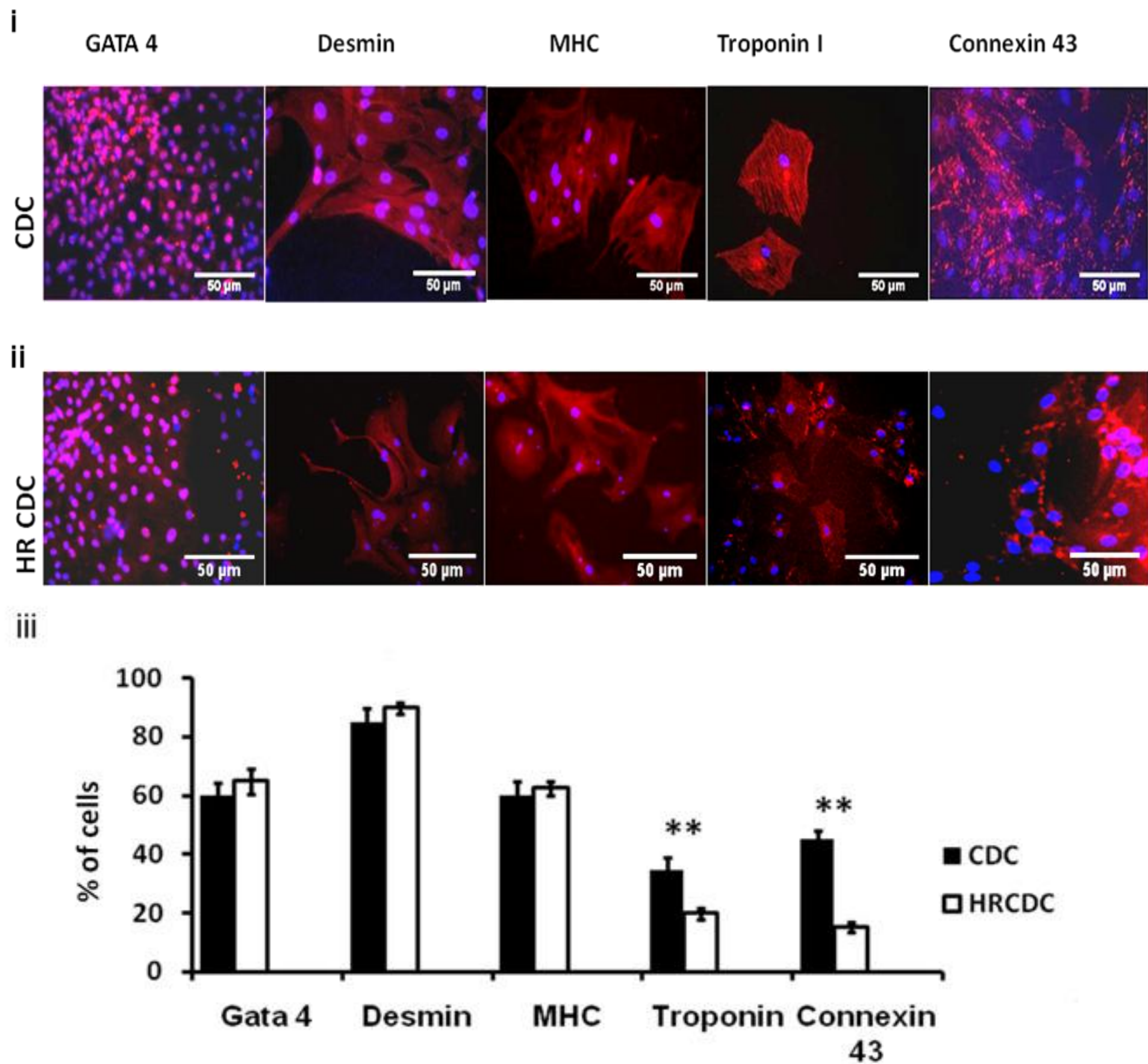


Figure 12: Effect of HR injury on the differentiation of CDCs into cells of cardiac lineage.

*Immunocytochemical staining for different cardiac markers, GATA 4, Desmin, Myosin heavy chain (MHC), cardiac Troponin I, Connexin 43, superimposed on 4, 6-diamidino-2-phenylindole (DAPI) staining for the nucleus (blue). After HR injury CDCs were maintained in cardiomyocyte differentiation medium for 15 days (ii). Cells without HR injury, with myocyte induction served as the control (i). iii) Quantitative assessment of myogenesis after HR injury, in comparison with normal CDCs. ** p<0.01 CDC Vs HR CDC. n=3*

IV.2.8 Effect of HR injury on endothelial tube formation of CDCs

The angiogenic ability of CDCs after HR injury was evaluated using an in-vitro tube formation assay (Fig 13). Both CDCs and HRCDCs formed capillary-like networks on Matrigel (BD Biosciences,) within 8h. There was a significant decrease in the number of branching points following HR injury, in CDCs(Figure 13,P<0.01). There appears to be a reduction in the tube formation pattern after hypoxic injury.

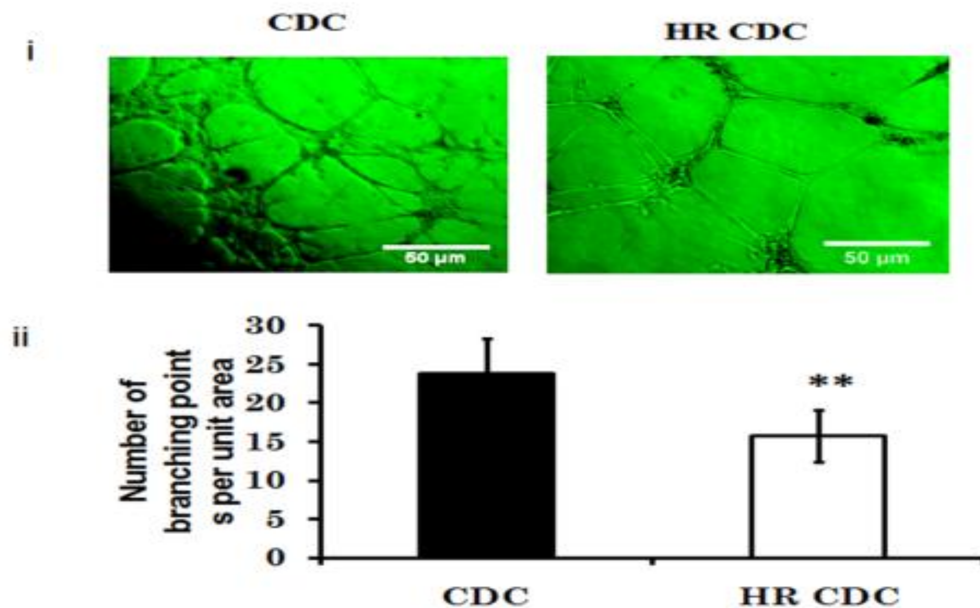


Figure 13: Endothelial tube formation after HR injury.

*The cells were plated at a density of 5x10^4 cells per well in 4-well plates coated with Matrigel (Becton Dickinson). After HR injury, cells on matrigel were again maintained in normal culture conditions for 10hrs. The cells maintained in the same condition without HR injury served as the control. 10 hrs after HR injury, tube formation was examined and branching points were counted using an inverted microscope. i) Representative images, ii) graphical representation. ** p<0.01 CDC Vs HR CDC, n=6.*

IV.2.9 Effect of HR injury on wound healing ability of CDCs

The effect of HR injury on wound healing ability of CDCs were evaluated by scratch wound assay (Fig 14). Confluent cultures were used for the experiment. Scratch-wounds were made in confluent cultures by linearly scraping using a 200-µl yellow pipette tip. The cells were washed with PBS to remove debris and replenished with the fresh medium without serum. CDCs in the HR group were given HR injury after making the scratch-wound. The migration was analyzed soon after HR injury, in the 8th and 24th hr. No significant difference in migration was observed after HR injury in CDCs.

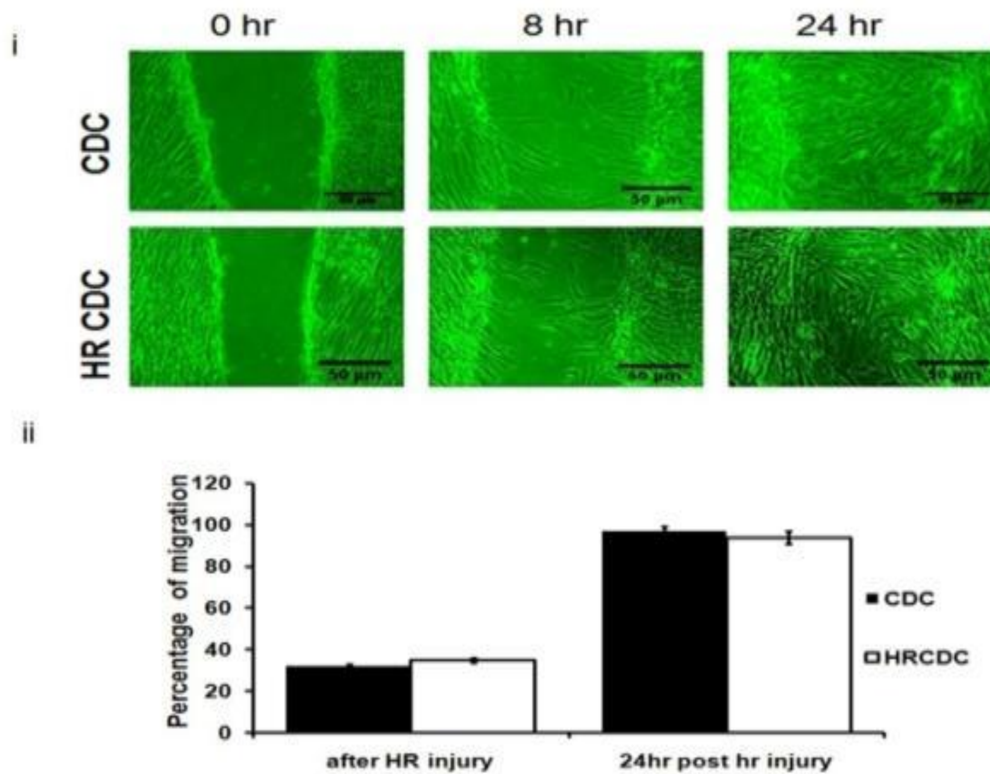


Figure 14: Migration of CDCs in response to hypoxic injury by Scratch- wound assay.

The width of the gaps was measured immediately after scratching, after 8hrs and 24 hrs. i) Representative images of the wound healing assay captured at 0th hr, 8th hr and 24th hr. ii) Graphical representation of wound healing assay, n=3.

CDCs have the capacity to withstand HR injury and promote tissue repair. However functional efficiency is compromised when stem cells are subject to HR injury

IV.3 Response of CDCs consequent to HR injury to myocytes

In the event of ischemic injury to the myocardium, factors released by the myocytes can have a compounding effect on stem cells.

IV.3.1 Effect of H9c2 conditioned medium on CDC – Cell cycle analysis

Hypoxically injured CDCs were treated with normal H9c2 conditioned medium and hypoxically injured H9c2 conditioned medium for 24 hrs. Flow cytometry analysis in conjunction with propidium iodide staining was carried out to ascertain the proportion of cells in various stages of cell cycle (Fig 15). Both normal H9c2 conditioned medium and injured H9c2 conditioned medium positively influenced G1-S progression of CDCs. This is possibly due to release of growth factors by proliferating H9c2 cells. However there was no significant difference between normal and injured H9c2 conditioned medium on HR injured CDCs. Hypoxic injury to H9c2 did not influence the proliferation of CDCs.

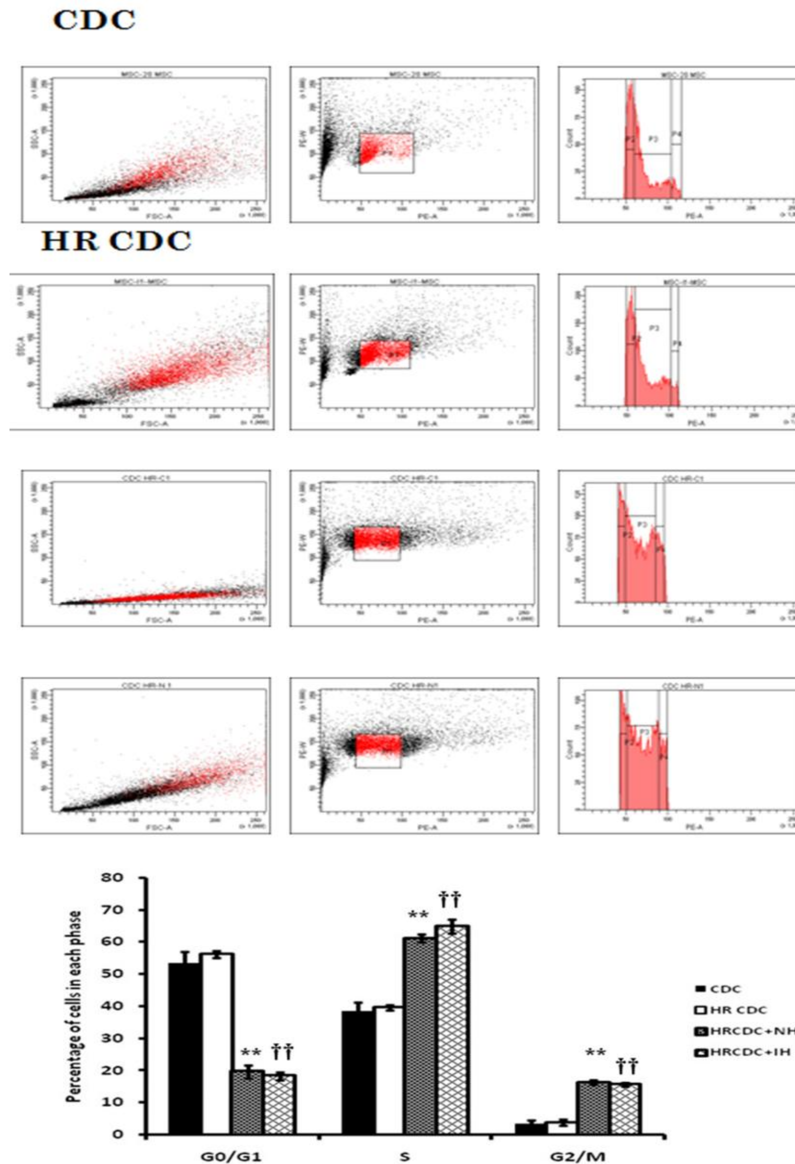


Figure 15: Effect of H9c2 conditioned medium on CDC – Cell cycle analysis

*FACS data showing the percentage of CDCs in each phase of the cell cycle, after treating with normal H9c2 conditioned medium and injured H9c2 conditioned medium 24 hrs after HR injury. Normal CDCs and HR CDCs served as the controls i) representative histograms of cell cycle analysis ii) graphical representation of percentage of cells in each phases of cell cycle. ANOVA p<0.01, **p<0.01 HRCDC Vs HRCDC+NH, †† p<0.01 HRCDC Vs HRCDC+IH, (NH- normal H9c2 conditioned medium, IH- injured H9c2 conditioned medium), n= 3.*

IV.3.2 **Migration of cells towards hypoxically injured tissue bit after HR** injury. Transwell migration assay was carried out to examine whether ischemic injury to the myocardium promotes the migration of stem cells towards hypoxically injured tissue. Myocardial explant cultures were subject to HR injury and the effect on migration of CDCs was assessed (Fig 16). Migration was stimulated when exposed to injured tissue bit compared to normal tissue bit in both normal and HR injured CDCs. Eventhough there was a reduction in the migration of HR CDCs compared to uninjured CDCs the difference was not statistically significant.

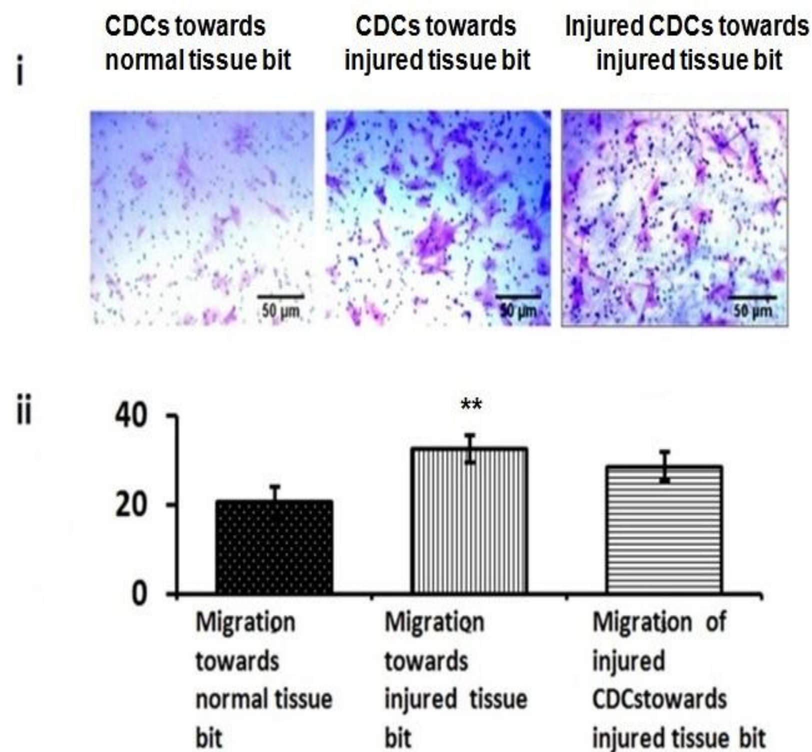


Figure 16: Migration of CDCs towards hypoxically injured tissue bit after HR injury. *Human myocardial tissue bit and surrounding cardiac outgrowth placed in the lower chamber was subject to hypoxic injury. Either normal cells or HR injured CDCs were placed in the upper chamber. CDCs and tissue explants without HR injury was used*

*as the control. CDCs in the upper chamber, that migrated to the lower surface of the filters were stained and counted. i) Representative images ii) Quantitative evaluation of cell migration towards injured myocardial tissue bit.ANOVA $p<0.01$, ** $p<0.01$, CDC migration towards normal tissue bit Vs injured tissue bit, n=3*.

IV.3.3 Migration of CDCs towards injured H9c2 cells

To examine specifically the effect of myocyte injury on migration of stem cells, a transwell migration assay was carried out to study the migration of normal and injured CDCs towards injured H9c2 (Fig 17). HR injury to H9c2 cells promoted the migration of both normal CDCs and injured CDCs. The migration of injured CDCs was significantly lower than that of uninjured CDCs. Transwell migration assay shows that HR injury affects the migratory capacity of stem cells. Myocardial injury stimulates homing in of CDCs.

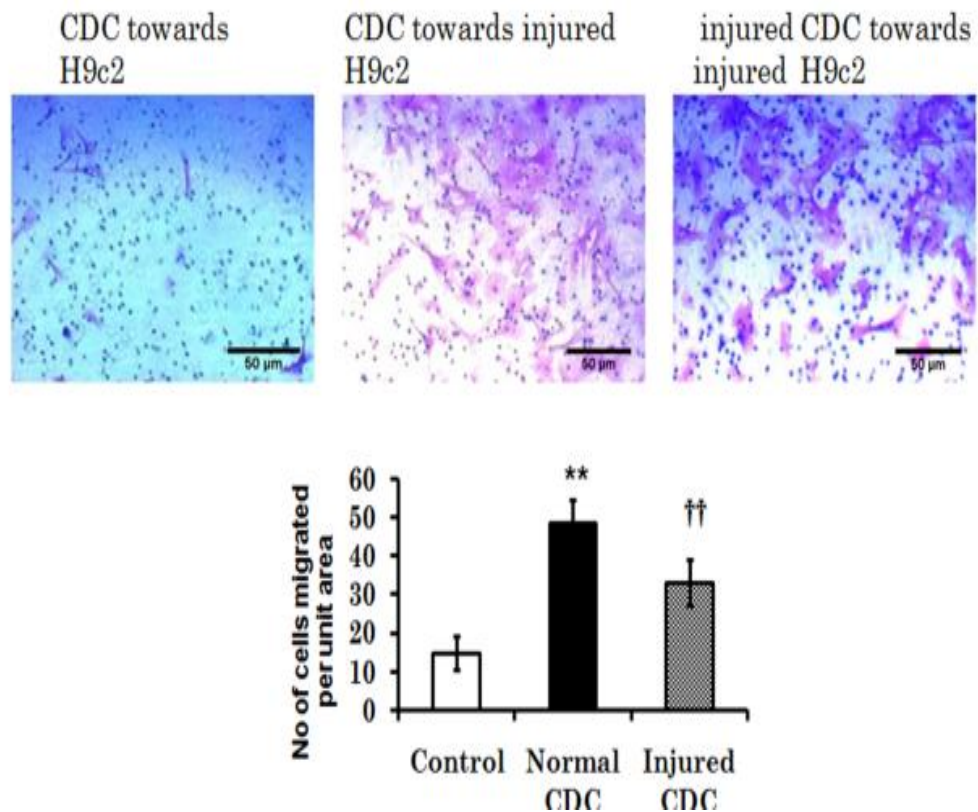


Figure 17: Migration of CDCs subjected to HR injury, towards injured H9c2.

*The migration of HR CDCs towards the lower chamber of a 24 well transwell system, with injured H9c2 cells were compared with the migration of normal CDCs towards injured H9c2cells. Migration of normal CDCs towards normal H9c2 cells was kept as the control. i) Representative images of transwell migration assay. ii) Quantitative evaluation of migration of cells subjected to HR injury. **p<0.01 migration of normal CDCs towards normal H9c2 cells Vs normal CDCs towards injured H9c2. †† p<0.01 migration of normal CDCs towards normal H9c2 cells Vs injured CDCs towards injured H9c2, ANOVA p<0.01, n=3.*

IV.4 Effect of injured stem cells on the surrounding myocytes

Ischemia reperfusion injury to the stem cells can influence the viability of surrounding cardiac myocytes. The response of H9c2 cells to treatment of culture filtrates from injured CDCs was examined by assessment of cell viability and effect on cell cycle.

IV.4.1 Effect of CDC conditioned medium on viability of injured H9c2

To examine whether stem cells can influence the survival of injured myocytes, HR injured H9c2 cells were cultured in CDC conditioned medium

Flowcytometric analysis for Annexin V-FITC/PI live/dead assay was done to evaluate the effect of CDC conditioned medium on viability of injured H9c2 (Fig 18). CDC conditioned medium was found to have a protective effect on injured cardiomyocytes. Hypoxic injury affected the viability of H9c2 cells ($p<0.001$), which was reduced by treatment with CDC conditioned medium ($P<0.01$) and injured CDC conditioned medium ($p<0.05$). HR injury to CDC was not found to induce any additive effect neither did it help in the prevention of cell death.

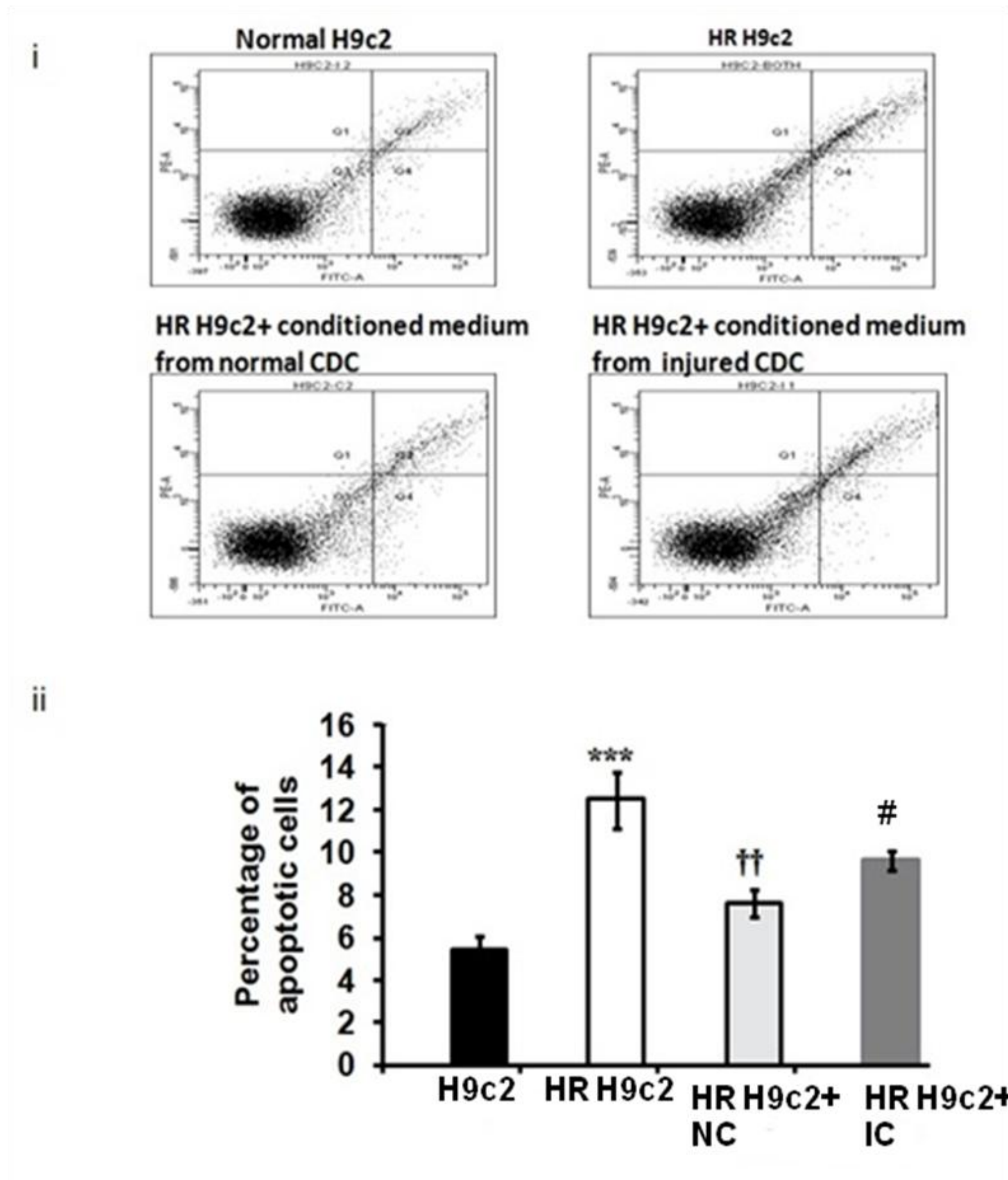


Figure 18: Influence of CDC conditioned medium on viability of injured H9c2

*Cultured H9c2 cells were subject to HR injury followed by treatment with either normal CDC conditioned medium or injured CDC conditioned medium. Apoptosis was quantified by FACS analysis after staining with Annexin V and propidium iodide (*PI). (i) *Representative histograms of Annexin V /PI live dead assay. ii) Quantitative data representing early apoptotic cells by annexin/PI live dead assay. *** $p<0.001$, H9c2 Vs HR H9c2, †† $p<0.01$ H9c2 Vs HR H9c2 + NC, #$p<0.05$, H9c2 Vs HR H9c2+ IC. (NC– normal conditioned medium, IC- injured conditioned medium) ANOVA $p<0.01$*

IV.4.2 Effect of H9c2 conditioned medium on CDC – Cell cycle analysis

Hypoxically injured CDCs were treated with normal H9c2 conditioned medium and hypoxically injured H9c2 conditioned medium for 24 hrs. Flow cytometry analysis in conjunction with propidium iodide staining was carried out to determine the proportion of cells in various stages of cell cycle (Fig 19). Both normal H9c2 conditioned medium and injured H9c2 conditioned medium positively influenced G1-S progression of CDCs. There was no significant difference between normal and injured H9c2 conditioned medium in HR injured CDCs. Hypoxic injury to H9c2 does not have a stimulatory effect on proliferation of CDCs.

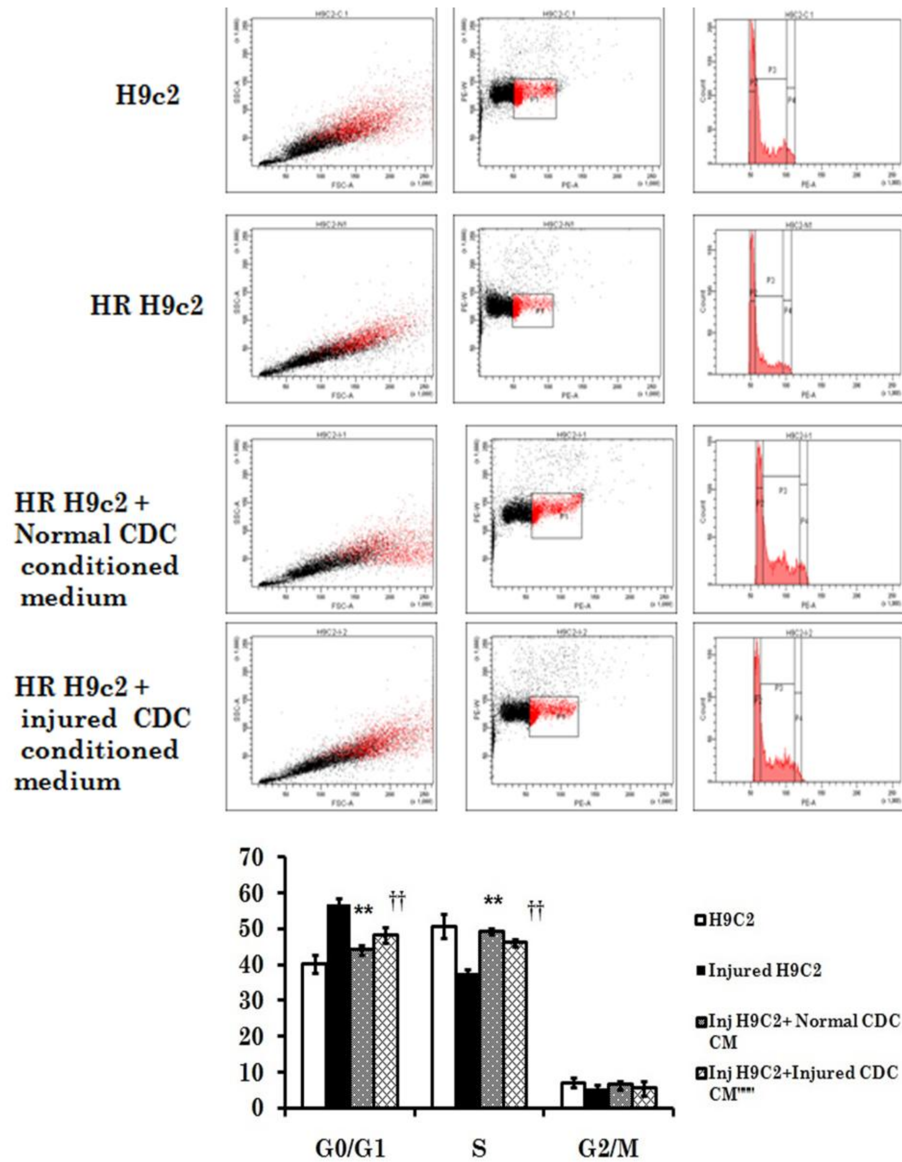


Figure 19: Role of CDC conditioned medium on cell cycle progression of injured H9c2 cells.

FACS data showing the percentage of HR H9c2 cells in each phase of the cell cycle, after treating with normal CDC conditioned medium and injured CDC conditioned medium, 24 hrs after HR injury. Normal CDCs and HR CDCs served as the controls i) representative histograms of cell cycle analysis ii) graphical representation of percentage of cells in each phases of cell cycle. ***p<0.01 HRH9c2 Vs HR H9c2+Normal CDC CM, †† p<0.01 HRH9c2 Vs HRH9c2+ Injured CDC CM, (CM- conditioned medium), n=3.ANOVA p<0.01*

IV.5 Comparison of CDCs with bone marrow mesenchymal stem cells

Bone marrow mesenchymal cells are popularly used for transplantation for tissue regeneration. Circulating MSCs are also suspected to be the autologous source of tissue repair as they are known to home in to the site of injury. Hence the survival, proliferation, differentiation and migration of MSCs as well as its capacity to withstand HR injury was compared with CDCs.

IV.5.1 Isolation of MSCs

Bone marrow mesenchymal stem cells were isolated by seeding the bone marrow aspirates on gelatin coated dishes Fig. 20 i). A fraction of the cells adhered to the culture surface and colony forming units (CFUs) were visible in one week (Fig 19.ii). The CFUs expanded into a confluent layer of MSCs within 14 days (Fig 19. iii). Subsequently, the MSCs were subcultured and passaged once in 7 days**.**

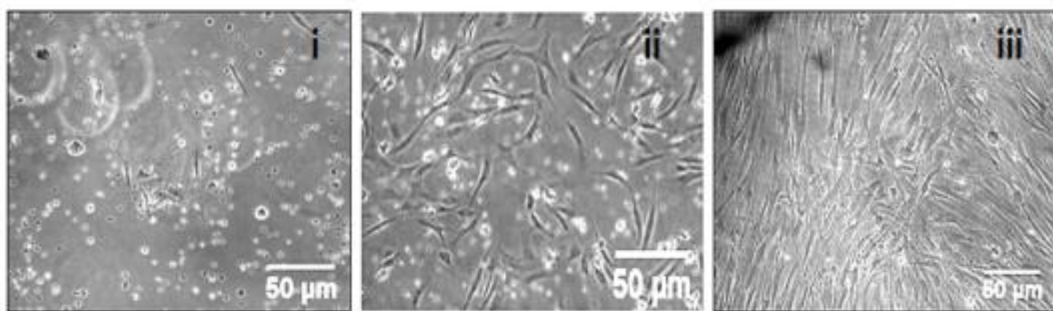


Figure 20: Isolation of Bone marrow mesenchymal stem cells

Cells from the bone marrow aspirate were plated in IMDM medium supplemented with 20% FBS, 100 U/ml penicillin G, and 100 µg/ml streptomycin. Representative images of i) Bone marrow derived cells after 72 hr in culture ii) Small colony forming units formed after 7 days. iii) Confluent P1 MSCs after two weeks.

IV.5.2 Characterization of MSCs

Subconfluent cultures of P4 MSCs were stained with primary antibodies for CD105, CD90, CD34, CD45, c-Kit, along with corresponding isotype control. MSCs were positive for CD 105, CD 90, CD 29, C kit (Figure 21, Table 5).

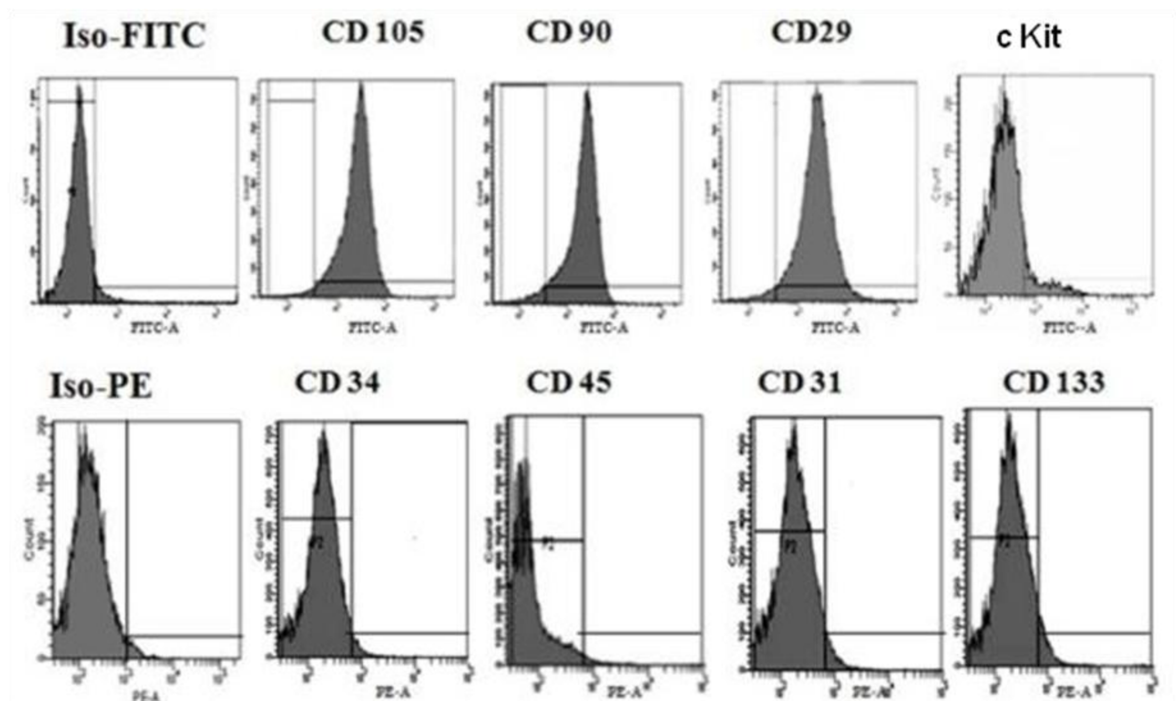


Figure 21: Representative FACS data of expression pattern of cell surface markers of MSC

Subconfluent cultures of P4 MSCs were stained with primary antibodies for CD105, CD90,CD29, cKi, CD34, CD4, c-Kit, along with corresponding isotype control. After incubation with appropriate fluorescent-labeled secondary antibodies, cells were washed, fixed with 4% paraformaldehyde and analyzed by flow cytometry.

Table 5: Distribution of surface markers of MSCs assessed by FACS. n=3.

Markers	Percentage
CD 105	98.3±0.5
CD 90	97.3±0.8
CD 29	99±0.16
c Kit	4.3±0.20
CD 34	1.3±0.2
CD 45	0.46±0.29
CD 31	0.7± 0.3
CD 133	1.2±0.34

IV.5.3 Comparison of surface marker expression of CDCs and MSCs

Flow cytometric analysis revealed differences in the expression of surface markers in CDCs and MSCs (Table 6). A highly significant difference was observed in the expression of CD 90, popularly known as Thy-1, a surrogate marker for various kinds of stem cells. While 97.3±0.8% of MSCs showed positive expression of CD90, only 16±1.2% of CDCs was positive for the marker. The cardiac stem cell marker, c Kit expression also ranged from 2.2 to 11% in CDCs but it remained at an average of 4.8 in MSCs. The expression of CD 105 or endoglin, a TGF-beta receptor ranged from 86% to 99% in CDCs and 92 to 99.5% in MSCs. Both the cell types showed very small

percentage (≤ 1.2) of other hematopoietic and endothelial markers CD 133, CD34, CD45, and CD 31.

Table 6: Comparison of expression of cell surface markers in CDCs and MSCs by Flow cytometry analysis, ,*p< 0.05 CDC Vs MSCs.

Markers	CDC (%)	MSC (%)
CD 105	94±1.5	98.3±0.5
CD 90	16±1.2	97.3±0.8*
CD 29	99±0.08	99±0.16
c Kit	6.3±0.8	4.3±0.20*
CD 34	1.1±0.3	1.3±0.2
CD 45	0.67±0.21	0.46±0.29
CD 31	1.06±0.15	0.7± 0.3
CD 133	0.93±0.16	1.2±0.34

IV.5.4 Comparison of the effect of Hypoxia- reoxygenation injury on cardiac and mesenchymal stem cells

To study the effect of hypoxia- reoxygenation injury on MSCs and CDCs, serum deprived cells were placed in a hypoxia incubator (0.5% O_2 for 2 hrs). After hypoxic injury the cells were reoxygenated in the presence of 10% serum containing medium for 6 hrs to

simulate ischemia-reperfusion injury. The effect of HR injury on CDCs and MSCs were assessed for cell survival, replication, migration and differentiation.

IV.5.5 Effect of Hypoxia- reoxygenation injury on apoptosis

Oxidative stress during hypoxia-reoxygenation is known to promote cell death. Flow cytometric analysis for Annexin V-FITC/PI live dead assay was carried out to evaluate the effect of HR injury on survival capacity of CDCs and MSCs (Fig 22). A small but significant increase in apoptosis (5.7±0.14%, $p<0.01$) after Hypoxia- reoxygenation injury was seen in CDCs compared to untreated cultures (3.5±0.37%). But, there was a six fold increase in the percentage of apoptotic cells in HR MSCs group compared to untreated MSCs ($p<0.01$). This data suggests that MSCs are relatively more susceptible to HR injury induced apoptosis.

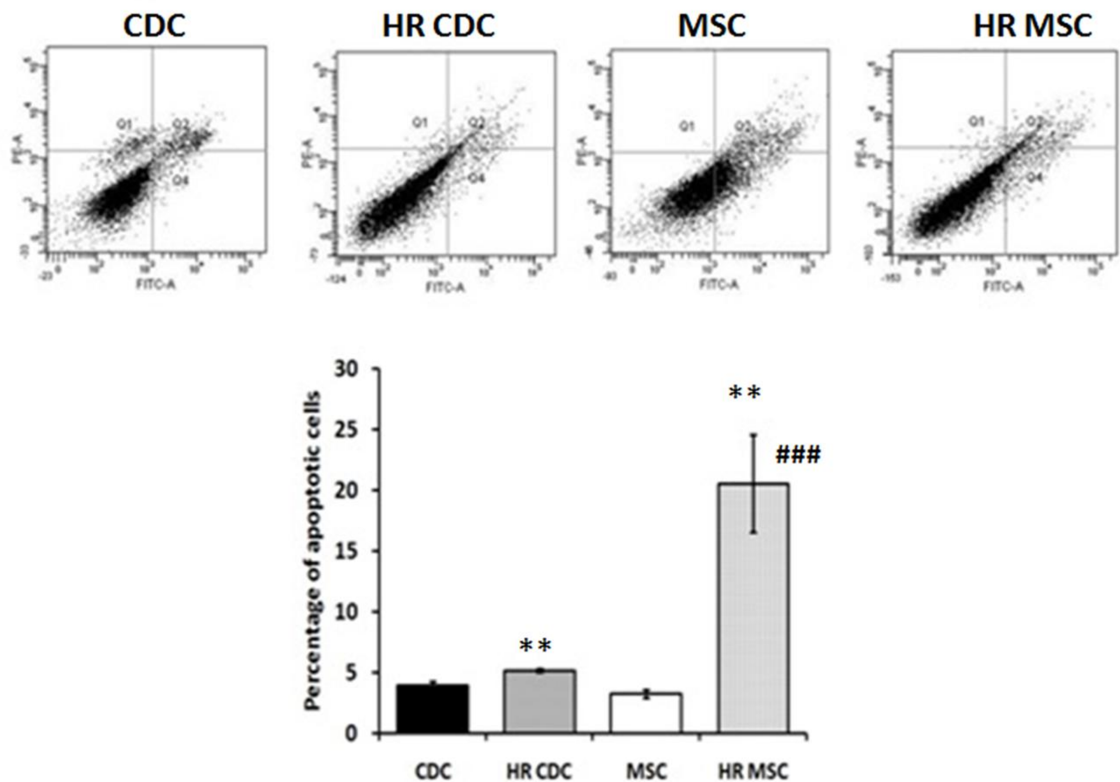


Figure 22: Effect of HR injury on apoptosis of CDCs and MSCs by Annexin V /PI live dead assay.

*Cultured cells were given HR injury. The level of apoptosis was quantified by FACS analysis after staining with Annexin V and propidium iodide (PI). (Ai)Representative histograms of Annexin V /PI live dead assay. ii) Quantitative data representing early apoptotic cells by annexin/PI live dead assay. ** p<0.01 compared to untreated control, ### p< 0.00 1 HR MSCs compared to HR CDCs, n=3, ANOVA p<0.01.*

IV.5.6 Effect of Hypoxia- reoxygenation injury on cell senescence

Cell cultures in the fourth passage were subject to HR injury and examined for the presence of senescent cells after 24 hrs in standard culture conditions. The proportion of

cells with positive basal SA-β-gal staining was negligible in control CDCs and MSCs (Fig 23). But, HR injury induced cell senescence in CDCs ($p<0.001$) as well as MSCs ($p<0.001$). The proportion of SA-β-Gal-positive cells was significantly higher in MSCs after HR injury compared to CDCs, ($p< 0.001$) indicating enhanced susceptibility of MSCs to HR injury.

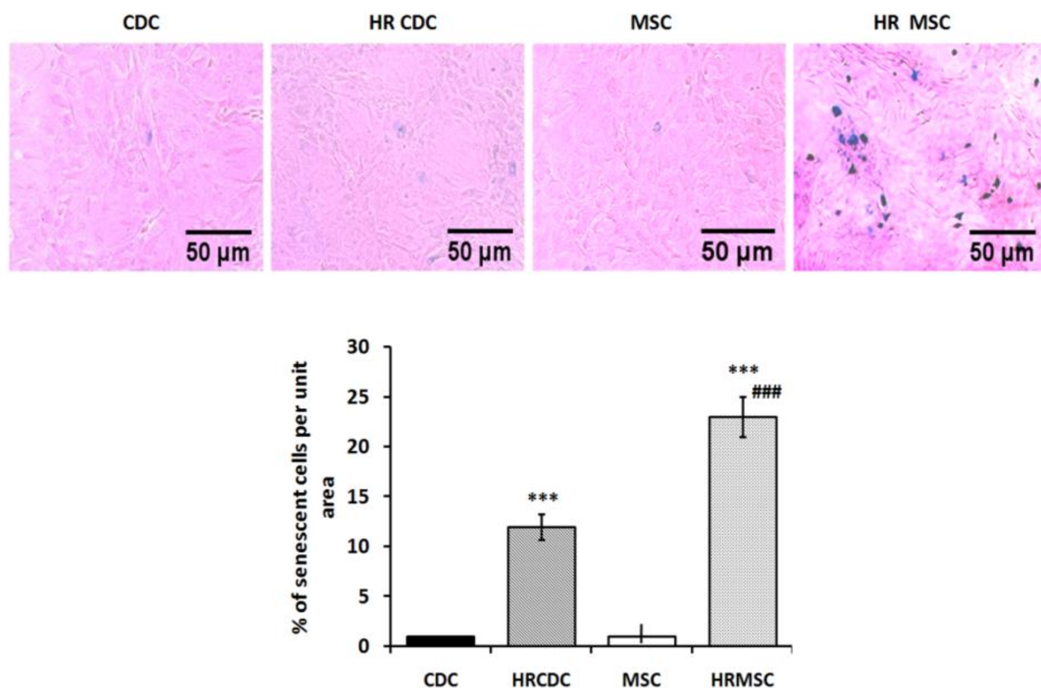


Figure 23: Senescence-associated β-galactosidase (SA-β-gal)-positive cells after 24hrs of HR injury in CDCs and MSCs from Passage 4.

*P4 MSCs and CDCs were seeded in triplicates at a density of $1x10^5$ cells/ ml and grown to 70% confluency. Twenty four hours after HR injury, wells were rinsed twice with PBS, fixed in 1 mL fixing solution, and incubated at room temperature for 15 min. Following washing with PBS, cells were reincubated overnight in freshly prepared SA β-gal staining detection solution. Senescent, SA-β-Gal-positive cells developed blue color. Staining was quantified by positive cell count i) representative images of (SA-β-gal)-positive cells. ii) Graphical representation of SA-β-gal positive cells. *** $p< 0.001$ compared to untreated control, ### $p< 0.001$ HR MSCs compared to HR CDCs, n=3, ANOVA $p<0.01$.*

IV.5.7 Effect of HR injury on the expression of prosurvival factors

CDCs and MSCs were subjected to HR injury. Prosurvival proteins expressed by the cells after HR injury were compared with those expressed in cells grown under standard conditions (Fig 24).The secretion of prosurvival factors in response to injury was assessed from the expression of HIF 1-α, pAkt, BCl2 and p21. Expression of the HIF-1α increased significantly in response to HR injury both in CDCs ($p< 0.05$) and MSCs. ($p<0.01$). Phosphorylated Akt (pAkt), a critical regulator of phosphatidylinositol 3 (PI3)-kinase-mediated cell survival, was stimulated in response to HR in CDCs but not in MSCs. ($p<0.01$CDC Vs HRCDCs, $p<0.001$, HR CDC Vs HR MSCs). Expression of the antiapoptotic protein Bcl2 ($p< 0.05$ CDC Vs MSCs) was significantly increased in hypoxic CDCs ($p< 0.01$, CDC Vs HRCDC) but not in hypoxic MSCs. The basal level of pAkt was higher and Bcl_2 lower in CDCs compared to MSCs. The results show improved survival of CDCs after HR injury than MSCs as seen from the upregulation of proteins involved in survival.

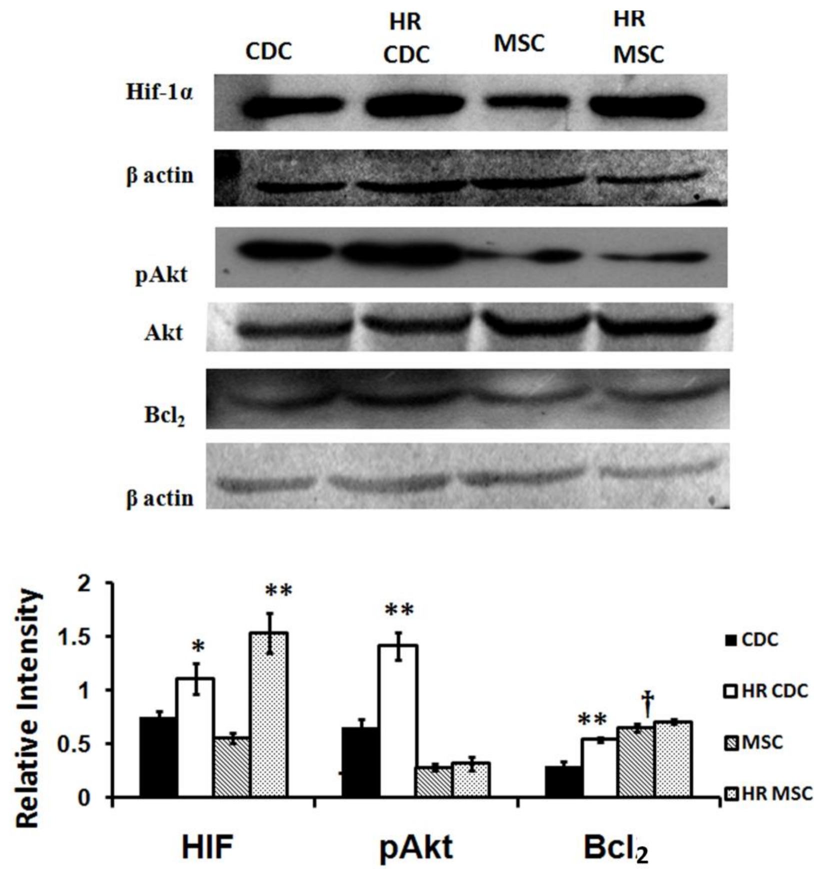


Figure 24: Expression of pro survival factors after HR injury.

Survival proteins expressed by CDCs and MSCs after HR injury was compared with those expressed in cells grown under normal conditions. β actin was used as the loading control. i) Representative Western blots. ii) Quantification of Western blot data. $p<0.05$, ** $p< 0.01$ *compared to untreated control,* † $p< 0.05$ *MSCs Vs CDCs, n=3. ANOVA* $p< 0.01$ *for Hif-1α and pAkt,* $p<0.05$ *for* Bcl_2.

IV.5.8 Effect of Hypoxia- reoxygenation injury on generation of Reactive Oxygen Species (ROS)

Intracellular ROS generation was assessed by DCFDA cellular ROS detection assay. Fluorimetric evaluation revealed no significant difference in ROS generation by CDCs and MSCs (Fig 25). In response to hypoxic injury, however, there was an increase in fluorescence of DCHFDA, in both cell types (P< 0.05 CDC Vs HR CDC and MSC Vs HR MSC) suggesting that hypoxia stimulates oxidative stress. Increase in ROS generation in response to HR injury was relatively more in MSC compared to CDC (P< 0.01).

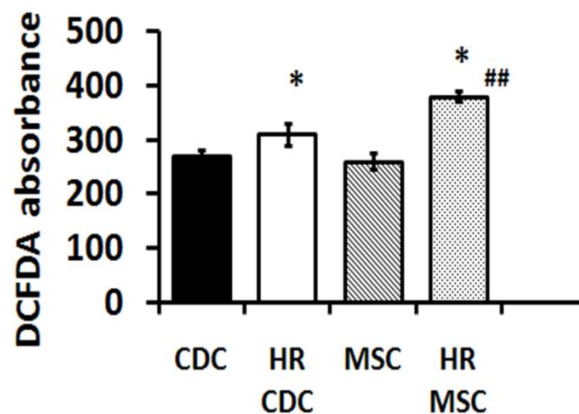


Figure 25: Effect of HR injury on ROS generation assessed by DCFDA FACS analysis.

*Cells from P4 were seeded at a density of 4 x10^4 cells per well in 96-well dark plates. After 24hrs cells were subject to HR injury. The cells were then incubated with 10 µM 2', 7'-dichlorodihydrofluorescein diacetate (DCFDA-Invitrogen) for 30 min. Normoxic cells served as control. The fluorescence of 2',7'- dichlorodihydrofluorescein (DCF) in cells was directly determined using FLx800™ Multi-Detection Microplate Reader with Gen 5 PC software (Biotek) at an excitation wavelength of 495 nm and an emission wavelength of 520 nm. Graphical representation of ROS generation after HR injury, quantified by FACS analysis after treating with DCFDA, *p< 0.05 compared to untreated control, ## p< 0.01 HR MSCs compared to HR CDCs, n=3. ANOVA p<0.05.*

IV.5.9 Effect of hypoxia- reoxygenation injury on cell cycle progression

Flow cytometry analysis in conjunction with propidium iodide staining was carried out to ascertain the proportion of cells in various stages of cell cycle (Fig 26). Cell cycle progression was determined 24 hrs after HR injury. The distribution of cells in the different stages was comparable for both the cell types in untreated cultures. Following HR injury, stimulation of cell proliferation was apparent in CDCs as seen from the increased proportion of cells in the S phase ($p<0.05$). Proportion of MSCs in G0/G1 phase was comparatively more ($p<0.01$ Vs MSC) and cells in S phase lower ($p<0.01$ Vs MSC), 24 hrs after HR injury.

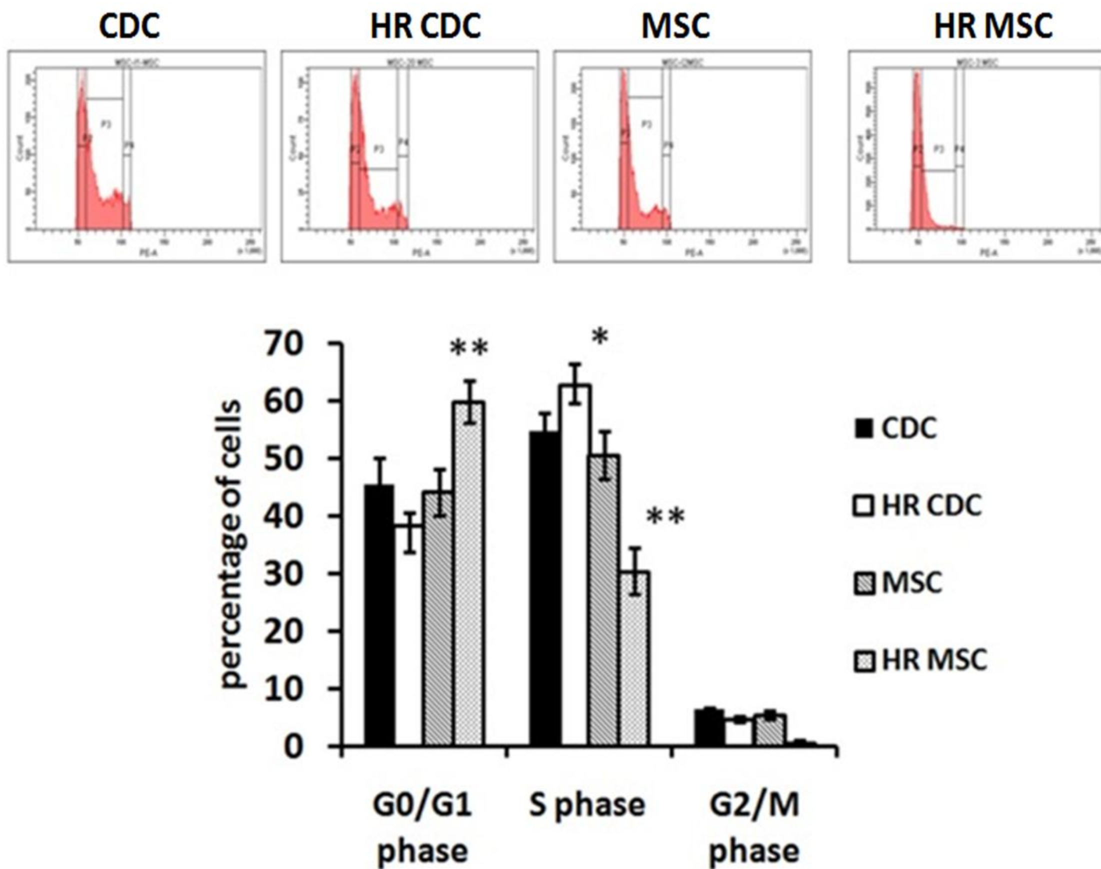


Figure 26: Effects of HR injury on the cell cycle progression of CDCs and MSCs

*Cells were plated at a density of 1x10^{5}cells/ml in a 35mm dish. After 48hrs in culture, cells were subject to HR injury. The cells were then harvested, washed twice with PBS, and fixed in 70% ice-cold ethanol. After RNase and propidium iodide treatment, each phase of the cell cycle was measured by FACS assay. Normal CDCs served as the control i) representative histograms of cell cycle analysis ii) graphical representation of percentage of cells in each phases of cell cycle, *p< 0.05, ** p< 0.01 compared to untreated control, n=3. ANOVA p<0.01.*

IV.5.10 Release of Hepatocyte growth factor after Hypoxia- reoxygenation injury

Hepatocyte growth factor (HGF) is a multifunctional growth factor which regulates DNA synthesis, cell growth and cell motility (Yan et al., 2001). HGF levels in culture supernatants were determined by ELISA (Fig 27). The basal levels of HGF was found to be relatively more for CDCs compared to MSCs (p<0.001). HR injury stimulated HGF release from both CDCs (p< 0.01) and MSCs (p< 0.05) with relatively higher proportion in the former.

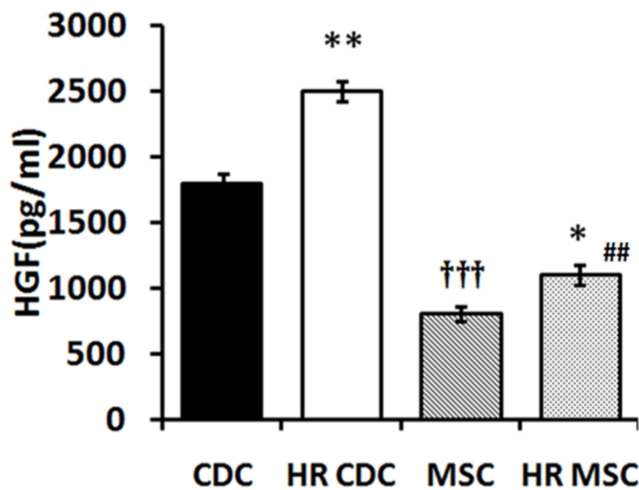


Figure 27: Levels of HGF secreted after HR injury measured by ELISA.

*P3 CDCs and MSCs were seeded in 35mm culture plates at a density of 2×10^5 cells and incubated for 3 days. The supernatants were collected and the concentration of HGF was measured using human specific ELISA kits (Abcam). Cells without HR injury were used as control. Data were expressed as Mean ± SD. *p<0.05, ** p< 0.01 compared to untreated control, ## p< 0.01 HR MSCs compared to HR CDCs, ††† p< 0.001 MSC compared to CDC, n=3.ANOVA p<0.01.*

IV.5.11 Effect of HR injury on directed myogenic differentiation

The stem cells were directed to differentiate into cardiomyocytesusing 5-azacytidine and TGF β. Differentiation potential was assessed by immunostaining for cardiac-specific markers (Fig 28). Fifteen days after azacytidine induction, differentiation to myogenic lineage was significantly higher for CDCs compared to MSCs as apparent from positive staining for GATA 4 ($p< 0.05$), troponin I ($p< 0.001$), Myosin heavy chain ($p< 0.001$), connexin 43 ($p< 0.001$).(Fig 28).The expression of desmin was comparable for both the cell types(Fig 28). Following HR injury and azacytidine induction, the effect of hypoxia and reoxygenation on differentiation of CDCs or MSCs towards a myocyte lineage was examined. The expression of early myogenesis markers like GATA 4, MHC and Desmin was unaffected by HR in CDCs but there was an apparent reduction in the expression of connexin 43($P\leq 0.01$) and cardiac troponin I ($P\leq 0.01$). Reduction in the expression of GATA 4($P\leq 0.05$) and MHC ($P\leq 0.05$) was observed in MSCs after HR injury. Expression of cTn1 and connexin 43 was totally absent in HR MSCs.

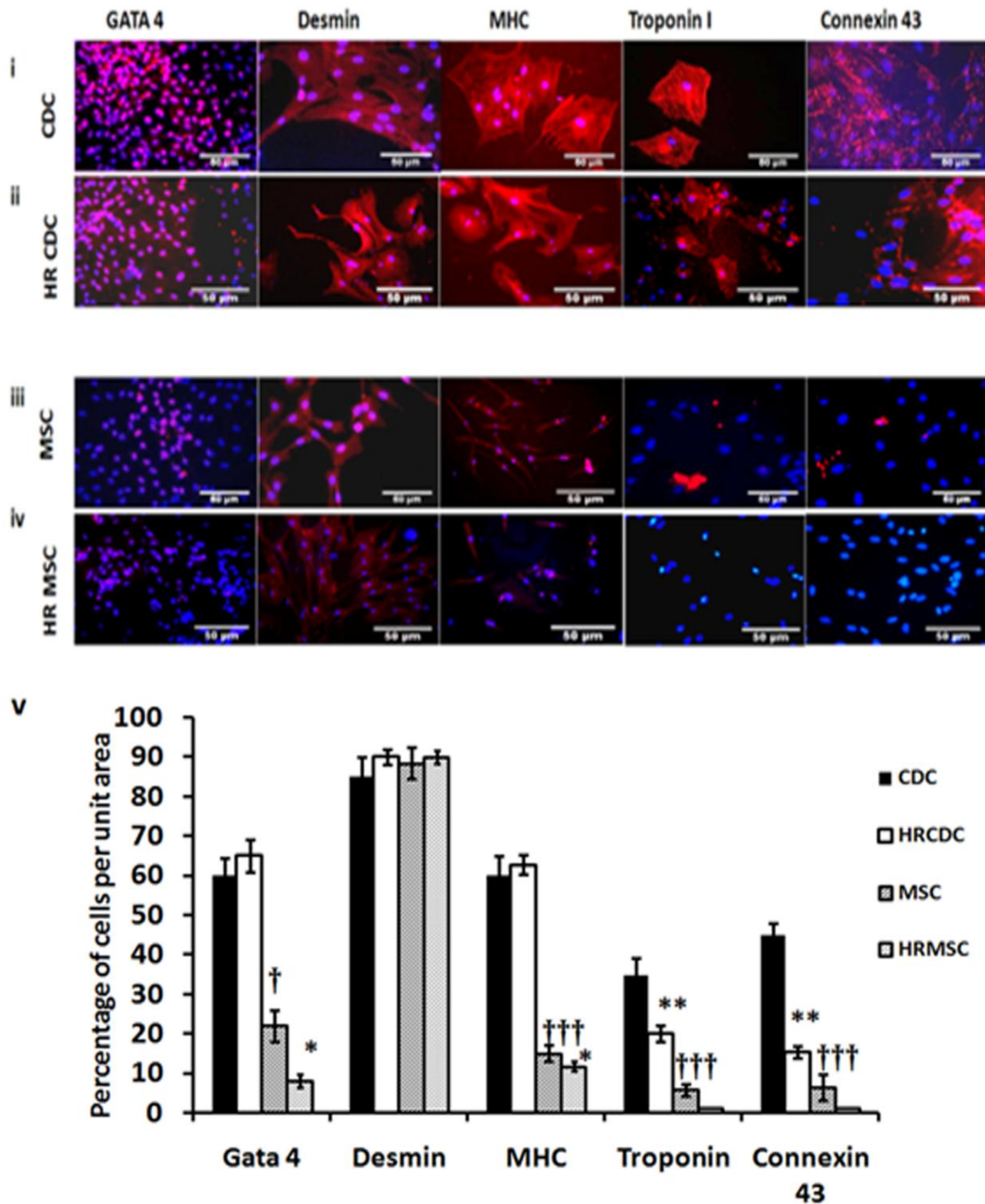


Figure 28: Effect of HR injury on directed differentiation of CDCs and MSCs into cells of cardiac lineage.

Immunocytochemical staining of different cardiac markers , GATA 4, Desmin, MHC, cardiac Troponin I, Connexin 43, subject to H/R injury. After HR injury CDCs (ii) and MSCs (iv) were maintained in cardiomyocyte differentiation medium for 15 days. Cells

*without HR injury, with myocyte induction were served as the control (i, ii). B) Quantitative representation of myogenesis after HR injury, in comparison with normal CDCs and MSCs. Data are expressed as Mean ± SD. *p< 0.05, ** p< 0.01 compared to untreated control, † p<0.05, ††† p≤0.001 MSC compared to CDC, n=3.*

IV.5.12 Effect of HR injury on spontaneous myogenic differentiation

Expression of early myocyte specific marker Mef 2C was assessed by western blot analysis (Fig 29). Without myogenic induction the expression of Mef 2C in CDCs was higher than that for MSCs (p<0.001) and was further enhanced in the former following HR injury (P< 0.01) compared to MSCs (p<0.05).

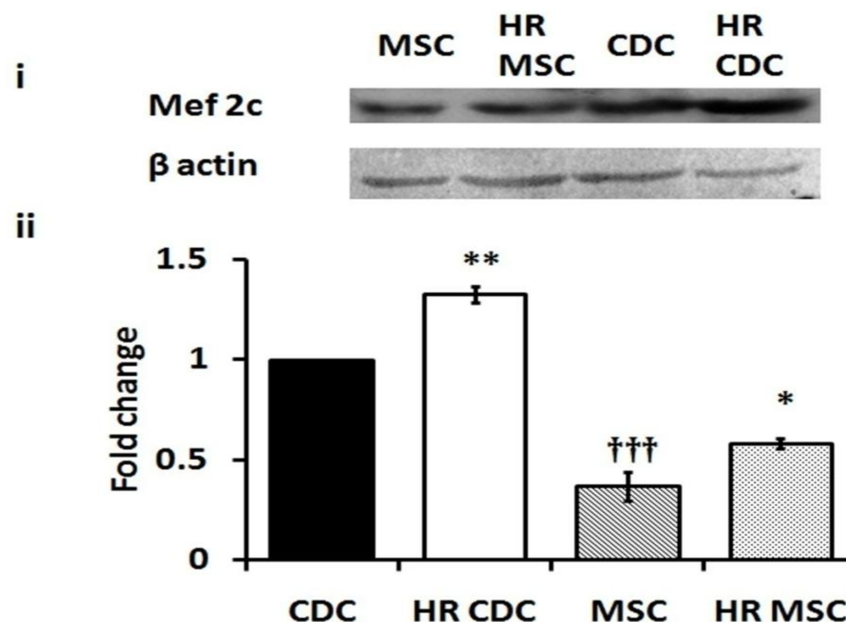


Figure 29: Expression of cardiac lineage marker Mef 2C after HR injury.

i) Western blot analysis for the expression of cardiac lineage marker Mef 2C soon after HR injury, without induction by differentiation medium. ii) Quantitative expression of Mef 2C soon after HR injury. Data expressed as Mean± SD of fold change normalized to the

*total level of CDC. *p<0.05, ** p< 0.01 compared to untreated control, ††† p≤0.001 MSC compared to CDC, n=3. ANOVA p< 0.05*

IV.5.13 Effect of HR injury on endothelial tube formation

The angiogenic ability of CDCs and MSCs was evaluated using in vitro tube-forming assay. Both cell types formed capillary-like networks on Matrigel (BD Biosciences) within 8h. Ten hours after HR injury, the tube forming ability was significantly lower for MSCs compared to CDCs (Fig 30). MSCs formed aggregates with short-protruding sprouts. Whereas, well organized, long interconnecting tubes were seen for CDCs. Quantitative analysis revealed that branching points of the capillary-like networks were greater in CDCs than in MSCs ($p < 0.001$). There was decrease in the number of branching points in both the cell types following HR injury, the difference being statistically significant for CDCs ($P< 0.01$). After HR injury, significantly lower number of cells attached to matrigel, a main cause for reduced endothelial tube formation in both cell types. Almost 25% cells were found floating on the matrigel surface after HR injury and these cells did not contribute for endothelial tube formation.

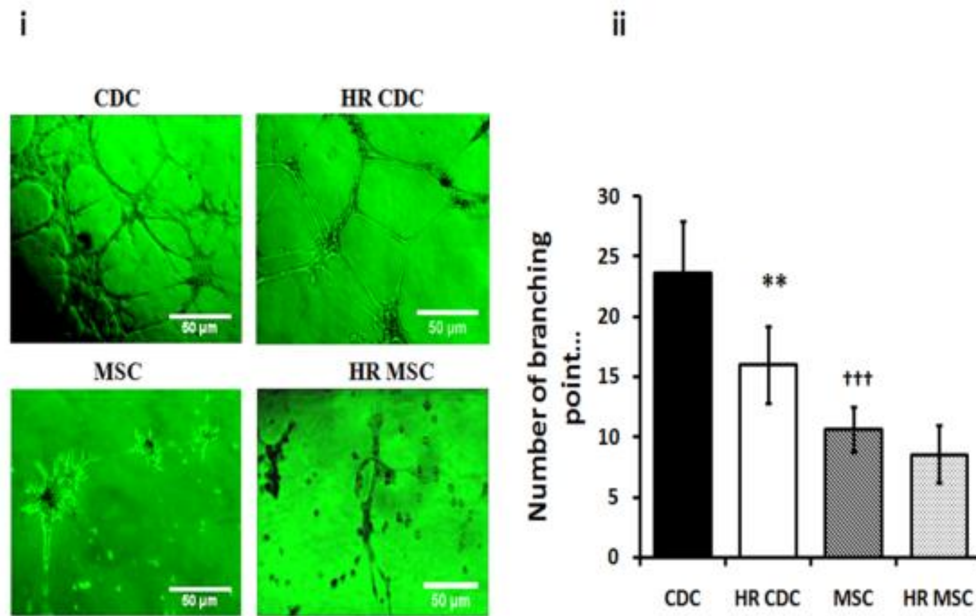


Figure 30: Endothelial tube formation after HR injury.

*The cells were plated at a density of 5x10^4 cells per well in 4-well plates coated with Matrigel (Becton Dickinson). Cells on matrigel were subject to hypoxia-reperfusion injury. The cells maintained in the same condition without HR injury served as the control. 10 hrs after, HR injury, tube formation was examined and branching points were counted using phase contrast optics. i) Representative images, ii) Quantitative representation of endothelial tubes formed after HR injury. ** $p<0.01$, compared to untreated control, †††$p\leq0.001$ MSC compared to CDC. n=6. ANOVA $p<0.01$.*

IV.5.14 Release of Vascular Endothelial growth factor after Hypoxia-reoxygenation injury

VEGF is important for promoting endothelial differentiation of cardiac stem cells (Xiao et al., 2014). The levels of VEGF secreted by CDCs and MSCs were measured by ELISA (Fig 31). The release of VEGF was comparatively higher for CDCs ($p<0.01$). HR injury stimulated release of VEGF in both CDCs and MSCs ($p\leq 0.05$).

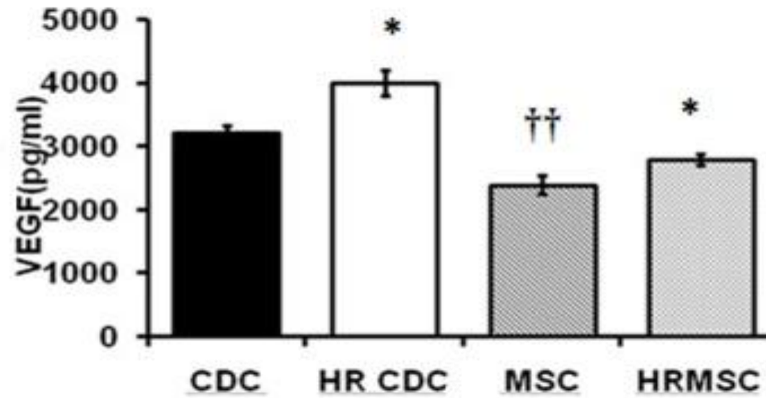


Figure 31: Expression of VEGF after HR injury

*Cells were seeded in 35mm culture plates at a density of 2× 10^5 cells and incubated for 3 days. The supernatants were collected and the concentration of VEGF was measured. Cells without HR injury were used as control. Levels of VEGF secreted by CDCs and MSCs and the effect of HR injury on VEGF secretion was evaluated using human specific ELISA kits (Abcam). Values represent mean ± SD. * $p < 0.05$ compared to untreated control, †† $p \leq 0.01$ CDC compared to MSC. N=6.ANOVA $p < 0.01$, n=6, ANOVA <0.05.*

IV.5.15 Effect of HR injury on Multi lineage differentiation potential

As multilineage differentiation potential is an indicator of stemness.Osteogenic and adipogenic differentiation potential was assessed

IV.5.15.1 Osteogenic differentiation

Osteogenic differentiation on stimulation was seen for both the cell types. MSCs showed a significantly higher potential to differentiate into osteocytes, when maintained in osteogenesis medium for 21 days as evidenced by the formation of calcium deposits

which stained red by alizarin red S (Fig 32). Hypoxic injury enhanced the osteogenic differentiation potential of both cell types (*p< 0.05 CDCs Vs HRCDCs, P≤ 0.05 MSC Vs HR MSCs, †† P≤ 0.01 CDC Vs MSC)

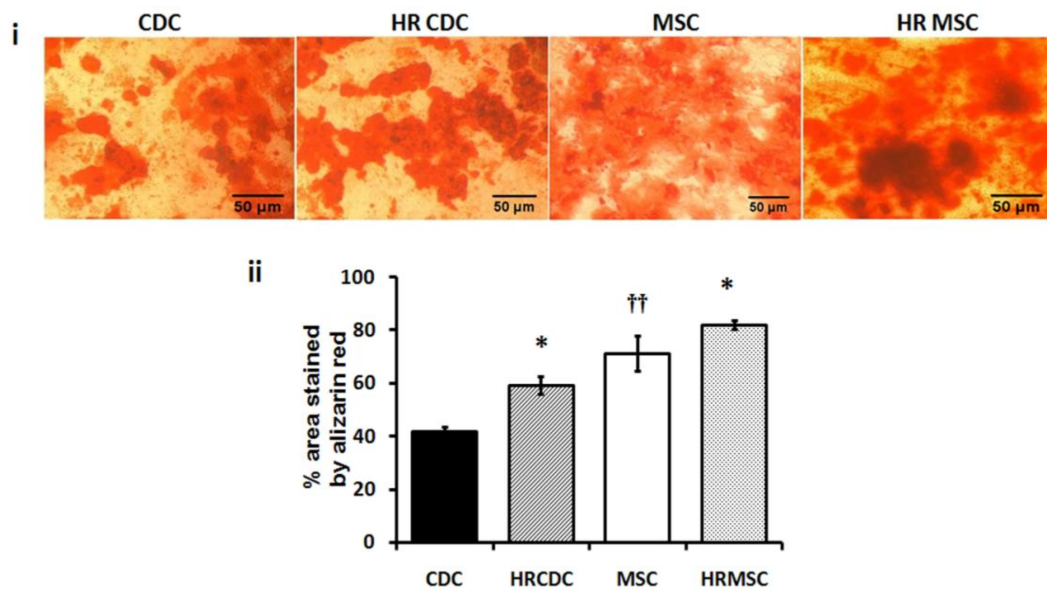


Figure 32: Osteogenic differentiation of CDCs and MSCs after HR injury.

*A) Effect of HR injury on osteogenic differentiation of CDCs and MSCs cultured in differentiation medium. Cells were subjected to HR injury. After HR injury CDCs and MSCs were maintained in osteogenic differentiation medium for 21 days. Cells without HR injury, with osteogenic induction served as control. (i) Representative images of calcium deposits, stained by Alizarin Red S. ii) Quantitative measurement of alizarin red staining using Image J software. Data were expressed as mean ± SD. * p<0.05, compared to untreated control, ††p≤0.01 MSC compared to CDC, n=6. ANOVA p<0.05.*

IV.5.15.2 Adipogenic differentiation

To assess their potential to differentiate to adipocytes, cells were incubated in the differentiation medium for 21 days. Positive staining with Oil Red O was significantly

higher for MSCs (p<0.001). Oil red O staining in CDCs was negligible compared to MSCs (Fig 33).Adipogenesis in MSCs was further potentiated by HR injury (p<0.05). A small degree of adipogenesis was observed in some cells of HRCDCs, with occasional appearance of oil filled vacuoles which stained positive for Oil red O (P<0.05). But complete adipogenesis with lipid vesicles thorough out the cells were absent in both CDCs and HRCDCs.

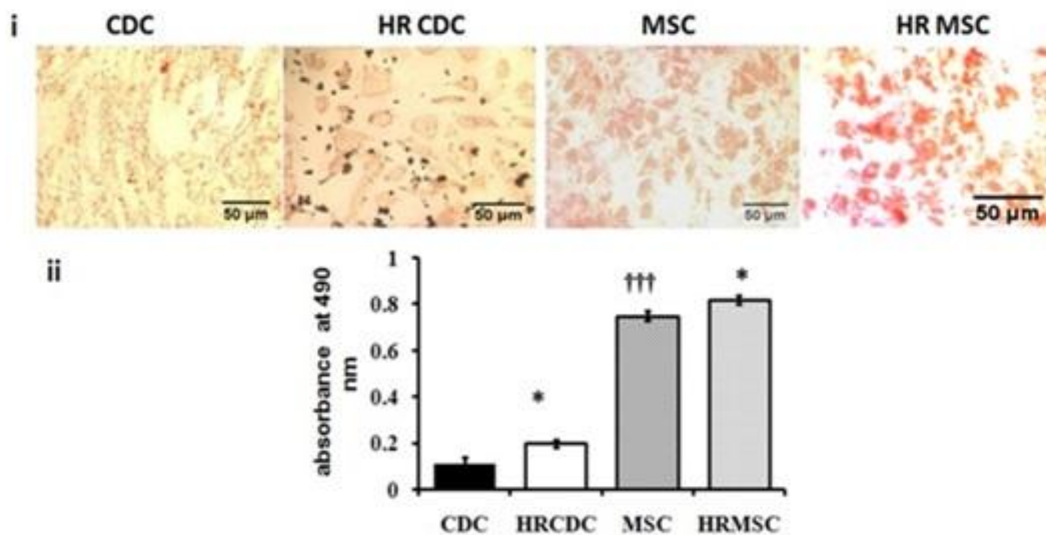


Figure 33: Adipognic differentiation of CDCs and MSCs after HR injury.

*After HR injury, CDCs and MSCs were maintained in adipogenic differentiation medium for 21 days. Normal CDCs without HR injury served as the control. (i) Representative images of Lipid droplets, stained by Oil red O. ii) Quantitative measurement of oil red O via isopropanol extraction method. Data were expressed as means ± SD. * p<0.05, compared to untreated control, †††p < 0.001 MSC compared to CDC, n=6.ANOVA p < 0.01*

IV.5.16 Effect of Hypoxia- reoxygenation injury on Migration of CDCs and MSCs:

Homing in of stem cells to the site of injury is essential for effective tissue repair. HR injury can influence the migration of stem cells. Concomitantly factors released from the surrounding tissue can also affect the migration of stem cells. Therefore, the migration of stem cells towards injured myocardial tissue as well as the behavior of the cells following HR injury was studied.

IV.5.16.1 Release of SDF after Hypoxia- reoxygenation injury

The levels of SDF secreted after HR injury was assessed by ELISA (Fig 34). Cells (10^5cells/ml) were cultured for 3 days and supernatants were collected, filtered and assayed for hSDF-1 levels. The CDCs were seen to secrete more SDF when compared to MSCs ($P<0.01$). There was an enhanced secretion of SDF after HR injury in both CDCs and MSCs ($p< 0.5$). The relative SDF secretion was more in HR CDCs than HR MSCs ($p< 0.001$).

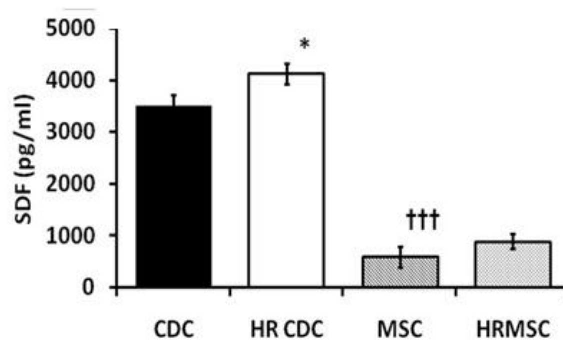


Figure 34: Expression of SDF after HR injury: *Cells were seeded in 35mm culture plates at a density of 2× 10^5 cells and incubated for 3 days. The supernatants were*

*collected and the concentration of SDF was measured using human specific ELISA kits (Abcam). CDCs without HR injury were used as control. Data represented as mean± SD. * $p<0.05$, compared to untreated control, †††$p< 0.001$ MSC compared to CDC, n=3. ANOVA $p<0.05$.*

IV.5.16.2 Migration of cells towards hypoxically injured tissue bit after HR injury.

Transwell migration assay was carried out to assess the migration of stem cells towards hypoxically injured myocardial explant cultures (Fig 35). Normal cells and HR injured cells were kept in the upper chamber. Cells and explant in both chambers, without HR injury was used as control. The stem cells in the upper chamber, that migrated the lower face of the filters were stained and counted. Migration was stimulated when exposed to injured tissue bits compared to normal tissue bit, both for CDCs ($p<0.001$) and MSCs ($p<0.05$). The basal migratory property was higher for CDCs ($p< 0.001$ Vs MSC), which increased proportionately in response to hypoxic injury to the tissue bit. No significant difference was detected between the number of migrated HRCDCs and HRMSC towards injured tissue bit, compared to respective non injured CDCs. The migratory property of stem cells was not much affected by hypoxic injury.

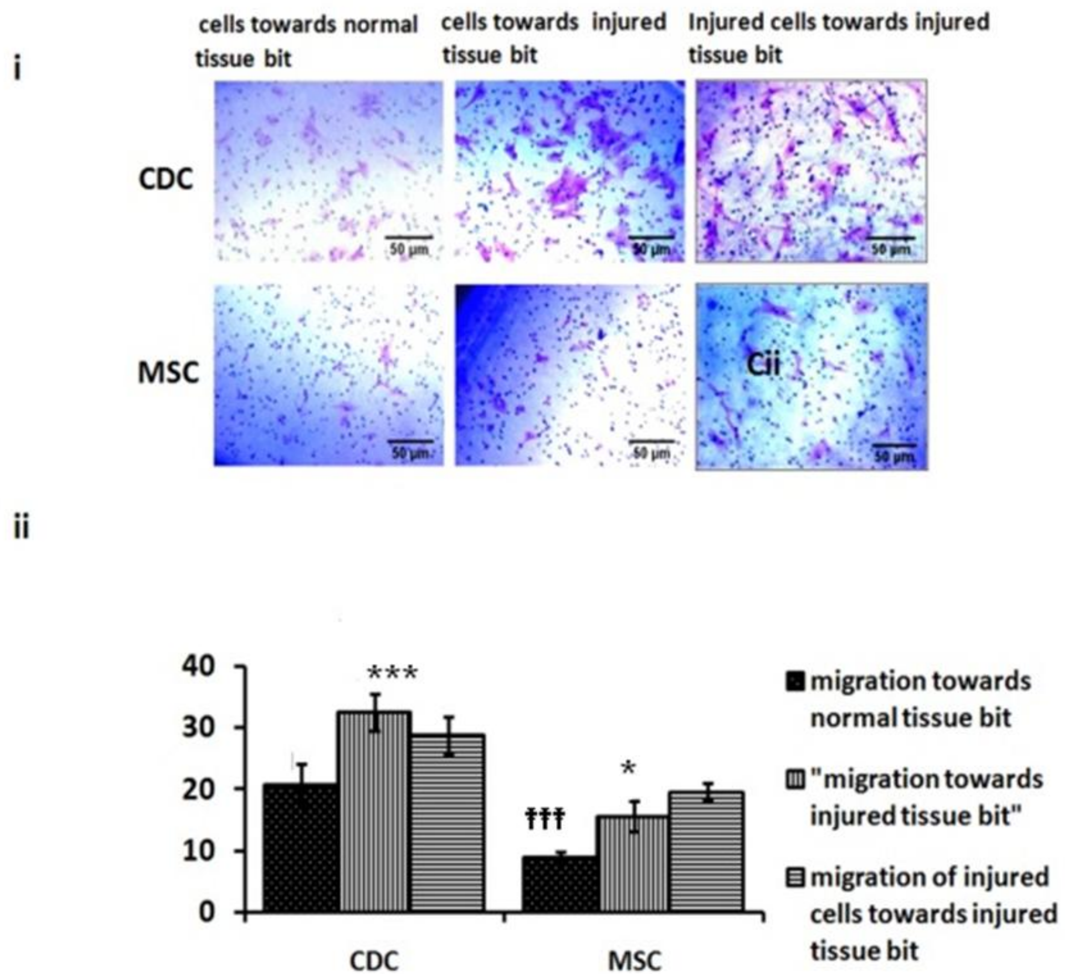


Figure 35: Migration of stem cells towards hypoxically injured myocardial explant culture.

*Human myocardial explant culture in the lower chamber of transwell migration assay plates was subject to severe hypoxic injury. i) representative images ii) Quantitative evaluations of cell migration towards injured myocardial tissue bit. Values represented as Mean ± SD of triplicate measurements, *** p<0.001 migration of CDCs towards normal tissue Vs migration of CDCs towards injured tissue bit, ††† migration of CDCs towards normal tissue bit Vs migration of MSCs towards normal tissue bit, * migration of MSCs towards normal tissue Vs migration of MSCs towards injured tissue bit, n=3. ANOVA p<0.01.*

IV.6 Identification of ideal culture conditions for exvivo expansion of human cardiosphere derived cells for transplantation for myocardial regeneration

The results shown thus far indicate that compared to MSCs, CDCs are better suited for transplantation by virtue of its capacity to withstand hypoxic injury as well as its proliferation efficiency and differentiation capacity to myogenic lineage. As observed in literature preclinical and clinical studies using CDCs have not yielded the expected results following transplantation of resident cardiac stem cells. Culture conditions during exvivo expansion prior to transplantation can have a significant influence on post transplantation survival and tissue regeneration. Majority of the studies carried out so far have used the conventional culture method, where cells are expanded in ambient O_2. The dissolved oxygen content of the culture medium is fivefold higher than that of the stem cell niche. The cells are then transplanted into the body where the O_2 levels are lower. The drastic changes in O_2 can significantly affect the efficacy of the stem cells. Therefore, simulating the physiological conditions by expanding the cells in O_2 levels present in niches prior to transplantation is expected to improve stem cell survival and function. With this objective, the behavior of CDCs cultured in 3% O_2 was compared with cells grown in ambient O_2.

IV.6.1 Cell migration from cardiac explants

Though an initial lag in the migration of cells from explants cultured in 3% O_2 was observed, compared to those in ambient O_2, within 7-8 days the outgrowth was comparable (Fig 36).

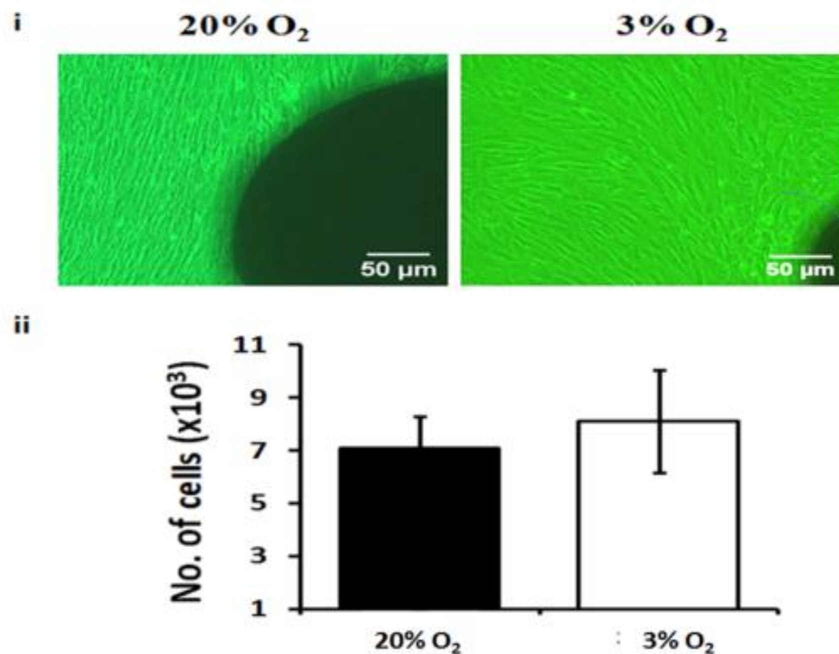


Figure 36: Outgrowth from explants maintained in ambient and 3% O_2

Tissue samples were minced into fine pieces of 1mm^3. After removing the adherent fat tissue, the tissue bits were placed in dishes coated with 2% gelatine and incubated in complete explant medium (CEM) at 37°C in 5% CO_2 and 99% humidity with either 20% O_2 or 3% O_2 i) Representative photomicrographs of outgrowths from explants on day 14 ii) Graphical representation of number of cells migrated per explant after 14 days in culture.

IV.6.2 Cardiosphere formation

When cells from 20% and 3% O_2 culture were seeded on poly-D-lysine-coated plates, in CGM, they aggregated to form cardiospheres within 12-48 hrs. The cells grown in 3% O_2 aggregated into spheres faster than those in ambient O_2, generally within 24hrs (Fig 37i). Though the number of cardiospheres formed was more in 20% O_2 culture (Fig 37.ii), there was a relative increase in the size of spheres in 3% O_2 (Fig37.iii).

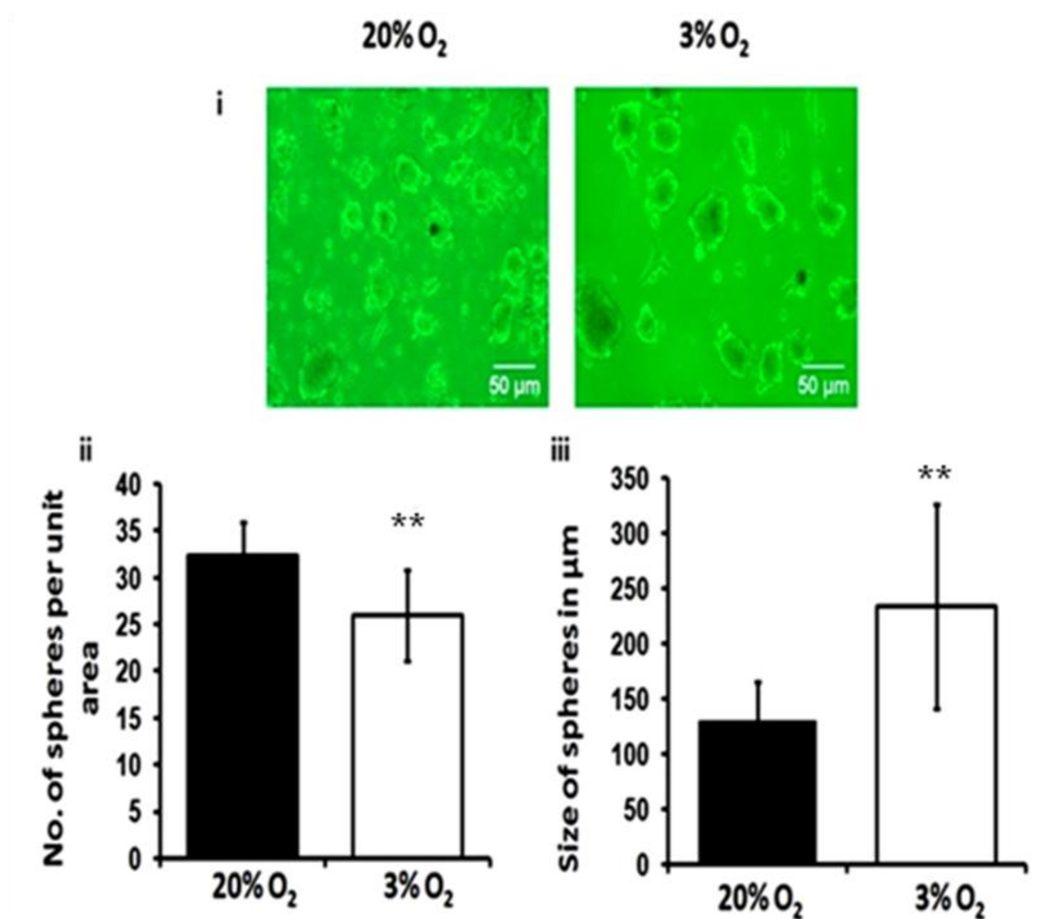


Figure 37: Cardiosphere formation in 20% and 3% O_2.

*i)Formation of cardiospheres 48 hrs after incubation in cardiosphere growing medium. ii) Graphical representation of number of cardiospheres formed per unit area, in 20% and 3% O_2.iii) graphical representation of size of cardiospheres in 20% and 3% O_2. Data represented as mean± SD. **$p<0.01$compared to 20% CDCs.*

IV.6.3 Culture of Cardiosphere Derived Cells

When plated on fibronectin coated dishes, the cardiospheres from both 20%O_2 and 3% O_2 adhered to the culture surface and started expanding into monolayer (Fig 38). The time to attain confluence was significantly higher for cells grown in 20%O_2. When cultures

reached passage 10, the cell morphology of CDCs changed to a more senescent appearance in 20% O_2 than 3% O_2.

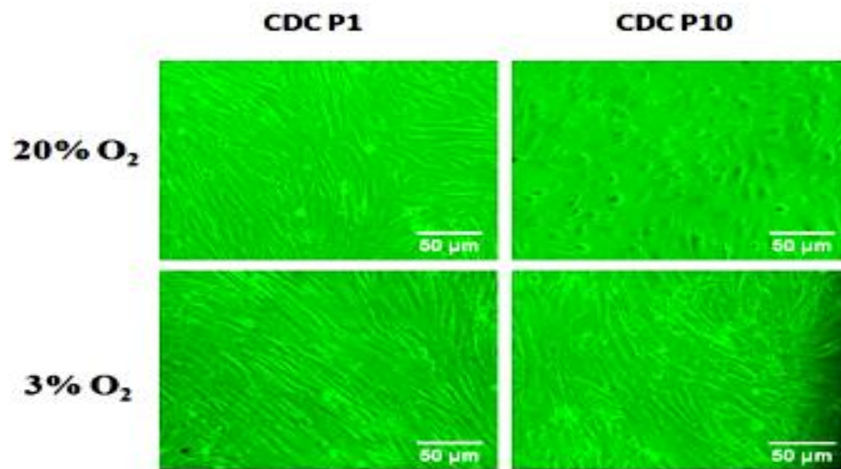


Figure 38: Cardiosphere derived cells from cultures maintained in 20% and 3% O_2.
Representative images of Cardiosphere derived cells (CDCs) from Passage 1 (CDC P1) and Passage 10 (CDC P10)

IV.6.4 Viability of CDCs Maintained in 3% and ambient oxygen- Annexin V/PI live dead assay

To check the influence of oxygen levels on the viability of expanded CDCs, apoptosis was assessed using the standard flow cytometry-based Annexin V/propidium iodide apoptosis assay. The percentage of viable (Annexin V and propidium iodide⁻) cells from 3% O_2 was comparable to that of CDCs cultured under 20% O_2 (Fig. 39). These data show that cell viability is unaffected by the oxygen levels.

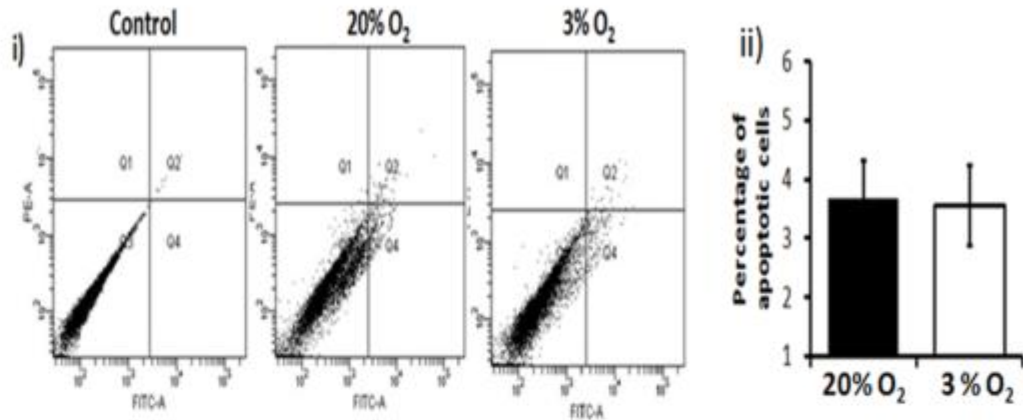


Figure 39: Effect of oxygen level on cell viability.

Apoptosis in CDCs cultured in 20% and 3% O_2 assessed by Annexin V/Propidium iodide based FACS assay (A) (i) Representative histograms (ii) Graphical presentation.

IV.6.5 Cell senescence in cultures maintained in ambient and 3% O_2

IV.6.5.1 β galectosidase cell senescence assay

Cell senescence was evaluated by the expression of p16INK4A, and SA-β-Gal staining. The fraction of SA-β-Gal-positive cells was lower in eighth-passage of CDCs expanded in 3% O_2 than in 20% O_2 (Fig40, $P < 0.01$).

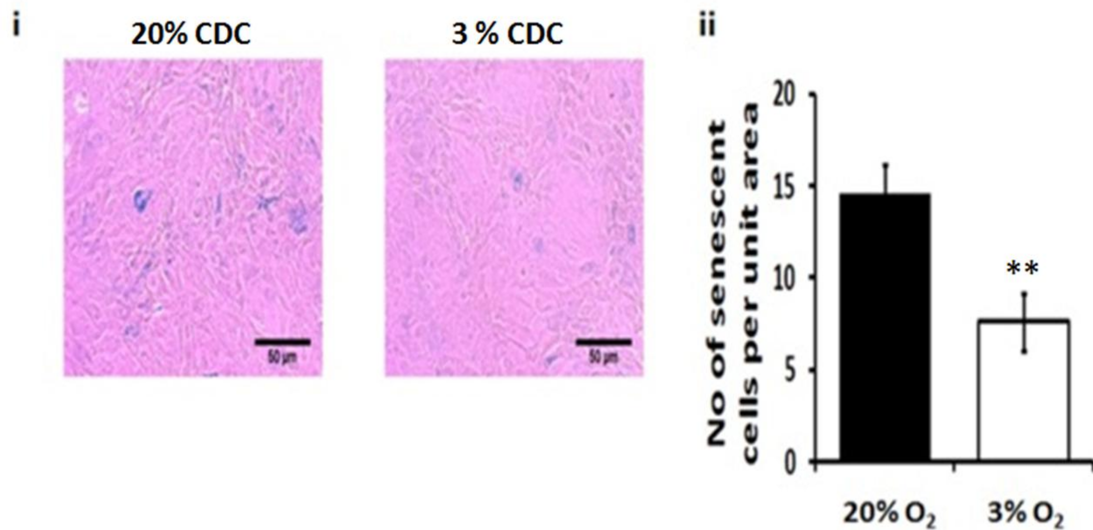


Figure 40: Senescent cells in CDCs from Passage 8, expanded in 20% O_2 and 3% O_2

CDCs from 20% O_2 and 3% O_2 were checked for the expression of senescence-associated β-galactosidase**.** (i) Representative photographs (ii) Graphical presentation. Data represented as mean±SD. ** p<0.01 compared to 20% CDCs.

IV.6.5.2 Expression of p16INK4A

The expression of p16^{INK4A} was lower (Fig41, P< 0.01) in CDCs maintained in 3% O_2.

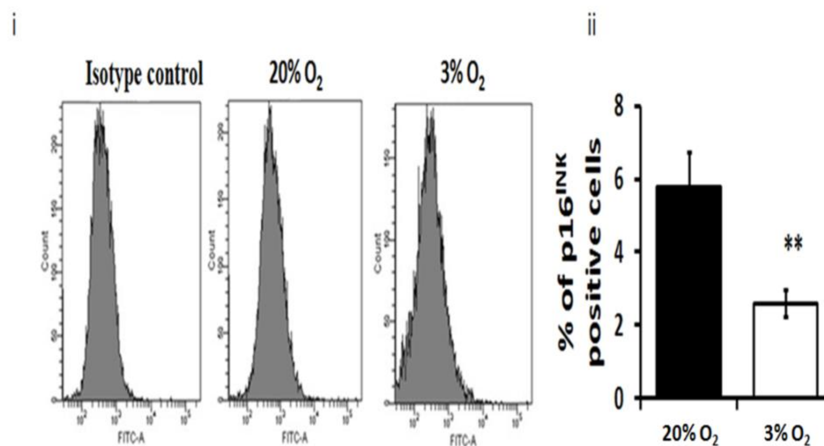


Figure 41: The percentage of p16INK4a positive cells in CDCs from passage 8 cultured in 20% and 3% O_2.

*Senescence assay of P8 CDCs based on flow cytometric analysis of p16INK4a (i) Representative histogram (ii) Graphical representation. *p<0.01 compared to 20% CDCs.*

Relative increase in the proportion of SA-β-Gal-positive cells and p16INK4a positive cells in cultures maintained in 20% O_2 indicate that maintaining stem cells at higher O_2 levels promotes stem cell aging.

IV.6.6 Proliferation of CDCs maintained in 3% and ambient O_2

IV.6.6.1 Population doubling time

The time taken for population doubling was lower in CDCs expanded in 3% O_2 (Figure 42) Significant difference was observed in the time taken to double the population. The population doubling time was lower for CDCs maintained in 3% O_2. Significant difference was not observed only for P2 and P3.

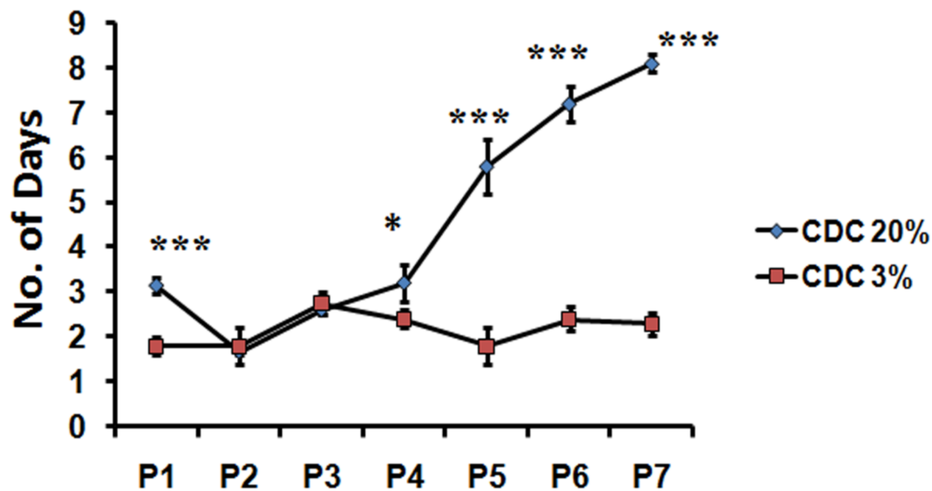


Figure 42: Population doubling time of CDCs maintained in 20% O_2 and 3% O_2.

Cardiosphere derived cells from the primary culture were seeded at a density of 2×10^5 *cells in 35 mm culture plates and incubated at 37°C with 5%* CO_2 *and 99% humidity for 48h. This was marked as the first passage (P1). At 48h cells were trypsinized and the cell count was determined. These cells were then replated at a density of* 2×10^5 *cells/ml and designated as P2. The cell count was determined after 48h and the process was repeated upto P7.Data represented as mean±SD. *p<0.05, ***p<0.001 compared to 20% CDCs.*

IV.6.6.2 Cell cycle analysis

Flow cytometry analysis in combination with propidium iodide staining was carried out to determine the proportion of cells in various stages of cell cycle, in both ambient and 3% O_2 culture. Sub confluent cultures maintained in different levels of O_2 were labeled with propidium iodide and subject to Flow cytometry analysis. A significant increase of cells in S and G2/M phases in 3% O_2 CDCs was found compared to CDCs in 20% O_2 (Fig 43), culture. Lower oxygen levels resulted in a greater percentage of cells in the S phase and a smaller percentage in the G0/G1 phase compared to cells maintained in ambient O_2.

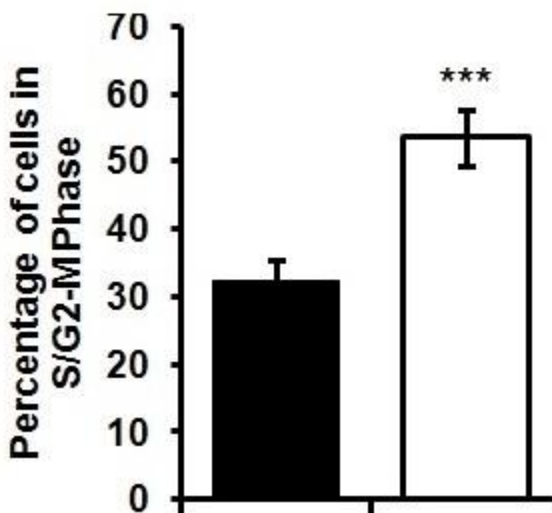


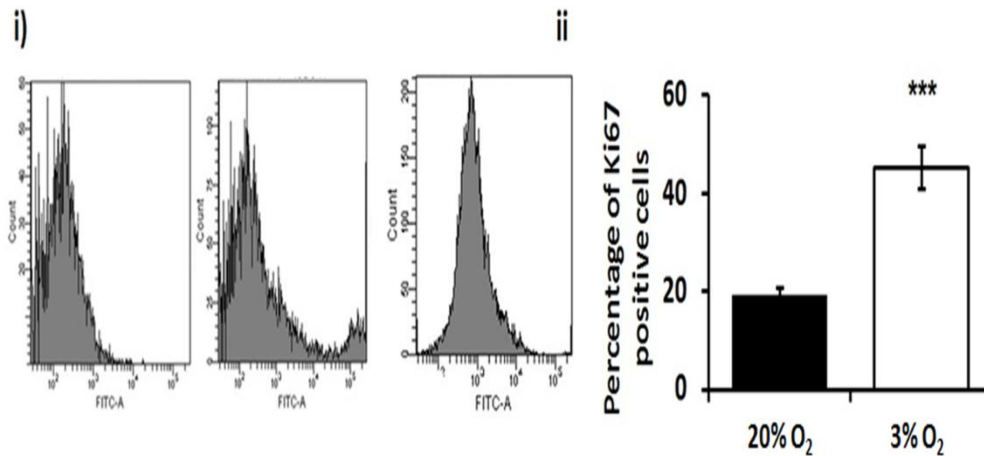
Figure 43: The effect of oxygen concentration on cell cycle profile examined by flow cytometric analysis.

*Both 20% O_2 and 3% O_2 CDCs were plated at a density of $1x10^5$cells/ml in a 35mm dish. After 48hrs in culture, cells were then harvested, washed twice with PBS, and fixed in 70% ice-cold ethanol. After RNase and propidium iodide treatment, each phase of the cell cycle was measured by FACS assay (i) Representative histograms of cell cycle analysis (ii) quantitative representation showing percentage of cells in S/G2/M phase of cell cycle. Data represented as mean± SD, *** $p<0.001$ compared to 20% CDCs, n= 3.*

IV.6.6.3 Flow cytometric analysis for Ki 67

Subconfluent cultures were stimulated using serum and labelled with Ki 67. Corresponding to the increased number of cells in S and G2/M phase of cell cycle as seen in Fig 43, the proportion of proliferating cells in 3% O_2 culture was higher, as evidenced by FACS analysis for Ki 67, (Figure 44, $P<0.05$),. There was a 2 fold increase in the number of CDCs in 3% O_2 culture. Ki-67 is a protein present in active phases of the cell cycle (G1, S, G2, and mitosis) but not in resting G0 cells and therefore used for

assessment of cell proliferation. On stimulation with serum, CDCs cultured under lower O_2 levels grew at faster rates and yielded higher total cell numbers as compared to CDCs cultured at 20% O_2.

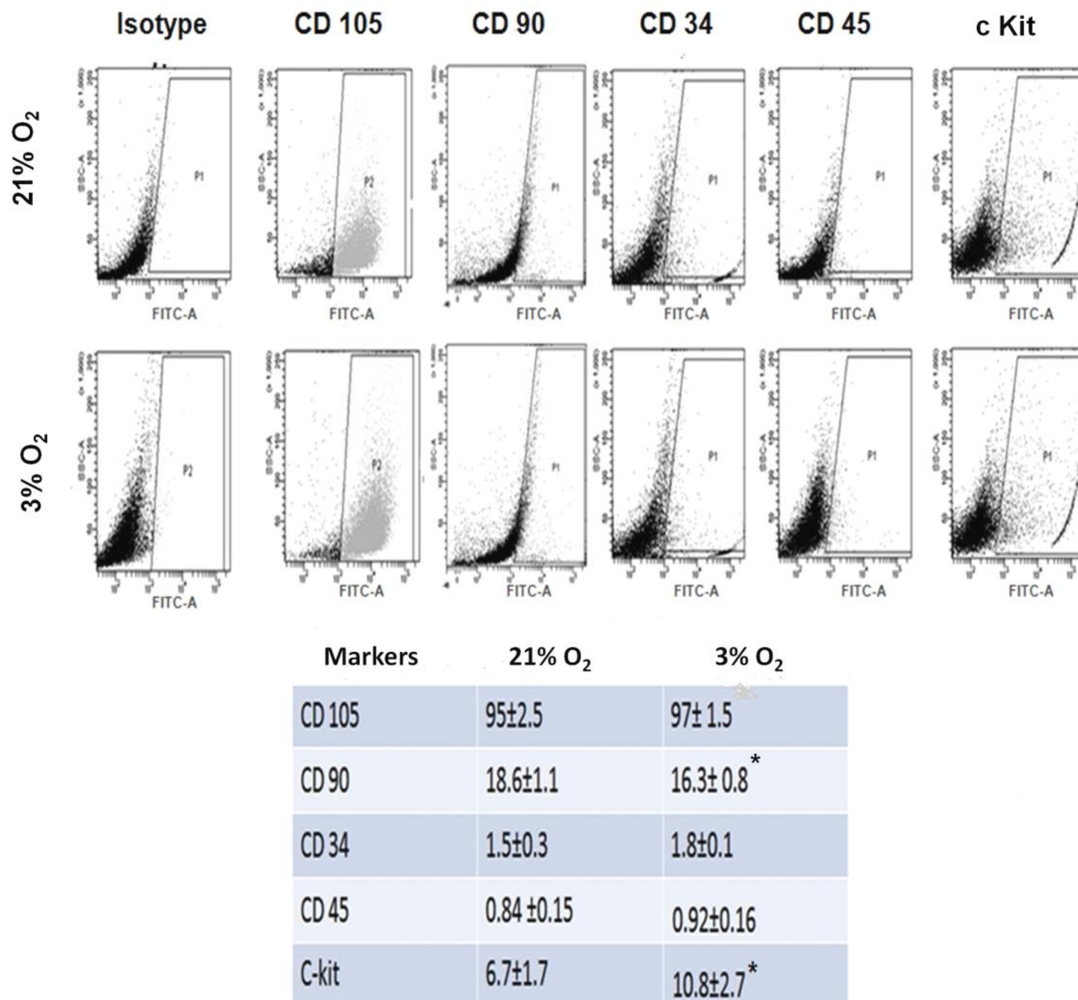


The Figure 44: Percentage of Ki 67 + cells in CDCs maintained in ambient and 3% O_2 quantified by FACS analysis.

*(i)Histograms showing the expression of Ki67 against isotype control. (ii) Graphical representation of Ki 67^{+} cells in both cultures. Data represented as mean±SD. *** $p<0.001$ compared to 20% CDCs, n= 3.*

IV.6.7 Surface phenotype characterization

Flow cytometry analysis showed that when cultured in 20% O_2 and 3% O_2, P4 CDCs were positive for the markers CD105, CD 90 and c-Kit and negative for CD45 and CD34 (Fig 45). No significant difference was seen in the surface marker expression level between the two populations except for CD 90 ($p<0.05$) and c-Kit ($p<0.05$).



Markers	21% O_2	3% O_2
CD 105	95±2.5	97± 1.5
CD 90	18.6±1.1	16.3± 0.8*
CD 34	1.5±0.3	1.8±0.1
CD 45	0.84 ±0.15	0.92±0.16
C-kit	6.7±1.7	10.8±2.7*

Figure 45: Effect of O_2 levels on surface marker expression.

(A) P4 CDC, from 20% O_2 and 3% O_2 were labeled with antibodies for CD 105,CD 90,CD 34,CD 45 and c-kit and analyzed by flow cytometry. (Ai) Representative FACS data of expression pattern of cell surface markers. Aii) summary of cell surface marker expression in 20% O_2 CDCs and 3%O_2 CDCs (n=3).

IV.6.8 Generation of reactive oxygen species by CDCs in ambient O_2 and 3% O_2

IV.6.8.1 Intracellular generation of ROS

Intracellular ROS was measured by DCFDA cellular ROS detection assay. FACS analysis revealed that the intracellular ROS level was lower in CDCs cultured in 3% O_2 than in ambient O_2-cultured CDCs (Fig 46, P <0.001).

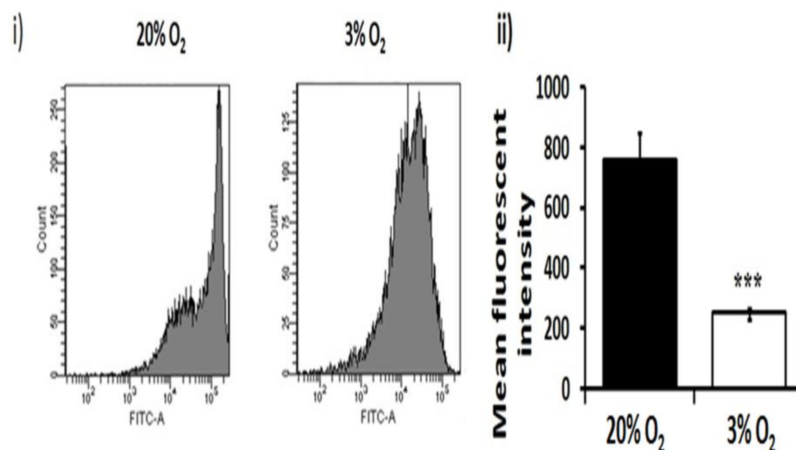


Figure 46: Effect of O_2 levels on intracellular generation of reactive oxygen species (ROS).

*ROS production by CDCs cultured in 20% O_2 and 3% O_2 measured by DCFDA fluorescence based FACS assay. (i)Representative histograms (ii)Graphical representation. *** $p < 0.001$, 20% O_2 Vs 3% O_2*

IV.6.8.2 Response to oxidative stress of CDCs cultured in ambient and subphysiological oxygen

The response of CDCs cultured in 3% O_2 to an external oxidizing agent was compared to cultures maintained in hyperoxia (20% O_2). After 24 h of exposure to 100 μM H_2O_2, apoptosis was measured by TUNEL assay. The proportion of apoptotic cells were lower

in 3% O_2 CDCs than in those grown in 20% O_2 (Fig 47, P <0.01). There was no significant difference in the percentage of apoptotic cells in untreated cultures maintained in 20% O_2 and in 3% O_2.

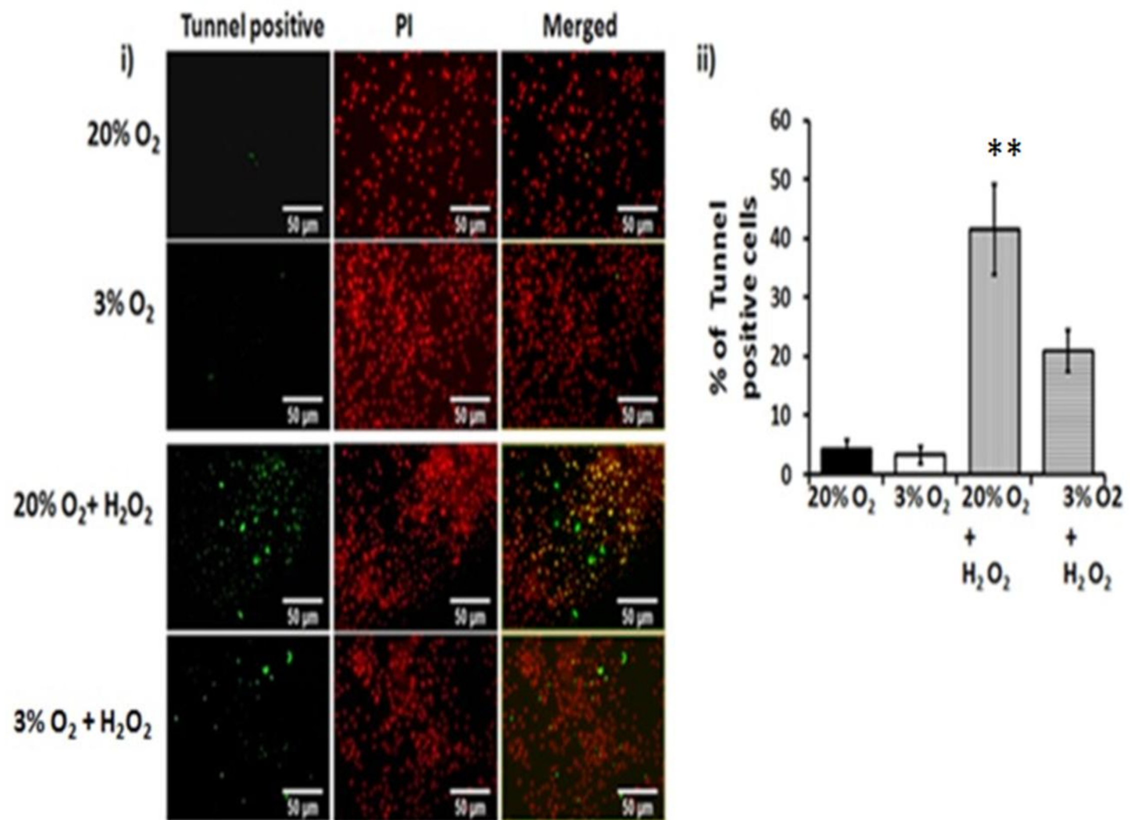


Figure 47: Effect of O_2 levels on resistance to oxidative stress.

*TUNEL-positive CDCs cultured in 20% O_2 and 3% O_2 after 24 h exposure to 100 µM H_2O_2. (i) Representative images (ii) Quantitative representation of TUNEL positive cells. **P<0.01 3%O_2+H_2O_2 Vs20%O_2 +H_2O_2. ANOVA p<0.001.*

IV.6.9 Differentiation potential of CDCs cultured in 3% and ambient O_2

IV.6.9.1 In vitro spontaneous myogenic differentiation

CDCs were maintained in low serum medium for assessment of spontaneous differentiation. Quantitative analysis by qRT-PCR showed that the spontaneous expression of lineage marker Nkx 2.5 (Fig 48i) and myocyte specific maker, cardiac troponin I (Fig 48ii) mRNA was upregulated in the 20% O_2 culture.

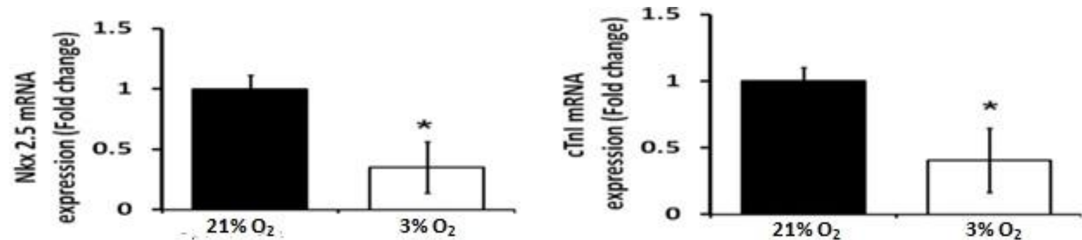


Figure 48: Spontaneous differentiation potential of CDCs cultured in 20% and 3% O_2.

Spontaneous differentiation- i) Quantitative real time PCR analysis for the expression of myocyte lineage marker, Nkx2.5 and (ii) cardiomyocyte structural protein Cardiac troponin Values are mean ±SD,<0.05, n=3.*

IV.6.9.2 Endothelial tube formation

IV.6.9.2.1 .Directed differentiation to endothelial lineage

In vitro angiogenesis was induced by culturing CDCs on matrigel in medium containing endothelial growth supplement and 2% serum for 12 hrs. CDCs expanded under both 3% O_2 and 20% O_2 could form capillary-like networks on matrigel in 8 h, in the presence of endothelial growth supplements. Branching points were significantly higher in 3% O_2

(Fig49).

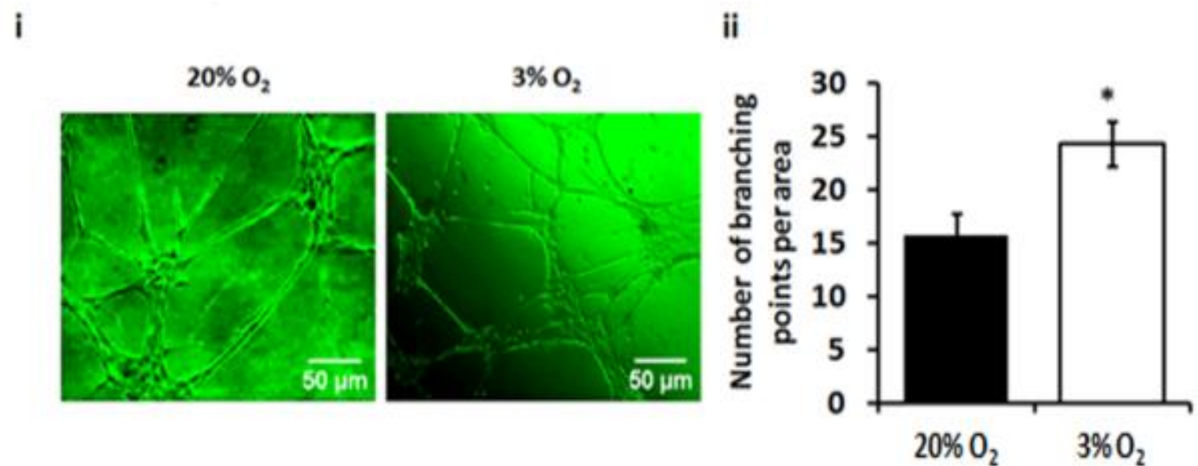


Figure 49: Endothelial growth supplement induced angiogenic differentiation potential of CDCs cultured in 20% and 3% O_2 assessed by endothelial tube formation

*(i) representative photographs showing morphology of tubes formed by CDCs maintained in 20% O_2 and 3% O_2 on matrigel ii) Number of branching points quantified for induced tube formation. Values are presented as mean ±SD; *P <0.05.n=6*

IV.6.9.3 Endothelial tube formation- spontaneous

In another set of experiments, spontaneous tube formation on matrigel was assessed, even without the addition of endothelial growth supplements. Tube formation and the number of branching points were significantly higher for cells maintained in 3% O_2 (Fig 50, $p<0.001$)

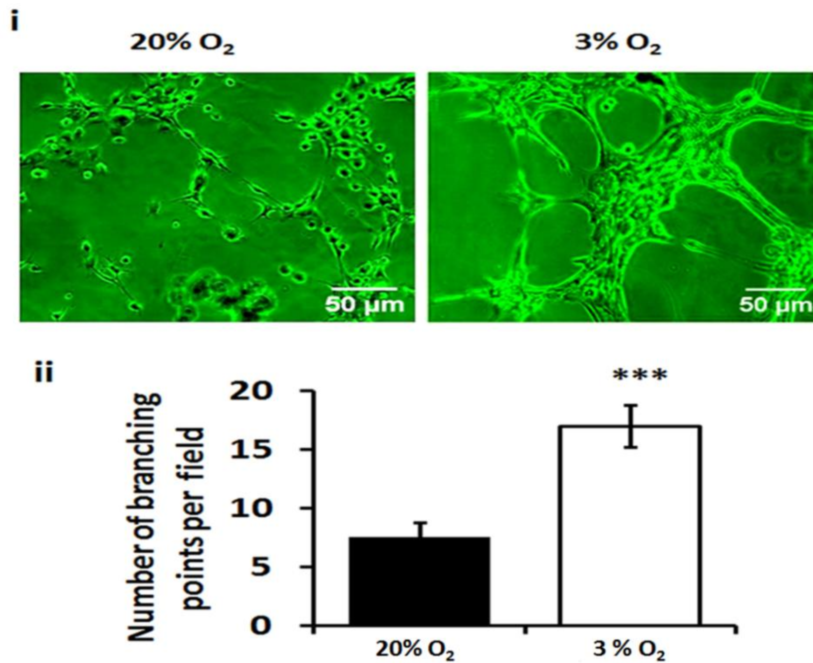


Figure 480: Spontaneous Differentiation potential of CDCs cultured in 20% and 3% O_2 assessed from endothelial tube formation

*(i) Representative photographs showing morphology of tubes formed from CDCs maintained in 20% O_2 and 3% O_2 on matrigel when incubated with medium alone. ii) Number of branching points quantified for induced and spontaneous tube formation respectively. Values are mean ±SD, ***P < 0.001Vs 20% O_2, n=6*

*Photographs showing morphology of tubes formed from CDCs maintained in 20% O_2 and 3% O_2 on matrigel when incubated with medium alone. ii) Number of branching points quantified for induced and spontaneous tube formation respectively. Values are mean ± SD, ***P < 0.001Vs 20% O_2, n=6*

IV.6.9.4 Osteogenic differentiation

To explore the influence of extended period of hypoxia on multi-lineage differentiation potential of CDCs osteogenic differentiation was induced at 20% and 3% oxygen. The osteogenic differentiation capacity was assessed in cells maintained both at 3% O_2 and 20% O_2 cells in oesteogenic differentiation medium for 21 days. Three fold increase in alizarin red staining (Fig 51, $p<0.01$) as indicated by Ca mineralization, in the initial stage of oesteogenesis was found in cells cultured under 20% O_2 .This shows that the propensity to undergo differentiation to non cardiac lineages decreases when maintained in 3% O_2.

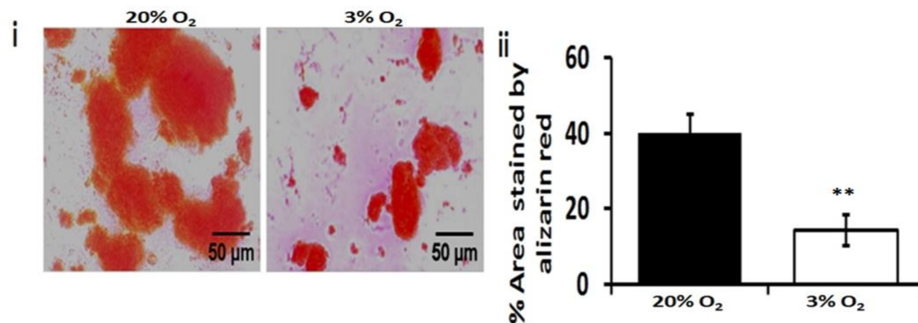


Figure 49: Osteogenic differentiation potential of CDCs cultured in 20% and 3% O_2

*CDCs incubated for 21 days in osteogenic differentiation medium (i) Representative images of Positive Alizarin Red staining for calcium deposits. (ii) The percentage of cells that stained positive for alizarin red. Values are mean ±SD; **$p<0.01$Vs 20% O_2*

IV.6.9.5 Adipogenic differentiation

To examine whether oxygen levels have any effect on adipogenesis, CDCs were cultured at 20% and 3% oxygen in adipogenic differentiation medium for 21 days. No evidence of Oil red O staining, indicative of adipogenesis was observed in both culture conditions (Fig 52).

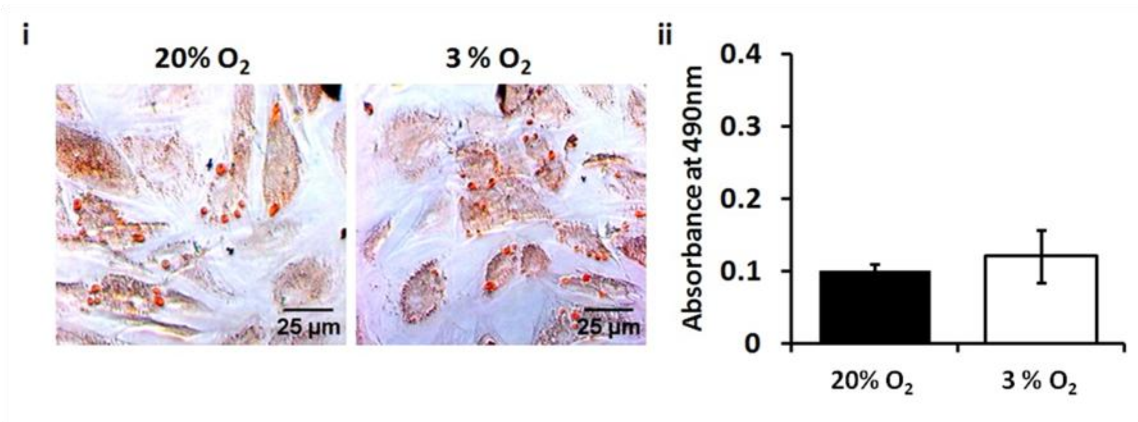


Figure 50: Adipogenic differentiation potential of CDCs cultured in 20% and 3% O_2

Differentiation of CDCs to adipocytes following incubation in adipocyte differentiation medium for 21 days. (i) Representative image of Oil Red O staining of intracellular lipid production. (ii) Percentage of total area that positively stained with alizarin red, quantified by Image J software.

IV.6.10 Proliferation and survival of cells in physiologic oxygen following culture in subphysiological (Niche) oxygen and ambient oxygen

Drastic changes in O_2 levels on ex-vivo expansion in ambient O_2 followed by transplantation to the tissue where the physiological O_2 level is lower can affect cell viability. Hence, viability of CDCs maintained in lower O_2 levels (3%) representing that present in Niches was shifted to physiological levels (5%) representing that present in myocardial tissue and compared with the cells maintained in ambient O_2 following the conventional cell culture system.

IV.6.10.1 Cell viability after the shifting CDCs maintained in 3% and ambient O_2 to physiological oxygen levels

Cells from both 20% O_2 and 3% O_2 were shifted to an incubator with 5% O_2, to simulate physiological oxygen condition of the heart. The cells were maintained in serum free medium as the cells are always transplanted in the absence of serum. There was a rapid increase in the necrotic cells (Annexin V+, PI+) in both sets of cells (Fig 53, $P<.001$). Necrosis was significantly lower in cells maintained in 3%O_2. The rapid increase in necrosis is possibly due to the shifting of cells from serum containing to serum free medium.

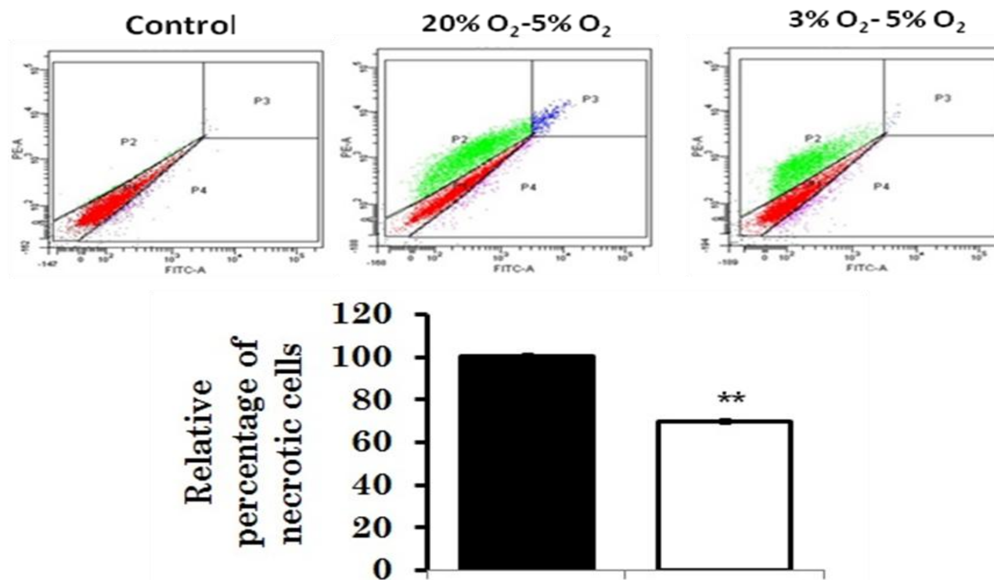


Figure 51: Effect of expansion in 20% and 3% O_2 on survival of cells in physiologic oxygen

*i) Apoptosis and necrosis were quantified by FACS after Annexin V-FITC and PI labeling. (i) Representative FACS data for 20% O_2 and 3% O_2 CDCs maintained in 5% physiological oxygen in the absence of serum for 24 hrs. (ii) Bar graph represents the mean percentages of apoptosis and necrosis. Data represented as mean±SD. **$p<0.01$ Vs 20%O_2 CDC*

IV.6.10.2 Serum stimulated proliferation after the Shifting of CDCs maintained in 3% and ambient O_2 to physiological oxygen levels (5% O_2)

CDCs maintained at the two different O_2 levels were shifted to 5% O_2 simulating the tissue levels. The cells were maintained in serum containing medium to assess the effect of shift in O_2 levels on proliferative capacity of the cells. Proliferation was assessed by FACS analysis for detecting the proportion of Ki67 positive cells

There was more than two fold increase in the percentage of cells undergoing proliferation when cells from 3% O_2 were shifted to 5% O_2 compared to cells maintained in ambient O_2.(Fig 54).

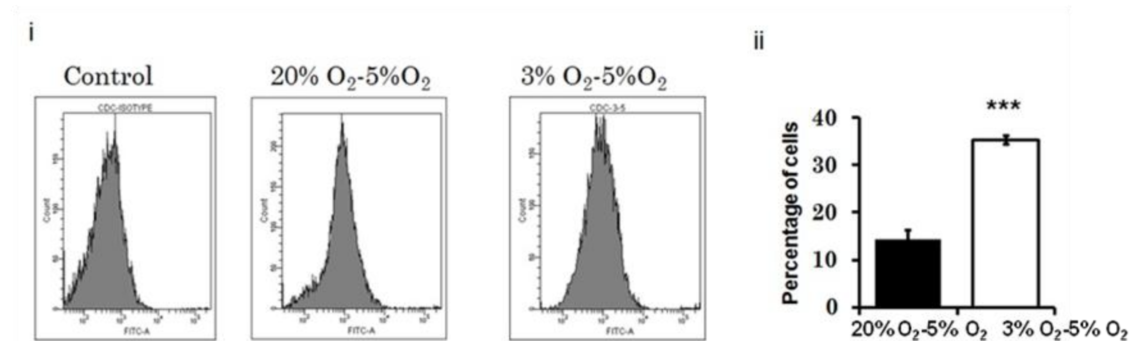


Figure 52: Effect of expansion in 20% and 3% O_2 on proliferation of cells in physiologic oxygen (5%) assessed by FACS analysis for proliferation marker Ki67

*(i) Representative histograms of CDCs maintained in 20% and 3% O_2 after shifting to Physiological oxygen (5% O_2) assessed from of Ki67 positive cells. (ii) Graphical representation of Ki67 positive cells. Data represented as mean±SD, ***$p<0.001$ Vs 20%O_2 CDC n=3*

The observations indicate that expansion of CDCs in lower O_2 levels confers selective advantage to the cells by preventing the environmental insult due to drastic changes in O_2 levels. Therefore a more viable and healthy population of CDCs will be obtained for transplantation if O_2 levels are maintained close to the physiological levels.

V. DISCUSSION

Permanent loss of cardiomyocytes and scar tissue formation after myocardial infarction (MI) results in an irreversible damage to the cardiac tissue and affects the normal functioning of the heart. Currently existing therapeutic strategies seek to limit the initial injury, prevent additional damage to the heart muscle and block maladaptive pathways. However, there is need to identify treatment strategies that repair the infarcted area by replacing the damaged myocytes. Stem cell based tissue regeneration therapies are now emerging as an effective therapeutic option with convincing results. Experimental studies in large and small animals using different stem and progenitor cell based therapy have recorded beneficial effect on the heart. The best cell type for transplantation into the ischemic heart; that survives and differentiates in the possibly hostile hypoxic environment remains a controversy because of the lack of consensus between the studies. Two promising cell types of autologous origin popularly identified for myocardial regeneration are the Bone marrow derived mesenchymal stem cells due to easy availability and adult resident cardiac stem cells because of cardiac commitment. Of the different forms in which cardiac stems cells can be transplanted, cardiosphere derived cells (CDCs) are advocated as the potential cardiac stem cell candidates due to superior paracrine potency and differentiation efficacy (Koninckx et al., 2011a, Li et al., 2012a, Cheng et al., 2014a). There is a lacuna in the information on the response of both these cell types to ischemia- reperfusion injury. The relative survival rate and differentiation potential of these two cell types consequent to reperfusion injury are important in choosing the ideal cell for myocardial regeneration. Post-transplantation, a large fraction of the stem cells are lost from the transplantation site. One possible reason can be the

sudden drop in the oxygen levels when cells cultured in ambient O_2 (20%), are transplanted to the myocardial tissue where the physiological O_2 level is lower (5% O_2). This study was therefore carried out with the objective of identifying factors that promote survival, differentiation and proliferation of resident cardiac stem cells in the event of ischemic injury and also identify the ideal cell type and culture conditions for effective stem cell based tissue regeneration. Stem cells were isolated from human atrial biopsies dissected out during CABG surgery. The cells were cultured as cardiosphere derived cells (CDCs) and used for experimental studies. The following studies were carried out:

i. Culture and characterization of CDCs for their capacity to proliferate and differentiate

ii. Response of CDCs to hypoxia-reoxygenation injury

iii Paracrine effects of injured stem cells on normal and injured myocytes and the stem cell response to myocyte injury

iv Comparison of the response of CDCs with the popularly used mesenchymal stem cells so as to identify the ideal cell type for transplantation

v. Expansion of CDCs at physiological oxygen levels to examine whether the latter confers selective advantage over cells cultured in the conventional ambient oxygen

V.1 Culture and Characterization of CDCs

Resident cardiac stem cells are expected to have a selective advantage over the other stem cells in differentiation to cardiac lineage. Cardiospheres and cardiosphere-derived cells (CDCs) from cardiac tissue are the major source of cardiac stem cells used for myocardial regeneration. Cardiospheres are formed from the phase bright cells that migrate from the explant, and cardiosphere derived cells are adherent cell cultures obtained from cardiospheres. The formation of cardiospheres and CDCs from atrial biopsies is in agreement with earlier reports (Aghila Rani et al., 2008) Shenje et al., 2008). Sphere forming ability is a characteristic of stem cells. Characterization of the CDCs showed that they expressed the cardiac stem cell marker c-Kit as well as mesenchymal cell markers (CD 105, CD 90,CD 29) (Figure 3, Table 4). Less that 1% of the cells stained positive for the hematopoietic marker CD45 (table 3) indicating the cardiac origin of the cells. This is further supported by the expression of lineage markers GATA4 and MEF 2c (Fig 4). Studies carried out earlier have confirmed that stem cells of cardiac origin have a greater capacity to differentiate into cardiomyocytes compared to the cells isolated from other organs (Oskouei et al., 2012). The population doubling time increased with passage (Fig 5) indicating that the proliferation efficiency of the cells decreases with passage. Smith et al suggest that the cells for transplantation be restricted to approximately five population doublings to obtain a healthy population of stem cells (Smith et al., 2007a). The authors suggest the possibility of age dependent and regional variations in the density of resident cardiac stem cells. They observed that cardiospheres derived from right ventricular endomyocardial biopsies when expanded to cardiosphere derived cells had regenerative

potential; and on transplantation increased the proportion of viable tissue within the infarct region (Smith et al, 2007a). Based on the observation in this study that an average of 3X 10^7 CDCs can be derived from 150mg of tissue from right atrial appendage, it is inferred that sufficient number of cardiosphere derived cells can be obtained for transplantation.

V.2 The effect of Hypoxia- reoxygenation injury on CDCs

Studies have reported the protective effect of stem cells on the myocardium in the event of ischemic injury. One aspect that has not received attention is that, ischemia reperfusion injury can also influence the behavior of resident cardiac stem cells and affect the fate of transplanted cells. There are no reports on the effect of ischemia-reperfusion injury on cardiac stem cells. To study the effect of ischemic injury on stem cells, the response of CDCs to hypoxia-reoxygenation (HR) injury was assessed. The effect of HR injury on cell survival, ROS generation, cell proliferation, myocyte differentiation, endothelial tube formation and migration was assessed.The percentage increase of apoptotic CDCs was about 30% higher (Fig. 6) after Hypoxia- reoxygenation injury.Sennescent cells were seen in CDCs exposed to HR injury (Fig 7). HR injury was accompanied by excessive intra-cellular free radical generation. (Fig. 8) This observation suggests that stem cells are independently affected by HR injury. This can affect endogenous tissue repair after an ischemic injury and also affect the efficacy of transplanted cells if the stem cells are transplanted soon after an ischemic insult. The negative response of CDCs to HR injury is possibly mediated by excessive intracellular ROS. The ability of stem cells to differentiate

to cardiac lineage is essential for myocardial regeneration. Reduced expression of cardiac troponin I and connexin 43 (1.5 fold reduction) and reduced number of branching points respectively (Fig. 12, 13) ($p<0.01$) suggest that both differentiation of cells to myocytes and angiogenesis are affected by HR injury A critical determinant of wound healing is the homing in of stem cells to the site of injury, which in turn is determined by the migratory capacity. Wound healing assessed by scratch-wound assay of CDC was unaffected following HR injury (Fig.14).

The protective effect following HR injury may be due to the release of cytokines that prevent apoptosis and promote cell proliferation. Observation of a two fold increase in the expression of cell survival factors pAkt and BCl_2; (Fig.9) and cell cycle arrest after HR injury suggests that, the stem cells do have defense mechanisms in the event of an ischemic injury. Cell proliferation increased along with the improved secretion of hepatocyte growth factor ($p<0.01$) (Fig.10), a mediator of growth and proliferation. The number of cells in the S-phase was also higher in the treated cells (Fig.11) Myocardial damage and compromised cardiac function incidental to ischemic injury illustrate that the endogenous repair mechanisms are not adequate for mending the repaired heart. Hence the viable option is the transplantation of stem cells to the site of injury to promote myocardial regeneration. From the therapeutic point of view the time of transplantation can also be critical due to the unfavourable milieu soon after ischemic injury.

V.3 Paracrine effects of injured stem cells on normal and injured myocytes and the stem cell response to myocyte injury

Stem cell response to HR injury can be mediated by autocrine effects as illustrated above, and also by paracrine signals from injured myocytes. Studies have also shown that intracoronary infusion of allogeneic CDCs improved ventricular function and stimulated myocyte proliferation, implicating stimulation of endogenous myocyte regeneration as the primary mechanism of repair (Suzuki et al., 2014). Therefore, the paracrine effects of injured stem cells on normal and injured myocytes and the stem cell response to myocyte injury was assessed using CDCs and the murine cardiomyocyte cell line H9c2 as well as human cardiac explants. The first step in the repair process is the migration of stem cells to the site of injury. Myocardial explants were exposed to ischemic injury and the migration of normal and injured CDCs towards the myocardial explants was assessed by transwell migration assay. Migration of normal as well as HR injured CDCs was stimulated when exposed to injured myocardial tissue (Fig.16). This suggests that in the event of myocardial infarction, cytokines are released that stimulates homing in of stem cells for endogenous tissue repair. To assess whether this is mediated specifically by cardiomyocytes, the experiment was repeated using H9c2 cells where it was observed that myocyte injury stimulates migration of stem cells (Fig.17). This was seen for both normal as well as hypoxically injured CDCs though there was mild retardation in the latter. This shows that even if the stem cells are exposed to HR injury, myocardial ischemia stimulates migration of stem cells to the site of injury. Homing in of bone marrow derived stem cells following myocardial infarction has been reported (Liao et al., 2007). This is a

paracrine response to cardiomyocyte injury; because injury to CDCs did not affect migration and wound healing in the absence of the influence of the myocardium. (Fig. 14) The effect of ischemia- reperfusion injury to the CDCs on the viability of surrounding cardiomyocytes and the stem cell response to myocyte injury was assessed by exposing the cells to conditioned medium respectively from CDCs and H9c2 cells. Conditioned medium from both normal CDCs and injured CDCs reduced apoptosis and increased proliferation (as from G1-S progression) of injured H9c2 cells (Fig. 18). In the same way, injury to H9c2 cells promoted migration and survival of cardiac stem cells. Both normal H9c2 conditioned medium and injured H9c2 conditioned medium positively influenced G1-S progression of CDCs irrespective of hypoxic injury. (Fig. 14, $p<0.05$)

Most of the reports have suggested the potential benefits of stem cell therapy for myocardial repair but a definitive answer is still underway. Identification of factors that stimulate migration and survival of stem cells in the presence of HR injury will help not only in promoting endogenous repair but also assist in transplantation where stem cells are transfused into an adverse environment. Hence, further studies are warranted to identify factors that would promote endogenous stem cell mediated myocardial repair, as well as facilitate myocardial regeneration by promoting the survival, proliferation and differentiation of transplanted stem cells.

Experimental studies in large and small animals using different stem and progenitor cells have recorded beneficial effect on the heart (Barbash et al., Ji et al., 2010, 2003b, Orlic et al., 2001b, Prasad et al., 2009b, Quevedo et al., 2009b). The stem cells for transplantation

can be either of autologous or allogenic origin. Two promising cell types popularly identified for myocardial regeneration are the Bone marrow derived mesenchymal stem cells due to easy availability and adult resident cardiac stem cells because of cardiospecificity. Mesenchymal stem cells (MSCs) have attracted attention as a source for cell transplantation therapy, because of their safety, multilineage differentiation potential (Pittenger et al., 1999), immunomodulatory (Boyle et al., 2010) and antifibrotic effects (Ian P. Counihan et al., 2012, Li et al., 2009). Of the different forms in which cardiac stem cells can be transplanted, cardiosphere derived cells (CDCs) are advocated as the potential cardiac stem cell candidatedue to superior paracrine potency and differentiation efficacy (Koninckx et al., 2011a, Li et al., 2012a, Cheng et al., 2014a, Pittenger et al., 1999, Ian P. Counihan et al.,2012.)

V.4 Comparison of characteristics of CDCs and MSCs for identification of the ideal stem cell for transplantation-

A comparison of the stem cell characteristics of cardiosphere derived cells and bone marrow mesenchymal stem cells (MSCs) isolated from the same patients was carried out. Characterization of the cell surface markers showed the expression of CD 105, CD 90, CD29 and c- Kit by both the cell types. But, the level of expression of CD 90 and c Kit was significantly different for the two cell types (Table 6). The expression of CD 90, the mesenchymal/ fibroblast marker was as low as 16% in CDCs, but was 97% among MSC. In a recent study Gago-Lopez has shown that CD 90^+ subpopulation from CDCs showed propensity for endothelial and SMC differentiation and higher expression of fibroblast-

associated genes with only partial expression to cardiac protein expressing cells without sarcomere formation. Whereas $CD90^{-}$ cells differentiated into SMCs, ECs, and CMs, with the formation of complete sarcomeres with spontaneous beating though at a very low frequency(Gago-Lopez et al., 2014b). Therefore, CDC have a greater propensity for cardiogenic differentiation and neovascularization compared to MSCs, making the former, the preferred cell type for myocardial regeneration, especially for autologous transplantation. Expression of CD 90 correlates with immunosuppressive activity in MSCs (Campioni et al., 2009). Therefore, MSCs have a selective advantage over CDCs in the event of allogenic transplantation.

The sensitivity of CDCs and MSCs to hypoxia-reoxygenation injury was evaluated. Cell survival, proliferation, resistance to oxidative stress and differentiation (myogenesis and endothelial tube formation) was examined. There was a six fold increase in the percentage of early apoptotic cells in MSCs compared to CDCs (Fig.22). Analogous to the increased proportion of apoptotic and senescent cells (Fig. 23) consequent to HR injury, the generation of ROS was also significantly higher in MSCs compared to CDCs (Fig. 23). Further, the protective effect on CDCs in the event of HR injury may be mediated by, enhanced Akt phosphorylation down stream to activated Hif1 α and upregulation of Bcl-2 and HGF (Fig.24). This possibly accounts for the increased potency of CDCs over MSCs for cardiac repair. Therefore, for transplantation, MSCs will be needed in higher numbers than CDCs. In addition to phenotypic differences, the higher population doubling time with the decreased expression of growth stimulant HGF-1 is another criterion that compromises the expansion of MSCs in short time period. HR injury was found to

increase the production of HGF (Fig. 27). Hepatocyte growth factor (HGF) is a pleiotropic cytokine which has implications on proliferation and migration. In a paracrine fashion it protects cells from apoptosis through a PI-3K/Akt/Bad pathway. Nicoleau et al, 2009 have demonstrated that HGF is required for self repair after MI (Nicoleau et al., 2009) (Nicoleau et al, 2009). HR injury retarded the cell cycle progression of MSCs with a reduced proportion of cells in the S-G2M phase (Fig.26). There was promotion of cell cycle progression in CDCs after HR injury. The activation of angiogenesis is a potential mechanism to counterbalance tissue hypoxia. The primary mechanism of oxidative stress-induced angiogenesis involves hypoxia-inducible factor/vascular endothelial growth factor (VEGF) signaling. The angiogenic action of VEGF includes an anti-apoptotic effect that promotes cell survival. The study has shown that, VEGF was increased by HR injury. The VEGF expression in CDCs was greater than that of MSCs (Fig. 31). Endothelial tube formation in CDCs was considerably advanced with well developed endothelial tubes, branching points and formation of interconnecting network (Fig 30). Both, the number of branching points and total tube length was high for CDCs. In case of MSCs they formed short sprouts and small tubes and very often, the tubes were not interconnected. The number of branching points was also lower. The reduced angiogenesis of MSCs is in accordance with the reduced secretion of VEGF in comparison with CDC. There was a decrease in the degree of angiogenesis after injury both in CDCs and MSCs though VEGF secretion was stimulated. The increased ROS production and the sequential upregulation of HIF-1 alpha possibly enhanced the VEGF secretion. These observations indicate that the angiogenic property is superior in CDCs than MSCs even after HR injury.

Exogenously expressed VEGF is reported to promote myocardial repair at least in part through SDF-1/CXCR-4 mediated recruitment of CSC (Tang et al., 2011). Cardiomyocyte differentiation of CDCs, in normal conditions and after HR injury was apparent with a significantly high expression of lineage marker, GATA4, (Fig.28) which has a tissue distribution limited to the heart. It is an early cardiomyocyte marker, playing an important role in transducing nuclear events that modulate cell lineage differentiation during development. A small percentage of MSCs expressed GATA 4, which was reduced after injury. CDCs expressed cardiomyocyte specific structural genes like myosin heavy chain, cardiac troponin I and connexin 43. Eventhough there was MHC expression, troponin I and connexin 43 was completely absent with both MSCs and HR MSCs. Both the cell types in all conditions expressed desmin. Increased myogenic potential in the injured CDC group was evident from the upregulation of Mef 2c, which regulates the expression of numerous cardiac structural and contractile proteins. Mef 2c, which is controlled by GATA 4 and Nkx2.5, was activated after injury to the CDCs without the addition of myocyte differentiation medium. Mef 2c also promotes endothelial cell proliferation and survival. It was also found to promote chondrocyte hypertrophy and vascularization of developing bones by triggering a network of transcription factors and signaling molecules involved in bone development (Potthoff and Olson, 2007). The basal expression of Mef2 in MSCs can be due to its multilineage differentiation potential. Apart from lineage commitment Heineke et al (2007) described that GATA-4 act as a mediator for angiogenesis through enhanced expression of VEGF (Heineke et al., 2007).

Multilineage potential of MSCs is one of the reasons for its use in regenerative medicine. MSCs are known to have the potential to differentiate into mesenchymal cell types like adipocytes, chondrocyte and osteocytes. Reports on multilineage differentiation potential of CDCs are limited. In this study, oesteogenic differentiation of CDCs was observed, when maintained in oesteogenic differentiation medium for 21 days (Fig. 32). This is in contrast to an earlier report, where osteogenic differentiation was not observed in CDCs (Koninckx et al., 2011b). The rate of oesteogenesis increased slightly after HR injury in both MSCs and CDCs. Adipogenesis was robust in MSCs and increased further after HR injury (Fig.33). Adipogenesis was not distinctly visible in CDCs, except for the presence of small vacuoles that stained positive for oil red O after maintaining in adipogenic differentiation media for 21 days. Fully matured oil droplets were not seen. But, after HR injury when the CDCs were grown in adipogenic medium more oil droplets were formed and there was partial adipogenesis in some areas. It is apparent that HR injury promotes differentiation of stem cells.

Migration of stem cells to the site of injury is an essential prerequisite for endogenous tissue repair. Stem cells generally home in to the site of injury. It was not known whether HR injury to the stem cells stimulates migration by an autocrine mechanism; or paracrine signals from the site of injury stimulate migration. Innate migratory potential was significantly higher for CDCs compared to MSCs (Fig. 34) .This is supported by relative increase in release of SDF-1 α by CDCs (Fig. 35). SDF-1α CXC is a chemokine known to play a critical role in the trafficking and has also been shown to increase cell survival during serum withdrawal, development, and differentiation (Hu et

al., 2007). Migration was unaffected by HR injury both in CDCs and MSCs when assessed by scratch-wound healing assay, as well as transwell migration assay (Fig. 35). To examine whether paracrine signals from injured tissue can stimulate migration, the migration of cells towards untreated myocardial explants was compared with hypoxically injured tissue explants. Transwell migration was significantly enhanced in cells exposed to injured tissue; the extent of stimulation being higher for CDCs compared to MSCs. (Fig. 36) The enhanced release of SDF-1 (Fig.35) corresponds to the pattern of migration consequent to exposure to hypoxically injured myocardial tissue.

Consolidating the observations, it is found that cardiospere derived cells are superior to bonemarrow mesenchymal stem cells by virtue of its capacity to greater survival and proliferation rate and better resistance to hypoxia- reoxygenation injury. One Preclinical study has shown that allogenic cell therapy is as effective as autologous cells for improvement in cardiac function in ischemic heart disease (Suzuki et al., 2014). Right atrial appendage when dissected as a part of surgical procedure can be maintained as cardiosperes or cardiospere-derived cells. This lends scope for providing potent cell products that can be used immediately as and when required.

V.5 Identification of the ideal culture condition for ex-vivo expansion of cardiosphere derived cells prior to transplantation

CDCs are found to be better candidates for myocardial regeneration both in terms of protective role on myocytes and in terms of survival and differention potential as observed

from the experiments illustrated above as well as earlier reports (Li et al., 2012b, Makkar et al., 2012, (Koninckx et al., 2011b). But, most of the transplanted cells are lost within two weeks and is best illustrated in a mouse myocardial infarction model, where Sca-1-positive CSCs did not provide long-term engraftment and benefit to cardiac function as determined by multimodality imaging (Li et al., 2009). The transplanted cells should survive and engraft for better results. Stem cells are maintained in specialized microenvironments called niches (Li and Neaves, 2006), that regulates stem cell function and protects stem cells from exhaustion (Drummond-Barbosa, 2008).Oxygen tension is animportant component of stem cell niches (2-4% O_2) that regulates proliferation, differentiation and plasticity of resident stem cells(Mohyeldin et al., 2010). The high oxygen gradient incidental to transplantation of the ex-vivo expanded cells from ambient oxygen (20% O_2 in the normal CO_2 incbator) to a relatively hypoxic environment (5% O_2 in the heart) can induce cell death and poor engraftment of transplanted cells. Repeated perturbations in oxygen tension can have significant implications on stem cell characteristics, as well as post transplantational survival and engraftment. Therefore, the advantages of maintaining physiological O_2 levels in the culture system, simulating the invivo conditions were examined based on the assumption that, that would be the ideal condition for obtaining an aliquot of healthy population of cells for transplantation compared to the conventional method of culture in ambient O_2.

Oxygen levels did not have a significant influence on the outgrowth of cells from atrial explants (Figure 36). There was difference in sphere formation capacity of cells depending on the oxygen tension. The spheres formed in 3% O_2 were larger in size

compared to conventional cultures that had smaller spheres though more in number (Figure 37). Cardiospheres bear resemblance to the "niches." Stem cell transplantation as cardiospheres is reported to have a significant advantage over single cells in terms of engraftment and improvement of myocardial function following implantation to the infracted heart (Li et al., 2010). Therefore, the observation that cultures grown in 3% O_2 forms larger spheres confers a selective advantage over conventional cultures for use in myocardial regeneration therapy. Adherent cultures obtained from cardiospheres also showed increase in cell yield. This is complemented by decrease in population doubling time significantly from Passage 4 (Figure 42). The proportion of proliferating cells was higher in 3% O_2 as reflected from the proportion of cells staining positive for Ki67 (< 2 fold, fig 44) and the cells in S/G2M phase (Figure 42). The cell yield from 7 passages was about 4 fold higher for cells cultured in 3%O_2. Improved viability in 3% O_2 is evident from the fewer number of apoptotic and senescent cells (Figure 40). The morphology of CDCs cultured in lower O_2 remained unaffected even after passage 10. Whereas, cells grown in 20% O_2 exhibited senescent phenotype (positive staining for β- galactosidase, increased percentage of $p16^{ink}$ positive cells) along with replicative senescence (increased population doubling time) (Figure 39, 40, 41). Increased expression of the p16 gene is reported to reduce the proliferation of stem cells and induces an early entrance to replicative senescence(Perez-Campo et al., 2014, Krishnamurthy et al., 2006).

Cell density is a crucial factor for the success of a transplant. Hence, expansion of CDCs in 3% O_2 helps in obtaining a higher yield of healthy cells in a shorter duration of time. As cardiospheres are reported to have better engraftment and survival, (Li et al., 2010) the

CDCs can be re-assembled to form cardiospheres. The advantage of culturing cells in lower O_2 levels is gaining importance. Enhanced proliferation of progenitor cells including mesenchymal stem cells (Grayson et al., 2007, Hung et al., 2012, Dos Santos et al., 2010), neural stem cells (Santilli et al., 2010) and embryonic stem cells (Forristal et al., 2010), in hypoxia has been reported. Yoshida et al (Yoshida et al., 2009) have shown that mild hypoxia is the optimal condition for both the generation and proliferation of induced pluripotent stem cells (iPSC) from human somatic cells 2α in comparison with 1% or 21% O_2(Yoshida et al., 2009). There is only one report on cardiosphere derived cells isolated from human ventricular biopsies, where Li et al (Li et al., 2011a) observed that CDCs expanded in 5% O_2 had lower number of aneuploid and senescent cells and increase in cell migration from explants(Li et al., 2011a). The effect of long-term culture in lower O_2 levels of CDCs derived from right atrial appendage has not been reported earlier.

The beneficial effects of long term culture in low O_2 levels are possibly mediated by reduction in oxidative stress. Generation of ROS was significantly lower in CDCs cultured in 3% O_2. Reduced ROS generation in 3% O_2 was evident by fluorometric analysis for DCFDA (Figure 46). Intracellular ROS act as signal molecules and maintenance of optimal levels are crucial for the proper functioning of cells. Cells cultured in lower O_2 levels were found to be more resistant to oxidative stress. When exposed to H_2O_2, a 2 fold increase in apoptotic cells was observed in cultures maintained in hyperoxia (Figure 47). Lower rate of senescence and higher resistance to oxidative stress of CDCs expanded in 5% O_2 has been reported (Li et al., 2011a, Li and Marbán,

2010). Therefore CDCs cultured in lower O_2 have survival advantage in an adverse microenvironment. Reactive oxygen species have a crucial role in cell differentiation. The proportions of c-Kit positive cells were significantly higher in CDCs expanded in 3% O_2 (Figure 45). Spontaneous differentiation to myocyte lineage was significantly higher for cells maintained in 20% O_2 (Figure 48). The stemness is therefore retained when cultured in 3% O_2 (Fig. 48). The CDCs were stimulated to differentiate into cells of different phenotypes. The cells differentiated into the desired phenotypes on stimulation (Figure 51, 52) indicating that the cells are pluripotent. Proangiogenic potential of cells preconditioned in 3% O_2 has been reported (Amirrasouli et al., 2013). Even in this study, endothelial tube formation was enhanced both spontaneously and in induced condition in 3% O_2 (Figure 49, 50). However, in 3% O_2 spontaneous myocyte formation was lower, as evident from the reduced expression of Nkx 2.5 and cardiac troponin T (Figure 48), which implies a better preservation of stemness in 3% O_2.

For therapeutic application, following ex-vivo expansion, the cells are to be transplanted to the tissue with physiological O_2 levels. The ex-vivo expansion condition can have significant influence on post transplantation survival and proliferation. Hence the effect of culture in 20% and 3% O_2 on survival and proliferation of CDCs was examined following transfer to physiological O_2 levels. The proportion of necrotic cells was significantly lower in CDCs expanded in 3% O_2 (Figure 53). The percentage of proliferating cells was significantly higher for CDCs expanded in 3% O_2. (Figure 54) This shows that the O_2 tension of cultures can have a significant influence on post transplantation engraftment and myocardial repair. Allogenic transplantation of CDCs

into the ischemic mouse and rat myocardium induced their spontaneous in vivo differentiation into cardiomyocytes(Li et al., 2012a, Cheng et al., 2012b, Malliaras et al., 2012). Extensive clinical and preclinical studies are required to establish the efficacy of allogenic CDCs for myocardial regeneration.

VI. SUMMARY AND CONCLUSIONS

Cardiomyocyte death and scar tissue formation after myocardial infarction in course of time leads to adverse ventricular remodeling; and the downward spiraling cascade of events finally precipitates into heart failure. Current treatment strategies do not reverse tissue remodeling. Though endogenous repair mechanisms are stimulated after tissue injury, the process appears to be inadequate for restoration of healthy myocardium. Resident cardiac stem cells are expected to play a significant role in tissue repair. However, inadequate tissue regeneration may be the result of limited availability of stem cells or the unfavorable milieu consequent to ischemia- reperfusion (IR) injury. Whether resident stem cells can withstand and preserve its innate characteristics after an ischemic insult in the heart is an unanswered question. Cardiosphere derived cells (CDCs) cultured from the right atrial appendage of patients undergoing coronary artery bypass graft surgery were used for the experimental studies. The cells were subjected to hypoxia-reoxygenation (HR) injury to simulate ischemia reperfusion injury in the heart and evaluated for their survival, proliferation and differentiation. Hypoxia- reoxygenation injury to CDCs was found to affect the survival of the cells. There was a significant increase in the proportion of apoptotic cells in CDCs subject to HR injury (6.7%) compared to untreated control (3.5%). Senescent cells (11%) were observed only in the treated CDCs. HR injury was also found to up regulate the expression of prosurvival factor HIF-1α, along with a two-fold increase in the expression of pAkt and Bcl-2. HR injury to CDCs was associated with increased oxidative stress. Cell migration was unaffected by HR injury, but cell differentiation to myocardial lineage was reduced, as assessed from the expression of myocyte differentiation markers connexin-43 and cardiac

troponin I and endothelial tube formation. No paracrine effects on CDCs in terms of survival and proliferation were observed consequent to HR injury to cardiomyocytes (H9c2), nor were the H9c2 cells affected by HR injury to CDCs. HR injury mediated damage to cardiac stem cells can be the underlying cause for inefficient endogenous repair of injured myocardium.

Endogenous repair mechanisms do not completely restore the damaged myocardium. Hence, transplantation of exvivo expanded stem cells in sufficient numbers appears to be the most viable option for myocardial regeneration. Of the different stem cells that can be used for transplantation, identifying the ideal cell type remains a debatable matter. The two cell types popularly used for transplantation are: Cardiosphere Derived Cells and Mesenchymal stem cells. Hence, the regenerative potential of the two cell types was indirectly assessed by measurement of proliferation, differentiation capacity and survival following HR injury. Cells isolated from the same patients were used for comparison. CDCs were found to have better survival capacity in culture, as assessed from the reduced apoptosis (3.5±0.37%), compared to MSCs (5.7±0.14%). When subject to HR injury, the proportion of apoptotic cells and senescent cells were significantly higher in MSCs compared to CDCs. Concomitantly, the expression of pro survival factors- pAkt and Bcl_2 were significantly higher in CDCs compared to MSCs. Stimulation of HIF-1α following HR injury was comparable for both the cell types. Exposure of the cells to injured myocardial tissue stimulated release of stromal derived factor as well as cell migration as

assessed by transwell migration assay. Spontaneous myogenic differentiation by CDCs was stimulated by HR injury and directed differentiation showed the expression of myocyte lineage markers and structural proteins. The angiogenic ability of CDCs and MSCs evaluated using in vitro tube-forming assay showed improved endothelial tube formation in CDCs even after HR injury. Compared to mesenchymal stem cells, cardiosphere derived cells are found to have greater resistance to HR injury, thereby validating the superiority of the latter cell type for use in transplantation for myocardial regeneration.

There are reports on the use of cardiosphere-derived cells for myocardial regeneration. However, the cell-based therapy has not met with the desired success, possibly due to the compromised survival and functioning of the transplanted cells. Prior to transplantation, the cells are expanded in vivo in ambient oxygen (20%) that is much higher than the tissue levels (5%). A large proportion of the stem cells are reported to be lost from the transplantation site. Sudden drop in the oxygen levels in the physiological condition compared to the ambient atmosphere in which they are grown can affect post transplantation survival of cells. Hence it was assumed that ex-vivo expansion of human CDCs in subphysiological oxygen (3% O_2) simulating the O_2 levels in niches can reduce cell loss and promote myocardial regeneration. Growth and survival of CDCs expanded in 3% and 20% O_2 were compared. Culture of cardiac stem cells in 3% O_2 stimulated cardiosphere formation. Compared to cells maintained in ambient oxygen, the proliferation rate was higher and the proportion of senescent cells lower for cells maintained in 3% O_2. There was a two-fold increase in the proportion of Ki-67 positive

cells in passages 3-4 and the cell yield from 7 passages was about 4 fold higher for cells cultured in 3% O_2. The proportion of c-Kit positive cells was also higher in 3% O_2, thereby ensuring better retention of stemness. This is possibly due to the fact that the generation of ROS was lower. The resistance to oxidative stress was higher when cultures were maintained in low oxygen. An interesting observation is that, when the CDCs maintained in 3% O_2 were shifted to physiological oxygen (5% O_2), simulating the oxygen levels that prevail in the tissue, the survival and proliferation efficacy of the former was relatively better than cells maintained in 20% O_2. Ex-vivo expansion of cardiosphere derived cells in physiological O_2 levels is therefore recognized as the ideal culture condition for improving engraftment and promoting myocardial regeneration.

Conclusion

Susceptibility of cardiac stem cells to ischemia-reperfusion injury possibly limits the endogenous repair of the damaged myocardium, leading to cardiac remodeling. Stem cell transplantation is therefore envisaged to promote myocardial regeneration. Cardiosphere derived cells have better survival capacity in culture, and greater resistance to hypoxia-reoxygenation injury compared to the popularly used mesenchymal stem cells. The differentiation to myocardial lineage was also better for the former. Cell cultures are conventionally maintained in ambient O_2. CDCs expanded in physiological O_2 levels, showed greater retention of stemness. Further, the viability of cells was highly compromised when cells maintained in higher O_2 levels were shifted to the physiological level. These findings lead to the conclusion that cardiosphere derived cells cultured in physiological levels of O_2 is ideal for transplantation for myocardial regeneration.

Preclinical studies are essential to validate the observations. Further studies should therefore be aimed at carrying out invivo experiments in animal models, by evaluating the consequence of stem cell transplantation following surgical induction of myocardial infarction. Because of the adverse extracellular milieu prevailing soon after ischemia-reperfusion injury and the observation of compromised function of CDCs consequent to HR injury, the ideal time for transplantation of CDCs have to be dertermined by invivo experimental studies.

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Papers presented

- Presented a poster titled "**Human cardiosphere derived cells have a selective advantage over bone marrow mesenchymal stem cells in terms of myocardial regeneration**" in 26th Kerala Science Congress- **Best poster award**

- Presented a paper titled **"Long term culture of cardiac stem cells in Physiological Oxygen levels retain stemness and promote survival",** in science Fete, SCTIMST, Trivandrum- **Best paper award**

- Presented a paper titled **"Expansion of human cardiac stem cells at physiological oxygen levels retains stemness and stimulates proliferation"** in International conference on Stem cells and Cancer, Haffkines institute, Mumbai.

- Presented a paper titled "**The effect of oxygen on cardiac stem cell survival**" in 25Th Kerala Science Congress

Publications

1. Deepthi RajendranNair Sreerengam, Karunakaran J, RenukaNair R. Differential response of human cardiac stem cells and bone marrow mesenchymal stem cells to hypoxia–reoxygenation injury. Molecular and Cellular Biochemistry, January 2017, Volume 425, Issue 1, pp 139–153.IF-2.61

2. Deepthi RajendranNair Sreerengam, Renuka Nair R. Hypoxia improves migration potential and limits differentiation of rat cardiac stem cells. International Journal of Innovative Research in Science, Engineering and Technology, Vol. 6, Issue 1, January 2017 ,IF- 6.2

3. Deepthi RajendranNair Sreerengam, Karunakaran J,Renuka Nair R, Effect of Subphysiological Oxygen Levels on Cardiac Stem Cells Isolated from Human Atrial Appendage. Molecular and Cellular Biochemistry, 2016. DOI 10.1007/s11010-017-3002-4,April 2017. IF-2.61