AGE-DEPENDENT VARIATION IN STEM CELL CHARACTERISTICS OF THE HEALTHY AND DISEASED HEART

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Ph.D. THESIS

2017

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY, TRIVANDRUM

THIRUVANANTHAPURAM

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A THESIS PRESENTED BY

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TO

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY, TRIVANDRUM

THIRUVANANTHAPURAM

IN PARTIAL FULFILMENT OF THE REQUIREMENT

FOR THE AWARD OF

DOCTOR OF PHILOSOPHY

2017

CERTIFICATE

I**, Sherin S,** hereby certify that I had personally carried out the work depicted in the thesis entitled, **"Age-dependent variation in stem cell characteristics of the healthy and diseased heart"** under the supervision of **Dr. R Renuka Nair,** except where external help was sought and acknowledged. No part of the thesis has been submitted for the award of any other degree or diploma prior to this date.

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This is to certify that Ms. Sherin S, in the Division of Cellular & Molecular Cardiology of this Institute, has fulfilled the requirements of the regulations relating to the nature and prescribed period of research for the PhD degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum. The study entitled **"Age-dependent variation in stem cell characteristics of the healthy and diseased heart"** was carried out under my direct supervision. No part of the thesis has been submitted for the award of any other degree or diploma prior to this date. Clearance was obtained from the Institutional Animal Ethics Committee for carrying out the study.

Date **Dr R. Renuka Nair**

The thesis entitled

AGE-DEPENDENT VARIATION IN STEM CELL CHARACTERISTICS OF THE HEALTHY AND DISEASED HEART

Submitted by

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For the degree of

Doctor of Philosophy

of

SREE CHITRA TIRUNAL INSTITUTE FOR

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ACKNOWLEDGEMENT

I consider myself privileged to have had the opportunity to carry out my doctoral studies in the Division of Cellular and Molecular Cardiology, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram. I thank Dr. K Radhakrishnan, Dr. Jaganmohan Tharakan and Dr. Asha Kishore, the former and present Directors, for extending support and excellent facilities provided for research programs in this Institute.

Words fail me in expressing my gratitude towards my mentor, Dr. R. Renuka Nair for her continued encouragement and constant support in my research work, which has helped me greatly in the successful completion of the doctoral program.

I express sincere gratitude to the members of my Doctoral Advisory Committee, Dr. Sreenivas and Dr. S Harikrishnan, for their support and suggestions. I express my sincere thanks to Dr. K Shivakumar, Division of Cellular and Molecular Cardiology for his encouragement and advice. I wish to express my thanks to Dr. V.S Harikrishnan, Division of Laboratory Animal Science, for the maintenance and supply of experimental animals.

My warm thanks to Mrs. K Remani for her technical support and assistance. I also appreciate the help rendered by Mrs. Sulochana, Mr. Sunil, Mr Najeeb and Ms. Sindhu.

I acknowledge the financial support received from INSPIRE, Department of Science and Technology (DST) and Board of Research in Nuclear Sciences (BRNS), India. I am thankful to Department of Biotechnology and British Council for Newton-Bhabha fellowship. I express my sincere gratitude to Prof. Sian Harding, staff and students at the Myocardial function, Hammersmith Campus, Imperial College, London for their support throughout my placement program.

I convey my gratefulness to the staff and students of the Departments of Biochemistry and Pathology for their help during the research program. I am thankful to Dr. Rajesh of Biogenix Research Centre for helping me with the comet assay analysis.

I appreciate the goodwill extended to me by all my colleagues which has been crucial in the progress of my studies. A special thanks to Dr. Ajay Godwin Potnuri for his encouragement and help extended to me in every aspect of this study. I would like to thank my ever-loving family and friends for their strength and support towards me at all occasions. Last but not the least; I thank my better-half for his endless support and my little boy for his patience.

Above all, I owe it to the Lord Almighty for granting me this opportunity and enabling me to achieve this goal.

Sherin S

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ABBREVIATIONS

ACE	Angiotensin Converting Enzyme
Ang-II	Angiotensin II
ANP	Atrial natriuretic peptide
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
bFGF	basic Fibroblast Growth Factor
BLSA	Baltimore Longitudinal Study on Aging
BNP	B-type natriuretic peptide
BP	Blood Pressure
BSA	Bovine Serum Albumin
CABG	Coronary Artery Bypass graft
cDNA	Complementary Deoxyribo nucleic acid
CHAPS	Dimethyl[3-(propyl]. azaniumyl}propane-1-sulfonate
CPCSEA	Committee for the Purpose of Control and Supervision of Experiments on Animals
CSC	Cardiac Stem Cell
cTnI	cardiac Tropinin I
CVD	Cadiovascular Disease
DAPI	4',6-diamidino-2-phenylindole
DCFDA	Dichloro-dihydro-fluorescein diacetate
DCFH	Dichloro-dihydro-fluorescein
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribo Nucleic Acid
EC	Excitation Contraction
ECM	Extracellular matrix

EDTA	Ethylenediaminetetraacetic acid
EF	Ejection fraction
EPC	Endothelial Progenitor Cell
ET-1	Endothelin 1
FACS	Fluoresecence Activated Cell Sorting
FAO	Fatty Acid Oxidation
FGF	Fibroblast Growth Factor
FITC	Fluorescein isothiocyanate
GPCR	G-protein Coupled Receptor
hCSC	human Cardiac Stem Cell
HEPES	Hydroxyethyl piperazineethanesulfonic acid
HF	Heart Failure
HFpEF	Heart Failure with a Preserved Ejection Fraction
HGF	Hepatocyte Growth Factor
HHD	Hypertensive Heart Disease
HSC	Haematopoietic Stem Cell
hUCMSC	Human umbilical cord mesenchymal stem cells
ICC	Immunocytochemistry
IGF-1	Insulin like Growth Factor-1
IHC	Immunohistochemistry
IMDM	Iscove's Modified Dulbecco's Medium
iPSC	Induced Pluripotent Stem cell
iPSC-CM	Induced pluripotent stem cell derived Cardiomyocytes
LAD	Left anterior descending coronary artery
LV	Left Ventricle
LVEF	Left ventricular Ejection Fraction
LVH	Left Ventricular Hypertrophy
LVM	Left Ventricular mass

MAPK	Mitogen-activated protein kinase
MDR	Multi Drug Resistant
MHC	Myosin heavy Chain
MI	Myocardial Infarction
MMP	Matrix metalloproteinase
MPI	Myocardial performance index
MSC	Mesenchymal stem cells
mTOR	mechanistic Target of rapamycin
NFAT	Nuclear factor of activated T-cells
NO	Nitric Oxide
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDGF	Platelet-derived growth factor
PDT	Population Doubling Time
PGC	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PI3K	Phosphatidylinositol-3-kinase
PSC	Pluripotent stem cells
RIPA	Radio-immuno-precipitation
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
RWT	Relative Wall Thickness
RyR	Ryanodine Receptor
SA β-gal	Senescence Associated β-galactosidase
SCF	Stem Cell Factor
SCIPIO	Stem Cell Infusion in Patients with Ischemic Cardiomyopathy
SDS	Sodium dodecyl sulfate

SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SERCA	Sarco/endoplasmic reticulum Ca^{2+}-ATPase
SHR	Spontaneously Hypertensive Rat
SMA	Smooth Muscle Actin
SPARC	Secreted protein Acidic and Rich in Cysteine
SR	Sarcoplasmic Reticulum
SRF	Serum response factor
TAC	Transverse Aortic Constriction
TBS-T	Tris Buffeed Saline-Tween 20
TCM	Takotsubo cardiomyopathy
TEMED	Tetramethylethylenediamine
TERT	Telomerase Reverse transcriptase
TF	Transcription Factor
TGF-β	Transforming Growth Factor-β
TIMP	Tissue Inhibitors of Metalloproteinase
TPG	Total Product generated
TRAP	Telomeric repeat amplification protocol
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organization
WST	Wistar rat
β_2-AR	β2-Adrenergic receptors

SYNOPSIS

Cardiovascular diseases are the leading cause of mortality and morbidity worldwide and accounts for 23 per cent of all deaths in India in the period 2010-13. Left ventricular hypertrophy consequent to pressure overload is recognized as the most important predictor of congestive heart failure and sudden death. Medical and surgical advances in the field of cardiovascular biology have brought relief to the patients with cardiac failure. However, no absolute cure has been established so far and the incidence of patients getting re-hospitalized is increasing. The heart was considered to be a post mitotic organ till scientists discovered cardiac specific stem cells in 2003. Of the different subsets of CSCs, c-kit^{+} CSCs are the most extensively investigated cell type and is widely used for pre-clinical and clinical trials. C-kit^{+} CSCs are clonogenic, self-renewing and multipotent. The role of stem cells in cardiac regeneration and its involvement in repair under diseased conditions are documented. Clinical trials using stem cells is gaining momentum with favourable clinical outcomes. One of the major risk factors associated with uncontrolled hypertension is left ventricular hypertrophy, leading to heart failure. In addition to pathological complications, the incidence of heart failure is higher in elderly population making age an independent risk factor. Reactive oxygen species is found to play a key role in cardiac remodeling associated with hypertension and aging. Functional impairment due to increased ROS levels in different types of stem cells has been reported. Aging is associated with decrease in number and loss of efficiency of stem cells. One human study has reported deterioration of c-kit^{+} CSCs with aging and heart failure. However,

a clear distinction between physiological and pathological aging was not possible. Based on this background, it was hypothesized that "***Progressive deterioration in efficacy of stem cells due to adverse microenvironment may be a possible cause for irreversible cardiac remodeling in hypertensive heart disease and aging.***" The study was carried out in cardiac stem cells isolated and cultured from hypertensive as well as normotensive rat heart of different age groups. Spontaneously hypertensive rat (SHR), a genetic model where cardiac pathology resembles the clinical course of hypertension and its transition to hypertrophy and failure; was used for assessment of the effect of pathological remodeling on CSCs. The effect of physiological aging was assessed in Wistar rat of different ages. Envisaging the significant role of oxidative stress in aging and hypertrophy, Spontaneously Hypertensive Rat in the compensatory phase of cardiac remodeling were treated with the antioxidant, tempol; to examine whether the treatment helps in prevention of stem cell aging and restoration of cardiac structure and function.

The study was designed with the following objectives:

i. Examine the effect of physiological aging on cardiac stem cells by characterizing the temporal variation in stem cell attributes in normotensive Wistar rat
ii. Examine the characteristics of cardiac stem cells in different stages of hypertensive heart disease using Spontaneously hypertensive rat as the experimental model in comparison with age matched Wistar rat

iii. Modulation of stem cell characteristics with antioxidant tempol to examine whether reduction of oxidative stress prevents stem cell aging

Cardiac stem cell characteristics were assessed in hearts of Wistar rats of different ages. Number, proliferation, survival, senescence and migration of CSCs were evaluated to examine the effect of physiological aging. To assess the effect of hypertensive heart disease on CSCs, comparison studies were carried out in male SHR and WST rats of different ages (newborn, 6 months, 12 months and 18 months) (n=3). The ages represent the various stages in progressive cardiac remodeling, from hypertension to hypertrophy and failure. The single cell suspension obtained from ventricular digest was used to determine the proportion of c-kit^{+} cells by FACS analysis. Atrial appendages were used for explant culture. The cells that migrated from the explant were trypsinised and subjected to immuno-magnetic isolation to sort out the c-kit^{+} cells. These CSCs were expanded in culture and used for further experiments. The stemness and purity of the cells at passage 3 was confirmed by Flow Cytometry, immunocytochemistry and clonogenecity studies. CSCs so obtained were used for evaluating the variation in proliferation, migration, differentiation, senescence and DNA damage. Proliferation potential was assessed based on growth kinetics and colony forming units. Growth rate and population doubling time were calculated. The migration potential was assessed by trans-well migration assay and scratch wound assay. The proportion of senescent cells was assessed by β-galactosidase staining using commercially available kit, as well as by expression studies of

$p16^{ink4a}$ and p21 by Western blot. Intracellular ROS was detected by H_2DCFDA fluorescence assay. The directed differentiation potential of the CSCs towards cardiovascular lineage were detected by stimulating CSCs with 5-azacytidine followed by the expression studies of cardiomyocyte and smooth muscle cell markers cardiac troponin I and smooth muscle actin. The effect of aging on DNA damage was assessed by Comet assay. Replicating efficiency was investigated by evaluating telomerase activity using commercially available kit and TERT mRNA levels by Real time PCR.

The observation of significantly increased levels of intracellular ROS in cardiac stem cells as early as 6 months of age in SHR and the prevalence of tissue oxidative stress led to the speculation that supplementation with antioxidants may regress stem cell aging. Tempol, a SOD mimetic, is a potent antioxidant. Six male SHR in the stable phase of hypertrophy (6 months) were treated with the antioxidant tempol. Age and sex matched normotensive WST and untreated SHR served as the control groups. The treatment involved intra-peritoneal injection of 20mg/kg/day of tempol for 2 weeks. Following the treatment, functional evaluation was done by echocardiography to confirm the efficiency of the treatment and the rats were sacrificed. Myocardial oxidative stress was assessed by immunocytochemistry to confirm the reduction of oxidative stress in response to the treatment. Having established the cardioprotective and antioxidant potential in response to the treatment, its effect on cardiac stem cells was

evaluated. CSCs, isolated and expanded from atrial explant cultures, were further assessed for their functional characteristics.

Major findings

Cardiac stem cell efficiency deteriorated with physiological aging

Cardiac stem cells isolated adult WST rats, when compared to pups showed signs of aging as confirmed by their decreased density and functional efficiency. Proliferation, survival and migration potential of CSCs declined with age.

Deterioration of stem cell attributes was accelerated in Hypertensive Heart Disease

Compared with the pups, the density of CSCs declined by 4-fold and 20-fold respectively in aged WST and SHR. The proliferation potential of CSCs was adversely affected by age as assessed by decreased growth rate, increased population doubling time and decreased number of colonies; and the decline was accelerated in SHR. The migration abitity of CSCs declined with age; and was severely affected in the SHR. The proportion of senescent cells was high in aged SHR, which was in concordance with the increased expression of $p16^{ink4a}$ and p21 proteins. The intracellular ROS levels were much higher in CSCs from SHR in all ages compared to WST counterparts. CSCs exhibited age associated increase in the ability to differentiate to cardiac lineage. No significant change was observed in the differentiation potential between CSCs of WST and SHR. Telomerase activity and TERT mRNA levels were reduced in CSCs of WST.

Whereas, their levels were critically low in CSCs of aged SHR. Extent of DNA damage was also significantly higher in CSCs of SHR compared to WST.

Antioxidant supplementation was found to be beneficial to CSCs

Cardiac stem cell efficiency was compromised in SHR as early as 6 months of age and the intracellular ROS levels were significantly higher. Envisaging the role of oxidative stress in hypertension induced hypertrophy, antioxidant supplementation was carried out. Following treatment for 14 days, the echocardiographic examination showed significant regression of hypertrophy and reduction of blood pressure. Immuunohistochemical analysis of the tissue sections showed decreased levels of oxidative stress. After validation of the effectiveness of the treatment, CSCs isolated from the three groups were evaluated. The treated group showed enhanced functional efficiency compared to untreated SHR and was comparable with WST. Antioxidant supplementation improved the proliferation potential and migration ability of CSCs. The treatment could also bring down the intracellular ROS levels. The expression of senescent markers decreased after the treatment. Tempol treatment, in addition to improving the general condition of the heart was found to be beneficial to the CSCs. Antioxidant supplementation at an early stage of hypertrophy is found to delay the hypertension induced pathological remodeling.

The observations from the present study provide insights into the physiological and pathological aging of CSCs. The deterioration of efficiency of CSCs of SHR

is possibly the critical determinant for transition from hypertrophy to failure. The findings provide positive cues for detailed investigation on the significant role played by CSCs in cardiac remodeling associated with disease as well as aging. The adverse milieu in the heart due to increased oxidative stress may negatively influence the efficiency of stem cells residing within the niches. The impairment of the stem cell attributes with aging can make the elderly population prone to heart failure. The beneficial role of antioxidant supplementation on CSCs provides scope for development of new therapeutic strategies for increasing the efficacy of stem cells, and delaying the progression to heart failure.

I. INTRODUCTION

Structural and functional changes occur in the heart adapting itself to the physiological and pathological demands. Cardiovascular diseases, affecting the heart and circulation, are the leading cause of mortality and morbidity. High blood pressure is a critical risk factor for CVDs and uncontrolled hypertension leads to hypertrophy, progressing to cardiac failure. Depending upon the duration and nature of the stimulus, hypertensive heart disease can either lead to concentric or eccentric hypertrophy with either a preserved or a reduced left ventricular ejection fraction (LVEF) ultimately resulting in malfunctioning of the heart. The progression of hypertension to hypertrophy occurs initially as a compensatory mechanism to maintain the cardiac output due to increased wall stress. The adaptive remodeling in course of time advances into maladaptive remodeling pushing the heart to a decompensated stage. The transition from compensatory phase of left ventricular hypertrophy to decompensatory phase remains enigmatic and still eludes an answer. Many physiological, cellular and molecular mechanisms have been proposed to explain the transition from hypertrophy to failure. The pathological changes present in patients with LVH include an increase in the size of the cardiomyocyte, alterations in the extracellular matrix with accumulation of collagen and fibrosis, and abnormalities of the intramyocardial coronary vasculature. The neuro-hormones, growth factors and cytokines also influence the progression of hypertrophy. LVH is a significant intermediate phenotype in the progression of hypertensive heart disease (Frohlich, 2000) and is associated with adverse outcomes (Levy *et al.*,

1990). Left ventricular hypertrophy (LVH) remains a powerful indicator of impending cardiac failure and hence finding a potential cause for the transition will pave the way for better treatment strategies.

Identification of resident cardiac stem cells (CSCs) in 2003 contradicted the paradigm that the myocardium is a post-mitotic organ. Heart harbors a small population of resident stem cells which are involved in cardiac homeostasis depending on the physiological and pathological demands (Beltrami *et al.*, 2003). Cardiac specific stem cells expressing various surface markers have been identified. This includes c-kit, Sca-1, MDR, Islet 1 and side population cells. Of these, c-kit$^+$ cells are the most widely explored cardiac stem cell type for regeneration and cell transplantation studies. The lineage negative c-kit$^+$ cells are self renewing, clonogenic and multipotent; having the ability to differentiate into cardiomyocytes, vascular smooth muscle cells and endothelial cells (Beltrami *et al.*, 2003; Bearzi *et al.*, 2007). As reported by Guo *et al.*, CSCs can migrate to the injured site in the myocardium and improve cardiac function by regeneration of the myocardium after ischemia/reperfusion injury (Guo *et al.*, 2014). Cardiac lineage commitment of c-kit$^+$ CSCs makes it a good candidate for stem cell therapy and is the first type of resident CSCs to be clinically tested. Ongoing first-in-human clinical trial, Stem Cell Infusion in Patients with Ischemic Cardiomyopathy (SCIPIO) used autologous c-kit$^+$ CSCs in patients undergoing CABG and the early outcomes seem to be encouraging in terms of scar size reduction and increase in viable myocardium (Bolli *et al.*, 2011; Chugh *et al.*,

2012). The positive response of the heart to CSC transplantation suggest that these cells are the primary source for myocardial repair (Messina *et al.*, 2004). Human heart undergoes myocyte turnover of 0.5 to 1% annually (Bergmann *et al.*, 2009) envisaging the role of CSCs in the maintenance of cardiac tissue homeostasis. Repeated utilization of stem cells can lead to decrease in number and decline in healthy stem cell pool. When the availability of stem cells is compromised, the tissue repair process can be inefficient, leading to cardiac decompensation. Several reviews have highlighted the association of stem cells with disease (Ho *et al.*, 2005; Oh *et al.*, 2014; Sharpless & DePinho, 2007), but the role of CSCs in maladaptive cardiac remodeling is less explored (Rupp *et al.*, 2012). A study on human CSCs comparing the stem cell characteristics of donor hearts with that of explanted hearts from patients with cardiac failure suggests that defects in the balance between cardiomyocyte mass and the pool of non-senescent stem cells may condition the evolution of the decompensated myopathy (Cesselli *et al.*, 2011). Due to lack of overlap between the ages of donor and explanted heart and due to lack of uniformity in etiology of cardiomyopathy, actual distinction of pathological and physiological aging and its influence on functional characteristics of CSCs could not be defined in this study. The differentiating features of CSCs in pathological and physiological aging have not been distinctly defined. In a recent study on the quality and quantity of Cardiosphere Derived Cells (CDC), age-associated increase in senescence markers was observed. However, no correlation was observed

between aging and growth rate, angiogenic ability and growth factor production of CDC (Nakamura *et al.*, 2016). These findings in human samples collectively underscore the requirement for a systematic analysis of variation in stem cell characteristics in both physiological and pathological changes of the heart.

Myocardial oxidative stress is implicated both in physiological and pathological cardiac remodelling (Sag *et al.*, 2014). Oxidative stress is one of the initial causes for hypertension induced hypertrophy (Purushothaman *et al.*, 2011). Further, ROS induced ROS production is characteristic of cardiac pathology (Zorov, *et al.*, 2014). There is good evidence that cardiac remodeling is associated with the generation and release of reactive oxygen species (ROS) indicating that oxygen-derived free radicals play an important role in the pathogenesis of heart failure (Maulik & Kumar, 2012). Oxidative stress is indicated as a major contributor for cardiac diseases and aging and is also implicated in myocardial ischemia and cardiac hypertrophy (Sawyer *et al.*, 2002; Takimoto & Kass, 2007). A proper balance between free radicals and antioxidant defense system is necessary for maintaining cardiac structure and function. LVH is associated with myocyte loss (Anversa *et al.*, 1990) and increased oxidative stress (Takimoto & Kass, 2007), both of which can affect the density and efficiency of stem cells. Increased oxidative stress in the surrounding milieu can influence the stem cell characteristics. Therefore, the quality and quantity of stem cells can decrease with age and pathological conditions, paving the way for progressive maladaptive changes. Concurrently, embryonic stem cells and mesenchymal

stem cells are found to be sensitive to oxidative stress and ROS is also implicated in the progenitor and stem cell dysfunction in cardiovascular diseases (Denu & Hematti, 2016; Guo *et al.*, 2010). Oxidative stress is found to be a causative factor for the shortened life span of stem cells and EPCs (Case *et al.*, 2008). These points suggests the vulnerability of CSCs to oxidative stress. Studies have shown that treatment with antioxidants at an early stage is beneficial to the ailing heart (Yusuf *et al.*, 2000). However, information regarding the dynamic effects of antioxidant therapy on CSCs is lacking. Tempol is a redox-cycling, metal-independent, and membrane permeable antioxidant and exhibits both SOD and catalase activity. Tempol treatment was found to reverse dexamethasone induced hypertension in rat (Schnackenberg & Wilcox, 1999). It has also been studied in several models of oxidative stress and is reported to improve the survival of skeletal myoblasts and cardiomyoblasts when delivered to the myocardium during cell transplantation (Avolio *et al.*, 2014; Batinić-Haberle *et al.*, 2010; Oh *et al.*, 2014). Given the potential link between CSC and oxidative stress, antioxidant treatment can possibly enhance the functional ability of CSCs and improve the repair of the injured myocardium, thereby preventing and/or attenuating the progression of adverse remodelling in hypertension. Many of the cardioprotective antihypertensives are also known to have antioxidant effect. The β-blocker metoprolol has been shown to increase the cardiac stem cell density in the left vetricle after myocardial infarction (Serpi *et al.*, 2009).

The present study is based on the hypothesis that "***Progressive deterioration in efficacy of stem cells due to adverse microenvironment may be a possible cause for irreversible cardiac remodeling in hypertensive heart disease and aging***." As cardiac remodelling progresses with age, the temporal variation in stem cell characteristics was assessed in normotensive Wistar rat (WST) and compared with the changes in Spontaneously hypertensive rat (SHR). The number, proliferation efficiency, migration potential, senescence and DNA damage profiles as well as intracellular oxidative stress of CSCs at different stages of cardiac remodeling were assessed. SHR is a genetic model of hypertension, where the cardiac sequelae bears similarity to hypertension in man (Trippodo & Frohlich, 1981) and is considered as the best model to study the transition from compensated ventricular hypertrophy to failure (Bing *et al.*, 1995). Considering the prominent role of oxidative stress in cardiac remodelling, and the possible beneficial effects of reduction of oxidative stress, the effect of antioxidant treatment was investigated in SHR.

The objectives of the study were:

i) Examine the effect of physiological aging on cardiac stem cells by characterizing the temporal variation in stem cell attributes in normotensive Wistar rat

ii) Examine the effect of pathological heart disease on CSCs using SHR as a model of hypertensive heart disease, and compare the temporal variation in CSCs at different stages of hypertrophy with physiological aging

iii) Modulation of stem cell characteristics with antioxidant tempol to examine whether reduction of oxidative stress prevents stem cell aging

The next chapter includes a brief description of the cardiac changes associated with age and hypertension. Literature on cardiac stem cells and their role in cardiac regeneration are also reviewed. Information on the role of oxidative stress in physiological and pathological aging is also included.

The third chapter deals with the design of study and experimental methodology.

The results are presented and the findings discussed in the light of available information in the fourth chapter.

The fifth chapter includes significant findings, observations, conclusion and scope for future studies.

The references cited in text are listed in 'Bibliography'.

The stem cell response to metoprolol, an established cardioprotective agent was also tested. The results are presented in Appendix I.

Report of work carried out under Newton-Bhabha Fellowship is presented in Appendix II.

II. REVIEW OF LITERATURE

Heart, the central organ of circulation, works invariably to provide oxygen and nutrients and assist in removing metabolic wastes. Being a dynamic organ, it is capable of adapting to developmental, physiological and pathological alterations in workload. Of all the accounted diseases, cardiovascular disease (CVD) is the leading cause of death globally. According to American Heart Association Stroke and Heart Statistics and World Health Organizations (WHO) fact sheet, 31% of all global deaths were due to CVDs (WHO fact sheets). In India and Kerala, CVDs accounted for 23% and 14% of all deaths respectively for the period 2010-2013 (Krishnan *et al.*, 2016).

II.1. Cardiac changes associated with hypertensive heart disease and age

II.1.1. Hypertensive Heart Disease

Hypertension is a key risk factor for left ventricular hypertrophy and in the development of clinical heart failure (Meijs *et al.*, 2007). Unattended high blood pressure is considered to be the major risk factor for coronary artery diseases and heart failure. Hypertension accounts for about one quarter of heart failure cases as reported by the Framingham Study (Kannel & Cobb, 1992) and are considered the leading cause of mortality by WHO. Arterial blood pressure is influenced by cardiac output and peripheral vascular resistance. Stroke volume and heart rate that control cardiac output is dependent on the myocardial contractility and size of the vascular compartment. The function and structure of blood vessels affect the vascular resistance. Prolonged, untreated hypertension can lead to maladaptive remodeling in the myocardial structure, coronary vasculature, and

conduction system of the heart. The high blood pressure causes the narrowing of the blood vessels leading to minimal or no blood flow to the heart muscles resulting in coronary artery disease. Consequent to pressure overload, the heart muscles thicken to counterbalance the increased stress and thus maintain the ejection fraction. This initial hypertrophic response of increased myocyte size in the heart occurs as a compensatory mechanism. On progression, the adaptive remodeling becomes maladaptive, leading to cardiac failure. Various hemodynamic, structural, neuroendocrine, cellular and molecular factors play integral roles in the development of hypertension and associated abnormalities (Patel, 2014). Abnormalities in the rennin-angiotensin-aldosterone system and sympathetic nervous system, endothelial dysfunction and vascular inflammation can independently contribute to hypertension (Sowers, 2002). Also oxidative stress that develops in the early stages of hypertension results in further release of ROS, contributing to hypertension induced irregularities (Csányi & Miller, 2014). The complications of hypertensive heart disease (HHD) include left ventricular hypertrophy, heart failure, coronary artery diseases, ischemic heart disease, arrhythmia, sudden cardiac death and stroke. β-blockers, calcium channel blockers, angiotensin converting enzyme (ACE) inhibitors, angiotensin receptor blockers and renin inhibitors are the commonly prescribed antihypertensive agents.

Hypertension plays a key role in the evolution of cardiac failure. In an attempt to compensate for the increased peripheral resistance noted in hypertension, the heart hypertrophies, with the left ventricular enlargement, accompanied by myocyte hypertrophy and fibrosis. Eventually, left ventricular failure occurs when the hypertrophied or fibrosed myocardium is no longer able to maintain normal cardiac output. HHD progresses with initial left ventricular hypertrophy leading to diastolic dysfunction followed by systolic dysfunction.

II.1.2. Cardiac hypertrophy

Heart responds to various environmental conditions by either enlargement or atrophy. The reactions will depend particularly on the type, duration and strength of the stimuli. In addition to cardiomyocytes - the muscle cells; heart also harbors smooth muscle cells, endothelial cells, fibroblasts, mast cells and resident cardiac stem cells. The cell to cell communication in the heart directs integrated biological responses to the altered environment with the help of various transcription factors and other signaling molecules. The autocrine and paracrine factors released acts on various cell types and bring about the desired expression of proteins. Congestive heart failure is a common end point of many abnormal cardiac conditions, including hypertrophy. When compensatory mechanisms that become activated during cardiac hypertrophy fail, congestive heart failure is manifested, and is associated with a grave prognosis. Heart failure subsequent to cardiac hypertrophy remains a major clinical problem, and the mechanism

associated with progression of a well-compensated hypertrophied heart to a decompensated stage is poorly understood.

Cardiac hypertrophy is one of the most prominent initial adaptive responses of the heart. It is different from hyperplasia in which the number of cells increases with no change in size. Hypertrophy has been noted as a significant er for diseases affecting the heart. Hypertrophy occurs as an adaptive response to various physiological and pathological stimuli. Hence, it is vital to understand and differentiate between various types of cardiac hypertrophy (Fig. 1).

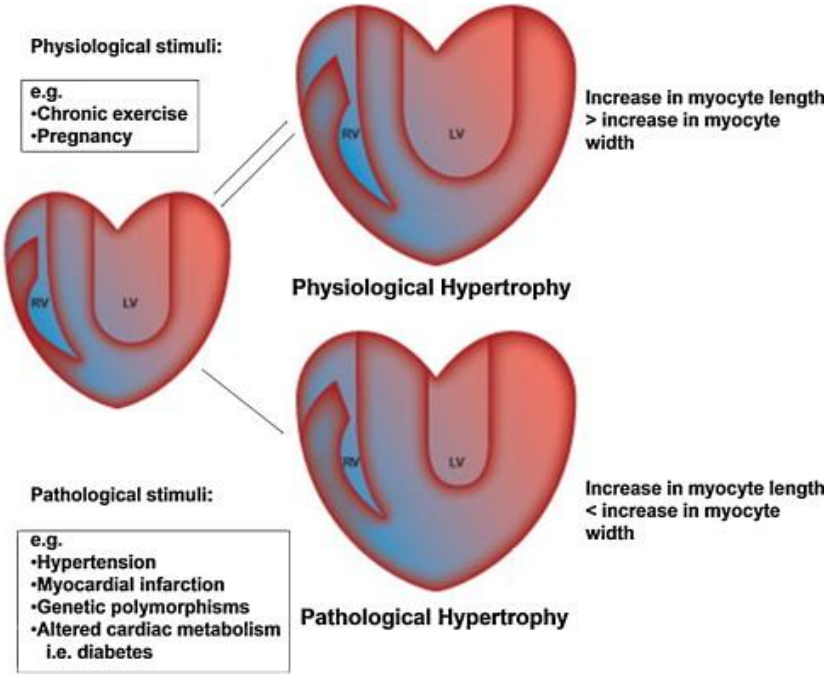


Figure 1: Classification of cardiac hypertrophy based on physiological and pathological stimuli *(Adapted and modified from Frey et al., 2004)*

II.1.2.1. Physiological cardiac hypertrophy

Physiological enlargement of the heart occurs during normal growth, pregnancy and exercise. It involves myocyte hypertrophy along with the expansion of capillary network. Hence, there is sufficient availability of nutrients to the hypertrophied cells to maintain its health and function. It is characterized with normal or enhanced contractile function while maintaining cardiac architecture. Hudlicka and Fahard observed that the heart preserves oxygen demand to match the proportional increase in myocyte area in the case of exercise-induced hypertrophy (Fagard, 2003: Hudlicka *et al.,* 1992). A linear relationship is maintained between body weight and cardiac weight during physiological hypertrophy. In humans, the increase in LV mass observed with maturation of the heart from infancy to childhood is necessary and is not deleterious. The requirement of increased stroke volume and cardiac output in pregnant women is accompanied by substantial increase in LV dimension and mass which regresses in the postpartum period (Mesa *et al.*, 1999). The concentric and eccentric hypertrophy occurring in athletes is consistent with normal systolic and diastolic function. Hence, in the clinical context, the increase in LV mass by itself must be carefully interpreted. The complex cross talk between various intracellular signaling molecules directs the specific pattern of hypertrophy. Mechanical forces and growth hormones induce physiological hypertrophy by transducing via various signaling molecules like Akt, mTOR and PI3K (Heineke & Molkentin, 2006; Molkentin & Dorn, 2001). The endothelium lining the

coronary vasculature and endocardium also play critical roles in regulating heart size (Tirziu & Simons, 2009). NO, ET-1, AngII, prostaglandins, procoagulant and anticoagulant factors, and various growth factors, including FGF, VEGF, and PDGF released by endothelium can affect numerous parameters of myocardial and vascular function. Reports showed that the increase in heart vasculature may not only support cardiomyocyte hypertrophy but may also induce this process (Tomanek & Busch, 1998; Karpanen *et al.*, 2008). Physiological hypertrophy is reversible in the instance of exercise or pregnancy-induced hypertrophy (Bernardo *et al.*, 2010). Cardiac metabolism in physiological hypertrophy is characterized by enhanced fatty acid and glucose oxidation (Gertz *et al.*, 1988).

II.1.2.2. Pathological cardiac hypertrophy

In contrast to physiological hypertrophy, pathological hypertrophy occurs in response to chronic pressure or volume overload. Pathological hypertrophy is mostly seen consequent to diseases like hypertension, valvular disease, myocardial infarction, ischemia, inherited genetic mutations or diabetes. Pathological hypertrophy is considered to be an intermediate stage in the transition to heart failure. Similar to physiological hypertrophy, pathological hypertrophy is also associated with increase in cardiomyocyte size and cardiac mass. In the initial phase of hypertrophy, in response to hypertensive stress, cardiac efficiency is maintained and is termed as the “adaptive phase” or “compensatory phase”. Here the myocytes increase in thickness in order to cop

up with the increased pressure on the ventricular wall and to preserve the cardiac output. Prolonged hypertension induced hypertrophy can result in the transition from adaptive to maladaptive remodeling which is characterized with reduced ejection fraction and left ventricular dilatation. Hypertrophy is associated with severe cardiomyocyte loss and fibrotic remodeling and is characterized by systolic and diastolic dysfunction which predisposes the development of cardiac failure or even sudden death (Levy *et al.*, 1990). Myocytes loss is mediated by apoptosis or necrosis and is replaced with collagen leading to fibrosis. The accumulation of collagen results in the stiffening of the ventricular wall leading to the impairment of contraction and relaxation. The electrical conduction is disturbed due to presence of extensive fibrotic tissue, leading to arrhythmias. Angiogenesis is compromised in pathological hypertrophy and hence capillary density is not in par with the increased myocyte size. The reduced capillary density increases oxygen diffusion distances leading to myocardial ischemia (Perrino *et al.*, 2006).

Normal heart relies on fatty acid oxidation for the production of ATP needed for the proper functioning whereas glucose and lactate oxidation accounts for only 30% of energy production. Heart is able to switch between the fuel molecules depending on the need and is considered as an adaptive response. However, in pathological hypertrophy, there is reduced fatty acid oxidation and increased dependence on glucose for ATP production. This is because, the oxygen supply is limited and the transport of fatty acid is impaired in pathological hypertrophy.

In advanced state of hypertrophy, glucose metabolism decreases reducing the overall ability of the heart to generate ATP (Neubauer, 2007).

II.1.2.3. Cellular and molecular mechanisms in pathological hypertrophy

Reactivation of fetal gene program is observed in pathological cardiac hypertrophy. Fetal recapitulation occurs with molecular changes similar to that observed during fetal cardiac development and includes stimulation of atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP) and genes for fetal isoforms of contractile proteins (Fig. 2). It is accompanied by downregulation of adult genes, such as α-MHC and upregulation of β-myosin heavy chain (MHC). The various transcription factors (TFs) mediating the reactivation of fetal gene program are NFAT, NF-*k*B, MEF2, GATA4, and SRF. These TFs are implicated to play a prominent role in embryonic development (Akazawa & Komuro, 2003). The expression of fetal genes can be used as the markers of adverse cardiac remodeling. The aberrant expression of genes involved in fetal contractile proteins and cardiac metabolism leads to progressive myocardial dysfunction and irreversible pathogenesis.

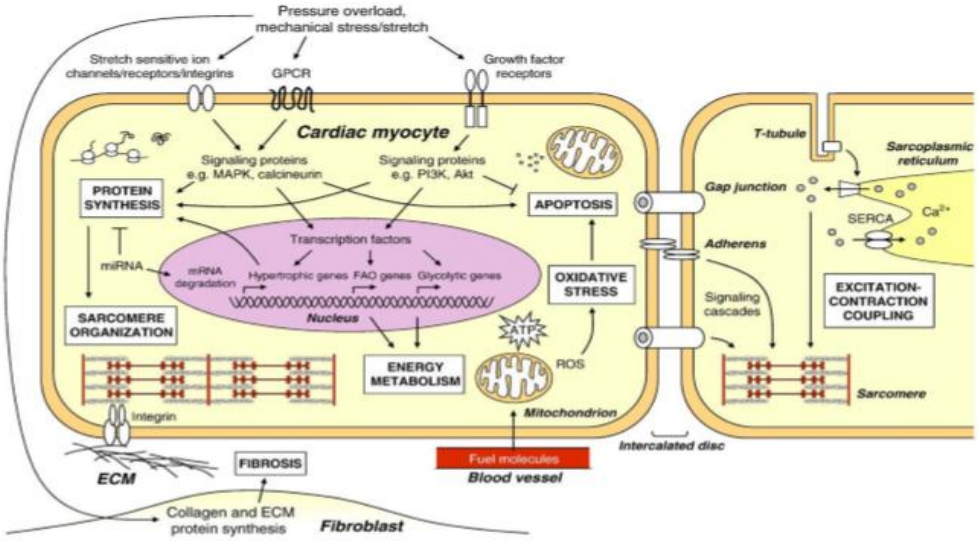


Figure 2: Cellular processes involved in the development of cardiac hypertrophy

ECM: extracellular matrix, FAO: fatty acid oxidation, GPCR: G protein-coupled receptor, MAPK: mitogen-activated protein kinase, PI3K: phosphoinositide 3-kinase, ROS: reactive oxygen species, SERCA: sarcoplasmic reticulum Ca^{2+} ATPase *(Adapted from Cavalcante et al., 2016)*

The irreversible changes of the myocardium occurring during pathological hypertrophy not only affect the parenchyma but also the stroma, which forms the extracellular matrix. The extracellular matrix is composed of fibroblasts which secrete collagen along with smaller amounts of elastin, laminin, and fibronectin. The fibrillar collagen provides structural integrity to adjoining myocytes, and aids in myocyte contraction that translates into efficient cardiac contraction (Weber *et al.*, 1994). Pathological hypertrophy is associated with increased interstitial and perivascular fibrosis. Compared to the normal myocardium, the extent of fibrosis is inversely proportional to the number of surviving myocytes

and is directly related to the degree of hypertrophy. Excessive collagen deposition replaces the myocyte loss due to apoptosis and necrosis. Disproportionate accumulation of collagen increases ventricular stiffnesss and altered microcirculation resulting in impaired cardiac function and adverse cardiovascular events. The augmented fibrosis and reduced capillary density increases the oxygen diffusion distance resulting in myocardial ischemia (Gradman & Alfayoumi, 2006).

II.1.2.4. Classification of hypertrophy

Depending on the initiating stimuli, cardiac hypertrophy has classically been subdivided as concentric or eccentric (Kehat & Molkentin, 2010) (Fig. 3). Concentric hypertrophy is observed in response to pressure overload pathological stimuli such as hypertension and aortic stenosis. An increase of relative wall thickness and cardiac mass, with a small reduction in chamber volume is observed with concentric hypertrophy. In contrast, eccentric hypertrophy leads to increase in cardiac mass due to increased chamber volume. Pathological conditions which induce volume overload such as aortic regurgitation and arteriovenous fistula causes diastolic wall stress leading to eccentric cardiac hypertrophy. Concentric hypertrophy is characterized by parallel addition of sarcomeres leading to an increase in width of individual myocytes. In eccentric hypertrophy, sarcomere units are added in series resulting in relatively increased length of the myocytes. Clinical studies suggest that eccentric cardiac

hypertrophy poses greater risk than concentric cardiac hypertrophy since it progresses to ventricular dilation and systolic failure (Berenji e*t al.*, 2005).

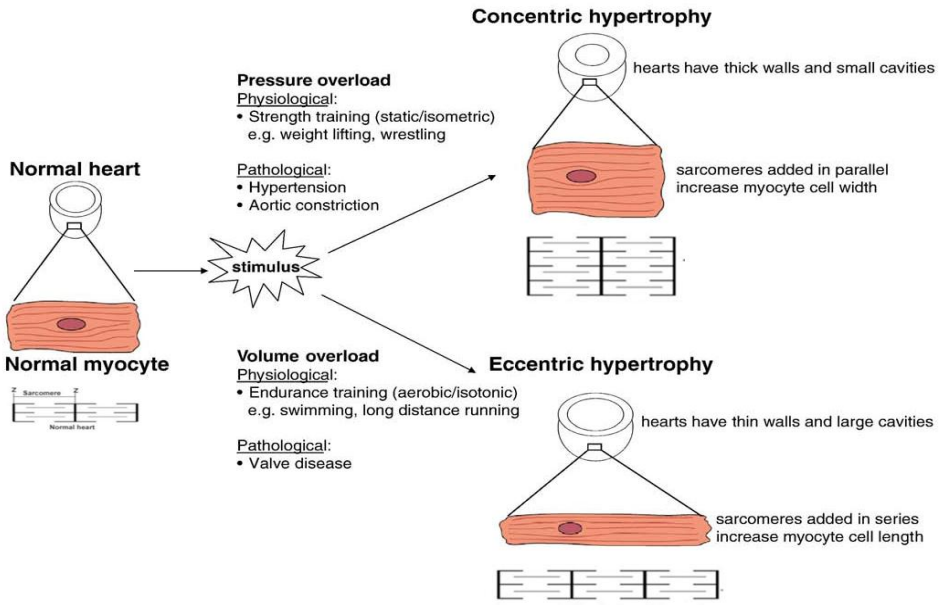


Figure 3: Classification of cardiac hypertrophy based on the phenotype of the heart and individual myocytes *(Adapted and modified from Bernardo et al., 2010)*

II.1.2.5. Transition from hypertrophy to failure

Hypertensive heart disease commonly results in concentric hypertrophy, a non-dilated thick-walled left ventricle typically with a normal left ventricular ejection fraction (LVEF), but is the common precursor to LV failure (Fig. 4). The transition from concentric LV hypertrophy to failure has been demonstrated in animal models including the Spontaneously hypertensive rat (Bing *et al.*, 1995), in surgical models of aortic banding, by transgenic manipulation and in humans

with aortic stenosis or familial hypertrophic cardiomyopathy (Berry *et al.*, 2007).

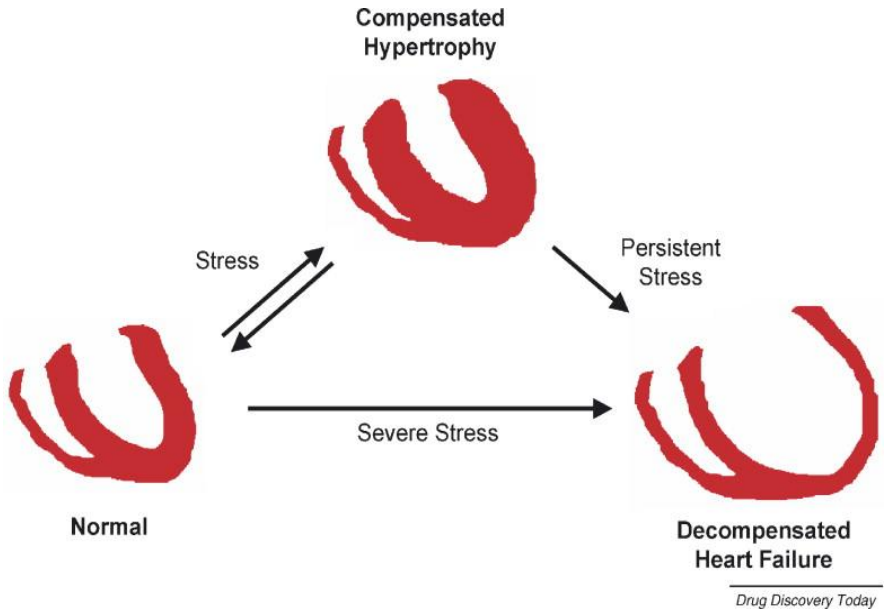


Figure 4: Transition from compensated hypertrophy to decompensated hypertrophy leading to heart failure *(Adapted from Berry et al., 2007)*

A number of factors are identified as mediators for the transition from hypertrophy to failure. PGC-1β deficiency is reported to accelerate the transition to heart failure in pressure overload. PGC-1α and PGC-1β regulate mitochondrial biogenesis and genes encoding for enzymes and proteins of mitochondrial metabolism and electron transport chain and are primarily expressed in oxidative tissues (Lehman *et al.*, 2008). The contribution of PGC-1β to mitochondrial function and gene expression in non-stressed hearts and its contribution to maintaining contractile function under pathological increased workload has been identified (Garnier *et al.*, 2003). PGC-1β and PGC-1α play partially overlapping but distinct roles in maintaining cardiac mitochondrial energetics in the

unstressed heart with both pathways regulating the expression of genes involved in oxidative phosphorylation whereas PGC-1α predominantly regulates fatty acid oxidation. However, both isoforms contribute to the maintenance of cardiac function in the context of pressure overload. The modulation of PGC-1β activity represents a promising target for limiting the transition from pressure overload cardiac hypertrophy to heart failure (Riehle *et al.*, 2011). This study also underscores the potential role of additional mechanisms such as oxidative stress that is exacerbated by deficiency of the transcriptional co-activators in contributing to the accelerated transition to decompensated heart failure.

Adverse remodeling of T-tubule in intact hearts is observed during compensated hypertrophy that is tightly associated with decline of myocardial function. T-tubule is a highly organized ultrastructural substrate for excitation- contraction coupling in ventricular myocytes. T-tubules are essential for the initiation and synchronous triggering of sarcoplasmic reticulum (SR) Ca^{2+} release and hence it is an important determinant of myocyte function (Wang *et al.*, 2001). A significant loss of T-tubule density with T-tubule disorganization in ventricular myocytes was first identified in a dog model with pacing induced heart failure (Yano *et al.*, 2005). Several discrepancies regarding T-tubule remodeling was resolved by a study in HF patients which found a pronounced T-tubule loss irrespective of the different cardiac etiologies. T-tubule remodeling is an important event that occurs before the onset of HF and is not a secondary modification after HF (Wei *et al.*, 2010). This study using spontaneously

hypertensive rats in HF also showed that T-tubule disorganization led to an increase in orphaned Ryanodine Receptors (RyR), which led to the loss of local control of Ca^{2+}-induced Ca^{2+} release resulting in decreased Excitation Contraction (EC) coupling efficacy and increased dyssynchrony of SR Ca^{2+} release. Progressive deterioration of T-tubule structure in ventricular myocytes was observed throughout the disease development. The strong correlation between LV function and T-tubule disorganization makes it a critical factor mediating the transition from compensated hypertrophy to HF.

A study in Dahl salt sensitive rat myocytes observed that alterations in EC-coupling occur in LV myocytes during the transition from compensated hypertrophy to heart failure (Nagata *et al.*, 1998). Prolongation of contraction and relaxation may be an early indicator of abnormalities in myocyte mechanical function during the transition. A shift in myosin heavy chain (MHC) isoforms from the V1 to the V3 isoform was observed during the development of cardiac hypertrophy or failure in rats (Gupta, 2007). The altered catalytic hydrolysis of ATP by changes in the activity of myosin ATPase will affect maximal rates of energy liberation during the cross-bridge cycle. The isoform shift may affect the time course of the contraction and represent a compensatory mechanism to maintain the contraction amplitude. A decrease in contractility will be observed when the prolongation of contraction time course can no longer preserve the adequate contractile function with disease progression.

Apoptosis of cardiac muscle cells (myocytes) is suggested to be one of the determinants of transition from compensated to decompensated hypertrophy leading to heat failure. The loss of myocardial cells through apoptosis has been considered one of the major contributing factors for cardiovascular failure (Narula *et al.*, 2006). Oxidative stress, implicated in hypertension and hypertrophy is known to trigger the mitochondrial death pathway (Sharma *et al.*, 2007). The organelle, mitochondria is the primary site of action for key apoptotic regulatory factors, including the Bcl-2 family of proteins. An increase in the ratio of Bax to Bcl-2 in the outer mitochondrial membrane, determines survival or death of a cell following an apoptotic stimulus (Oltvai *et al.,* 1993). This increase leads to collapse of the mitochondrial membrane potential and translocation of cytochrome-c into the cytoplasm, thereby triggering the caspase cascade and subsequent apoptotic cell death. Activation of caspase cascade and upregulation of Bcl-2 proteins during the transition to pressure overload-induced heart failure was reported in a sheep model (Moorjani *et al.*, 2007). Activation of apoptotic factors is also reported in guinea pigs during the transition from cardiac hypertrophy to failure (Sharma *et al.*, 2007).

Increased reactive oxygen species is an important and the main contributing factor for the downstream pathways involved in the development and progression of hypertrophy. An increase in free radicals or a relative deficit in the endogenous antioxidant reserve leading to oxidative stress can cause contractile dysfunction (Dhalla *et al.,* 1996) and has been suggested as one of the

contributing factors in the transition of compensated heart hypertrophy to the decompensated stage. The increase in cardiac workload observed in hyper-functional heart hypertrophy is associated with increased endogenous antioxidant enzyme activities (Hill & Singal, 1996). Heart failure due to a variety of conditions has been shown to be associated with oxidative stress indicated by reduced antioxidants, a depressed redox state and increased lipid peroxidation (Fig. 5).

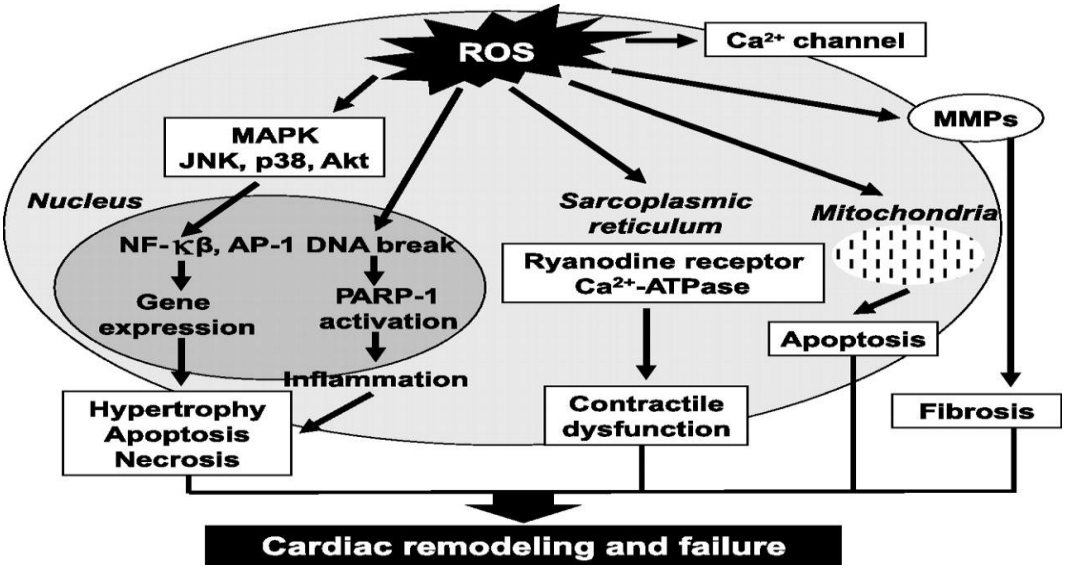


Figure 5: Role of oxidative stress in the transition from hypertrophy to heart failure

ROS: Reactive Oxygen Species, MAPK: Mitogen Associated Protein Kinase, MMP: Matrix metalloproteinase *(Adapted from Tsutsui, Kinugawa & Matsushima, 2011)*

The mechanisms by which the heart adapts to chronic pressure overload by undergoing adaptive remodeling and its transition eventually leading to heart

failure (HF), are still not well defined. Mining new mechanisms involved in the transition will help in developing new and efficient therapeutic strategies.

II.1.3. Cardiac changes associated with age

Aging, an unavoidable process, impairs different systems that serve our body and mind. Aging starts with birth and accelerates with advancing age. The alterations that occur during the aging process go unnoticed for a long time until it requires medical attention. The prevalence of diseases increases dramatically with age. Aging is a dominant risk factor for cardiovascular diseases and predisposes the heart to various adverse structural and functional alterations. A high prevalence of heart failure is noted in the older population, generally above the age of 65 (Strait & Lakatta, 2012). Significant changes in cardiac structure and function occurs with advancing age. Many of these changes are the consequence of cardiovascular diseases. Nevertheless, changes can occur even in the absence of clinical cardiac dysfunction.

Microscopic changes like increase in intimal thickness and deposition of collagen with aging ultimately leads to arterial wall thickening and stiffness. This is manifested as increased systolic blood pressure and pulse wave velocity as well as widening of the pulse pressure (Stern *et al.,* 2003). Arterial stiffness can lead to the development of left ventricular hypertrophy as a result of increased afterload and wall stress. In the heart, myocardial volume is found to be unaltered with age. 35% of the total myocytes in the ventricle is reported to be lost between

the ages 30 and 70 (Anversa *et al.*, 1990, North & Sinclair, 2012). To compensate for the cell loss, the remaining myocytes hypertrophy and this occurs along with the expansion of the non-myocyte compartment. Though the reason for the cell death is unclear, reduction in capillary density is noted to occur with aging leading to ischemic injury (Jesmin *et al.*, 2005).

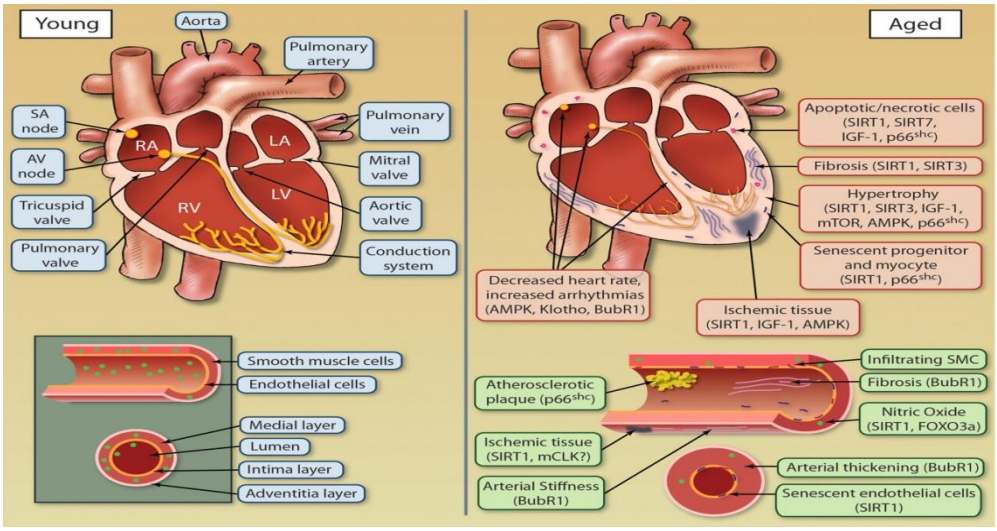


Figure 6: Characteristics of the aged heart *(Cited from North & Sinclair, 2012)*

An age dependent increase in the prevalence of left ventricular hypertrophy in healthy individuals has been reported in the Framingham Heart Study and the Baltimore Longitudinal Study on Aging (BLSA) without concomitant cardiovascular diseases. They have also shown age dependent decline in diastolic function, and relatively preserved systolic function at rest (Lakatta & Levy, 2003). A decline in exercise capacity and the prevalence of atrial fibrillation is also implicated with aging. In older population during exercise, increase in heart rate and Ejection Fraction (EF) indicates reduced cardiac reserve (Lakatta, 2001).

Myocardial performance index (MPI) increases with age which indicates that a greater fraction of systole is spent to cope with the pressure changes during isovolumetric phases. MPI has been shown to reflect both LV systolic and/or diastolic dysfunction (Tei *et al.*, 1997). These variations are independent of the conventional risk factors for heart disease such as smoking, hypertension, blood lipid levels, diabetes, etc. Hence they may be considered to be part of intrinsic cardiac aging. In the absence of systolic heart failure, diastolic dysfunction is increasingly seen in the elderly, a condition named as heart failure with preserved ejection fraction (HFpEF).

Mouse models are normally used for cardiac aging studies since they closely recapitulate the phenotypes of human cardiac aging (Dai & Rabinovitch, 2009, Zhang *et al.*, 1997). The relatively short lifespan and the availability of genetically modified mice are other advantages of using mouse model for studying molecular mechanisms of cardiac aging. Laboratory mice do not develop elevated blood pressure or adverse blood glucose and lipid profiles which allows the investigation on intrinsic cardiac aging without the added cardiac complications. The echocardiography performed on a mouse model exhibited phenotypic changes like increased LV mass, decreased diastolic function and worsened MPI parallel to human cardiac aging (Dai *et al.*, 2012).

The functional abnormalities of the aging myocardium occur not only due to structural changes but also due to cellular and molecular alterations. Initially, these changes occur as compensatory adaptive mechanisms for energy

preservation, which in course of time becomes maladaptive. Multiple molecular mechanisms are involved in the pathogenesis of cardiac aging.

Hypertrophy of the heart is a hall of cardiac aging. Nutrient and growth signaling in the heart are altered with advancing age due to hypertrophy. Two major signaling pathways implicated in cardiac hypertrophy and aging are mechanistic target of rapamycin (mTOR) and insulin-like growth factor-1 (IGF-1). mTOR regulates growth and has been shown to be a major modulator of aging and age-related diseases. Increased mTOR signaling impairs and reduced mTOR signaling improves resistance to cardiac aging in mouse models (Sciarretta *et al.*, 2014). One of the best characterized pathways of lifespan regulation in animal models is Insulin/IGF-1 signaling. Deficiency in insulin/IGF- 1 signaling attenuates age related cardiomyocyte dysfunction in mice. Age-dependent decline in serum IGF-1 levels in humans is associated with increased risk of heart failure. Unlike the observations in mice, interventions that increase IGF-1 signaling were beneficial in heart failure in rat. In aged rats, IGF-1 treatment was protective against mitochondrial oxidative stress (Higashi *et al.*, 2012). These studies confer the beneficial effects of IGF-1 to mitochondrial protection mechanisms.

The vicious cycle of ROS induced ROS release seen in dysfunctional mitochondria can lead to cellular and organ functional damage that limits lifespan (Harman, 1972). ROS production in mitochondria is found to increase with advancing age in heart. The mitochondria are usually enlarged with swelling

along with loss of cristae in aged cardiomyocytes. The inner membranes of mitochondria are destructed and are deficient in ATP production (Terman *et al.*, 2004).

One of the key regulators of mitochondrial biogenesis, Peroxisome proliferator-activated receptor coactivator 1α or PGC-1α enhances mitochondrial function in the heart (Liang & Ward, 2006). The expression of PGC-1α is repressed in the failing heart. The mitochondrial gene expression was reduced in PGC-1α knockout mice and they were found to develop cardiac dysfunction at 7 months of age (Dai *et al.*, 2010). Mitochondrial dysfunction and aberrant ROS production contribute to aging through both direct damage to cellular macromolecules and also by interfering with normal signaling and energetics.

Extra-cellular matrix is a complex collection of proteins located outside the cells. Their function is to provide structural and biological support to the surrounding cells. The primary source of cardiac ECM proteins are cardiac fibroblasts, which secretes proteins like collagen type I, II, III, IV, V, and VI, elastin, fibronectin, laminin, and fibrinogen (Frantz *et al.*, 2010). Even though the cardiac ECM properly aligns cardiomyocytes, excessive ECM deposition increases the stiffness of the myocardium and mediates diastolic dysfunction (Kwak, 2013). The synthesis and degradation of ECM proteins is dynamically regulated by matrix metalloproteinases (MMPs), Tissue Inhibitors of MMPs (TIMPS) and other proteases. Myocardial fibrosis and deregulation of ECM protein synthesis and degradation is observed in aging heart. A profibrotic factor, transforming

growth factor-β (TGF-β) has been shown to induce the expression of ECM proteins and also inhibit matrix degradation by MMPs (Bujak & Frangogiannis, 2007). The expression of another matricellular protein, Secreted protein acidic and rich in cysteine (SPARC), was found to be increased with age, and its deletion resulted in reduced fibrillar collagen in the LV and decreased LV diastolic stiffness (Bradshaw, 2016). These evidences suggest the critical role played by ECM proteins in diastolic dysfunction with age. MMPs are zinc-dependent enzymes that regulate ECM degradation whereas TIMPS regulate MMP proteolytic activity in the tissue (Klein & Bischoff, 2011). MMPs and TIMPs are differentially regulated by age but their roles in cardiac aging have not been established.

Cardiac aging is a complex process and involves intrinsic and extrinsic factors. Management of aging with well balanced diet and lifestyle will help to maintain heart young and healthy.

II.2. Antioxidant Supplementation for Cadiovascular Diseases

As mentioned in II.1.2.5, oxidative stress is a major contributor for hypertension induced hypertrophy and aging of the heart. The impairment of the delicate balance between ROS and antioxidant defense system results in the accumulation of ROS causing damage to macromolecules and activating several signaling pathways involved in the pathology. Oxidative stress has emerged as a central common pathway by which disparate influences may induce and exacerbate

cardiac remodeling. Though the role of oxidative stress in CVDs and aging is well-established, whether it is a cause or consequence still remains unanswered.

Antioxidants, as the name suggests constitute a diverse group of compounds which operate by inhibiting oxidant formation and repairing oxidant-induced injury. Reviews that appeared in the 90's claimed the prevention of CVDs by supplementing antioxidant vitamins and was found to be a promising area of research. The ability of antioxidants to inhibit the oxidation of low-density lipoprotein cholesterol was postulated. Several descriptive and cross-sectional studies, analytic investigations using case-control and prospective cohort study designs, and small randomized clinical trials have explored the antioxidant vitamin hypothesis. Though the findings from these studies are not totally consistent, they support the hypothesis that antioxidant vitamins reduce the risk of CVD (Manson *et al.*, 1993).

Dietary antioxidants including vitamin C, vitamin E and β-carotene have received the greatest attention with regard to coronary heart disease prevention. A number of trace elements also act as antioxidants and include selenium, copper, zinc, and manganese, which serve as cofactors for enzymes with antioxidant activity. Antioxidant intake was shown to be associated with reduced CVD risk. This generally has involved increased consumption of antioxidant-rich foods. Some of the results have suggested the possible importance of supplemental levels of antioxidants (Tribble, 1999).

The increased oxidative stress impairs endothelial function leading to an increase in intracellular free calcium, peripheral vascular resistance, and hypertension. Supplementation with the antioxidants vitamin C, E, or B6 lowerered blood pressure in animal models and humans. Hence, dietary supplementation with antioxidants is a beneficial and inexpensive, first-line alternate treatment modality for hypertension (Vasdev & Gill, 2005). Epidemiological and clinical data suggests that antioxidant-rich diets reduces blood pressure and cardiovascular risk whereas randomized trials and population studies using natural antioxidants have yielded disappointing results. Incomplete knowledge of the mechanisms of action of the antioxidants, lack of target specificity, and potential inter-individual differences in therapeutic efficacy can be the reason for the inconsistent observations that precludes from consuming any specific natural antioxidant for antihypertensive therapy (Kizhakekuttu & Widlansky, 2010). However, diets rich in fruits and vegetables continue to be the best strategy for non-pharmacological therapy in hypertension. Antioxidant supplementation has not shown consistently to be effective and improvement was not seen in blood pressure in subjects at high risk of cardiovascular disease after treatment with single or combination antioxidant therapy (Baradaran *et al.*, 2014).

However, in treating left ventricular hypertrophy and associated complications, antioxidants were found to be effective, given the potential role of ROS in maladaptive remodeling. The antioxidant N-2-mercaptopropionyl glycine

attenuated left ventricular hypertrophy in an in vivo murine pressure-overload model (Date *et al.*, 2002). When L-arginine, BH(4) and the combination of two antioxidants, superoxide dismutase and catalase, were administered in a swine left ventricular hypertrophy model to assess endothelium-dependent relaxations, they prevented endothelial dysfunction (Malo *et al.*, 2003). Edavarone, free radical scavenger 3-methyl-1-phenyl-2-pyrazolin-5-one, is used to treat patients with ischemic brain damage. When edavarone was administered to murine model of pressure overload-induced cardiac hypertrophy, it significantly attenuated pressure overload-induced cardiac hypertrophy and was mediated through its antioxidative function (Tsujimoto *et al.*, 2005).

Tempol, a potent antioxidant and superoxide dismutase mimetic was given to high-fructose diet fed mice with increased myocardial oxidative damage and exacerbated systolic dysfunction after transverse aortic constriction (TAC). Tempol significantly neutralised markers of cardiac hypertrophy, LV remodeling, contractile dysfunction, and oxidative stress in fructose-fed TAC mice (Chess *et al.*, 2008). In hypertensive cardiac remodeling, increased oxidative stress upregulates matrix metalloproteinases (MMPs) and transforming growth factor-β (TGF–β). When tempol was administered to hypertensive rats, it decreased oxidative stress, TGF-β levels, and gelatinolytic activity comparable to control levels. These findings support the assumption that antioxidants may help to prevent hypertension-induced cardiac hypertrophy (Rizzi *et al.*, 2013).

II.3. Cardiac Stem Cells (CSCs)

The general paradigm that the heart is a post mitotic terminally differentiated organ was challenged by the studies carried out in the late nineties and early twenties. Adult cardiac muscle cells were believed to have a life as long as that of the entire organism and were incapable of dividing. According to this view, the number of myocytes defined after birth is preserved throughout life till death of the organism; and myocyte loss leads to irreparable cardiac injury. In 2001, a study reported that the adult heart has a subpopulation of myocytes that are not terminally differentiated and these could reenter the cell cycle and undergo mitosis. Ki-67 labelling identified multiplying myocytes, underscoring the presence of undifferentiated cells in the heart, with the dividing myocytes being their progeny (Beltrami *et al.*, 2001). The existence of cycling ventricular myocytes in the normal and pathological heart and their increase in response to acute and chronic injury was established in the early part of this century. These results raised the question of the origin of the dividing cardiomyocytes. The presence of cell division suggests a continuous turnover of cells during the life span of the organism. In the following year, a clinical study was carried out to understand the interaction between donor and recipient cells during heart transplantation. Men who received sex mismatched donor hearts showed primitive cells bearing Y chromosomes which expressed the markers of cardiomyocytes, smooth muscle cells and endothelial cells (Quaini *et al.*, 2002). These primitive cells expressed on their surface stem-cell–related antigens

including c-kit, MDR1, and Sca-1 further pointing to the occurrence of resident cardiac stem cells in vivo that differentiate into myocytes. Rudnicki and colleagues made the first attempt to describe the resident cardiac stem cell (Hierlihy *et al.,* 2002). They isolated a Hoechst effluxing cell population which was similar to the side population identified in other organs (Asakura *et al.*, 2002; Bunting, 2002; Goodell *et al.,* 2005). They were negative for haematopoietic markers like CD34, c-kit, Sca-1 and Flk-2. In addition, they were clonogenic and were able to differentiate into cardiac muscle cell expressing connexin-43. This work introduced the concept of stem cell pool in adult heart. The presence of resident cardiac stem cells was first reported in rat heart by Beltami in 2003. They identified lineage negative, c-kit^{+} cells that were self-renewing, clonogenic and multipotent (Beltrami *et al.*, 2003). These cells were able to differentiate into all the three major cell types of the heart including cardiomyocytes, smooth muscle cells and endothelial cells in vitro and in vivo. This study opened new possibilities and opportunities for cardiac therapy using exogenous cardiac stem cells for functional repair of damaged myocardium. Subsequently, different types of adult stem cells with specific membrane markers were identified and investigated in detail for their utilization in heart disease management. Heart, a tissue which till recently was believed to lack self-renewal capability is found to be populated by different types of tissue-specific stem cells. Of the many different resident cardiac stem cells, c-kit^{+} CSCs are the most

extensively used and widely investigated for pre-clinical and clinical stem cell transplantation studies.

II.3.1. c-kit$^+$ Cardiac Stem Cells

CD-117 or c-kit also called as stem cell growth factor receptor is a receptor tyrosine kinase protein present on cell surface and is mainly used for the identification of progenitor cells. Binding of c-kit ligand to stem cell factor activates its intrinsic tyrosine kinase activity, which in turn phosphorylates and activates signal transduction pathways. Signalling through CD117 plays a role in cell survival, proliferation, and differentiation. c-kit$^+$ CSC was the first identified stem cell in the heart by a team led by Beltrami in 2003 and was found to exhibit all the typical features of stem cells (Beltrami *et al.*, 2003). Clones developed from individual cells expressed cytoplasmic markers specific for cardiac lineages like GATA 4, Nkx2.5 and MEF2. The myocytes produced in vivo by these Lin$^-$ c-kit$^+$ cells expressed N-cadherin and connexin-43 and were functionally integrated and electrically and mechanically coupled. Beltrami *et al*. also observed that the regenerating myocytes result from proliferation and differentiation of injected c-kit+ cells and not from cell fusion. Linke *et al.* in 2005 identified c-kit$^+$ stem cells in the dog heart, that were self-renewing, clonogenic, and multipotent and regenerated infarcted myocardium, improving cardiac function (Linke *et al.*, 2005). CSCs isolated from canine hearts expressed cell surface markers c-kit, MDR1 and Sca-1 in different proportions. More than

60% of CSCs expressed all the three antigens and CSCs were seen in the ratio of 1:18000 myocytes. CSCs were found to be recruited and activated by growth factors like HGF and IGF1 in canine hearts after infarction to invade the damaged tissue and promote regeneration. In the event of myocardial injury, CSCs differented to the desired cell type and improved ventricular hemodynamics, thereby mediating reverse remodeling. These studies prove that heart is a self renewing organ with a growth reserve of stem cells that can be coaxed to regenerate the damaged myocardium (Fig. 7).

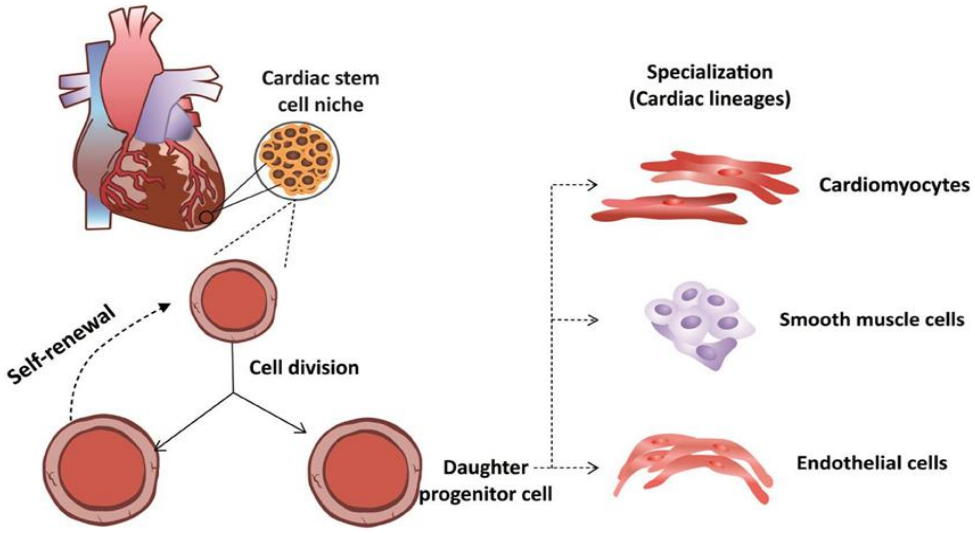


Figure 7: Cardiac stem cells: Stem cell Niche, Self–renewal, Assymetric Division and Multi-lineage differentiation *(Cited from Camila et al., 2015)*

Bearzi and his colleagues were the first to isolate cardiac stem cells from the human heart (Bearzi *et al.*, 2007). They identified clusters of hCSCs were intimately connected by gap junctions and adherens junctions to myocytes and fibroblasts. These CSCs were multipotent and their ability to create

cardiomyocytes and coronary vessels in vivo provides strong evidence in favor of their role in cardiac homeostasis and myocardial regeneration.

c-kit$^+$ cells colonize the yolk sac, liver and other organs which co-express the SCF, the ligand of the c-kit receptor, in the early stages of development (Kunisada *et al.*, 1998; Teyssier-Le Discorde *et al.*, 1999). Hence, it can be presumed that stem like cells are present in the heart from fetal life. Rapid increase in SCF during myocardial ischemia and the increased formation of myocytes in the acutely infracted heart strengthened the notion that SCF was involved in the activation of c-kit$^+$ cells (Frangogiannis *et al.*, 1998; Beltrami *et al.*, 1994). Taken together, the presence of undifferentiated cells along with early committed progenies suggests the role of CSCs as the critical modulators of the homeostasis of the normal and stressed myocardium.

In 2010, Miyamoto *et al.* investigated the stability of c-kit$^+$ cells in long term culture. c-kit$^+$ cells were isolated from adult rat hearts and were passaged for as many as 40 passages while retaining genes for stemness. They exhibited stem cell properties of single cell-derived clone formation, cardiosphere generation, and potential to differentiate in vitro into the three main cardiac lineages: cardiomyocyte, smooth muscle, and endothelial cells. However, after long term culture, increased GATA-4 expression was noticed in certain CSCs which resulted in enhanced cardiomyocyte differentiation and they also exhibited spontaneous differentiation into other lineages (Miyamoto *et al.*, 2010). These

findings justify the feasibility of culturing and maintaining c-kit^{+} cells in repeated passages without much variation in their characteristics.

A study led by Camila and her team focused on the involvement of stem cells in physiological cardiac hypertrophy induced by exercise training. Male mice which underwent swimming training protocol were used for the study which induced true physiological cardiac hypertrophy. The increase in the cardiac mass index occurred without any change in collagen deposition and was accompanied by proportional increase in microvascular density. The significant increase observed in the number of c-kit^{+} Lin^{-} CSCs in the trained animals could be due to increase in the proliferation of these cells or due to reduced apoptosis/necrosis as a result of the growth factors released during cardiac remodeling and growth (Leite *et al.*, 2015). Tracing the proper lineage of the so called cardiac stem cells and understanding their role in regeneration and repair of the normal and injured cardiac tissue will aid in better management of the cardiovascular diseases.

II.4. Aging of Stem Cells

Adult tissues harbor a small portion of embryonic stem cells from the inner cell mass/blastocysts and retain their developmental potential. These tissue specific stem cells play an important role in the normal homeostasis of the organ as well as aid in regeneration or repair during damage. Depending upon their tissue location, stem cells reveal a tremendous heterogeneity in growth kinetics, differentiation and survival. Appropriate balance between stem cells, precursor

cells and differentiated cells is maintained by various feedback mechanisms that regulate the proliferation, survival and differentiation signals. Stem cells are carefully regulated so as to meet the demands of the tissue in which they reside. Various intrinsic regulators like telomerase and Bcl-2 family members and extrinsic regulators like Notch signaling pathway, growth factors/cytokines and cell adhesion molecules influence the stem cell fate. Stem cells comprise only a very small population within a tissue which makes it hard to delineate the cellular and molecular changes that occur with aging. The difficulty of obtaining pure stem cell populations in sufficient quantities to perform various analyses is yet another barrier. However, considering the importance of stem cells in tissue homeostasis, analyses of these cells is imperative in aging research. Understanding the molecular pathways involved in age-dependent deterioration of stem cell function will be critical for developing therapeutic strategies for diseases of aging.

Studies have provided evidence about the diminished ability of stem cells to respond to environmental demands with aging. Haematopoietic stem cells (HSC) are the most widely used stem cell type for aging studies basically due to its abundance and ease in acquiring (Morrison *et al.*, 1996). Though the HSCs were five times more frequent in the bone marrow of old mice than the younger mice, only one−quarter was efficient at homing to and engrafting the bone marrow of irradiated recipients. It is unknown whether these changes are determined intrinsically or caused by the aging of their environment. Another study by

Chambers *et al.,* also found that hematopoietic stem cells are not protected from aging. With aging, the genes associated with the stress response, inflammation, and protein aggregation were up-regulated; whereas genes involved in the preservation of genomic integrity and chromatin remodeling were down-regulated. The loss of epigenetic regulation at the chromatin level may drive both functional attenuation of cells as well as other manifestations of aging (Chambers *et al.*, 2007). The role of both extrinsic and intrinsic factors in HSC aging was reviewed. The altered microenvironment/niche along with other regulatory intrinsic pathways primarily Wnt-pathway were found to be the major contributors of HSC aging (Akunuru & Geiger, 2016).

Bone-marrow derived circulating endothelial progenitor cells (EPCs) contribute to vascular haemeostasis. Aging poses one of the major risk factors for the development of cardiovascular disease. This can be explained in part by the reduced ability of circulating endothelial progenitor cells to contribute to vascular repair and regeneration. EPCs are subject to age-associated changes that results in decrease in their number in circulation and diminished function, thereby enhancing risk for vascular diseases. Kushner *et al.,* found a 57% reduction in early EPC telomerase activity in older men compared to younger counterparts. EPC senescence induced by telomere shortening may contribute to numerical and functional impairments of EPCs (Kushner *et al.*, 2009). The age-associated impairment of EPC number and function is due to a variety of environmental changes that impair their generation, mobilization, homing, and

function. The intracellular alterations within the cells with age induces a senescent phenotype in EPCs (Williamson *et al.*, 2012).

In 2011, a group of researchers examined the effect of aging and heart failure on cardiac stem cells. The properties of human Cardiac stem cells isolated from the explanted and donor hearts were examined. The hCSCs from explanted hearts exhibited lower proliferative capacity, lower migration rate, high expression of senescent markers and lower telomerase activity compared to hCSCs from donor hearts. However, a definite distinction between physiological and pathological aging was not possible since the donors were younger compared to older patients who underwent transplantation. There was also variation in the disease pathologies of the patients which made the comparison more problematic (Cesselli *et al.*, 2011). Though not conclusive, this study for the first time reported the age and disease associated decline in the functional ability of hCSCs.

An age-dependent study on the quality and quantity of cardiosphere derived cells (CDC) found that the impact of age on CDCs was quite limited though there was variation in CDC characteristics between patients. Though the growth rate, growth factor production and angiogenic potency of CDCs were not impaired with age, the CDCs from older patients exhibited senescent markers and damaged DNA (Nakamura *et al.*, 2016). These studies have important clinical implications for autologous stem cell transplantation in elderly patients. A detailed and critical investigation on the effect of aging on CSCs is imperative

using an appropriate animal model of cardiovascular disease to arrive at a definite conclusion.

II.5. Effect of antioxidants and antihypertensives on stem cells

Physiologically relevant levels of ROS are required for the initiation of stem cell proliferation and differentiation. However, high levels of ROS/low levels of antioxidants can interfere with the efficient functioning of stem cells. Studies have reported the importance of endogenous antioxidant level for the transplantation efficacy of stem cells. Oxidative stress is the main reason for the low efficiency of hUCMSCs (human Umbilical Cord Mesenchymal stem cells) transplantation. Pretreatment of the hUCMSCs with the antioxidant Edavarone decreased cell apoptosis in vitro and improved the expansion efficiency of stem cells in vivo (Zeng *et al.*, 2015).

Pre-treating male Sprague-Dawley rats with resveratrol maintained a reduced oxidative stress in myocardial tissue by over-expressing Nrf2 and Ref-1 resulting in an improvement in cardiac functional parameters. The improvement of cardiac function was accompanied by enhanced stem cell survival, proliferation and differentiation towards the regeneration of the myocardium after LAD (Left anterior descending coronary artery) occlusion in the resveratrol-treated hearts (Gurusamy *et al.*, 2010).

The antihypertensive, pravastatin improved function of hibernating myocardium by mobilizing c-kit^{+} bone marrow progenitor cells and promoted myocytes to

reenter the growth phase of the cardiac cell cycle (Suzuki *et al.*, 2009). The effect of a β-blocker, metoprolol, on left ventricular (LV) remodeling, c-kit$^+$ cells, proliferation, fibrosis, apoptosis, and angiogenesis was assessed using a model of coronary ligation in rats. Metoprolol treatment improved LV systolic function, increased the number of c-kit$^+$ cells and expression of Ki-67 (Serpi *et al.*, 2009). The beneficial effect of metoprolol can be associated with the increased cellular proliferation following MI. However, Losartan, an angiotensin receptor blocker, failed to exhibit these characteristics in the treated rats.

The cardioprotective effects of antioxidants and antihypertensives can possibly be mediated by prevention of deterioration in stem cell number and efficiency. These studies provide insight for a detailed investigation on the impact of antioxidants and antihypertensive on c-kit$^+$ CSCs.

III. METHODOLOGY

III.1. DESIGN OF THE STUDY

Age-associated cardiac remodelling leads to cardiac ailments in the elderly. Left ventricular hypertrophy, consequent to uncontrolled hypertension is an independent risk factor for cardiac failure. Many physiological, cellular and molecular mechanisms have been proposed to explain the transition from hypertrophy to failure. Cardiac stem cells, since its discovery in 2003, have been the centre of attention for use in cardiac regeneration and stem cell transplantation studies. The involvement of stem cells in physiological and pathological conditions in various organs is documented. However, information on the changes in cardiac stem cells and the distinction between pathological and physiological aging is lacking.

Reactive oxygen species and oxidative stress is implicated both in hypertension and aging. The imbalance in antioxidants and ROS is debilitating in nature and is found to affect several signalling pathways culminating in the expression of genes involved in adverse cardiac remodelling. High levels of oxidative stress in the heart of SHR is indicative of the adverse milieu in the myocardium, which can negatively influence the cardiac stem cells residing within the niches. Antioxidants are widely used as health supplements and are known to have an overall improvement on the wellbeing of the individual. The beneficial effect of vitamin E and other potent antioxidants on cardiac tissue has been recorded.

However, the beneficial effects of antioxidant supplementation in SHR, particularly its effect on cardiac stem cells remains to be explored.

The study was designed with three main objectives:

i. Assess the age dependent variation in the attributes of cardiac stem cells in different age groups of WST
ii. Determine the variation in cardiac stem cell characteristics with pathological aging in SHR, a model of hypertensive heart disease and compare with physiological aging
iii. Evaluate the effect of supplementation with the antioxidant, tempol, on CSCs of SHR

(i) To assess the effect of physiological aging on CSCs, the normotensive WST rat was used as SHR is derived from Wistar rat. Pups (1-week-old), 6, 12 and 18 month old rats were selected for the study to evaluate stem cell characteristics and heart was isolated and separated into ventricles and atria. The density of c-kit^{+} CSCs was assessed in the ventricular digests. Proliferation, differentiation, migration, senescence and oxidative stress - attributes known to be affected by aging were assessed in CSCs isolated from atrial explant cultures. Three animals were analysed in each group.

(ii) The animal model of hypertensive heart disease used for the study was Spontaneously Hypertensive Rat. Studies in SHR, the genetic model of

hypertension, has shown that it manifests the cardiac changes similar to clinical hypertension and exhibits the various stages of hypertensive heart disease, including compensated ventricular hypertrophy and its transition to cardiac failure (Bing *et al.*, 1995; Trippodo & Frohlich, 1981). Male SHR of different ages- pups (1-week-old), 6-months, 12-months and 18-months were selected representing various stages of cardiac remodelling (Doggrell & Brown, 1998; Sen *et al.*, 1974). No significant cardiac changes occur before 1 month of age, 6 months represents the stable phase of hypertrophy, and cardiac decompensation is initiated at 12 months and established by 18 months of age. Studies using aged SHR has shown typical features of heart failure reiterating the appropriateness of this model (Abbate *et al.*, 2006, Conrad *et al.*, 1995). The density of CSCs was determined in single cell suspension of ventricular digests. Migration, proliferation, differentiation, oxidative stress, DNA damage and senescence were evaluated in CSCs isolated from atrial explants. Three animals were analysed in each group. The data obtained was compared with that of physiological aging.

(iii) To ascertain the role of oxidative stress in modulation of CSC characteristics, the response to antioxidant supplementation was evaluated. Six month old SHR, which represents the stable phase of hypertrophy, were chosen for the treatment. Previous studies from our lab have shown that the myocardial oxidative stress is significantly high in SHR at 6 months of age compared to WST. In addition, characteristics of CSCs were also found to be

compromised as early as 6 months. To examine the effect of antioxidant, tempol on CSCs, twelve male SHR (6 months old) were randomly assigned into two groups of six rats each. One group received intra-peritoneal injection of 20mg/kg/day tempol for 2 weeks. Untreated SHR served as hypertensive control. Six age-matched male WST served as normotensive control. Untreated SHR and WST received the vehicle.

The study was approved by The Institutional Animal Ethics Committee. The housing care and the management of these animals were in accordance with the Committee for the Purpose of Control and Supervision of Experiments in Animals (CPCSEA) Guidelines.

III. 2. MATERIALS

III.2.1. Fine chemicals- The source is given in italics

Iscoves Modified Eagles Medium(IMDM) (*Himedia*), Foetal Bovine Serum (*Gibco*), basic Fibroblast Growth Factor (bFGF), B27 serum supplement (*Invitrogen*), Insulin selenium Transferrite, Gelatin, 5-azacytidine, 2',7'-dichlorodihydrofluorescein (DCFH), Bovine Serum Albumin (BSA), Collagenase (Type II), DNase I, Disodium hydrogen phosphate, Potassium chloride, Potassium dihydrogen phosphate, EDTA, Trypsin, Diethyl pyrocarbonate (DEPC), Protease inhibitor cocktail, Acrylamide, bisacrylamide, β-mercaptoethanol, TEMED, Ammonium persulphate, Color burst electrophoresis marker, Sodium dodecyl sulphate (SDS), Trizol reagent, Trizma base, Agarose, Paraformaldehyde, Crystal Violet solution, DAPI nuclear stain, Dimethyl Sulphoxide (DMSO) (*Sigma-Aldrich*), Nitrocellulose membrane (*Millipore USA*). Tempol (4-hydroxy Tempo) for animal experiments were purchased from *Sigma Aldrich.*

Antibodies: Anti c-kit antibody (Santa Cruz), Anti c-kit FITC, Anti $p16^{ink4a}$ antibody, Anti p21 antibody, Anti cTnI antibody, Anti 3-nitrotyrosine antibody (*Abcam*), Anti CD45 PE, Anti CD31 APC (*BD Pharmingen*), Anti SMA Antibody (*Cell Signalling Technologies*), Monoclonal Anti-β-actin antibody, Anti-mouse IgM antibody, monoclonal Anti-rabbit antibody, Rat anti- rabbit FITC (*Sigma-Aldrich*).

Kits: EasySep^{TM} FITC positive selection kit (*Stem Cell Technologies*), Coomassie protein assay reagent, Super signal West Femto Substrate kit (*Thermo scientific USA*), Senescence β-galactosidase staining kit (*Abcam*), TRAPeze Telomerase Detection Kit (*Millipore*), PrimeScript First-Strand cDNA Synthesis Kit (TAKARA), RNA isolation kit (*Qiagen*), Primers for real time PCR were obtained from *Sigma Aldrich*. Power SYBR Green for real time PCR was purchased from *Applied Biosystems.*

III.2.2. Routine Chemicals

Sodium chloride, chloroform, Propanol, ethanol, Methanol, Hydrochloric acid, glycerol, formalin, Isopropanol, Xylene were purchased from *Sisco Research Laboratories, Nice chemicals, Merck, India*.

III.2.3. Instruments used

EasySep^{TM} magnet (*Stem Cell Technologies*), Laminar Flow chamber (*Clas, India*), CO_2 water –jacketed incubator (Sanyo, USA), High speed refrigerated centrifuge (*Hitachi, Japan*), Eppendorf centrifuge 5415 R, Incubators (*Beston India; Kemi, India*), Phase contrast microscope (*Nikon, Japan*), Phase contrast microscope (*Olympus KX4, Philippines*) with camera (*Evolution LC, model no:PL-A662,Media cybernetics*), Weighing balance (*Sartorius, USA and Ohaus*), Deepfreezer -20^{o}C (*Vestfrost*), Deepfreezer -80^{o}C (*Sanyo*), Steam distillation unit (*Beston*), Microwave oven (*IFB*), Water bath (*LKB, Sweden*), Ice machine (*Hoshizaki, Japan*), pH meter (*Labindia*), Low speed magnetic

stirrer (*Remi, India*), Hot air oven (*Tempo, India*), EASY pure UV/UF compact reagent grade water system (*Barnstead, USA*), ELISA reader (*Bio-Tek instruments, USA*), Electrophoresis unit (*Biorad laboratories, USA*), Mini Blot (*Biorad laboratories, USA*), Programmable Thermal Cycler (*MJ Research Inc, USA*), Submarine electrophoresis unit (*Bangalore Genei, India*), UV-Transilluminator (*Bangalore Genei, India*), ABI Prism 7500 Sequence Detection System (*Applied Biosystem*) FACS (BD FACSAria™), Neubauer improved haematocytometer (*Sigma-Aldrich, USA*), Cell counter (*Biorad*), Fluorescent microscope (*Nikon eclipse TE 2000-U*), Multivolume spectrophotometer (*Nanodrop Bio-Tek*).

III.2.4. Software used

Image-Pro Plus 5.1 for image analysis (*Media Cybernetics*)

ImageJ (*NIH*)

Comet Assay Software

Zotero

III.3. COMPOSITION OF REAGENTS AND BUFFERS

III.3.1. Phosphate buffered saline (PBS) –pH 7.4

NaCl - 4g

KCl - 0.1g

KH_2PO_4 - 0.12g

Na_2HPO_4 - 0.78g

Dissolve and make upto 500mL using deionised water. Autoclave and refrigerate.

III.3.2. Tissue Dissociation solution

Collagenase (TypeII) - 40g

DNase I - 30mg

BSA - 30mg

Dissolve in 30mL sterile PBS. Prepare fresh before use.

III.3.3. Stem Cell Medium

IMDM - 90mL

FBS - 10mL

bFGF - 10µL

Insulin Selenium Transferrite - 1mL

III.3.4. Trypsinisation Solution

Trypsin	- 5mg
EDTA	- 2mg

Dissolve in 10 mL sterile PBS and store at 4^0C.

III.3.5. DEPC-treated deionized water

1 ml of DEPC in one litre of deionized water, stirred for 30 minutes at room temperature. Autoclave and store at 4^0C.

III.3.6. 10% Buffered formalin

NaH_2PO_4 (anhydrous)	- 3.5g
Na_2HPO_4 (anhydrous)	- 6.5g
Formalin	- 100ml
Distilled Water	- 900ml

III.3.7. RIPA buffer

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

Make up to 30 ml using distilled water. Store at 4^0C.

III.3.8. Electrode buffer (pH 8.3) for SDS–polyacrylamide gel electrophoresis (SDS- PAGE)

Tris base - 25 mM

Glycine - 192 mM

SDS - 0.1%

Deionized water - 100ml

III.3.9. SDS gel-loading buffer (6X)

SDS - 9% w/v

Bromophenol blue - 0.03%

β-mercaptoethanol - 9%

Glycerol - 50% v/v

1M Tris HCl- pH 6.8 -18.75 ml

III.3.10. Resolving gel buffer (pH 8.8)

Tris base - 18.165 g

Dissolve in 80 ml deionized water. Adjust pH to 8.8 using HCl and make upto 100 ml. Store at room temperature.

III.3.11. Stacking gel buffer (pH 6.8)

Measure about 30 ml of resolving buffer. Adjust pH 6.8 using HCl and make up to 45ml. Store at room temperature.

III.3.12. 30% Acrylamide solution

Acrylamide - 29.2g

bis acrylamide - 0.8g

Dissolve and make upto 100mL using distilled water. Store at room temperature and protect from light.

III.3.13. 10% Ammonium per sulphate solution

Ammonium per sulphate (APS) - 0.1g

Dissolve in 1ml distilled water. Prepare freshly before use.

III.3.14. 10% SDS solution

SDS - 1g

Dissolve in 10 ml distilled water.

III.3.15. Blocking buffer

Skimmed Milk - 0.5g

1x TBS- Tween 20 - 10ml

Mix by placing in Rotary shaker.

III.3.16. Towbin's buffer (Transfer buffer)

Tris base	- 3.027 g
Glycine	- 14.4 g
Methanol	- 200 ml
Deionized water	- 800 ml.

III.3.17. Tris borate EDTA buffer (TBE) (5X, pH 8.3)

Tris base	-54 g
Boric acid	-27.5 g
0.5 M EDTA (pH 8.0)	-20 ml

Dissolve and make upto 1L in deionized water.

III.3.18. RNA loading dye

Bromophenol blue	- 0.25%
Xylene cynol	- 0.25%
EDTA	- 1mM
Glycerol	- 50% in nuclease free water

III.3.19. Ethidium bromide (Stock solution)

EtBr - 1mg

Dissolve in 1ml water. To 20mL of 1% agarose gel electrophoresis, add 5µl of the stock solution.

III.3.20. Tris-buffered saline (TBS) (10X, pH 7.6)

Tris base - 24.2 g

Sodium chloride - 80 g

Dissolve and make upto 1L using distilled water.

III.4. EXPERIMENTAL STUDIES

Spontaneously hypertensive rat (SHR), a genetic model of hypertensive heart disease which manifests the clinical progression from hypertension to hypertrophy, was used for the characterization of resident CSCs in pathological hypertrophy and compared with age and sex matched normotensive Wistar (WST) rats. Spontaneously hypertensive rats were purchased from Animal Resource Centre, Perth, Australia and were housed at 22±2°C in 55±10% relative himidity in individually ventilated cages. Light levels measured at 1m height were less than 300 Lux and maintained a 12:12 hour dark: light pattern. Rats were fed with regular rat chow and had free access to drinking water *ad libitum.* Both SHR and the Wistar rats were maintained in the Division of Laboratory Animal Science of the Institute (SCTIMST).

III.4.1. Assessment of the age dependent variation in quantity and quality of CSCs of Wistar rat

The age groups used for the comparison were pups (1-week-old), 6 months, 12 months and 18 months. The selection of the ages represents the various stages of hypertrophy. Studies from our lab has reported the development of hypertension at about 4 weeks with evident oxidative stress and the initiation of left ventricular hypertrophy at 8 weeks of age (Purushothaman *et al.*, 2011).

The animals of the required age were procured from the Animal House at the time of experiment and sacrificed following the measurement of body weight. The animals were euthanized under deep surgical plane of anesthesia using 5mg/kg Xylazine and 70mg/kg Ketamine followed by 10mg/kg body weight of 1% Thiopentone sodium as i/p injections. The heart was immediately dissected out and placed in ice cold sterile PBS. Ventricles and atrium were separated aseptically and used for the further experiments.

III.4.1.1. Assessment of the density of c-kit^{+} CSCs

Ventricles, separated from the heart were used for determining the density of CSCs. Briefly, ventricles were washed in cold $Ca^{2+/}Mg^{2+}$ -free PBS and were minced with dissecting scissors into $\simeq 3mm^3$ pieces and digested in dissociation medium containing 2 mg/mL Collagenase type II in PBS at 37°C for 15 minutes with gentle agitation. After 15 minutes, enzymes were neutralized by adding twice the original volume of IMDM with 10% FBS, filtered through sterile 70µm

nylon mesh cell strainer, centrifuged at 300g for 5 minutes, and resuspended in hemolytic buffer (155mM NH_4Cl, 10mM $KHCO_3$ and 0.1mM EDTA in H_2O) for 5 minutes at room temperature. Enzymatic dissociation was continued until all tissue bits were digested completely. Then, cells were resuspended in PBS, and transferred to 1.5mL Eppendorf tubes for FACS analysis.

III.4.1.2. Assessment of the attributes of CSCs

The stem cell characteristics were evaluated in CSCs isolated from atrial explants.

III.4.1.3. Isolation and expansion of c-kit$^+$ CSCs from atrial explants

CSCs were isolated from the atrial explant culture as previously reported with minor modifications (Bearzi *et al.*, 2007; Messina *et al.*, 2004). After removing adherent fat, the atria were washed with cold $Ca^{2+/}Mg^{2+}$ -free PBS. Thereafter, tissues were minced into small pieces ($\simeq$2 mm^3). Washed extensively in fresh cold PBS and seeded onto the surface of 2% gelatin coated 100mm culture dishes and supplemented with Iscove's Modified Dulbecco's Medium (IMDM, with L-Glutamine and 25mM HEPES Buffer) containing 10% Fetal Bovine Serum, 100U/mL penicillin G and 100µg/mL gentamycin. Cells migrated from the explants and phase bright cells were seen over a layer of fibroblast like cells. The phase bright cells were dislodged by mild trypsinisation and sorted for c-kit$^+$ cells using EasySep^{TM} magnet and FITC positive selection kit. Briefly, trypsinised cells were incubated with rabbit anti-c-kit followed by FITC-

conjugated rat anti-rabbit IgG before sorting it using the magnet as per the manufacturer's protocol. Sorted c-kit^{+} small round cells were cultured in IMDM containing 10% FBS, 10ng/ml bFGF and 10µl/ml insulin-selenium-transferrin mixture at 37^{0}C in a humidified atmosphere with 5% CO_2. On becoming sub-confluent, the cells were subcultured and the cells obtained at passage 3 were subjected to phenotypic characterization using flow cytometry and immunocytochemistry. Clonogenecity studies were performed to confirm the purity of the stem cells.

On confirming the purity of cells at passage 3, they were used for further characterization studies.

III.4.1.4. Assessment of the proliferation potential of CSCs

Aging is known to affect the growth potential of stem cells. The proliferation potential of CSCs was evaluated by colony forming unit assay and growth kinetic studies. Growth rate was plotted and Population doubling time (PDT) was calculated.

III.4.1.5. Assessment of the migration potential of CSCs

Stem cells reside within niches and are recruited to the site of injury via chemotaxis. Hence, the ability to migrate forms an integral part of stem cell characteristics and is a measure of its functional efficiency. The migration potential of CSCs was assessed by trans-well migration assay and scratch wound assay.

III.4.1.6. Assessment of intracellular ROS in CSCs

Oxidative stress is implicated in cardiac ailments and its role in pathological cardiac remodeling is well defined. Intracellular ROS levels were detected by using DCFDA fluorescence.

III.4.1.7. Assessment of directed differentiation potential of CSCs

Stem cells residing within the adult tissues retain the ability to differentiate into the specific cells at times of demand. Stem cells also play an important role in maintaining the tissue homeostasis. The ability of the stem cells to undergo differentiation is a determinant of its ability to repair or regenerate the tissue depending on the need. The ability of the CSCs to undergo differentiation upon stimulation with 5-azacytidine was assessed by western blot analysis.

III.4.1.8. Cytochemical assessment of the senescence of CSCs

Cellular senescence is a hallmark of aging. Various stem cells have to been reported to acquire features of senescent cells with aging where the functionalities of stem cells is impaired. The senescence was assessed by staining for β-galactosidase using commercially available kit.

III.4.2. Comparison of age associated variation in stem cell characteristics of the hypertensive heart and normal heart

The age groups used for the comparison studies were pups (1-week-old), 6 months, 12 months and 18 months. The selected ages represent the various stages of hypertrophy, and were evaluated for the following variables.

III.4.2.1. Density of c-kit^{+} CSCs

The ventricular digests were subjected to FACS analysis and the proportion of CSCs was determined as explained in III.5.1.

III.4.2.2. Proliferation potential of CSCs

Proliferation potential was assessed by colony forming unit assay and growth kinetic studies. Population doubling time was also calculated (III.5.4).

III.4.2.3. Migration potential of CSCs

The migration ability was assessed by trans-well migration assay and scratch-wound assay.

III.4.2.4. Measurement of intracellular ROS levels in CSCs

The ROS levels in CSCs were detected by measuring the fluorescence intensity of DCFDA.

III.4.2.5. Directed differentiation of CSCs

The differentiation was stimulated by adding 5-azacytidine and cell specific proteins were analyzed by western blotting.

III.4.2.6. Senescence of CSCs

The senescent nature of CSCs was assayed by using β-galactosidase staining kit and expression levels of $p16^{ink4a}$ and p21.

III.4.2.7. Telomerase activity, TERT mRNA and DNA damage in CSCs

Aging is associated with decreased telomerase activity and TERT mRNA expression. These variables were analysed by using commercially available kit and Real time PCR respectively. DNA damage was detected by comet assay.

III.4.3. Assessment of the effect of antioxidant supplementation on CSCs

Aging of CSCs was found to be accelerated in diseased heart compared to the normal heart. The levels of intracellular ROS in CSCs were significantly high in SHR compared to WST, which indicates the presence of oxidative stress in the pathological heart. The prevalence of oxidative stress in hypertension induced hypertrophy and its role in transition to heart failure is well documented. To examine the effect of the antioxidant, tempol on CSCs, twelve six-month-old male SHR were randomly assigned into two groups of six rats each. One group received intra-peritoneal injection of 20mg/kg/day tempol for 2 weeks (Francischetti *et al.*, 2014). Untreated SHR served as hypertensive control. Six age-matched male Wistar rat served as normotensive control. Both untreated SHR and WST received the vehicle during the treatment period.

On completion of the experimental period, animals of all three groups were subjected to echocardiography to examine the regression of hypertrophy. Blood pressure was also recorded by the tail-cuff method. The animals were sacrificed as described earlier and hearts were dissected out. Ventricular sections were used for immunohistochemistry analysis for detection of myocardial oxidative levels by 3-nitrotyrosine staining.

Having confirmed the effectiveness of the treatment in the reduction of myocardial oxidative stress and reduction of hypertrophy, the stem cell response was assessed in CSCs obtained from atrial explant culture. The levels of

intracellular ROS, proliferation potential, migration ability and senescence of CSCs was examined in treated SHR and compared with untreated SHR and WST.

III.5. METHODOLOGY

III.5.1. Flow Cytometric Analysis

For FACS assay, the c-kit$^+$ cells were dissociated into single cell suspension and tested for the expression of cell surface markers (Ye *et al.,* 2013). The following primary conjugated anti-rat antibodies were used: anti c-kit FITC, anti CD31 APC and anti CD45 PE along with the corresponding isotype controls. The cells were incubated with antibodies for 25 min on ice, washed with PBS containing 0.2% BSA and analyzed by Fluorescent Activated Cell Sorting (FACS) Cabilur with CellQuest software.

III.5.2. Immunophenotyping

Cell phenotype was defined by immunocytochemistry (Hsiao *et al.,* 2014). Immunostaining was performed by incubation with specific primary antibody: rabbit anti-c-kit (diluted 1:100) at 4^0C overnight. Cells were then incubated with FITC-conjugated rat anti-rabbit IgG (diluted 1:200) at 25^0C for 1 h. Cells were also stained with conjugated antibodies against CD-31 and CD-45. Nuclei were stained with 1µl/ml Hoescht. The immunoreactions were observed under the fluorescent microscope.

III.5.3. Clonogenic assay

C-kit$^+$ CSCs were harvested using trypsin-EDTA solution at passage 3. The cell suspension was serially diluted to 50 cells in 10mL of complete medium and seeded into a 96-well plate at a density of 0.5 cell per well to generate single-cell clones (Hsiao *et al.*, 2014). After 4h, each well containing a single cell was identified under a phase-contrast microscope and examined for growing colonies twice weekly. After 2 weeks, the number of wells with clones derived from a single cell was counted. Clonogenicity was determined using the following formula:

Clonal efficiency (%) = (Total wells with clone/Total wells with single cell) x100.

III.5.4. Growth Kinetics and Population Doubling Time

A total of 10000 cells/ml of c-kit$^+$ CSCs was cultivated in 35 mm dishes for a period of 10 days (Zhang *et al.*, 2013). Cell numbers were determined at 48 h intervals using a Neubauer improved haematocytometer. Cell numbers were counted in triplicate.

Growth curve was plotted and the growth rate was calculated using the formula: $GR= \ln(N_t/N_0)/T$, where T is the incubation time, N_0 is the cell number at the beginning of the incubation time and N_t is the cell number at the end of the incubation time.

Population doubling time was calculated using the formula, $PDT= \ln(2)/GR$.

III.5.5. Colony forming unit assay

C-kit^{+} CSCs were seeded onto cell culture plates at a density of 500 cells per 60mm dish (Zhang *et al.*, 2013). Growth media was changed every 3–4 days. After 11 days, the cells were washed with PBS and stained with 3% crystal violet in methanol for 30 minutes at room temperature. The colonies larger than 2mm were counted.

III.5.6. Trans-well migration Assay

10000 c-kit^{+} CSCs suspended in serum free medium were seeded onto the upper chamber (BD Falcon, pore size - 8µm) of the trans-well system. IMDM supplemented with 10% FBS was placed in the lower chamber, where serum acted as the chemo-attractant. The chamber was incubated for 12 h at 37^{0}C in a humidified atmosphere with 5% $CO_{2,}$ after which the filter was removed and the un-migrated cells on the upper surface of the membrane was wiped away. The cells were fixed with 4% paraformaldehyde and stained with crystal violet solution. The number of migrated cells on the lower surface of the filter was counted under a microscope. On an average, six fields per chamber was counted.

III.5.7. Scratch wound Assay

Cells were grown to 90% confluence in a 35mm culture dish. A scratch gap was created in the monolayer by dragging a 10µl pipette tip. After PBS wash, cells were cultured in 0.5% serum containing medium. The wound closure pattern in

about 3- 4 fields were examined after 18 hours later and images were acquired using a Nikon inverted phase contrast microscope.

III.5.8. Detection of intracellular ROS by DCFDA

The intracellular ROS generation of cells can be measured using the $DCFH_2$-DA as an indicator to detect and quantify intracellular production of reactive oxygen species (LeBel *et al.,* 1992). The assay is based on the principle that oxidation of 2',7'-dichlorodihydrofluorescein (DCFH) converts it to a fluorescent 2',7'-dichlorofluorescein (DCF). Briefly, cells were washed with PBS and fresh medium was added. Fresh stock solution of carboxy-H_2DCFDA was prepared in sterile dimethylsulfoxide (DMSO) and 10µl was added to medium. Following incubation for 10 minutes, fluorescence values at different conditions were monitored by excitation at 498 nm and emission at 530 nm.

III.5.9. Directed differentiation of CSCs

The c-kit$^+$ CSCs were plated on 6-well plates. Cell differentiation was induced by incubation in differentiation medium (IMDM, 10% FBS and 10µM 5-azacytidine) for 24 hrs. The cells were washed and cultured in low serum medium for 21 days and the ability to differentiate was determined by analyzing the expression of cell-specific markers cardiac troponin I (cTNI) and smooth muscle actin (SMA) by western blot assay.

III.5.10. Senescence-Associated β-Gal Staining (Gal Staining)

Senescence-associated β-galactosidase staining (Gal staining) was used as a biomarker of senescence in c-kit^{+} CSCs. Gal activity was assessed with a histochemical staining kit (*Abcam*) as per the manufacturer's instructions, followed by light microscopy. The percentage of senescent cells was determined from the average number of stained cells in a total population of 100 cells.

III.5.11. Western blotting

Western blot analysis was carried out following the protocol described by Maniatis *et al.* (1982) with minor modifications. Cell pellet obtained after trypsinisation was homogenized in 1ml of RIPA buffer containing protease inhibitor cocktail. The extracts were kept in ice with intermittent vortexing for 60 min and centrifuged at 12,000rpm for 20 minutes at 4^{0}C. The supernatant was collected and protein concentration was determined using Coomassie protein assay reagent.

30µg of total protein was fractionated on 10% SDS-polyacrylamide gels at 100V and electroblotted to nitrocellulose membranes. To ensure that the transfer was successful, membrane was taken out from the assembly at the end of the transfer, washed with deionized water and stained reversibly with ponceau S. The stain was removed by washing the membrane twice with Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for 5'. The non-specific binding sites were blocked with 5% skimmed milk in TBS-T at room temperature. After blocking,

the membrane was incubated with the specific primary antibody: cTnI, SMA, $p16^{ink4a}$ or p21 (1: 1000 dilution) overnight at 4^0C in a shaker.

Following incubation, the membranes were washed three times in TBS-T and incubated with specific horseradish peroxidase-conjugated secondary antibody at room temperature for 1 hour. The immunoreactive bands were visualized using chemiluminescence detection kit (*Thermo*). The same membrane was re-probed with anti β-actin antibody, which served as the normalisation control. The images were captured on Syngene gel documentation system.

III.5.12. Telomerase activity assay

Telomerase activity in cells was detected by the modified TRAP assay using the TRAPeze Telomerase Detection Kit, which is based on the method described by Kim *et al.* (Kim *et al.*, 2013). The assay is a one-buffer, two-enzyme system using the PCR. In the first step of the assay, telomerase adds the 6-bp telomeric sequence (TTAGGG) onto the 3' end of a 5' end infrared dye–labeled oligonucleotide substrate (5'-AATCCGTCGAGCAGAGTT- 3') (TS Primer,Kit). In the second step, the extended products are amplified by PCR. Briefly, 10^6 cells of each sample were resuspended in 200µL of 3-[(3-cholamidopropyl)dimethyl-ammonio]-1- propanesulfonate (CHAPS) lysis buffer (Kit) and incubated for 30 min on ice. After the incubation, lysates were centrifuged at 12,000 *g* for 20 min at 4°C. The supernatant was recovered, and the protein concentration was measured using the Biciuchoriuic acid (BCA) test. Because the best

differentiation of the samples was seen with extracts containing 500ng protein, this concentration was used as the standard concentration. To heat inactivate the telomerase, 5µl of each sample extract was incubated at 85^0C for 10 min. TSR8 and CHAPS buffer (Kit) were used as positive and negative controls, respectively. After a 30-min incubation at 30°C, the samples were subjected to 33 PCR cycles of 94°C for 30s, 58.3°C for 30s and 72^0C for 1 min (PCR machine). The PCR products were separated by electrophoresis on non-denaturing polyacrylamide gels. The gels were stained with Ethidium Bromide and viewed under a trans-illuminator and photographed. Telomerase activity was measured on digital images by ImageJ software version 1.42q (NIH) as units of total product generated (TPG), calculated as TPG =[(x - x0)/c x100]/[(r - r0)/cR], where x is the signal of the region of the gel lane corresponding to the TRAP product ladder bands from non-heat-treated samples, x0 is the signal of the region of the gel lane corresponding to the TRAP product ladder bands from heat-treated samples, r is the signal of the region of the gel lane corresponding to the TRAP product ladder bands from TSR8 quantitation control, r0 is the signal of the region of the gel lane corresponding to the TRAP product ladder bands from 1X CHAPS lysis buffer-only control, c is the signal from the internal standard (S-IC) in non-heat-treated samples, and cR is the signal from the internal standard (S-IC) in TSR8 quantitation control. Each unit of TPG corresponds to the number of TS primers extended with at least 4 telomeric repeats by telomerase in the extract in a 30 min incubation at 30^oC.

III.5.13. Real Time PCR

Total RNA was extracted from CSCs using commercially available kit (*Qiagen*). Following DNase I treatment, 2µg of total RNA was transcribed to complementary DNA (cDNA) using PrimeScript First-Strand cDNA Synthesis Kit (*TAKARA*) under the following conditions: 37^0C for 15 minutes and 85^0C for 5 seconds. The primers were diluted 1:10 using sterilized distilled water. The real-time PCR reaction solution consisted of cDNA, SYBR green and forward and reverses primers for a final reaction volume of 20µl. All results are expressed relative to 18S rRNA. The threshold cycle (CT) for fluorescence development was used to calculate the fold change using the formula "$2^{-\Delta\Delta CT}$ method". All PCR reactions were performed using the ABI Prism 7500 Sequence Detection System (*Applied Biosystems*).

Table 1: List of genes and its primers used for real time PCR analysis

TERT	
Sense	5'AGTGGTGAACTTCCCTGTGG3'
Antisense	5'CAACCGCAAGACTGACAAGA3'
18S rRNA	
Sense	5'TCAAGAACGAAAGTCGGAGG3'
Antisense	5'GGACATCTAAGGGCATCAC3'

III.5.14. Comet assay

Comet assay was performed as described by Hermeto et al., (Hermeto, *et al.*, 2015). Briefly, cells were dissociated from the culture surface by trypsinization, and an aliquot was mixed with 10μL 0.75% low-melting point agarose at 37°C and layered onto a precoated slide with 1.5% regular agarose, and covered with a cover-slip. After a brief agarose solidification in the refrigerator, the cover-slip was removed and slides were immersed in lysis solution [2.5 M NaCl, 100 mM ethylenediaminetetraacetic acid (EDTA), 10 mM Tris-HCl buffer, pH 10, 1% sodium sarcosinate with 1% Triton X-100, and 10% dimethyl sulfoxide], and maintained in the dark at 4°C for 2 h. After lysis, the slides were left for 20 min in the electrophoresis buffer (300 mM NaOH, 1 mM EDTA) and electrophoresed for another 20 min at 25V and 300mA. After electrophoresis, the slides were neutralized with 0.4 M Tris-HCl, pH 7.5, for 15 min (3 times for 5 min each), dried and stained with Ethidium bromide. The comet evaluation was carried out based on the tail moment, a product of the tail length and the percentage of tail DNA, which gives a more integrated measurement of overall DNA damage in the cell.

Each cell had the appearance of a comet, with a brightly fluorescent head and a tail to one side formed by the DNA, which contained strand breaks that were drawn away during electrophoresis. Quantitative image analysis was performed using an intensified solid state CCD camera (Sony CCD-IRIS; I.S.S. Group, Manchester, U.K.) attached to the microscope and linked to the Comet Assay II

image analysis software (Perceptive Instruments, Haverhill, Suffolk, U.K.). Samples were run in duplicate, and 50 cells were randomly analyzed per slide for a total of 100 cells per sample and scored for Comet tail parameters as defined by Olive & Banath (Olive & Banath, 2006). Comet tail length is the maximum distance the damaged DNA migrates from the center of the cell nucleus, the percentage of tail DNA is total DNA that migrates from the nucleus into the comet tail, and the tail moment is a product of the tail length and the percentage of tail DNA. Comet assay was carried out at Biogenix Research Center for Molecular Biology and Applied Sciences, Poojappura.

III.6. STATISTICAL ANALYSE

All values are expressed as mean $\pm$ Standard Deviation (SD). ANOVA was carried out when there were more than 2 groups. When there was significant variation between groups, by ANOVA, the difference between two groups was assessed by two-tailed Student t-test. Values where $p<0.05$ was considered statistically significant.

IV. RESULTS

IV.1. Isolation and Expansion of c-kit^{+} CSCs

Atrial explant culture was established in gelatin coated dishes. Initially, fibroblast-like cells migrated from the explant in 3-4 days. In about 2 weeks small, round, phase bright cells migrated from the explant and were found loosely attached over the layer of fibroblast- like cells (Fig. 8).

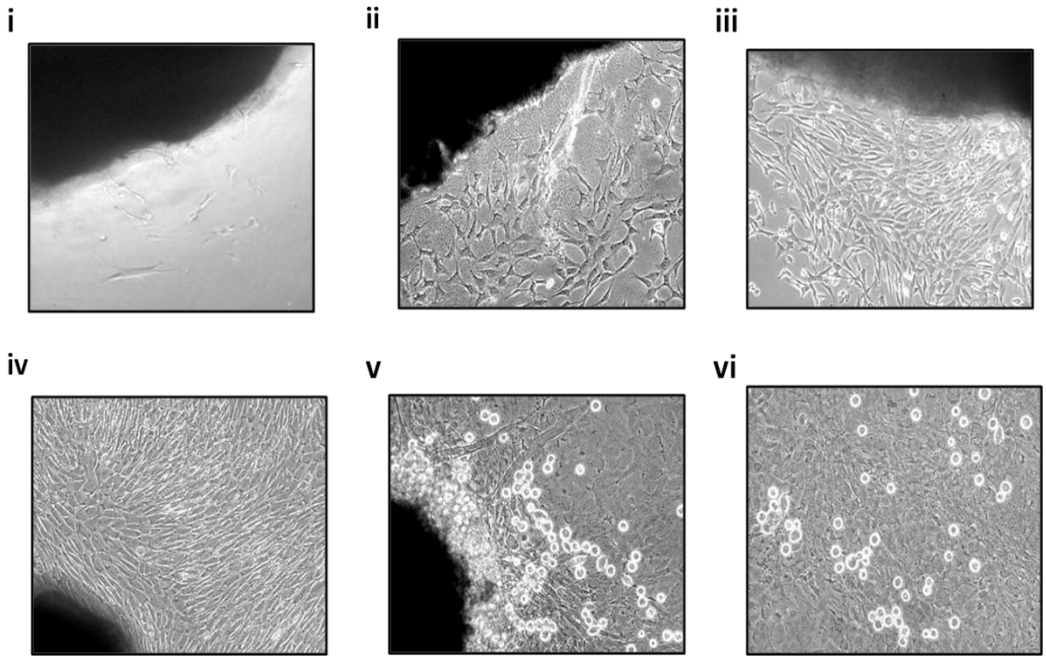


Figure 8: Representative phase-contrast images of atrial explant culture

Atrial explants were placed on gelatin coated dishes and supplemented with IMDM containing 10% FBS, B27 serum supplement and antibiotics. i-iii) 3rd, 5th and 7th Day – Atrial explants attached to the culture surface and cells started migrating from the explant iv) 9th Day – Monolayer of fibroblast like cells is seen around explants v-vi) 12th Day – Small, round, phase bright are seen migrating from the explant.

Once the explant culture became confluent, the cells were collected by trypsinization and subjected to immunomagnetic isolation to sort out the c-kit^{+}

CSCs. The specific cells were isolated using EasySep^{TM} Magnet using EasySep Selection kit^{TM} described in 'Methods' (III.4.1.3). The cells so isolated were incubated in IMDM along with the supplements and by the third day, bright spherical c-kit^{+} CSCs gradually attached to the culture plate, proliferated, and formed clusters (Fig. 9).

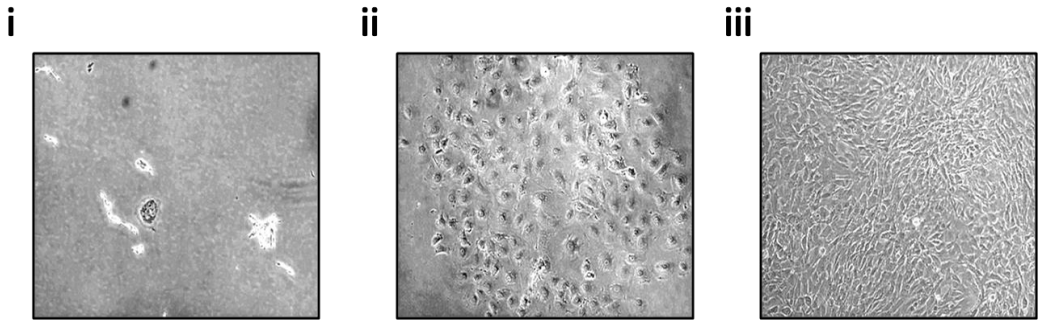


Figure 9: Culture of c-kit^{+} Cardiac Stem Cells isolated by immunomagnetic isolation

Cells that migrated from atrial explants were trypsinised and subjected to immunomagnetic isolation using anti c-kit FITC antibody. i) 3rd Day- Isolated round cells attached to the dish surface ii) 6th Day- Attached cells formed clusters iii) 12th Day- Confluent culture of CSCs

IV.1.1. Phenotypic Characterization of Cultured c-kit^{+} CSCs

The purity of the immuno-magnetically sorted cells were confirmed at the 3rd passage by flow cytometry and immunocytochemistry (ICC). By flow cytometric analyses, >90% of the cells were found to be positive for c-kit and negative for CD31 and CD45 (Fig. 10).

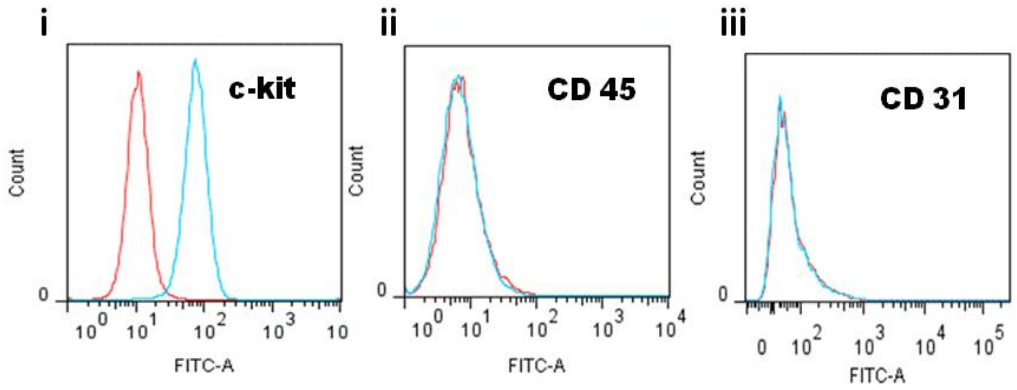


Figure 10: Representative FACS data for the expression pattern of cell surface markers

Cells at passage 3 were trypsinised and single cell suspension so obtained was incubated with primary antibodies for (i) c-kit (ii) CD45 and (iii) CD31 along with the corresponding isotype control. After incubation with appropriate fluorescent-labeled secondary antibodies, cells were washed, suspended in PBS and analyzed by FACS.

The cells were fixed in paraformaldehyde and subjected to immunocytochemistry to confirm the identity of the sorted cells (Fig. 11).

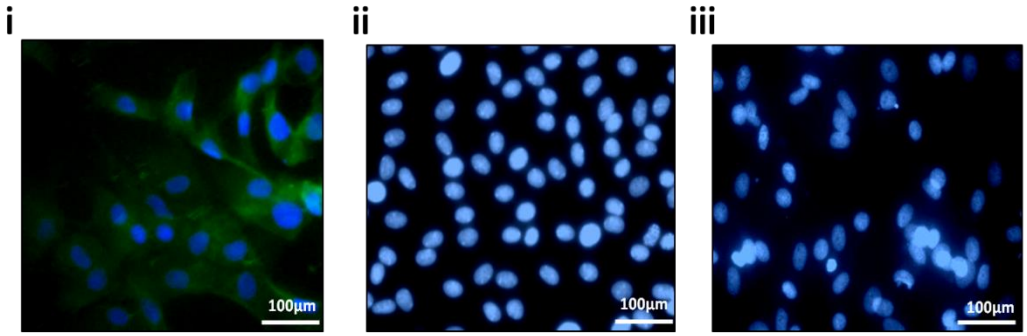


Figure 11: Representative images of immunocytochemistry for the expression of cell surface markers

(i) Merged image of CSCs expressing c-kit shown as green where nuclei is stained blue. (ii) Merged image of CSCs expressing CD45 where nuclei is seen as blue (iii) Merged image of CSCs expressing CD31 where nuclei is stained blue.

Retention of cellular identity despite repeated subculture was apparent from the observation that about 74% (Fig. 12) of the cells were c-kit^{+} at passage 10.

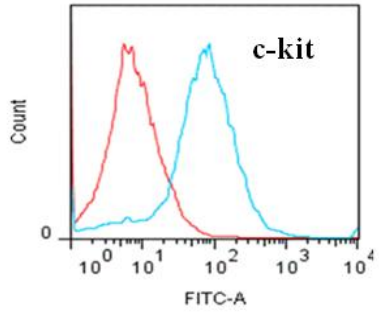


Figure 12: Representative FACS data for the expression pattern of c-kit^{+} CSCs at passage 10

Stemness was further confirmed by clonogenic assay in which single cells seeded in 96 well plates were found to form colonies. On an average, 96±2% of the cells that adhered to the culture surface formed colonies.

IV.2. Effect of Physiological Aging on CSCs from Wistar Rat

IV.2.1. Density of c-kit^{+} CSCs

Single cells obtained from ventricular tissue digests were subjected to FACS analysis following haemolysis. The density of CSCs declined with age as shown in Figure 13. No significant reduction was observed at 6 months compared to pups. However, the proportion reduced to ½ and ¼th at 12 and 18 months respectively when compared with the pups.

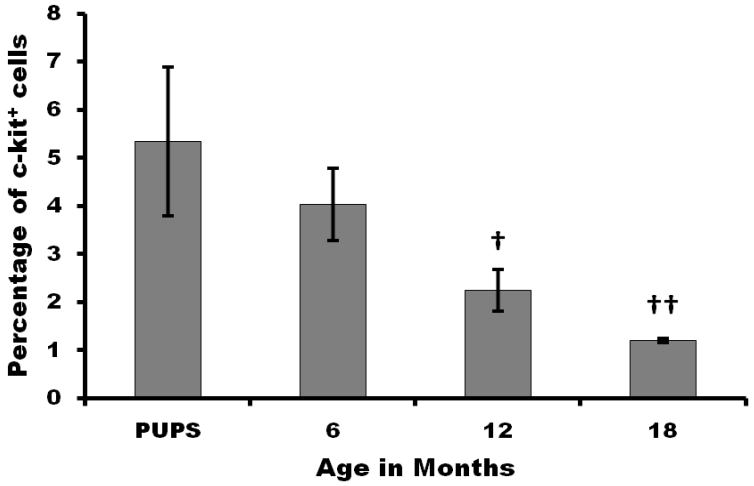


Figure 13: Age-associated variation in density of c-kit^{+} CSCs in ventricular tissue digest

FACS data is expressed as percentage of total cells in the ventricular tissue (n=3). Data presented as mean ± SD. †† p<0.01 and † p<0.05 WST of different ages compared to 1 week old pups.

IV.2.2. Self-renewal ability

The number of colonies formed by CSCs exhibited a significant reduction with aging as early as 6 months (Fig. 14). The CSCs from older rats formed only half

the number of colonies as compared with the pups. The size of the colonies formed by Wistar CSCs was in the range of 0.5 to 0.8cm.

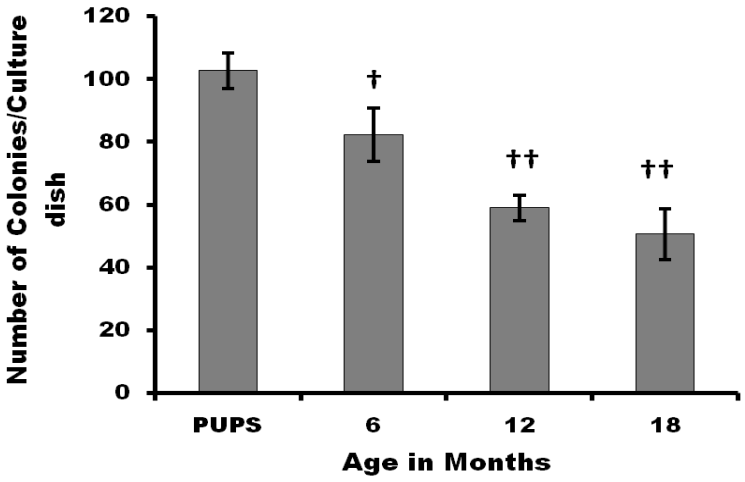


Figure 14: Age-associated variation in CFU of c-kit^{+} CSCs

CFU is represented as number of colonies/dish (n=3). Data presented as mean ± SD. †† p<0.01 and † p<0.05 WST of different ages compared to 1 week old pups.

IV.2.3. Growth Kinetics, Population Doubling Time (PDT) and Growth Rate

CSCs from WST rat exhibited a significant decline in growth kinetics from 6 months of age (Fig. 15A). The cell number obtained at the 10th day of culture from 6-month-old was 22% lower than that obtained from pups. The cell number obtained from CSCs of 18-month old rat on the 10th day of culture was only 38% of that from pups during the same duration in culture.

Concordant with the growth kinetics, growth rate and PDT also exhibited the same pattern of temporal variation. Growth rate declined (Fig. 15B) and PDT

increased (Fig. 15C) with age. The doubling time of CSCs from pups was ≈33hrs and that from older rats ≈40 hrs.

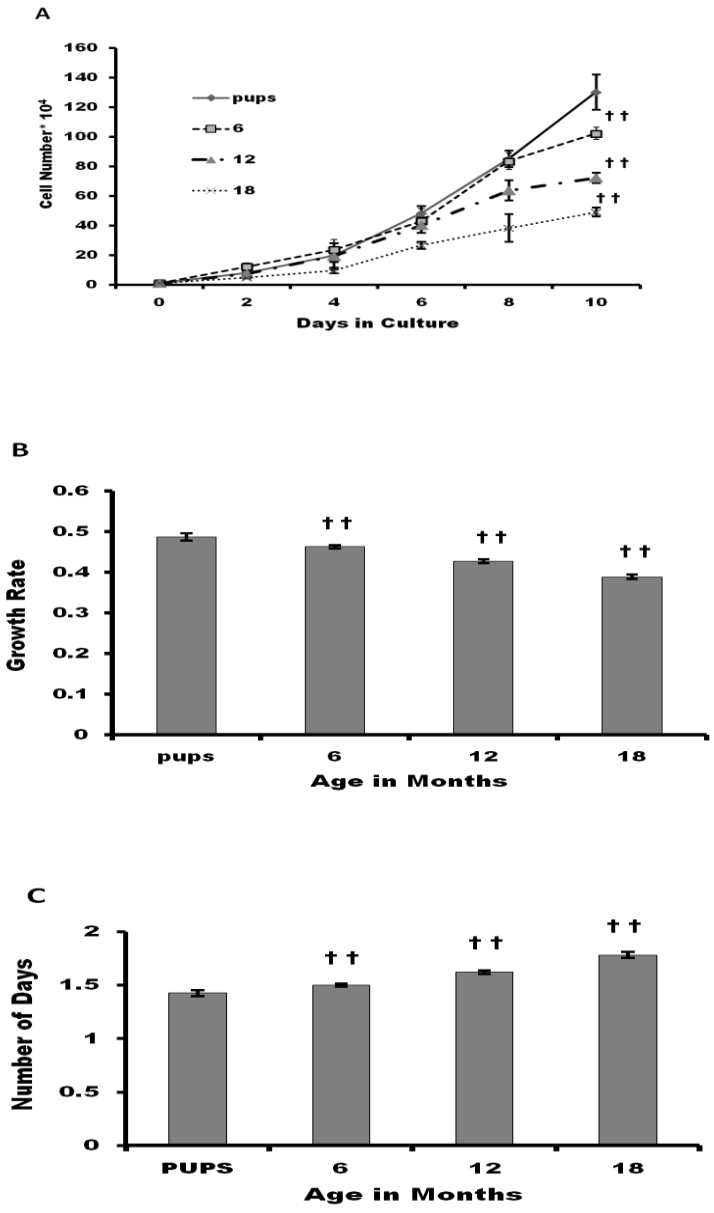


Figure 15: Age-associated variation in Growth Kinetics, Growth Rate and PDT of c-kit^{+} CSCs

A) Variation in Growth kinetics represented as cell number B) Variation in growth rate C) variation in PDT represented as number of days. (n=3). Data presented as mean ± SD. †† p<0.01 WST of different ages compared to 1 week old pups.

IV.2.4. Migration ability

The ability of cells to invade the tissue and migrate towards the injured myocardium is a determinant for proper tissue repair and regeneration. The migration ability as assessed by trans-well and scratch wound assay displayed age dependent decline (Fig. 16). Compared with the pups, the migration potential of CSCs from 6, 12 and 18 month ol rats decreased by 74%, 43% and 31% respectively.

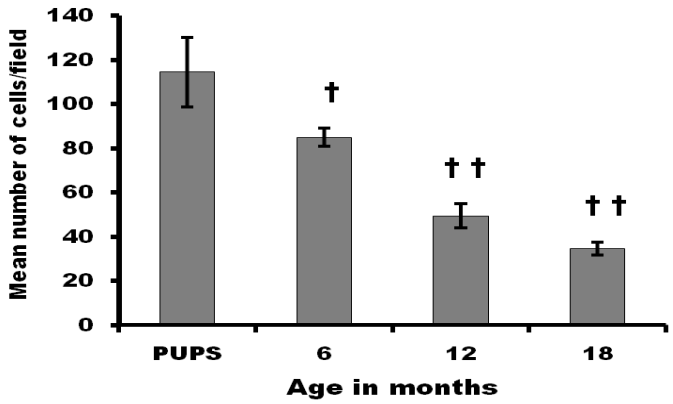


Figure 16: Age-associated variation in migration ability of c-kit^{+} CSCs with age

Migration ability is represented as mean number of cells/field (n=3). Data presented as mean ± SD. † p<0.05 and

†† p<0.01 WST of different ages compared to 1 week old pups.

IV.2.5. Intracellular ROS levels

Free radical stress is regarded as one of the reasons for physiological aging. Though tissue oxidative levels are well documented, the intracellular ROS levels in CSCs are still unexplored. With aging, the intracellular ROS levels of CSCs increased significantly from 12 months (Fig. 17). A 2 fold increase in ROS levels were observed in 18 month old rats compared to the pups. The oxidative stress in the extracellular milieu possibly affects the stem cells.

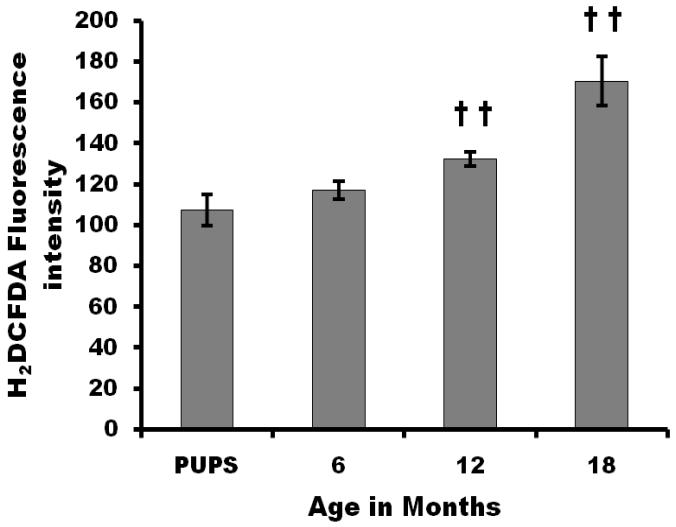


Figure 17: Age-associated variation in intracellular ROS levels of CSCs

Intracellular ROS levels are represented as fluorescence intensity. (n=3). Data presented as mean ± SD. †† p<0.01 WST of different ages compared to 1 week old pups.

IV.2.6. Senescence of CSCs

The somatic cells show senescent changes with physiological aging and undergo apoptosis. The number of senescent CSCs increased significantly with age in WST rat. A 6-fold increase of senescent cells was observed in 18 month old WST compared to pups.

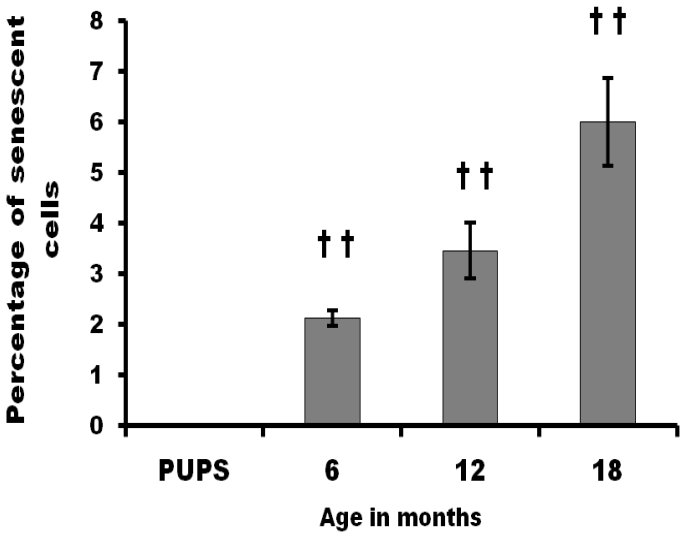


Figure 18: Age-associated variation in the number of senescent CSCs

Senescent cells were expressed as number of senescent cells/100 cells (n=3). Data presented as mean ± SD. †† p<0.01 WST of different ages compared to 1 week old pups.

IV.3. *Characteristics of cardiac stem cells in different stages of hypertensive heart disease using Spontaneously hypertensive rat as the experimental model in comparison with age matched Wistar rat*

IV.3.1. Density of c-kit$^+$ CSCs in ventricular tissue digest

The number of c-kit$^+$ cells decreased with age in both SHR and WST rat (Fig. 19). Stem cell density in the pups of both strains was comparable. C-kit$^+$ cells in 18-month-old SHR was about 1/20 of that present in pups. Whereas, in WST the decrease was relatively lower, the value in 18 month old rat being one-fourth of that seen in the pups. The density of c-kit$^+$ cells in 18-month-old WST was 6 times more than that in SHR.

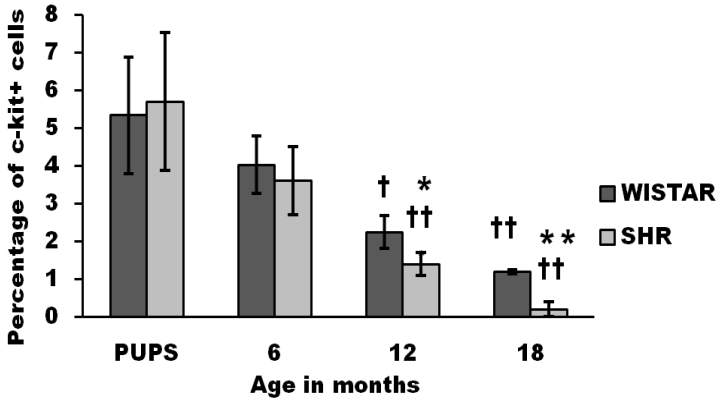


Figure 19: Comparison of age-associated variation in density of CSCs from SHR and Wistar rat

*FACS data is expressed as percentage of total cells (n=3). Data presented as mean ± SD. ** p<0.01 and * p<0.05 SHR Vs Age matched WST; †† p<0.01 and † p<0.05 WST & SHR of different ages compared to respective 1 week old pups. Two way ANOVA p<0.001.*

IV.3.2. Self-renewal capacity of CSCs

Self-renewal capacity of the cells was assessed as colony forming unit (CFU). Age associated decrease in colony forming ability was observed both in SHR and Wistar rat (Fig. 20). The number of CFU was comparable in the newborn rat of both the strains. But, significant decrease in CFU was observed in SHR compared to Wistar from 6 months of age. The number of colonies decreased by 50% in WST with age whereas in SHR it declined by about 75%.

The size of the colonies formed by CSCs in 6-month-old Wistar rats were larger (0.5- 0.8cm) when compared with that of SHR (0.1-0.4cm). The difference in colony size between SHR and Wistar was maintained at all ages.

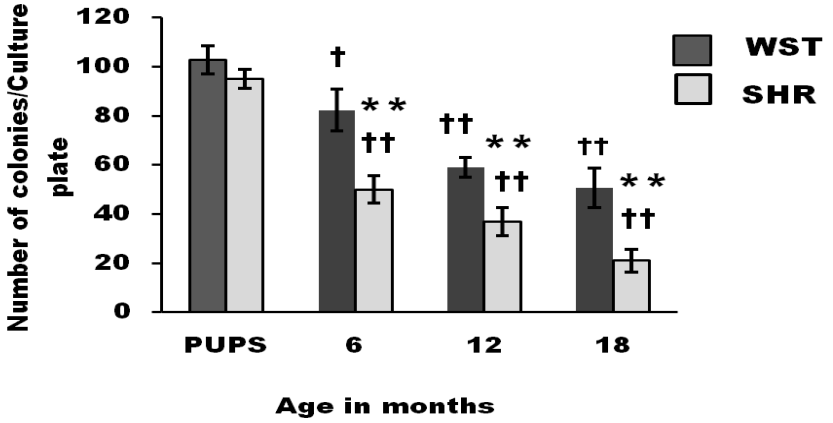
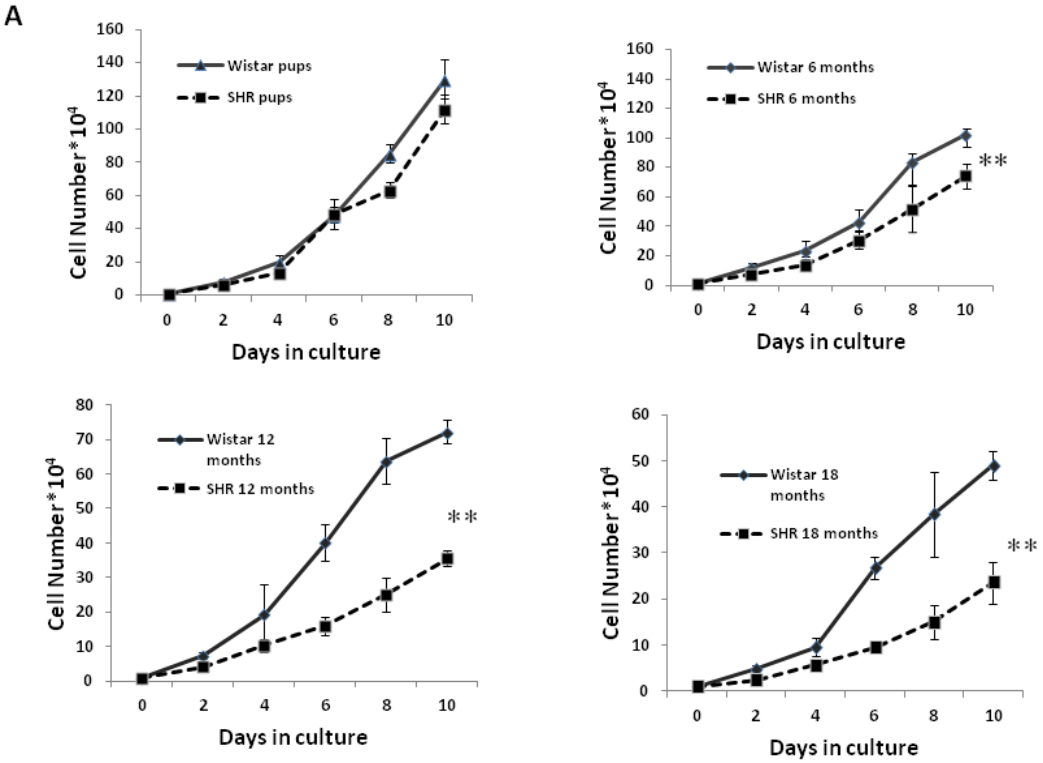


Figure 20: Comparison of age-associated variation in CFU of CSCs from SHR and Wistar rat

*Data represented as number of colonies per plate (n=3/group). Data presented as mean ± SD. ** p<0.01 SHR Vs Age matched WST; †† p<0.01 and † p<0.05 WST & SHR of different ages compared to respective 1 week old pups. Two way ANOVA p<0.001*

IV.3.3. Growth Kinetics, Growth Rate and Population Doubling Time (PDT) of CSCs

CSCs from SHR, like that from WST showed age dependent decline in cell yield. When c-kit^{+} cells were isolated on the 10th day of culture, the yield of CSCs from 18-month-old SHR (23.52±4.5*10^{4}) was half of that from age matched WST rat (49±3*10^{4}) ($p<0.01$) (Fig. 21A). Age associated decrease in growth rate (Fig. 21B) with consequent increase in population doubling time (Fig. 21C) was observed, the decline being significantly more for SHR. As early as 6-months of age, CSCs from SHR exhibited a significant difference in PDT and GR indicating a steady decline in growth kinetics on initiation of hypertrophy.



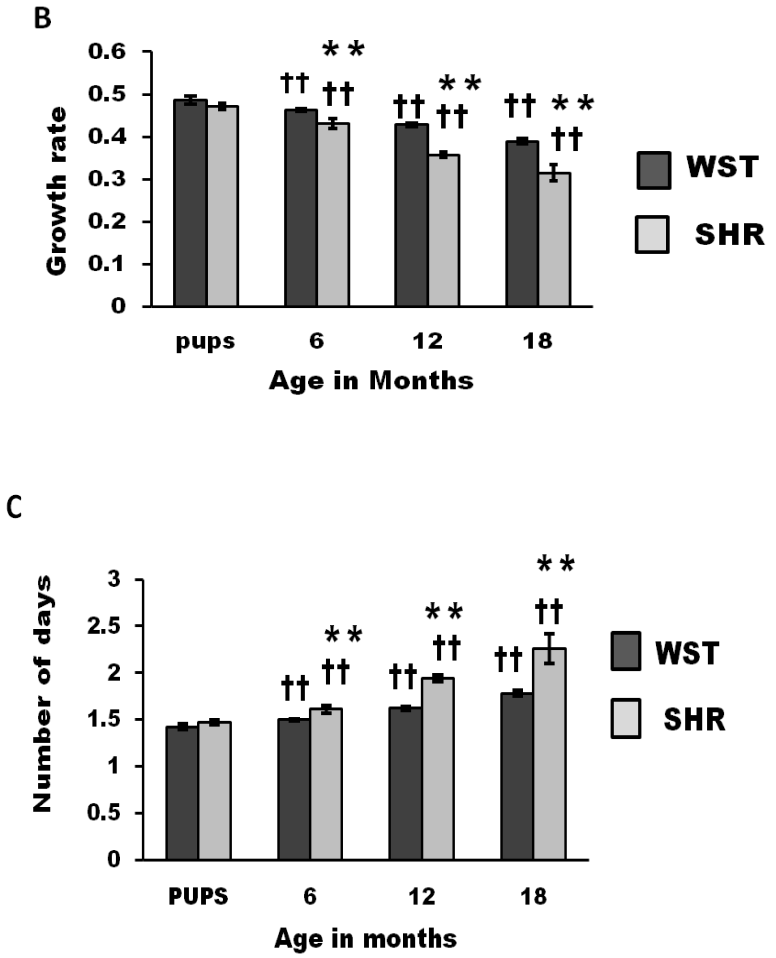
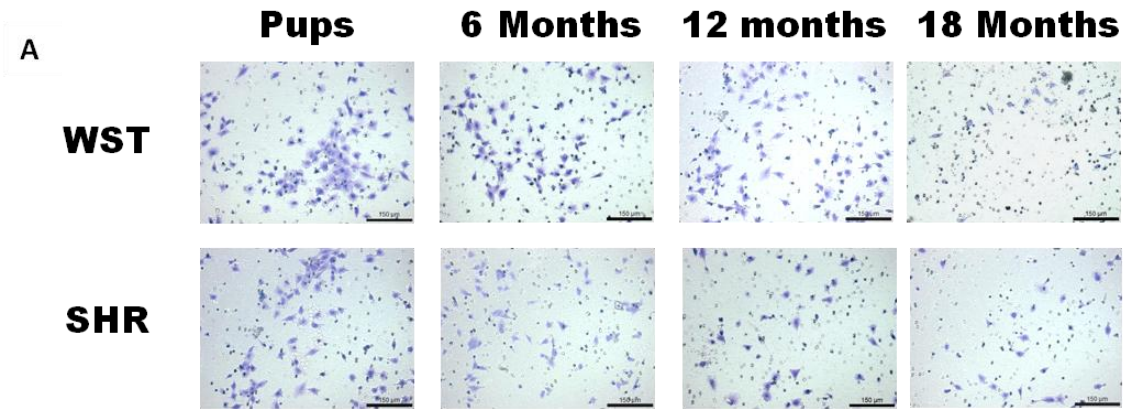


Figure 21: Comparison of age-associated variation in growth kinetics, growth rate and PDT of CSCs from SHR and Wistar rats

*(A) Variation in Growth kinetics represented as cell number (B) Variation in GR represented as growth rate (C) Variation in PDT represented as number of days. (n=3/group). Data presented as mean ± SD. ** p<0.01 SHR Vs Age matched WST; †† p<0.01 WST & SHR of different ages compared to respective 1 week old pups. Two way ANOVA p<0.001*

IV.3.4. Migration ability of CSCs

Trans-well migration assay of CSCs from SHR and Wistar exhibited an age dependent reduction in migration when serum was used as the chemoattractant. A significant decline in the migration ability was seen at 18 months when compared to the new born pups (Fig. 22). For WST rats and SHR, stem cell migration at 18 months was 30% and 17% respectively of that seen in pups. There was almost 2 fold difference in the number of migrated cells between WST and SHR at 6 and 12 months of age.



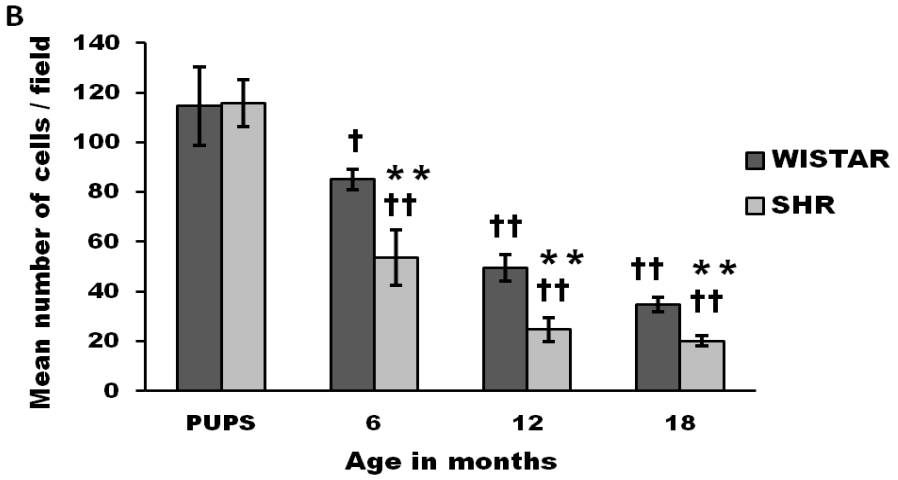


Figure 22: Comparison of migration ability of CSCs from SHR and WST rats

*Migration ability is represented as mean number of cells/field. (A) Representative images of the migrated cells at different ages (B) Graphical representation of the migrated cells. Data is represented as mean number of cells/field. (n=3/group). Data presented as mean ± SD. ** p<0.01 SHR Vs Age matched WST; †† p<0.01 WST & SHR of different ages compared to respective 1 week old pups. Two way ANOVA p<0.001*

A similar trend was observed by scratch-wound assay. The wound area covered by CSCs from WST was relatively more than CSCs from SHR at any given age except for the new born (Fig. 23)

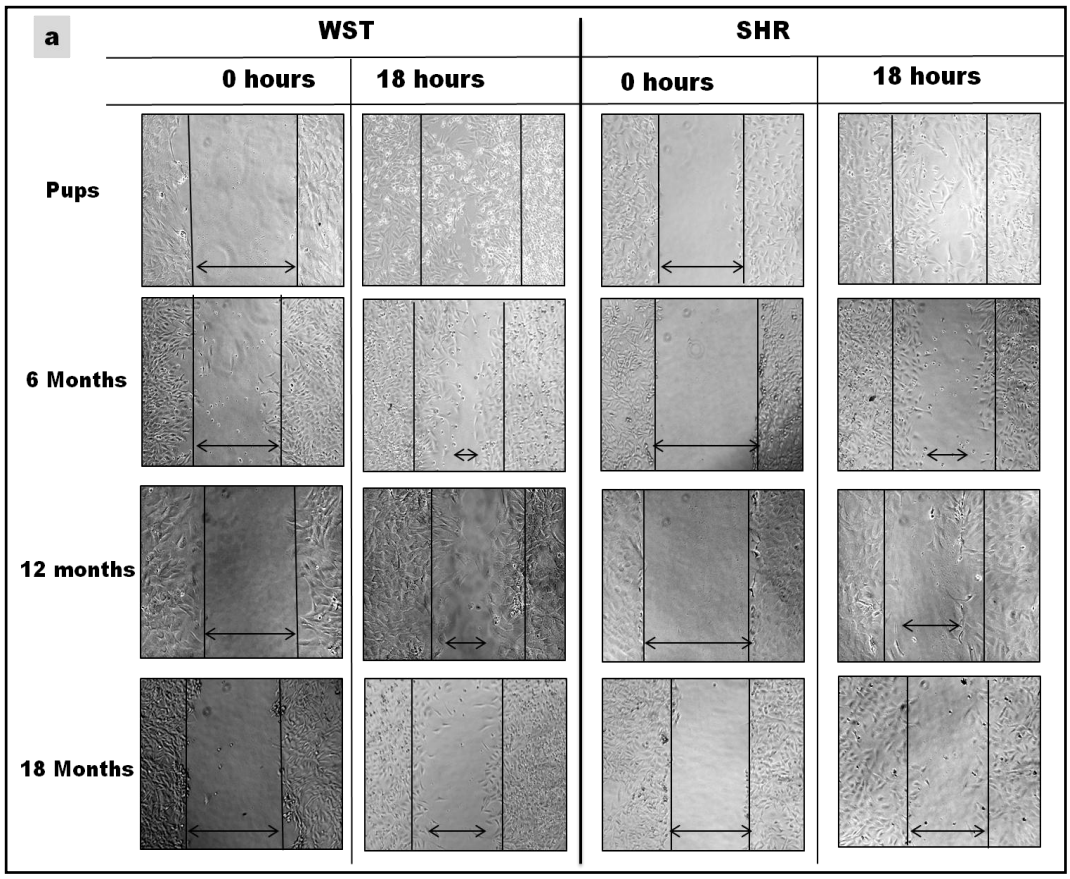


Figure 23: Representative images of scratch–wound assay of CSCs from SHR and Wistar rat

The observations confirm age associated decline in migratory capacity of stem cells, which can affect tissue repair. Decline in migratory efficiency was exaggerated in SHR.

IV.3.5. Intracellular ROS levels in CSCs

Intracellular ROS levels were measured using a fluorescent probe, dichlorodihydrofluorescein diacetate (H_2DCFDA). CSCs from older rats showed a significant increase ($p<0.01$) in intracellular ROS levels compared to pups (Fig. 24). ROS levels in CSCs from SHR of 6, 12 and 18 months were significantly

higher ($p<0.01$) compared to their age matched WST rats. The increased ROS levels in CSCs of SHR are indicative of an adverse microenvironment.

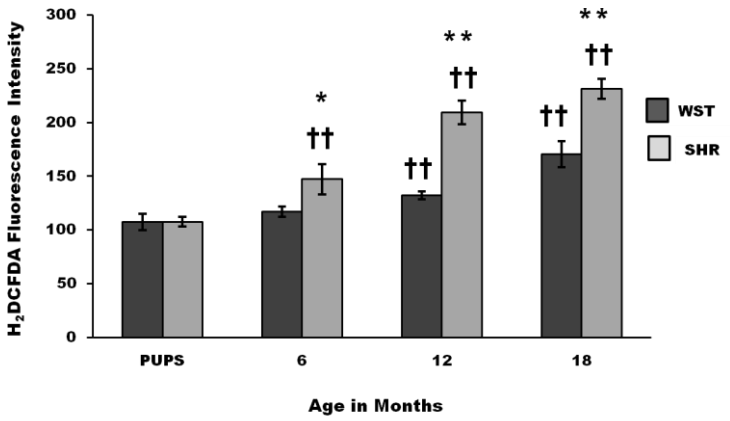


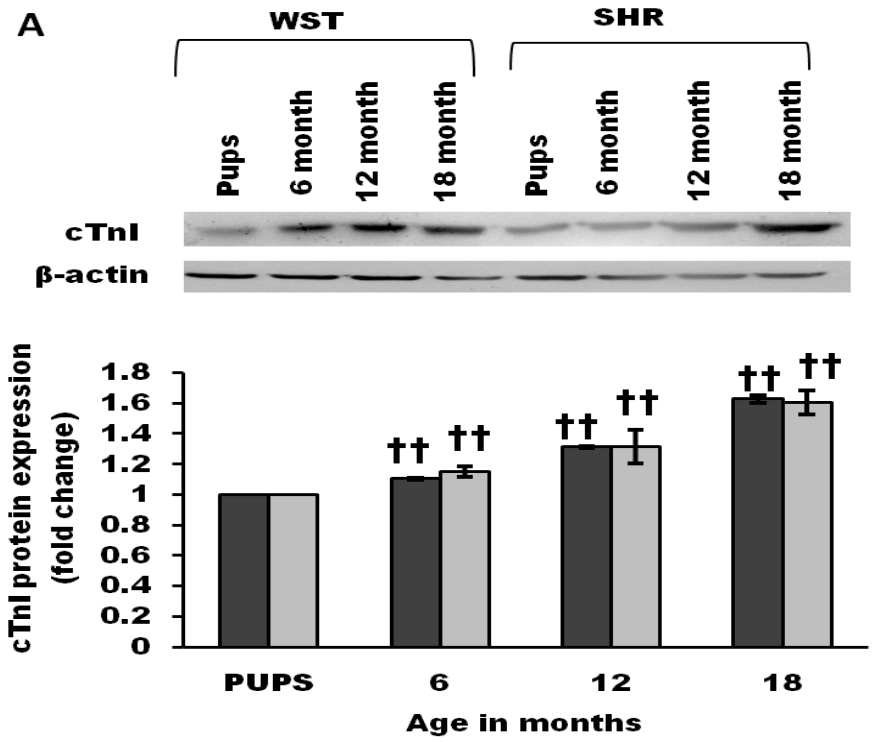
Figure 24: Comparison of intracellular ROS in CSCs of SHR and Wistar rat

*Intracellular ROS levels are represented as fluorescence intensity. (n=3/ group). Data presented as mean ± SD. *p<0.05 & ** p<0.01 SHR Vs Age matched WST; †† p<0.01 WST & SHR of different ages compared to respective 1 week old pups. Two way ANOVA p<0.001*

IV.3.6. Directed differentiation of CSCs

Following culture in differentiating medium containing 10µM 5-azacytidine, cytoplasmic proteins specific to smooth muscle cells (SMCs) and cardiomyocytes were identified by western blot. CSCs of WST and SHR expressed similar proportions of cardiac troponin and smooth muscle actin at specific ages, exhibiting no significant difference between the strains. However, with aging, CSCs from both WST and SHR exhibited a significant increase in the expression levels of both proteins ($p<0.01$) indicating that aged stem cells had an

enhanced ability to differentiate upon stimulation when compared to younger CSCs (Fig. 25).



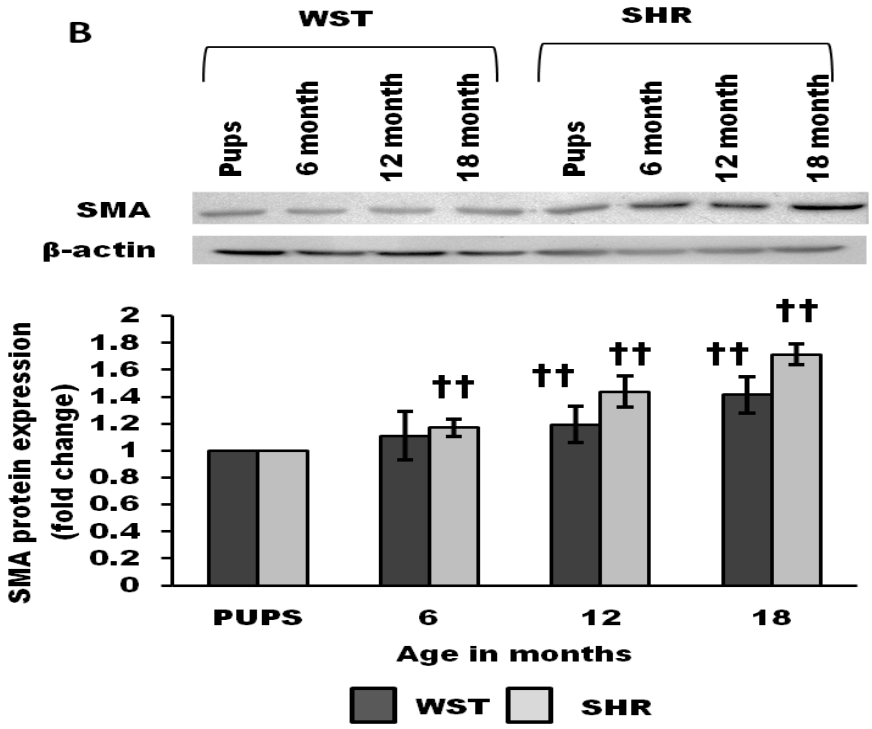


Figure 25: Comparison of directed differentiation potential of c-kit^{+} CSCs of SHR and Wistar rat

(A) Representative blots and graphical representation of expression of cardiac troponin I (cTnI) (B) Representative blots and graphical representation of expression of smooth muscle actin (SMA). Levels of cTnI and SMA denoted as fold change. (n=3/group). Data presented as mean ± SD. †† p<0.01 WST & SHR of different ages compared to respective 1 week old pups. Two way ANOVA p<0.001

IV.3.7. Evaluation of senescence in CSCs

B-galactosidase staining was used as a marker of senescence. CSCs from pups had no detectable senescent cells whereas the number progressively increased with age. Senescent CSCs were observed at 6 months where SHR showed a fold increase compared to WST ($p<0.01$) (Fig. 26). The number of senescent CSCs in

18 and 12 month old SHR was double that of age matched Wistar ($p<0.01$) indicating accelerated aging of CSCs in SHR.

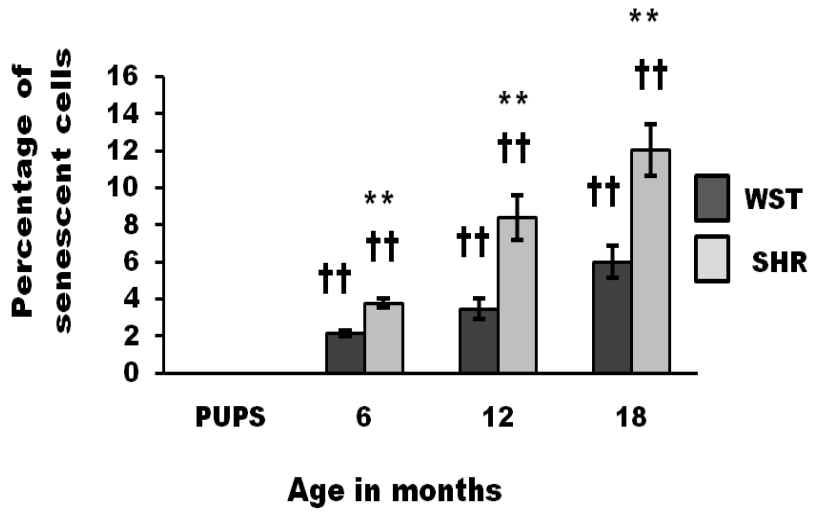


Figure 26: Comparison of proportion of senescent c-kit^{+} CSCs in SHR and Wistar rat

*Senescent cells are expressed as percentage. Graphical representation of senescent cells in different age groups. (n=3/group). Data presented as mean ± SD. ** p<0.01 SHR Vs Age matched WST; †† p<0.01 WST & SHR of different ages compared to respective 1 week old pups. Two way ANOVA p<0.001*

Further, cellular aging is also characterized by the expression of nuclear proteins involved in cell cycle inhibition and irreversible growth arrest. The protein expression analysis of two such proteins, p16^{ink4a} and p21 further confirmed the accelerated aging of CSCs in SHR compared to WST. There was a significant increase in the expression of p16^{ink4a} in CSCs of WST and SHR with age

(p<0.01), with a 2 fold increase in the expression level of p16^{ink4a} in CSCs from 18-month-old SHR compared to age matched WST rat (p<0.01) (Fig. 27).

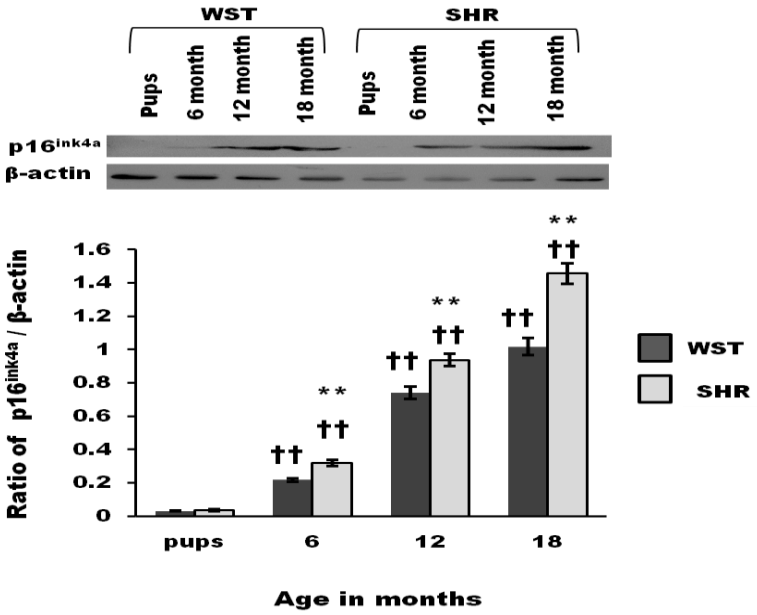


Figure 27: Comparison of the expression of senescent marker p16^{ink4a} in CSCs of SHR and Wistar rat

*Representative blots and graphical representation of expression of p16^{ink4a}. (n=3/group). Data presented as mean ± SD. ** p<0.01 SHR Vs Age matched WST; †† p<0.01 WST & SHR of different ages compared to respective 1 week old pups. Two way ANOVA p<0.001*

The expression of p21, which is a downstream effecter of p53, also exhibited a significant increase with age in both strains of rat (p<0.01) (Fig. 28). CSCs from 18-month-old SHR had an increased expression of p21 compared to that of CSCs from WST of same age (p<0.01).

All the variables studied indicate that, with aging, the expression of senescent markers increased; and the increase was amplified in SHR.

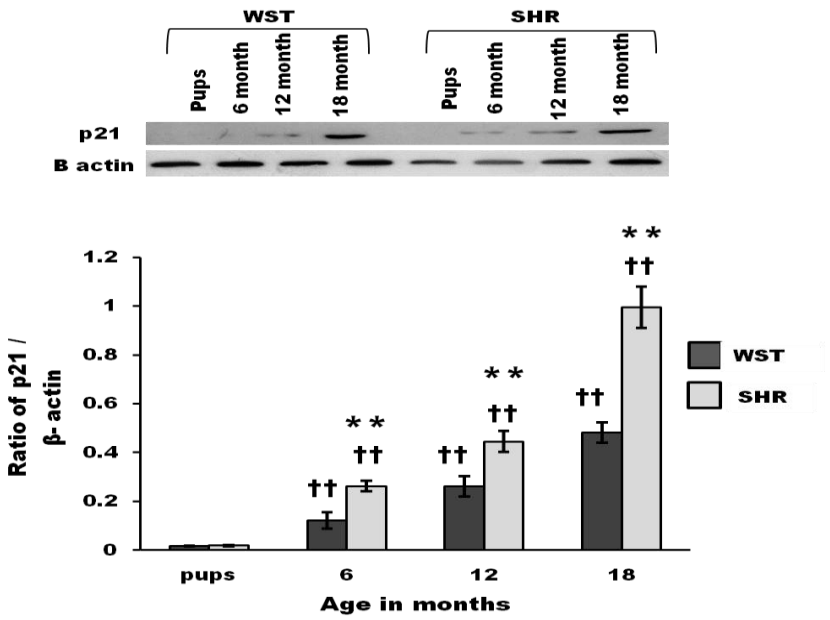


Figure 28: Comparison of the expression of senescent marker p21 in CSCs of SHR and Wistar rat

*Representative blots and graphical representation of expression of p21. (n=3/group). Data presented as mean ± SD. ** p<0.01 SHR Vs Age matched WST; †† p<0.01 WST & SHR of different ages compared to respective 1 week old pups. Two way ANOVA p<0.001*

IV.3.8. TERT mRNA levels in CSCs from SHR and Wistar rat

Real time PCR analysis was carried out to assess the level of TERT mRNA in CSCs of both SHR and Wistar. As shown in Figure 29 there was a significant decline in TERT expression levels in both SHR and Wistar rat upon aging ($p<0.01$). Between groups, SHR exhibited a more drastic decrease in TERT mRNA levels. The difference between SHR and Wistar was significant at 12 and 18 months ($p<0.01$). However, there was no statistically significant difference in

the TERT mRNA expression between CSCs from 6-month-old WST and SHR. Decrease in TERT mRNA expression suggests reduction in efficiency of cell proliferation.

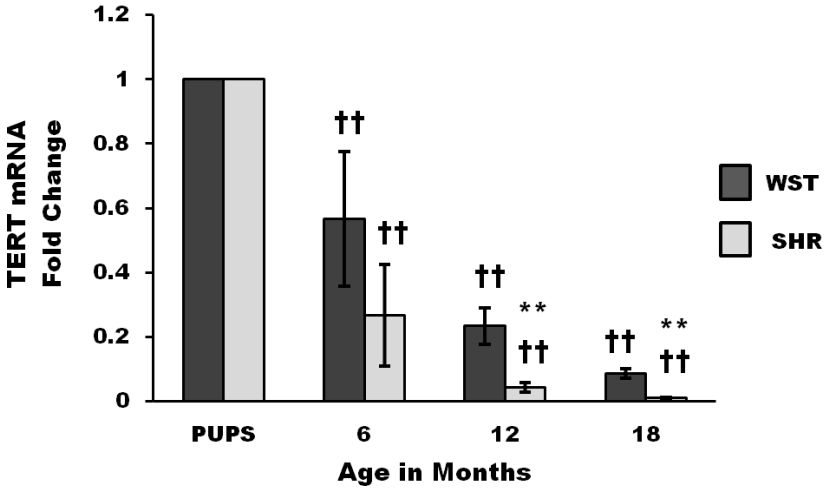


Figure 29: Comparison of TERT mRNA levels in CSCs of SHR and Wistar rat

*TERT mRNA levels are expressed as fold change. (n=3/group). Data presented as mean ± SD. ** p<0.01 SHR Vs Age matched WST; †† p<0.01 WST & SHR of different ages compared to respective 1 week old pups. Two way ANOVA p<0.001*

IV.3.9. Telomerase activity in CSCs from SHR and Wistar rat

Telomerase activity was assessed by TRAP assay kit in 12 and 18-month-old rats and compared with the level in pups (Fig. 30). A decrease to 25% and 16% respectively of telomerase activity in CSCs from 18-month-old Wistar and SHR was observed compared to the levels in the newborn ($p<0.01$). The telomerase activity in CSCs from 12 and 18-month-old SHR and Wistar rat showed a significant difference ($p<0.01$) between groups. The reduced activity of this

ribonucleoprotein in cells from older animals was consistent with TERT mRNA expression.

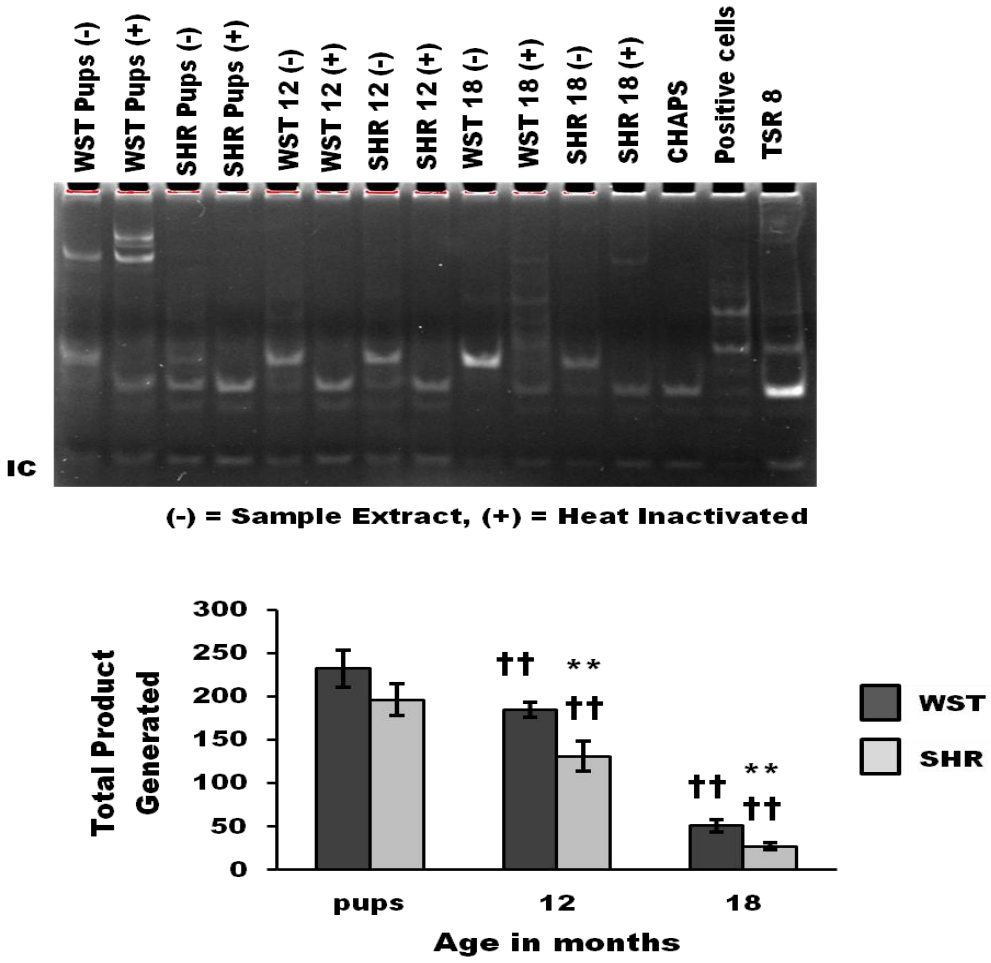


Figure 30: Comparison of telomerase activity in CSCs from SHR and Wistar rat

*Representative gel image and graphical representation of telomerase activity of CSCs. Telomerase activity is expressed as Total Product Generated. (n=3/group). Data presented as mean ± SD. ** p<0.01 SHR Vs Age matched WST; †† p<0.01 WST & SHR of different ages compared to respective 1 week old pups. Two way ANOVA p<0.001*

IV.3.10. DNA damage in CSCs from SHR and Wistar rat

Age dependent increase in the extent of DNA damage was noticed in CSCs of both SHR and WST (Fig. 31). Statistically significant increase in comet tail moment was seen in older animals compared to younger rat. Physiological aging resulted in 3 fold and 19 fold increase in tail moment in CSCs expanded from 12 and 18-month-old WST rat. However, the magnitude of tail moment was about 8 fold and 36 fold in CSCs from 12 and 18-month-old SHR, indicating exaggerated DNA damage in cells from hearts with pathological remodeling. As early as 6 months of age, there was significant increase in DNA damage in CSCs from SHR compared to WST ($p<0.01$).

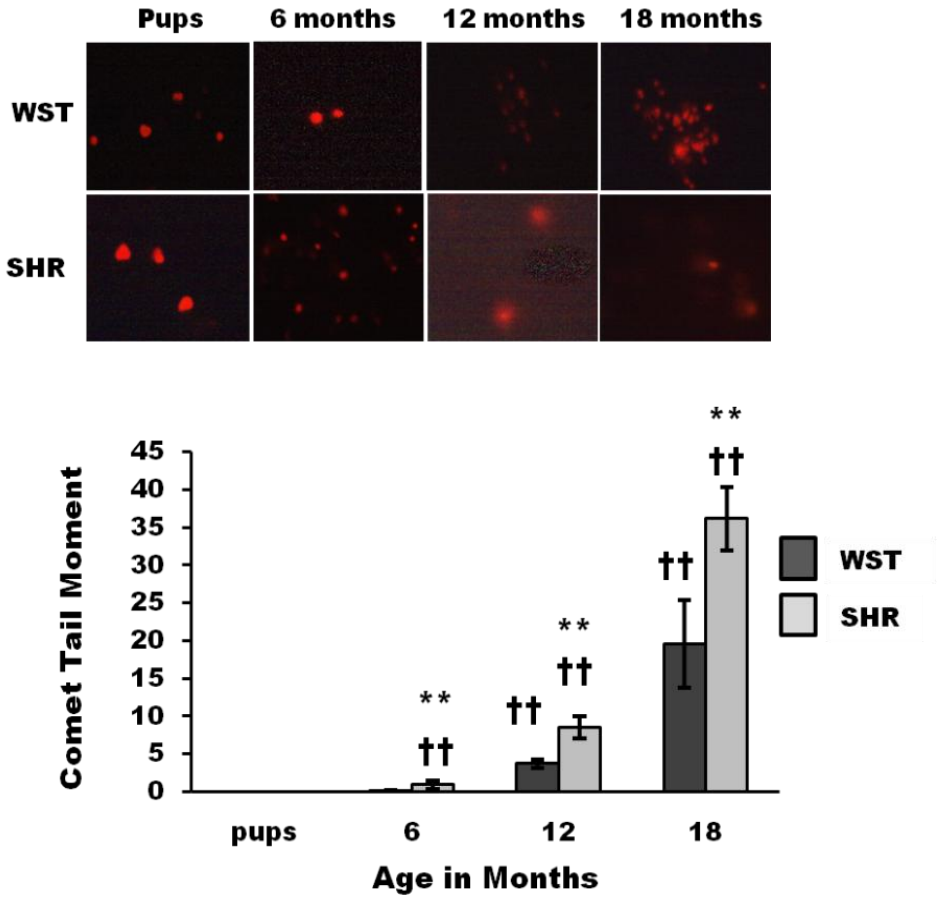


Figure 31: Comparison of DNA damage in CSCs from SHR and Wistar rat

*Representative images and graphical representation of DNA damage presented as tail moment. (n=3/group). Data presented as mean ± SD. ** p<0.01 SHR Vs Age matched WST; †† p<0.01 WST & SHR of different ages compared to respective 1 week old pups. Two way ANOVA p<0.001*

IV.4. Effect of supplementation with the antioxidant tempol on CSCs

IV.4.1. Cardiac response to treatment with tempol

Following supplementation with tempol for 14 days, the animals were subjected to blood pressure measurement and echocardiography to assess the regression of hypertrophy. The animals were sacrificed and heart was dissected out. Ventricular tissue was used for immunohistochemistry to assess the tissue oxidative levels.

	WISTAR	SHR	TEMPOL
SBP (mm Hg)	115.2±12.2	165±14.8**	131±11.5†
DBP (mm Hg)	76±9.8	114±14.3**	94±10.1†

Table 2: Effect of tempol on Blood Pressure as assessed by Tail Cuff method

*Data presented as mean ± SD;. Variation was analyzed by ANOVA followed by t-test. ** p<0.01 SHR Vs WST; † p<0.05 SHR Vs Tempol ANOVA p<0.01*

Blood pressure in SHR was significantly higher than WST (Table 2). Both systolic and diastolic BP was reduced significantly with tempol and the levels were comparable to WST.

Echocardiographic Analysis -Significant differences were observed in E/A ratios in SHR when compared to WST suggesting the presence of diastolic dysfunction. Increase in E/A ratio in response to tempol indicates improvement in diastolic function. Decrease in LV mass and RWT indicate regression of hypertrophy.

	WISTAR	SHR	TEMPOL
LV MASS (mg)	495±21	684± 37**	593±22††
RWT	0.68±0.05	0.83±0.06**	0.78±0.04††
E/A RATIO	2.1±0.34	1.12±0.27**	1.46±.22††

Table 3: Effect of tempol on LV function as assessed by 2D echocardiography

*Left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LVESD), posterior wall thickness (PW) and septal wall thickness (IVS) during diastole were measured using M mode, following the American Society of Echocardiography guidelines. LV mass (LVM) and Relative wall thickness (RWT) were calculated following standard protocol. Mitral flow was recorded at the tip of the mitral valve from an apical view using Doppler imaging. Maximal velocities of the E and A waves were recorded and E/A ratio calculated. (n=6/group). Data presented as mean ± SD;. Variation was analyzed by ANOVA followed by t-test. ** p<0.01 SHR Vs WST; †† p<0.01 SHR Vs Tempol ANOVA p<0.01*

Myocardial Oxidative Stress - Immunohistochemical analysis of the ventricular tissue confirmed the reduction of tissue oxidative stress on treatment with tempol. There was a significant increase in staining intensity for 3-nitrotyrosine

in the myocardial tissue of SHR when compared to WST (Fig. 32). In treated animals, the staining intensity was lower and was comparable with WST.

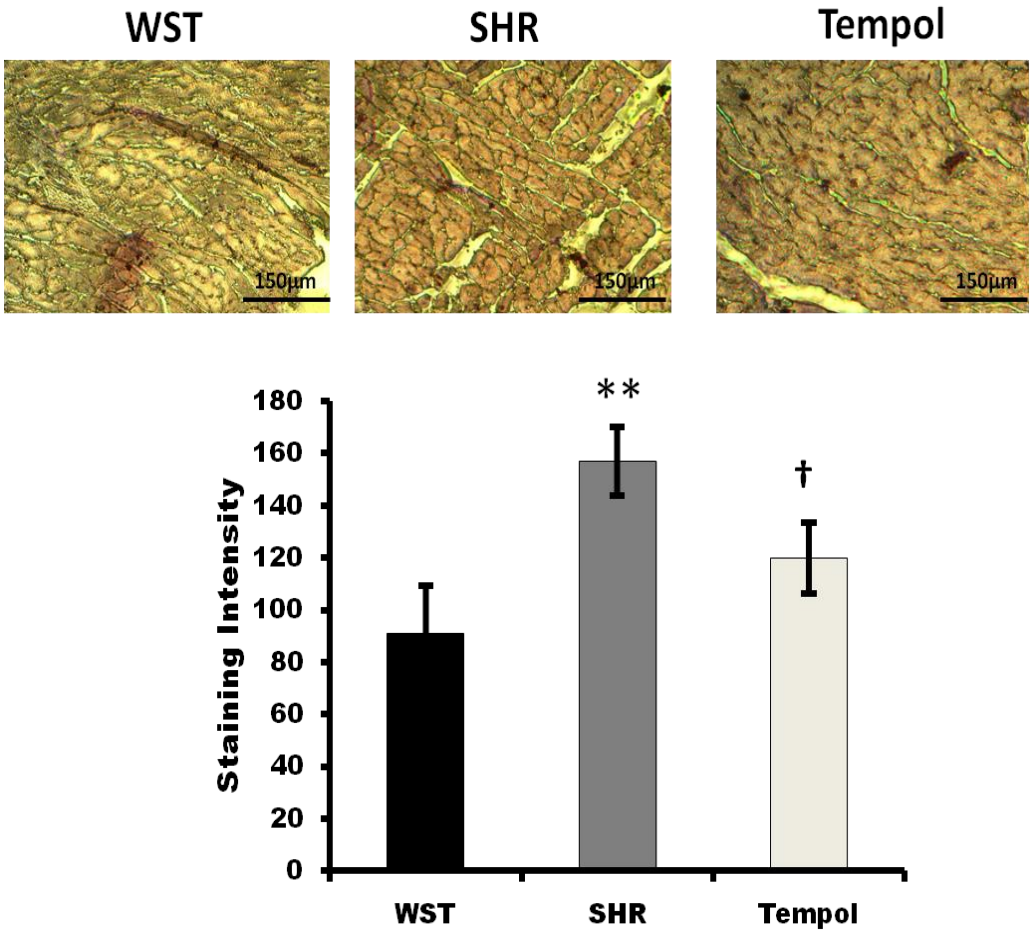


Figure 32: Effect of antioxidant supplementation on tissue oxidative stress

*Five micron thick ventricular sections was subjected to immuhistochemistry to stain for the presence of 3-nitrotyrosine, a marker for oxidative stress. Staining intensity was calculated using the software ImageJ. Representative images and graphical representation of staining intensity of 3-nitrotyrosine by IHC (n=6/group). Data presented as mean ± SD. Variation was analyzed by ANOVA followed by t-test. ** p<0.01 SHR Vs WST; † p<0.05 SHR Vs Tempol. ANOVA p<0.01*

IV.4.2. Effect of tempol treatment on self- renewal capacity of CSCs

Self renewal capacity of CSCs was significantly compromised in SHR. On treatment with tempol the ability of CSCs to form colonies increased and was comparable with that of WST (Fig. 33)

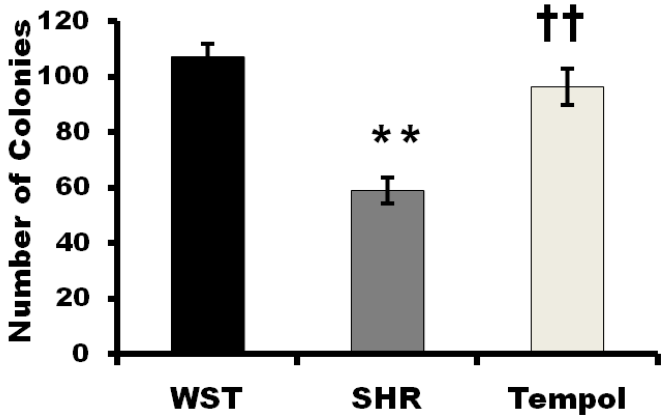


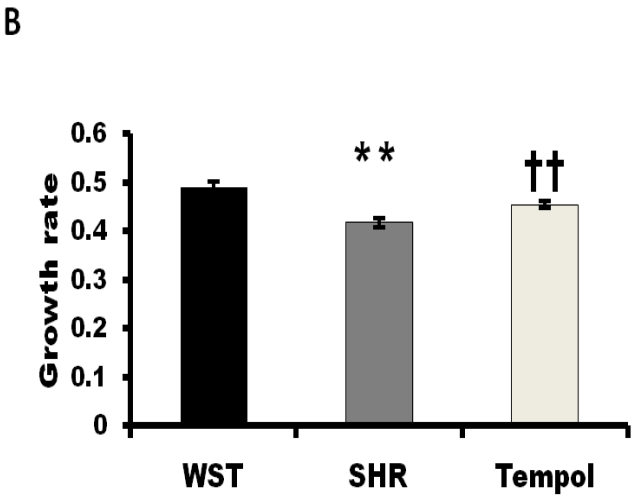
Figure 33: Effect of antioxidant supplementation on self-renewal capacity of CSCs

*CFU is represented as number of colonies/ plate. (n=6/group). Data presented as mean ± SD. Variation was analyzed by ANOVA followed by t-test. ** p<0.01 SHR Vs WST; †† p<0.01 SHR Vs Tempol. ANOVA p<0.01*

IV.4.3. Effect of tempol treatment on Growth Kinetics, PDT and Growth Rate of CSCs

The cell yield from WST following 10 days of culture was $134.33\pm14.04*10^4$ whereas, that of untreated SHR reached only $65.33\pm6.5*10^4$. Following treatment, cell yield from SHR was $111\pm 8.5*10^4$ after 10 days of culture, which was comparable with that of WST. Growth rate and Population Doubling Time (PDT) also exhibited a similar pattern upon treatment with the SOD mimetic

(Fig. 34). CSCs from SHR exhibited a significantly decreased growth rate and significantly increased PDT when compared with CSCs from WST. However upon treatment, GR and PDT of CSCs improved and were comparable with that of WST and significantly different when compared to CSCs from untreated SHR.



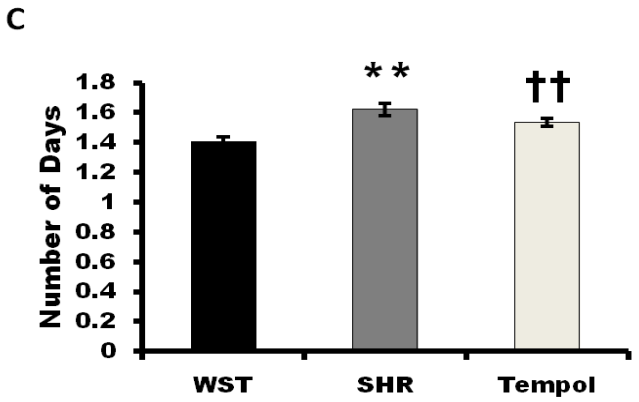
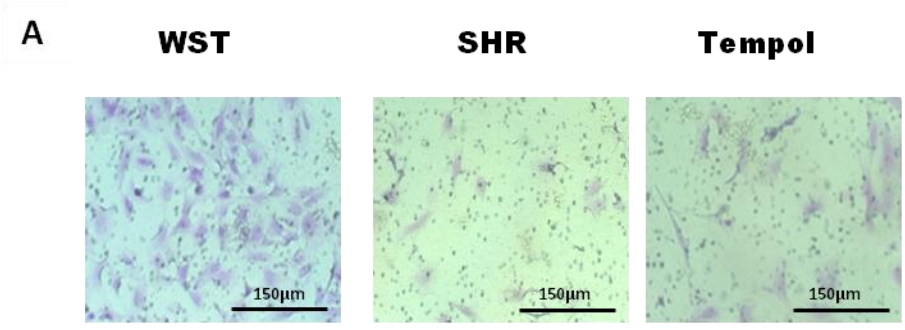


Figure 34: Effect of antioxidant supplementation on growth kinetics, growth rate and PDT of CSCs

*(A) Growth kinetics represented as cell number (B) GR represented as growth rate (C) PDT represented as number of days. (n=6/group). Data presented as mean ± SD. Variation was analyzed by ANOVA followed by t-test. ** p<0.01 SHR Vs WST; †† p<0.01 SHR Vs Tempol. ANOVA p<0.01*

IV.4.4. Effect of tempol treatment on migration ability of CSCs

CSCs from SHR exhibited 37% decline in the number of migrated cells when compared to the WST control and which was increased by two fold with the tempol treatment. Similar observations were obtained from scratch wound assay (Fig. 35).



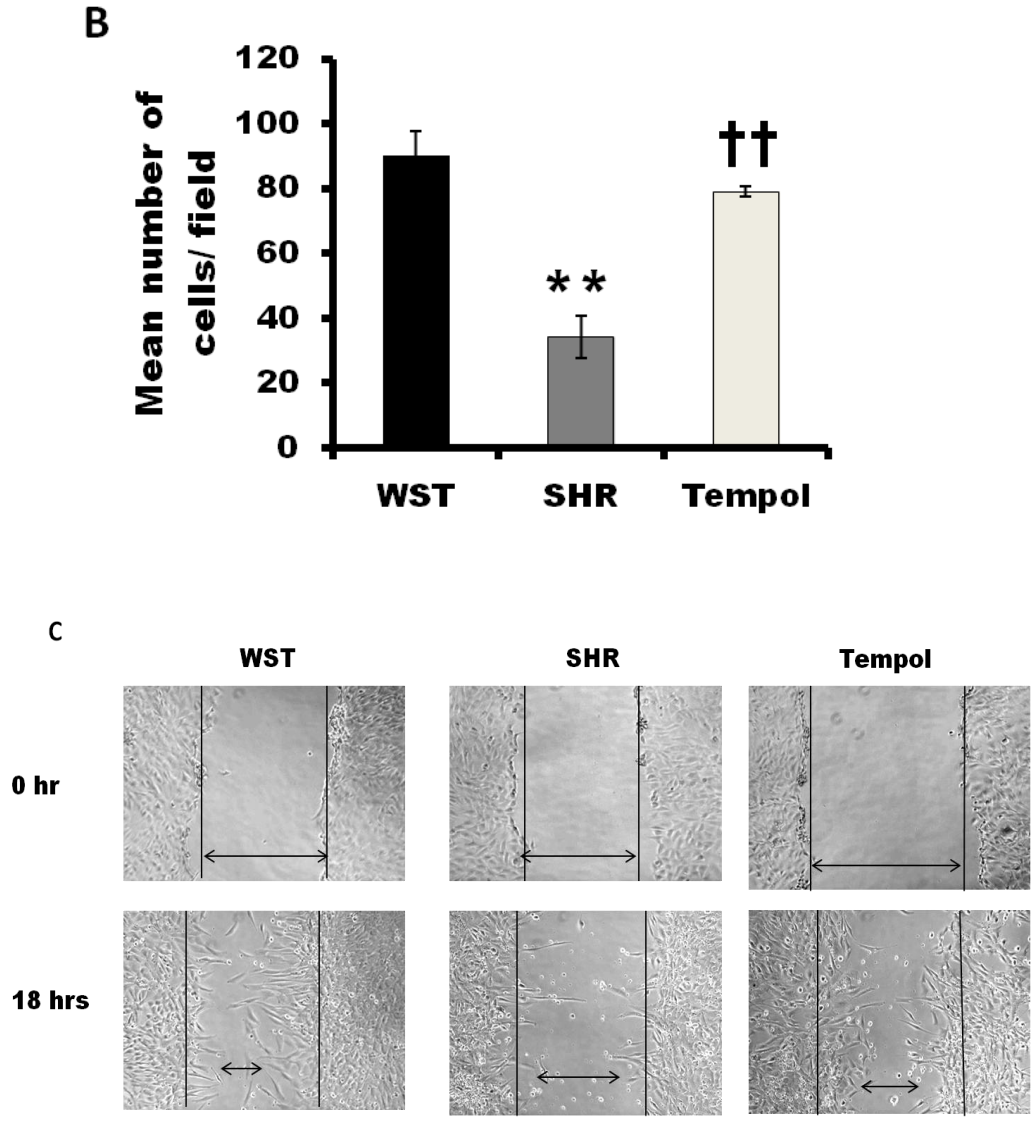


Figure 35: Effect of antioxidant supplementation on migration potential of CSCs

*(A) Representative pictures of membranes stained with Coomassive Brilliant Blue (B) Graphical representation of data, where migration ability is represented as mean number of cells/field. (C) Representative images of Wound-healing assay. (n=6/group). Data presented as mean ± SD; p<0.05 was considered to be statistically significant. Variation was analyzed by ANOVA followed by t-test. ** p<0.01 SHR Vs WST; †† p<0.01 SHR Vs Tempol. ANOVA p<0.01*

IV.4.5. Effect of tempol treatment on intracellular ROS levels in CSCs

Intracellular ROS levels were higher in CSCs from SHR; but decreased significantly in response to treatment with tempol (Fig. 36).

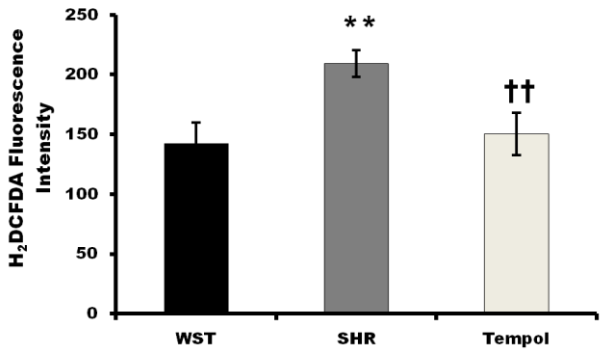


Figure 36: Effect of antioxidant supplementation on intracellular ROS levels in CSCs

*Intracellular ROS levels are represented as fluorescence intensity. (n=6/group). Data presented as mean ± SD.. Variation was analyzed by ANOVA followed by t-test. ** p<0.01 SHR Vs WST; †† p<0.01 SHR Vs Tempol. ANOVA p<0.01*

IV.4.6. Effect of tempol treatment on differentiation potential of CSCs

C-kit^{+} CSCs of 6 month old SHR and WST exhibited no statistical difference in the differentiation potential as evident from the western blots. Concomitantly, even the tempol treatment had no influence on the differentiation ability. The expression levels of cTnI and SMA were comparable in all three groups (Fig. 37).

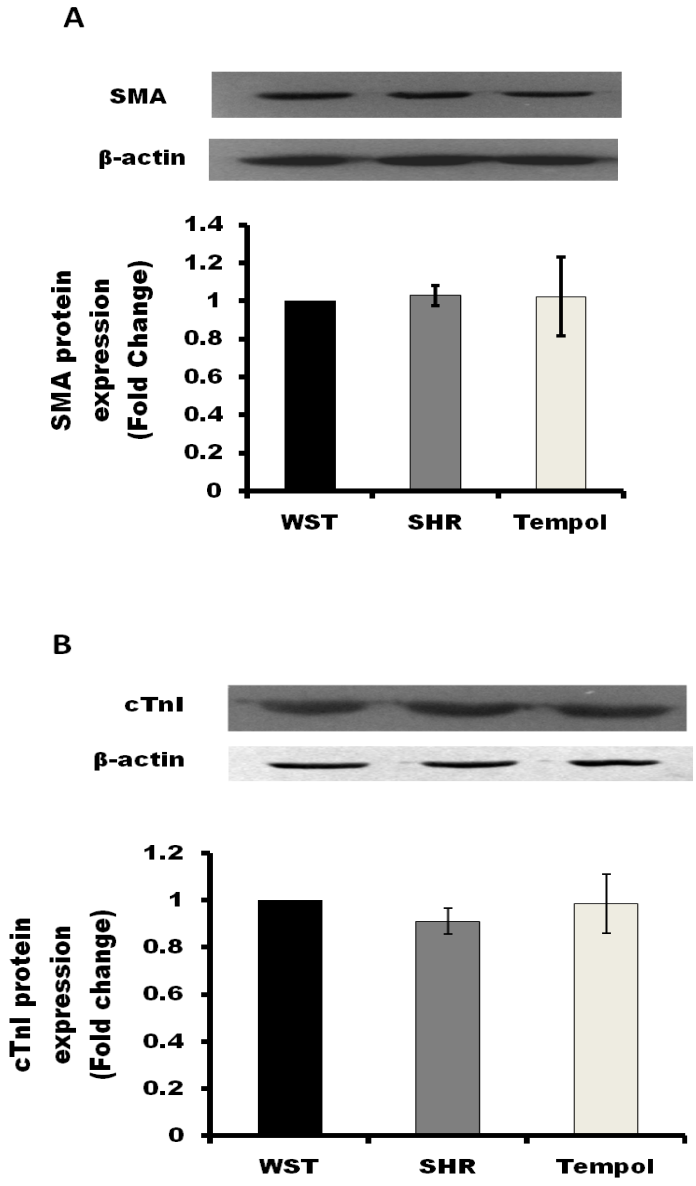
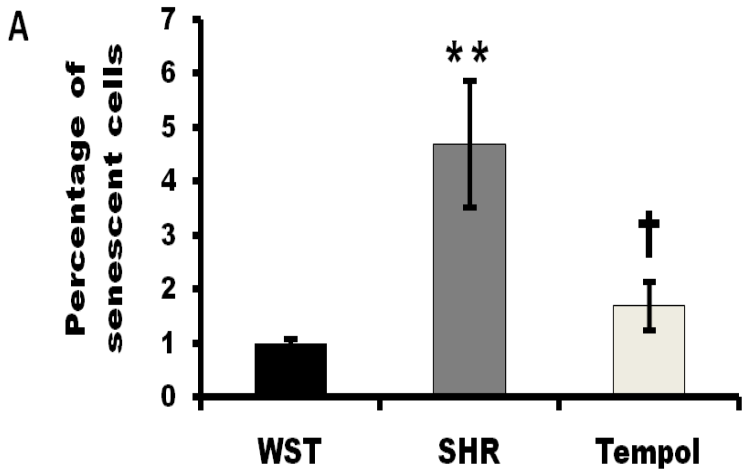


Figure 37: Effect of tempol on directed differentiation potential of CSCs

(A) Representative blots and graphical representation of expression of smooth muscle actin. (B) Representative blots and graphical representation of expression of cardiac troponin I. Levels of cTnI and SMA denoted as fold change. (n=6/group). Data presented as mean ± SD. Variation was analyzed by ANOVA. ANOVA $p>0.05$

IV.4.7. Effect of tempol treatment on proportion of senescent CSCs

A four-fold increase in the number of senescent cells was observed in CSCs of SHR when compared with the CSCs of WST. Upon treatment with tempol the number decreased to half and was comparable with that of WST. The expression of nuclear proteins involved in cellular aging, $p16^{ink4a}$ and p21 were found to be enhanced in SHR compared to WST. However, with the tempol treatment, the expression levels of both proteins decreased and were comparable with WST (Fig. 38).



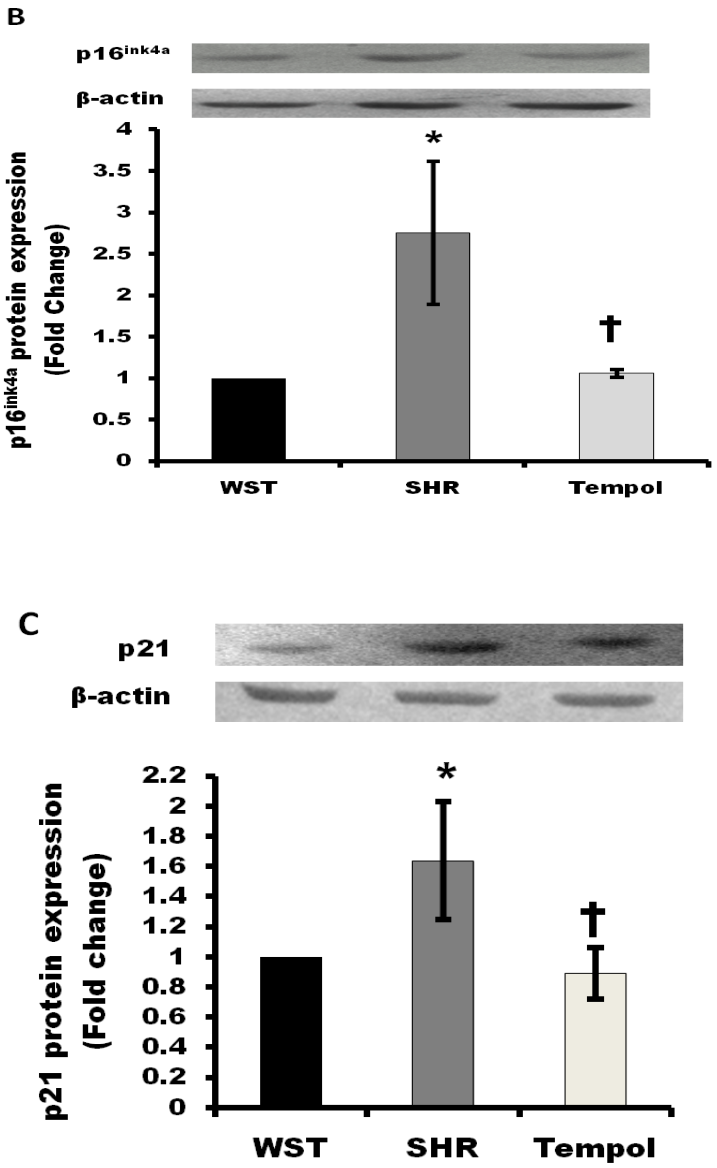


Figure 38: Effect of antioxidant supplementation on senescence of CSCs

*(A)Graphical representation of senescent cells represented as percentage. Protein was isolated and subjected to western blot analysis (B) Representative blots and graphical representation of expression of p16^{ink4a}. (C)Representative blots and graphical representation of expression of p21. (n=6/group). Data presented as mean ± SD. Variation was analyzed by ANOVA followed by t-test. * p<0.05 SHR Vs WST; † p<0.05 SHR Vs Tempol. ANOVA p<0.05*

V. DISCUSSION

Resident cardiac stem cells are vital for the maintenance of tissue homeostasis. In the event of cell loss due to tissue injury, stem cells home in to the affected site and enable tissue repair. In the normal course, 0.5-10% of cells are renewed every 6 months (Kajstura *et al.*, 2010, Soonpaa & Field, 1997, Bergmann *et al.*, 2009, Walsh *et al.*, 2010) envisaging the role of stem cells in regeneration and repair. In pathological conditions, in addition to increased myocyte damage, the adverse microenvironment such as enhanced oxidative stress can influence the properties of the resident cardiac stem cells. Stem cells have been implicated in cardiac failure based on analysis of human tissues from donor and explanted failing hearts obtained at transplantation (Cesselli *et al.*, 2011). A decrease in functionally competent CSCs in cardiac failure patients led to the postulation that this may be the underlying cause for the deterioration of cardiac function and onset of failure (Cesselli *et al.*, 2011; Urbanek *et al.*, 2005). However, a systematic analysis on the distribution and function of cardiac stem cells in association with age and disease, using an appropriate animal model has not been reported so far. Against this backdrop, the properties of cardiac stem cells from normotensive Wistar rat was analysed to evaluate the age associated changes in number and functional efficiency of the stem cells. In hypertensive heart disease, the cause for the transition from compensatory phase of left ventricular hypertrophy to decompensatory phase remains enigmatic. To understand the fate of stem cells in the pathological heart, age associated variation in CSCs were examined in Spontaneously Hypertensive Rat, a genetic model of hypertension,

in comparison with the normal heart. The study has shown that the number and efficiency of CSC declined with age, the progression being accelerated in the pathological heart. The present study was based on the hypothesis that decline in the number and/or efficiency of resident cardiac stem cells can possibly affect adaptive remodeling of the heart, leading to cardiac failure. As aging and disease is associated with oxidative stress, the study has also looked into the efficiency of antioxidant supplementation in the modulation of stem cell characteristics.

V.1. Isolation and characterization of c-kit$^+$ CSCs

C-kit$^+$ CSCs were immunomagnetically sorted from primary atrial explant culture (Fig. 8,9). FACS analysis and immunocytochemistry established the purity of the CSCs, as the cells were positive for c-kit and negative for lineage markers CD-31 and CD-45 confirming the absence of haematopoietic and endothelial stem cells (Fig. 10, 11). The cells were able to maintain the c-kit surface marker expression even at passage 10 (Fig. 12). This reiterates that stem cells from an adult can be passaged repeatedly under optimal culture conditions (Reynolds & Weiss 1996), to obtain a population of healthy stem cells with self renewing ability. This also implies that the cells sorted were cardiac stem cells and supports the notion that atria contain a pool of primitive CSCs expressing surface marker of stemness. Earlier studies have also reported the feasibility to isolate and expand CSCs from biopsies of myocardium (Bearzi *et al.*, 2007; Beltrami *et al.*, 2003, , Leri *et al.*, 2005). Despite the age associated decline in

number, CSCs from aged Wistar and SHR were obtained in sufficient quantities for the experiments at passage 3.

V.2. Age associated variation in cardiac stem cell number and efficiency

To determine whether the number and efficacy of CSCs decline with physiological aging, c-kit$^+$ cells were isolated from the heart of normotensive Wistar rat of different ages and assessed for variation in number, proliferation, migration, intracellular ROS and senescence. This is the first study to report the physiological aging of CSCs in an animal model. A gradual decline in the density of c-kit$^+$ cells in the ventricular tissue were observed with age (Fig. 13). A significant reduction in cell density with age indicates the lesser availability of healthy CSCs in older rats for regeneration in the event of an insult. These results are concordant with the reports on neural stem cells, where the number declined with age in adult rodents (Maslov *et al.*, 2004). With age, CSCs exhibited decline in growth rate, resulting in longer doubling time indicating their compromised proliferation capacity (Fig. 14, 15). The heart undergoes irreversible remodeling with physiological aging (Lakatta, 2001) implicating the deficient functionality of resident CSCs. Furthermore, the migration ability of CSCs declined with age (Fig. 16) which will have profound effect on the regeneration capacity of the heart. Reports on the functional decline of hematopoietic stem and progenitors cells with age are well documented. Defects in mobilization and homing to bone marrow by HSCs from older mice has been reported (Morrison *et al.*, 1996). The

role played by ROS in the maintenance of self-renewal, proliferation, and differentiation of mesenchymal stem cells (MSCs) and pluripotent stem cells (PSCs) is reported (Maraldi *et al.*, 2015, Yang, Park & Kang, 2015). Intracellular ROS levels of CSCs increased with physiological aging (Fig. 17). Though no significant difference in their levels was observed in 6-month old compared to the newborn, significant increase was observed at 12 and 18 months indicating the presence of adverse intracellular milieu in CSCs. Senescence, an intricate change with aging is noted not only in tissues, but also in stem cells residing within their niches. Haematopoietic stem cells are prone to senescence with aging (Geiger *et al.,* 2013). Same has been observed in the case of CSCs with physiological aging (Fig. 18).

V.3. Comparison of stem cell density in Hypertensive Heart Disease with Normotensive Heart

To understand the variation in stem cell characteristics with progressive cardiac remodeling in hypertensive heart disease, c-kit$^+$ cells were isolated from the heart of Spontaneously Hypertensive rat and assessed for growth, migration, differentiation and senescence. Age associated variation in SHR was compared with changes attributed to physiological aging. Age associated decline in the number of c-kit$^+$ cells (Fig. 19) implies that deficiency in the number of stem cells can adversely affect replenishment of lost myocytes for maintenance of tissue homeostasis and lead to cardiac failure. Studies have reported age associated increase in myocyte turnover, where stem cells play a significant role

(Kajstura *et al.*, 2010). The age associated decline in CSCs can also be accounted for the utilization of stem cells for replacement of lost myocytes. The number of stem cells in the compensatory phase of hypertrophy was found to be significantly lower than that of age matched normal heart indicating that decline in stem cell number is initiated at an early stage and lack of availability of the required stem cell population can account for the transition from the compensatory to decompensatory phase. Myocyte loss was observed in experimental models of hypertension induced hypertrophy (Okoshi *et al.*, 1997). Utilisation of resident cardiac stem cells for replacement of lost myocytes can result in a steady decline in stem cell number initiated from the stage of compensatory hypertrophy. Urbanek *et al.* observed that ischemic heart failure led to an increase in the hCSC number; and that in chronic infarcts, the number of CSCs was lower than in acute infarct. Homing of stem cells for tissue repair can account for the observation of increase in the number of stem cells in the acute stages, whereas decrease in stem cell population in chronic heart disease may be the consequence of cell loss due to repeated cycling (Urbanek *et al.*, 2005). CSCs actively take part in cardiomyocyte turnover (Bergmann *et al.*, 2009) and these cardiomyocytes undergo senescence with aging (Li *et al.*, 2014). Immunohistochemical analysis had shown that the number of c-kit^{+}, lineage-negative human CSCs was 3.2-fold higher in atria of normal donor hearts than of diseased explanted hearts in humans (Cesselli *et al.*, 2011). Age as well as disease can account for the lower proportion of cells in the explanted heart, as the

donor subjects were young. Apart from repeated cycling of CSCs to generate new cells, adverse microenvironment in the form of oxidative stress prevalent in the aged and diseased heart can affect the density of CSCs.

V.4. Comparison of characteristics of atrial CSCs in SHR with Wistar

CSCs from SHR exhibited lower growth kinetics than CSCs of WST, as apparent from the decreased growth rate and increased PDT (Fig. 21). Pathological remodeling appears to have affected proliferative capacity of CSCs.

Stem cells are important mediators of tissue maintenance and wound repair. Stem cells possess the ability to migrate to the region of injury and respond according to the needs of the surrounding milieu by either autocrine or paracrine mechanisms (Gnecchi, *et al.*, 2008, Stastna *et.al.*, 2009). CSCs from SHR were found to have lower migratory capacity when compared with the age matched WST as evident from trans-well assay and scratch-wound assay (Fig. 22, 23). This was consistent with the study on hematopoietic stem cells where functional ability changed with time (Rossi *et al.*, 2005). The intracellular signaling pathways involved in promoting movement may be impaired due to increased ROS and senescent nature of CSCs.

Aerobic metabolism, although central to life, produces reactive oxygen species (ROS) that have been implicated in both cardiovascular disease and aging. Excessive amounts of ROS can lead to cellular senescence, apoptosis and carcinogenicity (Balaban *et al.*, 2005). HSCs, NSCs and early progenitors

contain lower levels of ROS than their mature progeny; and these differences are critical for maintaining stemness (Le Belle *et al.*, 2011). Physiological levels of intracellular ROS are required to maintain genomic stability in cardiac and embryonic stem cells through activation of the DNA repair pathway (Diehn *et al.*, 2009, Li & Marbán, 2010). Therefore, the intracellular ROS levels in c-kit^{+} CSCs have implications on stem cell attributes. Intracellular ROS was detected using the non-fluorescent $DCFH_2$, which is converted to DCF through the action of intracellular oxidants like H_2O_2, $HO^{\bullet}$, $ROO^{\bullet}$. With aging, the increased ROS generation in CSCs led to enhanced DCF fluorescence intensity (Fig. 24). In SHR, ROS levels of CSCs were much higher than the age matched WST at any given age except for newborn reiterating the role of oxidative stress in compromised stem cell efficiency. Another impact of oxidative stress is the induction of cellular senescence as apparent from the expression of senescent markers and DNA damage leading to apoptosis of CSCs.

Normally, senescent somatic cells accumulate in the body with advancing age. The expression of senescence-associated markers like SA-β-gal staining, p21 and $p16^{ink4a}$ was higher in CSCs from aged rat (Fig. 26-28). This suggests that CSCs from aged rats are more prone to undergo senescence than those isolated from younger rats but at a faster pace in the diseased state. These observations are consistent with the report that senescence and death of CSCs increased with age in wild type mice impairing the growth and turnover of cells in the heart (Torella *et al.*, 2004). Hematopoietic and musculoskeletal systems are also known to

undergo degenerative changes with age (Jung & Brack, 2014; Zhuo *et al.*, 2010). Expression of senescence associated proteins $p16^{ink4a}$ and p21 was elevated upon aging which play important role in growth arrest. Senescent stem cells affect their microenvironment by decreasing regenerative potential of the entire stem cell pool, while also affecting neighboring myocytes and vasculature.

With both groups of CSCs, the expression pattern of cardiac troponin I and smooth muscle actin were found to be similar (Fig. 25). The ability to differentiate upon stimulation with 5-azacytidine into cells of cardiovascular lineage is an indication of the potential of the CSCs to differentiate to the desired phenotype. The differentiation potential assessed from the expression levels of cell specific proteins increased with age, possibly implicating the lineage commitment of CSCs, based on demand. The lineage commitment could also be the consequence of oxidative stress as ROS is required for differentiating into cardiomyocytes from embryonic, haematopoietic and cardiac stem cells (Bigarella *et al.*, 2014; Crespo *et al.*, 2010; Puceat, 2005). Lineage commitment therefore results in decrease of stemness.

Telomerase is a reverse transcriptase that extends the 3' chromosomal ends by using its own RNA as a template. Shortening of telomeres beyond a critical length triggers cellular senescence, which corresponds to irreversible growth arrest in the G1 phase with loss of specialized functions, including stem cell proliferation, migration, and differentiation. Telomerase protects chromosomes from telomere erosion, maintains cell replication, and opposes cell death

(Hiyama & Hiyama, 2007). Telomeres play an essential role in cellular aging (Hornsby, 2007) and is dictated by down-regulation of telomerase, and loss of telomere-related proteins (Flores & Blasco, 2010). Telomerase activity was found to be decreased, and CSC division was impaired by severe telomeric shortening and cellular senescence in humans with ischemic heart failure (Urbanek *et al.,* 2005). Similarly, TERT mRNA expression and telomerase activity were found to be decreased in CSCs from both groups upon aging (Fig. 29, 30). However, the decline was more profound in the CSCs from hypertensive heart, signifying the accelerated aging of CSCs. Comet assay indicated the presence of DNA damage in 12 and 18-month-old rat, which was significantly higher in SHR (Fig. 31). Though no information is available on the fate of CSCs, higher rate of senescence and lower rate of telomerase activity of endothelial progenitor cells from SHR compared to age matched WST has been reported (Imanishi *et al.,* 2008).

These results indicate that chronological age is a major determinant of the loss in growth reserve of the adult heart, dictated by a progressive decline in the qualitative and quantitative nature of CSCs. The decreased regenerative capacity appears to contribute to the aging of heart, thereby supporting the 'stem-cell hypothesis' for age-associated decline in cardiac function. The accelerated deterioration in stem cell efficacy in SHR can be linked to the transition from hypertrophy to failure.

V.5. Response of CSCs to antioxidant supplementation

This study for the first time demonstrates that CSCs deteriorate with age, which is further accelerated in SHR, thereby linking decrease in number of functionally efficient CSCs to pathological remodeling of heart leading to cardiac failure. The role of oxidative stress in pathological remodeling is postulated, whereby, supplementation with antioxidants is expected to have beneficial effect. The consequence of antioxidant supplementation on cardiac stem cells has not received much attention.

Oxidative stress, which is well established in hypertension induced hypertrophy, results from an imbalance between reactive oxygen species and antioxidant enzyme levels (Siwik *et al.*, 1999). Oxidative stress is observed as early as 2 months of age in SHR and can trigger left ventricular remodeling (Takimoto & Kass, 2007; Purushothaman *et al.*, 2011). Although, the adverse effects of reactive oxygen species and free radicals on pathological remodeling is well known, its effect on CSCs is least explored. Studies in non-cardiac stem cells have shown the deleterious effect of ROS with aging and disease (Case *et al.*, 2008). The response of CSCs to a potent antioxidant, tempol was investigated with the objective of assessing whether systemic oxidative stress affects the CSCs and also to examine whether antioxidants have a beneficial effect on CSCs.

Six-month-old Spontaneously Hypertensive Rats when treated with tempol for a period of 14 days, led to regression of hypertrophy and reduction of myocardial

oxidative stress (Tables 1, 2, Fig. 32). Following the treatment, atrial explants were established in culture and functional changes were examined in c-kit^{+} CSCs.

Growth kinetics of CSCs from SHR demonstrated that the cell number on the 10th day of culture was half of that seen in WST but on supplementation with tempol, CSCs recovered its growth potential and proliferated to attain values comparable to normotensive control (Fig. 34A). Growth rate and Population Doubling Time also exhibited a similar pattern of difference between SHR and WST that was restored on tempol supplementation (Fig. 34B, 34C). Such an enhancement in growth rate has been reported in adipose derived mesenchymal stem cells in the presence of antioxidants (Lin *et al.*, 2005).

Self renewing ability of cells as evaluated by Colony forming Unit that was significantly compromised in SHR increased by 37% and was comparable with the normotensive control (Fig. 33). Therefore, self renewal capacity of CSCs that deteriorated with hypertensive heart disease improved with the supplementation. Redox homeostasis is critically important in the regulation of stem cell self renewal and differentiation (Wang *et al.*, 2013).

Migration facilitates the homing of stem cells to the site of injury for tissue repair and is an indicator of stem cell efficacy. The beneficial effect on the heart consequent to tempol supplementation on adverse remodeling can also be due to increased recruitment of CSCs for repair. Upregulation of SDF during stress can promote homing of stem cells to heart (Askari *et al.*, 2003). Reactive oxygen

species were found to play a key role in the proliferation and migration of adipose derived stem cells (Kim *et al.*, 2013). The observations confirmed that the disease associated decline in migratory capacity of stem cells that can affect tissue regeneration was restored on treatment with the SOD mimetic (Fig. 35).

CSCs from untreated SHR exhibited increased intracellular ROS levels indicative of weakened antioxidant defense mechanism. The demonstration of reduction of intracellular ROS levels in CSCs in response to antioxidant supplementation (Fig. 36) has not been reported earlier. Reduction of oxidative stress will help the stem cells to remain in undifferentiated state and maintain the stem cell pool. ROS levels have a significant influence on the phenotypic nature of the CSCs.

ROS can act as signaling molecules and can induce differentiation of stem cells (Chaudhari *et al.*, 2014). Even though ROS levels were altered in CSCs upon treatment, no significant effect on the differentiation ability was detected. All three groups exhibited similar levels of cTnI and SMA protein expression (Fig. 37) which can be due to the fact that differentiation ability of CSCs were not much affected in the initial stages of hypertrophy. Senescence or cell cycle growth arrest can be driven by oxidative stress (Shao *et al.*, 2011) and can affect the typical behavior of stem cells. The presence of senescent CSCs in SHR is an indication of the adverse microenvironment compared to the WST. The decreased oxidative stress upon treatment is possibly responsible for the decrease in expression of the senescent markers $p16^{ink4a}$ and p21 as well as the proportion of β-galactosidase positive cells (Fig. 38).

These observations lead to the inference that tempol improves the functional efficiency of stem cells which was otherwise compromised in the diseased condition. The results suggest that ROS plays an important role in hypertension induced hypertrophy, a leading cause for cardiac failure. Tempol is a nonenzymatic, antioxidant possessing SOD-mimetic activity and can easily penetrate cell membranes with low toxicity, which possibly accounts for the efficiency of this antioxidant. Antioxidant treatment is expected to maintain the overall well being of the heart, where the functionally efficient CSCs promotes myocardial regeneration by formation of new myocytes and vascular cells in the event of chronic and acute injury. The observations suggest the possible use of appropriate antioxidants for therapeutic application.

The role of reduction of oxidative stress in prevention of stem cell damage was further confirmed by treating SHR with the β-blocker, metoprolol- a cardioprotective antihypertensive with antioxidant effect (Bao *et al.*, 2015). Deterioration in stem cell function was prevented on treatment with metoprolol (Appendix I, Fig. AIa, AIb). The cardioprotective effect of metoprolol is possibly mediated by restoration of stem cell efficiency.

VI. SUMMARY AND CONCLUSION

VI.1. Summary

Left ventricular hypertrophy is an independent risk factor for cardiovascular diseases. Cardiac abnormalities are predominant in the elderly, being a major shareholder of all mortalities globally. Treatment in the later stages of heart failure is not effective, necessitating identification of strategies for preventing disease progression. The discovery of resident cardiac stem cells (CSCs) and the observation of dividing myocytes contradicted the paradigm that the myocardium is a post-mitotic organ, and led to the postulation that CSCs have a role in the maintenance of cardiac homeostasis. In the event of myocardial injury, CSCs differentiate and replace the lost myocytes presumably contributing towards tissue repair. Though several reviews have implicated the role of stem cells in cardiac failure, the effect of age and disease on CSCs has not drawn much attention. Observations in human samples on the possible role of stem cells in cardiac remodeling underscore the requirement for a systematic analysis of the variation in stem cell characteristics using an appropriate animal model. Left ventricular hypertrophy (LVH) in chronic pressure overload is an adaptive change to maintain cardiac output. Though various morphological, physiological, cellular and molecular alterations have been implicated in the transition from compensatory phase of left ventricular hypertrophy to decompensatory phase, there is no conclusive basis for the progressive deterioration in cardiac efficiency. Oxidative stress is an important contributor to hypertension induced

hypertrophy and is also involved in the physiological aging process. Increased oxidative stress in the surrounding milieu can influence the stem cell characteristics. However, the involvement of ROS in determining the efficiency of CSCs is still enigmatic. The present study is based on the hypothesis that "***Progressive*** d***eterioration in efficacy of stem cells due to adverse microenvironment may be a possible cause for irreversible cardiac remodeling in hypertensive heart disease and aging***."

Age associated variation in stem cell characteristics was assessed in normotensive Wistar rat (WST) and compared with the changes in Spontaneously hypertensive rat (SHR) so as to get a clear distinction between pathological and physiological aging. SHR is a genetic model of hypertension where the cardiac changes are similar to that seen in clinical hypertension with transition from compensated ventricular hypertrophy to failure. CSCs isolated from the atrial explants of 1 week, 6, 12 and 18 month old WST and SHR were characterised for their proliferation, migration and differentiation abilities. Intracellular ROS levels and DNA damage studies were also carried out. The variation in the number of CSCs was assessed in the ventricular digests. To examine the effect of oxidative stress on stem cell efficiency, modulation of the characteristics of CSCs was assessed after treating 6-month-old SHR with tempol, a superoxide dismutase mimetic.

Significant findings of the study are:

(I) Age associated variation in CSCs of normotensive heart:

- *Density of CSCs declined in the ventricles with increasing age*
- *Self-renewing capacity, growth kinetics and migration ability of CSCs from atrial explants was compromised with age*
- *Intracellular ROS levels increased with age in CSCs*
- *Proportion of senescent CSCs were high in aged rats*

Inference: **Chronological age affects the efficiency of CSCs.**

(II) Comparison of age associated variation in stem cells of normal and pathological heart

- *Density of CSCs in ventricles significantly lower in hypertensive heart disease*
- *Accelerated decrease in self renewing capacity, growth kinetics and migration ability in SHR*
- *Considerable increase in the intracellular ROS levels, possibly due to the adverse microenvironment prevalent in HHD*
- *Proportion of senescent cells more in SHR and expression of senescent proteins* $p16^{ink4a}$ *and p21 higher in SHR*
- *Comet assay exhibited severe DNA damage in CSCs from SHR*
- *Loss of TERT mRNA was concordant with the decreased telomerase activity in SHR*

Inference: **Adverse microenvironment possibly leads to accelerated aging of CSCs in SHR compared to normotensive Wistar rat.**

(III) Modulation of CSCs with antioxidant supplementation

- *Supplementation with tempol regressed hypertension induced hypertrophy as evident from reduced blood pressure and improved left ventricular function*
- *Tempol improved the efficiency of CSCs which was otherwise deficient in untreated SHR*

Inference: **The observations of the experimental study implicate the role of oxidative stress in determining the functionality of CSCs and reiterate the beneficial effects of antioxidant supplementation.**

VI.2. Conclusion

Resident cardiac stem cells have a contributory role in cardiac regeneration and repair after myocardial infarction. Though stem cells in general are known to be affected with age and disease, the information regarding CSCs is lacking. This is the first systematic study to provide a clear distinction of the influence of physiological aging and pathological conditions on CSCs using an appropriate animal model. Chronological age affects the efficiency of CSCs, and, the deterioration is accelerated in hypertensive heart disease. Decline in stem cell attributes, initiated as early as the stable phase of hypertrophy can possibly mediate the transition from compensatory hypertrophy to cardiac failure. Supplementation with the antioxidant tempol restored the stem cell efficiency, highlighting the requirement for maintaining a conducive microenvironment for prevention of progressive remodeling. The observations of this study substantiate the role played by the extracellular milieu in determining the efficiency of cardiac stem cells and the requirement for maintaining an ideal microenvironment for keeping the heart young and healthy.

VI.3. Future Directions

1) Elucidate the signaling pathways mediating cardiac stem cell aging

2) Long term pre-clinical studies for confirming the positive response to antioxidants

3) Clinical correlation of experimental observations

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VIII. PUBLICATION

Publication

"Modulation of cardiac stem cell characteristics by metoprolol in hypertensive heart disease". **Sherin Saheera**, Ajay Godwin Potnuri, Renuka R Nair (Accepted- **Hypertension Research, In Press**).

Conference Proceedings

Oral Presentations

1. Sherin S, Renuka R Nair. Age Associated Decline in Cardiac Stem Cell Efficiency: A Possible Cause for Adverse Cardiac Remodelling. National seminar on Frontiers in Biotechnology, from 3rd - 5th March, 2017 at Inter University Centre for Genomics and Gene Technology, University of Kerala.
2. Sherin S, Renuka R Nair. Supplementation with antioxidants preserves stem cell attributes in Spontaneously Hypertensive rat. National seminar on Recent Biochemical Approaches in Therapeutics, RBAT-III (2017) from 15th - 17th February, 2017 at Department of Biochemistry, University of Kerala and **received the Prof. P.A.Kurup Memorial Award.**

Poster Presentations

3. Sherin S, Renuka R Nair. Decrease in efficacy of resident cardiac stem cells mediate age associated cardiac remodeling. Indo-Caladium symposium on heart failure- progress and prospects from 14th - 15th March, 2015 at Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram.
4. Sherin S, Renuka R Nair. Age Associated Decline in Resident Cardiac Stem Cell Efficacy – A Possible Cause for Adverse Cardiac Remodeling. Indo-French 'Women in Science' through CEFIPRA from 3rd - 5th February 2015, at Indian Institute of Science (IISc), Bangalore.

IX. APPENDICES

Appendix I

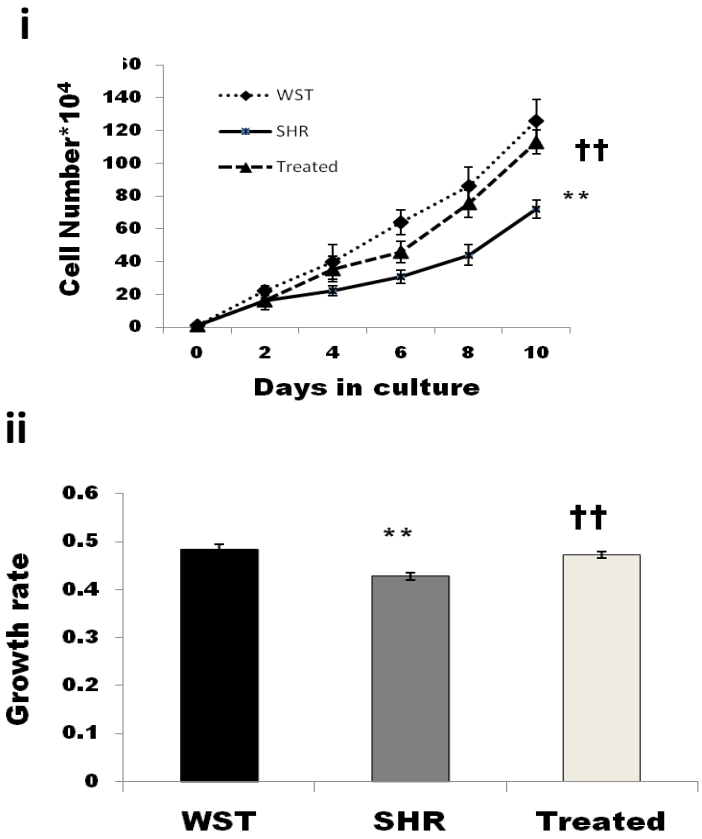
Effect of cardioprotective antihypertensive, metoprolol, on Cardiac Stem Cells

Background: Based on the assumption that stem cell function is compromised in hypertensive heart disease, the study was carried out to evaluate the efficiency of an antihypertensive metoprolol in the restoration of stem cell attributes in Spontaneously Hypertensive Rat. The β-blocker metoprolol is known to have antioxidant property and promote regression of hypertrophy.

Methods: Twelve adult male SHR were randomly assigned to two groups of six rats each. Untreated SHR served as hypertensive control and the other group received a daily oral dose of 50 mg.kg-1.day-1 of Metoprolol tartrate for 2 months. Sex and age-matched Wistar rat served as normotensive control. CSCs were isolated from atrial explant cultures and assessed for growth kinetics, migration, intracellular ROS and senescence.

Results: Treatment with metoprolol stimulated the proliferation of CSCs and the cell yield was comparable with that of WST (Fig. AIa i). Growth rate and population doubling time, both of which were negatively affected in SHR, was restored by the β-blocker treatment (Fig. AIa ii, iii). Metoprolol enhanced the migration potential of CSCs (Fig. AIb i) and decreased the intracellular ROS levels, comparable to that of normotensive rat (Fig. AIb ii). The number of senescent cells were significantly reduced (Fig. AIb iii).

Conclusion: Metoprolol plays a significant role in improving stem cell characteristics in addition to being a potent anti hypertensive and cardioprotective drug. Hence, the beneficial role of metoprolol in preventing progressive pathological remodeling can be attributed to the improvement in efficiency of cardiac stem cells.



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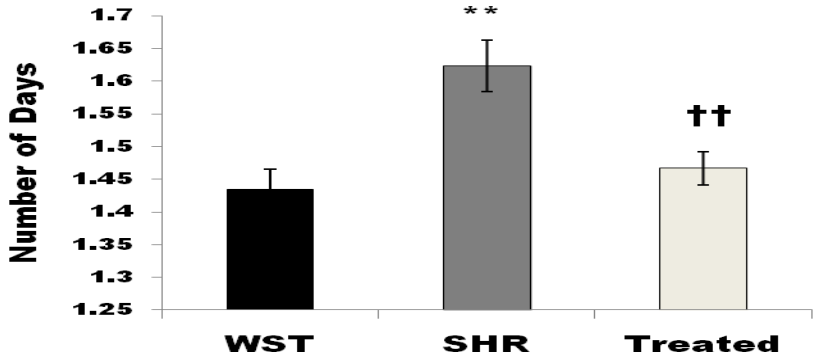
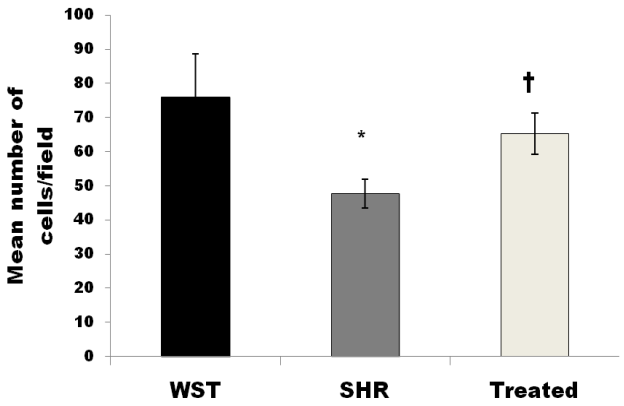


Figure AI a. Effect of metoprolol treatment on growth kinetics, growth rate and population doubling time of CSCs. (i) Growth kinetics of CSCs represented as cell number*10^4, (ii.) Age associated variation in growth rate of CSCs (iii) Age associated variation in Population doubling time (PDT) of CSCs represented as number of days. Data is represented as mean ± SD and $p<0.05$ was considered to be significant. Variation was analysed by one way ANOVA followed by student t-test. (**$p<0.01$ SHR Vs WST; †† $p<0.01$ SHR Vs Treated) One way ANOVA $p<0.01$ (n=6).

i



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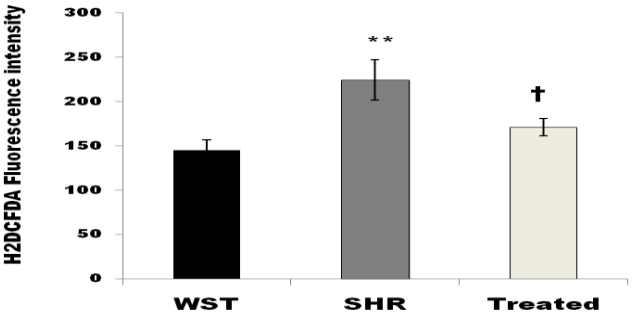

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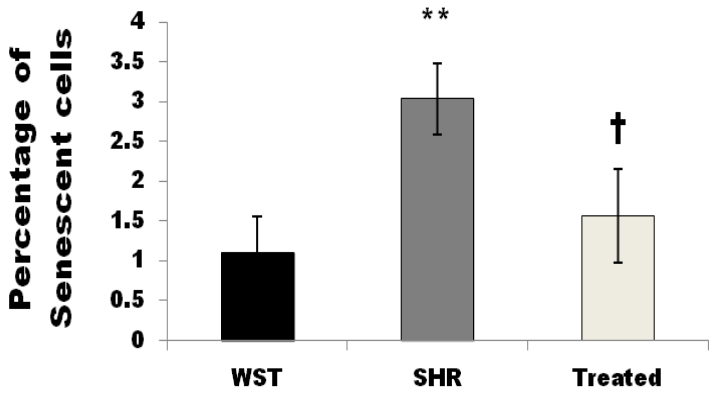

Figure AI b. Effect of metoprolol treatment on migration, intracellular ROS levels and senescence of CSCs. (i) Migration ability represented as mean number of cells/field (ii) Intracellular ROS levels in CSCs represented as H2DCFDA fluorescence intensity (iii) Proportion of senescent cell represented as percentages. Data is represented as mean ± SD and $p<0.05$ was considered to be significant. Variation was analysed by one way ANOVA followed by student t-test. (**$p<0.01$ SHR Vs WST; † $p<0.05$ SHR Vs Treated) One way ANOVA $p<0.01$ (n=6).

Appendix-II

Work done as part of Newton-Bhabha PhD Placement Programme-2016 at Imperial College, London

Investigate the effect of miRNAs implicated in Takotsubo Cardiomyopathy on induced Pluripotent Stem Cell derived cardiomyocytes

Background: Takotsubo Cardiomyopathy (TCM) is an endogenous catecholamine induced myocardial stunning and microinfarction. Physiological or physical stress is the major reason for TCM and epinephrine plays the central role in precipitating the disease. TCM is characterized by hypercontractile base relative to hypocontractile or akinetic apex leading to apical ballooning. This typical morphology of TCM got it the name "Octopus Pot". Two miRNAs, miR-16 and miR-26a were found to be upregulated in TCM and this distinguishes it from myocardial infarction. The study was aimed at investigating the effect of these miRNAs in cardiomyocytes derived from human induced pluripotent stem cells (iPSCs).

Objective: To transfect human induced pluripotent stem cell derived cardiomyocytes with miRNAs, and to test the hypothesis that this results in altered β2-adrenoceptor function.

Materials and Methods: The iPSCs were differentiated into cardiomyocytes following a standard protocol. The beating cardiomyocytes were dissociated and used for further experiments when they were around 30 days old. To standardize the transfection reagent, transfection efficiency and Live/dead assay was performed for Mirrus siRNA Quest, Lifofectamine RNAimax and Lipofectamine 3000. Following transfection with the miRs for 48hrs, the iPSC derived cardiomyocytes were subjected to IonOptix to record the chronotropic changes in contractility. Data were collected and the rate of contraction was recorded for the time periods required for plotting the graphs.

Results: Out of three transfection reagents used, LF 3000 was found to exhibit higher percentage of transfection efficiency and lesser number of dead cells (Fig. AIIa i, ii). On transfection with the miRs, the iPSC derived cardiomyocytes did not exhibit any significant change in the beating patterns as compared with the control (Fig. AIIb i-iv).

Conclusion: The chronotropic response to catecholamines was unaltered even after transfection with the miR-16 and miR-26a in the human iPSC derived cardiomyocytes. miR-16 and miR-26a modulation therefore does not affect β_2AR response in iPSC-CMs.

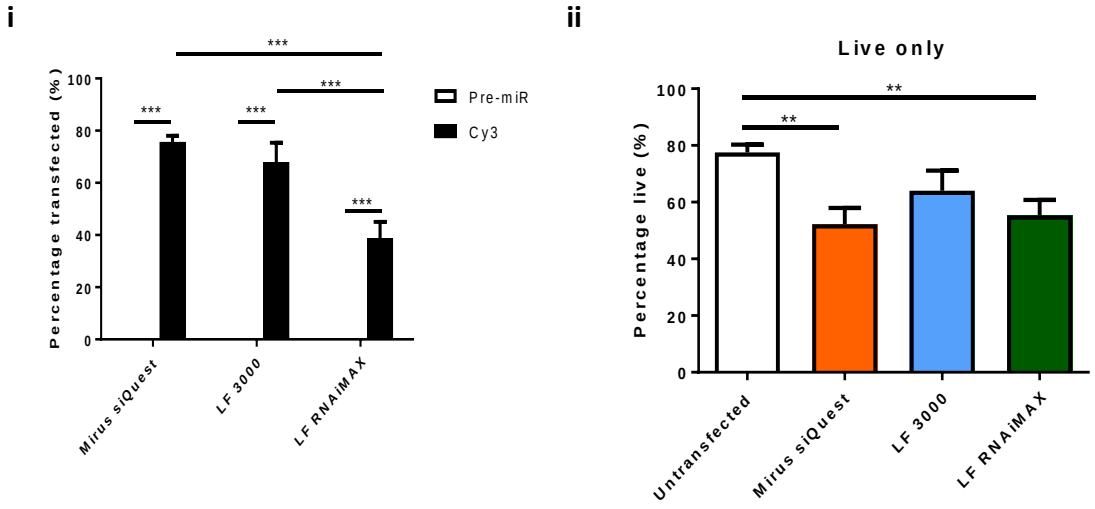

Figure A II a. Standardisation of the Transfection reagent (i) Transfection efficiency experiment (ii) Live/dead assay. Data presented as mean±SD (n=6). **p<0.01 and ***p<0.001 Control Vs Transfection reagent

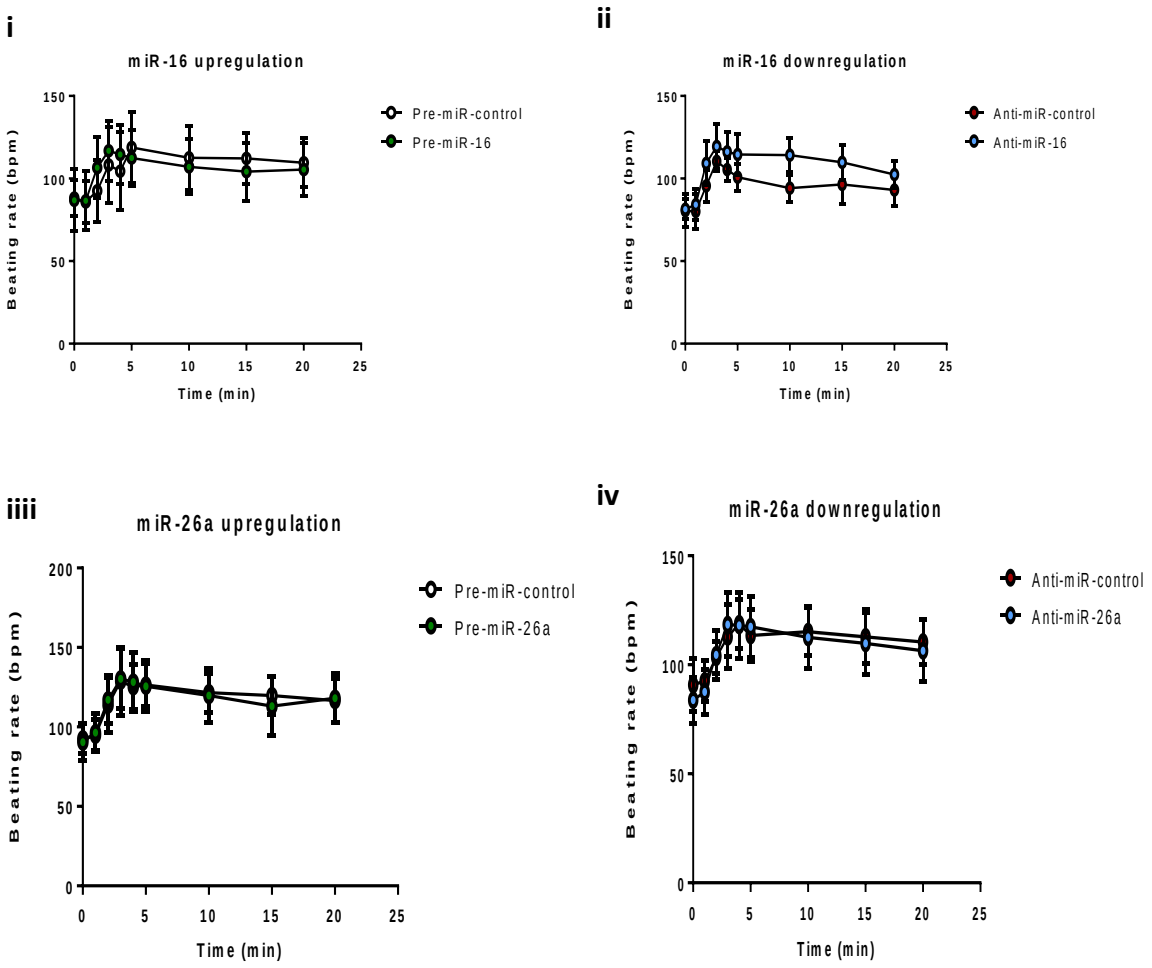

Figure AII b i-iv: IonOptix data for the chronotropic response of iPSC-CMs to up-regulation and down-regulation of miR-16 and miR-26a. Data presented as mean±SD. (n=7)