METABOLIC MODULATION BY MEDIUM CHAIN TRIGLYCERIDES PREVENTS CARDIAC REMODELING IN SPONTANEOUSLY HYPERTENSIVE RAT

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A THESIS PRESENTED BY

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CERTIFICATE

I**, Saifudeen Ismael,** hereby certify that I had personally carried out the work depicted in the thesis entitled, **"Metabolic modulation by medium chain triglycerides prevents cardiac remodeling in Spontaneously Hypertensive Rat"** 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.

Date **Saifudeen Ismael**

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This is to certify that Saifudeen Ismael, 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 **"Metabolic modulation by medium chain triglycerides prevents cardiac remodeling in Spontaneously Hypertensive Rat"** 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

METABOLIC MODULATION BY MEDIUM CHAIN TRIGLYCERIDES PREVENTS CARDIAC REMODELING IN SPONTANEOUSLY HYPERTENSIVE RAT

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ABBREVIATIONS

ACC	Acetyl-CoA carboxylase
AMP	Adenosine Monophosphate
AMPK	AMP activated protein kinase
Ang II	Angiotensin II
ANOVA	Analysis of Varience
ANP	Atrial Natriuretic Peptide
AP-1	Activated protein -1
BNP	Brain Natriuretic Peptide
BSA	Bovine serum albumin
CD36	Cluster of differentation 36
COUP-TF	Chicken ovalbumin upstream promoter transcription factor
CPCSEA	Committee for the Purpose of Control and Supervision of Experiments in Animals
CPT-1	Carnitine Palmitoyl Transferse -1
DEPC	Diethyl pyrocarbonate
DNPH	2,4-dinitrophenylhydrazine
ERK-MAPK	Extracellular signal-regulated kinases- Mitogen activated protein kinase
ERR	Estrogen-related receptor
ET-1	Endothelin1
FABP	Fatty acid binding protein
FACS	fatty acyl-CoA synthase
$FADH_2$	flavin adenine dinucleotide
FAO	Fatty acod oxidation
GLP-1	Glucagon-like peptide-1
GLUT	Glucose transporter
GPCR	G protein coupled receptor
GSK3β	Glycogen synthase kinase-3β

HCM	Hypertrophic cardiomyopathy
HDL-C	High density lipoprotein cholesterol
HIFα	hypoxia-inducible transcription factors-α
HRP	Horse raddish peroxidase
IGF-1	Insulin like growth factor-1
JNK-MAPK	Jun amino-terminal kinases Mitogen activated protein kinase
LCFA	Long chain fatty acids
LDH	Lactate dehydrogenase
LDL-C	Low density lipoprotein cholesterol
LVH	Left ventricular hyertrophy
LXR	Liver X receptors
MCAD	Medium chain acylCoA dehydrogenase
mCAT	mitochondrial targeted Catalase
MCFA	Medium chain fatty acid
MCT	Medium chain triglyceride
MDA	Malonedialdehyde
MEF-2	Myocyte enhancer factor-2
MHC	Myosine heavy chain
MMLV RT	Moloney Murine Leukemia Virus Reverse Transcriptase
MMPs	Matrix metalloproteinases
NADPH	Nicotinamide adenine dinucleotide phosphate
$NADH+H^+$	Nicotinamide adenine dinucleotide
NFAT	Nuclear factor of activated T cells
NF-*k*B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NRRE-1	Nuclear receptor response element-1
PCr	Phosphocreatine
PDH	Pyruvate dehydrogenase
PDK	Pyruvate dehydrogenase Kinase
PET	Positron emission tomography

PFK-1	Phosphofructokinase-1
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PI3K	Phosphoinositide 3-kinase
PKA	Protein Kinase A
PKC	Protein Kinase C
PKD	Protein Kinase D
PPARα	Peroxisome proliferator-activated receptor α
ROS	Reactive oxygen species
RXR	Retinoid X receptor
SDS	Sodium dodecyl sulphate
SHR	Spontaneously hypertensive rat
SRF	Diethyl pyrocarbonate
TBARS	Thiobarbituric acid reactive substances
TCA	Tricloro acetic acid
TEMED	Tetramethylethylenediamine
TIMPs	Tissue inhibitors of metalloproteinases
VLCAD	Very long chain acyl CoA dehydrogenase

SYNOPSIS

Left ventricular hypertrophy (LVH) is an adaptive mechanism triggered in the heart in response to pathological stimuli such as hypertension or aortic stenosis and is regarded as the major risk factor for adverse cardiovascular events and sudden death. Attenuation of LVH is associated with better maintenance of cardiac function and prevention of adverse cardiac remodeling. Two factors intimately linked with pathological hypertrophy are oxidative stress and change in cardiac metabolic profile. Oxidation of long-chain fatty acids are the major metabolic fuel for the healthy heart. In pathological cardiac hypertrophy, there is a decrease in the oxidation of long chain fatty acids and increased relative contribution of glucose for energy production, co-ordinated by down regulation of fatty acid oxidizing enzymes, consequent to diminished activity of Peroxisome proliferator-activated receptor–alpha (PPAR-α). PPAR-α is a ligand activated transcription factor that regulates the expression of genes involved in the fatty acid oxidation. Long chain fatty acids are the natural ligands of PPAR-α. The availability of the long chain fatty acids is limited when the sarcolemmal fatty acid transporter CD36 is defective. In patients with hypertrophic cardiomyopathy, defective CD36 results in the decreased PPAR-α activity. The transporter is also defective in Spontaneously hypertensive rat (SHR). Several studies have reported the association between reduced rate of fatty acid oxidation and development of cardiac hypertrophy. Hence it is anticipated that, restoration of fatty acid metabolism will help to maintain the metabolic status and prevent progressive cardiac remodeling. Medium chain triglycerides (MCT) have the capacity to bypass CD36 and serve as substrate for fatty acid oxidation.

Medium chain triglycerides are triglycerides of saturated fatty acids of chain length 8-12 carbon atoms, and their oxidation is independent of cytoplasmic and mitochondrial fatty acid transport systems such as CD36 and Carnitine palmitoyl transferase-1 (CPT-1). Hence, they are regarded as direct fuel for β-oxidation. Several studies have reported the beneficial effect of MCT on heart. Inclusion of MCT is reported to improve contractile function of the hypertrophic heart and its consumption is not associated with the development of coronary artery diseases. Hence, the study was based on the hypothesis that *stimulation* of *fatty acid metabolism by supplementation of medium chain triglyceride can prevent and reverse adverse cardiac remodeling in Spontaneously hypertensive rat.*

The study was designed with following objectives:

- Validate the suitability of the experimental model by screening for markers of cardiac hypertrophy, metabolic shift and oxidative stress in spontaneously hypertensive rat (SHR) in comparison with normotensive wistar rat (W)
- Compare the cardiac response to stimulation of fatty acid oxidation by supplementation with odd and even chain MCT
- Evaluate the effect of supplementation with MCT on cardiac remodeling and oxidative stress at the initial and established stages of cardiac hypertrophy

Spontaneously hypertensive rat (SHR), was used as the experimental model to investigate cardiovascular response to metabolic stimulation by MCT. The SHR replicates the clinical progression of hypertension in humans; wherein early development of hypertension is followed by a long stable period of compensated cardiac hypertrophy that slowly progresses to heart failure. Before initiation of experimental studies, it was confirmed that the stock of SHR available in the laboratory carries the essential features of hypertrophy and metabolic alteration. Adult SHR (6 months old) were compared with age and sex matched Wistar rat (W). Hypertrophy was quantified from hypertrophy index [Heart weight / body weight ratio (mg/g)], myocyte cross sectional area, myocardial fibrosis, mRNA expression of Brain natriureic peptide (BNP) and expression of Calcineurin A. Myocardial malonedialadehyde, protein carbonyl and 3-nitro tyrosine were used as the markers for myocardial oxidative stress. Metabolic remodeling was assessed from mRNA expression of PPARα, medium chain acylCoA dehydrogenase (MCAD) and phophofructokinase-1 (PFK-1).

MCT is available in two different forms, odd and even chain MCT based on the number of carbons. Odd chain MCT is reported to be anaplerotic in nature. Anaplerosis is the re-filling of the catalytic intermediates of the Citric acid cycle. Therefore, there is possibility of difference in their metabolic properties. A pilot study was therefore carried out to identify the type of triglyceride that induces better cardiac response. To select the ideal MCT 2-month-old SHR were supplemented with 5% (v/w) Ticaprylin or

Triheptanoate of the total feed for 2 months. Morphological and histological markers of cardiac hypertrophy and markers of oxidative stress were analyzed.

Experimental studies were carried out in SHR to investigate the cardiovascular response to metabolic stimulation by MCT supplementation. Animals at initial (2months old) and established (6 months old) stages of cardiac hypertrophy were treated with 5% MCT along with standard feed for 4 months and its cardiovascular response was analyzed from morphological histological and molecular markers. Effect of MCT on metabolic modulation was assessed from the expression of Peroxisome proliferator activated receptor (PPAR)-α, medium chain acylCoA dehydrogenase (MCAD) and phosphofructokinase-1 (PFK-1) mRNA by Real Time PCR. Hypertrophy was quantified from hypertrophy index [Heart weight / body weight ratio (mg/g)], myocyte cross sectional area, myocardial fibrosis, mRNA expression of Brain natriureic peptide (BNP) and expression of Calcineurin A. Myocardial malonedialadehyde, protein carbonyl and 3-nitrotyrosine were used as the markers for myocardial oxidative stress. Cardiac energy level was assessed by biochemical estimation of adenosine triphosphate (ATP) and phosphocreatine. Blood pressure was measured noninvasively. Commercially available kits were used for determination of lipid profile.

Comparison of the markers of cardiac hypertrophy, metabolic shift and oxidative stress in spontaneously hypertensive rat (SHR) with normotensive Wistar rat (W):

The study has confirmed that Blood pressure of SHR was significantly higher than that of Wistar. Morphological, histological and molecular markers confirmed the presence of LVH in SHR. A shift in substrate preference away from fatty acid oxidation towards glucose was seen as assessed from the expression of PPARα, MCAD and PFK-1. The decrease in fatty acid oxidation is possibly caused by non-availability of substrate due to down regulation of the fatty acid transporter CD-36. Hypertrophic response in SHR is associated with increased oxidative stress.

Cardiac response to supplementation with Odd chain and Even chain triglycerides:

Even chain and odd chain triglycerides have shown variable cardiac response. Although both sources of MCT decreased myocardial oxidative stress, prevention of hypertrophic response was relativel better with even chain MCT. Hence tricaprylin was used as the source of MCT for further experimental studies. Both treatments did not affect body weight and lipid profile.

Stimulation fatty acid oxidation in initial and established stages of cardiac hypertrophy:

Supplementation of MCT stimulated fatty acid metabolism in the initial and established stages of cardiac hypertrophy in SHR. Stimulation of fatty acid metabolism prevented

progressive cardiac remodeling independent of blood pressure. MCT modulated cardiac oxidative stress. The reduction of cardiac hypertrophy is possibly associated with decrease in oxidative stress. The study shows that stimulation of fatty acid metabolism by supplementation of MCT under hypertrophic condition is beneficial to the heart. The treatment did not induce dislipidemia or obesity.

The above observations lead to the conclusion that, contrary to the belief that stimulation of Fatty acid oxidation can be detrimental in the presence of hypertrophy, this study has shown that, restoration of the metabolic profile is beneficial to the heart. Supplementation of medium chain triglycerides prevented progressive cardiac remodeling in hypertrophic heart, possibly mediated by reduction of oxidative stress. As hypertension is associated with consequences other than hypertrophy, MCT can be used only as a supplement along with antihypertensives. Preclinical studies will help to determine the therapeutic implications of supplementation with MCT in patients with hypertensive heart disease.

I. INTRODUCTION

Left ventricular hypertrophy is an adaptive mechanism triggered in the heart in response to pathological stimuli such as hypertension or aortic stenosis and is regarded as the major risk factor for adverse cardiovascular events and sudden death. Attenuation of LVH is associated with maintenance of cardiac function and reduction in cardiac injury. Oxidation of long-chain fatty acids forms the major metabolic fuel for the healthy heart and supplies 60-70% of myocardial ATP. The remaining energy comes from glucose and lactate oxidation. In pathological cardiac hypertrophy, there is a decrease in the oxidation of long chain fatty acids and increased relative contribution of glucose for the energy maintenance. The decrease in the metabolic substrate preference for fatty acid is possibly caused by reduction in the expression of Cluster of Differentation-36 (CD36), a major sarcolemmal fatty acid transporter, that limits the availability of long chain fatty acids. A number of reports testify the role of CD36 deficiency in the metabolic alteration and the consequent cardiac remodeling. Tanaka et al reported the direct association between CD36 deficiency and development of hypertrophic cardiomyopathy in the Japanese population(Tanaka et al., 1997). Pharmacological inhibition of CD36 was associated with development of cardiac hypertrophy independent of blood pressure in rodents (Kusaka et al., 1995). Defective CD36 in spontaneously hypertensive rat (SHR) is identified as the primary determinant of altered myocardial metabolism and myocardial hypertrophy (Hajri et al., 2001). Deficiency of CD36 has been reported recently by Magida *et al* in mouse models with familial hypertrophic cardiomyopathy (Magida and Leinwand, 2014). These studies highlight the concept that decreased fatty acid oxidation is secondary to reduced CD36 expression in cardiomyocytes. As chronic

reduction in fatty acid availability can lead to pathological remodeling, it is presumed that restoration of fatty acid oxidation will help to maintain the metabolic status and prevent progressive cardiac remodeling.

Although enhanced glucose metabolism is more economical in terms of oxygen cost, chronic reliance on glucose metabolism is inefficient for maintaining contractile function under work overload. (Ingwall, 2009) Nevertheless, substantially enhanced insulin independent glucose utilization by GLUT-1 overexpression prevented the progression of cardiac dysfunction during hypertrophy (Liao et al., 2002a). However such enhanced glucose utilization by the approach of genetic modification is of less clinical relevance. Kolwicz *et al* reported that sustenance of myocardial fatty acid metabolism preserved myocardial energetics and prevented the development of cardiac hypertrophy under pressure overload, showing that that maintenance of inherent cardiac metabolic profile is beneficial under pathological conditions (Kolwicz et al., 2012). Similarly, studies have reported that high dietary fat could activate mitochondrial oxidative metabolism and alleviate adverse cardiac remodeling associated with development of obesity (Chess et al., 2008, 2009; Duda et al., 2008; Okere et al., 2005). The therapeutic strategies aimed at reduction of hemodynamic overload does not essentially modulate myocardial metabolism or regress cardiac remodeling. Hence, the study was designed to evaluate cardiac response to metabolic modulation by supplementation of Medium chain triglyceride (MCT) at the initial and established stages of cardiac hypertrophy

Medium chain triglycerides (MCT) have the capacity to bypass CD36 and serve as substrate for fatty acid oxidation. Medium chain triglycerides are triglycerides of saturated fatty acids of chain length 8-12 carbon atoms, and their oxidation is independent of cytoplasmic and mitochondrial fatty acid transport systems such as CD36 and Carnitine palmitoyl transferase-1 (CPT-1). Hence, they are regarded as direct fuel for β-oxidation. Several studies have reported the beneficial effect of MCT on heart. Inclusion of MCT is reported to improve contractile function of the hypertrophic heart and its consumption is not associated with the development of coronary artery diseases. Therefore, this study was based on the hypothesis that, "*Reactivation of fatty acid metabolism by supplementation of medium chain triglyceride can prevent and reverse adverse cardiac remodeling."* The study was carried out in spontaneously hypertensive rat, an animal model of chronic pressure overload induced left ventricular hypertrophy. The cardiac response to stimulation of fatty acid metabolism was examined in animals of two different age groups, as metabolic modulation using pharmacological ligands had yielded positive results in young SHR but not in older animals. (Purushothaman et al., 2011b). Further, the studies that reported positive response to MCT was carried out either in young SHR or surgical models.

MCT is available in two different forms, odd and even chain MCT based on number of carbons. Odd chain MCT is reported to be anaplerotic in nature. Anaplerosis is the re-filling of the catalytic intermediates of the Citric acid cycle. Therefore, there is the possibility of difference in their metabolic properties. A pilot study was therefore carried

out to identify the type of triglyceride that can induce a comparatively better cardiac response, and then use that for further experiments. This study was carried out with the following objectives

i. *Validate the suitability of the experimental model by screening for markers of cardiac hypertrophy, metabolic shift and oxidative stress in spontaneously hypertensive rat (SHR) in comparison with normotensive Wistar rat (W)*

ii. *Compare the cardiac response to stimulation of fatty acid oxidation by supplementation with odd and even chain MCT*

iii. *Assess cardiac response to stimulation of fatty acid oxidation by supplementation of medium chain triglycerides at the initial and established stages of cardiac hypertrophy*

SHR in the initial and established stages of cardiac hypertrophy were supplemented with 5% MCT (v/w) along with standard feed for 4 months and its cardiovascular response was analyzed for morphological, histological and molecular markers. Effect of MCT on metabolic modulation was assessed from the expression of Peroxisome proliferator-activated receptor (PPAR)-α, medium chain acylCoA dehydrogenase (MCAD) and phosphofructokinase-1 (PFK-1) mRNA by Real Time PCR. Evaluation of cardiac remodeling was based on morphological, histological and molecular markers of cardiac hypertrophy. Myocardial malonedialadehyde, protein carbonyl and 3-

nitrotyrosine were used as the markers for myocardial oxidative stress. Cardiac energy level was assessed by biochemical estimation of adenosine triphosphate (ATP) and phosphocreatine. Blood pressure was measured noninvasively. Commercially available kits were used for determination of lipid profile.

A brief description of the morphological and molecular changes associated with cardiac hypertrophy and the energy metabolism of the normal and hypertrophied heart are given in the next chapter. Literature on the role of CD36 in the development of cardiac hypertrophy and strategies adopted for modulation of energy metabolism in cardiac hypertrophy and the consequence are also reviewed.

The design of study and experimental methodology are given in the third chapter,

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

Salient observations of the study are listed in the fifth chapter. The conclusion and scope for further studies are also given.

The references cited in the text are listed in ‘Bibliography’.

II. REVIEW OF LITERATURE

II.1. CARDIAC HYPERTROPHY

The heart is a dynamic organ capable of self regulation and adaption in response to alterations in workload associated with developmental, physiological and pathological stimuli. As the postnatal growth of the heart is closely matched to its functional load, heart muscle enlarges its size and mass to counterbalance the chronic increase in wall stress resulting in cardiac hypertrophy. Cardiac hypertrophy is defined as the enlargement or overgrowth of cardiac mass due to an increase in size of its terminally differentiated myocytes and can broadly be classified as either pathological or physiological depending on the nature of the stimulus and the phenotype.

II.1.1. Pathological cardiac hypertrophy

Pathological cardiac hypertrophy is considered as an intermediate stage in the progression to heart failure. The heart undergoes remodeling in response to multitude of stimuli such as hemodynamic overload, neurohormonal activation and sarcomeric gene mutation. In response to hormonal, genetic and mechanical stimuli, ventricular myocytes hypertrophy by increasing the number of sarcomere units per cell without increase in cell number. Although the stimuli are different, the molecular and cellular events converge into a process called cardiac hypertrophy which is regarded as a compensatory phase (Kehat and Molkentin, 2010). According to Law of Laplace, under chronic hemodynamic overload, the heart tends to increase its wall thickness to diminish wall stress; and maintenance of cardiac output forms the basis for the adaptive nature of the cardiac growth. (Vidt and Prisant, 2005). Sustained hypertrophy is maladaptive and is an

independent risk factor for myocardial infarction, arrhythmia and sudden death (Nagendran et al., 2013). Regression of cardiac hypertrophy reduces the risk of heart failure and ventricular dysfunction independent of the treatment adopted (Esposito et al., 2002; Frey et al., 2004; Yusuf et al., 2000). The defining features of cardiac hypertrophy are an increase in cardiomyocyte size, enhanced protein synthesis, and reinduction of fetal cardiac gene programme. It is also associated with overproduction of reactive oxygen species (ROS) and reactivation of fetal cardiac metabolism: a shift in substrate preference from fatty acid to glucose (Barger et al., 2000; Takimoto and Kass, 2007).

Hypertension is the common cause for the development of left ventricular hypertrophy (LVH), where the heart must work harder to counterbalance the chronic increase in wall stress. As a result, the muscle cells within the heart enlarge leading to an increase in size and mass. However, prolonged wall stress results in ventricular dilation, contractile dysfunction and eventually leads to heart failure. LVH is also induced by various neurohormones such as angiotensin II, aldosterone, norepinephrine and insulin, independent of pressure overload (Db and Lj, 1996). In the 1960s, Meerson and colleagues classified development of pathological hypertrophy into different stages. The developing phase of hypertrophy, with ventricular hyperfunction where work load exceeds the work output is followed by a compensatory phase, where increased workload is compensated by the cardiac growth with maintenance of resting cardiac output. Finally, the decompensation phase with deterioration of cardiac output and filling

progressively leads to heart failure (Frey et al., 2004).The duration and progression of these stages depend on the magnitude and type of overload.

Pathological cardiac hypertrophy is accompanied with molecular changes that are observed during fetal cardiac development, such as reactivation of fetal gene program. Fetal recapitulation includes stimulation of atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP) and genes for fetal isoforms of contractile proteins, such as skeletal α-actin and β-myosin heavy chain (MHC). This fetal recapitulation is accompanied by downregulation of adult genes, such as α-MHC and the calcium-handling protein sarco(endo)plasmic reticulum Ca^{2+}-ATPase (Zhao et al., 2004). Heart parallely shifts its substrate preference from fatty acid towards glucose under cardiac hypertrophy, the hallmark of fetal cardiac metabolism (Huss and Kelly, 2005). The morphological, histological and biochemical changes associated with hypertrophy are illustrated in figure.1. The reactivation of fetal gene program is mediated by various transcription factors (TFs) such as NFAT, NF-*k*B, MEF2, GATA4, and SRF which play a prominent role in embryonic development. (Akazawa and Komuro, 2003; Oka et al., 2007). Aberrant expression of genes involved in fetal contractile proteins and cardiac metabolism leads to progressive myocardial dysfunction and irreversible pathogenesis. Decreased expression of fetal genes is accompanied by improvement of ventricular function. Hence, they can be used as the markers of adverse cardiac remodeling. The reactivation of fetal gene expression in the adult myocardium is mediated by the

combined action of transcription factors, chromatin remodeling and post transcriptional regulation at different levels of gene expression (Dirkx et al., 2013).

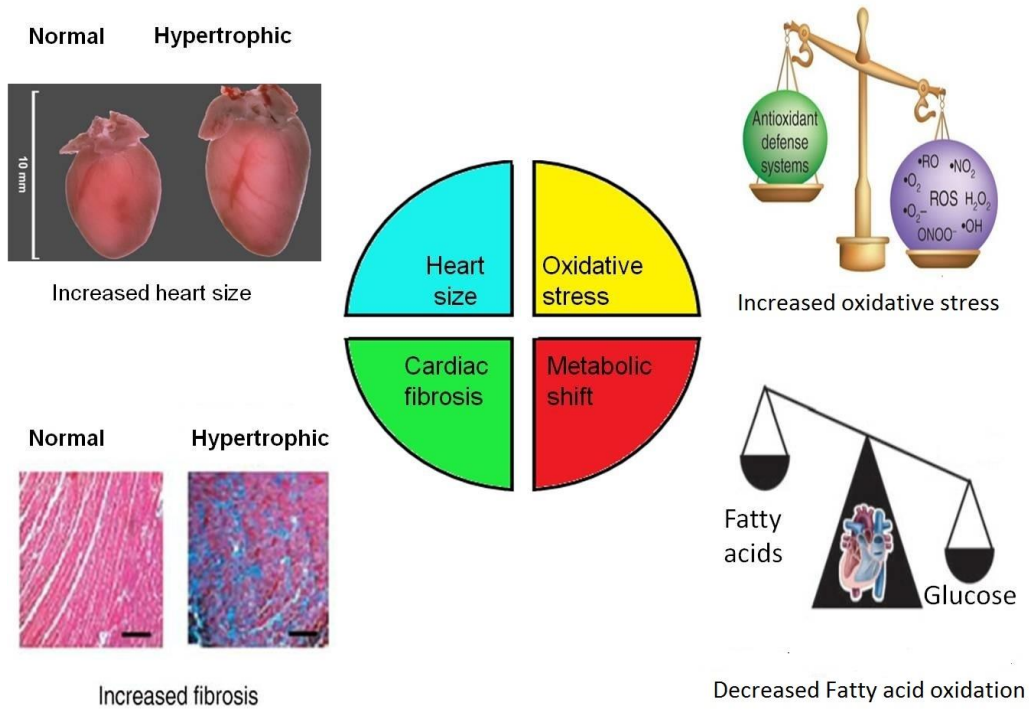

Figure 1: Charecteristic features of pathological cardiac hypertrophy

During cardiac hypertrophy, the myocardium undergoes structural remodeling to accommodate the hypertrophied myocytes by modulating extracellular matrix and myocardial capillary network. The extracellular matrix is principally composed of collagen with smaller amounts of elastin, laminin, and fibronectin. Although collagen types I, III, and V coexist in the myocardium, type I forms the major component (85% of

the total collagen). The extracellular fibrillar collagen provides structural integrity to adjoining myocytes, and aids myocyte contraction that translates into efficient cardiac contraction (Barsotti et al., 1993; Weber et al., 1994). Pathological LVH 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. Hypertensive cardiac remodeling involves initial deposition of type-III collagen, followed by type-I collagen. Disproportionate accumulation of collagen increases ventricular stiffnesss, contractile uncoupling, altered microcirculation and electrical uncoupling resulting in impaired cardiac function and adverse cardiovascular events. The augmented cardiac fibrosis and reduced capillary density increases the oxygen diffusion distance resulting in myocardial ischemia, and is likely to contribute to the progressive transition to heart failure (Gradman and Alfayoumi, 2006).

Based on the phenotype of the heart and the initiating stimuli, cardiac hypertrophy has classically been subdivided as concentric or eccentric (Kehat and Molkentin, 2010) (Fig.2). Concentric hypertrophy refers to an increase in relative wall thickness and cardiac mass, with a small reduction in chamber volume. Pathological stimuli such as hypertension and aortic stenosis produces systolic wall stress and results in concentric cardiac hypertrophy. In contrast, in eccentric hypertrophy the increase in cardiac mass is due to increased chamber volume. The stimuli such as aortic regurgitation and

arteriovenous fistula (volume overload) cause diastolic wall stress and leads to eccentric cardiac hypertrophy. At cellular level, concentric hypertrophy is characterized by parallel addition of sarcomeres leading to an increase in width of individual myocytes, where as 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 as it progresses to ventricular dilation and systolic failure (Berenji et al., 2005).

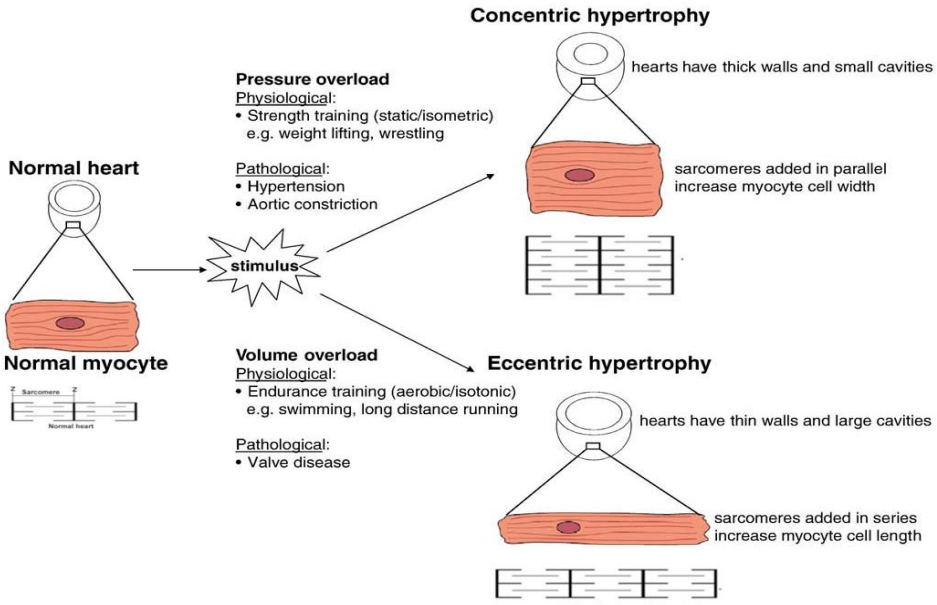

Figure 2: Classification of cardiac hypertrophy based on the phenotype of the heart and individual myocytes. *(adapted and modified from Bernardo* et al *2010)*

II.1.2. Physiological Cardiac hypertrophy

Physiological cardiac hypertrophy in contrast to pathological hypertrophy is advantageous and is distinct from the latter hypertrophy at the molecular, cellular and functional levels. Physiological hypertrophy develops in response to chronic exercise training with preserved or enhanced cardiac function, and does not lead to failure (Fagard, 1997). It is characterized by normal or enhanced cardiac function, improved cardiac metabolism, and absence of pathological features such as fibrosis and reactivation of fetal genes with enhanced fatty acid and glucose oxidation (McMullen and Jennings, 2007). Physiological cardiac hypertrophy is also observed during post natal growth, endurance exercise training and pregnancy and is reversible once the stimuli have been relieved. The mechanism by which the heart adapts differently to physiological and pathological stimuli remains mysterious. One school of thought is that most pathological stimuli are chronic while physiological stimuli are intermittent in nature, presuming that the duration of the stimulus determines the phenotype of the heart. Perrino *et al* applied pathological stimuli for intermittent periods to evaluate the role of duration of stimuli on this divergent cardiac phenotype, simulating the duration of exercise training (Perrino et al., 2006). Intermittent pathological stress induced cardiac abnormalities with diastolic dysfunction and vascular rarefaction. They demonstrated that the duration of stress determines the degree of cardiac hypertrophy whereas nature of the stress determines the cardiac phenotype whether it is physiological or pathological. The development of athletic heart is a beneficial adaptive response with decreased resting and submaximal heart rates and increased filling time and venous

return. These adaptations can facilitate the myocardium to satisfy the increased demands of exercise while maintaining or enhancing normal function (Iemitsu et al., 2001; Richey and Brown, 1998; Shapiro and Smith, 1983).

II.1.2.1. *Athletes heart and sudden cardiac death*

The athlete's heart has generally been defined as a benign increase in heart mass, representing a physiological adaptation to chronic exercise training. There are several media reports on increased incidence of sudden death in elite athletes due to cardiovascular abnormalities. United states based 12 year survey in high-school athletes reported that the frequency of sudden death is considerably low (1:200,000 per year) (Maron et al., 2009). Large autopsy-based studies revealed that, hypertrophic cardiomyopathy accounts for one-third of sudden death in US followed by congenital coronary artery anomalies and congenital malformations (Maron, 2003). To address the clinical consequence of cardiac remodeling in trained athletes, a study conducted in young Olympic athletes reported that extreme and uninterrupted endurance training over long periods of time (up to 17 years) does not induce cardiac structural and functional abnormalities or occurrence of cardiovascular events (Pelliccia et al., 2010). Thus, it is generally considered that cardiac hypertrophy in response to exercise training itself is not the cause for sudden death in elite athletes. However, a complete understanding of cellular check points that distinguishes physiological from pathological cardiac remodeling is critical for pharmacological targeting for prevention of the transition from compensated cardiac hypertrophy to failure.

II.1.3. Signaling cascades mediating cardiac hypertrophy

The signaling pathway mediating physiological cardiac hypertrophy is largely attributed to insulin-like growth factor-1(IGF-1) and growth hormones and is transduced by phosphoinositide 3-kinase (PI3K)/Akt signaling (Dorn and Force, 2005). The involvement of the IGF-1/PI3K/Akt signaling in mediating physiological hypertrophy was evaluated in mice with cardiac specific transgenic expression of constitutively active or dominant-negative mutant forms of signaling intermediates. Cardiac-specific deletion of the insulin-like growth factor-1 receptor prevented the development of exercise-induced cardiac hypertrophy (Kim et al., 2008). Mice with cardiac-specific expression of constitutively active PI3K resulted in larger hearts with the features of physiological cardiac hypertrophy (Shioi et al., 2000), whereas dominant-negative PI3K expression attenuated the development of exercise-induced physiological but not the pathological hypertrophy induced by pressure overload, demonstrating the role of PI3K pathway in the development of adaptive hypertrophy. Phosphoinositide 3-kinase (p110alpha) plays a critical role in the induction of physiological, but not pathological, cardiac hypertrophy. Similarly, *Akt1*$^{-/-}$ mice were resistant to exercise-induced cardiac hypertrophy, however, it developed greater cardiac hypertrophy in response to pathological stimuli such as aortic constriction demonstrating its critical role in mediating physiological cardiac hypertrophy (DeBosch et al., 2006).

The best characterized signaling cascades for mediating pathological cardiac hypertrophy are the G protein-coupled receptor (GPCR) mediated Gαq signaling

activated by Ang II, ET-1 and catecholamines with down stream activation of mitogen activated protein kinases (MAPKs), protein kinase C (PKC) and D (PKD) and calcineurin. In response to hypertrophic stimuli such as pressure overload, various paracrine and autocrine factors such as Ang II, ET-1 and noradrenaline (norepinephrine, NE) are released and plays a critical role in the development of pathological cardiac hypertrophy (Arai et al., 1995; Rapacciuolo et al., 2001; Schunkert et al., 1990; Yamazaki et al., 1999). These factors activate GPCR, causing dissociation and activation of Gαq/11 and downstream signaling proteins, including phospholipase C (PLC), MAPKs, PKC and protein kinase A (PKA). The role of $G\alpha q/_{11}$ in mediating pathological cardiac hypertrophy was evaluated in transgenic mouse models with cardiac specific over-expression of Gαq and inhibitory peptide specific for Gq-coupled receptor signaling (D'Angelo et al., 1997; Wettschureck et al., 2001). Downstream of Gq, mitogen-activated protein kinases (MAPKs) and some protein kinase (PK) C isoforms have been implicated in mediating pathological cardiac hypertrophy. MAPKs such as the extracellular signal-regulated kinases (ERKs), the c-Jun amino-terminal kinase (JNKs), and the p38-MAPKs are activated in cardiac myocytes in response to GPCR agonists (AT1 receptors, endothelin receptors and α1-ARs) and in pressure overload (Sadoshima et al., 1995; Sugden, 2001; Takeishi et al., 2001; Yamazaki et al., 1993). Pressure overload also stimulates GPCR mediated PKC and PKD activation to trigger hypertrophic responses (Dorn and Force, 2005; Harrison et al., 2006).

Another target of Gq is the calcium-dependent phosphatase, calcineurin that contributes to pathological cardiac hypertrophic gene transcription by desphosphorylating transcription factors known as nuclear factor of activated T cells (NFAT) (Wilkins and Molkentin, 2004). Calcineurin is activated by a sustained Ca^{2+} plateau and is insensitive to transient Ca^{2+} fluxes such as that which occur in response to cardiomyocyte contraction. Activated NFAT translocates to the nucleus, where it associates with other transcription factors such as GATA4 and myocyte enhancer factor 2 (MEF2) to initiate the transcription of hypertrophic gene program (Frey and Olson, 2003; Wilkins et al., 2002). Clinical studies revealed that calcineurin activity is increased in patients with LV hypertrophy and failure (Haq et al., 2001). Transgenic expression of activated calcineurin in mice developed profound cardiac hypertrophy which rapidly progressed to congestive heart failure and it was prevented by pharmacological inhibition of calcineurin (Molkentin et al., 1998). It is reported that calcineurin activity was elevated only in pathological models but not in the physiological models testifying its role in pathological cardiac remodeling (Wilkins et al., 2004).

II.1.4. Role of Oxidative stress in mediating Cardiac hypertrophy

Growing evidence highlights that oxidative stress is the contributing factor for the development and progression of cardiac hypertrophy (Li et al., 2002). Oxidative stress occurs when free radical synthesis outweighs the intrinsic antioxidant capabilities of the cell, and has been implicated in the genesis of pathological cardiac hypertrophy and heart failure (Takimoto and Kass, 2007). It is established that inhibition of endogenous

antioxidant enzymes can stimulate myocyte hypertrophy and was inhibited by the addition of antioxidants (Siwik et al., 1999). Extracellular stimuli such as Angiotensin II, Endothelin-I, tumor necrosis factor-α, α1-adrenergic agonists and mechanical stretch induced hypertrophic response mediated by stimulating ROS production in cardiac myocytes, which could be abolished by antioxidants. (Amin et al., 2001; Cheng et al., 1999; Nakamura et al.;1998; Pimentel et al., 2001). Angiotensin II induces the activation of NADPH oxidase, an enzyme primarily devoted for ROS production. Several extracellular stimuli are capable of inducing cardiac hypertrophy through various downstream signaling molecules such as PKC; the MAPKs p38, JNK, apoptosis-signaling kinase 1 (ASK-1), and ERK1/2; PI3K; Akt; several tyrosine kinases, NF-κB; and calcineurin; and these factors can be activated directly or indirectly by ROS (Giordano, 2005). ROS also induces activation of redox-sensitive protein kinases such as the mitogen activated protein kinase (MAPK) superfamily and activity of transcription factors like NF-*k*B, AP-1 (activated protein –I) (Li et al., 2002).

Increased ROS generation induces cardiac contractile dysfunction by modifying the activity of proteins involved in excitation-contraction coupling, such as sarcolemmal ion channels and exchangers and sarcoplasmic reticulum calcium release channels (Seddon et al., 2007). Superoxides produced by hypertrophic stimuli interact with nitric oxide (NO) to form peroxynitrite and decreases its bioavailability, leading to coronary vascular endothelial dysfunction (Shah and MacCarthy, 2000). ROS are involved in several processes such as an increase in fibroblast proliferation, transformation into matrix-

generating myofibroblasts, the expression of pro-fibrotic genes and alterations in the balance between the activities of MMPs and TIMPs. ROS also have a potent role in extracellular matrix remodeling by modulating the activity of matrixmetalloproteinases (Preeta and Nair, 2000; Siwik et al., 2001).

Although several antioxidants have shown a positive role in modulating oxidative stress and cardiac hypertrophy, their potential in maintaining mitochondrial redox status is poor. Mitochondria and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase a major site of intracellular ROS production, and NOX4 isoform of NADPH oxidase is located in mitochondria (Dai et al., 2011). Mitochondria targeted antioxidants such as Mito-Q and Szeto-Schiller (SS)-31 peptide have ameliorated cardiac hypertrophy and dysfunction (Dai et al., 2011; Graham et al., 2009). Furthermore, it is demonstrated that overexpression of mitochondrial targeted Catalase (mCAT) reduced age-dependent left ventricular hypertrophy and diastolic dysfunction (Dai et al., 2009).

II.2. CARDIAC METABOLISM

The heart has a tremendous capacity for ATP generation, allowing it to function as an efficient pump throughout the life of the organism. Cardiac function depends on the delicate equilibrium between work performance to meet requirements of the body and energy metabolism to maintain its contractile function. In the healthy heart the processes of ATP synthesis and breakdown are exquisitely matched such that there is never a

significant fall in ATP concentration, even with large increases in cardiac output (Stanley et al., 2005). Heart utilizes 60–70% of the ATP generated to fuel contractile function, and the remaining 30–40% is principally used by the sarcoplasmic reticulum Ca^{2+}-ATPase and other ion pumps (Gibbs, 1978).

The heart is considered as a metabolic omnivore with the capacity to oxidize wide range of substrates such as fatty acids, carbohydrates, ketone bodies, lactate and even amino acids. The contribution of each substrate to the overall production of ATP is tightly regulated, with each pathway possessing a considerable degree of plasticity and interdependence. The metabolic flexibility confers the advantage to the myocardium to adapt to a variety of physiological and pathological conditions by maintaining sufficient ATP level for sustaining cardiac function. Under normal aerobic conditions, the heart relies primarily on fatty acids as substrates for oxidative metabolism. Fatty acid β-oxidation normally contributes 60–70% of total ATP production in the healthy adult heart; the remainder is provided mainly by the oxidation of glucose and lactate oxidation, and a lesser extent by the oxidation of ketone bodies. Despite relatively limited lipid storage capacity, fatty acid oxidation serves as the primary myocardial ATP generating pathway.

Heart is a highly oxidative organ and derives 90% of ATP from oxidative phosphorylation in the mitochondria and the remainder is from glycolysis and to a lesser extent from the citric acid cycle (Krebs cycle). In the mitochondria, the high-energy

phosphate bond in ATP can be transferred to creatine by mitochondrial creatine kinase to form phosphocreatine (PCr). Due to its smaller molecular weight than ATP, PCr can diffuse through the mitochondrial membrane into the cytosol where it acts as a buffer to maintain constant ATP level by reactions catalyzed by the cytosolic creatine kinase. Because of its continuous mechanical work, the heart has a high rate of ATP hydrolysis (≈0.5 μmol/g wet weight per second). Nevertheless, the high-energy phosphate pool in the heart is relatively small and can be exhausted within a few seconds. Therefore, cardiac work depends essentially on ATP generation, and impairments in this process can rapidly induce contractile dysfunction.

Mitochondria occupy ~30% of the cardiomyocyte volume and are organized in rows between myofilaments, with constant diffusion distance within the core of the myofilaments, making them the cell type with the highest mitochondria content (Schaper et al., 1985). The $NADH+H^+$ and $FADH_2$ produced during fatty acid β-oxidation pathway, the citric acid cycle (TCA cycle), and to a lesser extent from the pyruvate dehydrogenase (glucose oxidation) reaction and glycolysis fuel the mitochondrial electron transport chain and synthesize ATP by oxidative phosphorylation. There is a coordinated link between the rate of oxidation of energy substrate and the contractile performance of the heart. Thus, an increase in contractile function results in a concomitant increase in the utilization of metabolic substrate for ATP production. (Fig.3)

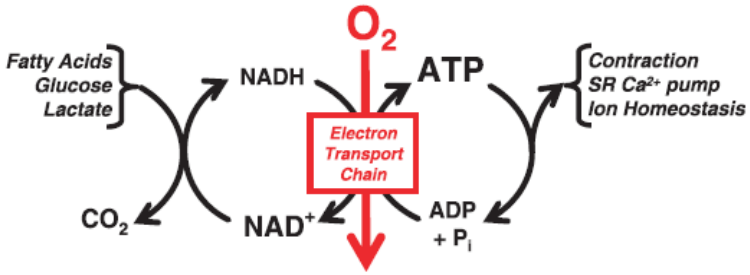

Figure 3: Linkages between cardiac power, ATP hydrolysis, oxidative phosphorylation, and NADH generation by dehydrogenases in cardiac metabolism. SR, sarcoplasmic reticulum. (Adapted from Stanley *et al*, 2005)

Myocardial fuel selection is highly influenced by the developmental stage and physiological/pathophysiological conditions; and is regulated by coordinated changes in the expression of genes involved in cellular fatty acid metabolism. Fetal heart utilizes glucose and lactate as the major energy substrate in a relatively hypoxic environment with limited ability for oxidation of long chain fatty acids. A switch in cardiac substrate preference from glucose to fatty acids occurs postnataly due to feeding of high fat containing breast milk, increased mitochondrial proliferation and increased myocardial oxygen consumption, accompanied by changes in the expression of the enzymes involved in the respective metabolic pathways.

An important feature of cardiac metabolism is that it is highly adaptable throughout the life cycle as well as under physiologic or pathologic stress. In utero, the fetal heart relies on carbohydrate substrates for ATP generation (Fisher, 1984). Clinical and experimental studies have shown that heart shifts its metabolic substrate preference away from fatty

acid towards glucose under various pathological conditions such as pressure- or volume overload–induced hypertrophy and also in heart failure, resembling the fetal metabolic program. Similarly, in the uncontrolled diabetic state, myocardium uses fatty acids almost exclusively to maintain energy level due to insulin resistance and high circulating free fatty acids (Wall and Lopaschuk, 1989). Conversely with aging, there is a decline in fatty acid metabolism, and the proportion of glucose metabolism to overall substrate metabolism increases (Kates et al., 2003; McMillin et al., 1993). A schematic representation of normal cardiac metabolism is given in fig.4.

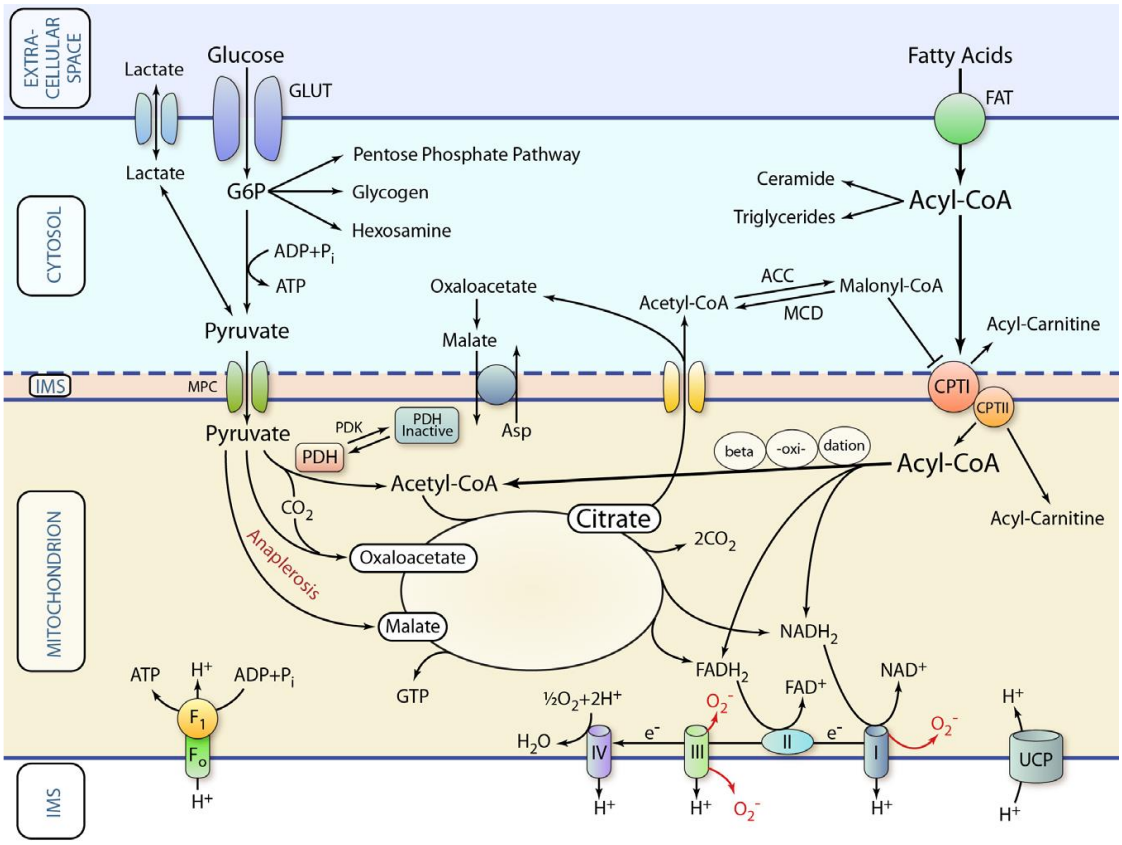

Figure 4: Schematic representation of normal cardiac metabolism.

ACC, acetyl-CoA carboxylase; CPT, carnitine palmitoyltransferase; FAT, fatty acid transporter; G6P, glucose 6-phosphate; GLUT, glucose transporter; IMS, mitochondrial intermembrane space; MCD, malonyl-CoA decarboxylase; MPC, mitochondrial pyruvate carrier; PDH, pyruvate dehydrogenase; and PDK, pyruvate dehydrogenase kinase (*Adapted Doenst et al 2013*)

II.2.1. Myocardial fatty acid metabolism

Myocardial fatty acid uptake is primarily determined by plasma concentration of nonesterified fatty acids. Free fatty acids are highly hydrophobic and associated with proteins or covalently bound with coenzyme A. Major sources of plasma fatty acid are triglycerides which are released from adipocytes, chylomicrons and very low density lipoproteins that are hydrolyzed by lipoprotein lipase bound to the capillary endothelial cells and cardiomyocytes. The major site of fatty acid oxidation is the mitochondrial matrix and is highly dependent on the intracellular delivery of fatty acids. Uptake of fatty acids into the cardiomyocyte is mediated either by passive diffusion or by protein-mediated fatty acid uptake from microvascular compartments; and is mediated by fatty acid translocase (FAT), or a plasma membrane fatty acid binding protein (FABPpm). A specific 88-kDa FAT protein called CD36 is abundantly expressed in skeletal and cardiac muscle and appears to be the predominant form of FAT in the heart.

Once fatty acids are transported into the sarcoplasm, the free fatty acids bind to FABP, the primary intracellular carrier of non esterified fatty acids and are then activated by fatty acyl-CoA synthase (FACS) to fatty acyl-CoA. FABP and FACS protein along with CD36 are assembled on the cytosolic side of the sarcolemmal membrane to ensure the immediate esterification of fatty acids entering into the sarcoplasm to fatty acyl-CoA, making them water soluble (Lopaschuk et al., 1994). Oxidation of fatty acids primarily occur in the mitochondria and to a small extent in peroxisomes. Hence fatty acids have to be transported into the mitochondrial matrix from cytosol. As inner mitochondrial

membrane is impermeable to fatty acyl-CoA, the transport of long chain acyl-CoA is mediated by carnitine dependent transport system, consisting of palmitoyl transferases (CPT) I and II and carnitine acyl translocase (CAT). CPT-I is present on the outer mitochondrial membrane. It binds to fatty acyl CoAs and catalyzes the formation of fatty acyl carnitines which are transported to the mitochondrial inter-membrane space. There, CAT translocates fatty acyl carnitines into the matrix (in exchange for carnitine), where CPT-II re-esterifies acyl carnitines into acyl CoAs. Within the matrix, acyl CoAs can then be progressively metabolized by fatty acid oxidation. CPT-1 is a regulatory enzyme that controls the mitochondrial uptake of long chain fatty acids with two different isoforms CPT-1α and β. CPT-Iα the liver isoform is found throughout the body except cardiac and skeletal muscle and brown adipose tissue. CPT-1β is the main isoform in the heart.

Once taken up by the mitochondria, fatty acyl-CoA undergoes β-oxidation, a process that repeatedly cleaves off two carbon acetyl-CoA units, generates energy in the form of reduced $NADH+H^+$ and $FADH_2$, that are subsequently utilized for the formation of ATP by electron transport chain and oxidative phosphorylation. Four main enzyme classes are involved in the mitochondrial β-oxidation. The first step is catalyzed by acyl-CoA dehydrogenase, followed by 2-enoyl-CoA hydratase, and then 3-hydroxyacyl-CoA dehydrogenase. The final step is 3-ketoacyl-CoA thiolase (3-KAT), which regenerates acyl-CoA for another round of β-oxidation. Acyl-CoA dehydrogenase and 3-hydroxyacyl-CoA dehydrogenase generate $FADH_2$ and $NADH+H^+$, respectively, and the

acetyl-CoA formed from β-oxidation is fed into citric acid cycle (CAC) and generates $NADH+H^+$, GTP and $FADH_2$.

II.2.2. Glucose Metabolism

Glucose that is used by the heart is derived from exogenous glucose and glycogen stores. Glucose transport into cardiomyocytes is mediated by the transmembrane glucose gradient and the glucose transporters (GLUTs) in the sarcolemma. Although GLUT1 is the major glucose transporter in the fetal heart and contributes to constitutive glucose uptake, in the adult heart, GLUT4 is the predominant isoform and mediates the bulk of basal myocardial glucose uptake and is sensitive to insulin stimulation. After uptake, free glucose is rapidly phosphorylated to glucose-6-phosphate (G6P), which subsequently enters many metabolic pathways. There is a translocation of glucose transporters from intracellular vesicles to the sarcolemmal membrane in response to insulin stimulation, increased work demand, or ischemia (Stanley et al., 1997; Young et al., 2000, 1997).

During glycolysis, glucose is converted into pyruvate with the net production of two molecules of ATP and two molecules of $NADH+H^+$. Under normoxic condition, pyruvate is oxidized by the pyruvate dehydrogenase (PDH) complex to form acetyl coenzyme A (CoA), which then feeds into the TCA cycle. Alternatively, under hypoxic condition, pyruvate can be converted to lactate by the enzyme lactate dehydrogenase (LDH), to regenerate the NAD^+ required to maintain glycolysis. The PDH complex is

rate-limiting for glucose oxidation, and is highly sensitive to product inhibition by Acetyl-CoA. When high rates of fatty acid oxidation is present, there is an increase in the concentration of Acetyl-CoA, which in turn can inhibit glucose oxidation.

The myocardium produces more amount of lactate under the conditions of ischemia and poorly controlled diabetes, when there is accelerated glycolysis in the face of impaired oxidation of pyruvate (Stanley et al., 1997). Lactate transport across the cardiac sarcolemma is facilitated by the monocarboxylic acid transporter-1. Lactate is extracted from the blood, converted to pyruvate in the cytosol, and further oxidized to acetyl-CoA in the mitochondrial matrix. In the normal healthy human heart, pyruvate is derived in approximately equal proportions from glycolysis and lactate uptake

Glycolytic enzymes are clustered together and arranged as complexes and are bound to sarcomere, and sarcoplasmic reticulam where they synthesize ATP which is readily available for ion pumps and other membrane structures (Pierce and Philipson, 1985). Glycolytically generated ATP is preferentially used by the sarcoplasmic reticulum to fuel Ca^{2+} uptake and by the sarcolemma to maintain ion homeostasis. (Entman et al., 1977; Weiss and Lamp, 1989)

Acetyl-CoA, the common end product of glucose and fatty acid oxidation, is further metabolized by citric acid cycle (tricarboxylic acid cycle or Krebs cycle) to generate GTP (or ATP), CO_2, and reducing equivalents such as $NADH+H^+$ and $FADH_2$. As the

intermediates of citric acid cycle are used for many biosynthetic pathways such as aminoacids and nucleic acids, the constant availability of the metabolic intermediates are critical. The reducing equivalents generated ($NADH+H^+$ and $FADH_2$) by glycolysis and citric acid cycle enter the electron transport chain (ETC) for oxidative phosphorylation.

II.2.2.1. *Accessory Pathways of Glucose Metabolism*

Glucose oxidation by glycolysis not only produces pyruvate for further oxidation but also yields metabolic intermediates which can enter into additional accessory pathways of biological significance that do not lend to ATP generation (Kolwicz et al., 2013). Glucose 6-phosphate (G-6-P) produced by the hexokinase reaction may also be channeled into glycogen synthesis or the pentose phosphate pathway (PPP). The PPP is an important source of reduced nicotinamide adenine dinucleotide phosphate (NADPH) and 5-carbon sugars. NADPH plays a critical role in the cellular antioxidant defense system by maintaining the level of reduced glutathione (Wu et al., 2004). The 5-carbon sugars formed such as ribose 5-phosphate becomes a substrate for nucleotide synthesis (Zimmer, 1992), and xylulose 5-phosphate has been suggested as a transcriptional signaling molecule (Doiron et al., 1996). In addition to the PPP a small amount of G-6-P enters into the hexosamine biosynthetic pathway yielding uridine diphosphate-N-acetylglucosamine, a monosaccharide donor for O-GlcNAcylation of proteins (Wells et al., 2001). Recent evidence suggests a role for these pathways in the pathophysiology of heart disease despite small fluxes (Doenst et al., 2013).

II.2.3. Ketone Body Metabolism

The heart extracts and oxidizes ketone bodies (β-hydroxybutyrate and acetoacetate) in a concentration dependent manner. Plasma ketone bodies are formed from fatty acids in the liver. The contribution of ketone bodies for myocardial energy production is considered to be minor as they are the minor substrate for the myocardium under normal physiological condition and its uptake is mediated by Monocarboxylate transporter (Halestrap and Price, 1999). During prolonged fasting, on high fat diet, poorly controlled diabetes and heart failure, blood level of ketone bodies increases and results in enhanced use by the heart (McNulty et al., 2000; Wentz et al., 2010). Elevated cardiac ketone body oxidation is said to inhibit the utilization of glucose and fatty acids in the heart presumably mediated through product inhibition on PDH by Acetyl-CoA (Kodde et al., 2007).

II.2.4. Regulation of Cardiac Metabolism

The cardiac metabolic machinery is designed to generate high amounts of ATP to meet the elevated energy demand during elevated workload. The control of these energy producing pathways is complex, but the different pathways normally work in perfect harmony to ensure that the energy requirement of the myocardium are met (Lopaschuk and Kelly, 2008). The utilization of fatty acids and glucose is tightly linked and coregulated. Use of one substrate may directly restrict the use of the other, and this reciprocal inter-regulatory relationship between glucose oxidation and fatty acid oxidation was originally described by Philip Randle, and is known as glucose/fatty acid

cycle or Randle cycle. The myocardial substrate selection is influenced by arterial carbon substrate concentration, hormone concentrations such as insulin, glucagon-like peptides (GLPs), and catecholamines, coronary flow, inotropic state, and the nutritional status of the tissue; and is tightly regulated at multiple levels. The heart employs different mechanisms to adapt to acute and chronic changes in energy demand. Acute changes in energy demand are met by coordinated activation or inactivation of enzymes and transporters and are achieved by modulating the level of protein phosphorylation, co-factors or allosteric compounds. These mechanisms allow for the rapid adaptation to acute metabolic stresses such as exercise, ischemia or fasting. Similarly, chronic changes in energy demand is maintained by adjustments in the rate of ‘‘metabolic’’ gene expression of key enzymes. Chronic changes in cardiac substrate preference occur during various physiological as well as pathophysiological conditions, such as postnatal development, cardiac hypertrophy, diabetes and ischemia; and are mediated by regulation of metabolic enzymes at a transcriptional and/or post-translational level (Taegtmeyer, 2000a).

Cardiac metabolic activity is regulated at transcriptional levels of metabolic enzymes by several nuclear transcription factors. Peroxisome proliferator–activated receptor (PPAR)-α nuclear receptor transcription factor regulates the expression of genes involved in fatty acid uptake and oxidation. Although PPARα is the predominant isoform in the heart, PPARδ and PPARγ isoforms may also modulate FA metabolism

(Leone et al., 1999). The increased fatty acid utilization by the activation of PPARα leads reciprocally to a decrease in glucose utilization. HIF1α (hypoxia-inducible transcription factors) is another nuclear receptor transcription factor which has several target genes that increase O_2 delivery or survival during hypoxia. These include genes involved in the upregulation of glucose metabolism (e.g transporters, dehydrogenases and kinases) (Semenza, 2000). HIF1α is one of the few transcription factors that regulates the myocardial adaptation to ischemia and hypoxia (Semenza, 2011).

The estrogen-related receptor (ERR) family is comprised of ERRα, ERRβ and ERRγ. Among them ERRα and ERRγ seem to be important mediators of myocardial metabolism. ERRα in association with PPAR-α activates over 90 distinct genes involved in multiple key energy producing pathways, including FA and glucose metabolism suggesting an intense interaction between both nuclear receptors (Huss et al., 2004). Myocardial ERRα expression increases considerably during the post-natal period, in parallel with the global upregulation of enzymes involved in cellular fatty acid uptake and mitochondrial oxidation. The transcriptional activities of PPARs and ERRs are potently induced by interacting with members of the PPAR-γ coactivator-1 (PGC-1) family. PGC-1α coactivates transcription factors which regulates mitochondrial biogenesis (Vega et al., 2000). It serves as the master modulator of oxidative energy metabolism.

AMP activated protein kinase (AMPK) is a highly conserved heterotrimeric enzyme that act as the energy sensor of the myocardium, activated by cellular stresses that deplete ATP and acts as an indicator of intracellular ATP/AMP (Heidrich et al., 2010). AMPK promotes energy-producing catabolic pathways and inhibits energy-consuming anabolic metabolism (Arad et al., 2007). Activated AMPK enhances uptake and oxidative metabolism of fatty acids and also glucose transport and glycolysis. AMPK stimulates the fatty acid metabolism by inhibiting acetyl-CoA carboxylase (ACC), thereby decreasing the myocardial level of malonoyl-CoA, an inhibitor of Carnitine palmitoyl transferase-I (CPT-I) (Dyck and Lopaschuk, 2006). Additionally, AMPK increases sarcolemmal expression of fatty acid transporter (FAT/CD36) and membrane-associated fatty acid binding protein (FABPpm) (Chabowski et al., 2006). AMPK also stimulates glucose metabolism by increased translocation of glucose transporters, GLUT1 and GLUT4, from intracellular reservoirs to the sarcolemma to mediate glucose uptake (Fryer et al., 2002). AMPK stimulates glycolysis directly by activating phosphofructokinases (PFK1), the primary regulatory enzyme in the glycolytic pathway by increasing the level of fructose-2,6-biphosphate, an allosteric stimulator of PFK1 (Marsin et al., 2000). AMPK is activated by various physiological and pathological conditions like exercise, hypoxia, ischemia, and neurohumoral factors.

II.3. METABOLISM IN CARDIAC HYPERTROPHY

Intricately associated with the defining features of cardiac hypertrophy such as increase in cardiomyocyte size, shift in myosin isoform, and reinduction of fetal cardiac gene program, altered expression of extra cellular protein and enhanced oxidative stress, myocardial metabolism undergoes a reprogramming in response to left ventricular hypertrophy. During hypertrophic response, the relative contribution of fatty acids to the overall energy production decreases and glucose becomes the favored fuel. (Barger and Kelly,1999). Increased glucose utilization, characterized by accelerated rates of basal glucose uptake and glycolysis, is not accompanied by correspondingly enhanced rates of glucose oxidation, suggesting an 'uncoupling' of glycolysis and glucose oxidation in cardiac hypertrophy. (Allard et al.,1994; Sambandam et al. 2002). A prominent decrease in fatty acid oxidation with enhanced glucose utilization suggests that, the pattern of energy substrate utilization in cardiac hypertrophy resembles that in fetal hearts called fetal recapitulation (Lopaschuk et al., 1991). Kagaya et al. demonstrated that the metabolic remodeling is not the consequence of work overload per se, but hypertrophic response itself is the ultimate cause (Kagaya et al., 1990). As the long-chain fatty acid oxidation is more efficient in terms of ATP synthesis per substrate molecule utilized, a substantial increase in glucose oxidation is required to balance the myocardial energy status in response to metabolic remodeling (Abdurrachim et al., 2015).

The cardiac phenotype due to defective fatty acid oxidation underscores the association between reduced rate of fatty acid oxidation and development of cardiac hypertrophy. Cardiac hypertrophy was provoked in rats by feeding fat-free diet (Panos and Finerty 1953). Humans with inborn errors in mitochondrial fatty acid oxidation enzymes often develop ventricular hypertrophy in the absence of stimuli such as hypertension (Kelly et al., 1992). Inhibition of mitochondrial fatty acid oxidation in animal models results in the development of cardiac hypertrophy (Bressler et al., 1989; Rupp and Jacob, 1992). Patients with idiopathic dilated cardiomyopathy have decreased myocardial fatty acid oxidation as assessed by PET (Dávila-Román et al., 2002). In Spontaneously hypertensive rat, a widely used experimental model for left ventricular hypertrophy (LVH), myocardial metabolism is characterized by increased glucose utilization and reduced palmitate oxidation (Christe and Rodgers, 1994a). Hypertensive left ventricular hypertrophy is associated with abnormal myocardial fatty acid metabolism (de las Fuentes et al., 2006). Cardiac specific deletion of Carnitine palmitoyl transferase-Iβ (CPT-Iβ) is associated with development of cardiac hypertrophy and increased mortality in mice (Haynie et al., 2014). Deficiency of long chain and very long chain acyl CoA dehydrogenase is associated with development of cardiac hypertrophy in mice (Cox et al., 2009). Though initially adaptive, prolonged low rate of fatty acid oxidation results in various cardiovascular consequences including increased oxidative damage. Modulating cardiac metabolism is therefore a therapeutic target for the treatment of heart disease.

Although alteration in metabolic phenotype may be adaptive under non stressful conditions with improvement in myocardial oxygen efficiency, this metabolic profile is inefficient in maintaining the myocardial energy level under increased work load, leading to depletion of myocardial energy status and reduced capacity to maintain myocyte lipid balance leading to cardiac dysfunction (Lehman and Kelly, 2002a; Neubauer, 2007) . The changes in substrate utilization may influence the heart's ability to withstand a subsequent metabolic stress, such as ischemia– reperfusion (Taegtmeyer, 2000b). PPARα null mouse heart (PPARα$^{-/-}$) with decreased fatty acid oxidation could not maintain myocardial ATP concentration under stressful condition, and showed impaired contractile performance when subjected to high workload challenge (Luptak et al., 2005; Watanabe et al., 2000). In an experimental study, a substantial increase in glucose metabolism by GLUT1 overexpression modulated the contractile performance of PPAR$^{-/-}$ mice under severe work overload, showing that inactivation of PPARα could be corrected by enhancing insulin-independent glucose utilization by overexpression of transporter GLUT1 in PPARα$^{-/-}$ hearts and prevented the development of heart failure under chronic pressure overload. (Liao et al., 2002b; Luptak et al., 2005). However, it is accepted that the ability to increase glucose utilization is limited in adult hearts, and is insufficient to sustain the contractile function in the face of severely decreased capacity to oxidize fatty acids. These findings imply that switching the substrate preference from fatty acid to glucose hampers the heart's ability to cope with subsequent metabolic stresses and myocardial energetics, and is therefore considered maladaptive (Taegtmeyer, 2000b).

II.3.1. Mechanism underlying decreased fatty acid oxidation in cardiac hypertrophy

Experimental studies in the last decade have revealed a number of molecular mechanisms that induces metabolic remodeling to adapt to hypertrophic condition, such as peroxisome proliferator-activated receptors (PPARs), adenosine monophosphate-activated protein kinase, and peroxisome proliferator-activated receptor-γ coactivator 1 (PGC1). The decline in fatty acid oxidation in the hypertrophic heart is the direct consequence of a transcriptional downregulation in the expression of the fatty acid oxidation genes (Sack et al., 1996; van Bilsen et al., 1998). Experimental studies in animal models have shown that the expression of genes involved in fatty acid uptake and metabolism was diminished under hypertrophic condition (Akki et al., 2008; de las Fuentes et al., 2003; Remondino et al., 2000). The decreased expression of genes involved in fatty acid metabolism is consequent to a decline in the activity and expression of the nuclear receptor PPAR-α, the transcription factor that plays a crucial role in the transcriptional regulation of genes involved in fatty acid metabolism (Huss and Kelly, 2004; van Bilsen et al., 2004). The $PPAR^{-/-}$ mice has shown reduced cardiac expression of genes involved in mitochondrial fatty acid oxidation (Leone et al., 1999; Watanabe et al., 2000). The cardiac mRNA expression of PPAR-α and PPAR gamma coactivator 1 (PGC-1α) were coordinately down regulated in the mouse model of pressure overload induced cardiac hypertrophy in parallel with the reduced expression of fatty acid oxidation enzyme genes (Barger et al., 2000). PGC-1α is a co-activator of

PPAR-α, and is involved in the transcriptional control of genes involved in fatty acid oxidation. These results demonstrate that one prominent mechanism involved in the downregulation of FAO enzyme expression in the hypertrophied heart involves repression of PPAR-α and PGC-1 gene expression (Lehman and Kelly, 2002a).

During cardiac hypertrophic growth, the DNA binding activity of PPARα was rapidly inhibited at the post-transcriptional level by a phosphorylation at the NH2-terminal AB domain by an extracellular-regulated kinase (Fig.5). The mitogen-activated protein kinase (ERK– MAPK), is an obvious candidate implicated in the hypertrophic growth; and pharmacological inhibition of ERK–MAPK activation with PD98059 prevented the decrease in fatty acid oxidation in cellular model of cardiomyocyte hypertrophy. Conversely, pharmacological inhibition of p38 and c-Jun N-terminal kinase (JNK)–MAPK did not prevent the decrease in fatty acid oxidation under hypertrophic condition (Barger et al., 2000).

Cardiac expression of mitochondrial fatty acid oxidation enzyme gene expression is antagonized in the hypertrophied heart by the transcriptional repressors COUP-TF, Sp1, and Sp3, with a concurrent decline in the nuclear protein levels of PPARα. (Fig. 5) The mRNA expression and activity of fetal nuclear receptor transcription factor chicken ovalbumin upstream promoter transcription factor I (COUP-TF) and Sp (Sp1 and 3) factors were found to be increased in the hypertrophied heart, accompanied with reciprocal expression pattern of PPARα. The enhanced DNA binding activities of Sp1,

Sp3, and COUP-TF on the NRRE-1/Site A is responsible for decreased expression of genes involved in fatty acid oxidation (Sack et al., 1997). NRRE-1 is a pleiotropic nuclear hormone receptor response element conferring bidirectional transcriptional regulation via activator (PPARα) and repressor (COUP-TF) transcription factors. Sp factors exerts its effect by binding to *site A* a sequence adjacent to NRRE-1 in the gene promoter (Lehman and Kelly, 2002b).

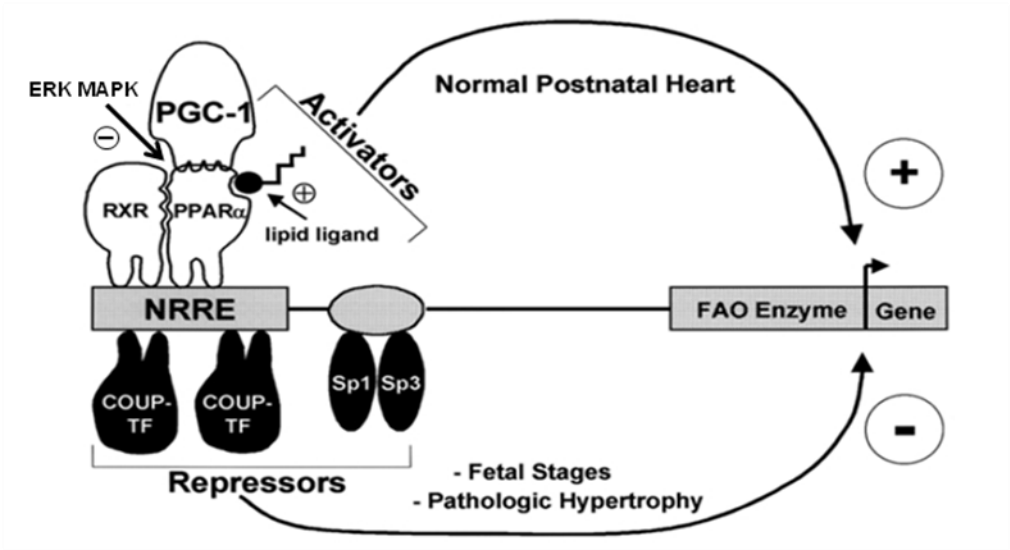

Figure 5: The transcriptional control of cardiac fatty acid oxidation in hypertrophic heart.

ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; RXR, retinoid X receptor; PGC-1, peroxisome proliferatoractivated receptorγ coactivator 1; PPARα, peroxisome proliferatoractivated receptor α; NRRE, nuclear receptor response element; COUP-TF, chicken ovalbumin upstream promoter transcription factor. *(Adapted from Lehman et al 2002)*

II.3.2. Mechanism of increased glucose utilization in cardiac hypertrophy

Cardiac glucose uptake is mediated by the glucose transporters GLUT-1 and GLUT-4. The increased utilization in cardiac hypertrophy is mediated through insulin independent glucose transporter GLUT-1, whereas insulin dependent glucose uptake through GLUT-4 is diminished (Ritchie and Delbridge, 2006). The enhanced myocardial basal glucose uptake associated with an elevation of the GLUT-1/GLUT-4 transporter ratio was observed in the hypertophic heart of SHR and was accompanied with diminished insulin stimulated glucose uptake (Paternostro et al., 1995). GLUT4-/- mice developed cardiac hypertrophy with reduced fatty acid oxidation (Abel et al., 1999; Stenbit et al., 2000).

It is proposed that the increased glycolytic flux during cardiac hypertrophy is mediated by the activation of an intracellular energy sensor Adenosine Mononucleotide Phosphate -activated protein kinase (AMPK) (Nascimben et al., 2004). AMPK, a metabolic sensor acts as a metabolic master switch under pathophysiological conditions. As the oxidation of long chain fatty acids is efficient in terms of ATP produced per molecule oxidized compared to glucose, prolonged low rate of fatty acid oxidation results in chronic depletion of energy reserve. AMPK acts as an energy sensor and, when the cytosolic AMP/ATP concentration increases, AMPK stimulates metabolic pathways that conserve ATP and inhibits pathways that consume ATP (Hardie et al., 1999; Tian et al., 2001). However, Allard et al demonstrated that the activation of AMPK during pressure overload is independent of the energy status of the heart. AMPK was activated in

hypertophic heart even in the absence of measurable change in energy level. (Allard et al., 2007).

AMPK was found to activate cardiac phosphofructokinase-2 (PFK-2) by phosphorylation, leading to augmented synthesis of fructose-2,6-bisphosphate (F-2,6-P2) from fructose-6-phosphte, an allosteric activator of the phosphofructokinase (PFK), the rate-limiting glycolytic enzyme (Marsin et al., 2000). Similarly, enhanced AMPK activity stimulates the translocation of the glucose transporters (GLUT) onto the plasma membrane and enhances glucose uptake (fig.6). Increased AMPK activity has been reported in rat model of pressure overload left ventricular hypertrophy (LVH) in response to chronic change in myocardial energetics manifested by increased insulin independent glucose uptake (Tian et al., 2001). In addition to regulation of myocardial glucose uptake, AMPK also regulates fatty acid metabolism. Pharmacological activation of AMPK increased fatty acid oxidation in rat skeletal muscle, mediated by inhibition of acetylCoA carboxylase (ACC), subsequent to malonyl-CoA mediated inhibition of CPT-I. (Merrill et al., 1997). In contrast, decreased fatty acid oxidation has been shown in parallel with increased AMPK activity in hypertrophied hearts under chronic pressure overload (Allard et al., 1994).

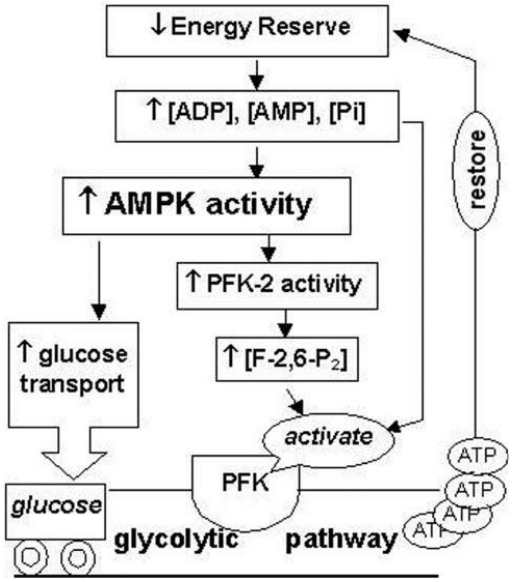

Figure 6: Proposed mechanism(s) for enhanced glycolysis in cardiac hypertrophy. (*Adapted from Nascimben et al., 2004*).

II.3.2.1. *Role of defective CD36 in mediating metabolic remodeling in cardiac hypertrophy*

Series of studies have reported that the metabolic remodeling is secondary to unavailability of metabolic substrate due to defective fatty acid transport. Defective fatty acid transport decreases the bioavailability of long chain fatty acids, a natural ligand of PPARα leading to its diminished activity, and inturn decreased fatty acid oxidation. Spontaneously hypertensive rat (SHRs) is the most widely studied animal model of chronic pressure overload, which develops hypertension as early as 4 weeks of age, and gradually acquires hypertensive cardiac hypertrophy leading to deterioration in cardiac function (Purushothaman et al., 2011a). SHRs exhibit a shift in myocardial energy production from the use of fatty acids toward the use of glycolysis. Pathological cardiac

hypertrophy in SHRs also results in an abnormal regulation of proteins and transcription of the key enzymes and transporters involved in myocardial energy metabolism (Coburn et al., 2000). Congenic mapping and microarray screening in SHR identified CD36 on rat chromosome 4 as a defective gene, resulting from recombination between wild type gene and Pseudo gene affecting post translational modification and membrane targeting of the protein (Aitman et al., 1999; Lauzier et al., 2011). Reduced expression of CD36 in SHR is associated with reduced long chain fatty acid uptake. Expression of wild type CD36 in SHR improved defective fatty acid oxidation without affecting hypertension (Pravenec et al., 2001). CD36 is a highly glycosylated integral membrane protein, that plays an important role as a LCFA-binding/transport protein that shuttles 40-80% of fatty acids from the extracellular compartment into the cytoplasm (Brinkmann et al., 2002). SHRs are characterized by a restricted utilization of exogenous LCFAs for energy production, which has been attributed to a genetic defect in CD36. In addition, the cytoplasmic content of fatty acid binding protein (FABP) is found to be decreased in SHR making SHR an ideal model of defective transporter mediated metabolic remodeling (Vork et al., 1992).

Clinical studies have reported the effect of defective fatty acid transport system on cardiac structure and function. Tanaka et al demonstrated the prevalence of association between CD36 deficiency and defective fatty acid uptake in patients with hypertrophic cardiomyopathy (HCM) (Tanaka et al., 1997). High prevalence of FAT/CD36 (478T→478C) point mutation has been reported in Japanese population resulting in

FAT/CD36 deficiency. The point mutation results in the replacement of Proline (^{90}Pro) with Serine (Ser) in CD36 resulting in post translational failure, with consequent shortening of the half life of the protein (Kashiwagi et al., 1995). Patients, homozygous for the 478T→478C substitution developed hypertrophic cardiomyopathy with reduced uptake of long chain fatty acid (Tanaka et al., 1997).This finding was the first clinical evidence of a possible causal association between defective fatty acid transporter and development of cardiomyopathy. Another study conducted in a larger patient population showed that 40% of the hypertrophic cardiomyopathic patients had CD36 mutation (Okamoto et al., 1998). These observations were challenged by Nakamura et al, who could not show any significant difference in cardiac phenotype between HCM patients with normal CD36 expression and those showing CD36 deficiency (Nakamura et al., 1999). Variable etiology can account for the discrepancy in the observations. Deficiency of CD36 has been reported recently by Magida *et al* in mouse models with familial hypertrophic cardiomyopathy (Magida and Leinwand, 2014). Defective CD36 did not affect the normal survival of the animals under basal conditions but showed reduced ischemic tolerance, increased susceptibility to arrhythmia and impaired response to adrenergic stimulation (Irie et al., 2003; Labarthe, 2004).

In addition to FAT/CD36 deficiency, direct interventions in cardiac fatty acid handling eventually entails myocardial hypertrophy. Cardiac hypertrophy in animal models showed decline in myocardial carnitine content (El Alaoui-Talibi et al., 1997). Pharmcological inhibition of carnitine acyl transferase-I (CPT-I) and Fatty acid

translocase (FAT/CD36) was associated with increased heart weight in rat compared to untreated control (Koundakjian et al., 1984; Kusaka et al., 1995). Similarly, cardiac specific fatty acid binding protein (H-FABP) knock-out mice developed exercise intolerance and cardiac hypertrophy at older age (Binas et al., 1999). The development of cardiac hypertrophy in juvenile visceral steatosis mice was alleviated by administration of carnitine (Horiuchi et al., 1993; Jalil et al., 2006). These observations justify that reduced substrate availability due to defective fatty acid transporter CD36 can be a leading cause for the development of cardiac hypertrophy independent of hypertension.

II.4. MODULATION OF ENERGY METABOLISM IN CARDIAC HYPERTROPHY

Pathologic cardiac hypertrophy is associated with the reappearance of the fetal metabolic profile characterized by decreased fatty acid oxidation and increased relative contribution of glucose as energy source (Barger et al., 2000). As cardiac hypertrophy is an important predictor for heart failure, the prolonged substrate preference for glucose is closely associated with impairment of cardiac energy balance and loss of contractile reserve (Neubauer, 2007). Cardiac interventions enhancing oxidation of carbohydrate over fatty acid were shown to enhance the function of the ischemic reperfused heart or the failing heart. The interventional studies in animals were carried out using drugs, which enhances glucose oxidation and inhibits fatty acid oxidation such as ranolazine,

oxfenicine, trimetazidine. However, it remains unanswered, how long this reduced fatty acid oxidation would be tolerated by the diseased heart, as it is observed that humans with genetic defects in fatty acid oxidation develop cardiomyopathies. Nevertheless, chronic decrease in fatty acid oxidation is associated with detrimental cardiac consequences such as intracellular lipid accumulation and energy deficit. Energy production from diminished fatty acid oxidation is not likely to be fully compensated by enhanced glucose use, under chronic workload. The diminished fatty acid oxidation leads to progressive deterioration of cardiac function during hemodynamic overload. Cardiac specific PPARα null mouse ($PPAR^{-/-}$) hearts with permissive increases in glucose oxidation was not able to maintain the myocardial energetics during high workload (Luptak et al., 2005). Cardiac specific overexpression of the insulin-independent glucose transporter (glucose transporter 1 [GLUT1]) to recapitulate metabolic profile of hypertrophic heart, has shown increased glucose oxidation, with reciprocal decreases in cardiac fatty acid oxidation (Liao et al., 2002). These mice were protected against contractile dysfunction and left ventricular dilation when subjected to pressure overload (ascending aortic constriction) with maintenance of myocardial energetics, demonstrating that the increased dependence on glucose per se is not detrimental to the heart under pressure overload. Similarly, transgenic mice with overexpression of liver X receptors (LXRs) was protected against cardiac remodeling induced by chronic pressure overload by stimulation of glucose oxidation (Cannon et al., 2015). These genetic modification approaches of metabolic modulations are of less clinical relevance because of its limited clinical applicability.

Partial inhibition of fatty acid oxidation by pharmacological inhibition of Carnitine palmitoyl transferase-I (CPT-I), a mitochondrial long chain fatty acid oxidation enzyme prevented adverse cardiac remodeling and progression of heart failure in rat model of pressure overload and in canine model of pacing induced heart failure (Lionetti et al., 2005; Rupp and Vetter, 2000). CPT-I inhibition prevented transcriptional downregulation of cardiac energy metablism enzymes and sarcoplasmic reticulum calcium uptake. However, prolonged CPT-I inhibition resulted in the aggravation of cardiac hypertrophy and development of cardiac lipotoxicity (Haynie et al., 2014; He et al., 2012). As the cardiac glucose oxidation is highly insulin dependent, impaired insulin signaling in combination with diminished fatty acid oxidation can result in severe limitations of substrate oxidation. These reports demonstate that the inherent cardiac capacity to increase glucose utilization is insufficient to sustain normal energy supply under stress when fatty acid oxidation is severely impaired (Allard et al., 1994). It has been reported that hypertrophic and failing heart are less responsive to insulin and insulin resistant, limit the utilization of glucose as the sole energy source. (Ashrafian et al., 2007; Paternostro et al., 1999, 1995; Witteles et al., 2004). However, administration of Glucagon like peptide-1(GLP-I) improved left ventricular performance in pacing induced dilated cardiomyopathy by increase in glucose uptake and oxidation, accompanied by stimulation of insulin secretion and insulin sensitivity (Nikolaidis et al., 2004).

Taken together, it is clear that enhancing glucose oxidation in cardiac hypertrophy improves cardiac function and energetics in short term experiments and are less promising for chronic use. (Kolwicz et al., 2013). Supporting this perception, several animal studies reported that sustenance of fatty acid metabolism in hypertrophic heart preserves cardiac function and energetics (Chess et al., 2009; Duda et al., 2008; Kolwicz et al., 2012).

Reactivation of fatty acid oxidation in hypertrophied hearts is expected to restore a more 'normal' metabolic physiology, thereby maintaining cardiac function. Modulating cardiac metabolism is therefore presumed as a therapeutic avenue for the treatment of heart disease. Modulation of fatty acid metabolism by pharmacologic intervention in cardiac hypertrophy has presented variable effects on the heart consequent to pharmacological stimulation of fatty acid metabolism. Fenofibrate attenuated left ventricular hypertrophy, fibrosis and improved diastolic dysfunction in young SHR and surgical models of cardiac hypertrophy (Chen et al., 2007; Li et al., 2009; Ogata et al., 2002a; Zou et al., 2013). Chronic activation of PPARα with fenofibrate did not influence Left ventricular dysfunction and dilation despite upregulation of fatty acid oxidation and markers of LV hypertrophy in infarct model of heart failure. (Morgan et al., 2006) However, Briqadeau et al. reported that fenofibrate reduced cardiac hypertrophy and slowed down the progression to heart failure on stimulation of fatty acid oxidation enzymes in pacing induced porcine model of heart failure (Brigadeau et al., 2007). Fenofibrate not only attenuated the development of hypertrophy and ventricular

dysfunction, but also prevented myocardial inflammation (Ichihara et al., 2006). In aldosterone induced hypertension, fenofibrate improved LV hypertrophy and fibrosis independent of blood pressure (Lebrasseur et al., 2007). Fenofibrate also improved cardiac remodeling and function in rat model of severe volume overload (Dhahri et al., 2013). Pharmacological reactivation of PPAR-α with fibrates have shown regression of cardiac hypertrophy and fibrosis along with increased life expectancy in SHR (Chen et al., 2007; Linz et al., 2009). Concurrently, severe depression of cardiac power and efficiency in the hypertrophied heart has also been reported (Young et al., 2001). Age associated variation in cardiac response on short term treatment with fenofibrate, with positive effect in young animals and negative response in older animals was also reported (Purushothaman et al., 2011b). Recently, we have observed ligand specific cardiac response to stimulation of fatty acid metabolism in spontaneously hypertensive rat on administration of fenofibrate and medium chain triglyceride (Ismael et al., 2015).

A series of studies in the past decade have suggested that high fat diet attenuates adverse cardiac remodeling. Dietary guidelines for cardiovascular diseases recommend high carbohydrate/low fat diet. However, Chess et al have reported that high dietary fat can activate PPAR-α and mitochondrial oxidative metabolism without inducing adverse cardiac remodeling in mice with transverse aortic constriction (Chess et al., 2008, 2009). High fat diet in 10 week old Dahl salt sensitive rat resulted in the attenuation of cardiac hypertrophy and improved contractile performance of the heart, inspite of increased myocardial triglyceride content (Okere et al., 2005). Similarly, consumption of a low

carbohydrate/high fat diet attenuated pressure overload induced LVH, remodeling and contractile dysfunction compared to a high carbohydrate/low fat diet in rats that underwent aortic banding (Duda et al., 2008). The altered gene expression such as increased atrial natriuretic factor mRNA, myosin heavy chain isoform switch, and decreased activity of citrate synthase and medium chain acyl-coenzyme A dehydrogenase associated with hypertrophy were attenuated by high-fat feeding in Dahl salt sensitive rat (Okere et al., 2006). In 5-6 week old SHR fed a low carbohydrate/ high fat diet, Bosse et al observed that the degree of cardiac hypertrophy was not affected despite reduction in blood pressure (Bosse et al., 2013). Adverse effects of high fat diet are also reported. Consumption of fat based diet is associated with increased myocardial triglyceride content, plasma fatty acids and development of obesity in rodents (Chess et al., 2009; Okere et al., 2005). In mice that underwent transverse aortic constriction, high fat diet worsened cardiac hypertrophy and dysfunction (Raher et al., 2008). Similarly, chronic high fat diet induced myocardial hypertrophy and fibrosis in 7-week old CD1 mice by inhibition of Glycogen synthase kinase-3β (Wang et al., 2015). Concomitantly, in 9-11 week old Dahl salt sensitive rat, diet based on simple sugars such as fructose and sucrose is shown to be associated with increased left ventricular dysfunction and mortality (Sharma et al., 2008, 2007). Conventionally, saturated fats are not recommend as its intake is presumed to increase the risk of cardiovascular diseases.

The contradictory observations from different approaches for stimulation of fatty acid metabolism raises the question whether reactivation of fatty acid metabolism is

detrimental during pathological cardiac hypertrophy. Kolwicz *et al* carried out an interesting experiment in mice with cardiac specific deletion of Acetyl-CoA carboxylase-2, as the latter is known to inhibit entry of long chain fatty acids into mitochondria by inhibition of malonyl coA production. In transgenic mice exposed to 8 weeks of pressure overload by transverse aortic constriction, hearts with elevated rate of fatty acid oxidation prevented myocardial hypertrophy and fibrosis (Kolwicz et al., 2012), suggesting that maintenance of inherent cardiac metabolic profile is beneficial under pathological condition.

II.4.1. Medium chain triglycerides, a metabolic substrate to bypass defective CD36

Medium chain triglycerides are esters formed from glycerol and saturated medium-chain fatty acids (MCFAs) with a chain length between 8-12 carbons. Coconut and palm oils are the dietary sources with the highest concentration of MCTs. Unlike long chain fatty acids, their structural and physiochemical properties confer unique metabolic characteristics (Odle, 1997). Their uptake is independent of cellular and subcellular fatty acid transport systems such as CD36 and CPT-1 making them direct fuel for β-oxidation (Labarthe et al., 2008). Naturally occurring medium chain fatty acids in oils and foods are caproic acid (hexanoic acid, C6:0), caprylic acid (octanoic acid, C8:0) and capric acid (decanoic acid, C10:0). The proportion of MCFAs is about 15%, 7.9%, 6.8%, 6.9%, 6.6% and 7.3% of total fatty acid in coconut oil, palm kernel oil, butter, milk, yogurt and cheese, respectively (Bach and Babayan, 1982; Nagao and Yanagita, 2010). They are isolated by lipid fractionation of edible fat and are

commercial formulations are mainly comprised of C8:0 and C10:0. They form a minor constituent of classical diet. The use of MCTs in food products is recognized as safe (GRAS status) by US Food and Drug Administration since 1994 (Traul et al., 2000).

In contrast to long chain triglycerides (LCT), MCTs are hydrosoluble and are rapidly hydrolysed to MCFAs that are absorbed into the portal vein and are rapidly metabolized by the liver. In the liver, MCFAs are directed towards oxidation rather than incorporation into lipids, showing its high propensity for oxidation. Excess acetyl-CoA formed by β-oxidation leads to ketone body synthesis. Acetoacetate and β-hydroxybutyrate, are the quantitatively most important MCFA-derived ketone bodies. Odd-carbon MCFAs also lead to the formation of the C-5 ketone body analogs β-ketopentanoate and β-hydroxypentanoate from propionyl-CoA metabolism

MCT were first introduced in the 1950s for the treatment of lipid absorption disorders (Bach and Babayan, 1982). Over the past decades, MCTs have also been used for the nutritional management of patients with inherited long chain fatty acid β-oxidation disorders. MCFAs, which bypass the enzyme defect and thereby restore energy production, is shown to improve clinical symptoms, particularly cardiac hypertrophy and dysfunction, in most patients associated with very long chain acyl-CoA dehydrogenase (VLCAD) deficiency (Brown-Harrison et al., 1996; Cox et al., 1998; Duran et al., 1991; Touma et al., 2001).

MCT is available in two different forms, odd and even chain MCT based on number of carbons atom (Fig.7). Odd chain MCT is reported to be more anaplerotic than even chain MCT. Anaplerosis is the re-filling of the catalytic intermediates of the Citric acid cycle. MCTs with an odd number of carbons were reported to be superior to even-carbon MCTs in improving the cardiomyopathy of one patient with genetic LCFA oxidation deficiency (Roe et al., 2002). The β-oxidation of one molecule of heptanoate (7 Carbon MCT) yields two acetyl-CoA and one propionyl-CoA molecules, while that of octanoate yields four acetyl-CoA units. The Propionyl-CoA, three carbon compound formed from heptanoate is further metabolized to succinyl-CoA, which is an anaplerotic reaction that feeds the pool of Citric acid cycle intermediates. Dietary regimen substituting tricaprylin (even chain MCT) with triheptanoin (odd chain MCT) improved cardiomyopathy in few patients with very long-chain acyl-CoA dehydrogenase deficiency. Triheptanoin intake enhanced ketone body synthesis without inducing propionyl-CoA overload (Roe et al., 2002). Recently, Nguyen *et al* demonstrated that odd chain MCT triheptanoin feeding improved diastolic function and cardiac hypertrophy in rodent model of pressure overload (Nguyen et al., 2015).

Several studies reported the beneficial effect of MCT on heart. 14 years of prospective study revealed that intake of short and medium chain fatty acids are not associated with the development of coronary heart disease (Hu et al., 1999). The impaired capacity of SHR hearts to withstand an acute adrenergic stress could be improved by increasing the contribution of energy production by supplementation with 0.2 mM octanoate (a eight

carbon triglyceride) (Labarthe et al., 2005). Octanoate reduced cardiac tissue damage in response to acute adrenergic stimulation represented by decreased LDH release and improved cardiac function. Inclusion of octonate in the perfusate normalized accelerated glycolysis, improved energy status and the contractile function in the hypertrophic working heart induced by aortic contriction (Allard et al., 2007). Similarly, addition of caprylic acid (8C) to the perfusate improved ischemic recovery of CD36 knock out working hearts, in comparison with long chain fatty acid palmitate (Irie et al., 2003). Additionally, supplementation of octanate enhanced contractile function of volume overload heart (Alaoui-Talibi et al., 1992). Supplementation of MCT prior to exercise training improved oxidative metabolism and decreased cardiac workload in patients with long-chain fatty acid oxidation defect (Behrend et al., 2012). Dietary administration of MCT improved LV dysfunction in mice with diabetic cardiomyopathy (Finck et al., 2003).

There are few studies reporting beneficial effects of MCT-enriched diet in SHR. Reactivation of fatty acid metabolism by supplementation of medium chain triglycerides (MCT) was found to prevent cardiac hypertrophy and improve cardiac function in young SHR with persistent hypertension (Hajri et al., 2001; Iemitsu et al., 2008; Rupp et al., 1995; Shimojo et al., 2004). However, data concerning the potential of MCT to promote reverse remodeling in older animals and the effect of supplementation of MCT on cardiac oxidative stress and energy status are not available.

III. METHODOLOGY

III.1. DESIGN OF THE STUDY

Oxidation of long-chain fatty acids is the major energy source for the healthy heart. Pathological cardiac hypertrophy is accompanied by reactivation of fetal cardiac metabolism, decreased oxidation of long chain fatty acids and increased relative contribution of glucose for the energy production. Prolonged low rate of fatty acid oxidation is associated with adverse cardiovascular consequences including increased oxidative stress. This metabolic preference is mediated by down regulation of the expression of genes involved in fatty acid metabolism. Studies have shown that the reduced fatty acid oxidation in patients with hypertrophic cardiomyopathy, and also in Spontaneously hypertensive rat (SHR) are due to reduced expression of the fatty acid transporter, CD36 (Cluster of differentiation-36) . Hence, it is hypothesized that supplementation of Medium chain triglyceride that by passes the defective fatty acid transport can maintain myocardial metabolism to prevent adverse cardiac remodeling.

Medium chain triglycerides are triglycerides of saturated fatty acids of chain length 8-12 carbon atoms, and their oxidation is independent of cytoplasmic and mitochondrial fatty acid transport systems such as CD36 and Carnitine palmitoyl transferase-1 (CPT-1). Hence, they are regarded as direct fuel for β-oxidation. Several studies have reported the beneficial effect of MCT on heart. MCT is known to improve contractile function of the hypertrophic heart. Consumption of MCT is not associated with the development of coronary artery diseases. MCT is available in two different forms, odd and even chain

triglycerides, based on number of carbons. Odd chain MCT is reported to be anaplerotic in nature. Anaplerosis is the re-filling of the catalytic intermediates of the Citric acid cycle. Therefore, there is possibility of difference in their metabolic properties.

The study is aimed at regression of cardiac hypertrophy for prevention of progressive cardiac remodeling. Experimental studies have shown that older animals are resistant to treatment. There are no reports on the use of MCT for regression of cardiac hypertrophy in chronic pressure overload associated with CD36 deficiency. Spontaneously hypertensive rat, an animal model of pressure overload was used as the experimental model. Cardiac remodeling in SHR follows the same pattern as the clinical course of hypertension induced cardiac hypertrophy and failure.

The study was designed with following objectives

i. Validate the suitability of the experimental model by screening for CD36 deficiency and markers of cardiac hypertrophy, metabolic shift and oxidative stress, by comparison of Spontaneously hypertensive rat (SHR) with normotensive Wistar rat (W)

ii. Compare the cardiac response to stimulation of fatty acid oxidation by supplementation with odd and even chain MCT

iii. Asses cardiac response to stimulation of fatty acid oxidation by supplementation of medium chain triglycerides at initial and established stages of cardiac hypertrophy.

(i) Prior to experimental studies, suitability of SHR as the experimental model for pathological cardiac hypertrophy was validated. Morphological, histological and molecular markers of cardiac hypertrophy, metabolic shift and oxidative stress of 6 month old SHR were compared with age and sex matched normotensive Wistar rat (W). Wistar-Kyoto rats were avoided as they have a tendency to develop cardiac hypertrophy. Blood pressure of the animals was monitored by tail cuff spygmomanometer. The expression of CD36 in Wistar and SHR was assessed by Western blotting. Cardiac index [heart weight/ body weight(mg/g)], myocyte cross sectional area, mRNA expression of Brain natriuretic peptide (BNP), calcineurin A expression were used as the markers of cardiac hypertrophy.

Cardiac metabolism in SHR was evaluated from mRNA expression of Peroxisome prolifrator-activated receptor-α (PPAR α) and one of its target genes Medium chain acylCoA dehydrogenase (MCAD), and Phosphofructokinase-1(PFK-1), a glycolytic enzyme by Real time PCR analysis. Myocardial fibrosis was evaluated from histological analysis of myocardial sections with Picrosirius red staining; and interstitial and perivascular fibrosis was quantified.

As oxidative stress is associated with cardiac hypertrophy, end products of oxidative damage such as malonedialdehyde (MDA), Protein carbonyl and 3-nitro tyrosine were used as the markers of myocardial oxidative stress. Myocardial MDA and Protein carbonyl were estimated biochemically and 3-nitrotyrosine was determined by immunohistochemistry.

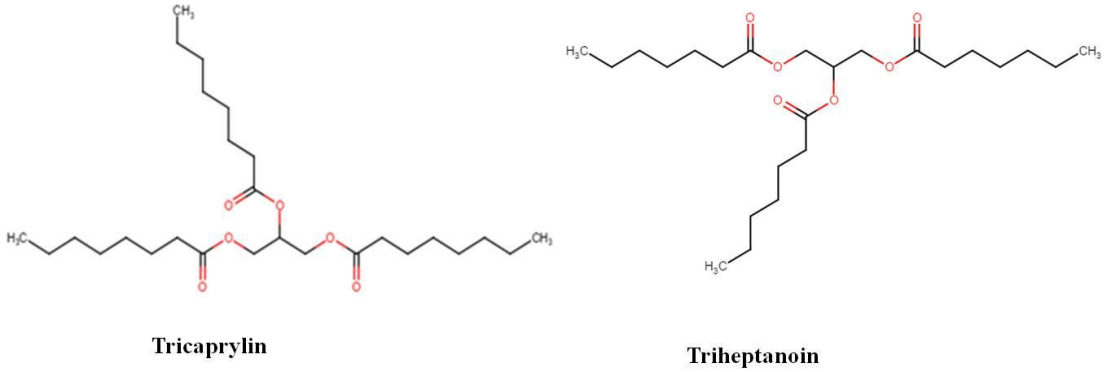

Figure 7: Structure of even (Tricaprylin) and odd (Triheptanoin) Medium chain triglyceride.

(ii) MCT is available as even chain and odd chain triglycerides (fig.7). Hence, a pilot study was carried out to identify the triglyceride with a superior cardioprotective effect. The ideal MCT would then be used for experimental studies. For this experiment, the feed of 2-month-old SHR was supplemented with either 5% (v/w) Ticaprylin (even chain)or Triheptanoate (odd chain)for 2 months. Morphological and histological markers of cardiac hypertrophy and markers of oxidative stress were analyzed. The cardiac response was evaluated from hypertrophy index, myocyte cross sectional area, and myocardial fibrosis. Biochemical estimation of malonedialdehyde (MDA) was used as

the marker of oxidative stress. Since fat based diet was used for experimental purpose, serum lipid profile was determined by commercially available kit (Agappe Diagnostics, India).

(iii) Cardiac response to stimulation of fatty acid metabolism by supplementation of medium chain triglycerides was assessed at initial and established stages of cardiac hypertrophy. Young, 2-months-old and older 6-months-old SHR were supplemented with Medium chain triglycerides (MCT) for 4 months, with the objective of preventing cardiac remodeling in the former, and promoting reverse remodeling in the latter group. Stimulation of cardiac fatty acid metabolism was substantiated from the expression of peroxisome proliferator-activated receptor alpha (PPAR-α) and medium chain acyl CoA dehydrogenase (MCAD -a key enzyme in β-oxidation of fatty acids) by real time RT-PCR analysis. Consequence of reactivation of fatty acid metabolism on glucose metabolism was evaluated from the mRNA expression of phosphofructokinase-1 (PFK-1), marker enzyme for glycolysis. Cardiac response to metabolic stimulation was evaluated based on hypertrophy index, cell size, morphometric analysis of ventricular collagen (interstitial and perivascular), expression of brain natriuretic peptide (BNP) and calcineurin A. Effect of MCT on oxidative stress was assessed, as the latter is associated with pathological hypertrophy. Myocardial malondialdehyde (MDA- a stable terminal metabolite of lipid peroxidation), 3-nitrotyrosine and protein carbonyl (end product of protein oxidation) were used as markers of oxidative stress. The energy level was

evaluated from biochemical estimation of ATP and phosphocreatine. Blood pressure and serum lipid profile were also determined.

The study was approved by The Institutional Animal Ethics Committee. The housing care and the management of these animals were in accordance with 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

Bovine Serum Albumin (BSA), Disodium hydrogen phosphate, Potassium chloride, Potassium dihydrogen phosphate, EDTA, glucose, Diethyl pyrocarbonate (DEPC), Protease inhibitor cocktail, Acrylamide, bisacrylamide, β-mercaptoethanol, TEMED, Ammonium persulphate, Color burst electrophoresis marker, Direct red, , Sodium dodecyl sulphate (SDS), Trizma base, Agarose, Glycine, Thiobarbituric acid, Tricloro acetic acid (TCA), Tris–HCl, Perchloric acid, Pottassium bicarbonate, Methyl orange, Triethanolamine, NADP, Ketamine and Xylazine, Glucose-6-phosphate dehydrogenase, Hexokinase, Creatine kinase, 2,4 dinitrophenyl hydrazine (DNPH), digitonin, ethyl acetate and streptomycin sulphate. (*Sigma-Aldrich*), Nitrocellulose membrane (*Millipore USA*). Odd and Even Chain MCT for initial experiments were gifted from *Jayant agro-organics limited, Mumbai, India.* MCT (Tricaprylin) for major experiments were purchased from *Sigma Aldrich.*

Antibodies: Monoclonal anti-calcineurin antibody (*Thermo*), Anti CD36 antibody (*Abcam*), monoclonal anti-beta actin antibody (Sigma), Trizol reagent, anti-mouse IgM antibody and monoclonal anti rabbit antibody (*Sigma-Aldrich*), Polyclonal anti dystrophin antibody (*Santa cruz Biotechnology*),

Kits: Coomassie protein assay reagent, DNase –I kit (amplication grade- *Sigma*), Super signal West Femto Substrate kit (*Thermo scientific USA*). The fine chemicals for cDNA synthesis including RT buffer, RNase inhibitor, oligo dT primers, dNTPs and M-MLV Reverse Transcriptase were purchased from *Promega*. Power SYBR Green for real time PCR was purchased from *Applied Biosystems,*

Total cholesterol, Triglycerides, HDL cholesterol and LDL Cholesterol estimation kits were purchased from *Agappe Diagnostics India.*

III.2.2. Routine Chemicals

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

III.2.3. Instruments used

Eppendorf centrifuge 5415 R, Incubators (*Beston India; Kemi, India*), Weighing balance (*Sartorius, USA and Ohaus*), Homogenizer (*IKA, Labortechnik, Germany*), Deepfreezer

-20ºC (*Vestfrost*), Deepfreezer -80ºC (*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*), UV- visible Spectrophotometer (*Shimadzu*), 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*). Small Animal Noninvasive Blood Pressure System (NIBP200A) (*Biopac Systems, USA*), BIOPAC Data Acquisition Unit (MP35/MP30), *Applied Biosystem* 7500 real time PCR system (*Applied Biosystem*).

III.2.4. Software used

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

BSL PRO for NIBP (*Biopac*)

ImageJ (*NIH*)

III.3. COMPOSITION OF REAGENTS AND BUFFERS

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

NaCl 137mM,

KCl 2.7mM,

KH_2PO_4 1.76mM,

Na_2HPO_4 10.14mM.

III.3.2. DEPC-treated deionized water

1 ml of DEPC in one litre of deionized water, stirred for 30 minutes at room temperature and autoclaved

III.3.3. 10% Buffered formalin

NaH_2PO_4 (unhydrous) – 3.5g

Na_2HPO_4 (unhydrous) – 6.5g

Formalin - 100ml

Distilled Water - 900ml

III.3.4. Harris Hematoxylin

Alum -100g

Hematoxylin - 5g

Ethanol -50ml

Mercuric oxide - 2.5g

Distilled water - 1000ml

III.3.5. Eosin Solution

Eosin	-1gm
Propanol	-100ml

III.3.6. Sirius Red stain

Sirius red (direct red)	- 0.1g
Saturated picric acid	-100ml

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

Made up to 30 ml with distilled water

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

in 80 ml deionized water. pH adjusted to 8.8 using HCl, made upto 100 ml and stored at room temperature.

III.3.11. Stacking gel buffer (pH 6.8)

30 ml from resolving buffer was measured, pH adjusted to 6.8 using HCl and made up to 45ml. Stored at room temperature.

III.3.12. 30% Acrylamide solution

29.2 g of acrylamide and 0.8g bis acrylamide dissolved in distilled water and made up to 100ml. Solution stored in dark at room temperature.

III.3.13. 10% Ammonium per sulphate solution

0.1 g Ammonium Per sulphate (APS) dissolved in 1ml distilled water.

III.3.14. 10% SDS solution

1g of SDS dissolved in 10 ml distilled water

III.3.15. Blocking buffer

Skimmed Milk	0.5g
1x TBS- Tween 20	10ml

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
Deionized water	1000ml

III.3.18. DNA/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)

1mg in 1ml water, 5ul of this stock solution added to 20ml of 1% agarose gel for electrophoresis.

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

Tris base	24.2 g
Sodium chloride	80 g
Distilled water	1000ml

III.3.21. Triethanolamine buffer (TEA) (pH 7.6)

Triethanolamine HCl	4.65g
Water	500 ml

pH was adjusted with NaOH

III.4. EXPERIMENTAL STUDIES

Spontaneously hypertensive rat (SHR) was used as the in vivo experimental model of left ventricular hypertrophy (LVH). It simulates the clinical progression of hypertension induced left ventricular hypertrophy. SHR develops hypertension at 4 weeks of age, left ventricular hypertrophy at 8 weeks followed by metabolic shift.

Spontaneously hypertensive rats were purchased from Animal Resource Centre, Australia and the Wistar rats were maintained in the Division of Laboratory Animal Science of the Institute (SCTIMST). The animals were housed at 22±2°C in 55±10% relative himidity in individually ventilated cages. Light levels measured at 1 meter height were less than 300 Lux and a 12:12 hour dark: light pattern was maintained. The rats were fed with standard pelletted feed and drinking water *ad libitum.*

III.4.1. Comparison of markers of cardiac hypertrophy, metabolic shift and oxidative stress in Spontaneously hypertensive rat (SHR) with normotensive wistar rat (W).

Prior to the experimental studies it was decided to confirm that the strain of SHR available at the Institute was deficient for CD36. The animals were characterized for markers of cardiac hypertrophy, metabolic shift and oxidative stress in SHR. Normotensive Wistar rat were used as the control for comparison. Morphological, histological and molecular features were analyzed in SHR and compared with Wistar rat. 6 months old male SHR were compared with age and sex matched Wistar rat.

III.4.1.1. *Assessment of markers of cardiac hypertrophy, oxidative stress and metabolic shift in SHR*

The expression of CD36 and indicators of cardiac hypertrophy, oxidative stress and metabolic shift in SHRs was assessed using morphological, histological, biochemical and molecular indicators compared with age and sex matched Wistar rat. Hemodynamic measurement of six month old animals 4 from each strain was recorded. After 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, ventricles separated from the atrium and weighed. Hypertrophy index was used as the morphological indicator of cardiac hypertrophy and it was compared with age and sex matched normotensive Wistar rat. Hypertrophy index was determined as the ratio of total ventricular weight to body weight [Ventricular weight in milligram (mg)/Body weight in gram (g)]. Protein expression of CD36 was carried out to confirm CD36 deficiency. Calcineurin A expression and mRNA expression of Brain natriuretic peptide (BNP) were used as the molecular markers of cardiac hypertrophy. Histological markers such as myocyte cross sectional area and myocardial fibrosis (interstitial and perivascular fibrosis) were determined and compared with Wistar rat. To determine myocyte cross sectional area heart sections were immunostained for dystrophin to obtain a clear outline of the cell. Dystrophin is a member of complex proteins which anchor muscular cytoskeleton to the extracellular matrix through plasma membrane. Cross sectional area of a minimum of 100 muscle fibers were measured from each ventricular

section and quantified by ImageJ 1.45s software (National Institute of Health). To maintain consistency, mid-ventricular region of the heart was excised and fixed in formalin for histological analysis.

As oxidative stress is an essential factor associated with cardiac hypertrophy, markers of end products of oxidative damage such as Malonedialdehyde (MDA), protein carbonyl and 3nitrotyrosine level were determined in SHR and compared with Wistar rat. Malonedialdehyde and protein carbonyls were biochemically measured from myocardial tissue and myocardial 3-nitrotyrosine were determined by immunohistochemistry.

Another factor intimately associated with cardiac hypertrophy is a shift in substrate preference from fatty acids towards glucose. The metabolic status in SHR was evaluated from the mRNA expression of Peroxisome priliferator-activated receptor (PPAR)-α, Medium chain acyl CoA dehydrogenase, (MCAD) and phosphofructokinase (PFK)-1 by Real time polymerase chain reaction. mRNA expression was quantified using $\Delta\Delta C_T$ method with β- actin as the loading control gene (Livak and Schmittgen, 2001).

III.4.2. Comparison of cardiac response to odd and even chain mct supplementation in SHR

III.4.2.1. *Evaluation of the cardiac response to Supplementation of even and odd chain MCT in SHR*

MCT is available in two forms, Even chain MCT and Odd chain MCT. Therefore, a pilot study was carried out to select the ideal MCT to reactivate fatty acid metabolism in SHR. Tricaprylin and triheptonoate were used as the sources of even chain and odd chain MCT respectively. As MCT is available in the liquid form, 50 ml of MCT was blended with 1000g of standard rat chow (5% MCT with standard feed). To evaluate the cardiac response, 2 months old male SHR were supplemented with 5% MCT. Age and sex matched untreated SHR served as the experimental control. SHR were parallely fed and each group contained 4 animals (n=4). Morphological and histological markers of cardiac hypertrophy were evaluated following 2 months of treatment. Hypertrophy index, myocyte cross sectional area and myocardial interstitial fibrosis were used as the markers for the evaluation of the cardiac hypertrophy. Myocardial malonedialdehyde , a marker of oxidative stress was also evaluated. To examine the consequence of fat based diet on lipid profile, serum triglyceride, total cholesterol and high density lipoprotein (HDL) cholesterol were evaluated. LDL was expressed as the calculated value.

III.4.3. Assessment of cardiac response to stimulation of fatty acid metabolism by supplementation of medium chain triglycerides at the initial stage and established stage of cardiac hypertrophy

Two months old SHR served as the model for initial stages of cardiac hypertrophy. This represents the stage prior to the occurrence of metabolic shift. Six months old SHR served as the model for the established stage of cardiac hypertrophy. This phase is the stable phase of cardiac hypertrophy. Age and sex matched untreated SHR served as the experimental control. Each group contained six animals. The feed of the SHR were supplemented with 5% MCT (v/w) (tricaprylin) for 4 months with the assumption that maintenance of fatty acid metabolism can prevent adverse cardiac remodeling. The animals were fed parallely. On completion of treatment, after measurement of body weight and hemodynamic evaluation, 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, ventricles separated from the atrium and weighed. Mid-ventricular region was excised and fixed in formalin for histological analysis. The ventricles were then rinsed in ice-cold saline, frozen in liquid nitrogen and stored at -80°C. Morphological, histological and molecular markers of cardiac hypertrophy were evaluated following 4 months of MCT supplementation. The markers of oxidative stress and myocardial metabolism were determined.

Evaluation of cardiovascular response to MCT supplementation.

III.4.3.1. ***Metabolic changes on supplementation of MCT in the initial and established stages of cardiac hypertrophy***

Cardiac hypertrophy in SHR is associated with a shift in energy substrate preference from fatty acid towards glucose. MCT was supplemented based on the assumption that it can maintain the fatty acid metabolism in SHR. Effect of MCT on cardiac metabolism was evaluated from the mRNA expression of PPAR-α, MCAD and PFK-1 as determined by Real time polymerase chain reaction. PPAR-α and MCAD are markers of fatty acid metabolism where as PFK-1 is marker for glucose metabolism.

III.4.3.2. ***Assessment of the effect of MCT supplementation on markers of cardiac hypertrophy***

Hypertrophy is characterized by increased myocardial mass with increased myocyte cross sectional area and interstitial fibrosis. Effect of metabolic stimulation on markers of cardiac hypertrophy was evaluated from the morphological, histological and molecular markers of cardiac hypertrophy. Hypertrophy index was calculated as previously described (Section III.4.1.1) and used as the morphological marker of cardiac hypertrophy. Myocyte cross sectional area was determined by immunostaining of myocardial sections with anti dystrophin antibodies to get the clear outline of the individual cell membrane. Myocardial fibrosis was assessed by picrosirius red staining of the myocardial sections and interstitial and perivascular fibrosis was determined. Protein expression of Calcineurin A and mRNA expression of BNP were used as the

molecular markers of adverse cardiac remodeling and the procedure described in III.5.2 and III.5.6 was followed.

III.4.3.3. *Consequence of MCT supplementation on markers of oxidative stress*

The effect of metabolic stimulation with MCT on oxidative stress was evaluated by biochemical estimation of cellular end products of oxidative damage such as molonedialdehyde and protein carbonyl. Myocardial level of 3-nitrotyrosine was also determined in tissue sections.

III.4.3.4. *Effect of MCT supplementation on blood pressure*

Effect of metabolic modulation on blood pressure was monitored by noninvasive tail cuff sphygmomanometer after warming and restraining the animals. Systolic and diastolic pressure was measured as described in the Section III.5.1

III.4.3.5. *Myocardial energy level in response to MCT supplementation*

Myocardial energy level was determined by biochemical evaluation of Adenosine Triphosphate (ATP) and Phosphocreatine(PCr).

III.4.3.6. *Effect of MCT supplementation on serum lipid profile*

Serum triglyceride, Total cholesterol, HDL were estimated by commercially available estimation kits (Agappe Diagnonotics. India).

III.4.3.7. *Hepatic lipotoxicty*

As fat based diets can cause hepatic lipid accumulation, liver tissue was histologically analyzed for any indication of lipotoxicity by hemotoxyline–eosin staining .

III.5. METHODOLOGY

III.5.1. MEASUREMENT OF BLOOD PRESSURE

On completion of the experiments, resting systolic and diastolic arterial pressure of the animals were measured. Blood pressure was measured using Noninvasive Blood Pressure Monitoring System for Small Animals (BIOPAC Systems.Inc) (Fig.7). The measurements were taken with a tail-cuff sphygmomanometer after restraining the animals.

The tail cuff was placed proximally on the tail to occlude the blood flow. Upon deflation of the cuff, blood pressure was determined which coincided with the restoration of caudal artery pulse. This was achieved by using a piezo electric pulse transducer placed distal to the cuff, which was coupled to the Data Acquisition System. For accurate noninvasive blood pressure measurement, the tail of the animal was pre-warmed to $32^{O}C$.

At least 6 determinations were made in every session of blood pressure measurement and the average was obtained. The systolic blood pressure value coincided with the

point of appearance of first pulse when the cuff is deflated and the point of the first maximum peak corresponds to diastolic blood pressure value.

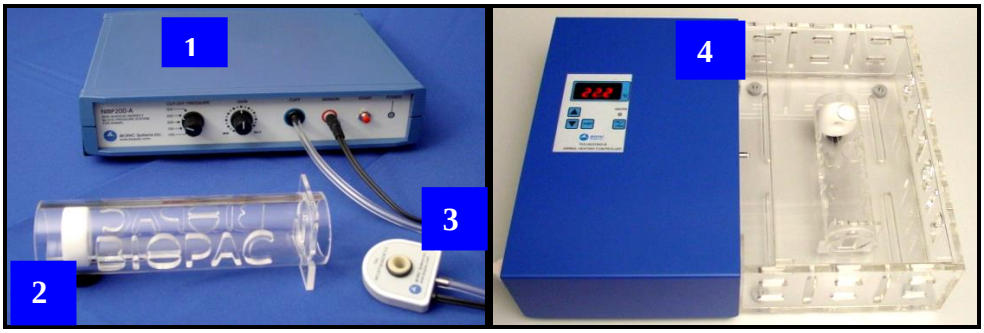

Figure 8: Set up for blood pressure measurement

1 NIBP200A Front Panel
2 Restrainer
3 Piezo electric pulse transducer (distal to cuff)
4 Animal heating chamber
5. A typical recording using BSL PRO software for NIB

III.5.2. GENE EXPRESSION ANALYSIS

III.5.2.1. *RNA Isolation*

The myocardial tissue for RNA isolation was snap frozen in liquid nitrogen and stored at -80^{0}C. Frozen myocardial tissue (100 mg) was thoroughly homogenized in 1ml Trizol reagent (Sigma) with a homogenizer (Ultra-Turrax). The homogenate was incubated at room temperature for 10 minutes for the dissociation of nuclear proteins. 0.2 ml of chloroform/ml of Trizol was added to the homogenate, mixed by vigorous shaking and centrifuged at 12000 rpm for 10 minutes. The aqueous layer was collected and 0.5 ml of Isopropanol was added to precipitate the RNA, followed by centrifugation at 12000 rpm for 15 minutes. The pellet formed was washed with 75% ethanol at 7500 rpm for 8 minutes and air dried. The final pellet was resuspended in 20µl nuclease free water. The RNA was quantified at A_{260nm} using Epoch spectrophometer (Biotek, USA). The purity of RNA was determined from the $A_{260/280}$ ratio spectroscopically. The integrity of the RNA was evaluated by 1% agarose gel electrophoresis. Genomic DNA contamination of RNA sample was removed by RNase free DNase-1 treatment according to manufacture's protocol.

III.5.2.2. *cDNA synthesis*

Rat-specific primers for the genes were designed by Oscimum Biosolutions (India). Real Time PCR was performed in a total volume of 20 µl, containing 60 ng of the cDNA derived from reverse transcription using Power SYBR Green technology. The threshold

cycle (C_T) for fluorescence development was used to calculate the fold changes applying the ‘‘$2^{-\Delta\Delta CT}$ method’’ (Livak and Schmittgen, 2001).

Construction of cDNA- 2 µg of each RNA sample was reverse transcribed to cDNA with MMLV reverse transcriptase using oligo dT primers. Briefly, to the 2 µg RNA , 2µl oligo dT primer was added and was incubated for 5’ at 70^0C for removing the unwanted secondary RNA structure and cooled immediately by placing it in ice for 5-10’ and centrifuged for 10 seconds. The sample was added to the reaction mixture containing dNTP, RNasin, MMLV-RT, RT buffer and water, and was incubated at 37^0C for an hour. The sample was kept at 90^0C for 5’ to inactivate reverse transcriptase enzyme.

III.5.2.3. *Real Time PCR analysis*

Real Time RT PCR was performed using Power SYBR Green technology on Applied Biosystem 7500 real time PCR system. Concentration of the primers were optimized by conventional polymerase chain reaction. PCR amplification was performed in a total volume of 20 µl, containing 60 ng of the cDNA derived from reverse transcription, 200-500nM of primer, and 10 µl of 2X Power SYBR Green mastermix. Each reaction was incubated for 2 min at 50°C and 10 min at 95°C and then subjected to 40 cycles, each involving denaturation at 95°C for 15s and annealing/extension at 60°C for 1 min. In this way, the threshold cycle (C_T) for fluorescence development was obtained. All samples were run in triplicate. The “$2^{-\Delta\Delta}C_T$ method” for comparing the relative expression results

between treatments in real-time PCR was applied following standard protocol (Livak and Schmittgen, 2001). The genes analyzed and the primers used are listed in Table 1.

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

BNP **Sense** **Antisense**	5' AGAGAGCAGGACACCATC 3' 5' AAGCAGGAGCAGAATCATC3'
PFK- I **Sense** **Antisense**	5'CTATGTTGGAGGCTGGACTG3' 5'TTATACTTGGTTATGTTGGCACTG3'
PPAR α **Sense** **Antisense**	5'AACTGACATTTGTGACTG3' 5'GTTTCCCATCTCTTGTAAG3'
MCAD **Sense** **Antisense**	5'TTTGCCAGAGAGGAAATAATC3' 5'CCAAGACCACCACAACTC3'
Beta actin **Sense** **Anti sense**	5'CGTGCGTGACATTAAAGAG3' 5'GCCACAGGATTCCATACC 3'

III.5.3. IMMUNOHISTOCHEMICAL ANALYSIS

The ventricular sections were deparaffinized in xylene for 15min (3 times), followed by rehydration in descending grades of alcohol. The hydrated sections were blocked for endogenous peroxidase with 3% hydrogen peroxide in methanol. The target antigen was retrived using 0.01M freshly prepared sodium citrate buffer (pH-6.0) at 95^0 C for 20 minutes. The sections were blocked with 3% BSA and incubated with primary antibody

at 4^{o}C overnight. The dilutions of the primary antibodies were - Dystrophin- 1:50 and CD36 and 3-nitro tyrosine-1:100. After washing with PBS, the sections were treated with HRP conjugated secondary antibody (dilution 1:200) for an hour at room temperature. After washing off unbound secondary antibody, the sections were treated with the chromogen, diaminobenzidine (DAB) (Sigma)

III.5.4. HAEMATOXYLIN AND EOSIN STAINING

Tissue sections are rehydrated in descending grades of alcohol and brought to the water. Hydrated sections were stained with hematoxylin for 3 minutes followed by differentiation in acid alcohol for 30 seconds. Bluing was done in running tap water. Then the sections were stained with Eosin for one minute. The dehydrated sections were mounted with DPX.

III.5.5. SIRIUS RED STAINING

The extent of myocardial fibrosis was determined by Sirius red staining of cardiac cross sections. Briefly, deparafinized and rehydrated cardiac sections were stained with 1% sirius red in saturated aqueous solution of picric acid for 90 minutes followed by wash with two changes of acidified water. Fibrosis was expressed as the percentage of stained area in a particular microscopic field. Perivascular and gap areas were excluded from the measurement of interstitial fibrosis. For the determination of Perivascular fibrosis percentage of stained area were normalized by vessel area and quantified by ImageJ.

III.5.6. WESTERN BLOTTING

Western blot analysis was carried out following the procedure described by Maniatis et al (1982). 100mg of tissue samples were homogenized in 1ml of RIPA buffer containing protease inhibitor cocktail (*Sigma*). The extracts were kept in ice with intermittent vortexing for 30min and centrifuged at 12,000rpm for 20 minutes at 4^0C. The supernatant was collected and protein concentration was determined using Coomassie protein assay reagent (*Sigma Aldrich*).

40 µg of total protein was fractionated on 10% SDS-polyacrylamide gels at 100 V and electroblotted to nitrocellulose membranes. The electrophoresis and transfer system used was the mini gel system from Biorad. At the end of transfer, membrane was taken out from the assembly and washed with deionized water and stained reversibly with ponceau S to ensure that the transfer was successful. The membrane was washed twice with Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for 5' to remove the stain. The nonspecific binding sites were blocked with 5% nonfat milk in TBS-T at room temperature. After blocking, the membrane was incubated with primary antibody solution (Santa Cruz, dilution 1:500) overnight at 4^0C in a shaker.

The membranes were then washed three times in TBS-T and incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody at room temperature for 1 hour. Immunoreactive bands were visualized using chemiluminescence detection kit (Thermo). The membrane was re-probed with anti-β actin antibody. The images were

captured on Syngene gel documentation system. Protein expression levels were normalized to β-actin.

III.5.7. MEASUREMENT OF MALONDIALDEHYDE

MDA levels were measured using the thiobarbituric acid reactive substances (TBARS) method of Buege and Aust (Buege and Aust, 1978). MDA level was assessed in cardiac tissue as well as in serum samples. The malondialdehyde concentration was determined colorimetrically from the intensity of the chromogen (color complex) formed when lipid peroxides resulting from oxidative stress reacted with thiobarbituric acid (TBA). Heart tissue was homogenized in 0.1M Tris HCl buffer (pH 7.5). 1ml of the homogenate was mixed with 2 ml of a TCA-TBA reagent (5% w/V) [TCA 0.375% (w/v) TBA, 0.25 mol/l HCl]. The complete mixture was heated for 15 minutes in boiling water bath and centrifuged at 3000 rpm for 10 minutes. The absorbance of the supernatant was determined at 535nm against a blank that contained all the reagents except the sample (tissue extract).

III.5.8. ASSAY OF PHOSPHOCREATINE (PCr) AND ADENOSINE TRIPHOSPHATE (ATP)

PCr and ATP levels were determined by the enzymatic method described by Heinz and Weiber (Heinz and Weiber, 1985). 100mg of fresh myocardial tissue was ground in 900µl of 6% ice cold Perchloric acid and centrifuged. The supernatant was neutralized with potassium bicarbonate using methyl orange as indicator and used for analysis. For

the measurement of ATP, the solutions were added to the sample in the following order. 0.4 ml 50mmol/l trietahnolamine buffer (pH 7.5), 0.01 ml 12.7 mmol/L NADP, 0.05 ml 0.01mol/l $MgCl_2$, 0.005ml 0.5 mol/l glucose and 0.005 ml 87.5 kU/l Glucose -6-phosphate dehydrogenase. The solution was mixed and read at 339 nm until a constant absorbance (A1) was obtained. 0.005 ml 70kU/l Hexokinase was added and absorbance (A2) was monitored. The change in absorbance (A2- A1) is proportional to the amount of ATP present in the sample. For the determination of Creatine phosphate, Absorbance (A1) was monitored after the addition of Hexokinase. Then 0.01 ml of 1900kU/l Creatine kinase was added to the reaction mixture and read (A2) at 339nm. The change in absorbance is directly proportional to the amount of PCr.

III.5.9. ASSAY OF PROTEIN CARBONYL

The protein carbonyl was assessed from end product of reaction between 2,4-dinitrophenylhydrazine (DNPH) with carbonyl to form a Schiff base which was analyzed spectroscopically (Levine et al., 1990). 100mg of heart tissue was minced and homogenized in 1 ml 50mM phosphate buffer (pH 7.4) containing 0.1% digitonin and incubated for 15minutes at room temperature. The contents were centrifuged at 6000g for 10 minutes at room temperature to remove debris. Inorder to remove nucleic acid, the supernatant was treated with 10% streptomycin sulphate at room temperature for 15 minutes. The sample was centrifuged at 6000g for 10minutes and supernatant was collected. Samples were taken in two vials and each was mixed with 0.4 ml 20% trichloro acetic acid (TCA), incubated at room temperature for 15 minutes and

centrifuged at 2800g for 5 minutes. To one vial 0.5ml Dinitrophenylhydrazine (0.2% in 2M HCl) was added, while to the other tube 0.5ml 2M HCl was added. The vials were kept at room temperature in dark for 1 hour. 0.5 ml 20% TCA was added and centrifuged at 3400g for 10 minutes and the supernatant was discarded. The pellet was washed three times with 1.5 ml of ethanol/ethylacetate (1:1) (v/v) to remove free DNPH and lipid contaminants. Finally the pellet was dissolved in 1.25ml 6M guanidine hydrochloride solution. The samples were read at 370nm using HCl treated sample as blank.

III.6. STATISTICAL ANALYSIS

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

IV. RESULTS

IV.1. COMPARISON OF MARKERS OF CARDIAC HYPERTROPHY, OXIDATIVE STRESS AND METABOLIC SHIFT IN SHR WITH NORMOTENSIVE WISTAR RAT

IV.1.1. Blood pressure

The cardiovascular characteristics of six months old SHR was compared with age matched normotensive Wistar rat. Evaluation of blood pressure by noninvasive tail cuff sphygmomanometer showed that systolic and diastolic blood pressure of the SHR were significantly higher ($p<0.01$) than that of the Wistar rat (Fig.9). Chronic pressure overload is regarded as the major causative factor for left ventricular hypertrophy.

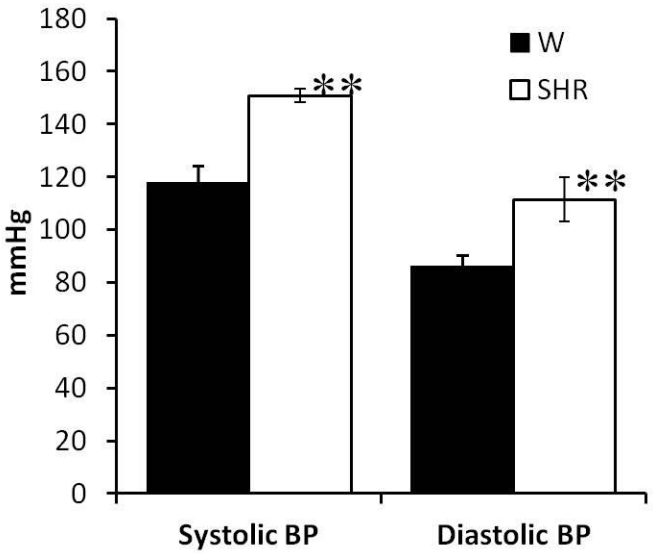

Figure 9: Systolic and diastolic blood pressure of 6 months old male SHR and normotensive Wistar (W) rat. (Data presented as Mean ± SD, n=4 per group. **p<0.01compared to Wistar rat)

IV.1.2. Expression of CD36

Metabolic changes in SHR is possibly the consequence of reduced expression of FAT/CD36. Inorder to evaluate the expression of CD36 in the stock of SHR used for the experimental studies, the protein expression was analyzed by Western blot analysis. SHR showed significantly reduced ($p<0.05$) expression of CD36 (fig.10). Immunohistochemical analysis also confirmed reduced expression of CD36 in SHR. (Fig. 11)

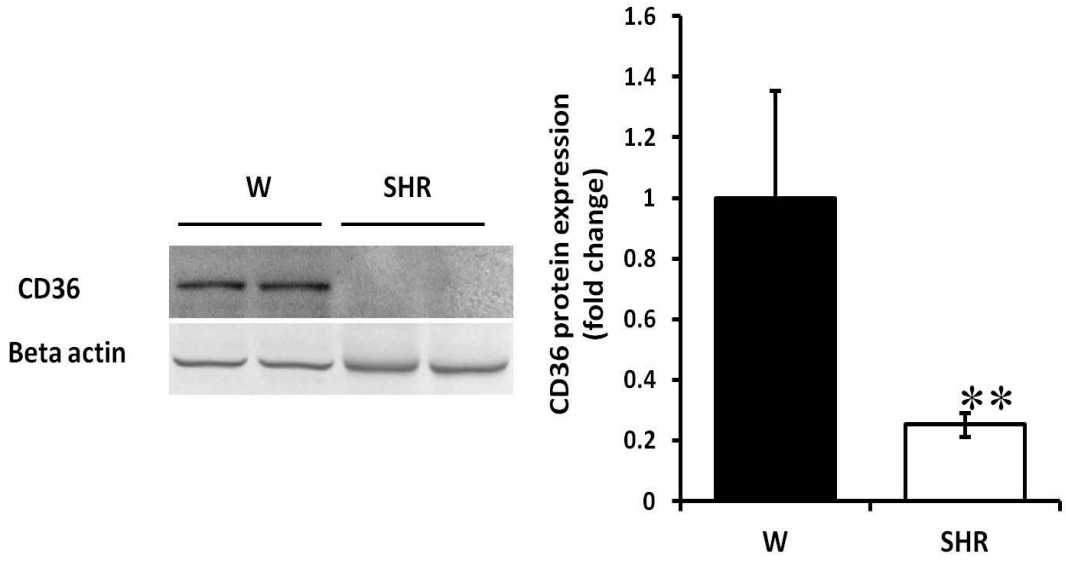

Figure 10: CD36 expression of the 6 months old SHR and normotensive Wistar (W) rat (Data presented as Mean ± SD, n=4 per group **p<0.01 compared to Wistar rat)

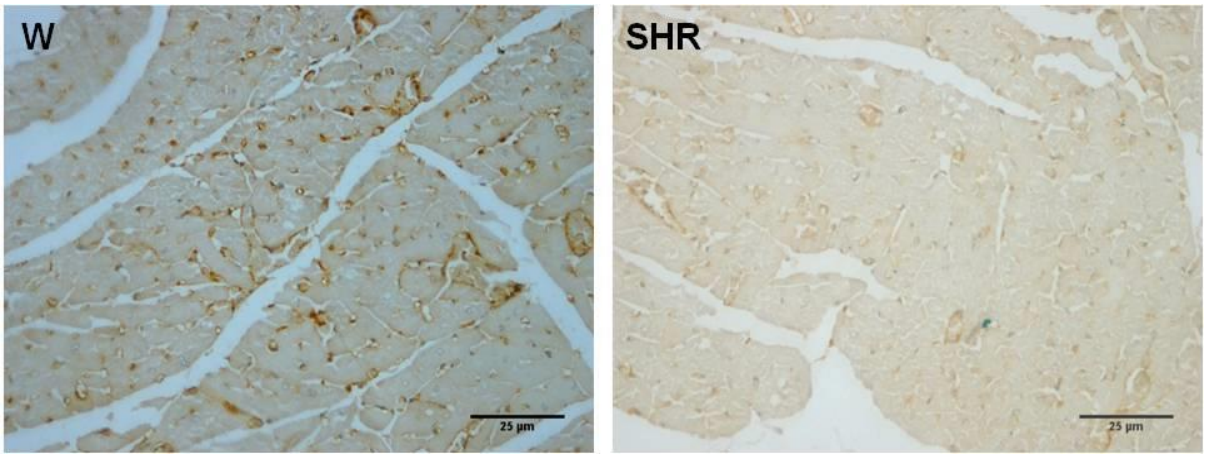

Figure 11: Immunohistochemical analysis of CD36 expression of the 6 months old SHR and normotensive wistar (W) rat – Representative photomicrographs

IV.1.3. Hypertrophy index

The ratio of heart weight (mg) to body weight (g) was used as the morphological marker of cardiac hypertrophy, and was found to be significantly higher ($p<0.05$) in SHR compared to Wistar rat (Fig:12)

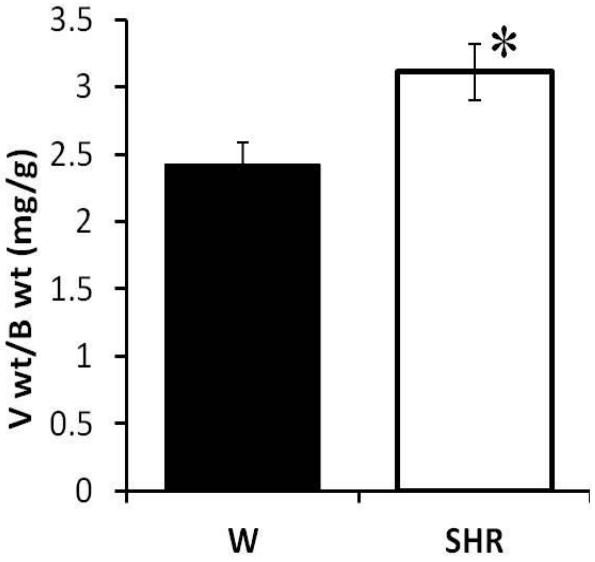

Figure 12: Hypertrophy index of the 6 months old SHR and normotensive Wistar (W) rat. (Data presented as Mean ± SD, n=4 per group. *p<0.05 compared to Wistar rat)

IV.1.4. Histological assessment for cardiac hypertrophy

Histological markers of cardiac remodeling such as myocyte cross sectional area and cardiac fibrosis were determined by Image J software. Myocyte cross sectional area was significantly higher ($p<0.01$) in SHR than Wistar rat (Fig.13). Myocardial fibrosis was measured as interstitial fibrosis and perivascular fibrosis. Extent of fibrosis for both the variables was significantly higher in SHR than that of Wistar rat (Fig. 14, 15).

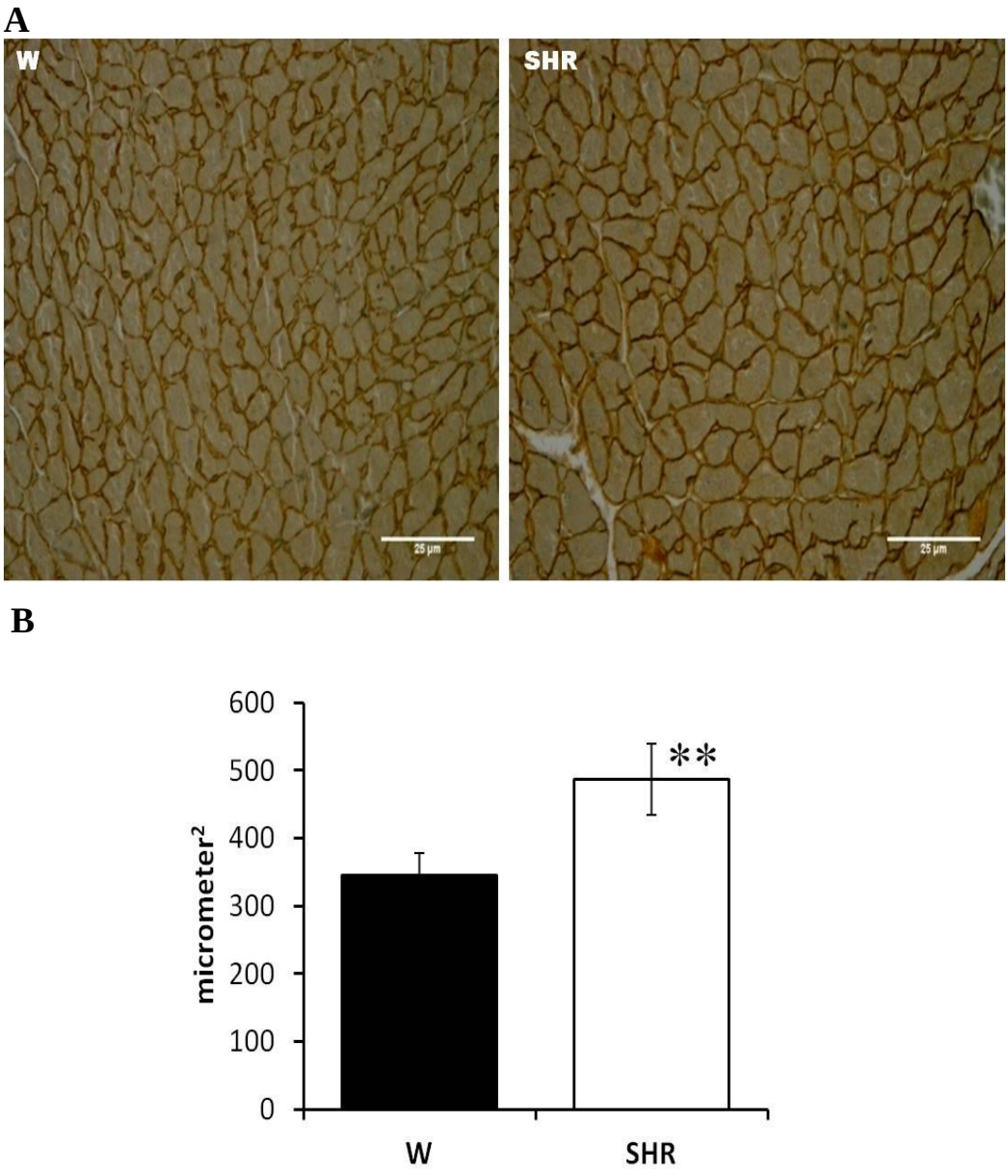

Figure 13: Myocyte cross sectional area of 6 months old SHR and normotensive Wistar (W) rat . Myocardial sections were immunostained with anti dystrophin antibody to get a clear outline of the cell membrane. (A) Representative photomicrographs. (B) Graphical representation of data. (Data presented as Mean ± SD, n=4 per group. ** $p<0.01$ compared to Wistar)

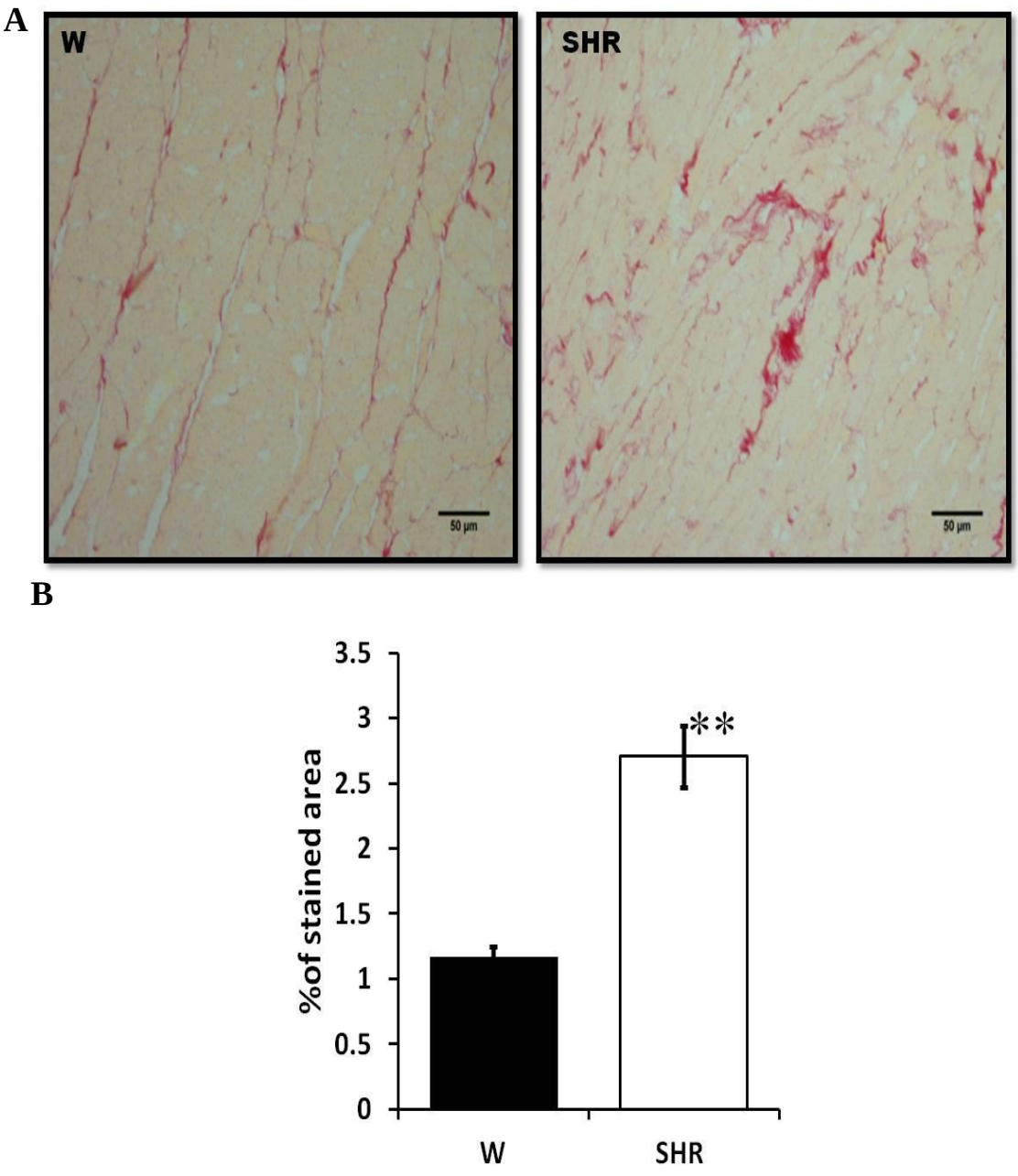

Figure 14: Myocardial interstitial fibrosis of 6 months old SHR and normotensive Wistar (W) rat . Myocardial sections were stained with Picrosirius red to visualize the interstitial fibrosis. Collagen appears red. (A) Representative photomicrographs (B) Graphical representation of data. (Data presented as Mean ± SD, n=4 per group. ** $p<0.01$ compared to Wistar)

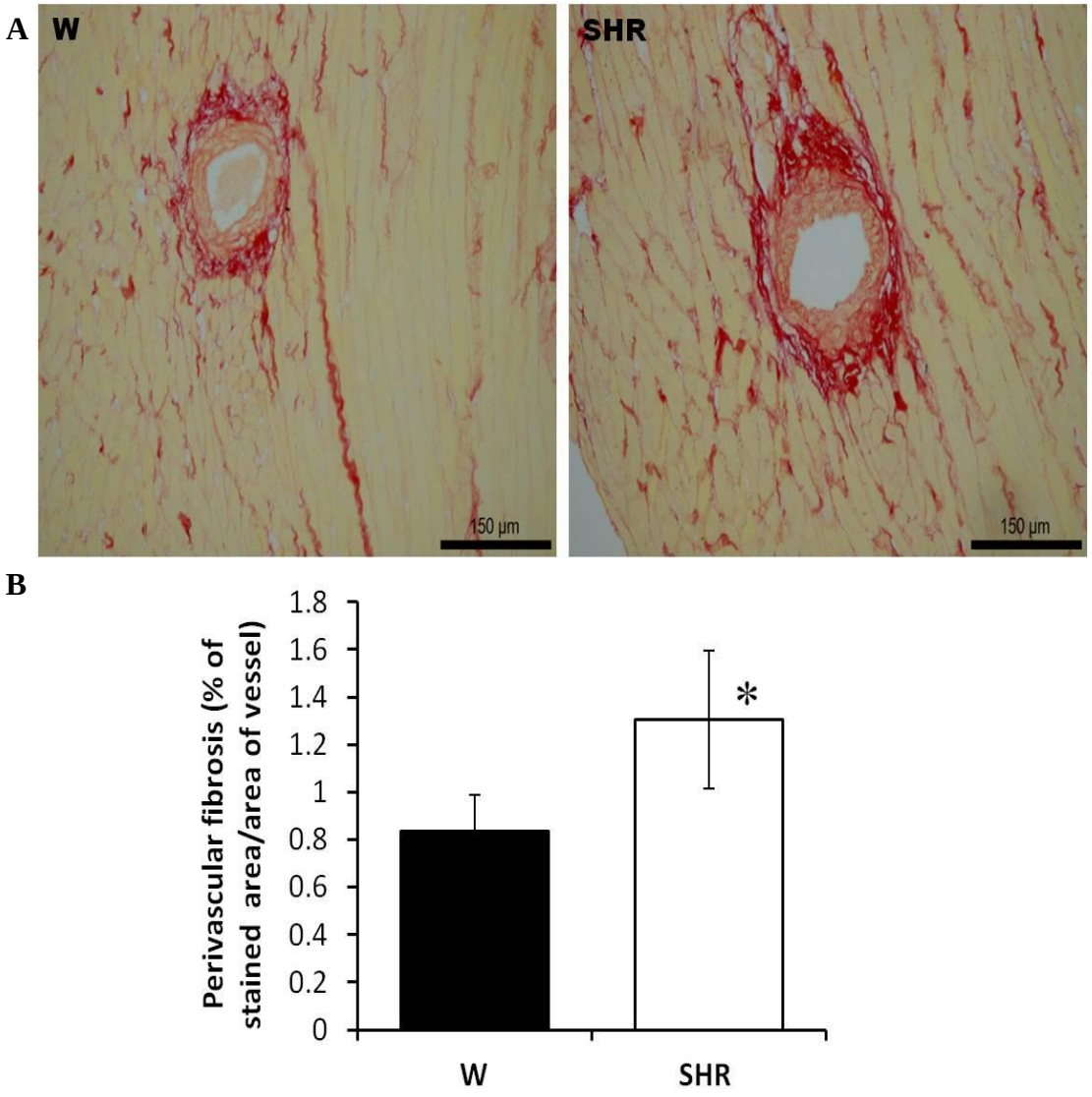

Figure 15: Myocardial perivascular fibrosis of 6 months old SHR and normotensive Wistar (W) rat. Myocardial sections were stained with Picrosyrius red to visualize the interstitial fibrosis. Collagen appeares red. (A) Representative photomicrographs. (B) Graphical representation of data. (Data presented as Mean ± SD, n= 4 per group * $p<0.05$ compared to Wistar)

IV.1.5. Molecular markers of hypertrophy

The mRNA expression of Brain natriuretic peptide (BNP) and protein expression of Calcineurin A were used as the molecular marker of cardiac remodeling. SHR heart showed significant increase in mRNA expression of BNP by Real Time PCR analysis (Fig.16). Western blotting analyses showed that the expression of Calcineurin A was significantly higher in SHR. (Fig. 17)

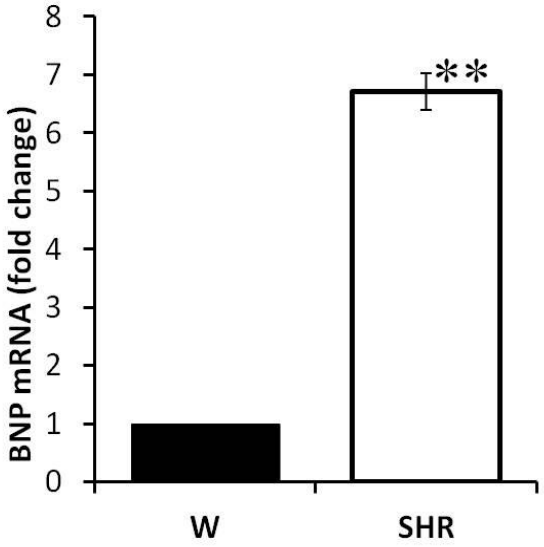

Figure 16: mRNA expression of BNP in 6 months old SHR and normotensive Wistar (W) rat determined by Real time PCR. (Data presented as Mean ± SD of the fold change in SHR compared to Wistar, n= 4 per group. **p<0.01 compared to Wistar)

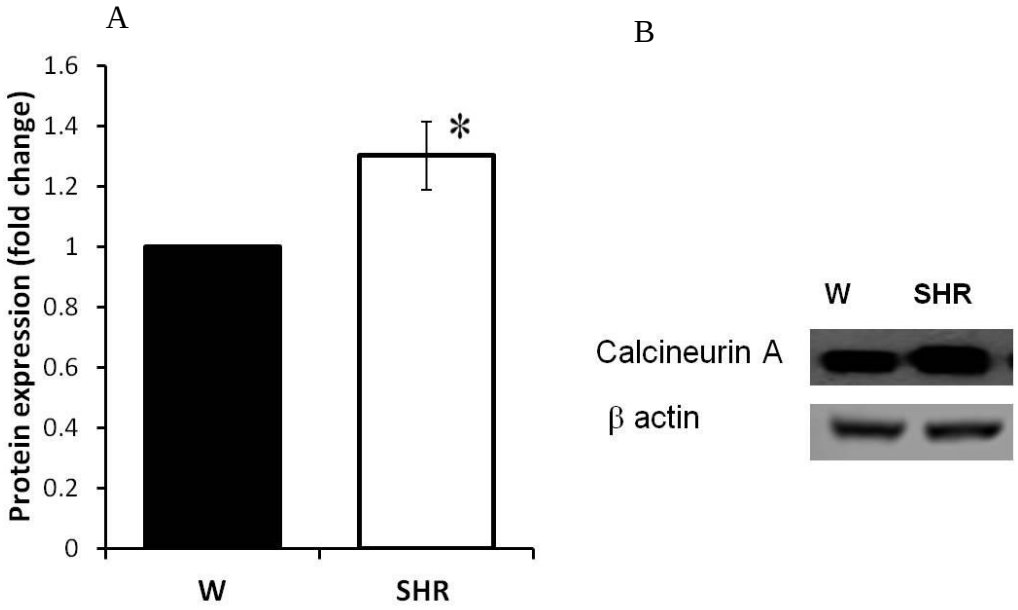

Figure 17: Protein expression of Calcineurin A in 6 months old SHR and normotensive Wistar (W) rat determined by Western blotting (A) Graphical presentation of data. (B) Representative blots showing expression of Calcineurin A and the loading control βactin (Data presented as Mean ± SD of the fold change in SHR compared to Wistar n= 4 per group, ∗p<0.05 compared to Wistar)

IV.1.6. Cardiac energy metabolism

The mRNA expression of PPAR-α, MCAD and PFK-1 were used as the molecular markers of cardiac metabolism. mRNA expression was evaluated by Real time PCR analysis. SHR showed significantly decreased expression of genes involved in fatty acid metabolism such as PPAR-α and MCAD ($p<0.01$) (Fig.18) and increased expression of the glycolytic enzyme PFK-1 ($p<0.05$) (Fig.19). The observations confirm the presence of shift in substrate preference from fatty acid to glucose in SHR.

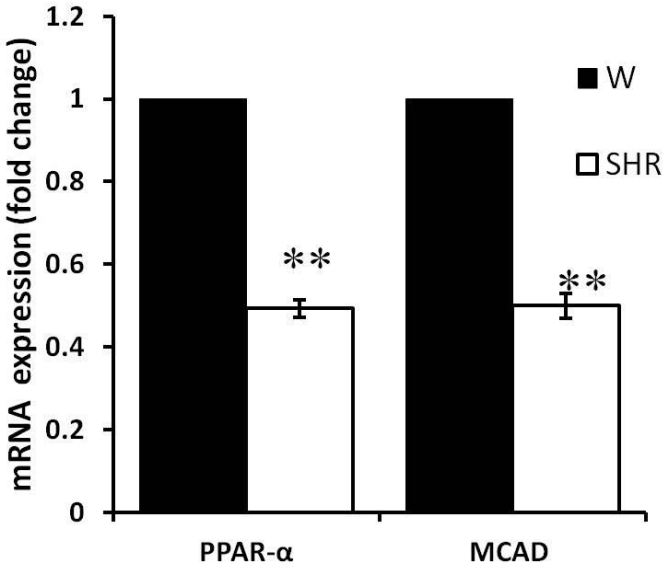

Figure 18: mRNA expression of PPAR-α and MCAD of 6 months old SHR and normotensive wistar (W) rat determined by Real time PCR (Data presented as Mean ± SD of the fold change in SHR compared to Wistar, n=4 per group. **p<0.01 compared to Wistar)

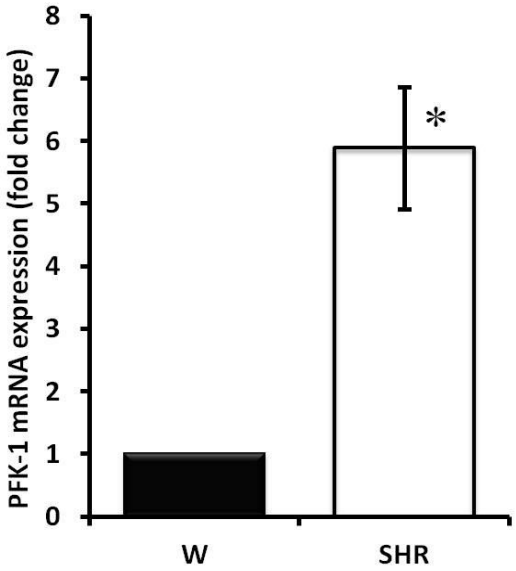

Figure 19: mRNA expression of PFK-1in 6 months old SHR and normotensive Wistar (W) rat determined by Real time PCR. (Data presented as Mean ± SD of the fold change in SHR compared to Wistar, n=4 per group. *p<0.05 compared to Wistar).

IV.1.7. Myocardial oxidative stress

Myocardial oxidative stress was evaluated by biochemical estimation of the end product of oxidative modification such as malonedialdehyde and protein carbonyl. Immunohistochemical analysis was used to measure the extent of myocardial 3-nitrotyrosine level. All the markers of oxidative stress such as malonedialdehyde, protein carbonyl (Fig.20), and 3-nitrotyrosine (Fig.21), were significantly higher ($p<0.05$) in SHR than wistar rat.

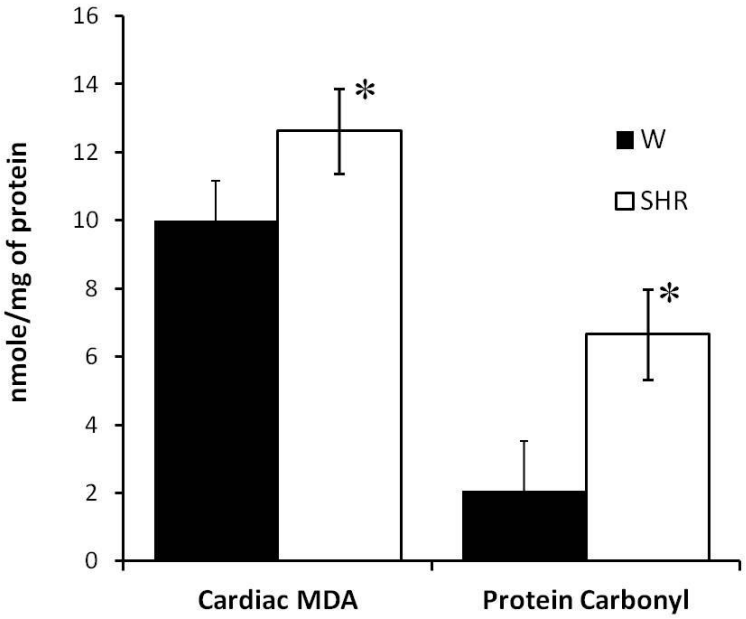

Figure 20: Cardiac malondialdehyde (MDA) and protein carbonyl levels of 6 months old SHR and normotensive Wistar (W) rat. (Data presented as Mean ± SD, n= 4 per group. ∗p<0.05 compared to Wistar)

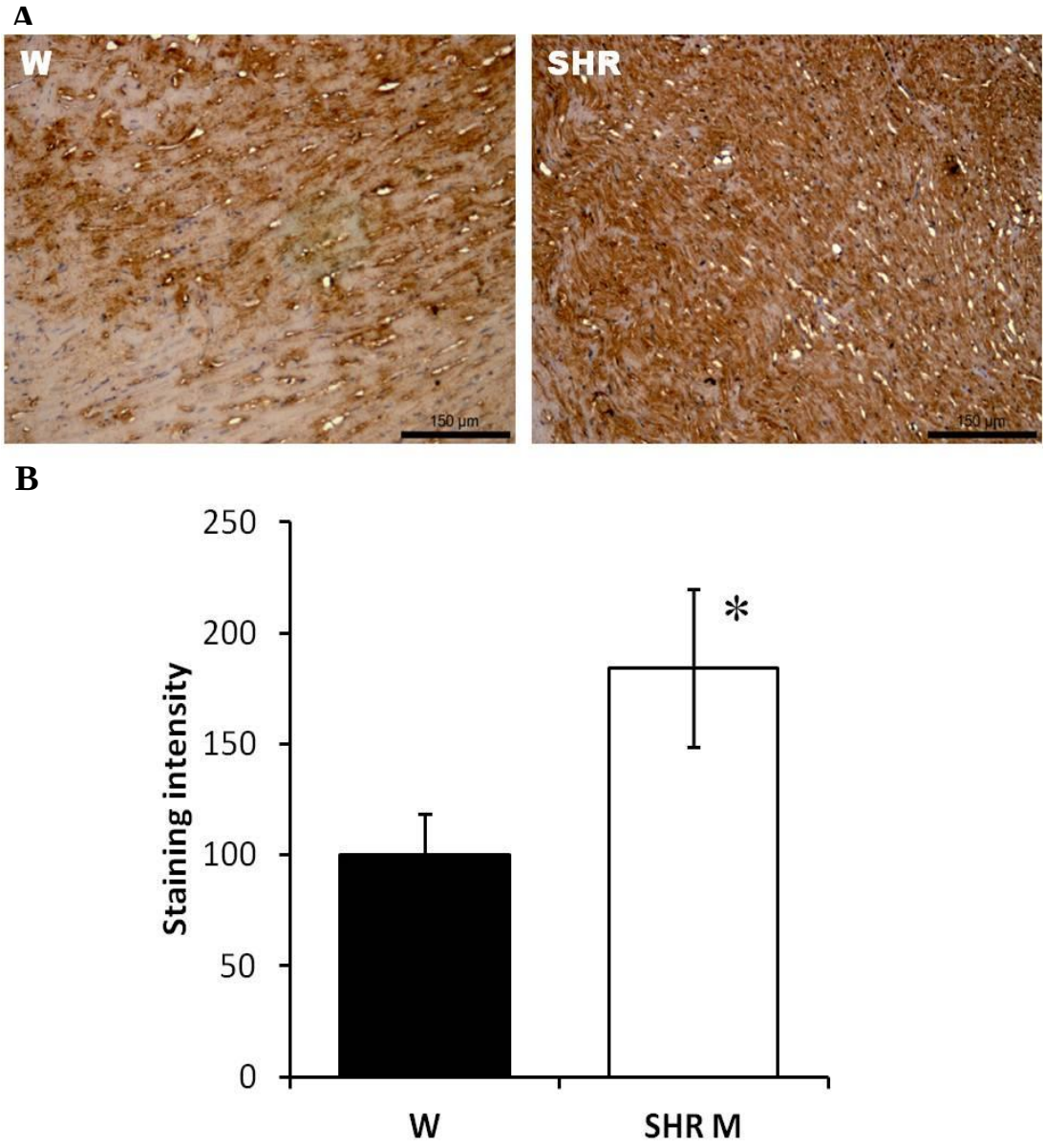

Figure 21: Myocardial 3-nitrotyrosine levels of 6 months old SHR and normotensive wistar (W) rat determined by immunohistochemistry. (A). Representative photomicrographs (B). Graphical representation of data. (Data presented as Mean ± SD, n= 4 per group. * $p<0.05$ compared to Wistar)

IV.2. CARDIAC RESPONSE TO EVEN CHAIN AND ODD CHAIN MCT SUPPLEMENTATION IN YOUNG SHR

MCT is available in two different forms, Even chain MCT and Odd chain MCT with difference in their metabolic properties. A pilot study was therefore carried out to select the type of triglyceride that would be relatively more effective. For this experiment, 2-month-old male SHR were supplemented with either 5% Tricaprylin or Triheptanoate (v/w) of the total feed for 2 months. Morphological and histological markers of cardiac hypertrophy and markers of myocardial oxidative stress were analyzed. SHR showed variable cardiac response to Even and Odd chain MCT. Hypertrophy index was significantly decreased ($p<0.05$) by Even chain MCT, but it was unaffected by Odd chain MCT (Fig.22). Myocyte cross sectional area and interstitial fibrosis were used as the histological markers of cardiac remodeling. Although cardiomyocyte cross sectional area was significantly decreased ($p<0.01$) by both types of MCT (Fig.23), interstitial fibrosis was significantly decreased ($p<0.01$) only with even chain MCT (Fig.24), Interstitial fibrosis was unaffected by odd chain MCT. There was no significant difference in the myocyte cross sectional area between Even chain and Odd chain MCT supplemented SHR. Myocardial malonedialdehyde was used as the marker of oxidative stress, and was found to be decreased by both types of MCT ($p<0.01$), with the extent of decrease being greater for even chain MCT (Fig.25). MCT did not induce obesity as assessed from the body weight in the treated and untreated groups (Fig.26). Supplementation of both type of MCT did not affect serum lipid profile of the SHR,

assessedby biochemical estimation of total cholesterol, triglyceride, HDL cholesterol and LDL cholesterol (Fig.27).

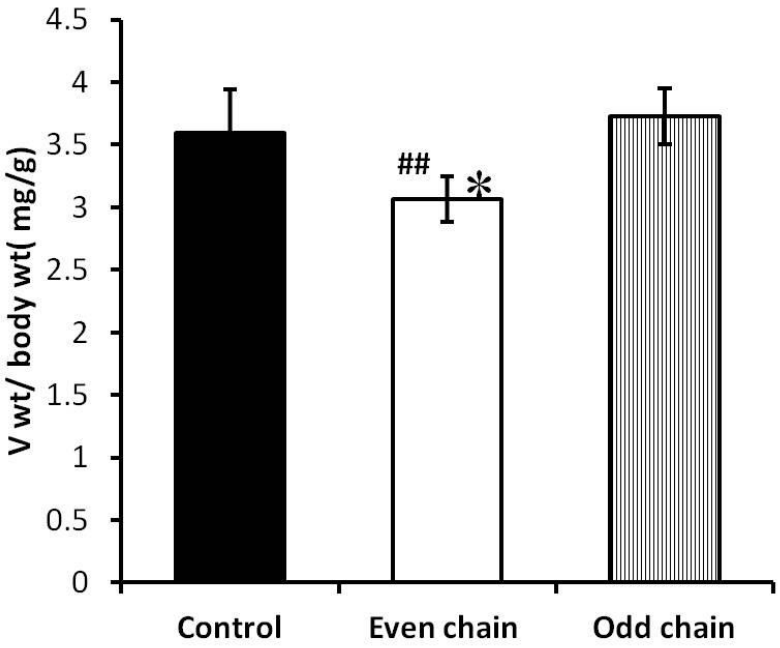

Figure 22: Hypertrophy index of 4 month old SHR following 60 days of supplementation with Even chain (Tricaprylin) or Odd chain (Triheptanoate) MCT (5% of the feed) compared with age and sex matched untreated SHR. (Data presented as Mean ± SD, n= 4 per group, ANOVA $p< 0.05$ $*p<0.05$ Even chain vs. Control, $^{##}p<0.01$ Even chain vs. Odd chain).

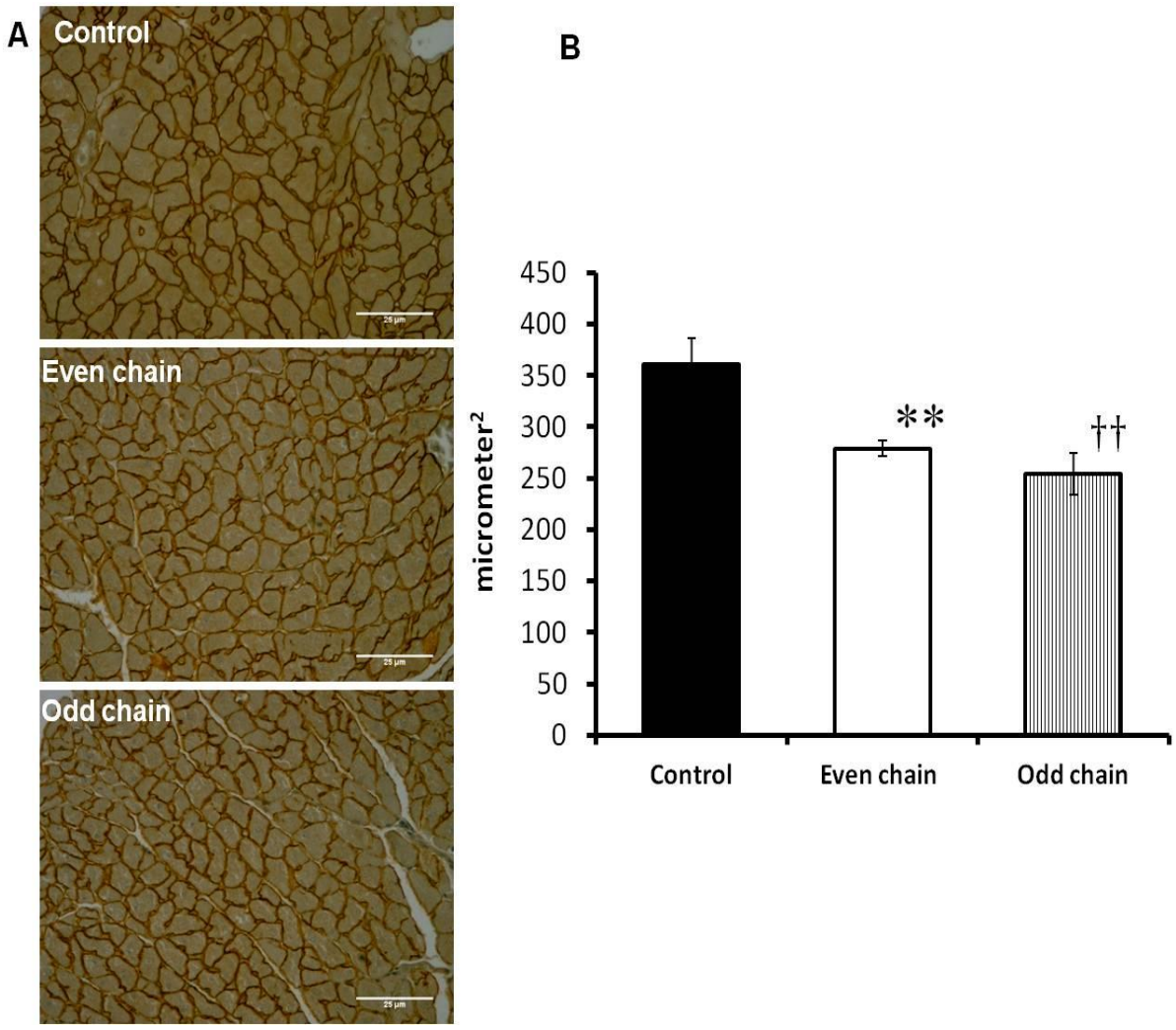

Figure 23: Myocyte cross sectional area of 4 month old SHR following 60 days of Even chain or Odd chain MCT supplementation (5% of the feed) compared with age and sex matched untreated SHR. (A) Representative photomicrographs. (B) Graphical representation of data. (Data presented as Mean ± SD, n= 4 per group, ANOVA $p< 0.05$ **$p<0.01$ Even chain vs. control, $^{\dagger\dagger}p<0.01$ odd chain vs. control)

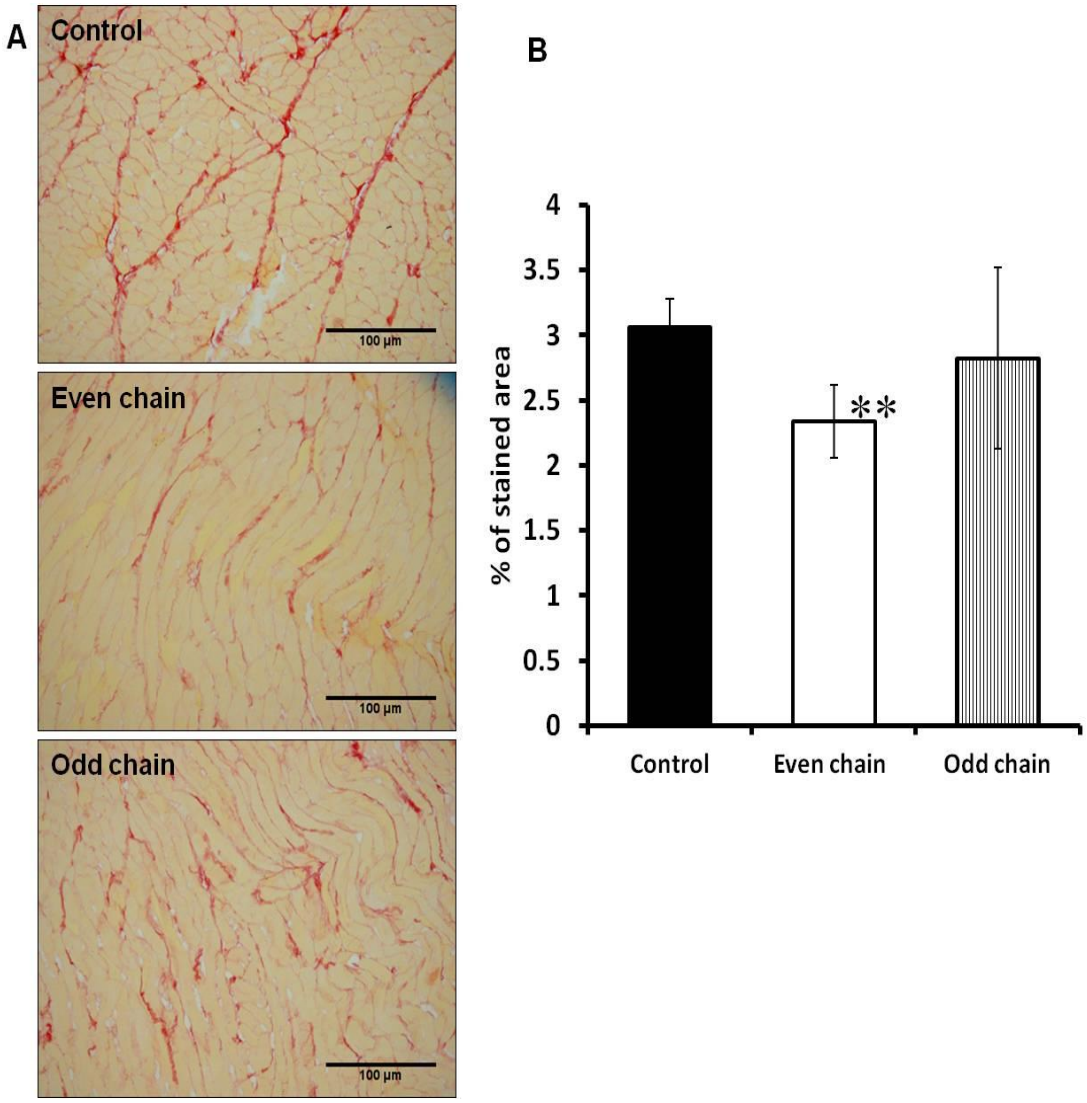

Figure 24: Myocardial interstitial fibrosis of 4 month old SHR following 60 days of Even chain or Odd chain MCT supplementation (5% of the feed) compared with age and sex matched untreated SHR. Myocardial sections were stained with Picro Siriusred, to visualize the collagen. Collagen appears red. (A) Representative photomicrographs. (B) Graphical representation of data. (Data presented as Mean ± SD, n= 4 per group, ANOVA $p< 0.05$, $^{**}p<0.01$ Even chain vs. control).

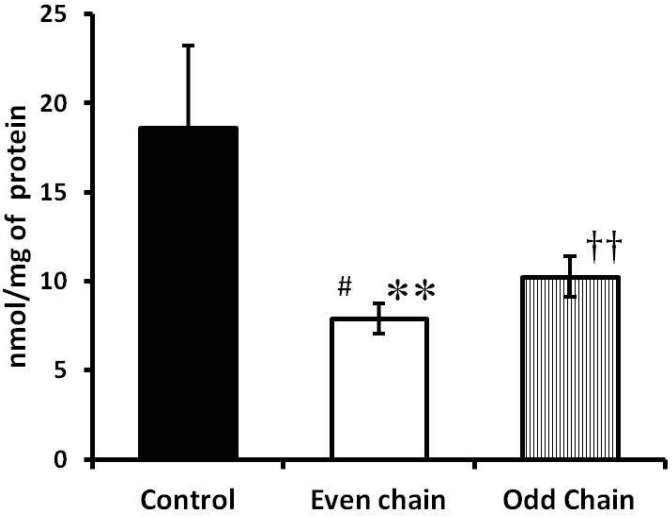

Figure 25: Myocardial Malone dialdehyde levels of 4 month old SHR following 60 days of supplementation with Even chain or Odd chain MCT (5% of the feed) compared with age and sex matched untreated SHR. (Data presented as Mean ± SD, n= 4 per group, ANOVA $p< 0.05$ $^{**}p<0.01$ Even chain vs. control, $^{††}p<0.01$ odd chain vs. control, $^{\#}p<0.05$ Even chain vs. odd chain)

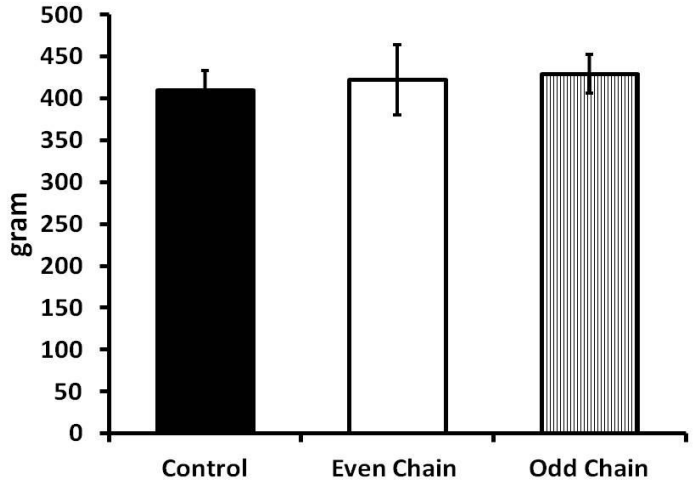

Figure 26: Body weight of 4 month old SHR following 60 days of supplementation with Even chain or Odd chain MCT (5% of the feed) compared with age and sex matched untreated SHR. (Data presented as Mean ± SD, n= 4 per group, ANOVA p=NS)

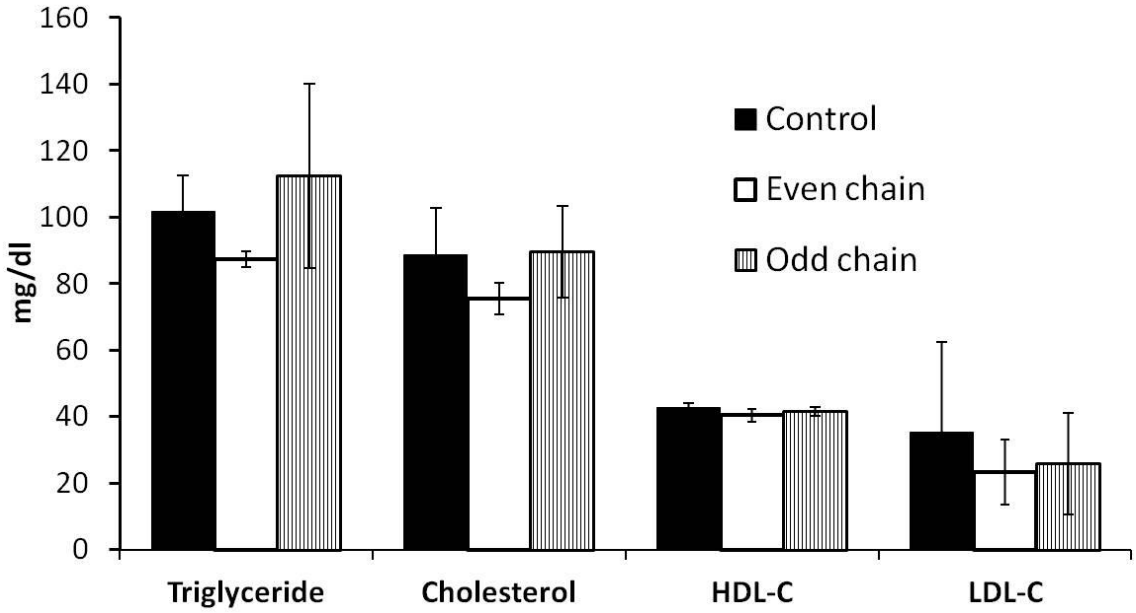

Figure 27: Serum lipid profile of 4 month old SHR following 60 days of supplementation with Even chain or Odd chain MCT (5% of the feed) compared with age and sex matched untreated SHR. (Data presented as Mean ± SD, n= 4 per group, ANOVA p= NS)

IV.3. CARDIAC RESPONSE TO STIMULATION OF FATTY ACID OXIDATION BY SUPPLEMENTATION OF MEDIUM CHAIN TRIGLYCERIDES AT INITIAL AND ESTABLISHED STAGES OF CARDIAC HYPERTROPHY

Male Spontaneously hypertensive rats (SHR) of two different age groups representing the initial and established stages of hypertrophy were used as the experimental model to stimulate fatty acid metabolism. The even chain fatty acid Tricaprylin was used for the experiments as the cardiac response was found to be relatively better than that of the odd chain fatty acid Triheptanoate. 2-months-old (initial stages of hypertrophy) and 6-months-old (established hypertrophy) SHR were supplemented with Medium chain

triglycerides (MCT) for 4 months to prevent hypertrophy in the former and regress cardiac remodeling in the latter group. The animals were sacrificed respectively at 6 months and 10 months of age for evaluation of the cardiovascular response to treatment. Cardiac response to MCT supplementation was evaluated based on cardiac morphology and the expression of genes associated with fatty acid oxidation (FAO). Hypertrophy index, cell size, morphometric analysis of ventricular collagen, expression of brain natriuretic peptide (BNP) and calcineurin A were used as markers of adverse cardiac remodeling. Myocardial malondialdehyde (MDA- a stable terminal metabolite of lipid peroxidation), 3nitrotyrosine and protein carbonyl (end product of protein oxidation) were used as markers of oxidative stress. Cardiac fatty acid oxidation was evaluated based on the expression of PPAR-α and medium chain acyl CoA dehydrogenase (MCAD -a key enzyme in β-oxidation of fatty acids) by Real time RT-PCR analysis. The energy level was evaluated from biochemical estimation of ATP and phosphocreatine.

IV.3.1. Effect of MCT on myocardial metabolism

To modulate the myocardial metabolism in SHR with early and established stages of cardiac hypertrophy 2 months and 6 months old SHR were supplemented with 5% MCT along with standard feed for 4 months. The effect of MCT on myocardial metabolism was determined from the mRNA expression of PPAR-α, MCAD and PFK-1 by Real time PCR analysis. PPAR-α and MCAD served as the molecular indicators for the stimulation of fatty acid oxidation in SHR. PPAR-α (*$p<0.01$) and MCAD (*$p<0.05$)

were significantly increased in initial and established stages of cardiac hypertrophy by supplementation of MCT (Fig.28,29). Increased PPAR-α expression along with increased MCAD mRNA indicates stimulation of fatty acid oxidation. The consequence of MCT supplementation on glucose oxidation was determined by mRNA expression of PFK-1. Expression of PFK-1 was not significantly affected by the treatment (Fig.30).

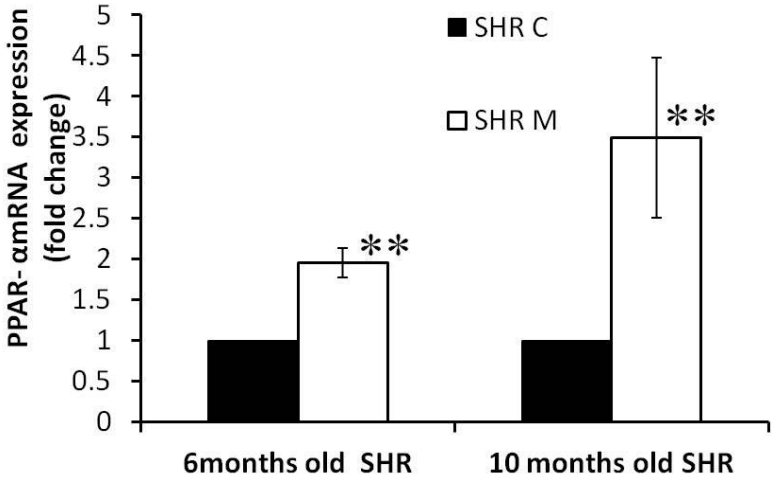

Figure 28: Fold change in the mRNA expression of PPAR-α in 6 and 10 months old SHR following 4 months of supplementation with MCT (5% of the feed) (SHR M) compared with age and sex matched untreated control (SHR C). (Data expressed as Mean ± SD of the fold change in treated compared to untreated SHR, n = 6/group; **p<0.01 compared to untreated control)

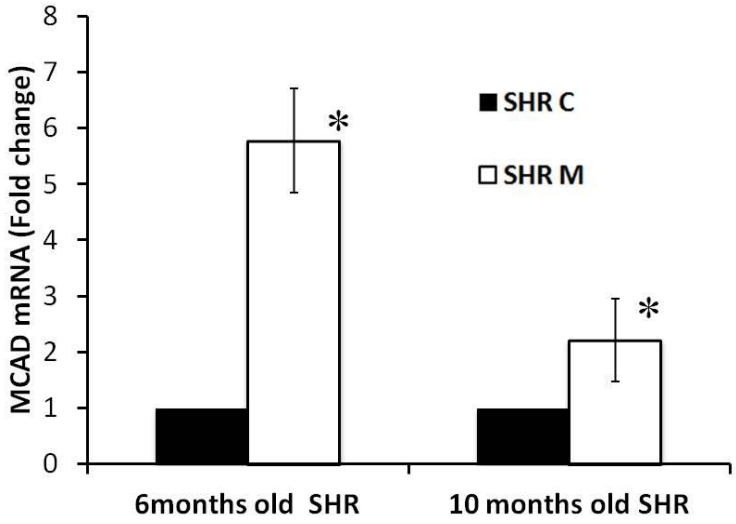

Figure 29: Fold change in the mRNA expression of MCAD in 6 and 10 months old SHR following 4 months supplementation with MCT (5% of the feed) compared with age and sex matched untreated control. (Data expressed as Mean ± SD of the fold change in treated (SHR M) compared to untreated SHR (SHR C), n = 6/group; *p<0.05 compared to untreated control)

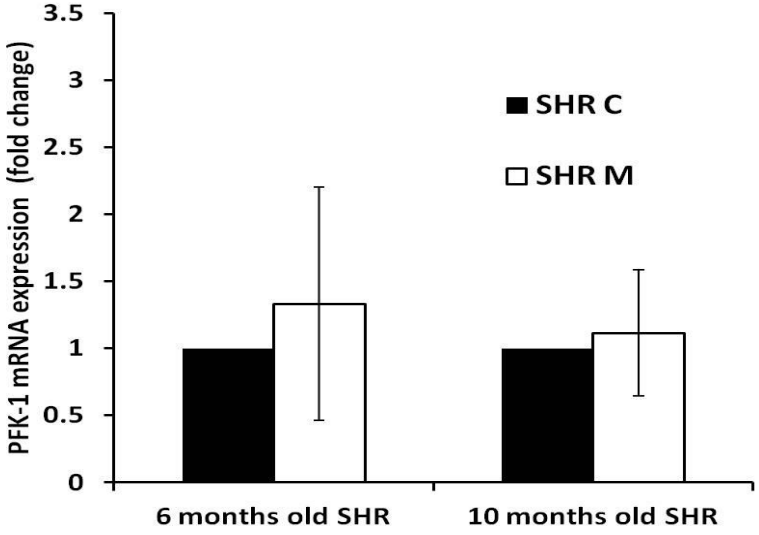

Figure 30: Fold change in the mRNA expression of PFK-1 in 6 and 10 months old SHR following 4 months of supplementation with MCT (5% of the feed) (SHR M) compared with age and sex matched untreated control (SHR C). (Data expressed as Mean ± SD of the fold change in treated compared to untreated SHR, n = 6/group)

IV.3.2. Cardiovascular response to stimulation of fatty acid oxidation

A. Blood pressure:

Blood pressure was monitored following 4 months of treatment. Irrespective of the age of the animal, supplementation of MCT did not cause any change in systolic or diastolic blood pressure in both the age groups. (Table 2)

Table 2: Mean (± SD) blood pressure of 6 and 10 months old SHR following 4 months of supplementation with MCT (5% of the feed) (SHR M) compared with age and sex matched untreated control (SHR C) (n=6/group)

Blood pressure	6 months old SHR		10 months old SHR	
	SHR C	SHR M	SHR C	SHR M
Systolic Blood Pressure (mm Hg)	150±2	151±3	183±6	187±8
Diastolic Blood Pressure (mm Hg)	114±8	117±6	126±5	129±8

B. Hypertrophy index:

Hypertrophy index was used as the morphological indicator of cardiac hypertrophy, represented as the ratio of heart weight to body weight. Reactivation of fatty acid metabolism by MCT significantly decreased hypertrophy index ($p<0.05$) in younger rat and was maintained in older rats in comparison with age-matched control SHR (Fig.31).

There was no difference in the body weight between control and the MCT treated group. The average body weight was 341±20 g and 367± 33 g respectively for control and treated 6 months old animals and 412±17 g and 400±34 g respectively for control and treated 10 months old animals indicating that the treatment did not induce obesity.

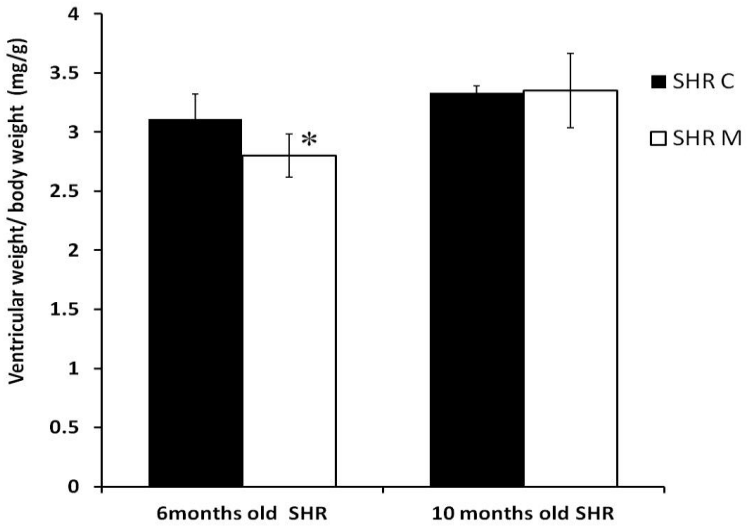

Figure 31: Hypertrophy index [(ventricular weight (mg)/ body weight (g)] of 6 and 10 months old SHR following 4 months of supplementation with MCT (5% of the feed) (SHR M) compared with age and sex matched untreated control (SHR C) (Data represented as mean ± SD, n= 6 /group, *p<0.05 compared to untreated control)

C. ***Myocyte cross sectional area and myocardial fibrosis:***

Increased cardiomyocyte cross sectional area and interstitial fibrosis are the histological features of myocardial hypertrophy. The sarcolemma of the individual myocytes was visualized by immunohistochemical staining for dystrophin and picro sirius red staining was used for assessment of fibrosis. The mean cross sectional area of cardiomyocytes and interstitial fibrosis decreased significantly with treatment in both the age groups (Fig.32,33). Perivascular fibrosis was unaffected in either of the age groups in response to MCT supplementation (Fig. 34).

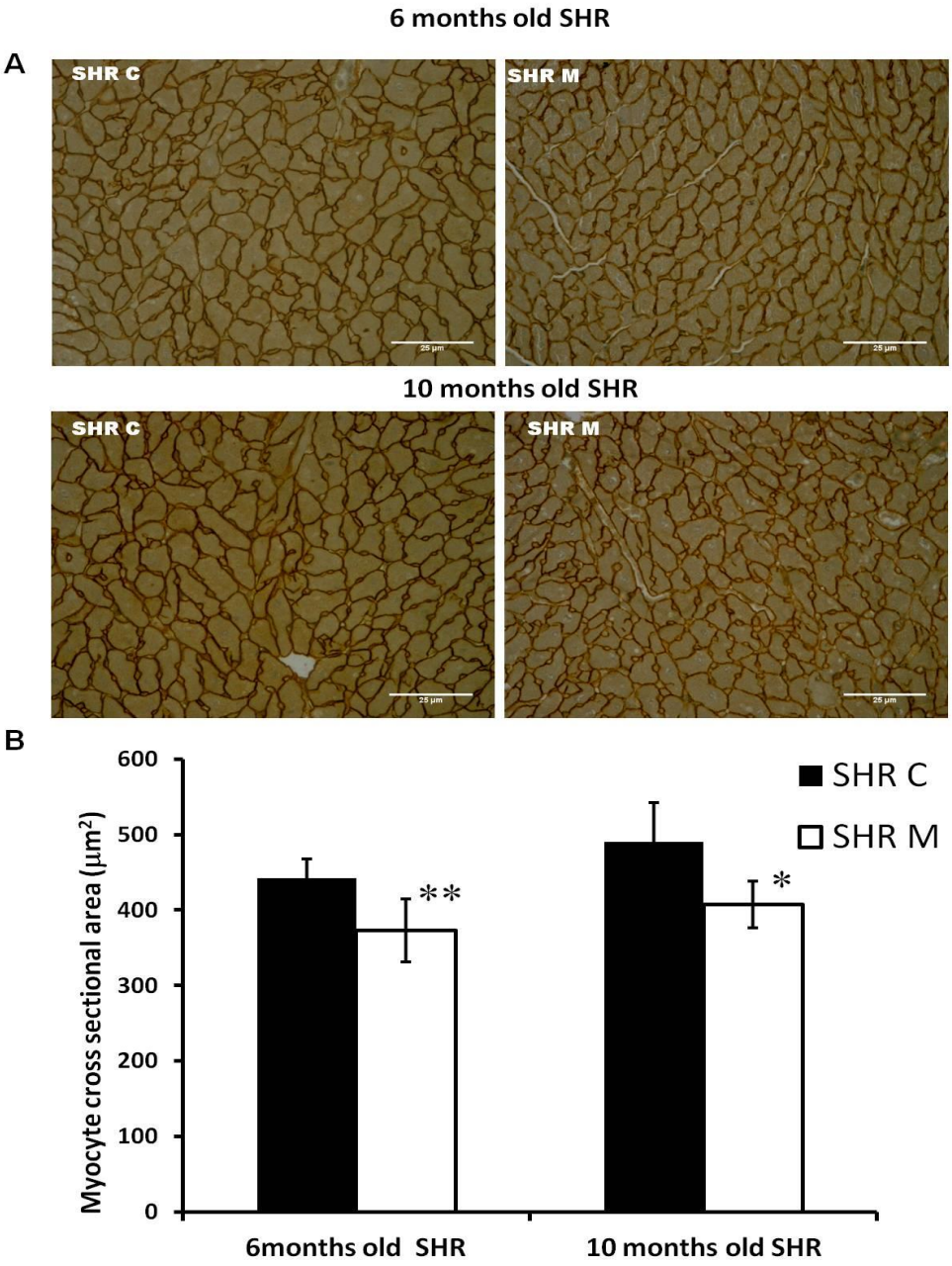

Figure 32: Myocyte cross sectional area of 6 and 10 months old SHR following 4 months of supplementation with MCT (5% of the feed) (SHR M) compared with age and sex matched untreated control. (SHR C)Mid ventricular cross sections were immunostained with dystrophin to get a clear outline of the cells. (A) Representative photomicrographs. (B) Graphical representation of data. (Data presented as Mean ± SD, n=6/group, ** $p<0.01$ and * $p<0.05$ compared to untreated control).

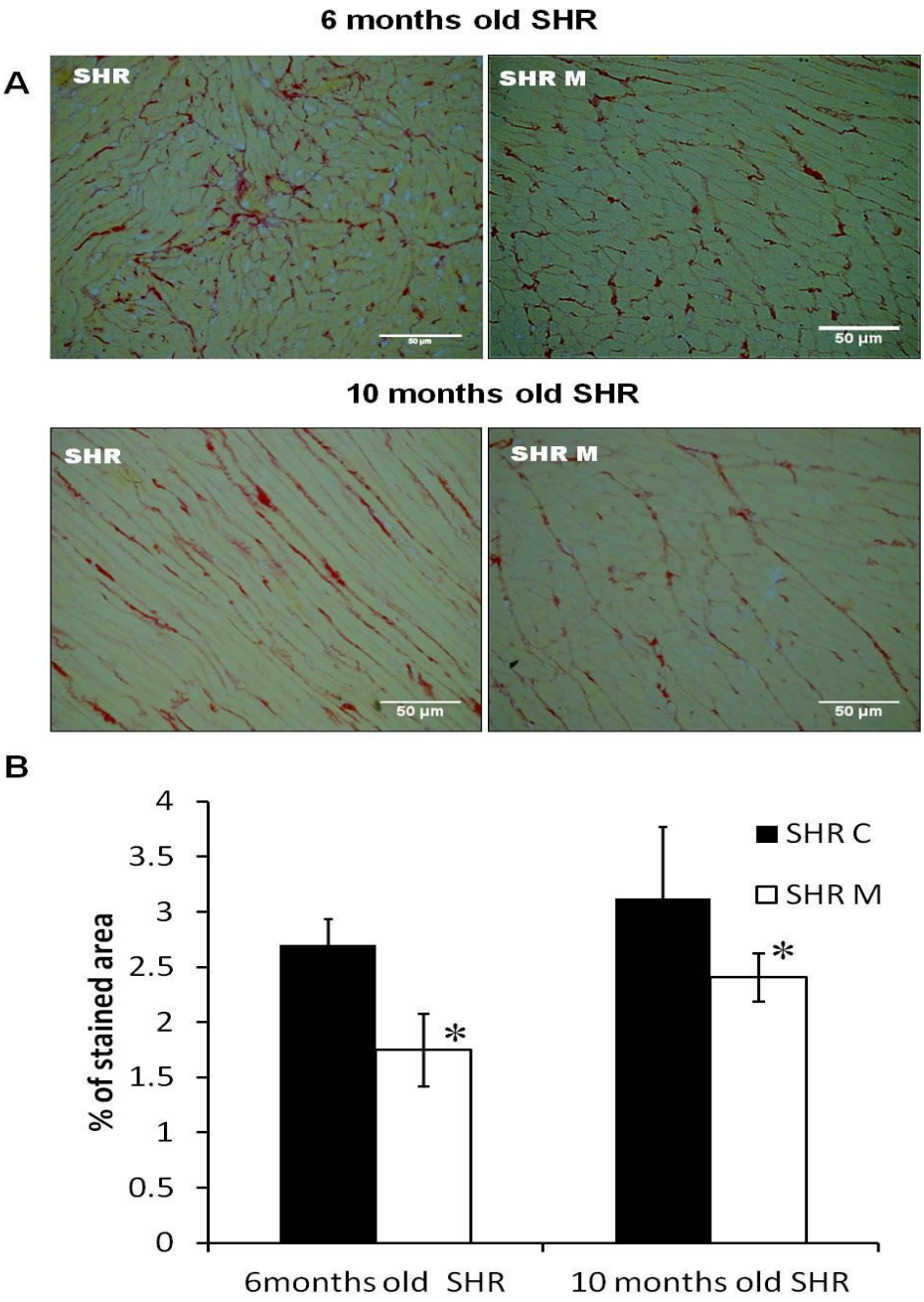

Figure 33: Interstitial fibrosis of 6 and 10 months old SHR following 4 months of supplementation with MCT (5% of the feed) (SHR M) compared with age and sex matched untreated control (SHR C) For the visualization of the fibrosis myocardial sections were stained with Picrosirius red. Collagen appears red. (A) Representative photomicrographs. (B) Graphical representation of data. (Data presented as Mean ± SD, n= 6/group. * $p<0.05$ compared to untreated control)

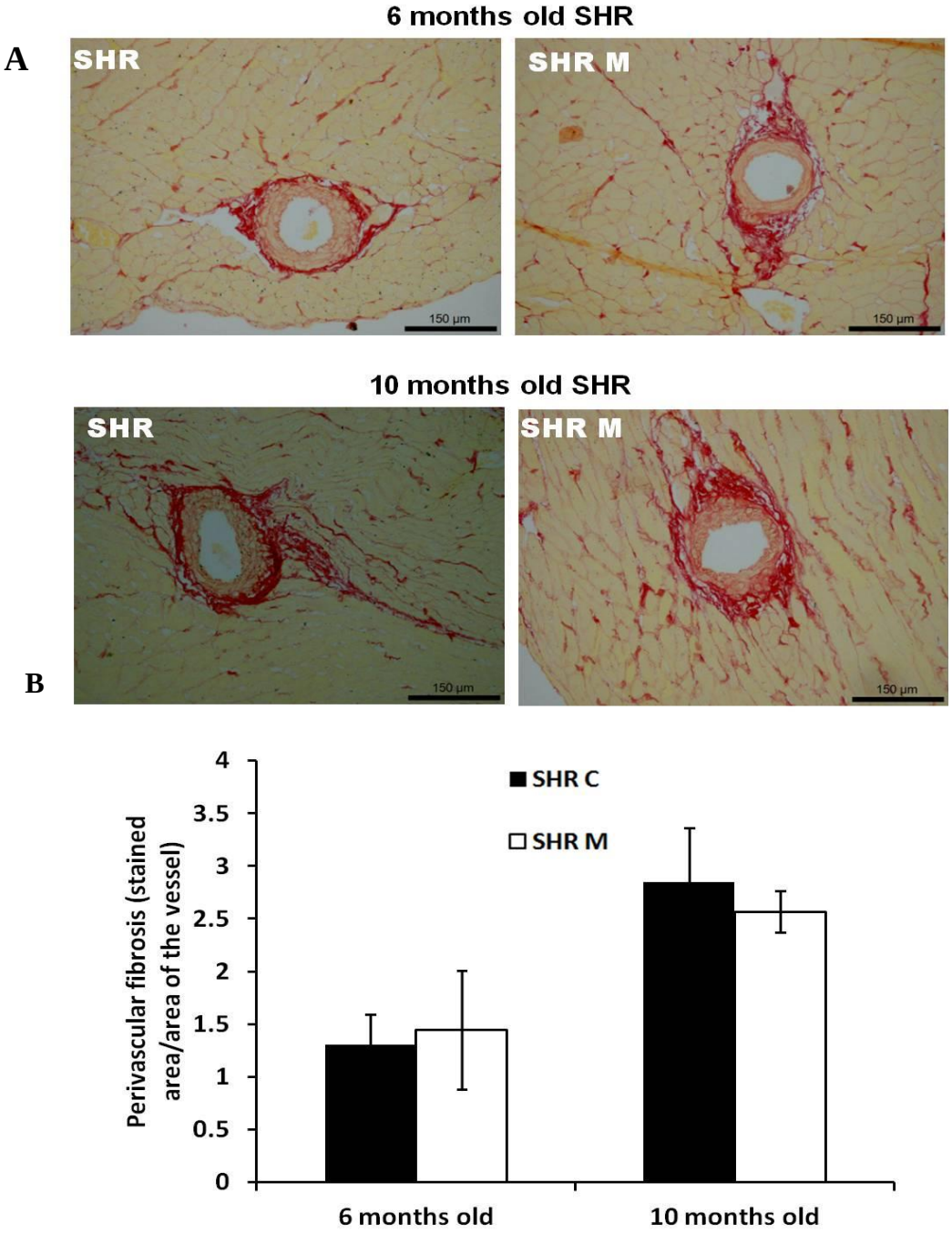

Figure 34: Perivascular fibrosis in 6 and 10 months old SHR following 4 months of supplementation with MCT (5% of the feed) compared with age and sex matched untreated control. For the visualization of the fibrosis myocardial sections were stained with Picrosirius red. Collagen appears red. (A) Representative photomicrographs. (B) Graphical representation of data. (Data presented as Mean ± SD, n= 6/group.)

D. *Expression of molecular markers of adverse cardiac remodeling*:

The mRNA expression of Brain natriuretic peptide (BNP) and protein expression of calcineurin A were used as markers for adverse cardiac remodeling. BNP and Calcineurin A were significantly decreased following reactivation of fatty acid oxidation in both the age groups (Fig 35,36).

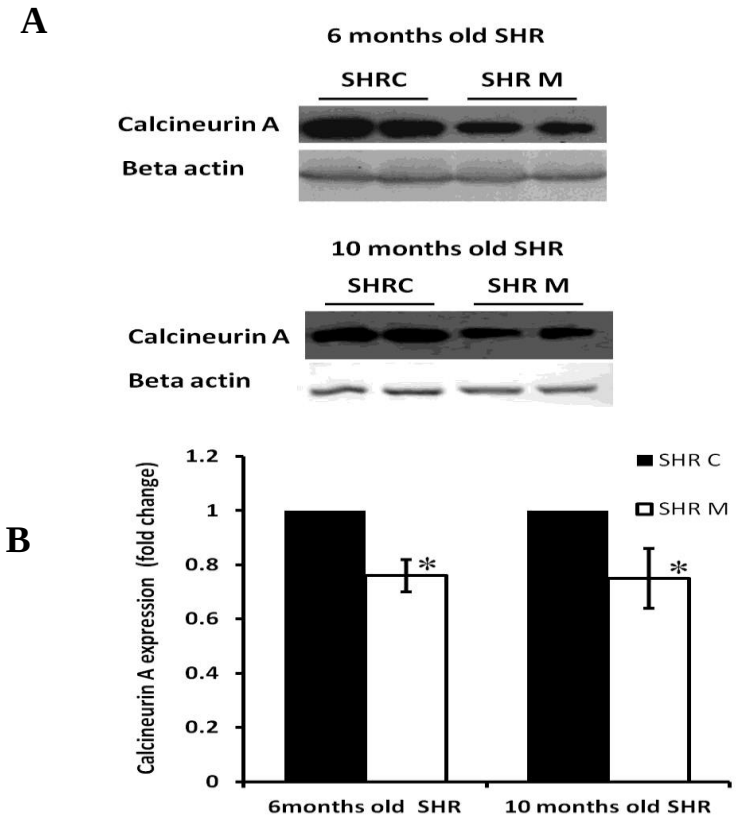

Figure 35: Western blot analysis of myocardial calcineurin A expression in 6 and 10 months old SHR following 4 months of supplementation with MCT (5% of the feed) (SHRM) compared with age and sex matched untreated control (SHRC) (A) Representative blots showing expression of Calcineurin A and the loading control β actin. (B) Graphical presentation of data. (Data presented as Mean ± SD, of the fold change in SHR M compared to SHRC, n=6/ group. ∗p<0.05 compared to SHRC).

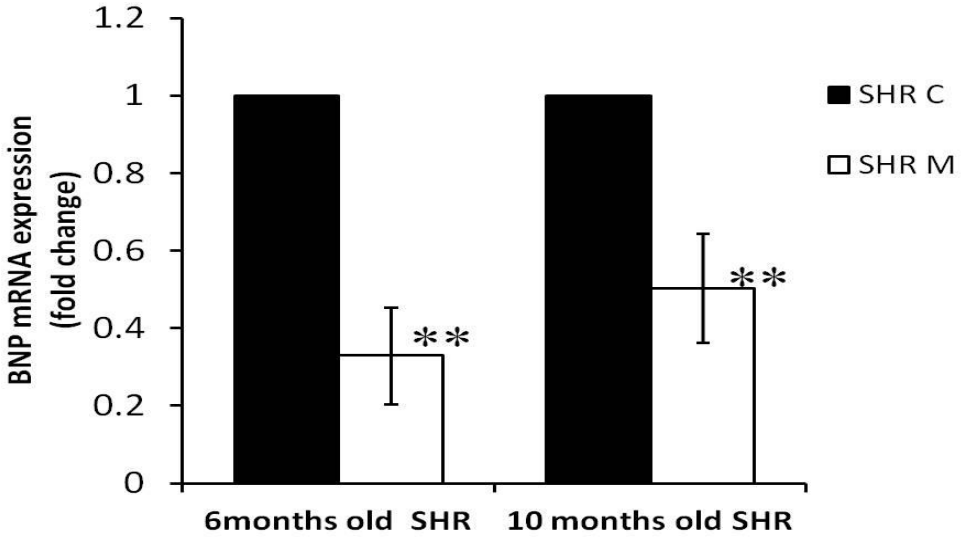

Figure 36: Fold change in BNP mRNA expression in 6 and 10 months old SHR following 4 months of supplementation with MCT (5% of the feed) (SHRM) compared with age and sex matched untreated control. (Data expressed as mean± SD of fold change in expression of SHRM compared to SHRC, n=6/ group.**p<0.01 compared to SHRC)

E. ***Effect of MCT supplementation on markers of oxidative stress:***

Malonedialdehyde (MDA), a stable end product of lipid peroxidation and protein carbonyl and 3- nitrotyrosine, end product of protein oxidation were used as the markers of oxidative stress. Increased lipid peroxidation is the expected consequence of high fat diet. However, MCT supplementation decreased myocardial lipid peroxidation, significantly in younger SHR (Fig.37). Myocardial protein carbonyl and 3-nitrotyrosine level were significantly reduced by metabolic stimulation in both the age groups (Figs 38,39).

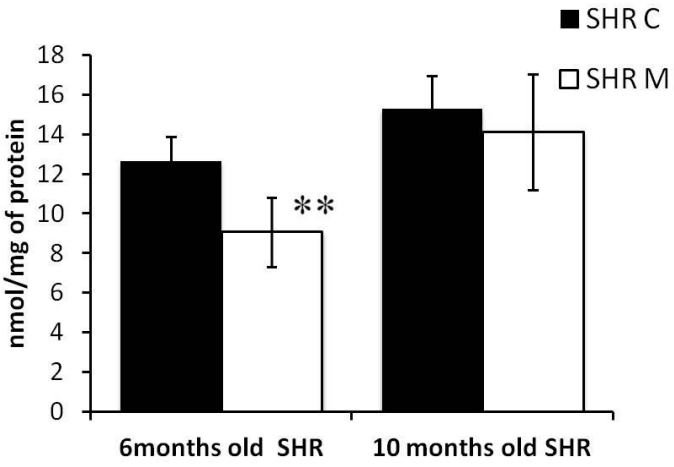

Figure 37: Cardiac Malondialdehyde levels of 6 and 10 months SHR following 4 months of supplementation with MCT (5% of the feed) (SHR M) compared with age and sex matched untreated control.(SHR C). (Data expressed as mean ± SD, n=6/group, **p<0.01 compared to untreated control)

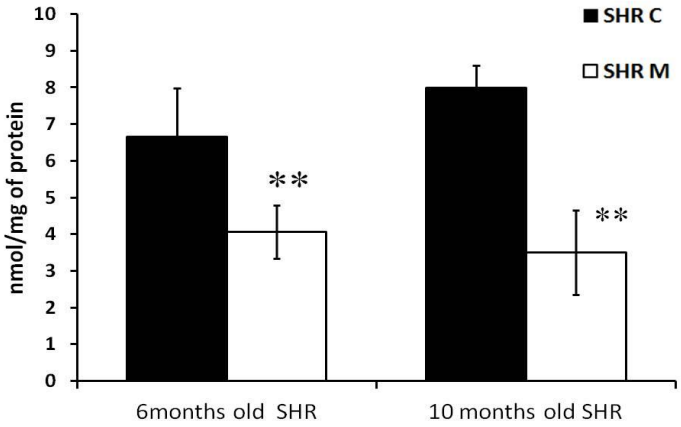

Figure 38: Cardiac protein carbonyl level of 6 and 10 months old SHR following 4 months of supplementation with MCT (5% of the feed) (SHR M)compared with age and sex matched untreated control (SHR C). (Data expressed as mean ± SD, n=6/group, **p<0.01compared to SHR C)

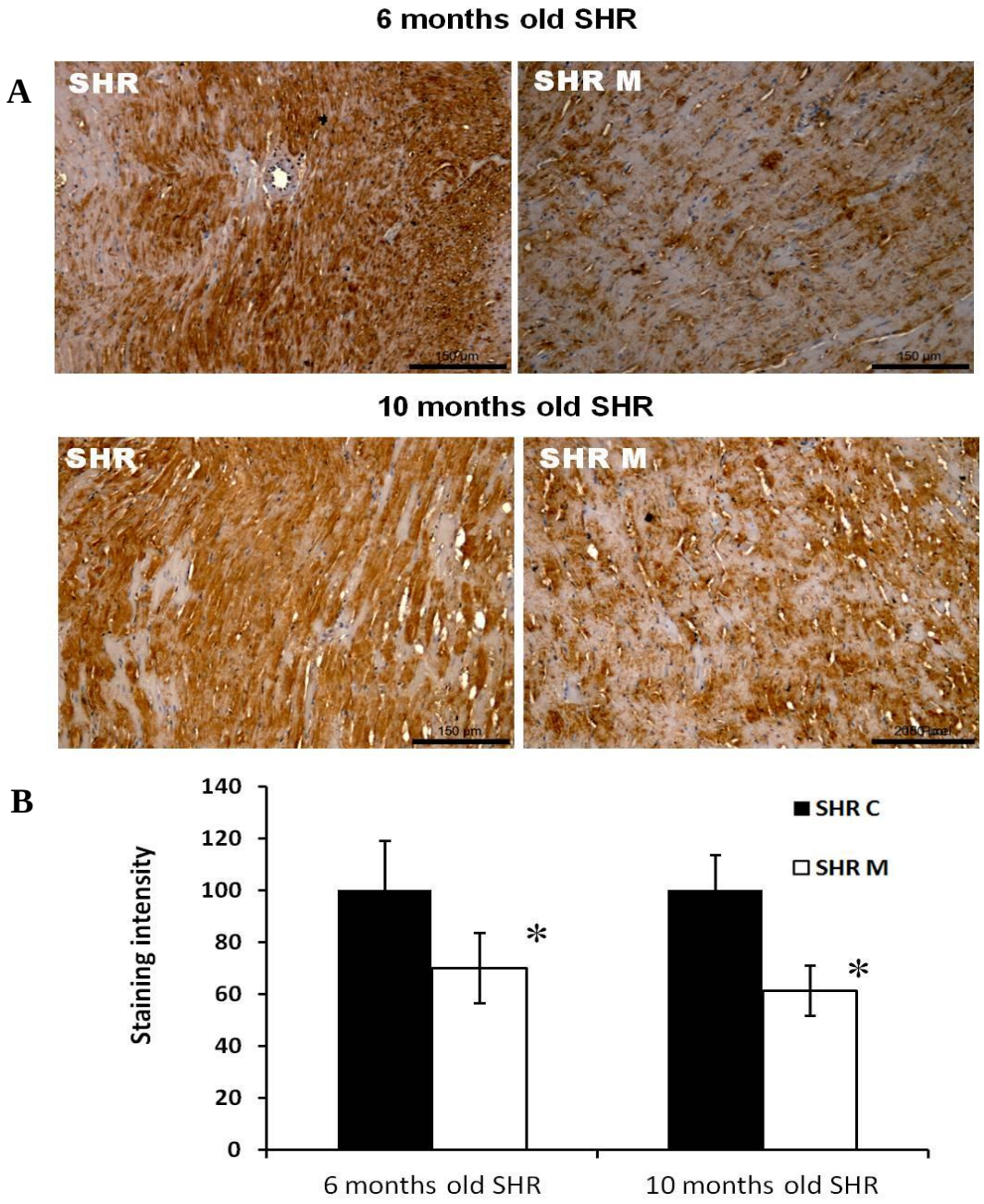

Figure 39: Cardiac 3nitrotyrosine level of 6 and 10 months old SHR following 4 months of supplementation with MCT (5% of the feed) (SHR M) compared with age and sex matched untreated control (SHR C) detected by immunohistochemistry of mid ventricular sections. (A) Representative photomicrographs. (B) Graphical representation of data. (Data presented as Mean ± SD, n= 6 * $p<0.05$ compared to untreated control).

F. Myocardial energy level:

Myocardial energy level was determined by biochemical estimation of ATP and phosphocreatine. MCT supplementation did not induce any change in myocardial ATP (Fig.40). However, phosphocreatine content was significantly increased in older SHR and maintained in younger SHR by MCT supplementation. (Fig.41). An age dependant decrease in phosphocreatine level was also observed in older SHR. The age associated decrease in ATP was not statistically significant (Fig.40).

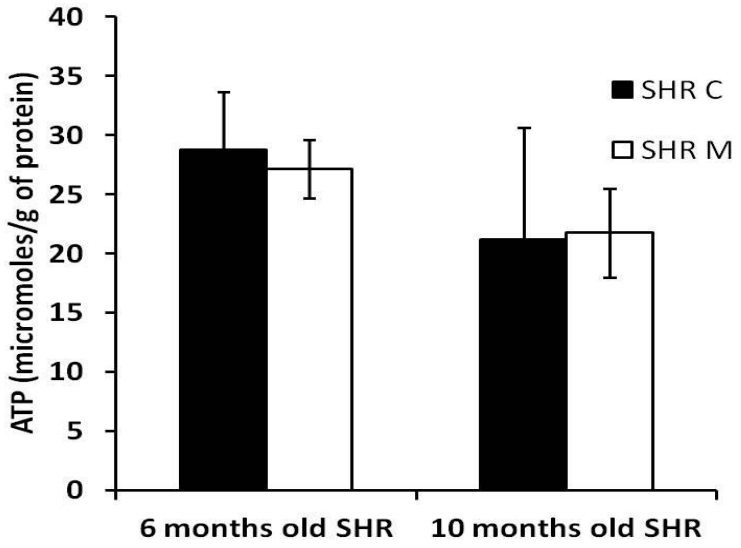

Figure 40: Myocardial adenosine triphosphate (ATP) level of 6 and 10 months SHR following 4 months of supplementation of MCT (5% of the feed) (SHR M) compared with age and sex matched untreated control (SHR C). (Data expressed as mean ± SD, n=6/group)

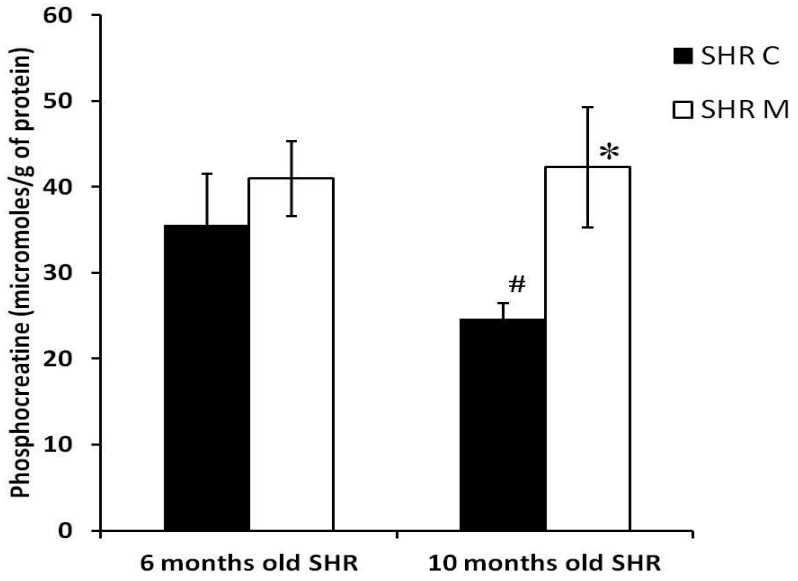

Figure 41: Myocardial Phosphocreatine (PCr) level of 6 and 10 months SHR following 4 months of supplementation of MCT (5% of the feed) (SHR M) compared with age and sex matched untreated control (SHR C). (Data expressed as mean ± SD, n=6/group, *p<0.05compared to SHR C, $^{\#}$p<0.05 compared to 6 months old SHR C)

G. Lipid profile and Hepatic Lipid accumulation:

MCT supplementation did not induce any change in serum lipid profile of the SHR. (Table.3). MCT maintained serum triglycerides, total cholesterol, HDL-C, and LDL-C in SHR. Consumption of MCT did not induce any hepatic lipid accumulation in comparison with control SHR (Fig.42).

Table 3. Lipid profile of SHR supplemented with MCT (5% of feed) (SHR M) compared with untreated control (SHR C) (Values are presented as mean ± SD, n=6/group)

Lipid profile	6 months old SHR		10 months old SHR	
	SHR C	SHR M	SHR C	SHR M
Triglyceride (mg/dl)	114.98±34.74	88.33±19.01	121±11.03	107.38±2.47
Total Cholesterol (mg/dl)	116.25±8.38	112.5±18.75	108.57±14.68	95.50±4.63
HDL-C (mg/dl)	49.35±6.47	48.38±9.51	42.63±1.53	40.41±1.90
LDL-C (mg/dl)	47.67±5.15	41.60±11.78	45.22± 12.66	33.61±5.72

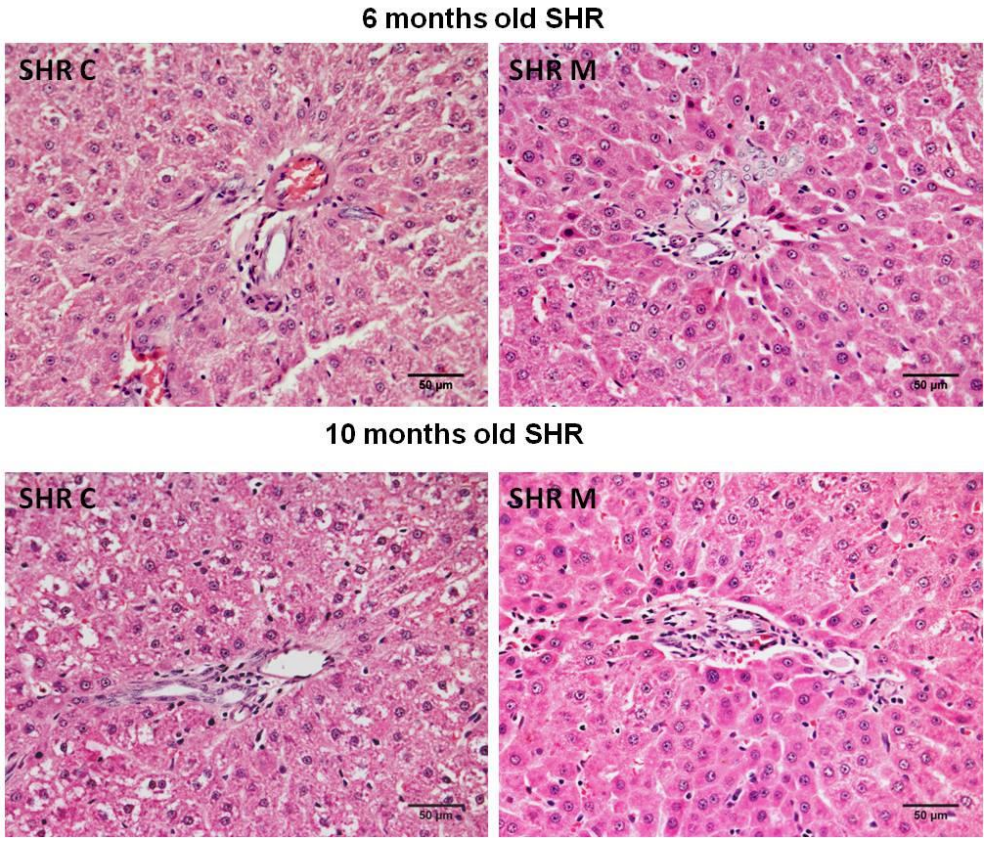

Figure 42: Representative photomicrographs of liver sections stained with Hematoxylin and eosin for the visualization of fatty changes in 6 and 10 months old SHR following 4 months of supplementation with MCT (5% of the feed). (SHR M) compared with age and sex matched untreated control (SHR C).

V. DISCUSSION

Left ventricular hypertrophy (LVH) is an adaptive mechanism triggered in the heart in response to pathological stimuli such as hypertension or aortic stenosis. However it is a major risk factor for adverse cardiovascular events and sudden death. Attenuation of LVH reduces the risk for cardiac failure and myocardial injury. During pathological hypertrophy, heart shifts its metabolic substrate preference from fatty acids to glucose (Allard, 2004; Allard et al., 2007). Chronic reliance on glucose metabolism is inefficient for maintaining contractile function especially under work overload (Ingwall, 2009). The decreased fatty acid utilization is possibly caused by reduction in the expression of Cluster of Differentation-36 (CD36), a major sarcolemmal fatty acid transporter that limits the availability of long chain fatty acids. A number of reports testify the role of CD36 deficiency in the metabolic alteration and the consequent cardiac remodeling. As chronic reduction in fatty acid oxidation can lead to pathological cardiac remodeling, it is presumed that restoration of fatty acid metabolism by bypassing the defective CD36 will help to maintain the metabolic status and prevent progressive cardiac remodeling. Hence, the study was carried out based on the hypothesis that reactivation fatty acid oxidation by supplementation of medium chain triglyceride (MCT) can prevent adverse cardiac remodeling in chronic pressure overload. The study was carried out in Spontaneously hypertensive rat, an animal model of chronic pressure overload induced left ventricular hypertrophy. Wistar (W) rats were used as the normotensive control.

V.1. COMPARISON OF MARKERS OF CARDIAC HYPERTROPHY, METABOLIC SHIFT AND OXIDATIVE STRESS IN SPONTANEOUSLY HYPERTENSIVE RAT (SHR) WITH NORMOTENSIVE WISTAR RAT (W)

The Spontaneously hypertensive rat (SHR) is a genetic model of hypertension induced left ventricular hypertrophy (LVH). SHR simulates the clinical progression of hypertension with a long term stable phase of left ventricular hypertrophy followed by progressive heart failure (Boluyt et al., 1995). Elucidation of mechanisms that restrict the development of cardiac hypertrophy in SHR may aid in the development of therapeutic strategies that prevent progressive cardiac remodeling.

Before initiating the experimental studies the presence of cardiac hypertrophy, metabolic shift and oxidative stress was validated in adult six-months old SHR (the established stage of cardiac hypertrophy) by comparison with age and sex matched normotensive Wistar rat. Wistar-Kyoto rats were avoided as they have a tendency to develop cardiac hypertrophy (Aiello et al., 2004). Morphological and histological analysis testified that, SHR is characterized by left ventricular hypertrophy. Hypertrophy index [heart weight (mg)/ body weight (g)] used as morphological marker of cardiac hypertrophy, was found to be significantly higher in 6 months old SHR compared to Wistar rat (W) (Fig.12).

Systolic and diastolic blood pressure of SHR, as determined by tail cuff sphygmomanometer was significantly higher in SHR (Fig.9). Histological assessment

showed that myocyte cross sectional area, interstitial and perivascular fibrosis (Fig.13-15) were significantly higher in SHR confirming the presence of cardiac hypertrophy.

Longitudinal studies in SHR had shown that SHR develop hypertension as early as one month of age, followed by hypertrophy in the 2nd month (Purushothaman et al., 2011a). Molecular markers of cardiac hypertrophy such as Brain natriuretic peptide (BNP) mRNA and Calcineurin A protein expression were significantly higher in SHR than normotensive Wistar rat (W) (Fig.16,17). Calcineurin is a Ca^{2+} dependant serine-threonine phosphatase that acts as a signaling molecule and mediates the development of pathological cardiac hypertrophy; and progressively increases with age in SHR (Zou et al., 2002).

Development of cardiac hypertrophy in SHR is associated with a shift in substrate preference from fatty acid to glucose mediated by downregulation of the expression of genes involved in fatty acid metabolism (Christe and Rodgers, 1994b). Previous studies have reported that the reduction in fatty acid oxidation in SHR is due to defective CD36, a major plasmalemmal fatty acid transporter (Hajri et al., 2001). The stock of SHR used for experimental studies also showed reduced expression of CD36 in heart, which was confirmed by Western blotting and immunohistochemistry (Fig.10,11). The reduced CD36 expression in SHR limits the availability of the long chain fatty acids, the natural ligand of PPARα. This is possibly the cause for the reduction in the fatty acid oxidation

as apparent from the reduced mRNA expression of PPARα and one of its target gene MCAD (Fig.18).

CD 36 is a highly glycolsylated integral membrane protein that shuttles 40-80% of fatty acid into the cardiomyocytes (Brinkmann et al., 2002). SHR carry a defective CD36 on rat chromosome 4, formed by recombination between wild type gene and Pseudo gene affecting post translational modification and intracellular localization changes the CD36's capacity to transport long chain fatty acids (Lauzier et al., 2011). Expression of wild type CD36 in SHR background improved defective fatty acid oxidation without affecting hypertension (Pravenec et al., 2001, 2003). SHRs are characterized by a restricted utilization of exogenous LCFAs for energy production, which has been attributed to a genetic defect in CD36. Age dependant downregulation of CD36 mRNA was also reported in SHR (Purushothaman et al., 2011b). The decreased fatty acid oxidation in SHR was accompanied by increased mRNA expression of PFK-1 (Fig.19), indicating accelerated glycolysis as a compensatory mechanism for sustenance of energy metabolism.

Apart from the shift in substrate preference from fatty acid to glucose, cardiac remodeling in hypertensive heart disease is associated with enhanced oxidative stress. Invitro and invivo studies carried out earlier have linked oxidative stress with cardiac hypertrophy (Adiga and Nair, 2008; Preeta and Nair, 2000; Purushothaman et al., 2011a). Oxidative stress in SHR was assessed by biochemical estimation of

malonedialdehyde (MDA) and protein carbonyl. Myocardial 3-nitrotyrosine was determined by immunohistochemistry. Malonedialdehyde, protein carbonyl and 3-nitrotyrosine levels were significantly higher in SHR (Fig.20,21), showing the association of oxidative stress with cardiac hypertrophy. Studies reported that use of antioxidants improved endothelial function and attenuated cardiac hypertrophy in young Stroke prone Spontaneously hypertensive rat (Graham et al., 2009). The observations ensure that the animals used for the experimental studies had cardiac hypertrophy, oxidative stress and shift in energy substrate preference.

V.2. COMPARISON OF CARDIAC RESPONSE TO STIMULATION OF FATTY ACID OXIDATION BY SUPPLEMENTATION WITH ODD AND EVEN CHAIN MEDIUM CHAIN TRIGLYCERIDES

MCT is available in two different forms, odd and even chain MCT based on number of carbons. Odd chain MCT is reported to be anaplerotic in nature. Anaplerosis is the re-filling of the catalytic intermediates of the Citric acid cycle. MCTs with an odd number of carbons were reported to be superior to even-carbon MCTs in improving the cardiomyopathy of one patient with genetic LCFA oxidation deficiency (Roe et al., 2002). Dietary regimen substituting tricaprylin (even chain MCT) with triheptanoin (odd chain MCT) improved cardiomyopathy in few patients with very long-chain acyl-CoA dehydrogenase deficiency. Triheptanoin intake enhanced ketone body synthesis without inducing propionyl-CoA overload (Roe et al., 2002). Recently, Nguyen et al. demonstrated that odd chain MCT triheptanoin feeding improved diastolic function and

cardiac hypertrophy in rodent model of pressure overload (Nguyen et al., 2015). Positive cardiac response to even chain triglycerides has also been reported (Labarthe et al., 2008). Hence, there exists the possibility of difference in their metabolic properties. A pilot study was therefore carried out to examine whether there would be a differential response depending on the type of triglyceride. For the experimental studies, 2-month-old SHR were supplemented with either Tricaprylin or Triheptanoate (5% of the total feed) for 2 months. Morphological and histological markers of cardiac hypertrophy and markers of oxidative stress were analyzed.

Even chain and odd chain triglycerides have shown variable cardiac response. Although both the sources of MCT decreased myocardial malonedialdehyde level, the decrease was more significant with even chain MCT (Tricaprylin) (Fig.25). Morphologial and histological markers of cardiac hypertrophy also showed divergent response. Although myocardial cross sectional area was significantly decreased by both the types of MCT (Fig.23), the hypertrophy index decreased only with even chain MCT (Fig.22). Similarly, myocardial fibrosis was decreased only with even chain MCT and not with odd chain MCT (Fig.24). Positive cardiac remodeling was significantly better with even chain MCT. Hence, tricaprylin was used as the source of MCT for further experimental studies. Both treatments did not affect body weight and lipid profile (Fig.26,27).

V.3. CARDIAC RESPONSE TO STIMULATION OF FATTY ACID OXIDATION BY SUPPLEMENTATION OF MEDIUM CHAIN TRIGLYCERIDES AT INITIAL AND ESTABLISHED STAGES OF CARDIAC HYPERTROPHY

Hypertension induced LVH is a convergent point for many risk factors leading to heart failure. During this hypertrophic response, the heart switches its substrate preference from fatty acid towards glucose. Several studies have reported the association between reduced rate of fatty acid oxidation and development of cardiac hypertrophy. Cardiac hypertrophy was provoked in rats on fat-free diet (Panos and Finerty, 1954). Humans with inborn errors in mitochondrial fatty acid oxidation enzymes often develop ventricular hypertrophy even in the absence of hypertension (Kelly et al., 1992). Inhibition of mitochondrial FAO in animals resulted in the development of cardiac hypertrophy (Rupp and Jacob, 1992). Patients with idiopathic dilated cardiomyopathy showed downregulation of FAO enzymes and/or decreased myocardial fatty acid oxidation as assessed by Positron emission tomography (Dávila-Román et al., 2002). Though initially adaptive, prolonged low rate of fatty acid oxidation is associated with cardiovascular consequences including increased oxidative damage and decreased energy reserve. Hence, restoration of fatty acid metabolism is expected to prevent adverse cardiac remodeling. Peroxisome proliferator-activated receptor-α (PPARα) is a critical regulator of myocardial fatty acid uptake and utilization. Studies reporting positive cardiac changes consequent to pharmacological stimulation of fatty acid metabolism were carried out in young rat or surgical models of cardiac hypertrophy (Chen et al., 2007; Li et al., 2009; Ogata et al., 2002b; Zou et al., 2013). Age associated

variation in cardiac response to ligand-mediated activation of PPARα was observed (Purushothaman et al, 2011b). Fenofibrate stimulated fatty acid oxidation and prevented development of hypertrophy in young SHR, but aggravated hypertrophy in older animals. An age associated downregulation of CD36 expression was observed in SHR (Purushothaman et al., 2011b). Variation in CD36 expression can account for this paradoxical cardiac response on pharmacological stimulation of fatty acid metabolism, as defective CD36 in SHR limits entry of long chain fatty acid into the cardiomyocytes. In the absence of metabolic substrate, on stimulation of fatty acid metabolism, this substrate deficiency is fulfilled by mobilization of the endogenous triglycerides that in turn results in the imbalance of the cellular redox status, leading to adverse cardiac consequences (Ismael et al., 2015). The lack of metabolic substrate availability consequent to defective CD36 is the primary determinant of altered metabolism and cardiac hypertrophy in SHR (Hajri et al., 2001). Defective CD36 did not affect the normal survival of the animals under basal conditions but showed reduced ischemic tolerance, increased susceptibility to arrhythmia under pressure overload and impaired response to adrenergic stimulation. (Irie et al., 2003; Labarthe, 2004).

A number of reports testify the role of CD36 deficiency in the metabolic alteration and the consequent cardiac remodeling. Tanaka et al. reported the direct association between CD36 deficiency and development of hypertrophic cardiomyopathy in the Japanese population (Tanaka et al., 1997). Pharmacological inhibition of CD 36 was associated with development of cardiac hypertrophy independent of blood pressure in rodents

(Kusaka et al., 1995). Altered myocardial metabolism and myocardial hypertrophy in SHR is attributed to defective CD36 (Hajri et al., 2001). Deficiency of CD36 has been reported recently by Magida *et al* in mouse models with familial hypertrophic cardiomyopathy (Magida and Leinwand, 2014). These studies highlight that the decreased fatty acid oxidation is secondary to reduced CD36 expression in cardiomyocytes. Therefore, it was assumed that, supplementation with medium chain triglyceride can overcome the maladaptive changes due to deficit in fatty acid oxidation as the substrate availability is independent of the fatty acid transporter CD36. Spontaneously hypertensive rats in the initial and established stages of cardiac hypertrophy were supplemented with 5% MCT, tricaprylin a triglyceride of caprylic acid along with standard feed for 4 months.

The study has shown that substrate mediated stimulation of fatty acid metabolism prevents progressive cardiac remodeling in SHR. Supplementation of MCT in SHR stimulated fatty acid metabolism as indicated by increased expression of PPAR-α and one of its target genes MCAD, on comparison with age matched untreated control (Fig. 28,29). Increased PPAR-α mRNA expression along with increased MCAD mRNA indicate stimulation of fatty acid metabolism by the activation PPAR-α. PFK-1 level was not affected by MCT supplementation (Fig.30).

Metabolic stimulation by MCT prevented the development and progression of adverse cardiac remolding in SHR, as indicated by decreased cardiomyocyte cross sectional area

and interstitial fibrosis compared with untreated SHR (Fig.32,33). Significant decrease in hypertrophy index was apparent in the younger age group (Fig.31). Interstitial fibrosis was also significantly reduced by the treatment. Studies have reported that, supplementation of short and medium chain triglycerides prevented the development of hypertrophy in young SHR (Hajri et al., 2001; Iemitsu et al., 2008; Shimojo et al., 2004). Stimulation of fatty acid oxidation was associated with a significant decrease in the expression of BNP mRNA and the protein Calcineurin A both of which serve as molecular markers for adverse cardiac remodeling (Fig.35,36). Pharmacological inhibition of calcineurin A is reported to attenuate cardiac hypertrophy and prevent transition to heart failure (Sakata et al., 2000). Morphological, histological as well as molecular indicators of hypertrophy were significantly reduced in SHR of both age groups, confirming that supplementation with MCT in the diet prevents cardiac hypertrophy.

It is now accepted that ROS are involved in the pathophysiology of myocardial hypertrophy and failure. Invitro and invivo studies carried out earlier have linked oxidative stress with cardiac hypertrophy (Adiga and Nair, 2008; Preeta and Nair, 2000; Purushothaman et al., 2011a). Superoxide at low levels stimulate hypertrophy, but chronic or high levels of superoxide result in cumulative cellular damage leading to secondary mitochondrial damage and eventual progression to heart failure(Sawyer et al., 2002). The direct correlation of oxidative stress with cardiac hypertrophy implies that reduction of oxidative stress is an essential mediator of positive cardiac remodeling.

Oxidative stress was assessed from cardiac malonedialdehyde (MDA), protein carbonyl and 3-nitrotyrosine levels. Protein carbonyl and cardiac 3-nitrotyrosine levels decreased significantly with the treatment, and cardiac MDA level was found to be significantly lower in young SHR, signifying that the metabolic stimulation by supplementation with MCT decreased oxidative stress (Fig.37-39). Reduction of oxidative stress is supported by the observation that intake of medium chain fatty acids prevents the accumulation of oxidatively damaged proteins and lipid in skeletal muscle by inhibition of reactive oxygen species (Montgomery et al., 2013).

There was no significant difference in diastolic or systolic blood pressure on MCT supplementation (Table 2), showing blood pressure independent attenuation of LVH. Perivascular fibrosis was unaffected by metabolic stimulation despite decrease in oxidative stress (Fig.34) possibly, due to the fact that blood pressure was unaffected by the treatment. This observation is supported by previous report by Iemitsu et al. (2008)

It is popularly known that cardiac decompensation consequent to shift in energy substrate preference from fatty acid to glucose is due to energy deficit. Pathological cardiac hypertrophy is manifested by depletion of phosphocreatine, the high energy reserve; with preserved ATP levels (Kolwicz et al., 2013; Liao et al., 1996). In the present study, an age dependant decrease in myocardial phosphocreatine content was observed in older SHR compared to younger animals, which was normalized by MCT supplementation (Fig.41). However MCT treatment maintained the cardiac ATP levels

in both age group (Fig.40). Studies have reported that, SHRs have similar cardiac high energy phosphate content at younger age (15 week old) compared to normotensive Wistar rat (Dodd et al., 2012), but diminishes gradually during sustained phase of cardiac hypertrophy (Perings et al., 2000; Shimamoto et al.,1982) These results imply that the positive cardiac response to the treatment seen in SHR is possibly due to maintenance of energy level as well as decrease in oxidative stress.

Apart from its effect on cardiac hypertrophy MCT are known to have other beneficial effects on the heart. Intake of short and medium-chain fatty acids was not associated with coronary artery disease; (Hu et al., 1999) and improvement in contractile function and ATP level of the hypertrophied heart and capacity to withstand acute adrenergic stress has been reported (Allard et al., 2007; Labarthe, 2004). Improved ischemic recovery of working hearts has also been reported on perfusion with caprylic acid (Irie et al., 2003). Additionally, supplementation of octanate enhanced contractile function of volume overloaded heart (Alaoui-Talibi et al., 1992). Supplementation of MCT prior to exercise training improved oxidative metabolism and decreased cardiac workload in patients with long-chain fatty acid oxidation defect (Behrend et al., 2012). Dietary administration of MCT improved LV dysfunction in mice with diabetic cardiomyopathy (Finck et al., 2003).

Dietary guidelines for cardiovascular diseases recommend high carbohydrate/low fat diet. High fat diet is reported to worsen cardiac hypertrophy and dysfunction. (Raher et

al.,2008). However, several studies have reported that high dietary fat could activate mitochondrial oxidative metabolism and alleviate adverse cardiac remodeling (Chess et al., 2009, 2008; Duda et al., 2008; Okere et al., 2006, 2005). It is also reported that high fat diet did not affect the degree of cardiac hypertrophy in SHR even with reduction in blood pressure (Bosse et al., 2013). Conventionally, saturated fats are not recommended as its intake is associated with elevated risk of cardiovascular diseases. Onge et al reported that MCT consumption at moderate level of 10-20% of energy intake does not increase the risk of cardiovascular disease (St-Onge et al., 2008). Consumption of MCT is not associated with the development of coronary artery disease (Hu et al., 1999). The current study has shown that supplementation of 5% of the diet with MCT does not produce increase in body weight or affect the lipid profile. On the other hand, it can be cardioprotective. There was no sign of hepatic lipid accumulation. Clinical studies that have reported dislipidemia on MCT consumption have used diet with more than 30% of calorie intake from MCT. (Hill et al., 1990; Swift et al., 1992)

The observations leads to the inference that, the positive cardiac changes in response to MCT are independent of blood pressure. Cardiac hypertrophy is regarded as an adaptation to pressure overload. The observations of the study indicate that decrease in blood pressure does not essentially lead to regression of hypertrophy, and that adverse cardiac remodeling can be prevented regardless of the maintenance of hypertension. The absence of a direct correlation of blood pressure with hypertrophy is supported by the

observation of blood pressure independent cardiac hypertrophy in Wistar-Kyoto rats (Aiello et al., 2004).

V.4. LIMITATION AND FUTURE DIRECTION

The beneficial effect of MCT supplementation would be appreciated with functional assessment, but this could not be done due to lack of facility

Future direction should be aimed at clinical validation of the experimental observation.

It would be interesting to examine whether reduction of oxidative stress can restore the original metabolic profile.

VI. SUMMARY AND CONCLUSION

VI.1. SUMMARY

The development of left ventricular hypertrophy (LVH) is an adaptive response to chronic pressure overload. Sustained hypertrophy is maladaptive and is a clinical predictor for heart failure. Oxidation of long-chain fatty acids serves as the major metabolic fuel for the healthy heart. In pathological cardiac hypertrophy, there is a decrease in the oxidation of long chain fatty acids and increased relative contribution of glucose for the maintenance of cardiac energy requirements. Prolonged lower rate of fatty acid oxidation is associated with adverse cardiovascular consequences. Pharmacological activation of Peroxisome proliferator-activated receptor α (PPARα) revealed an age dependent differential cardiac response in SHR possibly linked to the stage of hypertrophy; despite stimulation of fatty acid oxidation. Studies in Spontaneously hypertensive rat (SHR) and patients with hypertrophic cardiomyopathy leads to the postulation that, the reduced fatty acid oxidation is possibly caused by reduced expression of CD36, a major sarcolemmal fatty acid transporter. A temporal variation was observed in the expression of CD36, with decreased expression in older animals. As Medium chain triglycerides have the capacity to bypass CD36 and serve as substrate for fatty acid oxidation, it is presumed that restoration of fatty acid oxidation by supplementation of MCT will help to maintain the metabolic status and prevent progressive cardiac remodeling. Male Spontaneously hypertensive rat were used as the experimental model.

SHR in the initial and established stage of hypertrophy were supplemented with 5% Medium chain triglycerides (MCT) - Tricaprylin, along with standard feed for 4 months. Stimulation of fatty acid oxidation was assessed from the expression of PPARα and Medium chain acylCoA dehydrogenase (MCAD) mRNA by Real time polymerase chain reaction. Hypertrophy was assessed by hypertrophy index, myocyte cross sectional area, extent of cardiac fibrosis, mRNA expression of Brain natriuretic peptide (BNP) and expression of the protein calcineurin A. Malonedialdehyde and protein carbonyl assay, and histological analysis of 3-nitrotyrosine were used as the markers of oxidative stress. Myocardial energy level was determined by biochemical estimation of ATP and phosphocreatine.

The significant findings of the present study are

- **Comparison of morphological, histological and molecular markers of hypertrophy with age and sex matched Wistar rats confirmed that SHR used for the experimental studies developed Left Ventricular Hypertrophy**
- **Fatty acid oxidation was decreased as indicated by decreased mRNA expression of MCAD and PPARα and increased PFK-1 mRNA expression indicates increase in glucose oxidation in SHR in comparison with Wistar rat.**

- **SHR was characterized by reduced expression of the fatty acid transporter CD36, a possible cause for diminished Fatty acid oxidation.**

- **Hypertrophy in SHR was associated with increased oxidative stress as indicated by increased levels of cardiac malonedialdehyde, protein carbonoyl and 3-nitrotyrosine compared to Wistar rats**

- **The cardiac response to supplementation with MCT can depend on the type of fatty acid. Supplementation of Odd chain and Even chain triglycerides in 2-month-old SHR (5% of feed v/w) for 2 months indicated that decrease in hypertrophy index and interstitial fibrosis was significantly better with Even chain MCT compared to Odd chain MCT.**

- **Reduction of oxidative stress was apparent with both the treatments but was significantly greater with Even chain MCT.**

- **Supplementation with Even chain MCT in SHR in the initial and established stages of cardiac hypertrophy stimulated fatty acid oxidation as indicated by increased mRNA expression of MCAD and PPARα, with maintenance of PFK-1 mRNA expression.**

- **Stimulation of fatty acid oxidation by MCT attenuated progressive cardiac remodeling independent of blood pressure.**

- **Modulation of fatty acid oxidation increased myocardial energy level significantly in older SHR.**
- **Stimulation of Fatty acid oxidation by MCT was accompanied by decrease in cardiac oxidative stress.**
- **Reduction of cardiac hypertrophy is possibly the consequence of decrease in oxidative stress and maintenance of myocardial energy level.**
- **Consolidation of the observations lead to the inference that restoration of Fatty acid oxidation by MCT supplementation is beneficial to the hypertrophic heart**
- **Supplementation of MCT was not associated with the development of obesity and dislipidemia.**

VI.2. CONCLUSION

Contrary to the belief that stimulation of Fatty acid oxidation can be detrimental in the presence of hypertrophy, this study has shown that, restoration of FAO can be cardioprotective, irrespective of stage of the hypertrophy in SHR. The findings of this study is supported by the observations of Kolwicz *et al*, where they have shown that maintenance of inherent metabolic profile prevents adverse cardiac remodeling under work overload (Kolwicz et al., 2012). This is the first study to show that stimulation of fatty acid oxidation in older rat with chronic pressure overload confers beneficial effects on the hypertrophic heart. The observations can have therapeutic implications and provides insight for further studies; to obtain clinical correlates by supplementation of MCT in the diet of individuals with CD36 deficiency. As hypertension is associated with consequences other than hypertrophy, MCT can be used only as a supplement along with antihypertensives. Preclinical studies will help to determine the therapeutic implications for supplementation with MCT in patients with hypertensive heart disease.

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VIII. PUBLICATIONS

PUBLICATIONS

1. S. Ismael, S Lakshmi, K Remani, R.R. Nair, Metabolic Modulation by Medium Chain Triglycerides Reduces Oxidative Stress and Ameliorates CD36 Mediated Cardiac Remodeling in Spontaneously Hypertensive Rat in Initial and Established Stages of Hypertrophy, J. Card. Fail. (2016). doi:10.1016/j.cardfail.2016.08.001.

2. S. Ismael, S. Purushothaman, V.S. Harikrishnan, R.R. Nair, Ligand specific variation in cardiac response to stimulation of peroxisome proliferator-activated receptor-alpha in spontaneously hypertensive rat, Mol. Cell. Biochem. 406 (2015) 173–182. doi:10.1007/s11010-015-2435-x.

3. Mukund A Prabhu, S Ismael, Remani K, R.R Nair, S Harikrishnan. Association study of Transforming growth factor ß superfamily genes with non-regression of pulmonary hypertension following balloon mitral valvotomy: a pilot study. J Heart Valve Disease. (In press)

CONFERENCE PROCEEDINGS

1. Saifudeen Ismael, V S Harikrishnan, R Renuka Nair. Age dependant paradoxical effect of medium chain triglycerides on cardiac oxidative stress in spontaneously hypertensive rat. Abstract page number: 77-78. International seminar on "Recent biochemical approaches in therapeutics", Department of Biochemistry, University of Kerala, Thiruvananthapuram on January 2013.

2. Saifudeen Ismael, V S Harikrishnan, R Renuka Nair . Reactivation of fatty acid metabolism by medium chain triglycerides modulates oxidative stress and promotes cardiac antiremodeling in spontaneously hypertensive rat. Abstract page 34-35. 6th International Conference on Recent Advances in Cardiovascular Sciences organized by International Academy of Cardiovascular Sciences held at Delhi Institute of Pharmaceutical Sciences and Research (DIPSAR), New Delhi on January 2014 and received **Naranjan Dhalla Best Poster Award.**

3. Saifudeen Ismael, R Renuka Nair. Prevention of adverse cardiac remodeling in Spontaneously Hypertensive Rat: Ligand specific response to stimulation of Peroxisome proliferator-activated receptor-alpha. Abstract page number 59. Indo-Canadian Symposium on Heart Failure, at Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram on March 2015.