SUPEROXIDE DISMUTASE DELIVERY AND CARDIAC PROGENITOR CELL CHARACTERIZATION FOR MYOCARDIAL REGENERATION APPLICATIONS

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SUPEROXIDE DISMUTASE DELIVERY AND CARDIAC PROGENITOR CELL CHARACTERIZATION FOR MYOCARDIAL REGENERATION APPLICATIONS

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TO

My parents Seshadri & Banumathi,

My sister Priya Balaji &

My niece Smruthi

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LIST OF ABBREVIATIONS

AAR	Area at risk
AHA	American Heart Association
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BMC	Bone marrow derived stem cells
BSA	Bovine serum albumin
CDM	Cyclohexane dimethanol
CPC	Cardiac progenitor cells
CSC	Cardiac stem cells
DAPI	4',6-diamidino-2-phenylindole dihydrochloride
DHE	Dihydroethidium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
ED	End diastolic
EDTA	Ethylenediaminetetraacetic acid
EPC	Endothelial progenitor cells
ES	End systolic
ESC	Embryonic stem cells
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
FSOD	Fluorescein isothiocyanate conjugated SOD1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

GPX1	Glutathione peroxidase 1
GRAS	Generally recognized as safe
HAE	Hydroxyalkenals
hCPC	Human cardiac progenitor cells
HE	2-hydroxyethidium
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High performance liquid chromatography
HUVEC	Human umbilical vein endothelial cells
IGF1	Insulin like growth factor 1
IGFR	Insulin like growth factor 1 receptor
IGFR-	Insulin like growth factor 1 negative
IGFR+	Insulin like growth factor 1 postive
IL-12	Interleukin-12
IL-6	Interleukin-6
iPSC	Induced pluripotent stem cells
IR	Ischemia/reperfusion
KHB	Kreb's-4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer
LD50	Median lethal dose
LV	Left ventricle
MDA	Malondialdehyde
MDR1	Multiple drug resistance 1
MI	Myocardial infarction
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
NAD	Nicotinamide adenine dinucleotide

NADPH oxidase	Nicotinamide adenine dinucleotide phosphate-oxidase
NCX	Sodium calcium exchanger
NF κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIH	National Institute of Health
PBS	Phosphate-buffered saline
PCADK	Poly(cyclohexane-1,4-diyl acetone dimethylene ketal)
PGA	Polyglycolic acid
PK	Empty PCADK microparticle/ Polyketals
PK3	Poly(cyclohexane-1,4-diyl acetone dimethylene ketal -co-1,5-pentane-acetone dimethylene ketal)
PKFSOD	FSOD encapsulated PCADK
PK-p38i	p38 inhibitor encapsulated PCADK
PKSOD	SOD1 encapsulated PCADK
PLB	Phoshpholamban
PLGA	Poly(lactic acid), poly(lactic-co-glycolic acid)
PMA	Phorbol myristate acetate
PPADK	Poly(1,4-phenylene-acetonedimethylene ketal)
PVA	Poly vinyl alcohol
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Real time polymerase chain reaction
RYR	Ryanodine receptor
S/O/W	Solid in oil in water
SEM	Scanning electron/standard error of the mean
SERCA2a	Sarco/Endoplasmic reticulum calcium ATPase type 2A

SFM	Serum free media
siRNA	Short interfering ribonucleic acid
SOD	Superoxide dismutase
SOD1	Copper/Zinc superoxide dismutase
SOD2	Manganese superoxide dismutase
SOD3	Extracellular superoxide dismutase
TGFβ	Transforming growth factor beta
TNFα	Tumor necrosis factor alpha
TTC	2,3,5-triphenyltetrazolium chloride
TUNEL	Terminal deoxynucleotidyl transferase mediated 2´-deoxyUridine, 5´-Triphosphate Nick End Labeling assay
UV	Ultraviolet
W/O/W	Water in oil in water
XO	Xanthine oxidase
XXO	Xanthine/Xanthine oxidase

SUMMARY

Cardiovascular diseases are the leading cause of death throughout the world and various estimates predict that heart diseases will remain the number one killer in the world. Pharmacotherapies have not shown significant long term survival benefits to the patients. Therefore, alternate therapeutic strategies such as bioactive agent delivery and cell therapy based approaches are being investigated. One of the major causes of heart failure is the disease progression after an ischemic event and any successful therapy will be needed over the course of several days/weeks. Oxidative stress is greatly increased in the myocardium following infarction. This plays a significant role in cardiac disease progression and it has also been implicated in the failure of implanted cell therapy. Therefore, reducing oxidative stress in damaged tissue using antioxidants may have broad clinical implications for both the treatment of cardiac dysfunction and for cardiac regeneration applications. This dissertation work examines the effect of sustained delivery of endogenous antioxidant superoxide dismutase (SOD) to the rat myocardium following ischemia/reperfusion (IR) using polyketal polymers as drug carriers. The second major objective of this dissertation is to examine the effects of oxidative stress on cardiac progenitor cells – a promising endogenous adult stem cell in cardiac cell therapy applications.

Intracellular delivery of Cu/Zn SOD (SOD1) remains difficult and studies with SOD1 have had little success, mainly due to poor pharmacokinetics of the drug. Thus newer methods are required to improve the delivery of this rapidly-cleared protein. Biocompatible and neutral degradation products formation following the hydrolysis of poly(cyclohexane-1,4-diyl acetone dimethylene ketal) (PCADK) makes this polymer an attractive carrier to deliver drugs to treat chronic inflammatory heart diseases. We tested the in vitro and in vivo efficacy of SOD1 microencapsulated within PCADK polymer

(PKSOD) and found that (a) PKSOD efficiently scavenges both extra- and intra-cellular superoxide formed within RAW macrophages and (b) PKSOD but not free SOD1 treatment improves the acute cardiac function and decreases apoptosis in the ischemic myocardium following IR.

C-kit receptor positive cardiac progenitor cells (CPCs) have the potential to regenerate the myocardium following transplantation. However, one of the major bottle necks of cell based therapies is the poor survival of the transplanted cells and oxidative stress has been implicated in the death of the cells following transplantation. Additionally, oxidative stress and antioxidant levels control the differentiation, senescence and self renewal of stem cells. We studied the basal SOD levels and oxidative stress induced apoptosis of CPCs and found that (a) CPCs have significantly higher amounts of SODs compared to neonatal cardiomyocytes and (b) CPCs are resistant to oxidative stress induced apoptosis compared to myocytes. We have also studied the basal SOD levels of young and senescent phenotypes of human CPCs and found that young phenotype of CPCs have significantly higher activity of SOD2 compared to the senescent phenotypes.

CHAPTER 1

INTRODUCTION

Cardiac dysfunction following myocardial infarction is a leading cause of global mortality, and in the United States alone, there is roughly one death per minute due to a coronary event. In addition, according to the American Heart Association (AHA) report, the total financial burden to the US due to heart diseases runs in hundreds of billions of dollars each year outnumbering the direct and indirect costs associated with other major diseases. In the year 2007 alone, financial burden due to heart diseases in the US was about 180 billon dollars [1]. Thus, identifying new treatment options or improving the existing options could improve patient health and potentially reduce the enormous financial burdens.

1.1 Motivation

Ischemic heart diseases are a leading cause of global mortality, and the best method of preventing and controlling cardiac disease is to adopt a healthy life style [2]. However in current populations, there is increasing incidence of obesity, diabetes and high blood pressure, all of which increases the risk of cardiovascular diseases, coupled with sedentary lifestyles. Not surprisingly, various estimates predict that cardiac diseases will remain the leading cause of death in the world [1, 3, 4]. Currently, heart transplantation is the only definitive cure for patients suffering from end stage heart failure. While a viable cure, there is immense shortage of donor hearts in addition to other perioperative risks involved in this procedure. Thus, not many patients survive to receive a donor heart. The existing care procedures, both surgical and pharmacological interventions, have significantly improved the survival rate of patients after a first coronary event. However, most of these interventions focus on restoring blood flow, and

are not able to prevent the myocardial disease progression and chronic remodeling events following myocardial infarction (MI), which eventually lead to heart failure [5]. Widely prescribed pharmacotherapeutic drugs such as β-adrenergic receptor blockers, Angiotensin-Converting-Enzyme inhibitors, lipid lowering drugs and anti-platelet agents significantly improve the quality of life and reduce repeated hospitalization. However, these drugs have systemic effects with concerns for side effects and, importantly, these drugs do not provide permanent cure to the heart, and the heart remains more vulnerable for future cardiac events [6]. Therefore, identifying alternate, effective treatment options is essential.

Myocyte death following insults such as ischemia/reperfusion (IR) is one of the main reasons for the compromised ability of the heart to pump blood, leading to heart failure [7]. Further, these losses in the myocytes are mainly regional [8]. Therefore, a localized therapy reducing myocyte death or regenerating the lost myocytes could potentially provide a functional benefit to the heart. Although, previously heart was considered a post-mitotic organ with no regenerative potential, recent studies have revealed the existence of endogenous cardiac progenitor cells and evidences of cardiomyocyte renewal in the human heart [9, 10]. This led to the exploration of alternate therapeutic strategies by directly delivering bioactive molecules such as genes, micro-RNAs and proteins or whole cells to the heart with the aim of regenerating the myocardium [11]. One of the major bottlenecks in the progress of bioactive molecule based interventions is the lack of a suitable carrier or delivery vehicles, and in the same manner, the progress in cell based interventions is mainly hindered by poor survival and engraftment of the transplanted cells to the myocardium. Therefore, a part of this dissertation investigates the potential of a recently invented polymeric carrier in delivering soluble factors to the myocardium and the other part of this dissertation

presents few basic studies on survival of cardiac progenitor cells under physiological stress conditions.

1.2 Research Objectives

Following myocardial ischemia/reperfusion (IR), there is excessive reactive oxygen species (ROS) generation, cardiomyocyte apoptosis, and ultimately cardiac failure [12]. Roles of high oxidative stress in cardiac disease progression have been a subject of considerable interest, and it has been proposed as the unifying mechanism behind various risk factors of heart diseases [13]. The superoxide radical is thought to be a major contributor in initiating the cascade of events associated with reperfusion injury and levels of the endogenous superoxide scavenger – superoxide dismutase (SOD) – sharply decrease shortly after occlusion. Direct administration of SOD, overexpression and gene therapy studies – that increase bioavailability of the enzyme – protect the heart against post-ischemic injury, reduces infarct size and reperfusion damage [14-16]. In addition to the damage of ROS on cardiomyocytes directly, they can also have deleterious effects on resident stem and progenitor cell population. The total oxidative stress level and antioxidant levels within the progenitor cells directly affect their differentiation abilities [17]. Thus, antioxidant status in and around the cells of the myocardium could affect both the normal physiology of a healthy heart and the pathophysiology and recovery of a diseased heart.

Despite the large role of oxidative stress in cardiac dysfunction, successful antioxidant therapies have proved elusive, and many clinical trials have failed to show a significant benefit. It has been hypothesized that the therapy is limited by poor pharmacokinetics and inadequate delivery of the drug [18]. Although gene therapy with SOD is a good analytical tool, quantifying drug delivery and targeting tissues are very difficult with this method. Additionally, because of oncogenesis and other safety issues associated with it, gene therapy has not been met with much enthusiasm. Thus,

significant interest exists in developing better SOD delivery systems. Many modifications have been made on SOD protein mainly to improve the protein pharmacokinetics and for targeted delivery. Additionally, existing SOD therapies involve continuous injection or infusion of high levels of SOD protein following myocardial injury. Therefore, a treatment involving a single injection of SOD would be advantageous over existing treatment options. Polyketals, are recently described class of biomaterials and poly (cyclohexane-1,4-diyl acetone dimethylene ketal) (PCADK), is a polyketal polymer that hydrolyzes slowly at physiological pH values and degrades into non-toxic, FDA-approved compounds. Importantly, PCADK causes minimal tissue inflammatory response and, its ability to treat inflammatory heart diseases by delivering small molecule anti-inflammatory drugs to the myocardium is known [19]. In the first part of this research, PCADK is used to microencapsulate and deliver SOD1 to the myocardium. Initially, the efficacy of SOD1 encapsulated PCADK (PKSOD) in reducing the superoxide levels within the cells was evaluated in macrophage cell line, followed by investigations in vivo to study the effects of sustained reduction in excess superoxide levels on restoration of cardiac function following acute myocardial IR (Figure 1.1).

Cell based therapies benefit the myocardium either through direct differentiation of transplanted cells to cardiovascular lineage or through secretion of survival factors. Various candidates of stem cell populations such as bone marrow stem cells and embryonic stem cells exist, and evidences from many clinical trials suggest modest benefits of cell therapy to the myocardium [20]. A promising recent addition to these cell types is endogenous c-Kit positive myocardial progenitors. They exhibit robust cardiovascular lineage differentiation potential, and can be isolated during biopsies which can later be expanded in vitro for subsequent autologous administrations [10, 21]. Because of the promising regenerative potential of these cells, preclinical studies are already completed, and phase 1 clinical trials are in progress. However, transplanting

any cell type to the ischemic myocardium is a challenge because of the hostile tissue microenvironment with enhanced oxidative stress and the excessive inflammatory response after IR. As mentioned above, excessive oxidative stress levels and the antioxidant status of the cells affects both the survival and differentiation ability of progenitor cells. Compared to other cell types, CPCs are recent with their identification only in the year 2003. Although they are in clinical trials, many of its basic properties such as its response to physiological stresses remain unknown. Therefore, the second part of the thesis deals with understanding the response of CPCs when subjected to oxidative stress - one of the main physiological stresses in the ischemic myocardium (Figure 1.1).

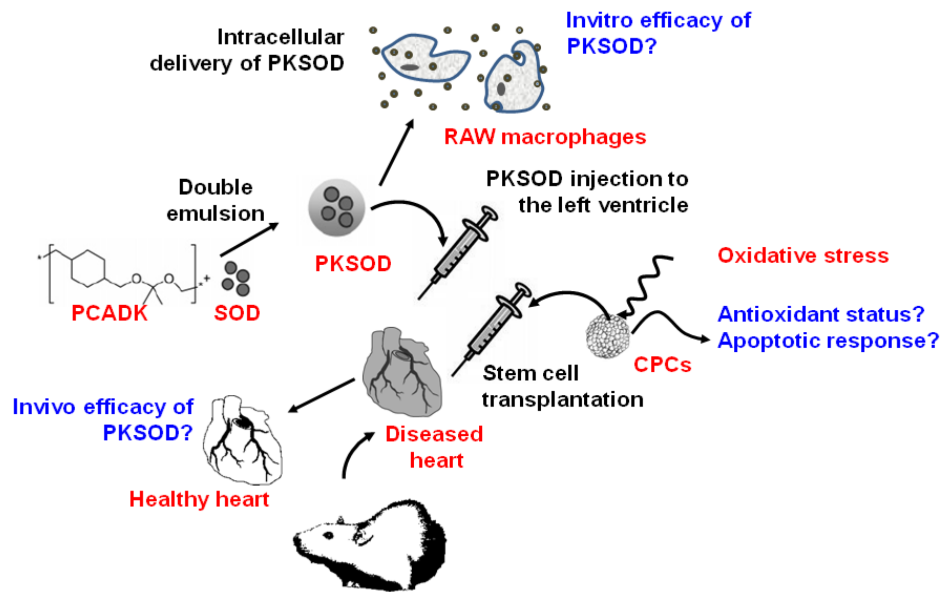

Figure 1.1. Schematic of the research objectives. Sustained delivery of SOD1 and stem cell transplantation has the potential in providing benefits to the diseased myocardium. In this research work, SOD1 will be encapsulated within PCADK polymer by double emulsion method. The efficacy of PKSOD in scavenging superoxide will be tested in RAW macrophage cell line. In vivo efficacy of PKSOD will be tested in a rat model of IR. Finally antioxidant SOD1 status and oxidative stress induced apoptotic response will be tested in CPCs.

1.3 Specific Aims

The central hypothesis of this dissertation is that retention of SOD1 within the infarct area over the course of several weeks reduces superoxide levels and restores function following myocardial IR and that the levels of SODs protects the progenitor cells from oxidative stress induced death. The objective of the dissertation will be completed by testing the central hypothesis using the following aims.

Specific Aim 1: Test the efficacy of SOD1 encapsulated PCADK microparticles (PKSOD) to scavenge superoxide in vitro. We hypothesize that micron-scale PKSOD can scavenge both intracellular and extracellular superoxide in vitro. In this aim, SOD1 was encapsulated within PCADK through a double emulsion process and the ability of PKSOD to reduce elevated superoxide levels in stimulated RAW macrophage cell line was assessed. Intracellular and extracellular superoxide was quantified using dihydroethidium – a superoxide specific probe – based HPLC analysis.

Specific Aim 2: Evaluate the ability of PKSOD to improve cardiac function. We hypothesize that PKSOD delivered to the myocardium following IR injury in rats, will scavenge excess superoxide following infarction and improve the cardiac function. In this study, oxidative stress levels in the myocardium were analyzed, and echocardiography based cardiac function was evaluated. Additionally, various histological analyses were performed to try to understand the cause of the observed functional effects of PKSOD.

Specific Aim 3: Assess the SOD levels and oxidative stress induced death in CPCs. We hypothesize that SOD levels within the cardiac progenitor cells will improve the survival of these cells subjected to oxidative stress. Initially, CPCs were isolated from

rat myocardium and expanded in vitro. Oxidative stress induced cell death was analyzed using propidium iodide and TUNEL assays. Later, SOD levels of CPCs was compared with other cell types and the role of SODs in offering protection to CPCs were established using siRNA based gene silencing studies.

CHAPTER 2

BACKGROUND

2.1 Myocardial Infarction and pathophysiology of ischemia/reperfusion

Recent AHA guidelines define heart failure as “a complex clinical syndrome that can result from any structural or functional cardiac disorder that impairs the ability of the ventricle to fill with or eject blood” [22]. One of the major causes of heart failure is the disease progression after an ischemic event. During ischemia, the myocardial oxygen demand is not met by the existing coronary blood perfusion due to events such as atherosclerotic plaques blocking the normal blood flow. While a mild ischemia leads to angina pectoris or chest pain, a severe ischemia and poor collateral distribution in the tissue bed causes irreversible injury to the cells leading to acute myocardial infarction (MI) – commonly termed as heart attack. MI following ischemia follows a “wavefront phenomenon” of cell death from sub-endocardial region to sub-epicardial region of the myocardium [23, 24].

Ischemia alters the normal biochemical, electrical and mechanical properties of the myocardium which eventually leads to severe myocardial injury. Because of decreased oxygen tension, the major mode of metabolism in the myocardium shifts from aerobic oxidative phosphorylation in mitochondria to anaerobic glycolysis through glycogen breakdown thus leading to a significant reduction in adenosine triphosphate (ATP) availability [25]. This reduces the activity of sodium/potassium ATPases, which alter the balance of crucial ions such as Na^+, K^+ and Ca^{2+}. These changes lead to altered myocyte contraction, impaired membrane integrity and cell swelling which ultimately causes irreversible injury and oncotic cell lysis within minutes to hours following the onset of ischemia [26, 27].

The extensive necrosis and acute damages caused by permanent occlusion of coronary artery are reduced when there is spontaneous, pharmacological, or percutaneous restoration of blood flow termed as reperfusion [28, 29]. However, the beneficial effects of reperfusion are countered by its equally damaging effects called reperfusion injury, observed initially in 1960 [30]. Subsequent investigations have revealed various cellular and sub-cellular changes that promote inflammation and chronic remodeling events following reperfusion. One crucial cellular response initiated by reperfusion is the recruitment and activation of inflammatory cells such as neutrophils [31]. Important subcellular changes observed after reperfusion include damage to mitochondria by forming mitochondrial permeability transition pores [32] and development of hypercontracture in the myocytes due to calcium overload [33]. Further, humoral factors such as complement systems are activated by IR [34]. Additionally, IR stimulates the production of inflammatory cytokines [35] and free-radicals [36]. All these changes induced by reperfusion causes damaging effects such as endothelial dysfunction [37], no-reflow phenomenon [38], apoptosis [39], contraction band necrosis [40], reperfusion arrhythmias [41] and myocardial stunning [42]. Excessive levels of molecular oxygen [43] and calcium ions [44] were thought to be the causative agent of many of these events leading to debates in oxygen paradox and calcium paradox and later to the hypothesis that both these paradoxes are the facets of the same problem – the problem of excessive reactive oxygen species production after reperfusion [45, 46].

2.2 Reactive oxygen species, oxidative stress and myocardial infarction

Reactive oxygen species (ROS) include reactive molecules formed from molecular oxygen. They include species derived from reduction of dioxygen: superoxide/hydroperoxyl radicals ($O_2^{-\cdot}$/$HO_2^{\cdot}$), hydroxyl radical ($OH^{\cdot}$) and hydrogen peroxide (H_2O_2); carbon-centered radicals with molecular oxygen: peroxyl ($ROO^{\cdot}$) and

alkoxy radicals ($RO^{\cdot}$); and free radical forming oxidants such as peroxinitrite ($ONOO^-$). They are all highly reactive entities with unpaired electrons in their valence shell. ROS are not always toxic, and their presence is essential for normal physiological functions of cells (Figure 2.1). Nitric oxide (NO), for example, is essential for regulating the tension of

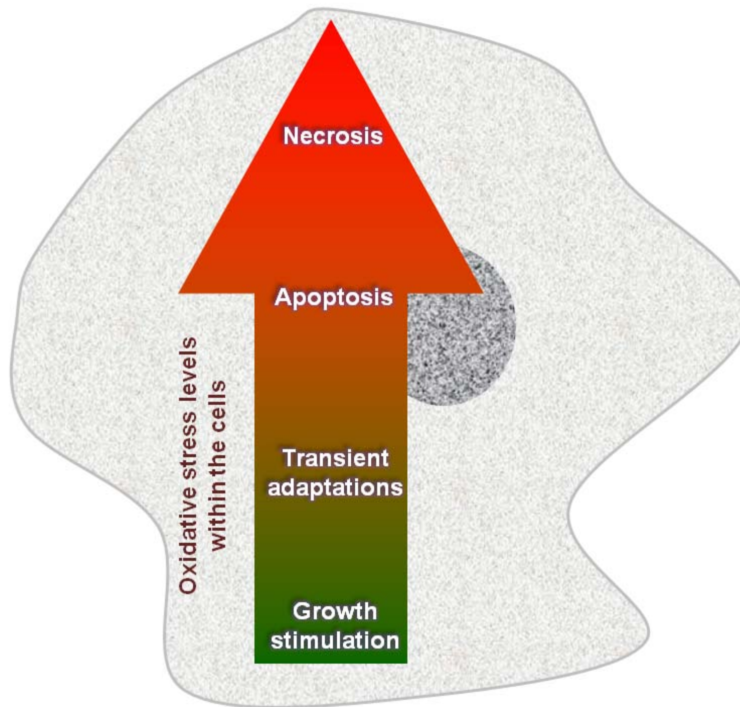

Figure 2.1. ROS play an important role in both physiology and pathology of a cell. Low oxidative stress (<15 μM H_2O_2) stimulates growth. However, high oxidative stress (>1mM H_2O_2) stimulates apoptosis and necrosis. Stress-activated genes induce the expression of many proteins within the cells as a transient adaptation to moderate oxidative stress.

blood vessels. Low ROS concentrations (< 15 μM H_2O_2) stimulate cell growth and proliferation, and act as important signaling molecules [47]; mild concentration arrests cell growth temporarily [48]. Moderate ROS levels alter gene expression profiles of many proteins such as heme oxygenase, catalase, mitochondrial superoxide dismutase (SOD2), and mitogen-activated protein kinases eventually inducing a transient cellular adaptation [49]. High ROS levels (~400 μM H_2O_2) arrest cell growth permanently [48].

Still higher concentrations (~ 1 mM H_2O_2) induce apoptosis and very high ROS levels (>5 mM H_2O_2) induce cell disintegration and necrosis [50, 51]. Thus, ROS play an important role in both physiology and pathology of a cell.

Tissues are usually protected from excess ROS by the presence of antioxidant enzymes such as superoxide dismutase (SOD) and catalase, or easily-oxidized organic compounds such as vitamins C and E. During disease states, the cells are continuously in a stressed state because the flux of free radicals is much higher than the scavenging ability of antioxidants. This stressed state called oxidative stress is considered to have an important role in ageing and pathogenesis of many diseases [52]. During oxidative stress, there is an imbalance in the pro and anti-oxidant level in the biological system. When the balance tips towards pro-oxidant mechanisms, extensive cellular damage occurs [13].

The role of oxidative stress in the pathology of heart diseases has been studied substantially [53-56]. Redox imbalance is implicated in many pathological states of the heart including hypertrophy [57], ischemia-reperfusion injury [58, 59] and myocardial stunning [60, 61]. Antioxidant levels in the heart are altered during pathological conditions. While preconditioning protocols of brief, repetitive IR increase the expression of antioxidants such as SOD2 and offer benefits to the myocardium [62], models of prolonged ischemia (> 20 min) followed by reperfusion, reduce the antioxidant levels in the myocardium [63]. For example, antioxidants such as ascorbate and ubiquinol declined in a systematically organized relationship under IR in a rat model [64]. Additionally, activities of major endogenous antioxidants such as SOD and glutathione peroxidase dropped by 43% and 39% after IR thus compromising the ability of myocardium to scavenge the excess free radicals [65, 66]. Oxidative stress is also known to upregulate factors such as TGFβ that induce myocardial fibrosis leading to hypertrophy and heart failure [67].

Scavenging ROS using antioxidants improves the functional recovery of myocardium after IR [68]. However, negative reports of antioxidant therapies are also documented [69]. Among the various free radicals, the role of superoxide anions in cardiac diseases have been implicated by both direct measurements and indirect inferences [70] in the pathogenesis of infarct development [71, 72], reperfusion injury [73] and eventual myocardial dysfunction [74]. Thus, a treatment option targeting the oxidative stress, specifically the superoxide radical, could potentially improve the recovery of the myocardium after IR.

2.3 Superoxide radical

Molecular oxygen (O_2) can add two additional electrons to its antibonding orbitals. Superoxide radicals ($O_2^{-\cdot}$) are the species formed when oxygen is reduced by a single electron. Under acidic environment (pH<4.8), they exist as hydroperoxyl radicals ($HO^{\cdot}_2$). Reaction rate constants of $O_2^{-\cdot}$ and $HO^{\cdot}_2$ with bioorganic compounds are in the range of 10^3 to 10^4 liters mole^{-1} s^{-1} [75]. Because of their moderately high reactivity, higher concentrations of superoxide radicals can cause toxicities such as inducing lipid peroxidation reactions in the cells [76, 77]. Importantly, superoxide radicals are the source for the generation of many other toxic ROS such as hydrogen peroxide (H_2O_2) and radicals such as peroxinitrite and hydroxyl (OH) which cause more damage to the biological system. Hydroxyl radicals, for example, are regarded as one of the very toxic and highly reactive free radicals [78]. Its rate constants in chemical reactions approach 10^8 to 10^{10} liters mole^{-1} s^{-1} [79] or in other words they are at least four orders of magnitude more reactive than superoxide radicals. However, formation of hydroxyl radical from superoxide has never been independently demonstrated in biological systems using classical chemical methods [80].

There are three main endogenous sources of superoxide. The first and an important source of superoxide in the heart is the aerobic respiratory oxidative chain in mitochondria, which converts 1% to 2% of molecular oxygen to superoxide radicals [81]. A second source that is relevant to reperfusion injury is produced by xanthine oxidase (XO). Ischemia triggers, a post translational modification of NAD^+- reducing xanthine dehydrogenase enzyme to ROS generating XO enzyme. Thus, when the myocardium is reperfused with oxygen laden blood after an ischemia, excess superoxide radicals are formed [82]. These reactive radicals elicit an inflammatory response by recruiting inflammatory cells such as neutrophils and macrophages that become a third source of superoxide during a phenomenon called respiratory burst [83]. During the respiratory burst, membrane bound NADP(H) oxidase supports the flow of electron from NADP(H) to oxygen to produce superoxide radicals. In addition to phagocytic cells such as macrophages, NADP(H) oxidase is also present in non-phagocytic cells such as fibroblasts and endothelial cells that can generate superoxide radicals in the myocardium [54]. Other than these physiological sources, superoxide can also be formed through exogenous influences, which include UV rays and industrial chemicals [77].

Superoxide radicals are formed rapidly within 10 s of reperfusion in the ischemic myocardium [59], and they are implicated in the development of subsequent cardiac pathology [54, 84]. Additionally, superoxide is known to be involved in development of other human diseases such as alzheimer's, parkinson's and amyotropic lateral sclerosis. Therefore, scavenging these radicals using superoxide dismutase – an endogenous superoxide scavenging enzyme – inhibits the propagation of oxidative cascade at an early stage thereby preventing the formation of many other toxic radicals and their associated pathologies [85].

2.4 Superoixde dismutases

SODs make up an important line of endogenous antioxidant defense systems against ROS, particularly superoxide. These metalloenzymes are widely distributed in prokaryotes and eukaryotes. Animal tissue contains at least three types of SODs: cytoplasmic Cu/Zn SOD (SOD1), mitochondrial Mn SOD (SOD2) and extracellular Cu/Zn SOD (SOD3) [86]. In aqueous solutions, superoxide reacts with itself spontaneously to give an oxidized and reduced form (dioxygen and hydrogen peroxide respectively). This self radical-quenching dismutation reaction of superoxide occurs at rates in orders of 10^5 liters $mole^{-1}$ s^{-1} at neutral pH conditions [75]. All isoforms of SODs catalyzes the dismutation of superoxide and increases this reaction rates to orders of 10^9 liters $mole^{-1}$ s^{-1} [75] thus aiding in the rapid quenching of these radicals.

SOD1

SOD1 is a copper and zinc containing enzyme and is a stable homodimer with a molecular mass of about 32 kDa. It is found in the cytoplasm, nuclear compartments and, lysosymes of mammalian cells [87]. Knockout studies suggest that although SOD1 knockouts were mild compared to lethal SOD2 knockouts, the absence of SOD1 produces subtle defects such as reduced reproductive potential [88]. Its presence is also very much essential for growth factor signaling. For example, it can act as a master regulator of extracellular signal-regulated kinase pathway by modulating the oxidation of protein tyrosine phosphatases [89]. mRNA level of SOD1 can be dramatically regulated by environmental conditions such as shear stress and UV radiation [87]. More importantly, the expression level and half life of its mRNA are reduced by physiological conditions such as hypoxia [90], which is relevant in diseased conditions such as myocardial ischemia. Ischemia and IR related pathologies in the myocardium increases the oxidative stress, reduces the activity of SOD [65] and its mRNA level [91], and SOD1 overexpression suppress these ischemia and reperfusion related injuries [15, 92]. It is

also known to create anti-apoptotic microenvironment within myocytes by modulating the activation of transcription factors such as NF κB [93]. Because of the positive effects of SOD1 in the heart, it remains an important therapeutic protein for the treatment of cardiac pathologies.

Other SOD isoforms:

SOD2 is a manganese-containing enzyme and is localized in the mitochondria [87]. The action of SOD2 is compartmentalized to the mitochondria, and diseases due to lack of SOD2 cannot be treated by other isoforms [94]. The importance of SOD2 in myocardium can be understood by the neonatal cardiomyopathy and mortality in SOD2 knockout mice [95] and, by the protection offered by SOD2 overexpressing mice against IR injuries [96]. SOD3 is a recently discovered copper/zinc containing enzyme. It has high affinity for heparin and is localized in the extracellular space and about one half of blood vessel SOD activity is due to SOD3. It has implications in cardiovascular diseases since it indirectly aids in smooth muscle relaxation by preventing the consumption of NO by superoxide radicals [18]. SOD3 administration is also known to reduce IR injuries [97].

SOD delivery

Gene-based SOD therapies are excellent analytical tools, but drug quantification is difficult, and prolonged overexpression of SOD could exacerbate cardiac dysfunction following IR [98]. Thus, delivery of SOD proteins becomes an important alternate treatment option to gene thearpy. In spite of the importance of SOD proteins, its therapeutic potential is not yet fully recognized due to its unfavorable pharmacokinetics. It has a rapid protein half-life: half life of circulating wild type bovine SOD in rat blood is about six minutes [99] and depending on the modifications made to the protein, its half life increases to about six hours [99-101]. Its bioavailability varies widely based on the route of administration; in rats, the bioavailability after oral administration is almost

nonexistent compared to subcutaneous administration [102], which is attributed to the poor absorption combined with its rapid degradation in the gastrointestinal tract. Many studies have modified the SODs to improve its half life and target it to specific tissue by coupling the SODs to various synthetic molecules such as polyethylene glycol and poly-styrene-co-maleic acid [103].

Thus, it can be understood that SODs have positive effects in the heart, and the role of SOD1 in particular is well established. Moreover, it can be inferred that delivery of SOD is an active area in therapeutic research and newer methods are required to improve the pharmacokinetic properties of this rapidly-cleared protein.

2.5 Controlled delivery of drugs

Widely practiced parenteral drug delivery methods to myocardium are minimally invasive. However, they require repeated drug administration and their effects are systemic. This problem could be resolved by employing a localized and targeted delivery platform to the myocardium such as by using drug eluting stents. However, drug eluting stents are cost effective only in limited circumstances [104]. Recent advances employ biocompatible polymers to achieve the same targeted and controlled release of drugs to the myocardium [105].

Polymeric carriers – both biodegradable and non-biodegradable – are frequently used in drug delivery. Non-degradable polymers are used as patches or inserts, where they can be recovered after the delivery of drug, or for oral ingestion when the polymer passes through the gastrointestinal system [106]. Biodegradable polymers – both natural and synthetic – have attracted much enthusiasm in controlled release technology because they require no intervention after implantation into the body. Some of the synthetic biodegradable polymers commonly used include polyesters, polyorthoesters, polyanhydrides, polyaminoacids and polyphosphazenes [107]. These systems are

designed such that they erode mechanically or degrade chemically or enzymatically to simpler compounds that can be eliminated through normal metabolic processes within the body or excreted [108]. Thus, it will be advantageous to use biodegradable polymers for treating cardiac diseases, since after the delivery of drug with a single injection; no further interventions will be required. In cardiovascular applications, both natual polymers such as alignate and, synthetic polymers such as poly lactic-glycolic acid (PLGA) polymers are used [105]. In this dissertation, polyketals – a recently identified biodegradable polymer – will be employed to deliver SOD1 to the myocardium. Because of the highly favorable property of polyketals undergoing acidic degradation to give neutral and biocompatible degradation products, they were chosen as a drug carrier in these studies and discussed in more detail, in 2.6 [109].

Controlled release technology uses active and passive modes of drug targeting through various delivery platforms including polymers and liposomes to achieve temporal- or distribution-based control. Temporal control systems deliver the drug over an extended duration or at specific time points and distribution control systems deliver the drug to specific sites in the body [106]. These systems are delivered as injectable hydrogels, or as sheets or patches of drug-containing polymer implanted to the site interest. Other drug delivery techniques based on strategies such as polymeric micelles, liposomes and dendrimers exist, and one of the widely used delivery strategies is to encapsulate the drugs within biodegradable polymers to create micro and nanoparticles.

Considerable interest exists in biodegradable microparticles, nanoparticles and microcapsules over the past few decades because of the ease of administration of a wide range of drugs in vivo through a syringe [107]. They are colloidal systems ranging in size from 10 nm to 1000 μm depending on the application involved [110]. Only few polymers are generally accepted for administration to human beings as microparticles. Polylactic acid, polyglycolic acid and their co-polymers are some of the best defined

polyester-based biomaterials for micro and nanoparticles-based delivery [108]. However, the hydrolysis and degradation products of microspheres based on these polymers are acidic, and they can lead to denaturation of pH sensitive proteins [111]. These acidic degradation products are also pro-inflammatory, which potentially limit their applicability in inflammatory diseases [19].

Retention of drug within extensive vasculature of heart is difficult. Additionally, drugs and other small molecule proteins are useful only when appropriate therapeutic regimen is followed which usually involves daily or multiple injections [111, 112]. As discussed above, microparticle based delivery has the ease of administration of a wide range of drugs in vivo through a syringe. Further, by suitable adjustment of the size of microparticles, they can be made to retain in the tissue for much longer time. Thus, considering different delivery platforms, microparticle-based delivery system appears more simple and apt for cardiac drug delivery applications.

2.6 Polyketal based delivery system

Cardiac dysfunction is an inflammatory disease, [113] and drug delivery with existing compounds such as PLGA microspheres may induce more inflammation due to acidic degradation products [114]. Thus, there is a need to explore other types of polymers with neutral degradation products. Polyketals (PK) are new biodegradable polymers designed for drug delivery. They hydrolyze into neutral compounds, which should therefore prevent the further recruitment of inflammation mediators including macrophages. Currently, polyketals such as poly-(1,4-phenylene acetone dimethylene ketal) (PPADK), poly (cyclohexane-1,4-diyl acetone dimethylene ketal) (PCADK) and PCADK copolymers (PK3) have been synthesized for drug delivery applications [109, 115, 116]. While the degradation product of PPADK has benzene dimethanol, a potentially toxic compound, the hydrolysis of PCADK generates only acetone and 1-4-

cyclohexanedimethanol, both of which have excellent biocompatibility. Hydrolysis of PKs is rate limited by the diffusion of hydronium ions/water into the polymer matrix. This can be inferred from the half life of 24 days at pH 4.5 for more hydrophobic PCADK and 2 days at the same pH for more hydrophilic PK3 [116]. Therefore, PK3 has been used for the treatment of acute inflammatory diseases such as acute liver failure. On the other hand, PCADK has increased half life and can release the drug of interest slowly over an extended period of time. Therefore, PCADK should be more suitable for chronic diseases including heart failure. Importantly, PCADK causes very little recruitment of CD45-positive cells, an inflammatory cell marker and other inflammation mediators such as TNFα, IL-6 and IL-12 compared to PLGA [19]. Therefore in this dissertation, cardiac dysfunction, which is an inflammatory disease modulated by free radicals, will be treated using SOD1 as drug and PCADK as the polymeric drug carrier.

2.7 Cardiac Regeneration and cell therapy

Heart failure affects 22 million people throughout the world, and heart transplant is currently the definitive therapy for heart failure. However, it is limited to about 4,000 recipients each year due to dearth in donor organ and other perioperative risks [3, 117]. Thus, cardiac repair and regeneration become an imperative alternative, and pharmaceutical-, gene- or cell therapy-based interventions are among the major treatment options being investigated [118-120]. Prevention of myocyte death is an important aspect in these therapies as their death results in loss of cardiac function and eventual heart failure [121]. Wound healing responses begin with acute inflammatory responses within the first day after cardiomyocyte death leading to increased neutrophil infiltration, cytokine activation and extra cellular collagen matrix degradation (Figure 2.2). The chronic phase of inflammation begins within two weeks of infarction, increasing the recruitment of macrophages and myofibroblasts [122]. Unfortunately, these reparative

responses only lead to fibrosis and scarring in human myocardium, unlike the healing and regenerative responses in the hearts of zebrafish [123]. Importantly, the endogenous regenerative mechanisms in human heart leading to effective healing are are inadequate. The rate of new myocyte formation is 1% per year at the age of 25, and by the age of 70, this rate decreases to 0.5% [9].

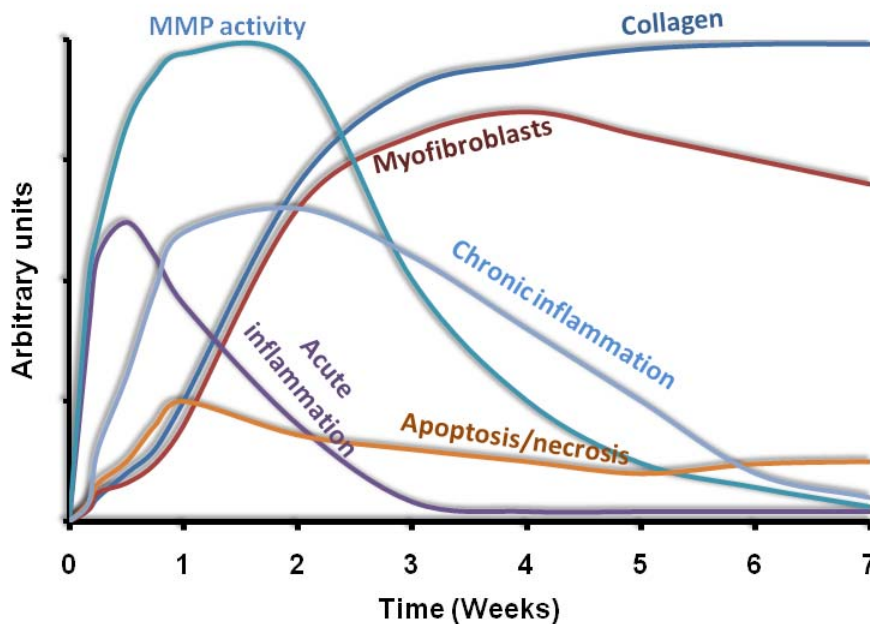

Figure 2.2. Wound healing responses in the myocardium post-infarction. Acute inflammation in the myocardium peaks within a day of infarction marked by recruitment of inflammatory cells such as neutrophils. Apoptosis and necrosis of myocytes begin within few hours after infarction. Chronic inflammatory phase begins within a day which increases the recruitment of macrophages. This phase prevails even five weeks post-infarction characterized by a large number of myofibroblasts and excessive collagen deposition. Extracellular matrix remodeling begins within a day of infarction marked by increased changes in the activities of matrix metallo proteinases (MMP) and tissue inhibitors of MMPs (not shown). Adapted from Jugdutt (2003) [122].

Recently, there is intense research in cardiac regeneration through cell therapy after the evidence that bone marrow cells can transdifferentiate into cardiomyocytes and vascular cells [124]. Cell based therapies have wide medical implications and its therapeutic applications range from its use as a drug delivery vehicle to its regenerative role in tissue engineering [125]. Cell types such as skeletal myoblasts, endothelial progenitor cells, mesenchymal stem cells (MSCs) and cardiac derived stem cells are few of the reported donor cells used for cardiac regeneration [10, 126-128]. For example, transplantation of MSCs is known to improve cardiac function following infarction [129]. Additionally, MSCs inhibit the functions of the dendritic cells [130] which suggest that their allogenic administration should be more favorable. EPCs are known to aid angiogenesis and in patients with myocardial infarction, the levels of circulating EPCs and its ability to migrate decreased [131]. Given that endothelial-cardiomyocyte interactions play a crucial role in cardiomyocyte development and repair [132], transplantation of EPCs may be useful in cardiac regeneration. Recently there is much enthusiasm on adult cardiac-derived stem cells (CSCs) in the field of cardiac regeneration since they have robust cardiovascular lineage differentiation ability. They have been identified by the expression of markers such as c-Kit, Sca-1 and MDR1 [10, 133]. The loss of functionally competent resident CSCs is suggested to be responsible for the onset of heart failure [134].

Irrespective of the type of cells used in transplantation, one of the largest hurdles in cell therapy is the limited survival of transplanted cells in the hostile ischemic myocardial environment [125]. Studies demonstrate that 70% to 90% of the transplanted cells die within the first few days [135]. Oxidative stress is implicated in the death of endogenous cardiomyocytes and implanted stem cells. For example, a recent study revealed that the average life span of cardiac stem cells was shortened by oxidative stress [136]. Additionally, oxidative stress is suggested to play a critical role in the

regulation of self-renewal and senescence of stem cells [137, 138]. Further, progenitor cells such as EPCs have higher expression of antioxidant enzymes, such as catalase and SOD2 to fight effectively against oxidative stress [139]. These suggest that maintenance of low oxidative stress levels is a stringent requirement in stem cells [138, 140].

2.8 Summary of background studies

In summary, it has become overwhelmingly clear that new treatment methods to repair and regenerate the heart after myocardial infarction are needed. Evolving treatment options include delivering bioactive materials and cells to the myocardium. Given the importance of oxidative stress in development of heart failure, targeting the excessive free radical levels, superoxide in particular, should correct much cardiac pathologies. SODs provide some benefit to myocardium; however, delivery of this protein with short circulating half life remains difficult. Therefore, identifying new delivery vehicles to SOD is essential. Cell based therapies are promising treatment alternatives to regenerate the myocardium. However, oxidative stress levels both inside and outside the cells affect the survival and efficacies of cell therapies. Hence, identifying the oxidative stress response of the transplanted cells could potentially benefit the cell based therapeutics.

CHAPTER 3

INTRACELLULAR SOD DELIVERY WITH POLYKETAL MICROPARTICLES

In this chapter, in vitro studies conducted to test the efficacy of SOD1 encapsulated PCADK microparticles (PKSOD) to scavenge superoxide will be discussed. Micron-size PKSOD was synthesized using a double-emulsion method, and based on the results from extra- and, intra- cellular quantification of superoxide levels, the ability of PKSOD to scavenge the superoxide radicals was determined. This chapter will demonstrate the potential of polyketal particles in intracellular delivery of drugs.

3.1 Introduction

Proteins are attractive as therapeutic agents due to their beneficial effects. However, they are difficult to deliver to the inside of cells since they are larger and their molecular weight ranges in thousands of Daltons. Cell membranes do not allow spontaneous entry of large molecules unless there is an active transport mechanism. In addition, due to the hydrophilic nature of many proteins, they are less permeable to hydrophobic cell membranes. Additionally, enzymatic proteins are less stable and lose their activity when their complicated three dimensional structures are altered. Therefore, delivery of proteins and other bigger hydrophilic compounds to the cells remain a challenge [111, 141].

SOD1 protein is an efficient, natural endogenous superoxide scavenger. However, similar to many other proteins, intracellular delivery of this enzyme remains difficult. Superoxide generation occurs both inside and outside cells. A simple intravenous administration of free SOD1 protein could potentially help in reducing the

extracellular superoxide levels. However, intracellular superoxide buildup cannot be adequately addressed by a simple parenteral SOD1 administration. In addition, sustained action of SOD1 cannot be seen due to its poor bioavailability and rapid serum half life [101]. Few studies show that SOD1 can directly traverse the membranes in cell types such as endothelial cells and hepatocytes through receptor mediated endocytosis [142]. However, less than 5% of internalized proteins are released to the cytoplasm following such internalization mechanisms [143]. Therefore, identifying better carriers for an effective and sustained delivery of SOD is essential to address both extra- and intra-cellular superoxide buildup.

Intracellular delivery of proteins can be achieved either by invasive or non-invasive delivery systems. Invasive methods such as microinjection [144], osmotic permeabilization [145] and electroporation [146] damage the cell membranes. Therefore, significant research is being conducted using non-invasive methods using carriers such as liposomes, micelles, cell penetrating peptides and, polymers to achieve intracellular delivery [141]. Polymer based carriers offer the advantage of freedom in the ease and range of chemistries that can be performed on them to custom fit the specific need. Biodegradable polymers have been studied for more than 30 years, and protein delivery using biodegradable microspheres is one of the widely used delivery systems. Microencapsulation is the process of enclosing micron sized solid or liquid in an inert shell that offers benefits such as controlled and targeted release of the encapsulated protein and, protection to the protein from the external environment [147]. Due to their efficacy and ease of manufacturing, many commercial formulations such as Zoladax® of Imperial Chemical Industry (now AstraZeneca) and Posilac® of Monsanto exist as drug encapsulated microspheres. Various polymers are used in this microencapsulation platform. For example, poly-d, l -lactide-co-glycolide (PLGA) is one of the most widely used polyester based polymers. However, their degradation products – lactic and

glycolic acid – are acidic, and therefore, may not be suitable to deliver pH sensitive bioactive compounds [148]. Polyanhydrides are other widely used polymeric carriers. They have water sensitive linkages and are useful in a short term rather than a sustained drug release. They also form acidic degradation products that may contribute to the inflammatory response. Other than these widely used polymers, various other carriers such as poly-amino acids, polyphosphazenes, and polyphosphoesters exist with their own pros and cons [106].

Recently acetal and ketal based polymers are used in drug delivery systems and unlike the acidic degradation products of other widely used carriers, acetals and ketals undergo acid hydrolysis to form neutral degradation products [149]. This property has been used for delivery of drugs [150] and to develop pH responsive delivery vehicles [151]. Additionally, polyacetals have previously been used for intracellular delivery of proteins such as ovalbumin to dendritic cells using microencapsulation platform [152]. Similarly, polyketals have been used as micro and nanoparticles for intracellular delivery of drugs [109]. Among the various reported polyketals, PCADK (poly (cyclohexane-1,4-diyl acetone dimethylene ketal) has slower hydrolysis kinetics which is advantageous for sustained delivery of drugs in chronic diseases. Additionally, it is known to have minimal inflammatory response compared to PLGA [19]. Therefore, identifying the ability of PCADK as an intracellular drug delivery vehicle is essential.

There are various techniques employing chemical, physicochemical or physico-mechanical processes to microencapsulate the drugs. Chemical techniques such as interfacial polymerization and in situ polymerization encapsulate the compounds simultaneously during the polymerization of monomer [153, 154]. Therefore, these methods are not convenient to encapsulate a protein on polymers synthesized using multiple synthetic steps. Physicochemical methods such as coacervation techniques could potentially produce effective microencapsulated drugs [155]. However, this

technique has the tendency to produce agglomerated particles. Importantly, since the amount of solvent used in this method is higher, there are issues with excessive residual solvent retention in the final microspheres [155, 156]. Spray drying is a physico-mechanical process that involves passing the microparticle through a heated drying chamber, and therefore, this technique is better for encapsulating non heat sensitive materials such as fragrances and flavors [147]. Solvent evaporation is another widely used physico-mechanical process to encapsulate both hydrophilic and hydrophobic drugs by employing emulsification techniques. While a single emulsification step is sufficient to encapsulate a hydrophobic drug, it is necessary to use double emulsions or solid-in-oil methods to encapsulate hydrophilic drugs [157]. Importantly, the ability of PCADK to microencapsulate SOD1 using a water in oil in water double emulsion process is known [115]. This double emulsion-solvent evaporation process briefly involves dispersing the aqueous phase of hydrophilic drug in an oil phase of solvent to create the first emulsion of water in oil. This emulsion is subsequently dispersed again in an external aqueous phase to create water in oil in water emulsion (Figure 3.1). The volatile solvent used in the process is evaporated in the water bath and finally, the drug encapsulated microparticles are obtained after the downstream washing and drying steps.

Cytoplasmic delivery of bioactive agents to macrophages remain difficult [158]. Further, they will be a relevant model system to investigate the efficacy of PKSOD since these cells play a vital role in all inflammatory diseases including cardiac diseases [159]. Therefore, in this chapter, the ability of PKSOD to scavenge the superoxide both inside and outside the macrophages is investigated. Initially, it will be shown that micron scale PKSOD particles can be created by double emulsion-solvent evaporation method. Subsequently it will be demonstrated that PKSOD can efficiently scavenge both intracellular and extracellular superoxide in a stimulated macrophage cell line. During

incubation of these micron-sized particles with macrophages, larger particles remain outside the cells while the smaller particles are internalized via phagocytosis.

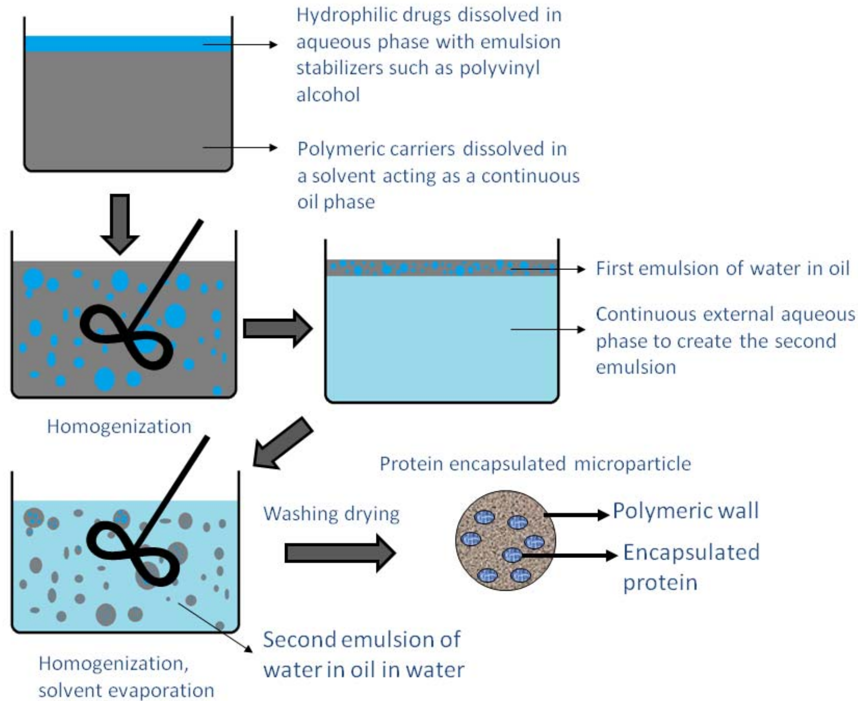

Figure 3.1. **Encapsulation of hydrophilic drugs using double emulsion – solvent evaporation technique.** Initially, the hydrophilic drug is dissolved in an aqueous phase containing emulsion-stabilizers such as polyvinyl alcohol (PVA). This water phase is emulsified in the polymer containing solvent acting as the oil phase using homogenization to create the first water in oil emulsion. This is further dispersed in an external aqueous phase containing PVA using homogenization. By altering the turbulence created in this step, the microparticle size can be easily adjusted. Finally, the volatile solvent is evaporated and the particles are thoroughly washed and lyophilized to yield the drug-encapsulated microparticles.

Distribution of the particles both inside and outside the cells should therefore reduce both intracellular and extracellular superoxide levels. However, the hydrophilic free SOD1 should remain outside the cells without encapsulation. This will be demonstrated by quantifying superoxide using dihydroethidium (DHE) and a high performance liquid

chromatography (HPLC) based assay. DHE is cell permeable. However, 2-hydroxyethidium (HE), a superoxide specific oxidation product of DHE, is cell impermeable. DHE oxidized to HE by extracellular superoxide will remain in treatment media that can be analyzed and quantified by HPLC. However, DHE that has crossed the cell membrane will be trapped as HE inside the cells after being oxidized by intracellular superoxide and it can be quantified separately.

3.2 Experimental Methods

Synthesis of PCADK

Poly(cyclohexane-1,4-diyl acetone dimethylene ketal) (PCADK) was synthesized as described previously [116]. Briefly, before beginning the reaction, the solvents including benzene and 2,2-dimethoxypropane were distilled in inert nitrogen atmosphere to remove traces of water. Additionally, p-toluenesulfonic acid was recrystallized prior to use. The reaction setup was begun by dissolving 1,4-cyclohexane dimethanol in benzene and heating it upto 100 ºC with constant stirring. A solution of p-toluenesulfonic acid in ethyl acetate was added to catalyze the acetal exchange reaction between 1,4-cyclohexane dimethanol and 2,2-dimethoxypropane. The ethyl acetate was allowed to boil off and subsequently 2,2-dimethoxypropane was added, in equimolar ratio to 1,4-cyclohexane dimethanol in benzene solution, to begin the polymerization reaction. Additional doses of 2,2-dimethoxypropane and benzene were subsequently added dropwise to compensate for the 2,2-dimethoxypropane and benzene that had been distilled off. The reaction was stopped after 48 hours by the addition of 500 μL of triethylamine. When lower molecular weight PCADK was made, the reaction was stopped in six hours. The polymer was precipitated in cold hexane (stored at -20°C) and separated out by vacuum filtration. The molecular weight was determined by Shimadzu gel permeation chromatography equipped with UV detector. Tetra hydro furan was used

as the mobile phase at a flow rate of 1 ml/min. The molecular weight of the resulting polymer was approximately 6 kDa with a mean polydispersity of 1.923. All reagents were purchased from Sigma-Aldrich.

PKSOD and empty PCADK (PK) particle preparation

PKSOD particles were made by a double emulsion-solvent evaporation process as described by Lee *et al.* [116] with slight modifications. Briefly, an aqueous solution of 15 mg SOD1 from bovine erythrocytes (Sigma-Aldrich) (75 KU) dissolved in 80 μl of 1% (w/v) polyvinyl alcohol (PVA), pH 8, was dispersed in 300 mg PCADK dissolved in 1 ml methylene chloride. Homogenization at (Power Gen 1000™, Fisher Scientific) 30,000 rpm for 30 seconds resulted in a first emulsion of water in oil (w/o). The w/o emulsion was redispersed at 7500 rpm for 1 minute in an external aqueous bath containing 5 ml of 4% (w/v) PVA. For creating sub-micron particles, following mechanical homogenization, the second emulsion was sonicated on ice with 30 pulses each of 0.5 s duration. The water in oil in water emulsion (w/o/w) was then poured into a bath containing 50 ml of 1 % (w/v) PVA and stirred for 5 hr at room temperature to allow the methylene chloride to evaporate. The solidified microparticles were washed four times with water by centrifugation (8,000 rpm, 10 minutes), and the slurry was finally freeze dried to yield white solid powder. PK particles were made in a similar manner without the addition of SOD1 protein. Solid in oil in water emulsion was made as described by Castellanos *et al* [160]. Briefly, SOD1 was co-lyophilized with polyethylene glycol (8 kDa) at a ratio of 1:4, and the lyophilized solids were emulsified in methylene chloride, to get the first emulsion of solid in oil. Further processing of this emulsion to create the solid in oil in water particles is the same as described for w/o/w.

Encapsulation efficiency

500 μl of methylene chloride and 500 μl of PBS was added to 1 mg of PKSOD or PK particles. After vortexing, the polymer dissolves and distributes predominantly in oil phase. The encapsulated SOD protein was extracted thrice to fresh aqueous phase by gentle mixing for one hour at room temperature. Protein content in the aqueous phase was determined by a micro BCA protein analysis kit. Encapsulation efficiency was expressed as the ratio of actual and theoretical SOD loading. Cumulative protein release was studied by suspending 2 mg of polymer in various tubes containing 1 ml of either acetate buffer (pH 4.5) or phosphate buffered saline (pH 7.5) at 37 ºC. The protein released to the buffer from the hydrolyzed polymer at various time points was quantified using micro BCA protein assay, and the percentage was calculated based on the total encapsulated protein levels.

Macrophage culture

RAW264.7 macrophages were maintained in DMEM (Fisher) supplemented with 10% fetal bovine serum (Hyclone), l-glutamine, and penicillin/streptomycin (Invitrogen). Kreb's Hepes buffer (KHB) containing (g/l): NaCl, 5.79; KCl, 0.35; $NaHCO_3$, 2.1; KH_2PO_4, 0.14; D(+) Glucose, 1.01; Na-HEPES, 5.21; $CaCl_2.2H_2O$, 0.37; $MgSO_4$. $7H_2O$, 0.30, was used as treatment media. For experiments involving PMA stimulation, cells were seeded on a 12 well plate (2 million cells/well) and, quiesced overnight in serum free DMEM. The media was then aspirated and replaced with treatment media containing SOD1 (50 U), PKSOD or PK, and incubated for 5 hr at 37 ºC to allow the particles to be engulfed by macrophages. Cells were then washed with ice cold KHB buffer followed by the addition of treatment media with or without 10 μM PMA for 20 min at 37 ºC in order to stimulate superoxide production.

Intracellular and extracellular superoxide measurement.

Superoxide was detected by separating the oxidation products of DHE using HPLC [161]. In the cell culture experiments, macrophages were stimulated with 10 μM PMA along with 20 μM DHE and were incubated at 37 ºC for 20 minutes protected from light. For extracellular superoxide measurement, 100 μl of reaction buffer was suspended in 300 μl methanol. For intracellular superoxide measurement, the cells were mechanically homogenized and suspended in 300 μl methanol. A small aliquot (50 µL) was saved for protein estimation, and the samples were injected into the HPLC system (Beckman coulter with system gold 125 solvent module) with C-18 reverse phase column and, equipped with Jasco FP-2020 plus fluorescent detector and Beckman coulter System gold 166 UV detector. Solvent A (water) and B (60% acetonitrile/0.1% trifluoracetic acid) were used as mobile phase with a linear gradient in pump to increase the acetonitrile concentration from 37 to 47% over 23 min at a flow rate of 0.5 ml/min. HE and ethidium were monitored by fluorescence detection with excitation 480 nm and emission 595 nm and in order to quantitate the analytes, the areas of the corresponding peaks were measured using the software provided with the HPLC system. The results were normalized to the protein concentration in the cell lysates, and fold of increase in HE with respect to control levels were calculated.

3.3 Results

Micron-scale, PKSOD particles can be created.

A double emulsion method was used to encapsulate SOD1 within the PCADK particles (PKSOD). Particle analysis with ImageJ software revealed that the size ranged from about 2 μm to 30 μm with a mean particle size of 11.4 ± 4.9 μm (Figure 3.2 A bottom

panel). Representative SEM images are shown in Figure 3.2 B. Empty particles (PK) were similar in size and morphology to PKSOD particles (10.8 ± 5.9 μm, Figure 3.2 A top panel). Micro-BCA protein analysis of the encapsulated protein revealed an encapsulation efficiency of 50% (expressed as the ratio of actual SOD1 loading to the theoretical maximum), corresponding to almost 100 U of SOD1 per mg of polymer (Figure 3.2 C). Cumulative release profile of the encapsulated protein, varied with the pH. Irrespective of the pH conditions about 60% of the protein was released within

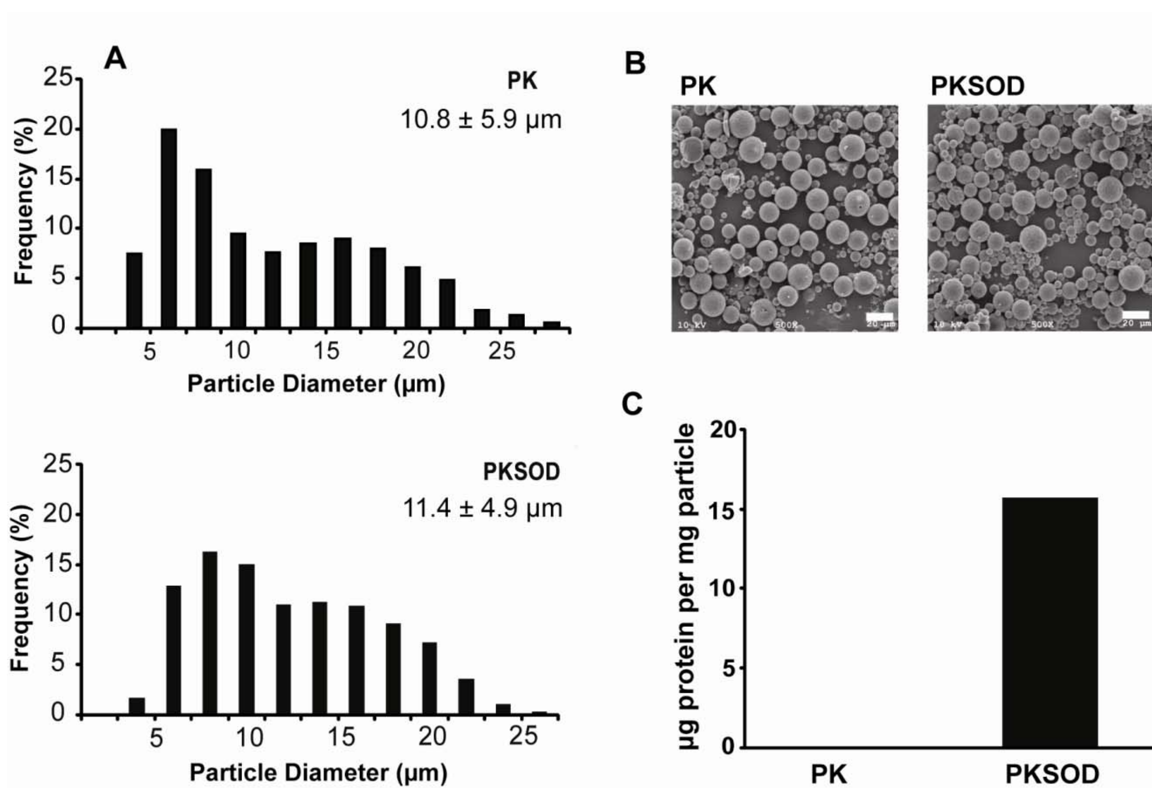

Figure 3.2. SOD can be encapsulated within PCADK to create micron sized particles. (A) Histogram of the microparticles analyzed with ImageJ software (B) SEM image (500X) of PKSOD made by a double emulsion technique that yields large microparticles. (Scale bar: 20 μm) (C) 50% protein encapsulation efficiency is obtained as measured by micro-BCA protein analysis

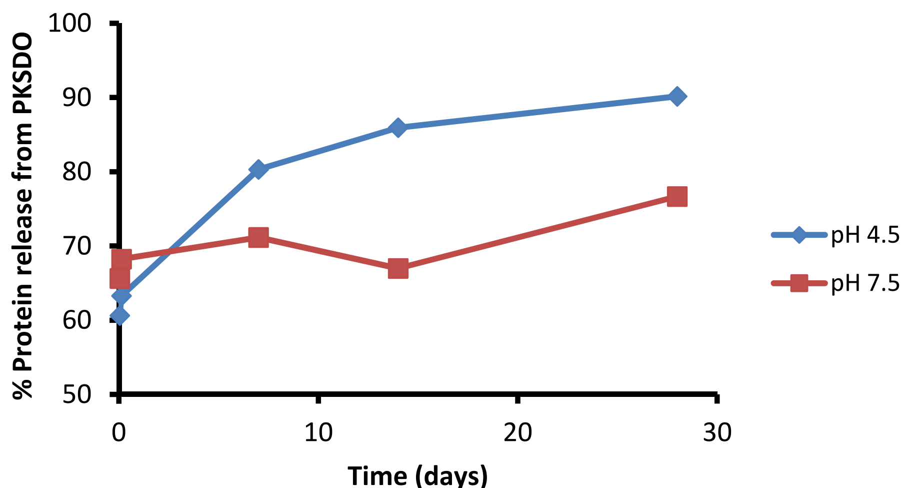

Figure 3.2 (Continued). SOD can be encapsulated within PCADK to create micron sized particles. (D) Cumulative SOD1 release profile from PKSOD at acidic and neutral pH conditions

six hours suggesting their surface presence and their easy dissolution into the surrounding medium (Figure 3.2 D). However, the remaining proteins were released with a release half- life of around 5 days at pH 4.5 and more than two weeks at pH 7.5. This suggests that the protein release from the in the inner core of the polymer occurs only when the PCADK undergoes acid hydrolysis

Encapsulation efficiency is affected by various factors

In order to compare the water in oil in water (w/o/w) double emulsion technique with solid in oil in water (s/o/w) technique, PCADK with a molecular weight of approximately 4 kDa was used with polymer to drug ratio of 10:1. s/o/w technique yielded only 5 % encapsulation of SOD1 and this percentage was increased by two fold by employing the w/o/w technique (Figure 3.3 A). In both techniques, more than 90 % of

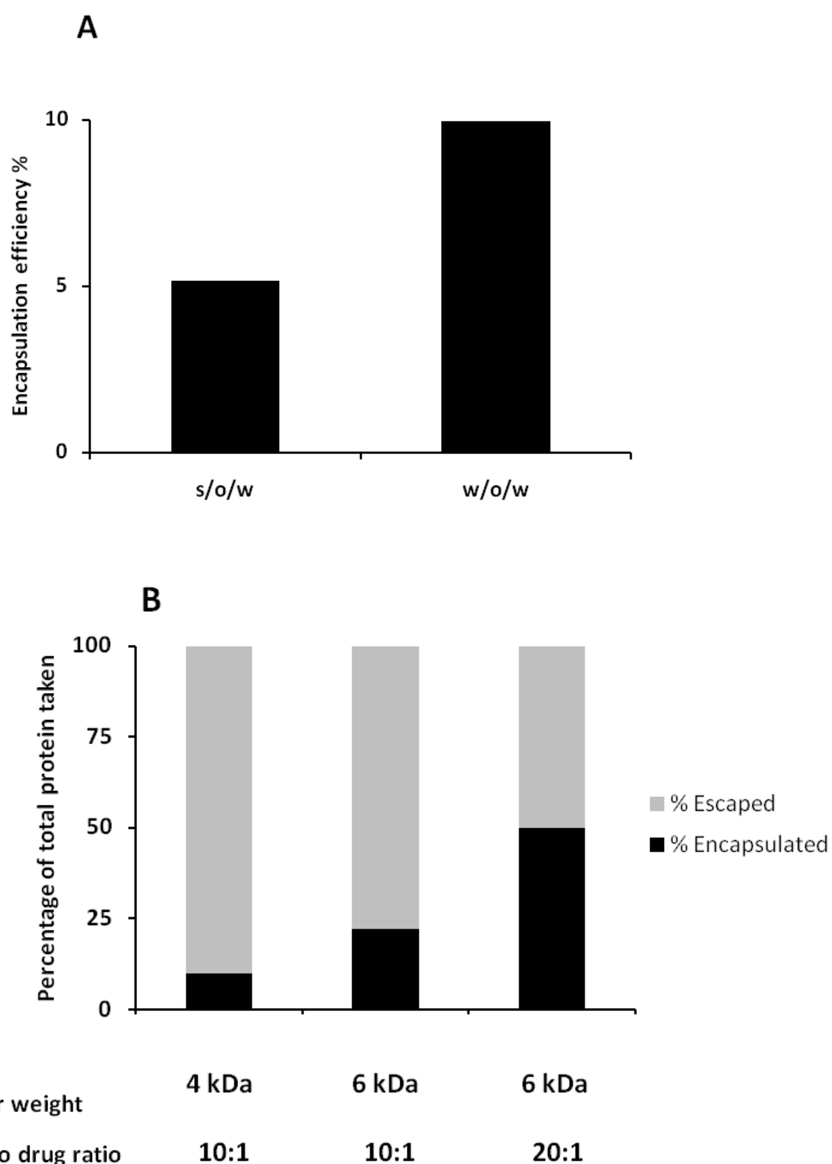

Figure 3.3. **PKSOD microparticle encapsulation is affected by various factors.** (A) w/o/w technique is better than s/o/w technique in encapsulating SOD1. (B) Increasing the molecular weight of PCADK and doubling the polymer to drug ratio (w/w) improves the encapsulation efficiency in PKSOD.

the protein was leaked into the aqueous solvent-evaporation bath. In order to address this issue, a higher molecular weight PCADK (6 kDa) was used in the w/o/w technique. This modification helped in decreasing the protein leak during solvent evaporation and

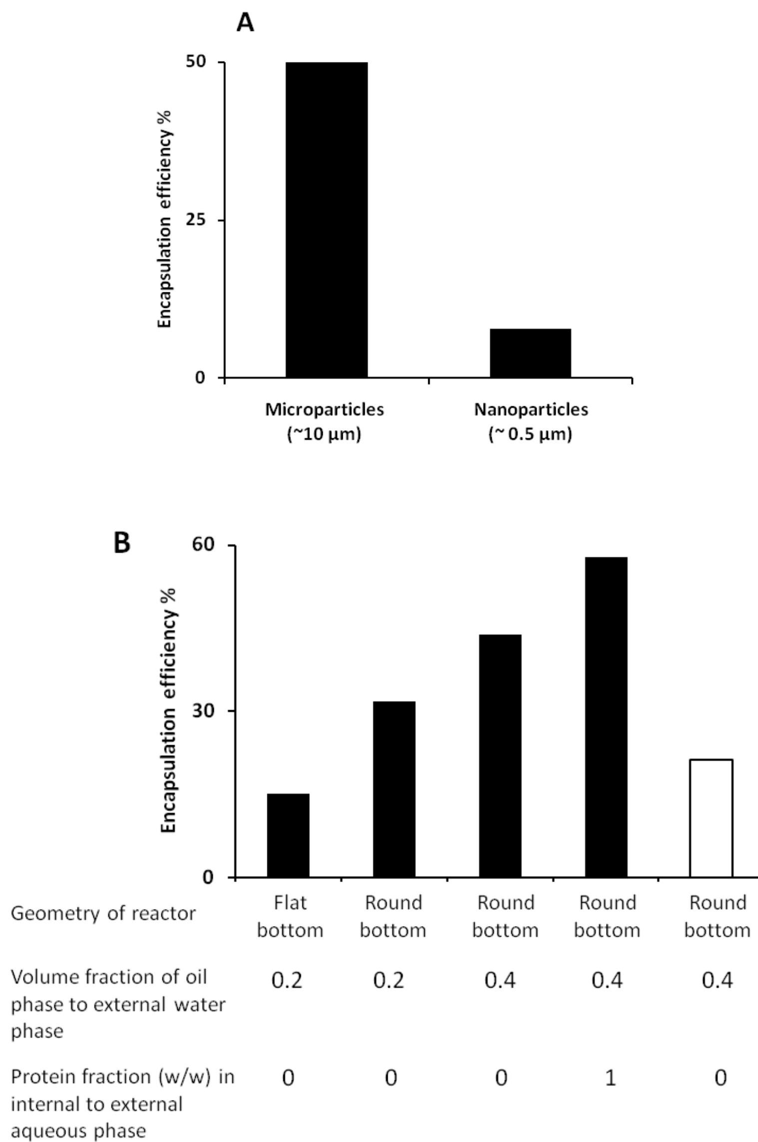

Figure 3.4. Encapsulation efficiency reduces when creating PCADK nanoparticles. (A) High molecular weight polymer at 20:1 polymer to protein ratio has a drastic reduction in encapsulation efficiency when the particle sizes are reduced to submicron levels. (B) Bovine serum albumin encapsulation in PCADK nanoparticles (~500 nm) increases by altering reactor geometry and volume fraction of oil to external water phase and by reducing the outer and inner protein concentration difference. Sub-micron PKSOD encapsulation (white box) was increased to 20% by implementing these changes.

aided in increasing the encapsulation efficiency to 20 % (Figure 3.3 B). This was further increased to 50 % by increasing the oil phase polymer concentration through higher (20:1) polymer to drug ratio (Figure 3.3 B).

When the particle size was reduced using sonication to submicron range (averaging 500 nm), the encapsulation efficiency dropped to 8 % (Figure 3.4 A). In our subsequent investigations on the factors that could improve the encapsulation efficiencies, bovine serum albumin (BSA) was used as a model protein as they were more economical. The encapsulation efficiency was doubled by changing the geometry of the reactor from flat bottomed to round bottomed tube (Figure 3.4 B). Additionally, the protein encapsulation was increased to 43% by increasing the ratio of oil phase to external aqueous phase by a factor of 2 (Figure 3.4 B). Further, addition of BSA to external aqueous phase decreases the protein concentration difference between the internal and external aqueous phase. This reduces the protein leakage into the solvent evaporation bath leading to 58% encapsulation of BSA (Figure 3.4 B). However, this last improvement may not be an economical option for one time encapsulation of costly proteins. Implementing these changes in the parameters while creating PKSOD nanoparticles (white bar, Figure 3.4 B) resulted in a 20% encapsulation efficiency compared to 7 % efficiency before the modifications (Figure 3.3 A).

PKSOD scavenges both intracellular and extracellular superoxide.

The ability of PKSOD particles to scavenge superoxide radicals was investigated in macrophage cell culture (RAW 264.7) using quantitative DHE-HPLC. PMA stimulation resulted in a 1.6-fold increase in extracellular superoxide production as compared to control cells ($p<0.01$). This increase was significantly inhibited by application of exogenous SOD1 (50 U/mL) ($p<0.001$; Figure 3.5 A). Cells pretreated with PK particles also showed a significant PMA-induced increase in superoxide levels ($p<0.01$ vs. vehicle treated control), whereas cells treated with PKSOD for 5 hours prior to PMA-stimulation

had no significant increase in superoxide levels compared to control cells. This inhibition was evident at both 0.25 mg and 0.125 mg of polymer per million cells (Figure 3.5 A).

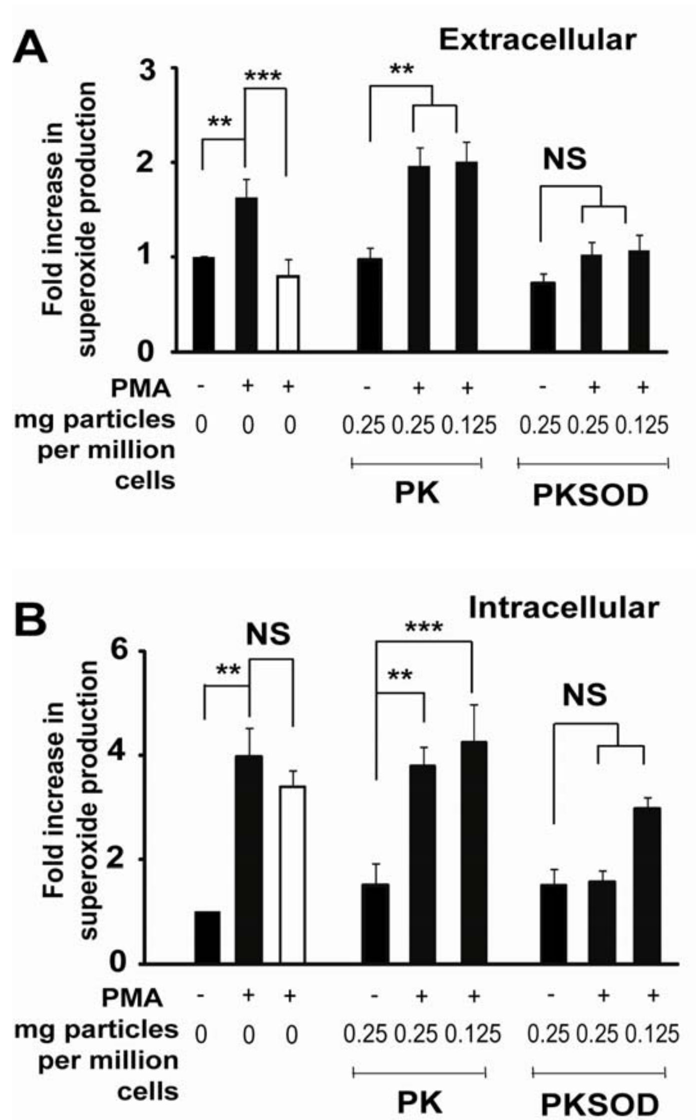

Figure 3.5 PKSOD dose-dependently decrease both intracellular and extracellular superoxide release in stimulated macrophage cell line. (A) Extracellular superoxide concentration from media per million cells represented after normalizing with respect to control. (B) Intracellular superoxide per mg protein represented after normalizing with respect to control. Only PKSOD reduced the PMA induced intracellular superoxide levels. Results are average of four experiments. **p<0.01, ***p<0.001 vs. PMA. ANOVA followed by Tukey's post-test.

Analysis of intracellular superoxide levels revealed that PMA stimulation significantly increased superoxide levels 3.9-fold as compared to control cells ($p<0.01$). In contrast to the results observed in extracellular superoxide measurement, treatment with exogenous SOD1 was not able to reduce PMA-induced intracellular superoxide production (Figure 3.5 B, $p<0.001$ vs control). Similarly, PK pretreatment also had no significant effect on intracellular superoxide levels ($p<0.01$ vs. vehicle treated control; Figure 3.5 B). Interestingly, PKSOD pretreatment was able to dose-dependently reduce the PMA-induced intracellular superoxide levels (Figure 3.5 B), suggesting efficient delivery of active SOD1 to the intracellular space.

3.4 Discussion

In this chapter, PCADK – a polyketal based biodegradable polymer – was used as a carrier to deliver SOD1 to scavenge both intra- and extra- cellular superoxide. Unlike polyester based biomaterials, these polymers do not have acidic degradation products, and they cause no significant inflammatory response [19]. Therefore, SOD1 encapsulated within these polymers were tested for its efficacy to scavenge superoxide radicals. Hydrophilic compounds can be microencapsulated using the widely used w/o/w solvent evaporation method. Although, few reports suggest better encapsulation of BSA in PLGA carriers using s/o/w technique [162], our results suggest that w/o/w technique is better than s/o/w in encapsulating SOD1 to PCADK. Our particle size analysis revealed that PKSOD microparticles in 2 to 30 μm size range can be made using a double emulsion method by employing low speeds during the homogenization step. This size distribution would be advantageous and could potentially be retained by a single injection in vascular tissues such as the myocardium, while still having particles small enough to be taken up by macrophages. In addition, our protein release data demonstrate that PCADK releases the drug slowly over a period of few weeks following

the initial rapid release of proteins from the surface. This property of slow protein release from PCADK in acidic conditions is previously reported in literature compared to other polyketals such as PK3 [115]. This property could potentially be used for sustained release of drugs in chronic diseases.

Many studies have shown that increasing the molecular weight of the polymer and polymer concentration increase the encapsulation efficiency of the protein [163, 164]. Therefore, the effect of molecular weight on PKSOD encapsulation efficiency was investigated. Similar to the literature reports, our results also demonstrate the PKSOD encapsulation increase with molecular weight and polymer concentration. PCADK molecular weight was not increased beyond 6 kDa in our studies; since beyond that range, the solubility of the polymer in methylene chloride decreases drastically. Macrophages can phagocytose particles larger than 20 μm [165]. However, fibroblasts and other cell types can phagocytose only sub-micron to less than six micron size particles [166]. Therefore, to achieve intracellular delivery of SOD1 to other cell types, it may be necessary to make nanoparticles of PKSOD. However, our results suggest that reducing the particle size reduces the amount of protein encapsulated. Turbulence is one of the important factors affecting the encapsulation. Geometry of the reactors [167] and, the volumes of oil phase and external aqueous phase, [157, 168] have profound effects on the turbulence. In addition, there is a huge difference in protein concentration from internal to external aqueous phase, and higher encapsulation efficiency could be obtained by reducing this concentration difference. Therefore, the effect of these factors on encapsulation efficiency was investigated. Our results show that, by modifying these parameters, about 25% of SOD1, is encapsulated within nano sized PCADK polymers. This has the potential for efficient intracellular delivery of SOD1 to other cell types.

Superoxide generation occur both inside and outside the cells, and because of its enhanced reactivity and short life time, it is difficult to assay these radicals in

physiological systems. Electron spin resonance is a widely used standard method for superoxide quantification. However, because of the complexity and cost of the technique, other agents such as cytochrome C, lucigenin and luminol are often employed for superoxide detection [169]. These compounds are not superoxide specific as they have side reactions with many other free radicals. However, the dye dihydroethidium (DHE) is reported to form a superoxide specific oxidation product - hydroxyethidium - along with other oxidation products [170]. Therefore, HPLC based separation of hydroxyethidium was performed in these studies for specific quantification of superoxide [161]. As expected, results for extracellular superoxide measurements demonstrate the ability of free SOD1 to scavenge the superoxide radicals. In addition, as large proteins do not cross the cell membrane, it is not surprising when our result shows that free SOD1 did not scavenge the intracellular superoxide. In stark contrast, our PKSOD particles were able to significantly reduce the superoxide levels intra- and extracellulary, suggesting the particles were taken up by macrophages and the contents released intact within. This finding suggests potential advantages of PKSOD to reduce the intracellular superoxide buildup. A simple parenteral administration of SOD1 is not adequate to scavenge the superoxide within the cells, and it requires modifications to increase cell permeability, such as coupling it to polyethylene glycol (PEG). Not only does this entail more synthetic steps, but PEG itself may induce an oxidative response [171]. Scavenging the superoxide within macrophages reduces the production of inflammatory cytokines such as tumor necrosis factor alpha [172], which has the potential to provide benefits to native cardiomyocytes [173].

CHAPTER 4

PKSOD DELIVERY TO THE MYOCARDIUM AND PROTECTION FROM MYOCARDIAL ISCHEMIA REPERFUSION INJURY

In this chapter, in vivo studies conducted to evaluate the ability of PKSOD to improve cardiac function are presented. Micron-size PKSOD were injected in the border of the cyanotic tissue of rat myocardium following IR, and based on the histochemical analysis and, functional evaluation of the heart, the effects of PKSOD on cardiac function were determined. This chapter will show the effects of sustained SOD1 therapy to the myocardium.

4.1 Introduction

Ischemic heart diseases, leading to myocardial infarction and heart failure, are a leading cause of global morbidity and mortality [3]. Loss of myocytes through necrosis and programmed cell death (apoptosis) following insults such as ischemia/reperfusion (IR) is mainly regional [174]; suggesting the potential benefit of a localized therapy in preventing the development of cardiac dysfunction. However, because the disease is progressive in nature, localized therapy must also be sustained to deliver a consistent amount of drug over relevant times. Oxidative stress has been proposed as the unifying mechanism behind various risk factors of heart diseases [13], and it is implicated in many pathological disease states of the heart including hypertrophy [57], IR injury [58] and, myocardial stunning [61]. Thus, a therapy that could address the high oxidative radicals over a sustained period of time will have great potential in prevention of cardiac dysfunction.

Direct measurements and indirect inferences have implicated the role of excess superoxide levels in the pathogenesis of infarct development, reperfusion injury and eventual myocardial dysfunction [71, 74]. Additionally, mRNA expression levels of superoxide dismutase (SOD), an endogenous superoxide scavenger, decrease significantly after myocardial infarction thereby potentially exacerbating superoxide levels [91]. Antioxidant therapy with SOD including transgenic overexpression and gene therapy studies improve cardiac function following infarction, but the clinical relevance of these studies is still unclear as questions still remain regarding the safety and efficacy of gene therapy [14, 15].

Despite the large role of oxidative stress in cardiac dysfunction, there is a strong lack of consistency in the efficacy of Cu/Zn SOD (SOD1) protein therapy and many large animal trials have failed to show a significant benefit [175, 176]. Possible factors contributing to these discrepancies are the unfavorable pharmacokinetics and the rapid protein half-life of SOD1. Half life of circulating wild type bovine SOD1 in rat blood is about six minutes [99] and depending on the modifications made to the protein, its half life can increase to about six hours [99, 101, 102]. Thus, there is significant interest in developing better SOD delivery systems, and many modifications have been made to the SOD protein to improve its pharmacokinetics and delivery including the addition of cell penetrating molecules and targeting sequences [103]. Although, these modified proteins have better efficacies than the native SOD protein, intracellular delivery of the drug remains a challenge. In addition, high doses of these modified SODs are used in most studies [103] which by itself can exacerbate the cardiac pathologies [98]. Thus, a single time administration of SOD1 that could sustain in the heart during disease progression, by using suitable drug carriers, could offer an advantage over existing treatment methods.

Various natural and synthetic polymeric drug carriers including collagen, fibrin, matrigel, PLGA, PGA and peptide nanofibres exist for use in cardiovascular applications [177]. Polyketal polymers are promising new candidates to treat inflammatory diseases since they form neutral degradation products upon acid hydrolysis. They are reported to be better a better drug delivery vehicle than the widely used PLGA polymers in the treatment of both acute and chronic inflammatory diseases [19, 115, 178]. As described in Chapter 2, various polyketals such as PPADK, PCADK and PK3 exist which differ in their degradation products and degradation rate. Among them, PCADK (poly (cyclohexane-1,4-diyl acetone dimethylene ketal) has half life of about three weeks at pH 4.5, and it degrades to 1-4-cyclohexanedimethanol, which is an FDA approved compound, and acetone, which is on FDA GRAS list. Due to these neutral and safe degradation products, PCADK is known to have minimal inflammatory response in the myocardium compared to PLGA [19]. Therefore, it will be advantageous to employ PCADK as a carrier of SOD1 to treat inflammatory heart diseases.

The aim of this chapter is to investigate the effects of a direct myocardial injection of SOD1 encapsulated within PCADK microparticles on oxidative stress levels in the myocardium and on cardiac functional recovery following IR injury. The study was performed in a rat model of myocardial IR by injecting PKSOD or PK particles in the myocardium immediately after IR. After the treatment time (3 or 21 days), the hearts were analyzed for oxidative stress levels and myocyte apoptosis. Functional improvement of myocardium was determined from echocardiography. Additionally, the effect of PKSOD on infarct size, collagen deposition, matrix metalloproteinase levels and mRNA levels of calcium handling proteins were analyzed. Based on these results, the effects of PKSOD delivery on cardiac function were determined.

4.2 Materials and methods

PKSOD and fluorescent PKSOD (PKFSOD) particle preparation

The preparation of PKSOD is described in section 3.2. PKFSOD was made by encapsulating fluorescein isothiocyanate (FITC) conjugated SOD (FSOD) within PCADK. FSOD was made by stirring SOD1 (5 mg/ ml pH 9.0 carbonate buffer) with FITC (1 mg/ml DMSO) overnight at 4°C. The conjugated protein was purified by dialysis (8k molecular weight cutoff) and lyophilized.

Animals

A randomized and blinded study was conducted using adult Sprague-Dawley rats (obtained from Charles River) weighing 250 g. Rats were divided into two time points (3 and 21 days) containing five groups (n = 7 to 12 per group) each. While one group was subjected to sham surgery, the other four groups received IR surgery (30 min. coronary artery ligation followed by reperfusion), with or without the injection of 100 μl of phosphate buffered saline (PBS) containing either 80U SOD1, 10 mg PK or PKSOD per ml of PBS with or without 5 mg SB239063 (p38 inhibitor) per ml PBS, into the perimeter of cyanotic ischemic zone (3 locations) through a 30-gauge needle immediately after reperfusion. Studies conducted to investigate the effect of higher concentrations of microparticles had 20 mg of either PK or PKSOD per ml of PBS. The animals were sacrificed after specific time points, and the hearts were molded in Optimal-Cutting-Temperature compound and, snap frozen in liquid nitrogen, or they were dehydrated and, embedded in paraffin for immunohistological analysis. In studies requiring real time analysis of gene products, the left ventricle of the myocardium was homogenized with Trizol® reagent and stored at -80 ºC until further processing. The investigation conformed to *The Guide for the Care and Use of Laboratory Animals* published by the

US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and all animal studies were approved by Emory University Institutional Animal Care and Use Committee.

Oxidative stress and superoxide detection following IR.

Reduction of cytochrome C by superoxide was measured as a change in absorbance at 550 nm as described previously [169]. Briefly, three days after surgery, the hearts were excised and the left ventricles were cut to small pieces and incubated for 15 minutes at 37 °C in KHB media with or without 50 U/ml SOD and supplemented with 100 μM partially acetylated cytochrome C (Sigma). Oxidative stress level in the myocardium was analyzed by measuring malondialdehyde and 4-hydroxyalkenals in the tissue homogenate using colorimetric lipid peroxidation assay kit (Oxford Biomedical Research) following the manufacturer's protocol. *In situ*-superoxide production was detected, using DHE as described previously with slight modifications [179]. Briefly, 10 μM DHE was topically applied over unfixed frozen 20 μm heart sections and incubated at 37 ºC in a light protected CO_2 incubator for 5 minutes. After 2 minutes of DAPI staining, the slides were mounted with antifading medium (Vectashield® HardSet™; Vector laboratories) and were analyzed with Axioscope fluorescence microscope with identical camera acquisition settings. DHE fluorescence intensity was quantified using ImageJ software (NIH).

Apoptosis detection.

Terminal transferase-mediated dUTP-fluorescein nick end labeling (TUNEL) assay kit (Roche diagnostics catalogue number 12 156 792 910) was used as per the manufacturer's protocol to determine the number of apoptotic cells in the tissue sample. Briefly, unfixed 5 μm frozen heart sections were washed with PBS and fixed with 2% paraformaldehyde for 30 min at room temperature. After washing with PBS, the heart

sections were permeabilized with ice cold 0.1 % sodium citrate containing 0.1% Triton-X 100 for 2 min. 50 μl solution containing 45 μl TUNEL label and 5 μl TUNEL enzyme (or 50 μl TUNEL label alone for negative control) were applied topically over the heart sections and incubated for 1 h at 37 ºC in CO_2 incubator. After washing the unbound TUNEL label with PBS, the nuclei were stained with 4, 6-diamidino-2-phenylindole (DAPI, 1 μg/ml) for 5 minutes at room temperature and washed once with PBS. The slides were then mounted with Vectashield® HardSet™ mounting medium and were imaged using Axioscope Fluorescence microscope at identical camera settings. The number of TUNEL positive cells were reported as a percentage of total cell count.

Echocardiography.

Anesthetized rats were subjected to echocardiography prior to and after 3 or 21 days of IR surgery. Short axis values of left ventricular end systolic (ES) and end diastolic (ED) dimension were obtained using Acuson Sequoia 512 echocardiography workstation with 14 MHz transducer. An average of 2 consecutive cardiac cycles was used for each measurement and were made three times in an investigator-blinded manner. Changes in fractional shortening (calculated as (ED-ES)/ED) were determined after normalizing each rat to its own baseline value.

Infarct size measurement.

Infarct size was measured using the methods previously reported using Evan's blue dye and 2,3,5-triphenyltetrazolium chloride (TTC) [93]. Briefly, after the hearts of the animals were excised, the LAD was re-occluded at the same location and was perfused with Evan's blue dye to define the area at risk. The left ventricle (LV) was then sliced into transverse sections (~2 mm) and incubated with TTC for two minutes to stain the viable myocardium. The sections were then fixed in 4% paraformaldehyde and photographed for analysis. Noninfarcted tissue was identified by deep blue staining, ischemic but viable

myocardium was identified by deep red staining, and necrotic LV tissue was identified by white coloration. Planimetry was performed with ImageJ software (NIH) by tracing the three areas in all sections. Area at risk was determined as the percentage of area with red plus white coloration in relation to the total LV area (red plus white plus blue). Infarct size was determined as the percentage of area with white coloration in relation to the area with red plus white coloration. These values were determined from all the slices and averaged.

Immunofluorescence, immunohistochemistry and in-situ zymography

Collagen deposition was determined by Picrosirius Red (Sigma) staining as described previously [19]. Briefly, 5 µm paraffin embedded heart sections from 21 days treatment groups were dewaxed with Histo-Clear (National Diagnostics) and subjected to gradual hydration with aqueous ethanol. The tissue sections were subsequently stained with pico-sirius red solution (0.5 g of sirius red F3B in 500 ml of saturated aqueous solution of picric acid) for one hour and washed with two changes of acidified water (5 ml glacial acetic acid in 995 ml of distilled water). Finally, the sections were dehydrated, mounted in a resinous medium and were imaged in brighfield using Axioscope microscope at identical camera settings. Fibrotic area was reported as percentage of red area in total area. For immunohistochemistry, the tissue sections were subjected to antigen retrieval with citrate based antigen unmasking solution (Vector laboratories) and probed with an antibody to C-Kit (Santa Cruz Biotechnology), then with a fluorescent secondary antibody (Alexa Fluor® 568, Invitrogen) before mounting with Prolong Gold mounting medium (Invitrogen). For isolectin staining, 1 µg/ml Alexa Fluor® 488 conjugated isolectin GS-IB4 (Invitrogen) was incubated for 1 hour with proteinase K (20 µg/ml) treated (10 minutes) tissue sections. In situ zymography to detect matrix metalloproteinase activity in the heart sections subjected to three days of IR with or without treatments was performed by incubating the frozen sections for 12 h at 37 ºC

with 10 µg fluorescein conjugated DQ collagen, type IV (Invitrogen) per ml of zymogram developing buffer (Invitrogen). All tissue sections for immunofluorescence were imaged with Axioscope fluorescence microscope with identical acquisition settings.

Real time polymerase chain reaction.

mRNAs were extracted using Trizol® reagent (Invitrogen) according to manufacturer's protocol from the left ventricle of the myocardium subjected to 3 days of IR with or without treatments. c-DNAs were synthesized by reverse transcribing the mRNA using Superscript reverse transcriptase (Invitrogen) along with oligo dT and random hexamers as primers. Real time polymerase chain reactions were run on StepOnePlus™ Real-Time PCR System (Applied Biosystems) with SYBR Green PCR master mix (Applied Biosystems) using primers (Appendix) for SOD1, SOD2, SERCA-2a, ryanodine receptors, sodium calcium exchanger and phospholamban. All the reaction products were normalized to the cDNAs of 18s primers.

Statistics.

All statistical analyses were performed using Graphpad Prism software as described in the figure legends. All data are expressed as mean $\pm$ SEM. p values of less than 0.05 were considered significant.

4.3 Results

PKSOD retention in the myocardium

In order to determine the ability of PKSOD to remain in the heart after IR, either fluorescent SOD1 (FSOD) or PKFSOD were injected intramyocardially (n=3) and analyzed by confocal microscopy three days following IR surgery (Figure 4.1). Three-dimensional reconstruction of images showed bright fluorescence from PKFSOD particles scattered in the left ventricular infarct zone of the myocardium (Figure 4.1 A). There was no fluorescence in the right ventricular region that is far from the site of particle injection. Additionally, data obtained in our laboratory demonstrates retention of

the particles for up to 10 days in native myocardium [19]. This fluorescence was totally absent in FSOD injected hearts (Figure 4.1 B).

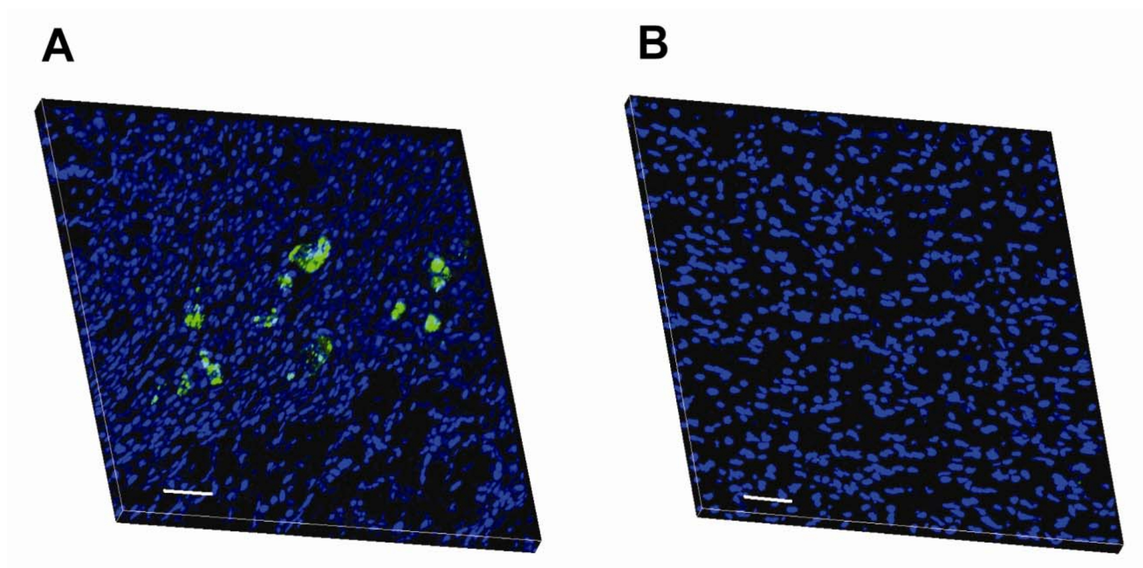

Figure 4.1. **PKSOD microparticles are retained in the heart.** Three-dimensional rendering of a confocal fluorescent microscope image of infarcted myocardium section injected with either free FITC-SOD1 (FSOD) or polyketal-encapsulated FITC-SOD1 (PKFSOD). (A) Bright green fluorescent PKFSOD particles can be observed in the myocardium three days following ischemia-reperfusion surgery. (B) No green fluorescence can be observed in myocardial tissue injected with FSOD. (Blue is DAPI counter staining, white bar represents 50 μm).

In vivo superoxide scavenging ability of PKSOD.

In preliminary studies, a significant increase in superoxide production was observed at 3 days following IR as measured by SOD inhibitable increase in the absorbance of cytochrome C reduction at 550 nm. This reduction was fully inhibited by PKSOD treatment (Figure 4.2 A). Malondialdehyde (MDA) and 4-hydroxyalkenals (HAE) generated during lipid peroxidation are an indirect indicator of oxidative stress levels. Our analysis of lipid peroxidation levels suggested a trend of decreased MDA and HAE in the PKSOD treatment group compared to IR alone (Figure 4.2 B). To better localize

the superoxide generation/scavenging, DHE-based in situ superoxide detection was performed as described in methods. Our results show that

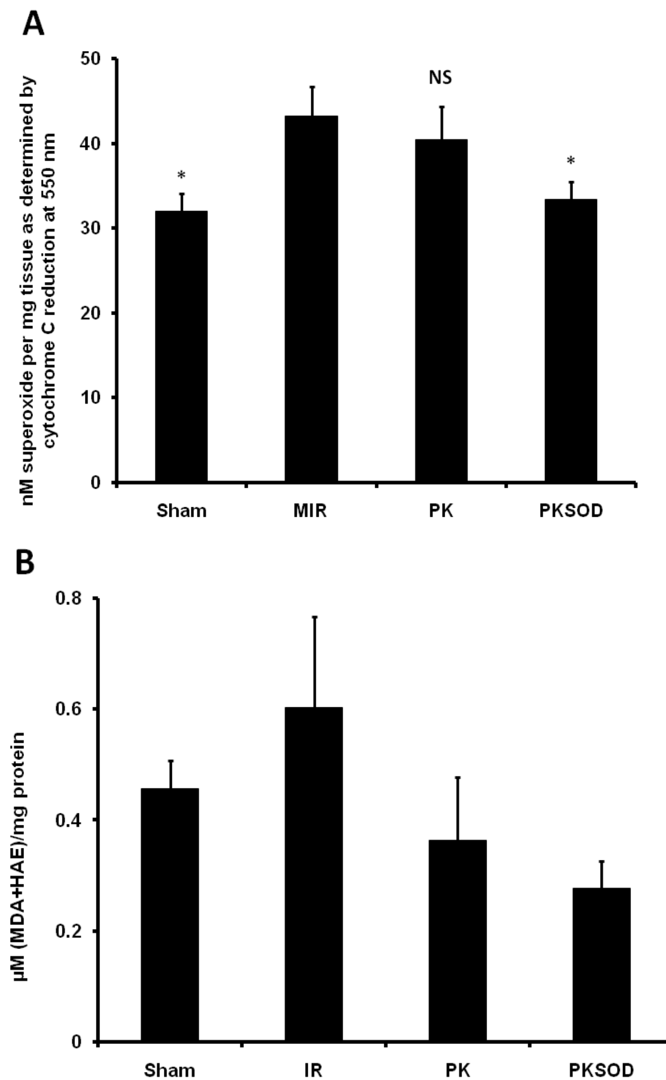

Figure 4.2. PKSOD scavenges superoxide in the ischemic myocardium following ischemia-reperfusion. (A) Superoxide production as measured by cytochrome C assay is enhanced following IR which is inhibited significantly only by PKSOD treatment, n=3 per group. (B) There is a trend of reduced lipid peroxidation due to PKSOD treatment in the left ventricle of the myocardium, n=3 per group. (Figure continued)

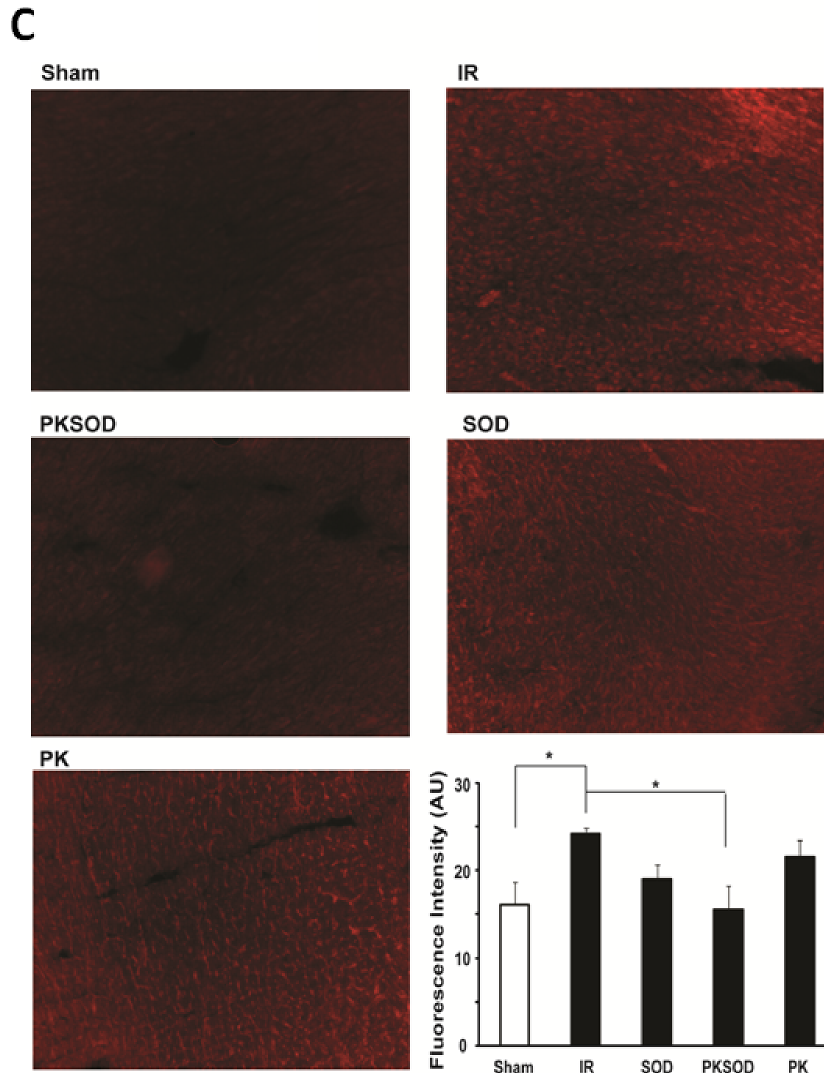

Figure 4.2 (Continued). PKSOD scavenges superoxide in the ischemic myocardium following ischemia-reperfusion. (C) Pictures shown are representative images of DHE fluorescence in the myocardium, imaged with identical camera settings. Individual images were quantified for red fluorescence intensity using ImageJ software in a blinded manner. $n \geq 5$ per group. Chart shows that only PKSOD significantly reduced DHE fluorescence 3 days following IR, with no effect of empty PK or free SOD1. *$p<0.05$ vs. IR; NS: Not significant vs. IR; ANOVA, followed by Dunnett's multiple comparison test.

superoxide levels were significantly increased in the ischemic left ventricular region surrounding the borders of excessive infiltration ($p<0.05$). Treatment with PK or free SOD1 protein was not able to reduce the increase in DHE fluorescence while PKSOD treatment was significantly ($P<0.05$) able to reduce it to basal levels (Figure 4.2 C). Examination of the infarct core demonstrated no significant increase in DHE fluorescence with IR (data not shown).

Effect of PKSOD on apoptosis in the myocardium following IR

In order to measure apoptosis, rats subjected to different treatments were sacrificed after three days and TUNEL staining was performed on 5 μm frozen sections, and the percentage of TUNEL positive cells was determined by manual counting in a blinded manner. Within the left ventricle infarct zone outside the borders of scarred infiltrated region, IR injury significantly ($p<0.05$) increased the percentage of TUNEL positive cells greater than 4-fold as compared to sham operated animals. There was no significant decrease in apoptosis with either PK or free SOD1 treatment; however, PKSOD treatment significantly ($p<0.05$) reduced the TUNEL-positive myocyte count (Figure 4.3 A). However within the core of the infarct containing heavy infiltration, there was no significant difference in apoptosis among the treatment groups (Figure 4.3 B).

Effect of PKSOD on infarct size after reperfusion injury

To determine the effect of prolonged superoxide scavenging on development of infarct size, the animals were subjected to IR surgery with or without particle injection. Infarct sizes were measured in a blinded manner by TTC and Evan's Blue staining 3 days following IR surgery. Infarct size in IR animals with vehicle treatment was 38% of the area at risk (AAR) and treatment with PK did not significantly reduce the infarct size (25% of AAR). Conversely, a significant inhibition of infarct size was observed with both free SOD1 (15 % of AAR) and PKSOD treatments (12% of AAR) as compared to IR or PK treated groups ($p<0.05$, Figure 4.4).

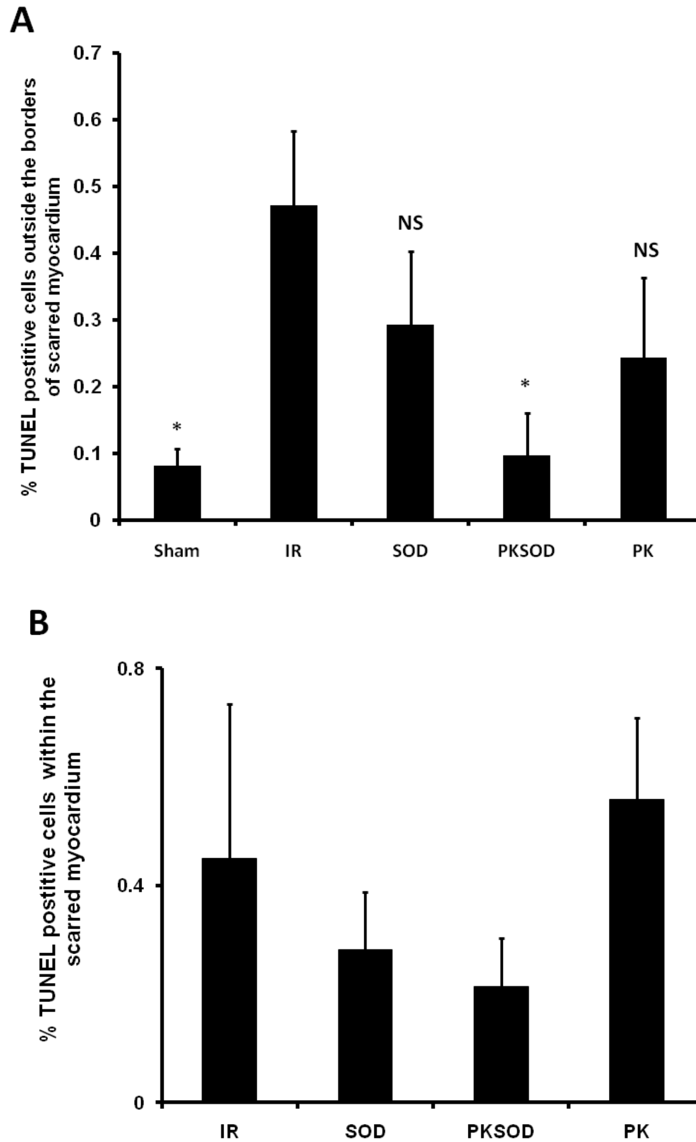

Figure 4.3. PKSOD treatment decreases apoptosis in the myocardium. (A) Three days after surgery in the borders of scarred myocardium in the left ventricle, IR injury significantly increased the apoptosis compared to sham operated animals that was not attenuated by empty polyketal (PK) or free SOD1 treatment. In contrast, PKSOD treatment was able to significantly reduce the TUNEL-positive count. (B) TUNEL counts within the infiltrated region were not significantly different between treatment groups. $n \geq 6$ per group. *$p<0.05$ vs. IR; NS Not significant vs. IR; ANOVA followed by Dunnett's multiple comparison test.

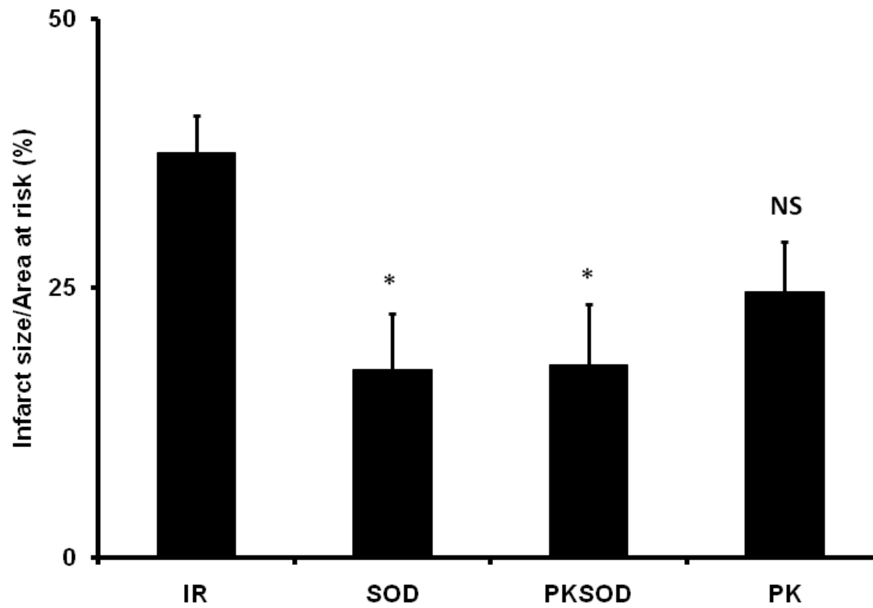

Figure 4.4. Acute SOD1 and PKSOD treatment decreases the infarct size. Three days after surgery, the hearts were stained with Evan's blue and TTC to identify the infracted area in the myocardium. Infarct area normalized to areas at risk (AAR) was reported as percentage. While IR and PK treatment has about 30% infarct area in the AAR, immediate injection of SOD1 and PKSOD after reperfusion was able to reduce the infarct size significantly. n$\geq$6 per group *p<0.05 vs. IR. ANOVA followed by Dunnet's multiple comparison test.

Effect of PKSOD on acute cardiac function following IR

To determine the effect of sustained SOD delivery on acute cardiac function, echocardiography data were collected. This method was chosen over the more accurate technique of measuring end systolic pressure volume relationships mainly because the latter method is fully invasive requiring multiple surgeries on animals. Prior to surgery, echocardiography data were collected to determine basal function, and then again 3 days after surgery as described in methods. As shown in Figure 4.5, IR significantly reduced cardiac function (p<0.01) as measured in absolute change in fractional shortening from baseline to 3 days post-injury. In addition, both SOD1 and PK groups

had a significantly reduced cardiac function ($p<0.01$, not shown in figure) compared to sham operated animals, and they did not function better than IR (Figure 4.5). In stark contrast, treatment with PKSOD significantly ($p<0.05$) improved function to sham levels (Figure 4.5).

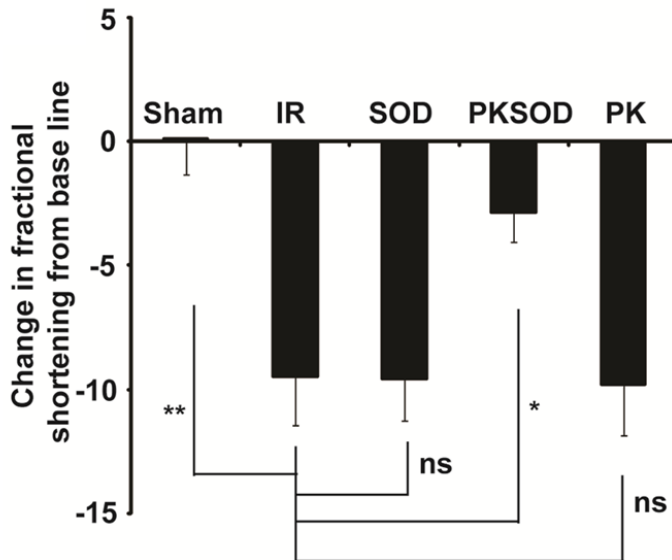

Figure 4.5. PKSOD treatment improves acute cardiac function following IR. Adult male Sprague-Dawley rats subjected to sham or IR surgery with or without indicated treatments were subjected to echocardiography prior to surgery, and 3 or 21 days post-infarction. Change in fractional shortening was normalized to baseline values of each rat before surgery. PK SOD treated rats had significantly improved cardiac function as compared to ischemia-reperfusion (IR) alone 3 days following infarction, with no effect of empty PK or free SOD1. $n \geq 10$ per group, *$p<0.01$, **$p<0.001$.

Effect of PKSOD on chronic cardiac function and fibrosis following IR

To investigate the effect of free SOD1 and PKSOD on chronic cardiac function, baseline and 21 days post-surgery echocardiographic data were collected. IR-injury significantly impaired chronic cardiac function as measured by absolute change in fractional shortening from baseline to 21 days ($p<0.01$). A trend with cardiac functional improvement was observed in PKSOD treated animals as compared to PK or free SOD1

injection. However, the rats were not significantly improved compared with IR alone (Figure 4.6 A). Further, chronic collagen deposition was higher in IR

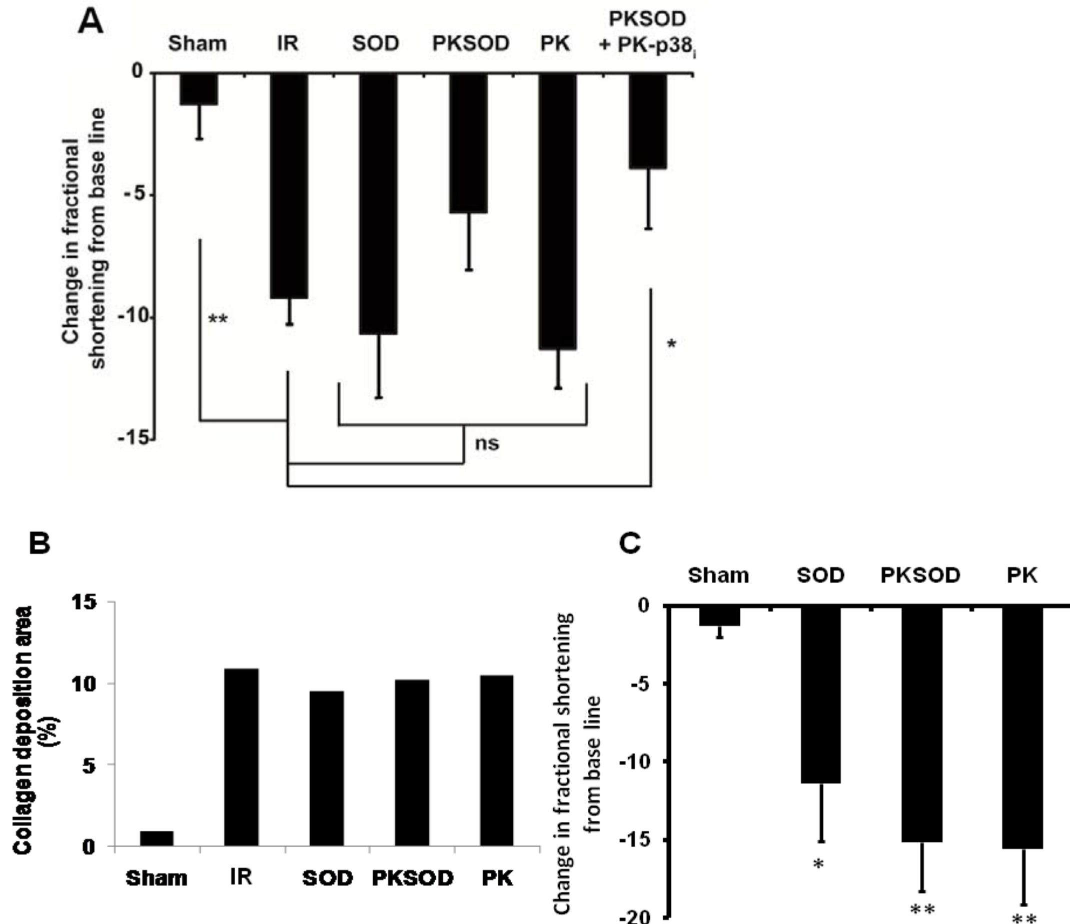

Figure 4.6. PKSOD treatment does not improve chronic cardiac function following IR. (A) Only a dual treatment with PKSOD and PK-p38i significantly improved cardiac function 21 days following infarction as compared to IR alone. No significant effect was seen with PKSOD, empty PK, or free SOD 1. n≥10 per group (B) Collagen deposition as measured by pico-sirius red staining show a trend of increased collagen deposition in all treatment groups compared to sham animals. (C) Doubling the units of free SOD1 injected or increasing the amount of delivered particles (2mg instead of 1 mg) to the myocardium did not offer any improvement in chronic function following IR. In B and C, n ≥ 4 per group. *p<0.05, **p<0.001 vs. sham, ANOVA followed by Dunnet's post-test

compared to sham operated animals and neither SOD1, PK nor PKSOD treatment were able to reduce the extent of fibrosis (Figure 4.6 B). These observations were again not statistically significant. In addition, doubling the dosage in all the treatment groups did not have any additional benefit in chronic cardiac function (Figure 4.6 C). Thus, we investigated whether dual delivery with particles containing a p38 inhibitor (PK-$p38_i$) that, in prior published studies had no acute effect but improved chronic cardiac function due to inhibition of fibrosis [19], could improve function in these rats. Interestingly, dual treatment with PKSOD and PK-$p38_i$ significantly improved cardiac function ($p<0.05$) compared to IR alone (Figure 4.6 A), suggesting the need for multiple therapeutics to combat the different phases of the disease.

Effect on angiogenesis and stem cell recruitment

Isolectin B4 (IB4) from *Griffonia simplicifolia* binds to terminal α-galactosyl residues expressed by endothelial cells [180]. Therefore, fluorescently tagged IB4 were used to quantify angiogenesis in the left ventricle of each hearts, 3 days post-surgery (Representative image in Figure 4.7 A). IR shows a trend of increased angiogenesis (23 endothelial cells per hundred nuclei) compared to sham (18 cells) (Figure 4.7 A). Both PK and free SOD1 treatment had similar trends of increased angiogenesis (24 and 23 cells respectively per hundred cells) compared to sham. However, endothelial cell count in PKSOD treatment was not increased (16 cells) after surgery (Figure 4.7 A). These data however did not reach any statistical significance. In addition to angiogenesis, the effect of sustained PKSOD delivery on stem cell recruitment was studied by staining the hearts 21 days post-surgery for c-kit positive cells. All the treatment groups were similar to sham animals and all the groups had roughly equal percentage of c-kit positive cells (about 7 cells for every thousand cells, Figure 4.7 B).

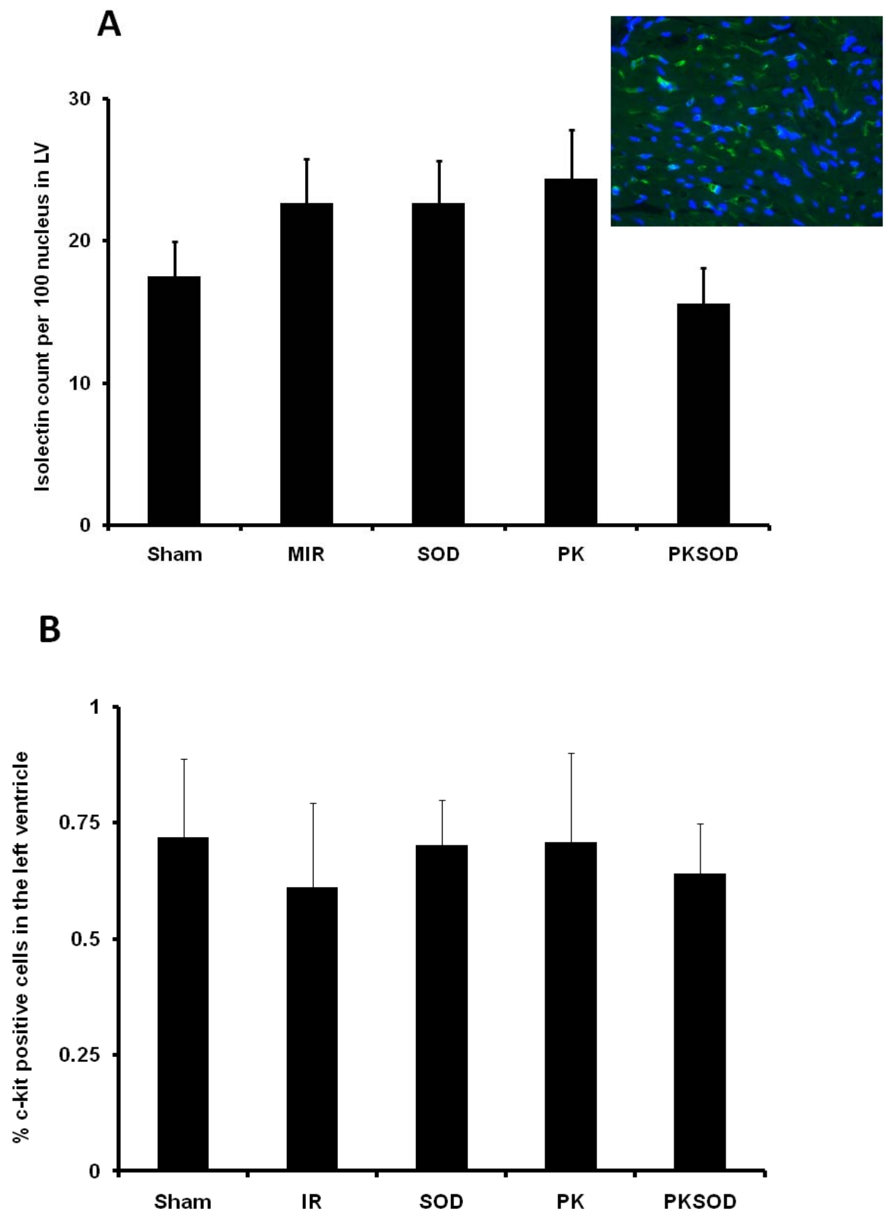

Figure 4.7. Effect of PKSOD delivery on angiogenesis and stem cell recruitment. (A) Endothelial cells were stained in heart sections 3 days post-surgery using Alexa-Fluor 488 tagged isolectin B4 (staining shown in representative picture). On an average IR, PK and SOD1 treatment had about 30% higher endothelial cell count compared to sham or PKSOD treated animals. (B) Immunohistochemistry performed to identify c-kit positive cells in the myocardium 21 days post-surgery shows no differences between groups. n ≥ 4 per group. ANOVA revealed no significant difference between groups.

Effect on calcium handling and antioxidant gene expression

To investigate if there are any changes in the gene expression levels of major calcium handling proteins following SOD1 delivery, real time polymerase chain reaction (RT-PCR) was conducted on cDNA obtained from the left ventricle of the rat myocardium, 3 days post-surgery. Except expression of sodium calcium exchanger (NCX) mRNA which did not vary significantly among any treatment groups compared to control (Figure 4.8), the expression of other major calcium handling proteins including

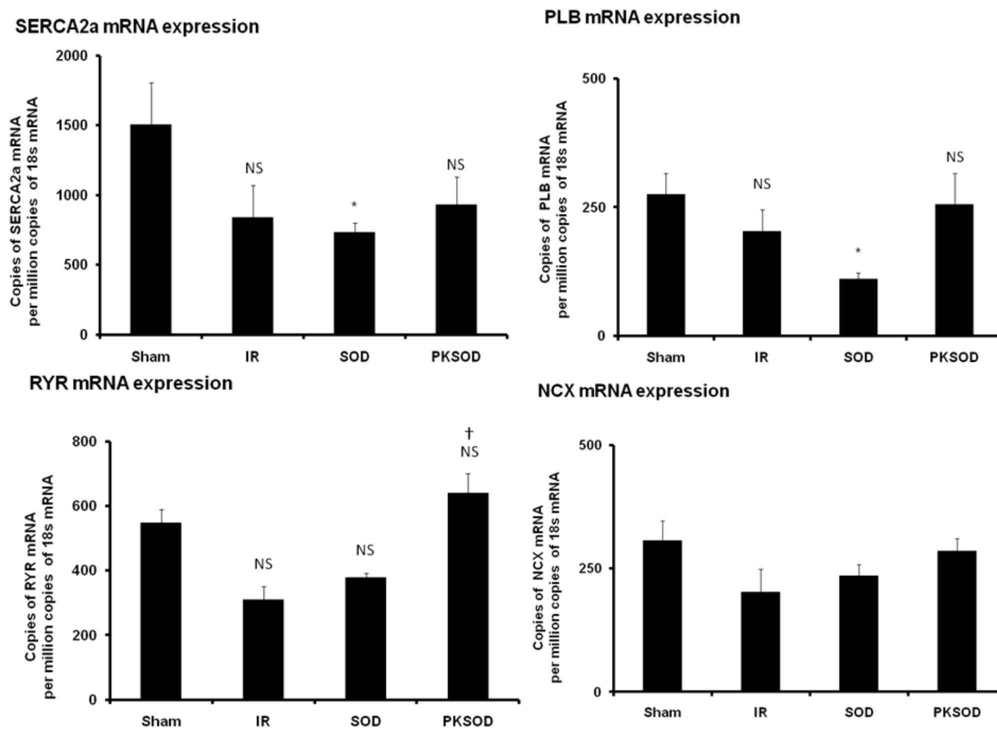

Figure 4.8. Effect of sustained SOD1 delivery on gene expression of major calcium handling proteins. RT-PCR analysis performed on c-DNA isolated from the left ventricle of myocardium 3 days post-surgery shows that SERCA2a and PLB mRNA level decrease significantly only in SOD1 treated animals. NCX mRNA levels did not vary among any treatment groups. Interestingly, mRNA level of RYR did not drop in PKSOD treatment compared to sham and its expression level remained significantly higher compared to IR. All data normalized to 18s mRNA expression, n ≥ 4 per group. NS Not significant vs. Sham, * $p<0.05$ vs. Sham, † $p<0.05$ vs. IR.

sarco-endoplasmic reticulum calcium ATPase-type 2A (SERCA2a), phospholamban (PLB) and ryanodine receptors (RYR) varied significantly between groups (Figure 4.8). There is a trend of decreased SERCA2a mRNA expression following IR and PKSOD treatment. However, following SOD1 treatment SERCA2a mRNA expression decreased significantly ($p<0.05$) compared to control. The same trend is observed for the expression of PLB mRNA where significant reduction ($p<0.05$) occurs only with SOD1 treatment following IR. RYR mRNA level decreases following IR and SOD1 treatment. However, it did not reach statistical significance. Interestingly, RYR mRNA expression did not decrease in PKSOD treatment compared to control and it maintained a significantly higher ($p<0.05$) expression level compared to IR (Figure 4.8).

In addition to analyzing the gene expression of calcium handling proteins, mRNA levels of major antioxidant proteins including SOD1, SOD2, catalase and glutathione peroxidase1 (GPX1) were also quantified using RT-PCR. Only SOD2 mRNA had a trend of decreased expression following IR, SOD1 or PKSOD treatment compared to sham operated animas (Figure 4.9). However, statistical significance was not reached. The expression of SOD1, catalase and, GPX1 mRNAs did not show any specific trend, and none of their expression differed significantly within treatment groups (Figure 4.9)

Effect on cardiac remodeling proteins

In order to study the effect of sustained SOD1 delivery on remodeling, in situ zymography was performed on heart sections three days post-surgery to analyze the activity levels of matrix metalloproteinases (MMP) in the left ventricle of the myocardium. In order to do that, DQ collage IV substrate with heavy fluorescein conjugation was incubated with the tissue sections. Initially, because of an excessive number of fluorescent dyes attached to the substrate, the fluorescence signal is almost non-existent. Collagen hydrolysis following MMP activity in the tissue sections results in the separation of the dye molecules from one another and the fluorescence signal increases

(Figure 4.10

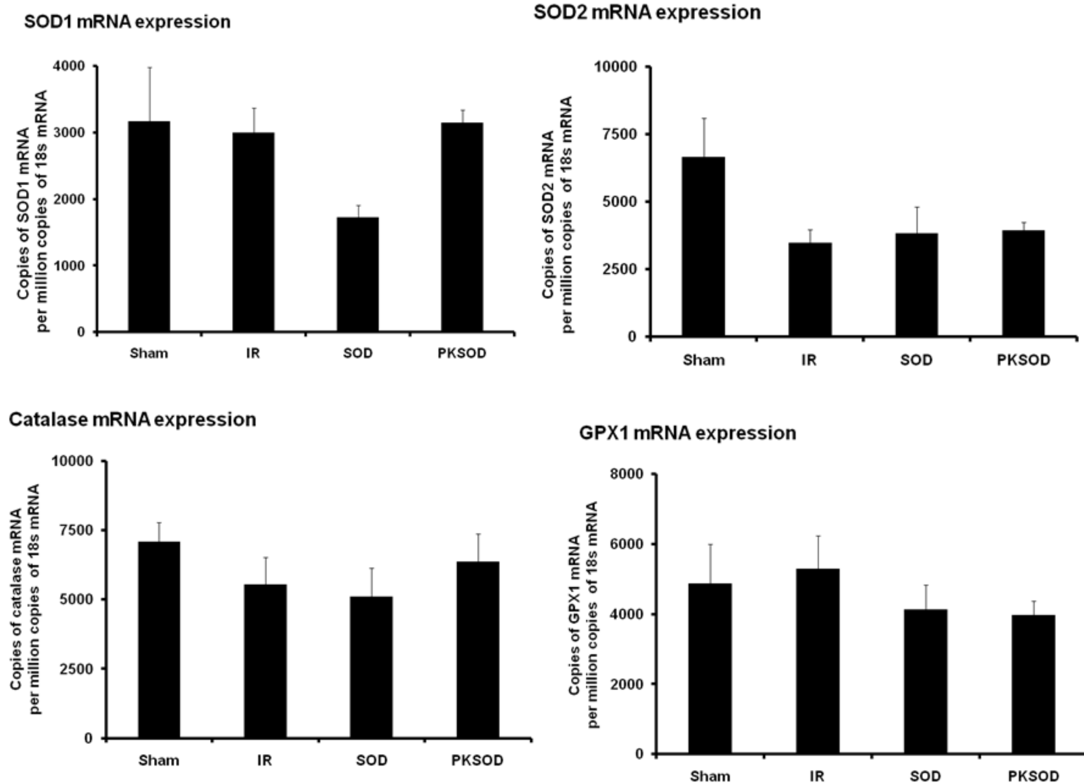

Figure 4.9. Effect of sustained SOD1 delivery on gene expression of major antioxidant proteins. RT-PCR analysis was performed on c-DNA isolated from the left ventricle of myocardium 3 days post-surgery to investigate the expression of SOD1, SOD2, catalase and GPX1 expression levels normalized to 18s mRNA expression. Data suggest that only SOD2 mRNA levels had a trend of decreased expression post IR in all treatment groups compared to sham. Whereas, the gene expression of SOD1, GPX1 and catalase did not show any specific trend and were not different vary within the treatment groups. n ≥ 4 per group. ANOVA showed no statistical difference between treatments.

representative picture). In each field of the image, areas with MMP induced fluorescence was calculated after subtracting out the areas with non-specific signals from matched sections stained with DQ collagen in the presence of MMP inhibitor (EDTA). The analysis shows that IR results in a trend of having more areas with MMP activity in left

ventricular region, compared to sham animals (Figure 4.10). Areas with MMP activity did not increase in SOD1 and PKSOD treatment compared to control (Figure 4.10). However these observations were not statistically significant.

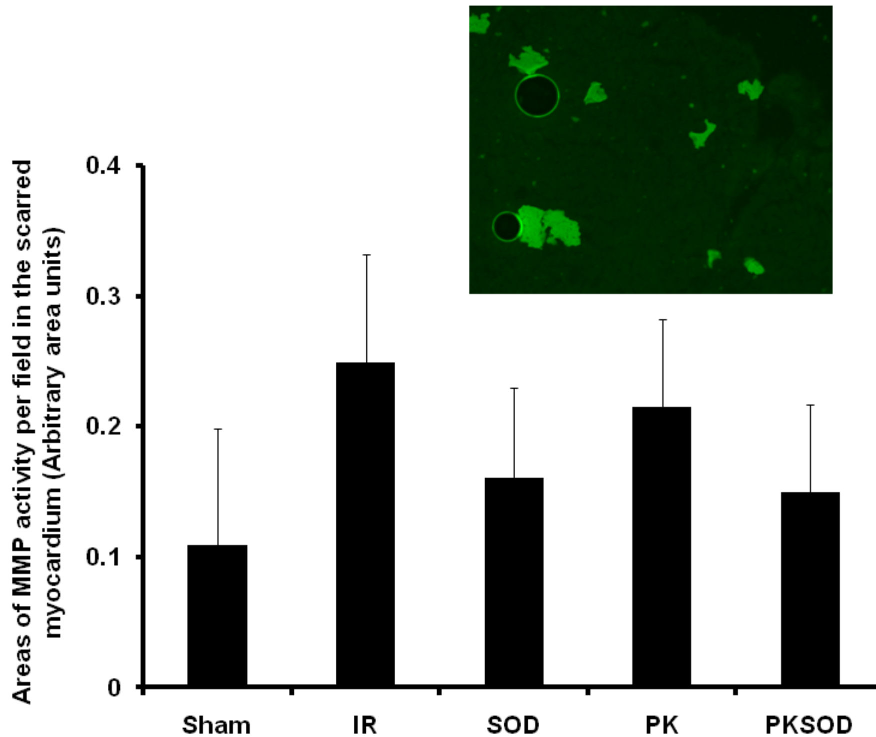

Figure 4.10. Effect of sustained SOD1 delivery on MMP activity. In situ zymography was performed using fluorescein tagged DQ collagen substrate to measure the MMP activity levels (shown in representative picture) in the frozen sections of the hearts subjected to 3 days of treatment. Although IR shows a trend of more areas with MMP activity in left ventricular region, there was no statistical significance between various treatment groups. n ≥ 4 per group.

4.4 Discussion

The overproduction of superoxide plays a central role in the progression of ischemia/reperfusion (IR) injury and causes a rapid loss of cardiomyocytes and decreased cardiac function. In spite of convincing studies on the role of superoxide in

causing cardiac pathologies and the ability of SOD1 to reverse this [15, 92], conflicting results exist on the efficacy of SOD treatment [16, 175] likely due to its poor stability. The findings in this chapter indicate that PCADK microparticles can be used for sustained delivery of SOD1 protein, which improves cardiac function during the acute phase of reperfusion injury.

In these studies, total particle injected (1 mg) constitutes only 0.0004% by body weight, and if the average heart volume is 2 ml (119), the volume injected (100 μl) constitutes only 5% of heart volume. Higher amount of particle injection (> 2mg) was not chosen in the studies since previous observations have suggested reduced metabolic activity of the cells at high doses [181]. In addition, no attempts were made to identify the lowest possible beneficial PKSOD dosage in these studies. While administering the drug, PKSOD hydrolyses to acetone (LD50 = 5800 mg/kg rat) and cyclohexane dimethanol (CDM) (LD50 = 3200 mg/kg rat). Here, 1 mg of PCADK is injected, which can generate about 0.29 mg acetone and 0.71 mg CDM per 250 g rat that corresponds to 1.16 mg acetone/ kg rat and 2.84 mg CDM/kg rat for spontaneous total degradation of polymer. Therefore, given the degradation time of the polymer ranges from weeks to months, the actual concentration of the hydrolysis products are well below the toxic limits. Additionally, chronic but not acute treatment with 0.25 M acetone is known to increase the generation of oxygen radicals in rat liver microsomes (117). Here, even if spontaneous total degradation of polymer is assumed, the maximum local acetone concentration in the zone of injection will be around 5 μM. Thus, it can safely be assumed that the degradation products will have only minimal if any effect on modifying the free radical levels.

The heart is a highly vascularized organ, and as such most small molecules and proteins are rapidly cleared [182]. Therefore, many therapies usually involve daily or multiple injections of drugs and proteins over extended time periods [100, 111]. Although

gene therapy could provide a solution for this problem [183], targeting specific tissues and cell types is very difficult through this method. Further, prolonged overexpression of drugs such as SOD1 could exacerbate cardiac pathologies [98]. Intracellular delivery of SOD1 using cell penetrating peptides may protect the myocardium against ischemic insult [184]. However, through this method the protein was delivered systemically, increasing the chances for nonspecific effects and protein loss. Thus, given the fact that intramyocardial injections are considered safe [185], a one-time localized administration of drugs that could sustain antioxidant levels in the heart during disease progression could offer advantage over existing treatment methods. Initially, to determine whether micron-sized PCADK particles are retained in the heart, fluorescent dye (rubrene) loaded particles were injected to healthy myocardium. Confocal microscopy showed the particles to be scattered in the myocardium three days following surgery and even over a week later [19]. Additionally, prior studies from this laboratory have demonstrated that the half life of these particles was quite stable in neutral environments with no inflammatory response. Thus, the particles can be expected to remain stable for several weeks in the myocardium [19, 116]. Subsequently, the retention of PKSOD particles in ischemic myocardium was determined by injecting fluorescent PKFSOD or FSOD intramyocardially following IR. The confocal images clearly show the local retention of PKFSOD in the myocardium three days after IR surgery.

To determine the effect of prolonged SOD1 retention with polyketals, PKSOD particles were injected in a randomized and blinded study to the myocardium of IR injured rats, injecting the treatments immediately after reperfusion. Cytochrome C reduction assay shows that, PKSOD administration reduces the oxidative stress levels in the left ventricle of the myocardium. Additionally, DHE staining shows that 3 days following IR, excess superoxide is generated along the borders of scarred regions in the ischemic left ventricle. This increase in superoxide is not affected by administration of

free SOD1 protein, but completely inhibited by intramyocardial injection of PKSOD several days after injection. This finding is striking not only due to the prolonged effect of PKSOD administration, but also because of the dose given (single injection of 1 mg per rat, corresponding to 80U of SOD1). This dose is several orders of magnitude lower than previous published reports demonstrating infusion of over 1,000 U/kg prior to infarction followed by continuous infusion of over 1,000 U/kg thereafter [71, 72]. We speculate that our treatment was more effective due to the localized nature of the superoxide generation (border zones only) and the fact that our microparticles are retained within the injection site. These conclusions are supported by our imaging data demonstrating that the native protein is not retained in the myocardium following injection. Thus, large doses of free SOD1 would be needed to maintain pharmacologically relevant doses in the affected area.

Superoxide generation is known to cause cardiomyocyte apoptosis [67], which is generally held to be responsible for progressive loss of cardiomyocytes after IR, in spite of wide variations in the percentage of apoptotic myocytes (0.05 to 35%) [186, 187]. The majority of cardiomyocyte apoptosis occurs within the first 72 hours of reperfusion, thus it was hypothesized that, the sustained reduction of superoxide by PKSOD administration would be beneficial in reducing early cardiomyocyte apoptosis. Interestingly only PKSOD treatment significantly reduced cardiomyocyte apoptosis, suggesting that prolonged superoxide scavenging plays an important role in the survival of endogenous cardiomyocytes. This is in contrast to some studies showing free SOD1 infusion reduces apoptosis and infarct size, however it should be noted that the levels used to correspond to our PKSOD treatment (80U) were much lower than levels used in those studies [71].

Decreases in cardiomyocyte apoptosis may lead to a reduction in infarct size [188]. In addition, overexpression of SOD is known to reduce infarct size in mice [93].

Further, considerable collateral flow exist in rat myocardium [189] which can create zones of sub-optimal flow in the ischemic zone that can be salvaged by appropriate treatment. Thus, we performed a separate study to determine the effects of PKSOD on infarct size. Three days after IR, we found an infarct size of roughly 30% of the area at risk (AAR) in vehicle treated rats. Interestingly, both free SOD1 and PKSOD treatment significantly reduced the infarct size to about 15% of AAR. Because the presence of free SOD1 was relatively short lived, this result suggests that, scavenging the excess superoxide generated immediately after IR plays a significant role in reducing infarct size, and that other processes, such as autophagy and necrosis, and not just apoptosis, may play an equally important role in determining infarct size [190].

Next, the effect of prolonged superoxide scavenging by PKSOD on cardiac function was measured by echocardiography. Change in fractional shortening from baseline echocardiograms demonstrated a significant improvement in function due to PKSOD treatment three days following treatment, with function similar to sham operated animals. In contrast, no functional improvements were observed with free SOD1 or PK treatment suggesting a critical need for sustained therapy. During the chronic phase (21 days following IR), no significant improvement in cardiac function was observed with PKSOD. Though there was a trend toward improved function with PKSOD treatment (30% better than IR alone) as compared to PK and free SOD1, this result did not achieve statistical significance. Additionally, there was no difference in the extent of fibrosis between IR and PKSOD treatment. It is possible that PKSOD dosage at 1 mg was completely consumed in the early phase. Therefore, a higher dose of PKSOD (2 mg) was injected to the myocardium to investigate if it offers any benefit. However, no chronic functional improvement was observed even at higher concentrations. It may be possible that increased superoxide alone does not determine the fate of orchestrated set of events happening in myocardial disease progression. Thus a single antioxidant alone

may not be sufficient to rescue function in the chronic phase of disease development. Supporting this, our recent study using polyketals to deliver small molecule inhibitors demonstrated no acute effect of p38 pathway inhibition, though sustained delivery had a striking effect on chronic remodeling. To address this interesting possibility, we delivered both PKSOD and PK-p38$_i$ following reperfusion and measured function at baseline and 21 days following injury. The combined treatment was not significantly different compared to PKSOD alone. However, the combined treatment significantly improved the cardiac function as compared to IR alone, suggesting the potential need for multiple therapies to treat this progressive disease.

Prior studies have shown that just a change of 0.023% in apoptosis in the myocardium is sufficient to induce a lethal cardiomyopathy [187, 191]. The data in this study also show that there is a low percentage of apoptotic cells (about 0.5%) after IR and a reduction of that percentage to about 0.1% by PKSOD treatment was able to show a significant functional recovery. Whereas inhibiting initial apoptosis is critical in maintaining normal function and homeostasis, the sustained inhibition of superoxide levels may additionally play important role outside of cell survival, such as improving vasculogenesis [192], expression of contractile proteins [193] and, recruitment of regenerative cells [192], which could potentially improve functional recovery synergistically with apoptosis reduction. Therefore, the effects of PKSOD on these other roles were investigated. None of the treatment groups had significant effect on angiogenesis. However, IR, PK and SOD1 treatment had a trend of expressing about 30% more endothelial cell count compared to sham and PKSOD treated rats. ROS generation following IR helps in promoting angiogenic response, and therefore, scavenging the ROS can prevent angiogenesis [194, 195]. Adding more animals to the groups could help in inferring about the significance of this trend and could potentially provide information on the amount of SOD that could be used beneficially without

compromising on the angiogenic response. Indirect inferences suggest vulnerability of c-kit positive cardiac stem cells to oxidative stress [196]. Therefore, it was analyzed to see if there are any changes in the percentage of c-kit positive cells existing in the myocardium following PKSOD treatment. However, no significant changes were observed between treatment groups. While initially discouraging, it should be noted that these snapshots merely represent one time point in selected areas and inferences should not be made about an entire population.

SERCA2a, PLB, NCX and, RYR are major calcium handling proteins affecting the contractility of the myocardium [197]. Additionally, their activity levels are redox mediated [198]. Therefore, their gene expression levels were analyzed. Following PKSOD treatment, the ryanodine receptors expression levels remained similar to sham level while significantly differing from a trend of decreased RYR expression in IR. RYR play a vital role in calcium induced calcium release [197], and therefore, their expression level could potentially alter the calcium transients within the myocardium [199]. mRNA levels of SERCA2a and PLB decreases during end stage heart failure [200, 201]. Our analysis show that the expression of SERCA2a and PLB genes drop significantly in SOD1 treated animals. It is possible that significant modifications in the expression of these important calcium handling proteins affect the contractility of SOD1 treated animals, in spite of infarct reduction. Further in depth investigations could provide an answer to this possibility. Since oxidative stress could alter the expression of antioxidant proteins, the effect of SOD1 delivery, on gene expression of major antioxidant proteins, was investigated. However, the results did not show any significant changes between treatments.

Oxidative stress affects remodeling in the myocardium by modulating the MMP activity [202]. Therefore, in situ zymography was performed to investigate MMP activities in the heart sections. However, zymogrpahy results showed that the differences in MMP

activities between various treatment groups were not statistically significant. It should be noted that this observation is based on collagen IV degrading MMP activity. Future studies with additional MMP substrates including gelatin, collagen I and IV subtypes could help us get a better picture of variations in total MMP activities following PKSOD treatment.

This chapter has demonstrated that following PKSOD delivery to rat heart subjected to IR, oxidative stress level is decreased, and its acute functional ability is improved. Although, the effects of PKSOD delivery on various factors were analyzed, only apoptosis reduction following PKSOD treatment could offer possible reason for the observed acute functional improvement. Oxidative stress affects many reactions in the biological systems. Therefore, to fully understand if sustained SOD1 delivery offer beneficial or harmful effects to the myocardium more investigations are needed.

CHAPTER 5

OXIDATIVE STRESS INDUCED DEATH AND INTRACELLULAR SOD LEVELS IN CARDIAC PROGENITOR CELLS

In this chapter, experimental findings on the effects of oxidative stress on the survival and antioxidant response of c-kit positive cardiac stem cells are presented. Endogenous c-kit positive cardiac progenitor cells (CPCs) were separated from the rat myocardium using immunomagnetic isolation technique. These cells were expanded and subjected to xanthine/xanthine oxidase induced oxidative stress and analyzed for apoptosis and antioxidant response. Later, SOD levels of CPCs were compared with myocytes and the role of SODs in offering protection to CPCs from oxidative stress was determined.

5.1 Introduction

The heart was considered a post-mitotic organ without regenerative potential, and the post-natal growth was attributed to cardiomyocyte hypertrophy [203]. However, recent studies show the existence of cardiomyocyte mitosis and endogenous regenerative capability within the myocardium [9, 204, 205]. Moreover, evidence began to accumulate showing that injection of stem cells to the heart can benefit the myocardium [124]. Additionally, the existence of cardiac stem cells with myocardial regenerative ability was identified in 2003 [10]. These paradigm changing observations have led to intense research in this field, and delivery of stem cells to the diseased myocardium has the potential of becoming a promising therapeutic strategy to repair and regenerate the heart.

Different stem cells – both exogenous and endogenous in origin – are being investigated to regenerate the myocardium. Exogenous pluripotent cell lines such as embryonic stem cells (ESC) can enhance the function of the infarcted myocardium [206]. However, ESCs have ethical issues and problems of teratoma formation and immunological rejection. Induced pluripotent stem cells (iPSCs) are multipotent and form functional cardiomyocytes [207], and like ESCs these cells also have problems with teratoma formation and immunological rejection. More basic understandings of the development and differentiation of autologous iPSCs could solve these issues and potentially help in utilizing iPSCs in regenerative therapies. Adult stem cells such as bone marrow derived stem cells (BMCs) provide modest benefits to the myocardium [20]. However, bone marrow derived cells such as hematopoietic stem cells do not transdifferentiate to cardiomyocytes [208]. Similarly, the transplantation of mesenchymal stem cells offers benefits to the myocardium despite its poor cardiomyocte differentiation ability [209, 210]. The benefits proffered by these cells are attributed to paracrine effects [211]. Endothelial progenitor cells also benefit the myocardium upon transplantation [212]. However, their cardiovascular differentiation ability has not been established, and long-term functional benefit in humans is questionable [213]. Large scale studies on the transplantation of adult skeletal myoblasts to the myocardium did not show any cardiac functional improvement [214].

Endogenous cardiac stem cells represent another pool of cells with promising potential for their applications in myocardial regeneration. A variety of endogenous myocardial stem cell populations exist that vary in their expression of signature markers on its surface. Hierlehy et al (2002) identified cells with stem cell-like activity in the postnatal myocardium. These cells excluded the dyes such as Hoechst 33342 and rhodamine 123 due to high efflux mediated by the expression of ATP-binding cassette transporters such as multidrug-resistance member 1 (MDR1). Thus they were termed as

side population (SP) cells [215]. Although cardiomyogenic potential of SP cells with Sca-1 expression is established in vitro [216], there is limited information about the in vivo regenerative potential of these cells. Endogenous progenitor cells positive for the LIM-homeodomain islet-1 transcription factor (isl+ cells) are another variety of progenitors existing in neonatal myocardium with multipotent cardiogenic differentiation potential [217]. However, there are no reports on the existence of isl+ cells in adult myocardium. Following gentle enzymatic digestion of rat myocardium or human cardiac biopsies, another multipotent fibroblast-like population with cardiogenic potential termed cardiospheres was obtained. They are positive for markers such as c-kit, Sca-1 and CD-34 [218]. However, functional benefits offered by these cells to the myocardium are not known. Finally, there are resident, clonable c-kit positive cells which are observed in rat, dog and human myocardium and characterized by the presence of markers including c-kit, Sca-1 and MDR-1 [10, 219]. Termed cardiac progenitor cells (CPCs), they improve ventricular function following transplantation [220] and differentiate to cardiomyocytes, smooth muscle cells, endothelial cells and fibroblasts [221, 222]. In addition, they can be obtained from human endomyocardial biopsies [223]. Due to their potential in cardiac regeneration, currently two phase I clinical trials are ongoing using cardiac stem cells (CADUCEUS, clinical trials identifier NCT00893360; and SCIPIO, clinical trials identifier NCT00474461).

Irrespective of the cell types used, poor survival and, engraftment of cells are two of the major limitations of cell transplantation therapy. There is a poor survival of cells in hostile ischemic myocardium with increased inflammatory cytokines and oxidative stress. For example, the survival of skeletal myoblasts and MSCs are 1% [224] and 0.5% respectively of the transplanted cells in the ischemic myocardium [225]. However, if the cells are transplanted a week after ischemic injury, the survival significantly improves [226]. Additionally, reducing the oxidative stress levels by SOD delivery in the

myocardium during transplantation increases the survival of the cells [227]. Further, enhanced expression of antioxidants such as SOD2 protects endothelial progenitors during oxidative stress [139]. Intracellular ROS levels and antioxidants regulate other important properties of stem cells such as their self-renewal and senescence [137, 138]. However, many of the basic properties of CPCs such as their antioxidant levels and their response to physiological stresses such as oxidative stress remain unknown. Therefore, additional research in these areas will improve our understanding and potentially aid in successful implantation of c-kit positive CPCs in myocardial regenerative therapies.

Therefore, the aim of this chapter is to investigate the effects of oxidative stress on the in vitro survival and on the antioxidant response of c-kit positive cardiac progenitor cells. Additionally, the basal SOD levels of rat CPCs compared to neonatal rat myocytes were determined, and the role of SODs in offering protection to CPCs from oxidative stress was investigated. Further, the SOD status between two subtype populations of human derived CPCs was compared, and their apoptotic response to oxidative stress was studied.

5.2 Materials and methods

C-kit coated magnetic beads for cell isolation

Micron sized magnetic beads (Dynabeads®, Invitrogen), were used as solid supports to bind antibodies targeted against c-kit cell surface receptors as per manufacturers protocol. Briefly, 50 μl beads were washed with 2 ml of sterile phosphate buffered saline containing 0.1% (w/v) bovine serum albumin (PBS/BSA) and the beads were separated from unbound antibody using Dynal® magnetic particle concentrator. The beads were incubated for 2 h at 37 °C in 2 ml of PBS/BSA containing 5 μl of H-300 c-kit antibody (Santa Cruz Biotechnology). The antibody coated beads were washed twice and stored at 4 °C in 2 ml of PBS/BSA and used within two weeks.

Isolation of cardiac progenitor cells

Endogenous cardiac resident progenitor cells were isolated from rat myocardium as previously described [10] with slight modifications. Healthy adult Sprague-Dawley rats (Charles River Labs) were euthanized; hearts were excised and washed with sterile Hank's balanced salt solution (HBSS) and their ventricles were minced to small pieces. The extracellular matrices in the minced hearts were digested for 30 minutes at 37 °C using 50 mg of collagenase type-2 dissolved in 50 ml of sterile HBSS. The digested tissue suspension was passed through a 70 μm cell strainer and centrifuged. The cell pellet was resuspended with 2 ml of c-kit coated magnetic beads for 2 h at 37 °C. C-kit positive fractions of the cells bound to the beads were separated using magnetic particle concentrator and washed three times with sterile PBS/BSA to remove non-specifically bound cells. Finally, the bead-bound cells were grown in culture media and expanded. Human CPCs isolated from endomyocardial biopsies as previously described [223] were kindly provided by Dr. Piero Anversa's laboratory (Brigham and Women's Hospital, Harvard Medical School).

Immunocytochemistry

The isolated cells were characterized using immunocytochemistry. Briefly the cells were fixed with cold 70% ethanol; permeabilized with 0.1% triton (Sigma) in PBS; blocked with 3% BSA in PBS and labeled with primary and fluorescent secondary antibodies. Primary antibodies used were: c-kit, sca-1, MDR-1, CD34, CD45, Nkx2.5 and GATA4 (Santa Cruz Biotechnology). The labeled cells were then subjected to flow cytometric analysis to measure the percentage of population positive for the labeled markers.

Isolation of cardiomyocytes

Cardiomyocytes were isolated from 1-2 days old Sprague Dawley rat pups (Charles River Labs) as previously described [228]. Briefly, the rat pups were

decapitated, and their hearts were washed with sterile HBSS and minced to a size of about 1 mm^3; followed by extracellular matrix digestion in 1 mg/mL trypsin solution in a rotating shaker at 4°C for 6h. Following centrifugation, the supernatant was removed, and the pellet was digested at 37 °C for 10 min with 0.8 mg/mL collagenase solution. The tissue suspension was passed through 70 μm cell strainer to remove the undigested tissues. Rapidly adhering cells such as vascular smooth muscle cells and endothelial cells were removed by plating the suspension for 1h in fibronectin-coated T75 flasks. The non-adhered fractions consisting of cardiomyocytes were removed and cultured in fibronectin-coated 6 well plates.

Cell culture and cloning

C-kit positive cells from rat myocardium were cultured in growth media that have Ham's F-12 media (Cellgro) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS) (Hyclone), l-glutamine, penicillin/streptomycin (Invitrogen), 10 ng/ml leukemia inhibitory factor (Sigma) and 10 ng/ml basic fibroblast growth factor (Fisher Scientific). Human cardiac stem cells were cultured using the same growth media without the addition of leukemia inhibitory factor. Before commencing the experiments the cells were grown to 80% confluence, serum starved in starvation media containing Ham's F-12 media supplemented with, l-glutamine, insulin / transferrin / selenium (ITS) serum supplement (Cellgro) and penicillin/streptomycin. Single cell cloning was performed to test the clonability of cells. Briefly, c-kit positive cells were counted and subjected to serial dilution until there is no more than 1 cell per ml of growth media. The diluted cell suspension was distributed to the wells of 24 well plates, such that only 12 wells of the 24 wells received a cell. Cells growing from a single colony were subsequently expanded and tested for cardiac progenitor cell markers using flow cytometry.

Cardiomyocytes were mantained in DMEM (Cellgro) supplemented with 10% FBS (Hyclone), l-glutamine, and penicillin/streptomycin (Invitrogen). They were serum starved overnight before experiments in DMEM supplemented with l-glutamine, and penicillin/streptomycin. Human umbilical vein endothelial cells (HUVECs), kindly provided by Dr. Hanjoong Jo's laboratory, were cultured in 0.1% gelation coated plates with Meidum 199 (Cellgro) supplemented with 20% (v/v) heat inactivated fetal bovine serum (Hyclone), l-glutamine, and penicillin/streptomycin (Invitrogen), 50 μg/ml endothelial cell growth supplement (BD Biosciences) and 20 U/ml Heparin (Sigma). They were quiesced overnight in Medium 199 supplemented with 2% fetal bovine serum, ITS, l-glutamine, and penicillin/streptomycin.

Xanthine/Xanthine oxidase treatment

Oxidative stress was induced in the medium using xanthine/xanthine oxidase system (XXO). Briefly, Ham's F12 supplemented with l-glutamine, ITS serum supplement (Cellgro) and, penicillin/streptomycin was used as the treatment media for the cells. Before the experiments, 1 mM xanthine and 10 U/ml catalase dissolved in the treatment media were added to the quiescent cells. Catalase was used to have increased flux of superoxide in the XXO system. The formation of superoxide radicals in XXO system was measured using DHE staining as described in section 3.2. In addition, the flux of ROS generated was measured using Amplex red assay kit (Invitrogen) as per the manufacturer's protocol. The experiments with XXO were terminated at same time point (48 h) and at 0 h, 6 h, 12 h, 24 h and 48 h before the termination of experiments; the treatment medium was subjected to 10 mU/ml xanthine oxidase addition. For groups receiving oxidative stress more than 24 h, the treatment media was subjected XXO addition once every 24 h to maintain the free radical levels. For time matched control experiments, each experiment had various indicated termination points with each time point having its own respective control and XXO treated cells.

Apoptosis detection

Sub-G1 cell fractions containing fragmented DNAs are stained with propidium iodide using protocols previously described to detect apoptosis in the cells [229]. Briefly, the cells were fixed in cold (-20 °C) 70% ethanol, washed with PBS and centrifuged at 250 g. DNA was extracted with 0.2 M Na_2HPO_4 containing 0.004% Triton (Sigma) followed by staining for 1 h at 37 °C with 1 ml PBS containing 20 µg propidium iodide (Sigma) and 200 µg DNAse free RNAse (Sigma). The stained cells were analyzed by flow cytometry; single cells were gated and, the percentage of sub-G1 fraction was quantified. Additionally, in independent experiments, cells were fixed with 4% paraformldehyde and apoptosis was determined using TUNEL assay as described in section 4.2.

SOD activity assay

Total SOD activity was measured using SOD assay kit (Dojindo Molecular Technologies) using manufacturer's protocol. This assay is based on inhibition of color forming reaction between superoxide and water soluble tetrazolium (WST) in the presence of active superoxide dismutase. Briefly, proteins were extracted by lysing the cells overnight at 4 °C using cell lysis buffer (1 mM EDTA, 150 mM NaCl, 10 mM KH2PO4, 10 mM Tris-HCl, 1% NP-40) containing cocktails of protease and, phosphatase inhibitors (Sigma) and stored at -20 °C until further analysis. During the day of analysis, the cell lysates and WST were loaded onto the well of 96 well plate and the absorbance at 450 nm was measured kinetically at 37 °C for 30 min. SOD2 activity was calculated using the same protocol in the presence of 4 mM potassium cyanide which inhibits the activities of SOD1 and SOD3. Since the amount of extracellular SOD inside the cells is minimal [18], the difference between the activities of total SOD and SOD2 is estimated as the activity of SOD1. All the activities were reported after normalizing to the

protein content in the samples. Protein content was measured using micro BCA protein assay kit (Thermo Scientific) according to manufacturer's protocol.

Western blotting

Equal amounts of denatured proteins from cell lysate were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, as described previously[230]. The separated protein were blotted onto a polyvinylidene fluoride (PVDF) membrane using wet transfer procedure as described previously [231]. Protein blotted PVDF membranes were blocked with 4% milk in tris buffered saline, containing 0.1% Tween, and probed with antibodies for SOD1, SOD2, GAPDH or beta-actin (Santa Cruz Biotechnology). The membranes were incubated with horse radish peroxidase-bound secondary antibodies and subjected to chemiluminescence reaction, and the signals were developed and quantified on Kodak Image station 4000 MM PRO and, Carestream MI software.

Real time polymerase chain reaction.

mRNAs were extracted from CPCs using Trizol® reagent (Invitrogen) according to manufacturer's protocol. c-DNAs were synthesized by reverse transcribing the mRNA using Superscript reverse transcriptase (Invitrogen) along with oligo dT and random hexamers as primers. Real time polymerase chain reactions were run on StepOnePlus™ Real-Time PCR System (Applied Biosystems) with SYBR Green PCR master mix (Applied Biosystems) using primers (Appendix) for, SOD1, SOD2, catalase, and GPX1. All the reaction products were normalized to the cDNAs of 18s primers.

siRNA transfection

Lipofectamine RNAiMax (Invitrogen) was used to transfect the cells with specified siRNAs as per manufacturer's protocol. Various duplexes of siRNAs for SOD1, SOD2 gene silencing and non specific scrambled siRNA (Appendix) were obtained from IDT. 10 nM siRNAs were reverse transfected to CPCs using RNAiMax, and the duplexes were tested for si knockdown after 24 h of transfection using RT-PCR. The duplex giving

the best gene silencing was chosen for further experiments. During the experiments, CPCs were transfected with chosen siRNAs and allowed to grow for 48 h before beginning serum starvation and XXO treatment.

Statistics.

All statistical analyses were performed using Graphpad Prism software as described in the figure legends. All data are expressed as mean $\pm$ SEM. p values of less than 0.05 were considered significant.

5.3 Results

Isolation of c-kit positive cardiac progenitor cells from adult rat myocardium

Heart cells were separated from 8-10 weeks old Sprague Dawley rat myocardium after digesting the extracellular matrix. C-kit positive cell population was isolated from the homogenous cell mixture using immunomagnetic separation by incubating with anti-c-kit H-300 antibody that recognize the N-terminal domain (corresponding to amino acids 23-322) of c-kit receptors on the cell membrane. The cells bound to the magnetic beads were cultured for about three weeks, and the colonies were subsequently expanded. Immunocytochemistry followed by flow cytometric analysis show that the isolated cells are >90% c-kit positive (Figure 5.1). Additionally, more than 80% of the population was positive for MDR-1, Sca-1, CD-34 and early cardiac transcription factors Nkx-2.5 and GATA-4 (Figure 5.1). However, these cells were negative for CD-45 (Figure 5.1). Analysis of these cells from passage number four to fifteen shows that, during population doublings, the percentage of C-kit, MDR-1, Sca-1, CD-34, Nkx-2.5 and GATA-4 remain greater than 70% (data not shown). For subsequent experiments, CPCs were used from passage 5 to passage 14. In addition, c-kit positive clonal cells can be expanded (Figure 5.1) from the CPCs following serial dilution procedure.

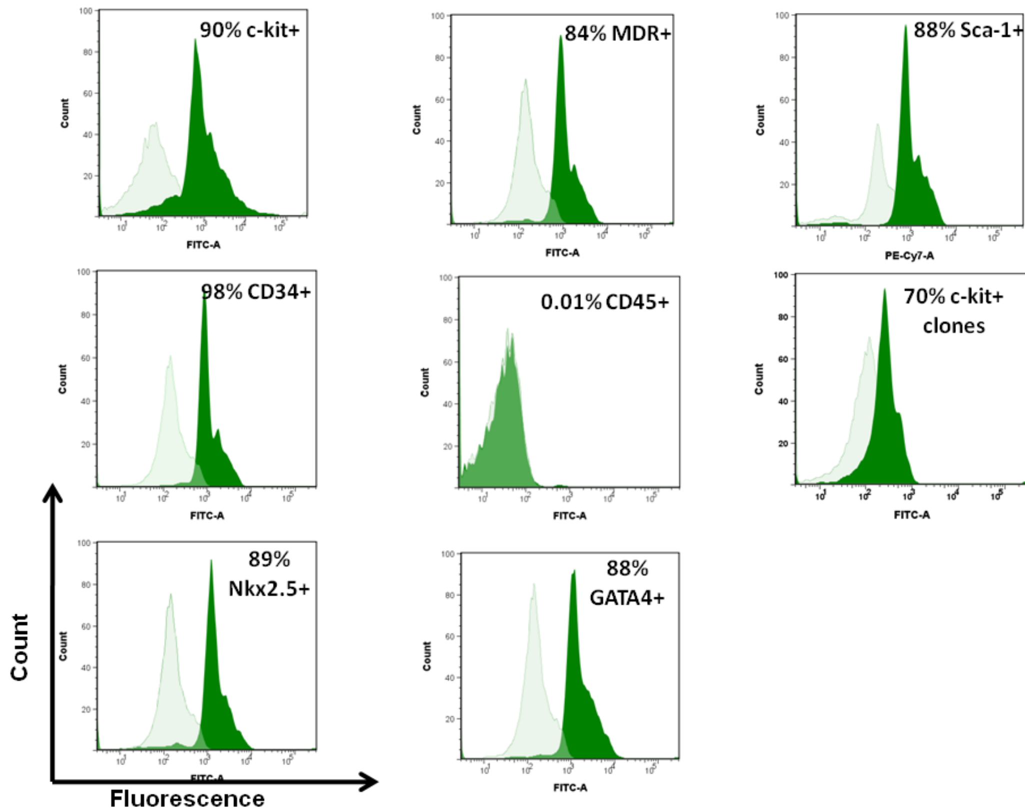

Figure 5.1. Isolation of c-kit positive CPCs from rat myocardium. Adult male Sprague-Dawley rat hearts were subjected to enzymatic digestion and incubated with c-kit coated magnetic beads. Cells attached to the beads were cultured and expanded. Flow cytometric analysis showed that the isolated cells were characterized by the presence of c-kit, Sca-1, MDR1 and CD-34 and by the absence of CD-45. They also expressed early cardiac transcription factors Nkx2.5 and GATA4. Additionally the clonal cells also expressed c-kit markers.

CPCs are more resistant to oxidative stress induced apoptosis

In our preliminary experiments, CPCs were subjected to xanthine/xanthine oxidase (XXO) or lumazine/xanthine oxidase induced oxidative stress. Various concentrations of xanthine (200 µM to 2.5 mM) were added to CPC treatment media with or without 10 mU/ml xanthine oxidase (XXO). Following 10 h of oxidative stress, DNA of

CPCs were stained with propidium iodide (PI) and the sub-G1 DNA fractions in the cells were quantified using flow cytometry. At various substrate concentrations of xanthine, there was no significant change in the sub-G1 fractions between control and XXO treated CPCs (Figure 5.2 A). Replacing xanthine with lumazine as a substrate to

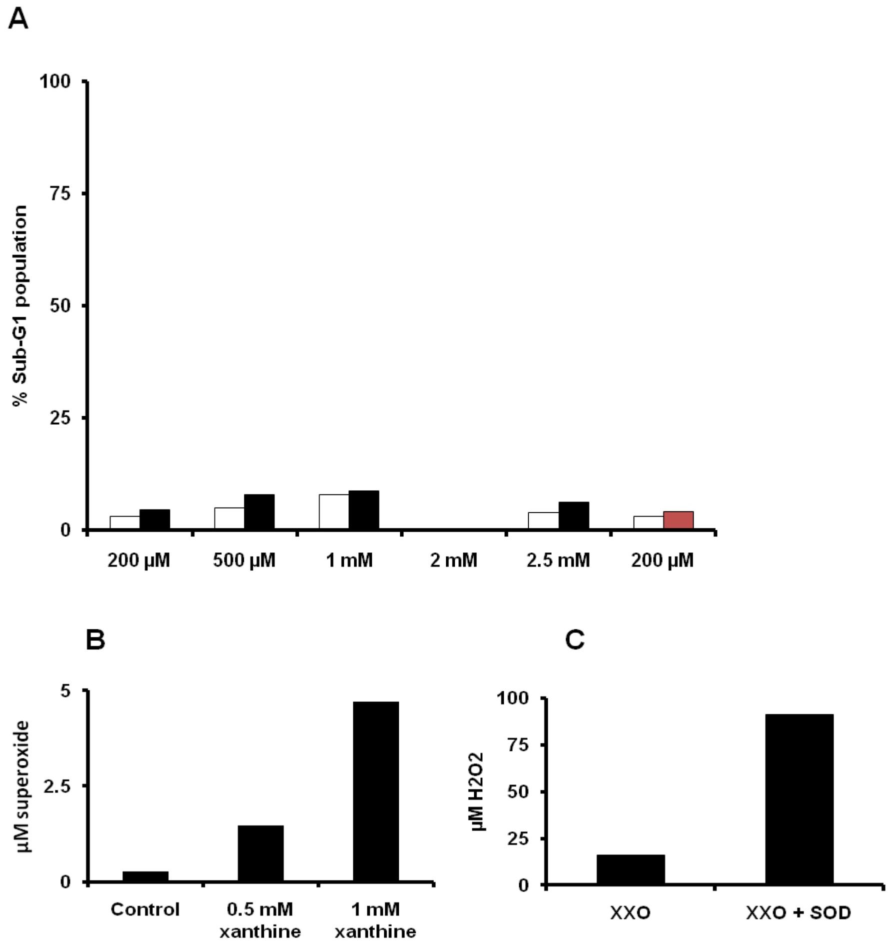

Figure 5.2. Effect of XXO treatment on CPCs. (A) Analysis of sub-G1 fraction of CPC DNA following propidium iodide staining show that following 10 h of treatment of CPCs with 10U/ml of xanthine and varying concentrations of xanthine or 200 µM of lumazine (brown bar) did not induce cell death compared to control cells without XXO treatment. (B) DHE based HPLC quantification of superoxide in 0.5 or 1 mM xanthine treatment media 15 h after 10 mU/ml XXO addition. (C) H_2O_2 analysis using Amplex red assay using XXO media with and without SOD shows that about 75 µM of superoxide flux is generated in the treatment media within 15 h.

increase the flux of superoxide generation had no effect on sub-G1 fractions (brown bar in Figure 5.2 A). In our subsequent studies, treatment media containing 1mM xanthine, 10 U/ml catalase and 10 mU/ml XXO was used to generate the oxidative stress. In those conditions, DHE based HPLC analysis showed the presence of superoxide in the media even 15 h after XXO addition (Figure 5.2 B). In addition, preliminary studies with amplex red assay show that around 75 μM of superoxide flux was generated over 15 h using this substrate/enzyme concentration (Figure 5.2 C).

Subsequently, using this treatment condition, we compared oxidative stress induced apoptosis in CPCs and neonatal cardiomyocytes and analyzed the data using two-way ANOVA. Similar to preliminary observations, CPCs had no significant difference in the sub-G1 fraction between 12 h of XXO treated cells and time matched controls. In stark contrast, myocytes subjected to the same treatment conditions had 25 % more sub-G1 fractions compared to the control cells (Figure 5.3 A). Due to the differences in basal cell death between the cell types, apoptosis was also quantified using TUNEL assay in independent experiments conducted using same the treatment conditions. CPCs had about 15% basal cell death, and addition of XXO did not increase the percentage of TUNEL positive cells. However, addition of XXO in myocytes significantly increases ($p<0.05$) its TUNEL positive cell count by 80% compared to control (Figure 5.3 B).

CPCs have higher basal SOD levels compared to HUVECS and myocytes

CPCs, HUVECs, and myocytes were cultured to 80% confluence; quiesced overnight and the basal SOD activities in the cell lysates were assayed using water soluble tetrazolium based assay. The SOD1 activity in CPCs was about 3.8 U per mg of protein (Figure 5.4 A). However, the activity level of SOD1 in both HUVECs and neonatal cardiomyocytes were two fold lower ($p<0.01$) compared to CPCs (Figure 5.4 A). Similarly, the basal activity levels of SOD2 followed the same trend in these three cell

types. While CPCs had about 0.3 U of SOD2 activity per mg of protein, the activity

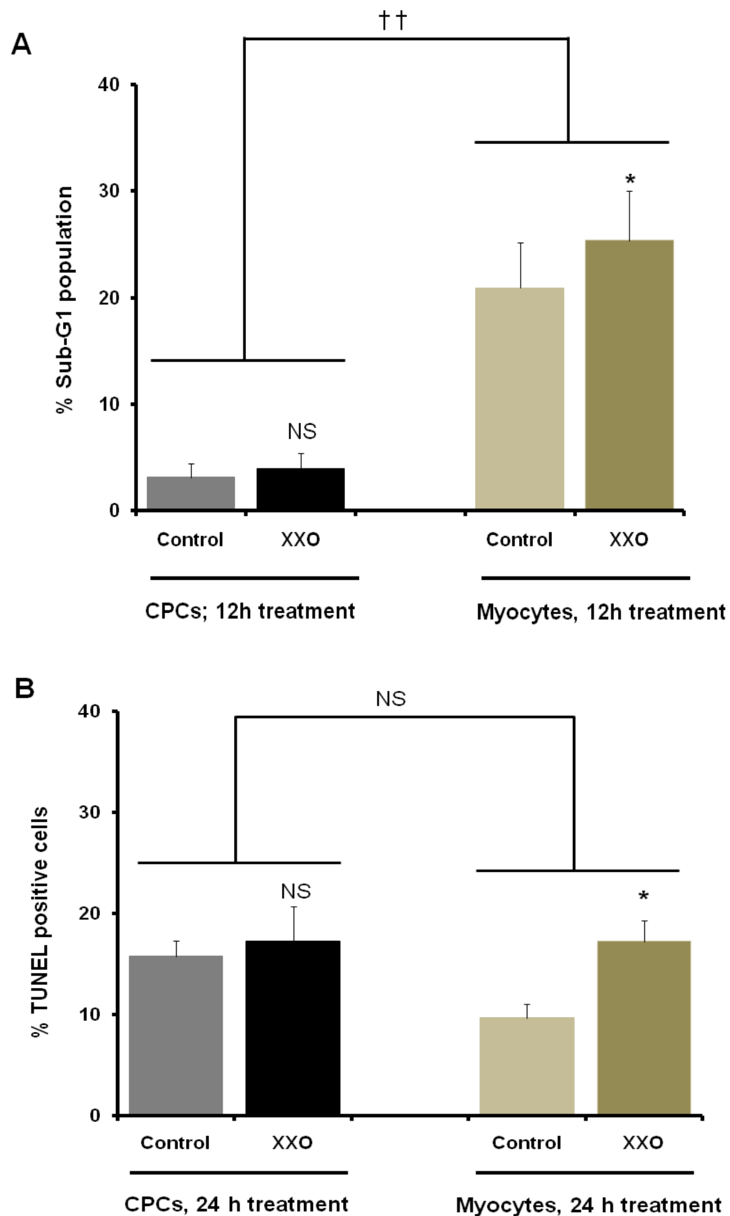

Figure 5.3. CPCs are resistant to apoptosis following XXO treatment compared to myocytes. (A) PI staining shows that only myocytes and not CPCs have significantly higher apoptosis following 12 h of XXO treatment compared to control. (B) TUNEL staining shows that only myocytes and not CPCs have significantly higher apoptosis following 24 h of XXO treatment compared to control. n=4, *$p<0.05$ vs. control, ††$p<0.01$ between cell types, two-way ANOVA followed by Bonferroni post test.

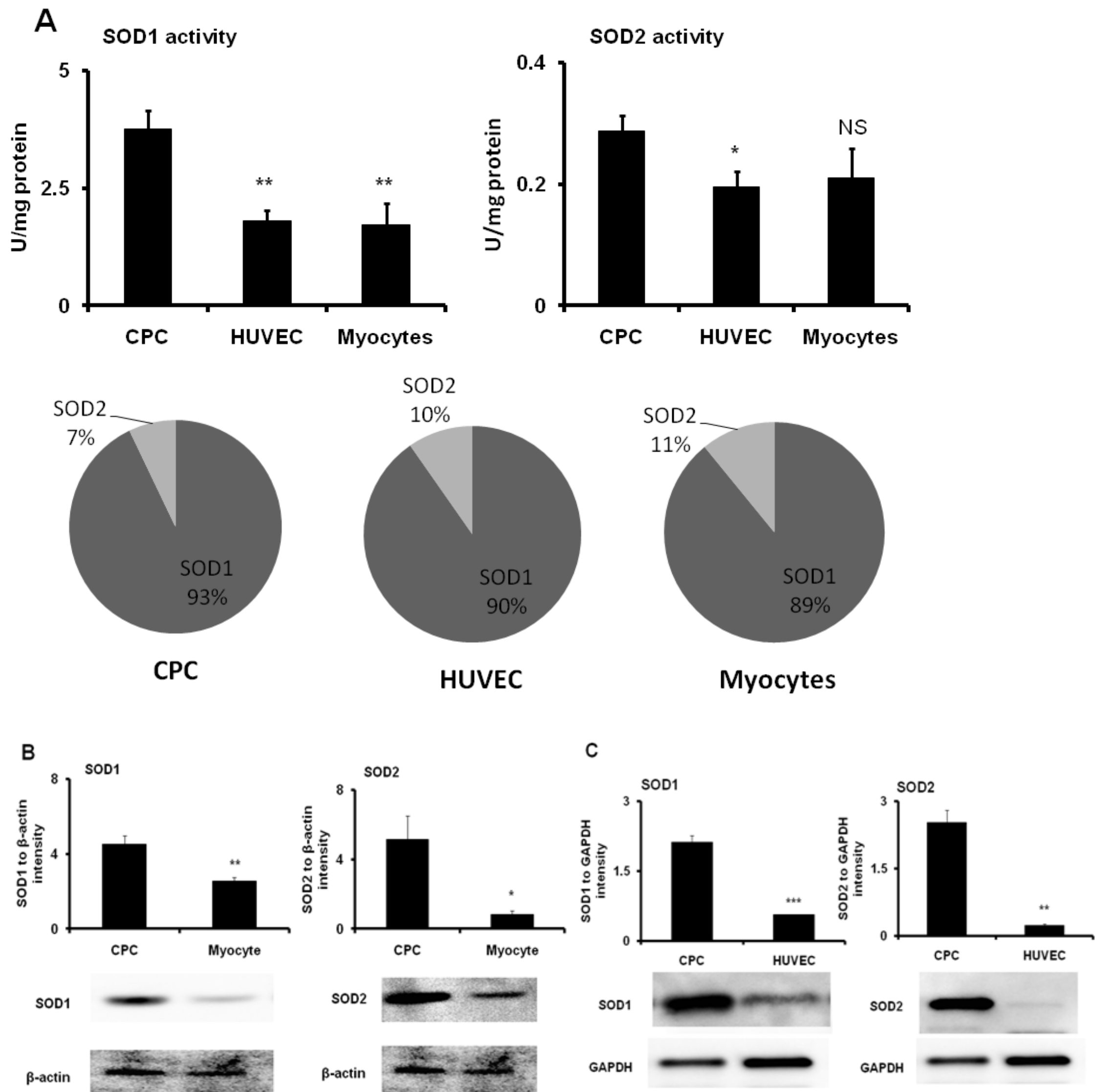

Figure 5.4. CPCs have higher basal SOD activity and protein levels compared to myocytes and HUVECs. (A) Quiesced CPCs have higher SOD1 (left panel) and SOD2 (right panel) activity compared to quiesced HUVECs and myocytes. n≥4, **p<0.05 vs. CPC, *p<0.05 vs. CPC, ANOVA followed by Dunnett's post test. Pie chart shows that in all the three cell types, 90% of total SOD activity is due to SOD1 and the remaining is due to SOD2. Western blot analysis shows that CPCs have significantly higher basal SOD1 and SOD2 protein levels normalized to β-actin vs. myocytes (B) and normalized to GAPDH vs. HUVECs. n≥4, *p<0.05 vs. CPC, t-test

levels of SOD2 were about 30% less ($p<0.05$) in both HUVECs and myocytes compared to CPCs (Figure 5.4 A). In these three cells, only about 10% of total SOD activity is due to SOD2, while 90% of total activity is due to SOD1 (Figure 5.4 A, pie charts). In addition to the activity levels, the basal protein levels of both SOD1 and SOD2 were analyzed semi-quantitatively using western blotting technique. Figure 5.4 B shows that the SOD1 and SOD2 protein levels in myocytes were significantly less ($p<0.01$ and $p<0.05$ respectively) compared to CPCs. In the same fashion, HUVECs had significantly less protein levels of both SOD1 and SOD2 ($p<0.001$ and $p<0.01$, respectively) compared to CPCs (Figure 5.4 C).

XXO treatment increases the expression of SOD mRNAs

RT-PCR was conducted to investigate the antioxidant gene expression of CPCs subjected to XXO treatment. The experiment was conducted as single end point experiment where cells without XXO addition act as control for other treatment groups (Figure 5.5 A). After 3 hours of XXO addition, the expression of SOD1 mRNA increases ($p<0.01$) by 1.75-fold and at later points, the expression returned back to control levels (Figure 5.5 B). In a similar manner, after 6 h of XXO addition, SOD2 mRNA expression was significantly ($p<0.05$) increased by 1.70-fold compared to control (Figure 5.5 B). However, the mean catalase gene expression level did not vary among the treatments following XXO addition (Figure 5.5 C). There was a trend toward an increase (1.3-fold) in catalase mRNA levels after 6 h of XXO addition though it was not statistically significant (Figure 5.5 C). Dunnet's comparison of GPX1 mRNA level of control cells with the treatment group 6 h after XXO addition shows a significant ($p<0.05$) 1.6-fold increase (Figure 5.5 D). However, the mean GPX1 gene expression levels among all the treatment groups remained the same with no significant difference.

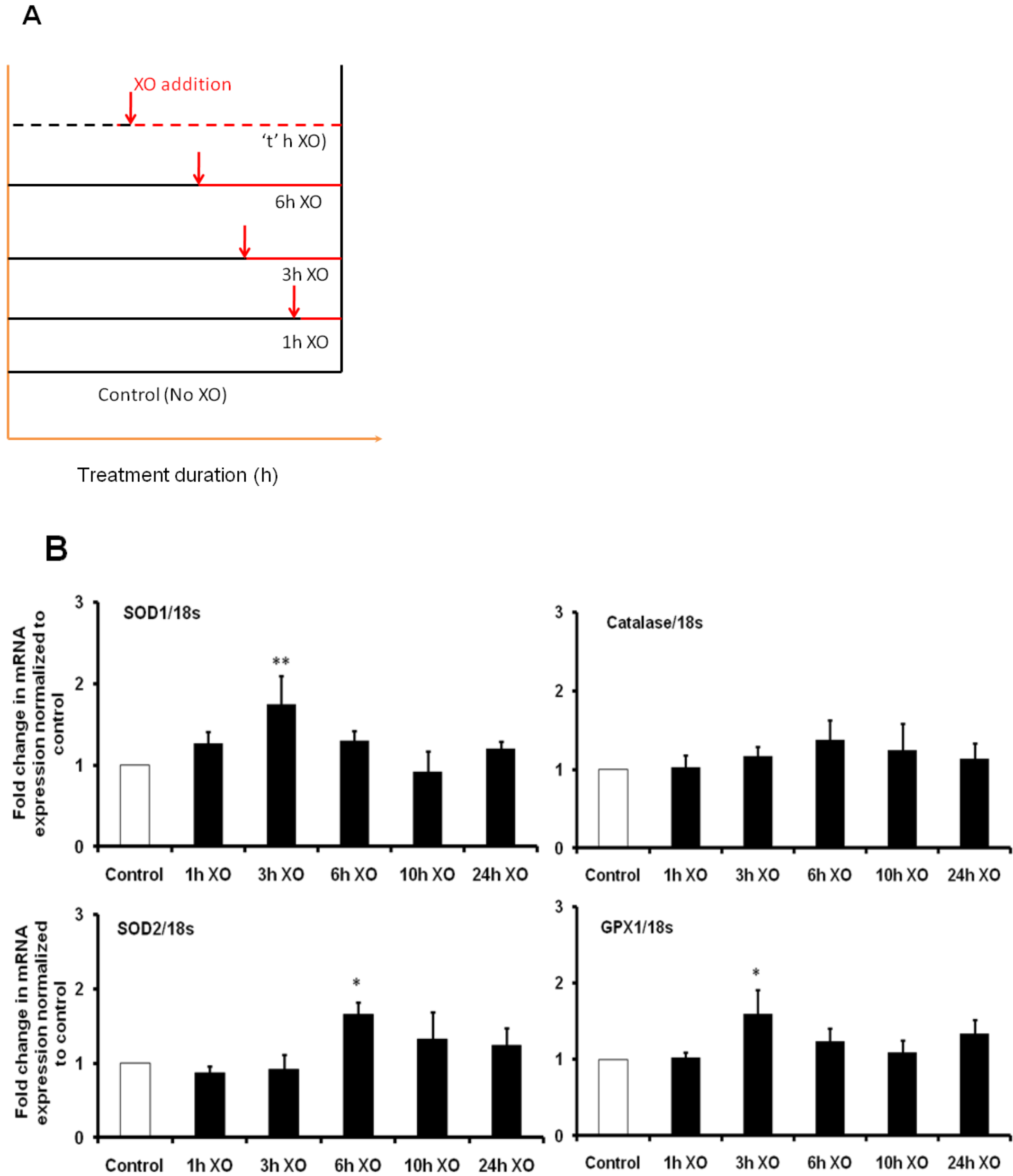

Figure 5.5. Effect of XXO treatment on antioxidant enzyme expression in CPCs. (A) In single end point experiments, all groups were subjected to same treatment duration in media containing xanthine but differing in the duration of XXO added 't' hours before the end of the experiment. (B) SOD1, SOD2 and GPX1 mRNA expressions are significantly increased following XXO addition compared to control cells without XXO. n≥7, ANOVA followed by Dunnett's post test, *$p<0.05$ vs. control, **$p<0.01$ vs. control.

SOD activity increases in CPCs after XXO treatment

To investigate the changes in total SOD activities in CPCs following XXO treatment, the cells were cultured to 80% confluence and quiesced overnight. Subsequently during the 48 h treatment of CPCs, they were subjected to various durations of oxidative stress by the addition of XXO. Total SOD activity in the cell lysate dropped significantly ($p<0.01$) by 50% within six hours of XXO addition (Figure 5.6 A). 12 h after the addition of XXO, the activity remained 40% lower ($p<0.05$) than control levels. Gradually, this drop of total SOD activity was compensated back to control levels by 24 h following XXO addition and with increasing duration of the treatment (48 h after XXO addition), there is a trend of 30% higher SOD activity compared to control cells (Figure 5.6 A). In contrast, when the SOD response of neonatal cardiomyocytes was investigated, their total SOD activity did not drop after XXO treatment. Instead, they had a trend of gradual increase in the SOD activity level following XXO treatment (Figure 5.6 B). When both CPCs and myocytes were compared using two-way ANOVA for their total SOD activity 48 h after XXO addition, they did not differ from each other in their SOD response. Both CPCs and myocytes had significantly higher ($p<0.0001$ and $p<0.01$ respectively) SOD activity 48 h after XXO addition, compared to their respective controls (Figure 5.6 C).

In addition to total SOD activity, SOD1 and SOD2 activities were also measured in CPCs with time-matched controls to understand the changes in the variations in basal SOD activities during the treatment. Two-way ANOVA analysis of the data shows that the basal SOD1 activity of the control group dropped by 40% within 48 h. However, this drop was not statistically significant (Figure 5.7 A). However, following 48 h of XXO treatment in CPCs, SOD1 activity increases significantly ($p<0.001$) by 1.4-fold compared to its time matched control (Figure 5.7 A). Basal SOD2 remains similar to control levels during the treatment (Figure 5.7 B). Further, similar to changes in SOD1 activity, SOD2

activity also increases significantly (p<0.01) by about 2-fold compared to the time matched controls of CPCs 48 hours after XXO addition (5.7 B).

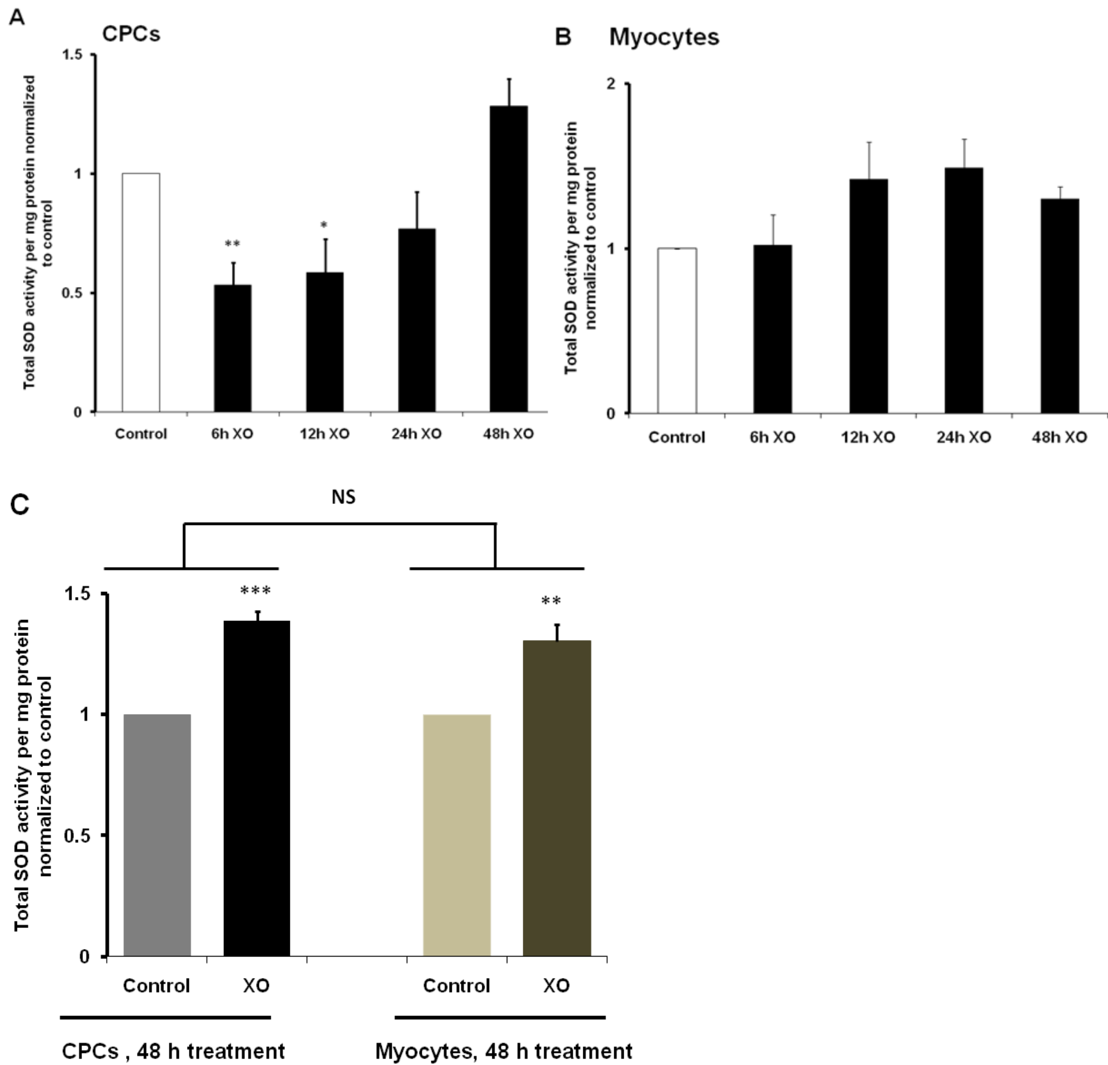

Figure 5.6. Effect of XXO treatment on total SOD activity of CPCs. (A) Total SOD activity of CPCs drops significantly within 6 h compared to control. However, it compensates back as XXO treatment duration is increased. n≥5, *p<0.05 vs. control. ANOVA followed by Dunnets post test. (B) Total SOD activity variation in myocytes following XXO treatment. (C) Two-way anova of CPCs and myocytes show that by 48 h of XXO treatment, total SOD activity of both these cell increase significantly. n=4, NS not significant, ***p<0.0001, **p<0.01 vs. respective control.

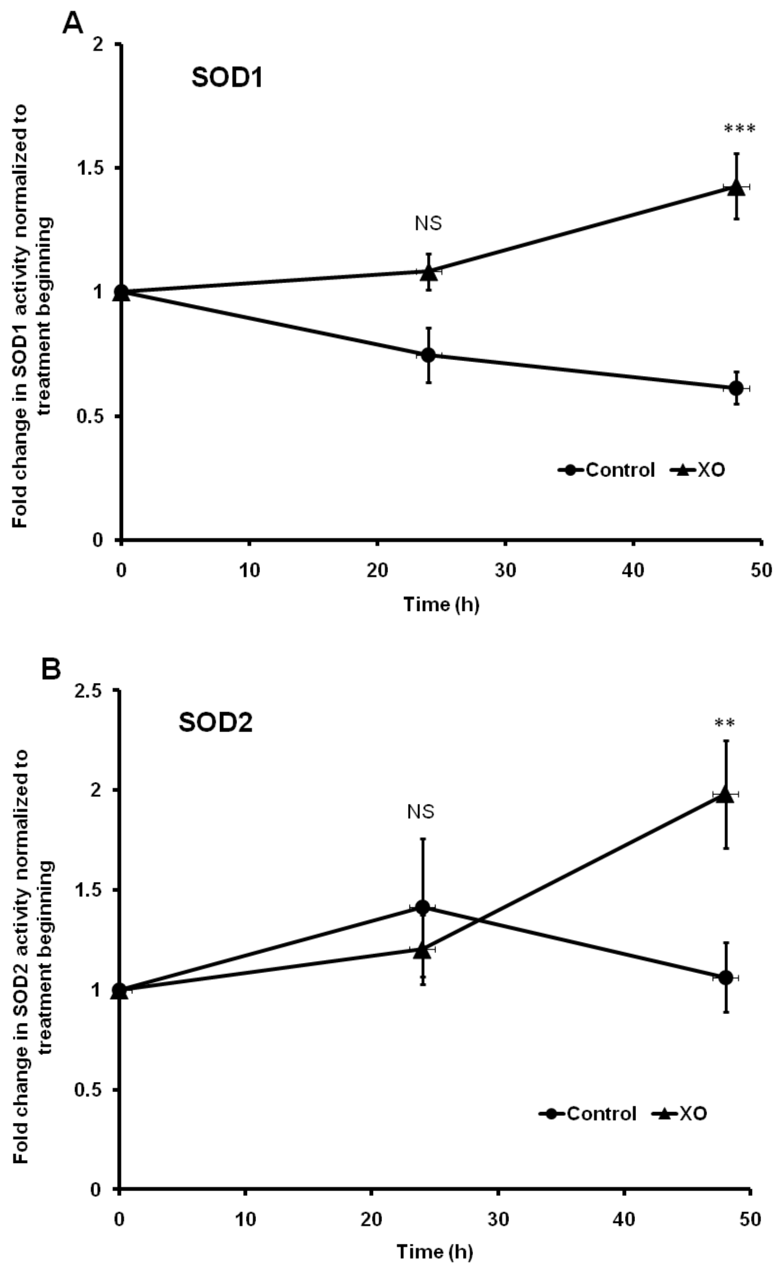

Figure 5.7. Effect of XXO treatment on SOD1 and SOD2 activities of CPCs. Both SOD1 and SOD2 activities represented as fold change normalized to treatment beginnings are significantly increased within 48 h following XXO treatment compared to its time matched controls. n≥4, Two-way ANOVA followed by Bonferroni post test, **p<0.01, ***p<0.001 vs respective time matched controls

SOD2 protects CPCs from apoptosis following XXO treatment

CPCs were subjected to siRNA-induced gene silencing experiments. Following siRNA screening, two siRNAs that give selective and efficient silencing of SOD1 and SOD2 expression were selected. After 24 h of transfection with SOD1 siRNA, the copy numbers of SOD1 mRNA were reduced by 95% compared to transfection-alone control (Figure 5.8 A). SOD2 mRNA levels were not significantly affected due to SOD1 siRNA transfection (Figure 5.8 A). In a similar manner, transfection of CPCs with SOD2 siRNA reduced the expression SOD2 mRNA levels by 95% compared to transfection-alone control, while the copy levels of SOD1 mRNA were unaffected (Figure 5.8 B). Following 48 h of siRNA transfections, TUNEL assays were conducted in the presence or absence of XXO, to investigate the oxidative stress induced apoptosis in CPCs. Results from duplicated experiments show that, 48 h after the tranfection of scrambled siRNA, the percentage of TUNEL positive CPCs did not increase basally nor following 12 h of XXO treatment, compared to its own time matched controls (Figure 5.8 C). SOD1 siRNA transfection showed a trend of increased percentage of TUNEL positive cells following XXO treatment. However, the variability was high between the experiments, and therefore, was not statistically significant (Figure 5.8 C). Interestingly, XXO treatment induces a significant ($p<0.05$) 2.5-fold increase in the percentage of TUNEL positive cells, following SOD2 siRNA treatment, with no effect on basal cell death (Figure 5.8 C).

SOD levels in human CPCs and effects of XXO treatment

Human CPCs (hCPCs) were obtained from myocardial biopsies and two subtypes of hCPCs were cultured: young hCPC phenotype containing IGF1 receptors (IGFR+ hCPC) and, senescent hCPCs containing lower percentage of IGF1 receptors (IGFR- hCPC) [232]. In order to determine the SOD activities levels in hCPCs, the cells were quiesced overnight and the proteins were extracted using lysis buffer. There was about 1.8 U of basal SOD1 activity per mg of protein in IGFR+ hCPCs (Figure 5.9 A).

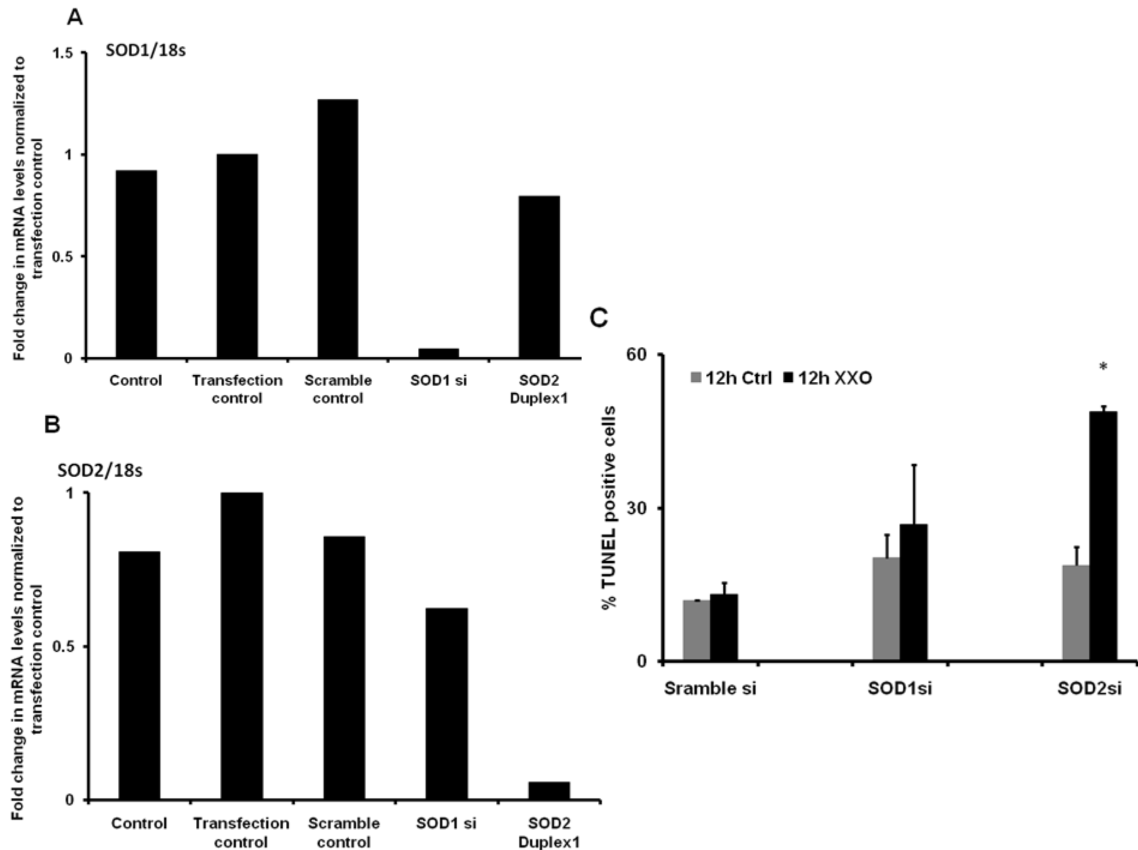

Figure 5.8. Effect of XXO treatment on siRNA induced gene silencing of SOD1 and SOD2 in CPCs. (A) SOD1 si but not SOD2 si reduces the expression of SOD1 mRNA normalized to 18 mRNA in CPCs. (B) SOD2 si but not SOD1 si reduces the expression of SOD2 mRNA normalized to 18 mRNA in CPCs. (C) 12 h of XXO treatment following 48 h of si RNA transfection show that the percentage of TUNEL positive cells increases significantly only in SOD2 silenced CPCs. n=2, two-way ANOVA followed by Bonferroni post test.

Although, this was not significantly different from the SOD1 activity of IGFR- hCPCs (1.13 U/mg protein), the activity was about 60% higher than the SOD1 activity of IGFR- hCPCs. On the other hand, the basal SOD2 activity of IGF- hCPCs was about 0.1 U/mg protein which is significantly ($p<0.01$) lower than the SOD2 activity of IGF+ hCPCs (0.22 U/mg protein) (Figure 5.9 B).

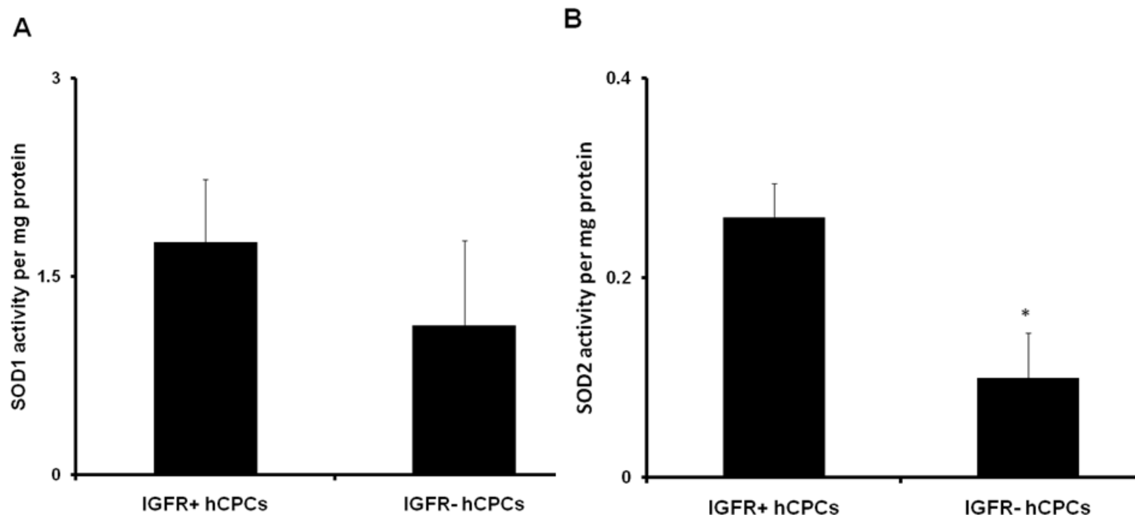

Figure 5.9. Basal SOD levels in human CPCs. (A) Basal SOD1 activity of both IGFR+ hCPCs and IGFR- hCPCs did not differ significantly from each other (B) SOD2 activity of IGFR- hCPCs are significantly lesser than SOD2 activity of IGFR- hCPCs. n≥4, unpaired t-test, *p<0.05 vs. IGFR+ hCPC.

When both IGFR- and IGFR+ hCPCs were subjected to similar oxidative stress levels as that used for treating rat CPCs (1 mM xanthine and 10 mU/ml XXO), >90% the cells died within 6 hours of treatment (data not shown). Therefore, hCPCs were subjected to treatment with 500 µM xanthine and 5 mU/ml XXO for six hours and the apoptosis was measured using TUNEL assay. There was a significant difference (p<0.01) between the cell types. IGFR+ hCPCs had lower basal cell death compared to IGFR- hCPCs. Following the addition of XXO, there was no significant increase in TUNEL count in IGFR+ hCPCs, compared to its own control (Figure 5.10 C). Similarly, TUNEL count was not significantly increased in IGFR- hCPCs following XXO treatment. However, the data show a trend of increased cell death in IGFR- cells following XXO addition (Figure 5.10)

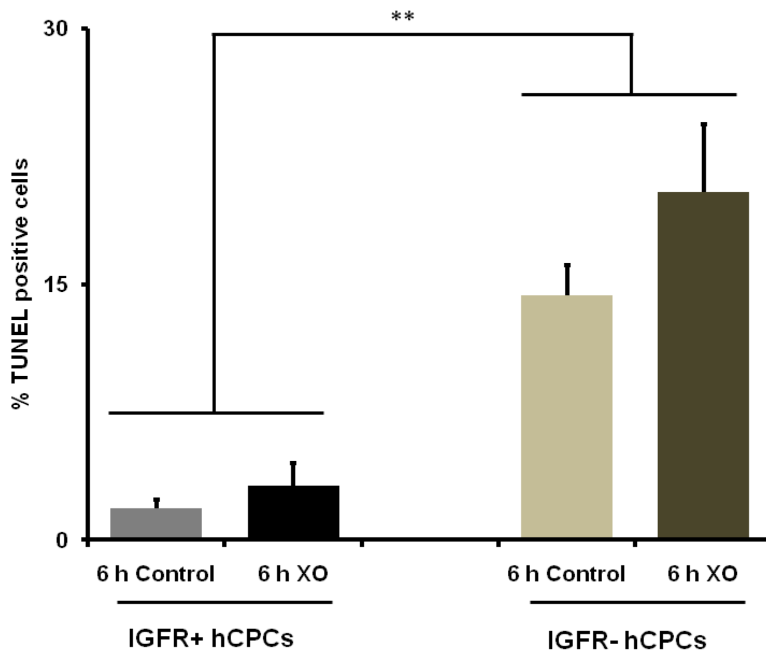

Figure 5.10. Apoptosis in human CPCs following XXO treatment. IGFR- hCPCs have higher basal cell death after 6h treatment with or without 500 μM xanthine and 5 mU/ml XO treatment following serum starvation. n=4 patients, two-way paired ANOVA followed by Bonferroni post test, **p<0.01 between cell types.

5.4 Discussion

Endogenous c-kit positive cardiac progenitors are a promising cell type for myocardial regenerative therapies. They have robust cardiovascular differentiation abilities and direct intramyocardial delivery of these cells structurally integrates and improves the performance of rat myocardium [221]. In addition, autologous CPCs can be obtained from human myocardial biopsies [21, 223]. Therefore, in vitro expansion and transplantation of cardiac progenitor cells is a potential therapeutic strategy to regenerate the myocardium. However, one of the major roadblocks of cell transplantation therapy is survival and engraftment of cells in the ischemic myocardium.

Oxidative stress is increased following an ischemic event and the survival, senescence, and differentiation of stem/progenitor cells are affected by intracellular ROS levels [233, 234]. Further indirect evidences suggest the vulnerability of CPCs to oxidative stress [196]. The findings in this chapter indicate that rat CPCs have higher amount of basal SODs compared to adult myocytes and endothelial cells. Further, rat CPCs are more resistant to oxidative stress-induced cell death compared to myocytes. In addition, our studies show that basal SOD levels were higher in healthy rat CPCs compared to human CPCs isolated from unhealthy patients and thus may not make the optimal test bed for in vitro testing. Additionally, we also found significant differences in SOD levels between young and senescent phenotypes of human CPCs.

Various cell surface markers are used in the isolation of cardiac stem cell population [235]. The population identified by the presence of c-kit markers has robust cardiovascular differentiation ability [10]. Therefore, we isolated c-kit positive cells expressed in the myocardium using an immunomagnetic method. The current isolation yielded a population that is greater than 90% c-kit positive and the percentage vary from 60 to 90% from passages 4 to 15. Although, the exact reason for variation in the percentage of c-kit expression is not known, c-kit receptors are known to undergo ligand induced dimerization and internalization [236]. C-kit expression is essential for the promotion and regulation of cardiac stem cell differentiation [237]. However, lower percentage (20%) of c-kit expression does not reduce its cardiogenic potential [221]. The c-kit expression is not exclusive to cardiac stem cells. C-kit is a receptor tyrosine kinase identified after a series of studies conducted in white spotting loci and steel loci of mice [238]. These receptors are also found in many other progenitors including hematopoietic lineage and mature cell lines such as mast cells, astrocytes, sweat glands and, breast glandular epithelial cells [239]. Therefore, the c-kit positive cells were tested for the presence of other markers such as Sca-1, MDR-1 and CD-34 that are reported to

exist in cardiac stem cells and also tested for its clonability [235]. Our analysis shows that the isolated cells are clonable and are positive for cardiac stem cell markers Sca-1, MDR-1 and CD-34 and negative for hematopoietic marker CD-45. Additionally, the cells used in this study were positive for the expression of early cardiac transcription factors such as Nkx-2.5 and GATA-4. Therefore, they were termed as cardiac progenitor cells instead of cardiac stem cells that lack the expression of these transcription factors [10].

Xanthine/xanthine oxidase (XXO) is a physiologically relevant system to generate reactive oxygen species – particularly superoxide and hydrogen peroxide [240]. Our preliminary experiments confirmed the generation of free radicals in the treatment media following XXO addition. Further, XXO is known to induce apoptosis in many cell types [241, 242]. Therefore, in our studies, XXO was used to investigate the effects of oxidative stress on CPC apoptosis. Following propidium iodide staining of the DNA, increased fractions of fragmented DNA, generated due to cell death, can be observed behind the diploid peak of normal, non-fragmented DNA. Quantification of this hypodiploid or sub-G1 fraction gives a simple estimate of what are believed to be apoptotic cells [229]. While previous reports show apoptosis with 100 μM xanthine and 10mU/ml XXO in HUVECs [241], our PI staining studies in CPCs show that there is no difference in the sub-G1 fraction between XXO treated cells and control cells even at higher xanthine concentrations. This interesting observation prompted us to compare the oxidative stress induced apoptosis in CPCs and neonatal cardiomyocytes isolated from rat pups. Independent apoptosis quantification using PI staining and TUNEL assay show increased apoptosis in myocytes following XXO treatment with no effect of XXO in CPCs. The data in Figure 5.3 A also show differences in basal cell death with propidium iodide. In order to make legitimate comparisons between cells, both cell subtypes were quiesced in serum-free conditions. It is likely that primary neonatal myocytes, being a terminally differentiated cell, are more sensitive to serum depletion in this assay that

may measure global cell death; however, TUNEL assays confirmed the effect of XXO on apoptosis.

SODs are one of the major endogenous antioxidant enzymes providing first line protection against oxidative stress. For example, higher SOD2 and catalase levels in circulating progenitors protect them from oxidative stress induced apoptosis compared to related cell types [139]. In addition, enhanced expression of antioxidant genes is considered one of the ‘stemness’ trait in many progenitor cells [243]. Therefore, the basal SOD protein and activity levels of CPCs were compared with other adult cardiac cell types. Interestingly, CPCs had significantly higher protein levels of both SOD1 and SOD2 enzymes compared to HUVECs and myocytes suggesting this as a mechanism by which CPCs were protected from oxidative stress, unlike cardiomyocytes. SOD activity assays confirmed Western analysis, though SOD2 levels were not significantly lower in cardiomyocytes compared to CPCs, despite the large difference in protein levels. As SOD2 activity is dependent on other co-factors, in addition to protein levels (Mn, for example), this could be an area for future study.

Oxidative stress is known to have a modest effect on the expression of antioxidant enzymes in many cell types such as epithelial cells and fibroblasts [244-246]. Therefore, the effect of oxidative stress on the antioxidant enzyme gene expression in CPCs was investigated. SOD2 is known to be regulated by external factors while SOD1 is often constitutively expressed in various cell types [247]. RT-PCR analysis of CPC mRNAs showed that XXO treatment induced about 2-fold higher expressions of SOD1, SOD2 and GPX1 mRNAs in 3-6 hours, perhaps suggesting a compensatory mechanism. SOD proteins are known to undergo proteasome-induced degradation [248]. Further their levels drop within 2 hours of oxidative stress [249]. SOD activities measured in CPCs show that the cells lose about 50% of total SOD activity within six hours of oxidative stress. In contrast, myocytes did not exhibit this drop in activity after

oxidative stress. However, within 48 h of oxidative stress, both CPCs and myocytes had significantly higher total SOD activity levels, compared to control cells. Additionally, 2-way ANOVA conducted on CPCs with time matched controls demonstrate significantly higher activity of SOD1 and SOD2 following 48 h of XXO treatment suggesting that the increased SOD mRNA expression following oxidative stress may have led to the generation of new SOD protein.

To determine if enhanced SOD levels were responsible for the resistance of CPCs to oxidative stress-induced apoptosis, siRNA based gene silencing experiments were conducted to decrease the expression of SOD1 and SOD2 mRNAs in CPCs. While decreased SOD1 had no significant effect on protection against XXO-induced apoptosis, our results showed that by decreasing the expression of SOD2, CPCs had significantly higher apoptosis following XXO treatment. This result is interesting given that total SOD2 activity is only 10% of total activity is CPCs. The result also implicates the importance of maintaining the mitochondrial homeostasis and oxidative stress level in CPCs.

Recently, CPCs isolated from human cardiac biopsies have demonstrated the presence of young and senescent phenotypes of cardiac stem cells marked by the presence or absence respectively of insulin like growth factor receptor 1 (IGFR1) [232]. Previous studies and our studies show that, following serum starvation, IGFR+ hCPCs have lower basal apoptosis compared to IGFR- hCPCs. Serum deprivation induces oxidative stress [250] and our siRNA studies suggest that, in rats, SOD2 levels can provide resistance to oxidative stress induced apoptotis. Therefore, basal SOD1 and SOD2 levels of IGFR+ and IGFR- hCPCs were determined. IGFR+ hCPCs had higher SOD1 levels than IGFR- cells. While this was a 60% increase, it was not statistically significant. More interestingly, IGFR- hCPCs had significantly less SOD2 levels compared to IGFR+ hCPCs. These results suggest the importance of endogenous SOD

levels especially SOD2 in offering protection to human CPCs during oxidative stress. Further studies on the role of these enzymes in CPCs could potentially help in improving the survival of these cells during transplantations.

CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

6.1 Summary and conclusions

The motivation of this dissertation work was to identify new treatment options or improve the existing options to treat myocardial diseases. Pharmacotherapeutic options to treat the post-ischemic myocardium do not shown significant, long term benefits. Therefore, cell based therapies and alternate therapeutic strategies such as delivering bioactive molecules including micro-RNAs and proteins are being investigated [11]. Further, oxidative stress is increased in ischemic heart disease and antioxidant status in and around the cells of the myocardium could affect both physiology and pathophisiology of the myocardium [70]. In the third chapter of this work, our experimental findings on the potential of a recently invented PCADK polymer to deliver SOD1 protein intracellularly are presented. In the fourth chapter of this dissertation, the effects of PKSOD delivery to the ischemic myocardium were investigated. In the fifth chapter, studies conducted on antioxidant status of CPCs and the effects of oxidative stress on the apoptosis of CPCs are presented.

Intracellular SOD1 delivery

Delivery of SOD1 to inside of the cells remains difficult. Using a double emulsion – solvent evaporation method, SOD1 encapsulated PCADK (PKSOD) microparticles were made. Our HPLC based quantification of superoxide specific DHE products showed that free SOD1 delivery scavenged only extracellular superoxide; however, it was not adequate to scavenge the superoxide within the cells. Pre-incubation of RAW macrophage cells with PKSOD reduced both intracellular and extracellular superoxide within the cells. The key finding in the third chapter of this dissertation was that micron-

sized PKSOD particles efficiently scavenged both extracellular and intracellular superoxide generated following PMA stimulation in cultured macrophages.

Sustained SOD1 delivery to the myocardium following IR

Superoxide anion radicals are implicated in reperfusion injury and eventual myocardial dysfunction [74]. Although SOD1 helps in scavenging the superoxide, the delivery of this enzyme is difficult due to its poor plasma half life. Therefore, SOD1 was encapsulated within micron sized PCADK particles and delivered to the rat myocardium following IR. PKSOD particles were found to remain in the myocardium even a week after the particle injection, indicating the potential for sustained delivery. Additionally, preliminary cytochrome c assay and DHE staining showed that superoxide levels were less in PKSOD treated myocardium following IR. Free SOD1 infusion at low doses is known to reduce infarct size [98] and our published study demonstrated that infarct size was reduced by both free SOD1 and PKSOD administration. However, apoptosis after IR was reduced significantly only by PKSOD treatment. In addition, only PKSOD treatment improved acute cardiac function. There was neither significant chronic functional improvement nor reduction of fibrosis in the myocardium due to PKSOD treatment. Published studies from our lab demonstrated that sustained delivery of a p38 inhibitor (PK-$p38_i$) significantly improved long-term function and reduced fibrosis [19]. We found that a combination treatment with PKSOD and PK-$p38_i$ improved both the acute and chronic cardiac function. No significant changes were observed in angiogenesis, or in the gene expression of major antioxidants and, calcium handling proteins following PKSOD treatment. Additionally, PKSOD did not induce significant changes in MMP activity following IR. The main finding in the fourth chapter of this thesis is that early PKSOD delivery to the rat myocardium restores the acute cardiac function following myocardial ischemia-reperfusion. The study also shows that PKSOD reduces apoptosis in the ischemic myocardium.

Antioxidant and apoptotic response of CPCs subjected to oxidative stress

CPCs are promising a promising candidate for cell based approaches to repair the myocardium due to their cardiovascular lineage differentiation ability [222]. While clinical trials are underway, many of their basic properties remain unknown. In this study, c-kit positive cardiac progenitor cells were isolated from adult rat myocardium using an immunomagnetic isolation technique. The isolated cells were negative for hematopoietic stem cell marker CD-45 but positive for cardiac progenitor cell markers such as c-kit, Sca-1, MDR-1 and CD-34. They also expressed early cardiac transcription factors such as Nkx2.5 and GATA4. Propidium iodide and TUNEL staining showed that the isolated CPCs did not undergo apoptosis following xanthine/xanthine oxidase (XXO)-induced oxidative stress. In contrast, XXO treatment induced apoptosis in myocytes. Analysis of basal SOD activity and protein levels showed that CPCs had higher basal levels of both SOD1 and SOD2 compared to myocytes and HUVECs. RT-PCR data showed that both SOD1 and SOD2 mRNA levels were significantly increased following XXO treatment. Additionally, both SOD1 and SOD2 activity levels were significantly increased 48 h following XXO treatment compared to their time-matched controls. siRNA-based gene silencing experiments demonstrated that reduced expression of SOD2 mRNA increased oxidative stress-induced apoptosis in CPCs. Finally, the studies with human CPCs showed that the “young” phenotype of CPCs marked by the presence of the IGF-1 receptor had significantly higher SOD2 levels compared to a “senescent” phenotype of CPCs that lack the IGF-1 receptor. The key finding in the fifth chapter of this dissertation is that CPCs have significantly higher basal expression of SOD1 and SOD2 compared to adult cardiac cells. Our study also demonstrates that CPCs are more resistant to oxidative stress induced apoptosis compared to myocytes.

6.2 Perspectives and future directions

Intracellular SOD1 delivery with polyketal microparticles

During these investigations, various other protocols for improving the encapsulation efficiency were tested. Those results show that the efficient encapsulation of SOD1 depends on various process parameters. Additionally, since each protein varies in its stability, they need to be optimized separately to identify optimal encapsulation parameters. Enhanced encapsulation of SOD1 was achieved by altering the turbulence and by increasing the molecular weight of the polymer. Altering the turbulence is easier to control as molecular weights of the polymer can vary depending on a large number of variables. Current polyketal synthesis protocols yield polymers around 5 kDa. This is far less than the molecular weights (~100 kDa) of widely used polyester based polymers [251]. Additionally, increasing the molecular weight of polyketals beyond 6 kDa reduces its solvent solubility drastically (observation from preliminary studies). Therefore, polyketal synthesis techniques should be refined to obtain soluble high molecular weight polymers. One possibility is to increase the branching in the polymer or to include side groups in the polymer backbone to improve its solubility characteristics [252].

Our studies indicate that PCADK efficiently encapsulates SOD1 and the model bovine serum albumin protein efficiently using a double-emulsion technique. However, this observation cannot be generalized to all proteins. For example, when we attempted to encapsulate catalase using the same technique within PCADK, less than 2% of the protein was encapsulated. Additionally, catalase activity was found to decrease drastically following mechanical homogenization. Therefore, to encapsulate unstable and sensitive proteins within polyketals, methods with minimal mechanical interferences such as coacervation techniques could be attempted [155]. Nevertheless, using the lessons from PKSOD project, we have observed that polyketals can be used to encapsulate various other bioactive agents including transcription factors. In our collaborative work

with the laboratory of Dr. Young-sup Yoon (Emory University), we have achieved about 25% encapsulation of induced pluripotent stem cell transcription factors such as Oct4, Sox2, c-Myc and Klf4 within polyketal microparticles.

Following the publication of intracellular SOD1 delivery in macrophages using PCADK microparticles in *Biomaterials* [253], the encapsulation and delivery of various other bioactive agents using polyketal micro and nanoparticles were investigated. For example, while our early data demonstrated efficient uptake of polyketals by macrophages, new modifications were made to deliver encapsulated drugs to the inside of cardiomyocytes - a relatively non-phagocytic cell. This was achieved by encapsulating the drugs after surface modification of polyketals with N-acetyl-glucosamine [254]. Altering the particle size also affects the internalization by the cells. For example, in our preliminary studies with cardiac progenitor cells (CPC), we had observed that while microparticle (> 10 μm) internalization by CPCs was minimal (<15%), the internalization was higher (> 50%) when the particle size was reduced below 2 μm. Another project on polyketal based delivery of bioactive agents involves siRNA delivery. Ion-pairing methods are currently being examined to encapsulate and deliver siRNA to reduce levels of ROS generating enzymes within macrophages, similar to work presented in this dissertation. This work will hopefully provide additional insights on the effects of reducing the levels of ROS in the ischemic myocardium following the polymer-siRNA delivery.

Sustained delivery of SOD1 with polyketal particles to the myocardium

This chapter demonstrates how the sustained retention of SOD1 through PKSOD delivery improved acute cardiac function. During the study, the IR model was chosen due to its clinical relevance and since the model is known to generate excessive superoxide radicals within 10 seconds of reperfusion [59]. Additionally, IR is known to cause myocardial disease progression [27]. Therefore, PKSOD was injected immediately after IR, to see if it provided benefits to the myocardium compared to empty

particle and free SOD1 delivery. Injection of 1 mg of PKSOD significantly reduced the oxidative stress levels in the ischemic myocardium. This led to an improvement in acute cardiac function; also, this significantly reduced the apoptosis in the myocardium. However, chronic function was not improved. These results suggest that sustained-reduction of superoxide radicals has beneficial effects on the heart, during the acute phase of the disease, but the chronic phase is likely more complex.

The role of free radicals on the myocardium may be positive or negative depending on the endpoint examined. For example, while superoxide scavenging reduced myocyte apoptosis, more in-depth studies may suggest that free radical generation is necessary for proper chronic remodeling given the role of oxidative stress on extracellular matrix regulation. Therefore, in addition to considering the scavenging the absolute concentration of free radicals, the temporal and spatial aspects of ROS generation also need to be considered before a conclusion can be reached on the role of sustained SOD1 delivery on the myocardium. For example, in a related project conducted in our laboratory, the deleterious effect of H_2O_2 to myocardium was found to vary depending on the time of onset of cardiac catalase overexpression [255].

In addition to the delivery of SOD1 with polyketal particles, our laboratory has used polyketal particles to deliver other drugs to treat cardiac dysfunctions. For example, Sy et al., have used the PCADK polymer to deliver the hydrophobic drug SB239063 (p38 inhibitor) to the myocardium (PK-$p38_i$) [19]. PK-$p38_i$ delivery improved chronic cardiac function following ischemia in rat myocardium. Our combination therapy with PKSOD and PK-p38i to the myocardium was undertaken based on this observation. This combination therapy showed significant improvements in both acute and chronic functions compared to IR alone. However, there was no significant difference between the chronic functional improvements of PKSOD and PKSOD/PK-$p38_i$ group. Adding more animals to the groups may help us understand the significance and real benefit of

the combination therapy. In addition to delivering drugs to treat chronic inflammatory cardiac diseases, polyketals have been used to treat acute inflammatory diseases. Yang, et al. delivered imatinib (NF κB inhibitor) using PK3 microspheres to treat acute liver failure [115]. PK3 has also been used to deliver SOD to lungs to reduce the fibrosis in lungs following bleomycin treatment [178]. Thus, delivery of antioxidants with polyketal microparticles may have wider-reaching implications in many diseases.

As the levels of myocytes apoptosis following IR were modest, other factors were analyzed in an attempt to identify alternative causes of the observed improvement in acute function. Since the expression of calcium handling proteins, controlling the contractility of the heart, is redox mediated [198], we measured the mRNA expression levels of major calcium handling proteins. The levels of ryanodine receptors, sodium-potassium exchanger, phospholamban, and SERCA remained unchanged due to PKSOD treatment following IR. However, myocardial contractility is affected by the balance of calcium handling proteins and more importantly on the actual transients of calcium within the myocardium. Additionally, superoxide is known to affect the calcium transients in myocytes [256], and therefore, future studies should be attempted to measure calcium transients in the myocardium to establish the role of PKSOD in myocardial calcium handling.

Oxidative stress is known to affect the expression of collagen and MMPs [202]. Our zymographic MMP activity result did not show any statistical significance although both SOD and PKSOD treatment group had a trend of reduced MMP activity than IR group in the ischemic myocaridum. Further continuation of zymographic tests with other substrates of MMPs such as gelatin and, type 1 collagen could potentially reveal the role of PKSOD delivery on MMP activation. In addition to the role of oxidative stress on MMPs, ROS are also known to alter the collagen expression [202]. Additionally, the expression level of collagen 1 and collagen 3 subtypes varies in cardiomyopathies [257].

Our preliminary RT-PCR analysis showed that mRNA level of collagen 1A – the most abundant subtype (>80%) in the myocardium [258] – decreased significantly following IR and SOD1 treatment. However, following PKSOD treatment, the mRNA expression of this subtype was not reduced. This could potentially offer a benefit to myocardial contractility [258] though more work needs to be done to examine other collagen subtypes and temporal changes.

In addition to remodeling, oxidative stress can potentially alter the oxidation, reduction and, degradation of proteins, for example, by causing perturbations in endoplasmic reticulum-associated functions [259]. This can be observed in our preliminary evidence from our collaborative work with the laboratory of Dr. Barbara Boyan (Georgia Tech). RT-PCR analysis of the expression of protein disulfide isomerase – an enzyme reductase and chaperone in the endoplasmc reticulum – was increased by 50% following IR similar to previously observed results [260, 261]. However, this increase was prevented by PKSOD addition, which suggests a potential beneficial effect of PKSOD to the myocardium. Future studies should be conducted in this area, to understand this relatively unstudied aspect of the relationship between oxidative stress and endoplasmic reticulum stress on the function of the myocardium.

Cardiac diseases are complex and multifactorial clinical syndromes, and oxidative stress is implicated in all the risk factors of this complex syndrome [13]. In addition to the possible targets that we have investigated, oxidative stress also has various other targets in the myocardium. For example, it alters energy metabolism, protein phosphorylation and signaling cascades, cytokine production and recruitment of cells [262] and each one of these will be an interesting arena to explore.

Antioxidant and apoptotic response of CPCs subjected to oxidative stress

Our original aim regarding CPCs was initially developed to test whether PKSOD delivery to ischemic myocardium could improve CPC transplantation and eventual

regeneration of the myocardium. However, during the course of this study, we interestingly observed that CPCs were resistant to oxidative stress induced apoptosis compared to myocytes. Since SODs are the major first line of defense against oxidative stress, basal SOD levels in the CPCs were analyzed and compared with myocytes and endothelial cells – the related cell types in the myocardium. Our data demonstrate that CPCs have higher SOD levels and are also resistant to oxidative stress induced apoptosis than the related cardiac cell types. However, all these observations were made in quiesced and ~80% confluent cells. These situations may not exist in the in vivo environments. Changes in confluence or the proliferative state of the cells can alter the antioxidant metabolism within the cells [263]. Therefore, CPCs may not have the same antioxidant status and these cells may be vulnerable to oxidative stress at other phases of its cell division. Our preliminary data have shown this interesting possibility. When 40-50% confluent CPCs in proliferative state were subjected to serum starvation induced oxidative stress, we observed significant cell death within 10 hours. It is equally possible that the observed death was due to anoikis which is apoptosis induced by inadequate or inappropriate cell-matrix interactions [264]. Additional studies in this intriguing aspect will potentially reveal interesting properties of CPCs that can be utilized during in vitro expansion and transplantation of these cells.

siRNA results based on duplicated experiments show that, following SOD2 silencing, CPCs were susceptible to XXO-induced apoptosis. This result suggests that enhanced SOD2 expression could be the reason for resistance of CPCs to oxidative stress induced apoptosis, when compared to adult cardiac cells. Similar observations exist in the literature which show that SOD2, but not SOD1, is critical to protect the cells against oxidative stress induced apoptosis [265]. SOD2 is known to maintain mitochondrial membrane integrity which when disrupted leads to cytochrome release and begin the chain of events leading to apoptosis [266]. Therefore, with this initial

evidence, further investigations should be conducted to establish the role of SOD2 in the survival of CPCs. In addition to SOD, the oxidative stress induced apoptosis may also depend on catalase – one of the major antioxidant enzymes in the cells. This was investigated in our laboratory, in a separate project on microRNA mediated CPC differentiation induced by H_2O_2. In those studies, it was observed that although 100 μM H_2O_2 did not induce apoptosis in CPCs, inhibiting the catalase using 3-aminotriazole made the CPCs vulnerable to H_2O_2-induced apoptosis.

As our studies were conducted in healthy rat CPCs, and human clinical trials are underway, we sought to determine the role of SOD in apoptosis of human CPCs (hCPCs). Recent studies, from the laboratory of Dr. Piero Anversa (Brigham and Women's Hospital), have identified subpopulations of hCPCs, that demonstrated "young" and "senescent" phenotypes, based on the presence or the absence of IGF-1 receptor [232]. Apoptosis measurements in hCPCs with and without the IGF-1 receptor show that IGFR+ hCPCs are more resistant to serum deprivation induced apoptosis than IGFR- hCPCs. Additionally, IGFR1+ cells had significantly higher activity of SOD2 compared with IGFR- hCPCs. Further, following 6 h of XXO treatment, IGFR- hCPCs had 40% higher death than control cells. However, this was not statistically significant because of high standard deviation. In the light of our evidences from rat CPCs on the importance of SOD2 in the survival of CPCs, the studies with human CPCs should be continued to help us understand the significance of low SOD2 levels in "senescent" phenotype of hCPCs.

Previous studies have shown that addition of IGF-1 or activation of the downstream AKT signaling reduces the apoptosis following serum deprivation induced oxidative stress [267, 268]. Additionally, AKT signaling pathway is known to control the expression of SOD1 and SOD2 [249, 269]. Further, following oxidative stress AKT is known to be activated within 10 minutes [270]. Our preliminary evidences also have

demonstrated that AKT is activated with 10 minutes of XXO treatment. Additionally, we have observed that by blocking AKT with LY29004, CPCs became vulnerable to apoptosis following XXO treatment. These preliminary studies open an interesting question of what is the relationship between CPC survival and AKT, IGF1 receptors and antioxidant levels, and more research needs to be done to explore this area.

Since their identification in 2003, the interest in CPC biology has begun to increase tremendously, as evidenced by the number of research articles published. However, many of the basic behaviors such as the antioxidant and oxidative stress response of CPCs is still remaining a less studied area in CPC biology. The interesting findings from this study should be a good starting point to explore this exciting area.

6.3 Concluding remarks

"The heart . . . is the beginning of life; the sun of the microcosm. . . (heart) is indeed the foundation of life, the source of all action" reported William Harvey in 1628. Since then, humanity have come a long way after many success and failures to understand about this critical pumping machine. Presently, the quality of life following a cardiac event is tremendously improved. Pharmacological interventions using agents such as anti-coagulants, beta-blockers and ACE inhibitors are still 'life savers' in emergency situations. However, other than cardiac transplantation, a long term cure for heart failure is yet to be identified. In recent years, alternate therapeutic strategies are being investigated to identify a permanent cure for the heart. These include bioactive agent delivery and cell delivery based regenerative approaches. This dissertation work was an attempt to provide some additional information to improve these promising treatment options. With recent improvements in science and technology, we will one day achieve the goal of finding a permanent cure for heart diseases. But until then…prevention is better than cure.

APPENDIX A

LIST OF PRIMERS

Gene	*NCBI Nucleotide reference number*	*Primer sequences*	
PLB	NM_022707.1	Forward	CATCGTCGTGAAGGGTCACGATTT
		Reverse	ACTGAGGAAACGGGCAGCTACATT
		Product size	117
NCX1	NM_019268.2	Forward	GAATCGGCGTGGCCTGGTCC
		Reverse	TGGCCTCCGCCGATACAGCA
		Product size	181
RYR	NM_032078.1	Forward	ACAGCACAAGCCATTCTGCAAGAC
		Reverse	ACCCAGACGTTAGCTGGTTCTTGT
		Product size	139
18s	M11188	Forward	TTCCTTACCTGGTTGATCCTGCCA
		Reverse	AGCGAGCGACCAAAGGAACCATAA
		Product size	132
SOD1	NM_017050.1	Forward	GGTGTGGCCAATGTGTCCATTGAA
		Reverse	CAATCCCAATCACACCACAAGCCA
		Product size	178
GPX1	NM_030826	Forward	AGTTCGGACATCAGGAGAATGGCA
		Reverse	AGGCATTCCGCAGGAAGGTAAAGA
		Product size	159
Catalase	NM_012520.1	Forward	TTGACAGAGAGCGGATTCCT
		Reverse	GGCATCCCTGATGAAGAAAA
		Product size	283
SOD2	NM_017051	Forward	ACGCGACCTACGTGAACAATCTGA
		Reverse	TCCAGCAACTCTCCTTTGGGTTCT
		Product size	193

SERCA2a	NM_058213	Forward	TCTGTGTGGCTGTCTGGCTTATCA
		Reverse	CACGATGGCGTTCTTCTTTGCCAT
		Product size	200
SOD1 siRNA	NM_017050.1	5'-3'	GGAAAUGAAGAAAGUACAAAGACTG
		3'-5'	CACCUUUACUUCUUUCAUGUUUCU GAC
SOD2 siRNA	NM_017051	5'-3'	AGAAUGUUAGCCAAAGAUACAUAGT
		3'-5'	CCUCUUACAAUCGGUUUCUAUGUAU CA
Scrambled siRNA	Not applicable	5'-3'	CGUUAAUCGCGUAUAAUACGCGUAT
		3'-5'	AUACGCGUAUUAUACGCGAUUAACG AC

REFERENCES

1. Roger, V.L., et al., *Heart disease and stroke statistics--2011 update: a report from the American Heart Association.* Circulation, 2011. **123**(4): p. e18-e209.

2. Smith, S.C., Jr., et al., *AHA/ACC Guidelines for Preventing Heart Attack and Death in Patients With Atherosclerotic Cardiovascular Disease: 2001 update. A statement for healthcare professionals from the American Heart Association and the American College of Cardiology.* J Am Coll Cardiol, 2001. **38**(5): p. 1581-3.

3. Mathers, C.D. and D. Loncar, *Projections of global mortality and burden of disease from 2002 to 2030.* PLoS Med, 2006. **3**(11): p. e442.

4. Deaton, C., et al., *The global burden of cardiovascular disease.* J Cardiovasc Nurs, 2011. **26**(4 Suppl): p. S5-14.

5. Ertl, G. and S. Frantz, *Healing after myocardial infarction.* Cardiovasc Res, 2005. **66**(1): p. 22-32.

6. McAlister, F.A., et al., *Randomised trials of secondary prevention programmes in coronary heart disease: systematic review.* BMJ, 2001. **323**(7319): p. 957-62.

7. Whelan, R.S., V. Kaplinskiy, and R.N. Kitsis, *Cell death in the pathogenesis of heart disease: mechanisms and significance.* Annu Rev Physiol, 2010. **72**: p. 19-44.

8. Kunapuli, S., S. Rosanio, and E.R. Schwarz, *"How do cardiomyocytes die?" apoptosis and autophagic cell death in cardiac myocytes.* J Card Fail, 2006. **12**(5): p. 381-91.

9. Bergmann, O., et al., *Evidence for cardiomyocyte renewal in humans.* Science, 2009. **324**(5923): p. 98-102.

10. Beltrami, A.P., et al., *Adult cardiac stem cells are multipotent and support myocardial regeneration.* Cell, 2003. **114**(6): p. 763-76.

11. Puceat, M., *Pharmacological approaches to regenerative strategies for the treatment of cardiovascular diseases.* Curr Opin Pharmacol, 2008. **8**(2): p. 189-92.

12. Griendling, K.K. and G.A. FitzGerald, *Oxidative stress and cardiovascular injury: Part I: basic mechanisms and in vivo monitoring of ROS.* Circulation, 2003. **108**(16): p. 1912-6.

13. Madamanchi, N.R. and M.S. Runge, *Oxidative Stress*, in *Principles of Molecular Cardiology*, M.S. Runge and C. Patterson, Editors. 2005, Humana Press. p. 549-561.

14. Li, Q., et al., *Gene therapy with extracellular superoxide dismutase protects conscious rabbits against myocardial infarction.* Circulation, 2001. **103**(14): p. 1893-8.

15. Wang, P., et al., *Overexpression of human copper, zinc-superoxide dismutase (SOD1) prevents postischemic injury.* Proc Natl Acad Sci U S A, 1998. **95**(8): p. 4556-60.

16. Jolly, S.R., et al., *Canine myocardial reperfusion injury. Its reduction by the combined administration of superoxide dismutase and catalase.* Circ Res, 1984. **54**(3): p. 277-85.

17. Diehn, M., et al., *Association of reactive oxygen species levels and radioresistance in cancer stem cells.* Nature, 2009. **458**(7239): p. 780-3.

18. Fukai, T., et al., *Extracellular superoxide dismutase and cardiovascular disease.* Cardiovasc Res, 2002. **55**(2): p. 239-49.

19. Sy, J.C., et al., *Sustained release of a p38 inhibitor from non-inflammatory microspheres inhibits cardiac dysfunction.* Nat Mater, 2008. **7**(11): p. 863-8.

20. Abdel-Latif, A., et al., *Adult bone marrow-derived cells for cardiac repair: a systematic review and meta-analysis.* Arch Intern Med, 2007. **167**(10): p. 989-97.

21. Smith, R.R., et al., *Regenerative potential of cardiosphere-derived cells expanded from percutaneous endomyocardial biopsy specimens.* Circulation, 2007. **115**(7): p. 896-908.

22. Hunt, S.A., et al., *2009 Focused update incorporated into the ACC/AHA 2005 Guidelines for the Diagnosis and Management of Heart Failure in Adults A Report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines Developed in Collaboration With the International Society for Heart and Lung Transplantation.* J Am Coll Cardiol, 2009. **53**(15): p. e1-e90.

23. Reimer, K.A. and R.B. Jennings, *The "wavefront phenomenon" of myocardial ischemic cell death. II. Transmural progression of necrosis within the framework of ischemic bed size (myocardium at risk) and collateral flow.* Lab Invest, 1979. **40**(6): p. 633-44.

24. Reimer, K.A., et al., *The wavefront phenomenon of ischemic cell death. 1. Myocardial infarct size vs duration of coronary occlusion in dogs.* Circulation, 1977. **56**(5): p. 786-94.

25. Danforth, W.H., S. Naegle, and R.J. Bing, *Effect of ischemia and reoxygenation on glycolytic reactions and adenosine-triphosphate in heart muscle.* Circ Res, 1960. **8**: p. 965-71.

26. Jennings, R.B., *Early phase of myocardial ischemic injury and infarction.* Am J Cardiol, 1969. **24**(6): p. 753-65.

27. Buja, L.M., *Myocardial ischemia and reperfusion injury.* Cardiovasc Pathol, 2005. **14**(4): p. 170-5.

28. Ginks, W.R., et al., *Coronary artery reperfusion. II. Reduction of myocardial infarct size at 1 week after the coronary occlusion.* J Clin Invest, 1972. **51**(10): p. 2717-23.

29. Maroko, P.R., et al., *Coronary artery reperfusion. I. Early effects on local myocardial function and the extent of myocardial necrosis.* J Clin Invest, 1972. **51**(10): p. 2710-6.

30. Jennings, R.B., et al., *Myocardial necrosis induced by temporary occlusion of a coronary artery in the dog.* Arch Pathol, 1960. **70**: p. 68-78.

31. Chatelain, P., et al., *Neutrophil accumulation in experimental myocardial infarcts: relation with extent of injury and effect of reperfusion.* Circulation, 1987. **75**(5): p. 1083-90.

32. Honda, H.M., P. Korge, and J.N. Weiss, *Mitochondria and ischemia/reperfusion injury.* Ann N Y Acad Sci, 2005. **1047**: p. 248-58.

33. Shen, A.C. and R.B. Jennings, *Kinetics of calcium accumulation in acute myocardial ischemic injury.* Am J Pathol, 1972. **67**(3): p. 441-52.

34. Homeister, J.W. and B.R. Lucchesi, *Complement activation and inhibition in myocardial ischemia and reperfusion injury.* Annu Rev Pharmacol Toxicol, 1994. **34**: p. 17-40.

35. Furuichi, K., et al., *Interleukin-1-dependent sequential chemokine expression and inflammatory cell infiltration in ischemia-reperfusion injury.* Crit Care Med, 2006. **34**(9): p. 2447-55.

36. Zweier, J.L. and M.A. Talukder, *The role of oxidants and free radicals in reperfusion injury.* Cardiovasc Res, 2006. **70**(2): p. 181-90.

37. Tsao, P.S., et al., *Time course of endothelial dysfunction and myocardial injury during myocardial ischemia and reperfusion in the cat.* Circulation, 1990. **82**(4): p. 1402-12.

38. Kloner, R.A., C.E. Ganote, and R.B. Jennings, *The "no-reflow" phenomenon after temporary coronary occlusion in the dog.* J Clin Invest, 1974. **54**(6): p. 1496-508.

39. Gottlieb, R.A., et al., *Reperfusion injury induces apoptosis in rabbit cardiomyocytes.* J Clin Invest, 1994. **94**(4): p. 1621-8.

40. Miyazaki, S., et al., *Quantitative analysis of contraction band and coagulation necrosis after ischemia and reperfusion in the porcine heart.* Circulation, 1987. **75**(5): p. 1074-82.

41. Manning, A.S., *Reperfusion-induced arrhythmias: do free radicals play a critical role?* Free Radic Biol Med, 1988. **4**(5): p. 305-16.

42. Bolli, R. and E. Marban, *Molecular and cellular mechanisms of myocardial stunning.* Physiol Rev, 1999. **79**(2): p. 609-34.

43. Hearse, D.J., et al., *Ultrastructural damage associated with reoxygenation of the anoxic myocardium.* J Mol Cell Cardiol, 1975. **7**(5): p. 315-24.

44. Zimmerman, A.N. and W.C. Hulsmann, *Paradoxical influence of calcium ions on the permeability of the cell membranes of the isolated rat heart.* Nature, 1966. **211**(5049): p. 646-7.

45. Hess, M.L. and N.H. Manson, *Molecular oxygen: friend and foe. The role of the oxygen free radical system in the calcium paradox, the oxygen paradox and ischemia/reperfusion injury.* J Mol Cell Cardiol, 1984. **16**(11): p. 969-85.

46. Hearse, D.J., S.M. Humphrey, and G.R. Bullock, *The oxygen paradox and the calcium paradox: two facets of the same problem?* J Mol Cell Cardiol, 1978. **10**(7): p. 641-68.

47. Burdon, R.H., *Superoxide and hydrogen peroxide in relation to mammalian cell proliferation.* Free Radic Biol Med, 1995. **18**(4): p. 775-94.

48. Wiese, A.G., R.E. Pacifici, and K.J. Davies, *Transient adaptation of oxidative stress in mammalian cells.* Arch Biochem Biophys, 1995. **318**(1): p. 231-40.

49. Griendling, K.K., et al., *Modulation of protein kinase activity and gene expression by reactive oxygen species and their role in vascular physiology and pathophysiology.* Arterioscler Thromb Vasc Biol, 2000. **20**(10): p. 2175-83.

50. Pryor, W.A., et al., *Free radical biology and medicine: it's a gas, man!* Am J Physiol Regul Integr Comp Physiol, 2006. **291**(3): p. R491-511.

51. Buetler, T.M., A. Krauskopf, and U.T. Ruegg, *Role of superoxide as a signaling molecule.* News Physiol Sci, 2004. **19**: p. 120-3.

52. Finkel, T. and N.J. Holbrook, *Oxidants, oxidative stress and the biology of ageing.* Nature, 2000. **408**(6809): p. 239-47.

53. Takimoto, E. and D.A. Kass, *Role of oxidative stress in cardiac hypertrophy and remodeling.* Hypertension, 2007. **49**(2): p. 241-8.

54. Sorescu, D. and K.K. Griendling, *Reactive oxygen species, mitochondria, and NAD(P)H oxidases in the development and progression of heart failure.* Congest Heart Fail, 2002. **8**(3): p. 132-40.

55. Kumar, D., H. Lou, and P.K. Singal, *Oxidative stress and apoptosis in heart dysfunction.* Herz, 2002. **27**(7): p. 662-8.

56. Seddon, M., Y.H. Looi, and A.M. Shah, *Oxidative stress and redox signalling in cardiac hypertrophy and heart failure.* Heart, 2007. **93**(8): p. 903-7.

57. Nakamura, K., et al., *Inhibitory effects of antioxidants on neonatal rat cardiac myocyte hypertrophy induced by tumor necrosis factor-alpha and angiotensin II.* Circulation, 1998. **98**(8): p. 794-9.

58. Ferrari, R., et al., *Occurrence of oxidative stress during reperfusion of the human heart.* Circulation, 1990. **81**(1): p. 201-11.

59. Zweier, J.L., J.T. Flaherty, and M.L. Weisfeldt, *Direct measurement of free radical generation following reperfusion of ischemic myocardium.* Proc Natl Acad Sci U S A, 1987. **84**(5): p. 1404-7.

60. Li, Q., et al., *Gene therapy with extracellular superoxide dismutase attenuates myocardial stunning in conscious rabbits.* Circulation, 1998. **98**(14): p. 1438-48.

61. Li, X.Y., et al., *Demonstration of free radical generation in the "stunned" myocardium in the conscious dog and identification of major differences between conscious and open-chest dogs.* J Clin Invest, 1993. **92**(2): p. 1025-41.

62. Das, D.K., R.M. Engelman, and Y. Kimura, *Molecular adaptation of cellular defences following preconditioning of the heart by repeated ischaemia.* Cardiovasc Res, 1993. **27**(4): p. 578-84.

63. Ferrari, R., et al., *Oxygen-mediated myocardial damage during ischaemia and reperfusion: role of the cellular defences against oxygen toxicity.* J Mol Cell Cardiol, 1985. **17**(10): p. 937-45.

64. Haramaki, N., et al., *Networking antioxidants in the isolated rat heart are selectively depleted by ischemia-reperfusion.* Free Radic Biol Med, 1998. **25**(3): p. 329-39.

65. Shlafer, M., C.L. Myers, and S. Adkins, *Mitochondrial hydrogen peroxide generation and activities of glutathione peroxidase and superoxide dismutase following global ischemia.* J Mol Cell Cardiol, 1987. **19**(12): p. 1195-206.

66. Guarnieri, C., F. Flamigni, and C.M. Caldarera, *Role of oxygen in the cellular damage induced by re-oxygenation of hypoxic heart.* J Mol Cell Cardiol, 1980. **12**(8): p. 797-808.

67. Li, P.F., R. Dietz, and R. von Harsdorf, *Superoxide induces apoptosis in cardiomyocytes, but proliferation and expression of transforming growth factor-beta1 in cardiac fibroblasts.* FEBS Lett, 1999. **448**(2-3): p. 206-10.

68. Ambrosio, G. and I. Tritto, *Reperfusion injury: experimental evidence and clinical implications.* Am Heart J, 1999. **138**(2 Pt 2): p. S69-75.

69. Tanaka, M., et al., *Superoxide dismutase plus catalase therapy delays neither cell death nor the loss of the TTC reaction in experimental myocardial infarction in dogs.* J Mol Cell Cardiol, 1993. **25**(4): p. 367-78.

70. Dhalla, N.S., et al., *Status of myocardial antioxidants in ischemia-reperfusion injury.* Cardiovasc Res, 2000. **47**(3): p. 446-56.

71. Ambrosio, G., et al., *Reduction in experimental infarct size by recombinant human superoxide dismutase: insights into the pathophysiology of reperfusion injury.* Circulation, 1986. **74**(6): p. 1424-33.

72. Chi, L.G., et al., *Effect of superoxide dismutase on myocardial infarct size in the canine heart after 6 hours of regional ischemia and reperfusion: a demonstration of myocardial salvage.* Circ Res, 1989. **64**(4): p. 665-75.

73. Tamura, Y., et al., *Superoxide dismutase conjugated to polyethylene glycol provides sustained protection against myocardial ischemia/reperfusion injury in canine heart.* Circ Res, 1988. **63**(5): p. 944-59.

74. Bolli, R., et al., *Direct evidence that oxygen-derived free radicals contribute to postischemic myocardial dysfunction in the intact dog.* Proc Natl Acad Sci U S A, 1989. **86**(12): p. 4695-9.

75. Bielski, B.H.J., et al., *Reactivity of HO2/O-2 Radicals in Aqueous-Solution.* Journal of Physical and Chemical Reference Data, 1985. **14**(4): p. 1041-1100.

76. Cai, H. and D.G. Harrison, *Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress.* Circ Res, 2000. **87**(10): p. 840-4.

77. Pryor, W.A., *Oxy-radicals and related species: their formation, lifetimes, and reactions.* Annu Rev Physiol, 1986. **48**: p. 657-67.

78. Biemond, P., et al., *Superoxide dependent iron release from ferritin in inflammatory diseases.* Free Radic Biol Med, 1988. **4**(3): p. 185-98.

79. Buxton, G.V., et al., *Critical-Review of Rate Constants for Reactions fo Hydrated Electrons, Hydrogen-Atoms and Hydroxyl Radicals (.OH/.O-) in Aqueous-Solution.* Journal of Physical and Chemical Reference Data, 1988. **17**(2): p. 513-886.

80. Gutteridge, J.M. and B. Halliwell, *Free radicals and antioxidants in the year 2000. A historical look to the future.* Ann N Y Acad Sci, 2000. **899**: p. 136-47.

81. Bergamini, C.M., et al., *Oxygen, reactive oxygen species and tissue damage.* Curr Pharm Des, 2004. **10**(14): p. 1611-26.

82. Hearse, D.J., et al., *Xanthine oxidase: a critical mediator of myocardial injury during ischemia and reperfusion?* Acta Physiol Scand Suppl, 1986. **548**: p. 65-78.

83. Meneshian, A. and G.B. Bulkley, *The physiology of endothelial xanthine oxidase: from urate catabolism to reperfusion injury to inflammatory signal transduction.* Microcirculation, 2002. **9**(3): p. 161-75.

84. Molavi, B. and J.L. Mehta, *Oxidative stress in cardiovascular disease: molecular basis of its deleterious effects, its detection, and therapeutic considerations.* Curr Opin Cardiol, 2004. **19**(5): p. 488-93.

85. Greenwald, R.A., *Therapeutic usages of oxygen radical scavengers in human diseases: myths and realities.* Free Radic Res Commun, 1991. **12-13 Pt 2**: p. 531-8.

86. Johnson, F. and C. Giulivi, *Superoxide dismutases and their impact upon human health.* Mol Aspects Med, 2005. **26**(4-5): p. 340-52.

87. Zelko, I.N., T.J. Mariani, and R.J. Folz, *Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression.* Free Radic Biol Med, 2002. **33**(3): p. 337-49.

88. Matzuk, M.M., et al., *Ovarian function in superoxide dismutase 1 and 2 knockout mice.* Endocrinology, 1998. **139**(9): p. 4008-11.

89. Juarez, J.C., et al., *Superoxide dismutase 1 (SOD1) is essential for H2O2-mediated oxidation and inactivation of phosphatases in growth factor signaling.* Proc Natl Acad Sci U S A, 2008. **105**(20): p. 7147-52.

90. Jackson, R.M., G. Parish, and Y.S. Ho, *Effects of hypoxia on expression of superoxide dismutases in cultured ATII cells and lung fibroblasts.* Am J Physiol, 1996. **271**(6 Pt 1): p. L955-62.

91. Khaper, N., et al., *Antioxidant enzyme gene expression in congestive heart failure following myocardial infarction.* Mol Cell Biochem, 2003. **251**(1-2): p. 9-15.

92. Tanaka, M., et al., *Overexpression of human copper/zinc superoxide dismutase (SOD1) suppresses ischemia-reperfusion injury and subsequent development of graft coronary artery disease in murine cardiac grafts.* Circulation, 2004. **110**(11 Suppl 1): p. II200-6.

93. Yang, J., et al., *Genetic redox preconditioning differentially modulates AP-1 and NF kappa B responses following cardiac ischemia/reperfusion injury and protects against necrosis and apoptosis.* Mol Ther, 2003. **7**(3): p. 341-53.

94. Copin, J.C., Y. Gasche, and P.H. Chan, *Overexpression of copper/zinc superoxide dismutase does not prevent neonatal lethality in mutant mice that lack manganese superoxide dismutase.* Free Radic Biol Med, 2000. **28**(10): p. 1571-6.

95. Li, Y., et al., *Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase.* Nat Genet, 1995. **11**(4): p. 376-81.

96. Chen, Z., et al., *Overexpression of MnSOD protects against myocardial ischemia/reperfusion injury in transgenic mice.* J Mol Cell Cardiol, 1998. **30**(11): p. 2281-9.

97. Hatori, N., et al., *Effects of recombinant human extracellular-superoxide dismutase type C on myocardial reperfusion injury in isolated cold-arrested rat hearts.* Free Radic Biol Med, 1992. **13**(2): p. 137-42.

98. Omar, B.A., et al., *Cardioprotection by Cu,Zn-superoxide dismutase is lost at high doses in the reoxygenated heart.* Free Radic Biol Med, 1990. **9**(6): p. 465-71.

99. Hallewell, R.A., et al., *Genetically engineered polymers of human CuZn superoxide dismutase. Biochemistry and serum half-lives.* J Biol Chem, 1989. **264**(9): p. 5260-8.

100. Jadot, G., et al., *Clinical pharmacokinetics and delivery of bovine superoxide dismutase.* Clin Pharmacokinet, 1995. **28**(1): p. 17-25.

101. Ogino, T., et al., *Chemical modification of superoxide dismutase. Extension of plasma half life of the enzyme through its reversible binding to the circulating albumin.* Int J Pept Protein Res, 1988. **32**(2): p. 153-9.

102. Regnault, C., et al., *Pharmacokinetics of superoxide dismutase in rats after oral administration.* Biopharm Drug Dispos, 1996. **17**(2): p. 165-74.

103. Muzykantov, V.R., *Targeting of superoxide dismutase and catalase to vascular endothelium.* J Control Release, 2001. **71**(1): p. 1-21.

104. Hill, R.A., et al., *Drug-eluting stents: a systematic review and economic evaluation.* Health Technol Assess, 2007. **11**(46): p. iii, xi-221.

105. Nelson, D.M., et al., *Intra-myocardial biomaterial injection therapy in the treatment of heart failure: Materials, outcomes and challenges.* Acta Biomater, 2011. **7**(1): p. 1-15.

106. Uhrich, K.E., et al., *Polymeric systems for controlled drug release.* Chem Rev, 1999. **99**(11): p. 3181-98.

107. Park, J.H., M. Ye, and K. Park, *Biodegradable polymers for microencapsulation of drugs.* Molecules, 2005. **10**(1): p. 146-61.

108. Mainardes, R.M. and L.P. Silva, *Drug delivery systems: past, present, and future.* Curr Drug Targets, 2004. **5**(5): p. 449-55.

109. Heffernan, M.J. and N. Murthy, *Polyketal nanoparticles: a new pH-sensitive biodegradable drug delivery vehicle.* Bioconjug Chem, 2005. **16**(6): p. 1340-2.

110. Allemann, E., J. Leroux, and R. Gurny, *Polymeric nano- and microparticles for the oral delivery of peptides and peptidomimetics.* Adv Drug Deliv Rev, 1998. **34**(2-3): p. 171-189.

111. Sinha, V.R. and A. Trehan, *Biodegradable microspheres for protein delivery.* J Control Release, 2003. **90**(3): p. 261-80.

112. Lee, J.C., et al., *Inhibition of p38 MAP kinase as a therapeutic strategy.* Immunopharmacology, 2000. **47**(2-3): p. 185-201.

113. Chen, D., et al., *Cytokines and acute heart failure.* Crit Care Med, 2008. **36**(1 Suppl): p. S9-16.

114. Kim, M.S., et al., *An in vivo study of the host tissue response to subcutaneous implantation of PLGA- and/or porcine small intestinal submucosa-based scaffolds.* Biomaterials, 2007. **28**(34): p. 5137-43.

115. Yang, S.C., et al., *Polyketal copolymers: a new acid-sensitive delivery vehicle for treating acute inflammatory diseases.* Bioconjug Chem, 2008. **19**(6): p. 1164-9.

116. Lee, S., et al., *Polyketal microparticles: a new delivery vehicle for superoxide dismutase.* Bioconjug Chem, 2007. **18**(1): p. 4-7.

117. Taylor, D.O., et al., *Registry of the International Society for Heart and Lung Transplantation: twenty-fourth official adult heart transplant report--2007.* J Heart Lung Transplant, 2007. **26**(8): p. 769-81.

118. Maulik, N. and M. Thirunavukkarasu, *Growth factors and cell therapy in myocardial regeneration.* J Mol Cell Cardiol, 2008. **44**(2): p. 219-27.

119. Levonen, A.L., et al., *Antioxidant gene therapy for cardiovascular disease: current status and future perspectives.* Circulation, 2008. **117**(16): p. 2142-50.

120. Molin, D. and M.J. Post, *Therapeutic angiogenesis in the heart: protect and serve.* Curr Opin Pharmacol, 2007. **7**(2): p. 158-63.

121. Anversa, P., J. Kajstura, and G. Olivetti, *Myocyte death in heart failure.* Curr Opin Cardiol, 1996. **11**(3): p. 245-51.

122. Jugdutt, B.I., *Ventricular remodeling after infarction and the extracellular collagen matrix: when is enough enough?* Circulation, 2003. **108**(11): p. 1395-403.

123. Poss, K.D., L.G. Wilson, and M.T. Keating, *Heart regeneration in zebrafish.* Science, 2002. **298**(5601): p. 2188-90.

124. Orlic, D., et al., *Bone marrow cells regenerate infarcted myocardium.* Nature, 2001. **410**(6829): p. 701-5.

125. Dove, A., *Cell-based therapies go live.* Nat Biotechnol, 2002. **20**(4): p. 339-43.

126. Shintani, S., et al., *Mobilization of endothelial progenitor cells in patients with acute myocardial infarction.* Circulation, 2001. **103**(23): p. 2776-9.

127. Menasche, P., et al., *Myoblast transplantation for heart failure.* Lancet, 2001. **357**(9252): p. 279-80.

128. Kawada, H., et al., *Nonhematopoietic mesenchymal stem cells can be mobilized and differentiate into cardiomyocytes after myocardial infarction.* Blood, 2004. **104**(12): p. 3581-7.

129. Strauer, B.E., et al., *Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans.* Circulation, 2002. **106**(15): p. 1913-8.

130. Jiang, X.X., et al., *Human mesenchymal stem cells inhibit differentiation and function of monocyte-derived dendritic cells.* Blood, 2005. **105**(10): p. 4120-6.

131. Schmidt-Lucke, C., et al., *Reduced number of circulating endothelial progenitor cells predicts future cardiovascular events: proof of concept for the clinical importance of endogenous vascular repair.* Circulation, 2005. **111**(22): p. 2981-7.

132. Hsieh, P.C., et al., *Endothelial-cardiomyocyte interactions in cardiac development and repair.* Annu Rev Physiol, 2006. **68**: p. 51-66.

133. Oh, H., et al., *Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction.* Proc Natl Acad Sci U S A, 2003. **100**(21): p. 12313-8.

134. Urbanek, K., et al., *Myocardial regeneration by activation of multipotent cardiac stem cells in ischemic heart failure.* Proc Natl Acad Sci U S A, 2005. **102**(24): p. 8692-7.

135. Ott, H.C., B.H. Davis, and D.A. Taylor, *Cell therapy for heart failure--muscle, bone marrow, blood, and cardiac-derived stem cells.* Semin Thorac Cardiovasc Surg, 2005. **17**(4): p. 348-60.

136. Torella, D., et al., *Cardiac stem cell and myocyte aging, heart failure, and insulin-like growth factor-1 overexpression.* Circ Res, 2004. **94**(4): p. 514-24.

137. Higashi, Y., et al., *Oxidative stress, endothelial function and angiogenesis induced by cell therapy and gene therapy.* Curr Pharm Biotechnol, 2006. **7**(2): p. 109-16.

138. Yao, E.H., Y. Yu, and N. Fukuda, *Oxidative stress on progenitor and stem cells in cardiovascular diseases.* Curr Pharm Biotechnol, 2006. **7**(2): p. 101-8.

139. Dernbach, E., et al., *Antioxidative stress-associated genes in circulating progenitor cells: evidence for enhanced resistance against oxidative stress.* Blood, 2004. **104**(12): p. 3591-7.

140. Case, J., D.A. Ingram, and L.S. Haneline, *Oxidative stress impairs endothelial progenitor cell function.* Antioxid Redox Signal, 2008. **10**(11): p. 1895-907.

141. Torchilin, V.P., *Recent approaches to intracellular delivery of drugs and DNA and organelle targeting.* Annu Rev Biomed Eng, 2006. **8**: p. 343-75.

142. Dini, L. and G. Rotilio, *Electron microscopic evidence for endocytosis of superoxide dismutase by hepatocytes using protein-gold adducts.* Biochem Biophys Res Commun, 1989. **162**(3): p. 940-4.

143. Berg, K., et al., *Photochemical internalization: a novel technology for delivery of macromolecules into cytosol.* Cancer Res, 1999. **59**(6): p. 1180-3.

144. Arnheiter, H. and O. Haller, *Antiviral state against influenza virus neutralized by microinjection of antibodies to interferon-induced Mx proteins.* EMBO J, 1988. **7**(5): p. 1315-20.

145. Chakrabarti, R., et al., *Incorporation of monoclonal antibodies into cells by osmotic permeabilization. Effect on cellular metabolism.* J Biol Chem, 1989. **264**(14): p. 8214-21.

146. Yuan, T.F., *Electroporation: an arsenal of application.* Cytotechnology, 2007. **54**(2): p. 71-6.

147. Jyothi, N.V., et al., *Microencapsulation techniques, factors influencing encapsulation efficiency.* J Microencapsul, 2010. **27**(3): p. 187-97.

148. van de Weert, M., W.E. Hennink, and W. Jiskoot, *Protein instability in poly(lactic-co-glycolic acid) microparticles.* Pharm Res, 2000. **17**(10): p. 1159-67.

149. Cordes, E.H. and H.G. Bull, *Mechanism and catalysis for hydrolysis of acetals, ketals, and ortho esters.* Chemical Reviews, 1974. **74**(5): p. 581-603.

150. Tomlinson, R., et al., *Polyacetal-doxorubicin conjugates designed for pH-dependent degradation.* Bioconjug Chem, 2003. **14**(6): p. 1096-106.

151. Murthy, N., et al., *Bioinspired pH-responsive polymers for the intracellular delivery of biomolecular drugs.* Bioconjug Chem, 2003. **14**(2): p. 412-9.

152. Kwon, Y.J., et al., *Directed antigen presentation using polymeric microparticulate carriers degradable at lysosomal pH for controlled immune responses.* Mol Pharm, 2005. **2**(1): p. 83-91.

153. Saihi, D., et al., *Microencapsulation of ammonium phosphate with a polyurethane shell. Part II. Interfacial polymerization technique.* Reactive & Functional Polymers, 2006. **66**(10): p. 1118-1125.

154. Kage, H., et al., *Effects of core material, operating temperature and time on microencapsulation by in situ polymerization method.* Advanced Powder Technology, 2002. **13**(4): p. 377-394.

155. Thomasin, C., et al., *Drug microencapsulation by PLA/PLGA coacervation in the light of thermodynamics. 1. Overview and theoretical considerations.* J Pharm Sci, 1998. **87**(3): p. 259-68.

156. Tewes, F., F. Boury, and J.P. Benoit, *Biodegradable Microspheres: Advances in Production Technology*, in *Microencapsulation: methods and industrial applications*, S. Benita, Editor. 2006, CRC Press: Boca Raton, FL. p. 1-54.

157. Li, M., O. Rouaud, and D. Poncelet, *Microencapsulation by solvent evaporation: state of the art for process engineering approaches.* Int J Pharm, 2008. **363**(1-2): p. 26-39.

158. van Rooijen, N. and A. Sanders, *The macrophage as target or obstacle in liposome-based targeting strategies.* International Journal of Pharmaceutics, 1998. **162**(1-2): p. 45-50.

159. Woollard, K.J. and F. Geissmann, *Monocytes in atherosclerosis: subsets and functions.* Nat Rev Cardiol, 2010. **7**(2): p. 77-86.

160. Castellanos, I.J., R. Crespo, and K. Griebenow, *Poly(ethylene glycol) as stabilizer and emulsifying agent: a novel stabilization approach preventing aggregation and inactivation of proteins upon encapsulation in bioerodible polyester microspheres.* J Control Release, 2003. **88**(1): p. 135-45.

161. Fink, B., et al., *Detection of intracellular superoxide formation in endothelial cells and intact tissues using dihydroethidium and an HPLC-based assay.* Am J Physiol Cell Physiol, 2004. **287**(4): p. C895-902.

162. Castellanos, I.J., et al., *Encapsulation of bovine serum albumin in poly(lactide-co-glycolide) microspheres by the solid-in-oil-in-water technique.* J Pharm Pharmacol, 2001. **53**(2): p. 167-78.

163. Witschi, C. and E. Doelker, *Influence of the microencapsulation method and peptide loading on poly(lactic acid) and poly(lactic-co-glycolic acid) degradation during in vitro testing.* J Control Release, 1998. **51**(2-3): p. 327-41.

164. Yang, C.Y., S.Y. Tsay, and R.C. Tsiang, *An enhanced process for encapsulating aspirin in ethyl cellulose microcapsules by solvent evaporation in an O/W emulsion.* J Microencapsul, 2000. **17**(3): p. 269-77.

165. Cannon, G.J. and J.A. Swanson, *The macrophage capacity for phagocytosis.* J Cell Sci, 1992. **101 (Pt 4)**: p. 907-13.

166. Grinnell, F., *Fibroblast spreading and phagocytosis: similar cell responses to different-sized substrata.* J Cell Physiol, 1984. **119**(1): p. 58-64.

167. Maa, Y.F. and C. Hsu, *Microencapsulation reactor scale-up by dimensional analysis.* J Microencapsul, 1996. **13**(1): p. 53-66.

168. Jeyanthi, R., et al., *Effect of processing parameters on the properties of peptide-containing PLGA microspheres.* J Microencapsul, 1997. **14**(2): p. 163-74.

169. Dikalov, S., K.K. Griendling, and D.G. Harrison, *Measurement of reactive oxygen species in cardiovascular studies.* Hypertension, 2007. **49**(4): p. 717-27.

170. Zhao, H., et al., *Superoxide reacts with hydroethidine but forms a fluorescent product that is distinctly different from ethidium: potential implications in intracellular fluorescence detection of superoxide.* Free Radic Biol Med, 2003. **34**(11): p. 1359-68.

171. Zhao, L., et al., *Nitric oxide protects against polyethylene glycol-induced oxidative damage in two ecotypes of reed suspension cultures.* J Plant Physiol, 2008. **165**(2): p. 182-91.

172. Ndengele, M.M., et al., *Superoxide potentiates NF-kappaB activation and modulates endotoxin-induced cytokine production in alveolar macrophages.* Shock, 2005. **23**(2): p. 186-93.

173. Kaur, K., et al., *Interplay of TNF-alpha and IL-10 in regulating oxidative stress in isolated adult cardiac myocytes.* J Mol Cell Cardiol, 2006. **41**(6): p. 1023-30.

174. Buja, L.M. and D. Vela, *Cardiomyocyte death and renewal in the normal and diseased heart.* Cardiovasc Pathol, 2008. **17**(6): p. 349-74.

175. Uraizee, A., et al., *Failure of superoxide dismutase to limit size of myocardial infarction after 40 minutes of ischemia and 4 days of reperfusion in dogs.* Circulation, 1987. **75**(6): p. 1237-48.

176. Griendling, K.K. and G.A. FitzGerald, *Oxidative stress and cardiovascular injury: Part II: animal and human studies.* Circulation, 2003. **108**(17): p. 2034-40.

177. Spadaccio, C., et al., *Drug releasing systems in cardiovascular tissue engineering.* J Cell Mol Med, 2009. **13**(3): p. 422-39.

178. Fiore, V.F., et al., *Polyketal microparticles for therapeutic delivery to the lung.* Biomaterials, 2010. **31**(5): p. 810-7.

179. Collin, B., et al., *Increased superoxide anion production is associated with early atherosclerosis and cardiovascular dysfunctions in a rabbit model.* Mol Cell Biochem, 2007. **294**(1-2): p. 225-35.

180. Benton, R.L., et al., *Griffonia simplicifolia isolectin B4 identifies a specific subpopulation of angiogenic blood vessels following contusive spinal cord injury in the adult mouse.* J Comp Neurol, 2008. **507**(1): p. 1031-52.

181. Sy, J.C., et al., *Surface functionalization of polyketal microparticles with nitrilotriacetic acid-nickel complexes for efficient protein capture and delivery.* Biomaterials, 2010. **31**(18): p. 4987-94.

182. Davis, M.E., et al., *Custom design of the cardiac microenvironment with biomaterials.* Circ Res, 2005. **97**(1): p. 8-15.

183. Agrawal, R.S., et al., *Pre-emptive gene therapy using recombinant adeno-associated virus delivery of extracellular superoxide dismutase protects heart against ischemic reperfusion injury, improves ventricular function and prolongs survival.* Gene Ther, 2004. **11**(12): p. 962-9.

184. Zhang, Y.E., et al., *In vivo protein transduction: delivery of PEP-1-SOD1 fusion protein into myocardium efficiently protects against ischemic insult.* Mol Cells, 2009. **27**(2): p. 159-66.

185. Mearns, B.M., *Intramyocardial injections are safe.* Nat Rev Cardiol, 2009. **6**(7): p. 441-441.

186. Cheng, W., et al., *Programmed myocyte cell death affects the viable myocardium after infarction in rats.* Exp Cell Res, 1996. **226**(2): p. 316-27.

187. Palojoki, E., et al., *Cardiomyocyte apoptosis and ventricular remodeling after myocardial infarction in rats.* Am J Physiol Heart Circ Physiol, 2001. **280**(6): p. H2726-31.

188. Zhao, Z.Q., et al., *Inhibition of myocardial apoptosis reduces infarct size and improves regional contractile dysfunction during reperfusion.* Cardiovasc Res, 2003. **59**(1): p. 132-42.

189. Maxwell, M.P., D.J. Hearse, and D.M. Yellon, *Species variation in the coronary collateral circulation during regional myocardial ischaemia: a critical determinant of the rate of evolution and extent of myocardial infarction.* Cardiovasc Res, 1987. **21**(10): p. 737-46.

190. Gustafsson, A.B. and R.A. Gottlieb, *Autophagy in ischemic heart disease.* Circ Res, 2009. **104**(2): p. 150-8.

191. Wencker, D., et al., *A mechanistic role for cardiac myocyte apoptosis in heart failure.* J Clin Invest, 2003. **111**(10): p. 1497-504.

192. Ceradini, D.J., et al., *Decreasing intracellular superoxide corrects defective ischemia-induced new vessel formation in diabetic mice.* J Biol Chem, 2008. **283**(16): p. 10930-8.

193. Costa, V.M., et al., *Adrenaline and reactive oxygen species elicit proteome and energetic metabolism modifications in freshly isolated rat cardiomyocytes.* Toxicology, 2009. **260**(1-3): p. 84-96.

194. Maulik, N. and D.K. Das, *Redox signaling in vascular angiogenesis.* Free Radic Biol Med, 2002. **33**(8): p. 1047-60.

195. Skyschally, A., et al., *Attenuation of ischemic preconditioning in pigs by scavenging of free oxyradicals with ascorbic acid.* Am J Physiol Heart Circ Physiol, 2003. **284**(2): p. H698-703.

196. Rota, M., et al., *Diabetes promotes cardiac stem cell aging and heart failure, which are prevented by deletion of the p66shc gene.* Circ Res, 2006. **99**(1): p. 42-52.

197. Kawase, Y. and R.J. Hajjar, *The cardiac sarcoplasmic/endoplasmic reticulum calcium ATPase: a potent target for cardiovascular diseases.* Nat Clin Pract Cardiovasc Med, 2008. **5**(9): p. 554-65.

198. Kuster, G.M., et al., *Redox-mediated reciprocal regulation of SERCA and Na+-Ca2+ exchanger contributes to sarcoplasmic reticulum Ca2+ depletion in cardiac myocytes.* Free Radic Biol Med, 2010. **48**(9): p. 1182-7.

199. Go, L.O., et al., *Differential regulation of two types of intracellular calcium release channels during end-stage heart failure.* J Clin Invest, 1995. **95**(2): p. 888-94.

200. Mercadier, J.J., et al., *Altered sarcoplasmic reticulum Ca2(+)-ATPase gene expression in the human ventricle during end-stage heart failure.* J Clin Invest, 1990. **85**(1): p. 305-9.

201. Linck, B., et al., *Messenger RNA expression and immunological quantification of phospholamban and SR-Ca(2+)-ATPase in failing and nonfailing human hearts.* Cardiovasc Res, 1996. **31**(4): p. 625-32.

202. Siwik, D.A., P.J. Pagano, and W.S. Colucci, *Oxidative stress regulates collagen synthesis and matrix metalloproteinase activity in cardiac fibroblasts.* Am J Physiol Cell Physiol, 2001. **280**(1): p. C53-60.

203. Karsner, H.T., O. Saphir, and T.W. Todd, *The State of the Cardiac Muscle in Hypertrophy and Atrophy.* Am J Pathol, 1925. **1**(4): p. 351-372 1.

204. Kajstura, J., et al., *Myocyte proliferation in end-stage cardiac failure in humans.* Proc Natl Acad Sci U S A, 1998. **95**(15): p. 8801-5.

205. Hsieh, P.C., et al., *Evidence from a genetic fate-mapping study that stem cells refresh adult mammalian cardiomyocytes after injury.* Nat Med, 2007. **13**(8): p. 970-4.

206. Laflamme, M.A., et al., *Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts.* Nat Biotechnol, 2007. **25**(9): p. 1015-24.

207. Zhang, J., et al., *Functional cardiomyocytes derived from human induced pluripotent stem cells.* Circ Res, 2009. **104**(4): p. e30-41.

208. Murry, C.E., et al., *Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts.* Nature, 2004. **428**(6983): p. 664-8.

209. Rose, R.A., et al., *Bone marrow-derived mesenchymal stromal cells express cardiac-specific markers, retain the stromal phenotype, and do not become functional cardiomyocytes in vitro.* Stem Cells, 2008. **26**(11): p. 2884-92.

210. Silva, G.V., et al., *Mesenchymal stem cells differentiate into an endothelial phenotype, enhance vascular density, and improve heart function in a canine chronic ischemia model.* Circulation, 2005. **111**(2): p. 150-6.

211. Mirotsou, M., et al., *Secreted frizzled related protein 2 (Sfrp2) is the key Akt-mesenchymal stem cell-released paracrine factor mediating myocardial survival and repair.* Proc Natl Acad Sci U S A, 2007. **104**(5): p. 1643-8.

212. Iwasaki, H., et al., *Dose-dependent contribution of CD34-positive cell transplantation to concurrent vasculogenesis and cardiomyogenesis for functional regenerative recovery after myocardial infarction.* Circulation, 2006. **113**(10): p. 1311-25.

213. Tendera, M., et al., *Intracoronary infusion of bone marrow-derived selected CD34+CXCR4+ cells and non-selected mononuclear cells in patients with acute STEMI and reduced left ventricular ejection fraction: results of randomized, multicentre Myocardial Regeneration by Intracoronary Infusion of Selected Population of Stem Cells in Acute Myocardial Infarction (REGENT) Trial.* Eur Heart J, 2009. **30**(11): p. 1313-21.

214. Menasche, P., et al., *The Myoblast Autologous Grafting in Ischemic Cardiomyopathy (MAGIC) trial: first randomized placebo-controlled study of myoblast transplantation.* Circulation, 2008. **117**(9): p. 1189-200.

215. Hierlihy, A.M., et al., *The post-natal heart contains a myocardial stem cell population.* FEBS Lett, 2002. **530**(1-3): p. 239-43.

216. Pfister, O., et al., *CD31- but Not CD31+ cardiac side population cells exhibit functional cardiomyogenic differentiation.* Circ Res, 2005. **97**(1): p. 52-61.

217. Moretti, A., et al., *Multipotent embryonic isl1+ progenitor cells lead to cardiac, smooth muscle, and endothelial cell diversification.* Cell, 2006. **127**(6): p. 1151-65.

218. Messina, E., et al., *Isolation and expansion of adult cardiac stem cells from human and murine heart.* Circ Res, 2004. **95**(9): p. 911-21.

219. Linke, A., et al., *Stem cells in the dog heart are self-renewing, clonogenic, and multipotent and regenerate infarcted myocardium, improving cardiac function.* Proc Natl Acad Sci U S A, 2005. **102**(25): p. 8966-71.

220. Dawn, B., et al., *Cardiac stem cells delivered intravascularly traverse the vessel barrier, regenerate infarcted myocardium, and improve cardiac function.* Proc Natl Acad Sci U S A, 2005. **102**(10): p. 3766-71.

221. Bearzi, C., et al., *Human cardiac stem cells.* Proc Natl Acad Sci U S A, 2007. **104**(35): p. 14068-73.

222. Kajstura, J., et al., *Cardiac stem cells and myocardial disease.* J Mol Cell Cardiol, 2008. **45**(4): p. 505-13.

223. D'Amario, D., et al., *Functionally competent cardiac stem cells can be isolated from endomyocardial biopsies of patients with advanced cardiomyopathies.* Circ Res, 2011. **108**(7): p. 857-61.

224. Pagani, F.D., et al., *Autologous skeletal myoblasts transplanted to ischemia-damaged myocardium in humans. Histological analysis of cell survival and differentiation.* J Am Coll Cardiol, 2003. **41**(5): p. 879-88.

225. Toma, C., et al., *Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart.* Circulation, 2002. **105**(1): p. 93-8.

226. Hu, X., et al., *Optimal temporal delivery of bone marrow mesenchymal stem cells in rats with myocardial infarction.* Eur J Cardiothorac Surg, 2007. **31**(3): p. 438-43.

227. Suzuki, K., et al., *Dynamics and mediators of acute graft attrition after myoblast transplantation to the heart.* FASEB J, 2004. **18**(10): p. 1153-5.

228. Aso, S., et al., *Effective uptake of N-acetylglucosamine-conjugated liposomes by cardiomyocytes in vitro.* Journal of Controlled Release, 2007. **122**(2): p. 189-198.

229. Riccardi, C. and I. Nicoletti, *Analysis of apoptosis by propidium iodide staining and flow cytometry.* Nat Protoc, 2006. **1**(3): p. 1458-61.

230. Laemmli, U.K., *Cleavage of structural proteins during the assembly of the head of bacteriophage T4.* Nature, 1970. **227**(5259): p. 680-5.

231. Kurien, B.T. and R.H. Scofield, *Western blotting.* Methods, 2006. **38**(4): p. 283-93.

232. D'Amario, D., et al., *Insulin-like growth factor-1 receptor identifies a pool of human cardiac stem cells with superior therapeutic potential for myocardial regeneration.* Circ Res, 2011. **108**(12): p. 1467-81.

233. Moldovan, N.I., *Emerging roles of reactive oxygen and nitrogen species in stem/progenitor cells.* Antioxid Redox Signal, 2005. **7**(11-12): p. 1409-12.

234. Jang, Y.Y. and S.J. Sharkis, *A low level of reactive oxygen species selects for primitive hematopoietic stem cells that may reside in the low-oxygenic niche.* Blood, 2007. **110**(8): p. 3056-63.

235. van Vliet, P., et al., *Isolation and expansion of resident cardiac progenitor cells.* Expert Rev Cardiovasc Ther, 2007. **5**(1): p. 33-43.

236. Jahn, T., et al., *Analysing c-kit internalization using a functional c-kit-EGFP chimera containing the fluorochrome within the extracellular domain.* Oncogene, 2002. **21**(29): p. 4508-20.

237. Li, M., et al., *c-kit is required for cardiomyocyte terminal differentiation.* Circ Res, 2008. **102**(6): p. 677-85.

238. Chabot, B., et al., *The proto-oncogene c-kit encoding a transmembrane tyrosine kinase receptor maps to the mouse W locus.* Nature, 1988. **335**(6185): p. 88-9.

239. Ashman, L.K., *The biology of stem cell factor and its receptor C-kit.* Int J Biochem Cell Biol, 1999. **31**(10): p. 1037-51.

240. Fridovich, I., *Quantitative aspects of the production of superoxide anion radical by milk xanthine oxidase.* J Biol Chem, 1970. **245**(16): p. 4053-7.

241. Dimmeler, S., et al., *Upregulation of superoxide dismutase and nitric oxide synthase mediates the apoptosis-suppressive effects of shear stress on endothelial cells.* Arterioscler Thromb Vasc Biol, 1999. **19**(3): p. 656-64.

242. Siwik, D.A., et al., *Inhibition of copper-zinc superoxide dismutase induces cell growth, hypertrophic phenotype, and apoptosis in neonatal rat cardiac myocytes in vitro.* Circ Res, 1999. **85**(2): p. 147-53.

243. Ramalho-Santos, M., et al., *"Stemness": transcriptional profiling of embryonic and adult stem cells.* Science, 2002. **298**(5593): p. 597-600.

244. Stralin, P. and S.L. Marklund, *Effects of oxidative stress on expression of extracellular superoxide dismutase, CuZn-superoxide dismutase and Mn-superoxide dismutase in human dermal fibroblasts.* Biochem J, 1994. **298 (Pt 2)**: p. 347-52.

245. Shull, S., et al., *Differential regulation of antioxidant enzymes in response to oxidants.* J Biol Chem, 1991. **266**(36): p. 24398-403.

246. Franco, A.A., R.S. Odom, and T.A. Rando, *Regulation of antioxidant enzyme gene expression in response to oxidative stress and during differentiation of mouse skeletal muscle.* Free Radic Biol Med, 1999. **27**(9-10): p. 1122-32.

247. Gwinner, W., C.C. Tisher, and H.S. Nick, *Regulation of manganese superoxide dismutase in glomerular epithelial cells: mechanisms for interleukin 1 induction.* Kidney Int, 1995. **48**(2): p. 354-62.

248. Yen, H.C., et al., *Global protein stability profiling in mammalian cells.* Science, 2008. **322**(5903): p. 918-23.

249. Nogueira, V., et al., *Akt determines replicative senescence and oxidative or oncogenic premature senescence and sensitizes cells to oxidative apoptosis.* Cancer Cell, 2008. **14**(6): p. 458-70.

250. Kops, G.J., et al., *Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress.* Nature, 2002. **419**(6904): p. 316-21.

251. Husmann, M., et al., *Polymer erosion in PLGA microparticles produced by phase separation method.* Int J Pharm, 2002. **242**(1-2): p. 277-80.

252. Schacht, E., et al., *Polyacetal and poly(ortho ester)-poly(ethylene glycol) graft copolymer thermogels: preparation, hydrolysis and FITC-BSA release studies.* J Control Release, 2006. **116**(2): p. 219-25.

253. Seshadri, G., et al., *The delivery of superoxide dismutase encapsulated in polyketal microparticles to rat myocardium and protection from myocardial ischemia-reperfusion injury.* Biomaterials, 2010. **31**(6): p. 1372-9.

254. Gray, W.D., et al., *N-acetylglucosamine Conjugated to Nanoparticles Enhances Myocyte Uptake and Improves Delivery of a Small Molecule p38 Inhibitor for Post-infarct Healing.* J Cardiovasc Transl Res, 2011.

255. Pendergrass, K.D., et al., *Temporal effects of catalase overexpression on healing after myocardial infarction.* Circ Heart Fail, 2011. **4**(1): p. 98-106.

256. Rueckschloss, U., M. Villmow, and U. Klockner, *NADPH oxidase-derived superoxide impairs calcium transients and contraction in aged murine ventricular myocytes.* Exp Gerontol, 2010. **45**(10): p. 788-96.

257. Pauschinger, M., et al., *Differential myocardial abundance of collagen type I and type III mRNA in dilated cardiomyopathy: effects of myocardial inflammation.* Cardiovasc Res, 1998. **37**(1): p. 123-9.

258. Weber, K.T., et al., *Collagen network of the myocardium: function, structural remodeling and regulatory mechanisms.* J Mol Cell Cardiol, 1994. **26**(3): p. 279-92.

259. Groenendyk, J., et al., *Biology of endoplasmic reticulum stress in the heart.* Circ Res, 2010. **107**(10): p. 1185-97.

260. Martindale, J.J., et al., *Endoplasmic reticulum stress gene induction and protection from ischemia/reperfusion injury in the hearts of transgenic mice with a tamoxifen-regulated form of ATF6.* Circ Res, 2006. **98**(9): p. 1186-93.

261. Huang, T.S., et al., *Stress-induced expression of protein disulfide isomerase associated 3 (PDIA3) in Atlantic salmon (Salmo salar L.).* Comp Biochem Physiol B Biochem Mol Biol, 2009. **154**(4): p. 435-42.

262. Chakraborti, T., et al., *Targets of oxidative stress in cardiovascular system.* Mol Cell Biochem, 1998. **187**(1-2): p. 1-10.

263. Bravard, A., et al., *Modifications of the anti-oxidant metabolism during proliferation and differentiation of colon tumor cell lines.* Int J Cancer, 1994. **59**(6): p. 843-7.

264. Frisch, S.M. and R.A. Screaton, *Anoikis mechanisms.* Curr Opin Cell Biol, 2001. **13**(5): p. 555-62.

265. Fukui, M. and B.T. Zhu, *Mitochondrial superoxide dismutase SOD2, but not cytosolic SOD1, plays a critical role in protection against glutamate-induced oxidative stress and cell death in HT22 neuronal cells.* Free Radic Biol Med, 2010. **48**(6): p. 821-30.

266. Murakami, K., et al., *Mitochondrial susceptibility to oxidative stress exacerbates cerebral infarction that follows permanent focal cerebral ischemia in mutant mice with manganese superoxide dismutase deficiency.* J Neurosci, 1998. **18**(1): p. 205-13.

267. Wang, L., et al., *Regulation of cardiomyocyte apoptotic signaling by insulin-like growth factor I.* Circ Res, 1998. **83**(5): p. 516-22.

268. Yamashita, K., et al., *Reperfusion-activated Akt kinase prevents apoptosis in transgenic mouse hearts overexpressing insulin-like growth factor-1.* Circ Res, 2001. **88**(6): p. 609-14.

269. Miao, L. and D.K. St Clair, *Regulation of superoxide dismutase genes: implications in disease.* Free Radic Biol Med, 2009. **47**(4): p. 344-56.

270. Rojo, A.I., et al., *Regulation of Cu/Zn-superoxide dismutase expression via the phosphatidylinositol 3 kinase/Akt pathway and nuclear factor-kappaB.* J Neurosci, 2004. **24**(33): p. 7324-34.

VITA

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Iyer, Gokulakrishnan (Gokul) Seshadri was born in Singanallur, a small town in Coimbatore district of Tamil Nadu, India. He received his early educations from schools in and around Singanallur. Although, as a kid he had interest in health sciences, for bachelors he chose engineering and received his Bachelor of Technology in Chemical Engineering from Coimbatore Institute of Technology in 2003. Gokul gradually moved into the field of life sciences, during his Masters studies in Biotechnology from Indian Institute of Technology Madras, in 2006, and in the same year, Gokul joined Georgia Institute of Technology to pursue his doctoral studies in Bioengineering. Outside of his research, Gokul has developed keen interest in music, poetry and painting and he spends his time with family and friends.