

**Institute Reference:** Project No.5198

**Information on Grant-in-Aid project of the LSRB sanctioned to  
Sree Chitra Tirunal Institute for Medical Sciences and Technology  
Thiruvananthapuram- 695011**

1.	Reference of LSRB initial sanction	DLS/81/48222/LSRB-179/ID/2009
2.	Project title	Modulation of energy metabolism in prevention of cardiac remodeling: Stimulation of peroxisome proliferator-activated alpha receptor
3.	Name of Principal Investigator	Dr. R.Renuka Nair
4.	Duration of the Project	3 years
5.	Date of completion	28 <sup>th</sup> July. 2012
6.	Total Funds for Duration of the project	Rs. 19,76,090

**Project Completion Report**

**(a) Aim of the Project-** Hypertension-induced pathological cardiac hypertrophy is an important risk factor for cardiac failure. In the hypertrophic heart, there is a switch in energy substrate preference from fatty acids to glucose. Alteration in energy metabolism is considered to play a role in the development of cardiac hypertrophy and its progression to failure, although the sequence of events remains to be elucidated. Long-term low rates of fatty acid oxidation will lead to energy deficit and accumulation of free radical species that might be toxic to the heart. The metabolic shift is facilitated by down regulation of the nuclear hormone receptor, peroxisome proliferator-activated receptor (PPAR) $\alpha$ . Hence, it was hypothesized that, “Stimulation of PPAR $\alpha$  can reestablish a more ‘normal’ metabolic physiology, thereby maintaining cardiac function and preventing cardiac failure.” The aim of the study was to examine whether stimulation of fatty acid metabolism using PPAR $\alpha$  agonists can prevent pathological cardiac remodeling.

**(b) Key words:** Hypertension, Cardiac hypertrophy, Cardiac failure, PPAR $\alpha$ , Fenofibrate, Medium chain triglycerides

**(c) Classification of Project:** Basic Research

**(d) Introduction:** Hypertensive heart disease is a progressive condition in which the compensatory left ventricular hypertrophy (LVH) that initially helps in the maintenance of cardiac output leads in course of time to morbidity and mortality. Clinical studies have demonstrated that reduction of cardiac mass reduces the risk of adverse cardiac events, independent of the treatment adopted. Though the currently used hemodynamic agents

are helpful in the management of hypertension and provide symptomatic relief with increase in lifespan, the prognosis remains poor and disease progression is not arrested. This necessitates identification of novel strategies for prevention of pathological hypertrophy and failure.

Cardiac hypertrophy is associated with a shift in energy metabolism from predominantly fatty acid to glucose. Though initially adaptive, this leads in course of time to energy depletion and cardiac dysfunction.

The shift to predominantly glucose oxidation is mediated by down regulation of PPAR $\alpha$ . Based on the assumption that reinvigorating fatty acid metabolism can prevent adverse cardiac remodeling, the cardiac response to PPAR $\alpha$  stimulation was examined in the rat model of hypertensive heart disease. PPAR $\alpha$  ligands are used in therapeutics as a lipid-lowering agent, but the cardiac response has not been evaluated with respect to the genetic makeup or pathophysiological condition. The project was therefore designed with the following objectives:

1. Determine the changes in energy metabolism and oxidative stress in different stages of cardiac remodeling
2. Assess the stage specific response to PPAR $\alpha$  stimulation in different stages of heart disease
3. Examine the cardiac response to agonist and substrate mediated stimulation of fatty acid metabolism

**(e) Material and methods:**

***Experimental Design***

Experimental Model- Experiments were carried out in spontaneously hypertensive rat. Spontaneously hypertensive rat (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 (HF).

Age at manifestation of hypertrophy and metabolic changes- The first step in the study was to identify ages at which hypertrophy and metabolic shift occur. The objective of this was to select the age at which treatment has to be effected.

Stimulation of fatty acid metabolism- The shift in energy metabolism from predominantly fatty acid to glucose is mediated by down regulation of the PPAR $\alpha$  gene. Studies in SHR showed that hypertrophy manifested at 2 months of age and shift in energy metabolism occurred at 4 months. It is expected that the stimulation of PPAR $\alpha$

gene will help to restore fatty acid metabolism in the heart and prevent adverse cardiac remodeling and also promote reverse remodeling.

***Experimental protocol:***

Identification of the age at manifestation of hypertrophy and metabolic shift- To identify the age at which structural and metabolic remodeling occurs in SHR, the expression of genes associated with hypertrophy and metabolism in SHR of 1, 2, 4, 6, and 8 months was compared with that of age-matched Wistar rats.

Blood pressure was measured and cardiac index was assessed as a morphological indicator of hypertrophy in animals of different ages. Size of myocytes and extent of fibrosis was measured histochemically.

Oxidative stress is known to be associated with hypertrophy and metabolic shift. Hence, cardiac and systemic oxidative stress was evaluated biochemically.

A minimum of six animals was analyzed in each group.

Metabolic modulation: Restoration of fatty acid metabolism was attained by: (i) ligand-mediated stimulation of the PPAR $\alpha$  gene and (ii) supplementation with medium chain triglycerides (MCT). Stimulation of fatty acid oxidation for prevention and regression of hypertrophy was studied, respectively, in 2- and 6-month-old male SHRs as hypertrophy was apparent at 2 months of age and metabolic shift at 4 months of age.

Fenofibrate was used as the ligand for the stimulation of the PPAR $\alpha$  gene. Fenofibrate is commonly used in the treatment of dislipidemia. Fenofibrate was administered orally by gavage at a dose of 100 mg/kg body weight (BW) for 60 days.

Restoration of fatty acid metabolism was also attained by supplementation of the feed of SHR with MCT (5%).

The morphological and molecular markers of hypertrophy, expression of genes associated with metabolism, and indicators of oxidative stress were studied to examine the myocardial response to stimulation of fatty acid metabolism. Cardiac response to the treatment was evaluated by comparison with age-matched untreated SHR.

Six animals were analyzed in each group.

### ***Techniques***

Measurement of Blood Pressure- Blood pressure was recorded by the tail-cuff method using a noninvasive blood pressure monitoring system for small animals (Biopac Systems, United States) in conscious rats after restraining them.

Measurement of Cardiac Index- The body weight of the animals was recorded, and the hearts were excised. Hypertrophy index was determined as the ratio of total ventricular weight to Body weight (BW) [ventricular weight (milligrams)/BW (grams)].

Measurement of Cardiomyocyte Cross-sectional Area- Mid-ventricular cross sections of the heart (5–6 mm) were taken and immunostained for dystrophin to get a clear outline of the cells. An area of 50 cross-sectioned cardiomyocytes in at least 5 different fields was measured using image analysis software.

Measurement of fibrotic changes in the left ventricular wall- The extent of fibrosis was evaluated by histomorphometry of sections of left ventricular wall stained with Sirius red. Sirius red (0.1%) stains interstitial collagen and Fast green FCF (0.1%) stains the non-collagenous proteins. Fibrosis was quantified from 5 different randomly chosen fields using Image-Pro Plus 5.1 and expressed as the percentage area stained red (collagen) in a particular microscopic field, without any gap areas. The sections were also immunostained for collagen subtypes 1 and 3.

Measurement of Serum Lipid Peroxidation- Malondialdehyde (MDA), a stable terminal metabolite of lipid peroxidation was used as a measure of oxidative stress. MDA levels were measured as thiobarbituric acid (TBA) reactive substances. MDA concentration was determined calorimetrically from the intensity of the chromogen formed when lipid peroxides resulting from oxidative stress reacts with TBA. Blood was collected before killing the animals, and serum was frozen. Analysis was carried out within a fortnight.

The serum was mixed with 2 mL of a trichloroacetic acid– TBA reagent [trichloroacetic acid 0.375% (wt/vol) TBA, 0.25 mole/L HCl], the mixture was heated for 15 minutes in a boiling water bath, centrifuged, and the intensity of supernatant color complex measured at 535 nm.

In addition to MDA levels, expression of NFκB was used as a marker of oxidative stress.

Measurement of serum cholesterol level- Fenofibrate is used clinically as lipid lowering drug and Medium chain triglyceride is a fatty acid, both of which can influence the lipid profile. Hence, total serum cholesterol was measured using cholesterol estimation kit. 10µl of serum was mixed with 1ml reagent and incubated for 10 minutes at 37<sup>0</sup>C. The absorbance of quinoneimine formed by the enzymatic hydrolysis and oxidation was measured at 505nm in AUTO CHEM NEXGEN Auto analyzer.

Oil Red O Staining for the Assessment of Tissue Lipid Accumulation- Cryostat sections (8–10 mm) were washed in distilled water and immersed in 100% propylene glycol for 2 minutes. The sections were incubated at 60<sup>0</sup>C for 30 minutes with constant stirring in filtered and preheated Oil red O stain (0.7%), transferred to 80% propylene glycol, washed in distilled water, counterstained with hemotoxylin and mounted in glycerol jelly.

Expression of Molecular Markers of Hypertrophy and Fatty Acid Metabolism -

Brain natriuretic peptide (BNP) is a well-documented marker of cardiomyocyte hypertrophy. The expression of NFκB was studied as a marker of oxidative stress. Cluster of differentiation 36 (CD36)—a long chain fatty acid transporter, carnitine palmitoyl transferase 1β (CPT 1β)—a mitochondrial transporter protein and medium chain acyl-Co-A dehydrogenase (MCAD)—a key enzyme in beta-oxidation of fatty acids were selected as indicators of fatty acid metabolism. Relative quantitative expression analysis was carried out using realtime polymerase chain reaction (PCR) to study the expression of these genes. The transcriptional level of PPARα was assessed by real-time PCR analysis and the protein level was evaluated by western blotting.

#### *Gene Expression Analysis*

RNA Isolation - Frozen myocardial tissue was homogenized in Trizol reagent (50–100 mg of tissue per milliliter of Trizol reagent). Total RNA was extracted in chloroform, precipitated with isopropanol, and washed with 70% ethanol. Genomic DNA contamination in the RNA sample was removed by treating with DNase (RNAase free, amplification grade; Sigma) followed by phenol–chloroform extraction.

cDNA Synthesis- RNA samples were reverse transcribed to cDNA using MMLV reverse transcriptase and oligo dT primers. RNA (2 µg) was incubated with 3 mL oligo dT for 5' at 70<sup>0</sup>C to remove unwanted secondary RNA strands, transferred to a reaction mix

containing dNTP, RNasin, MMLV-RT, and RT buffer; and incubated at 37°C for 1 hour and 90°C for 5'. The cDNA was stored at -20°C. Rat specific primers for the genes were designed and synthesized by Oscimum Biosolutions (India).

The sequences of oligonucleotide primers were as follows:

**BNP** forward - 5' AGAGAGCAGGACACCATC 3'  
reverse - 5' AAGCAGGAGCAGAATCATC3'

**NF-κB** forward - 5'TCCCCTCATCTTTCCCTCAG3'  
reverse - 5'GCCCTCGCACTTGTAACG3'

**MCAD** forward - 5'TTTGCCAGAGAGGAAATAATC 3'  
reverse - 5'CCAAGACCACCACAACCTC3'

**CPT 1β** forward - 5'CCACAGACCGAGACACTTC3'  
reverse - 5' AGGAGACGAACACAGATAGC 3'

**CD 36** forward- 5'CAGAGGAAGTGGCAAAGAATAG3'  
reverse - 5'CAGTGAAGGCTCAAAGATGG3'

**PPARα** forward - 5'-GAC GCT GGG TCC TCTGGT-3'  
reverse - 5'-GTC TTG GCT CGC CTC TAA -3'

**Beta actin** forward - 5'CGTGCGTGACATTAAAGAG3'  
reverse - 5'GCCACAGGATTCCATACC,

Relative quantitative PCR (RQ PCR) was performed using Power SYBR Green dye on Applied Biosystems 7500 real time PCR system. RQ PCR was performed in a total volume of 20 µL, containing 100 ng of cDNA, 25 nmoles of forward primer and 50 nmoles of reverse primer, and 10 mL of Power SYBR Green PCR Master Mix. The PCR conditions comprised incubation at 95°C for 10 minutes (for denaturation) followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute (for annealing and extension). This was followed by a melt curve analysis (95°C for 15 seconds, 60°C for 1 minute, 95°C for 30 seconds and 60°C for 15 seconds) to ensure the absence of primer dimer formation in the reaction mix. Each experiment was repeated 2–3 times, and each PCR reaction was performed in triplicate with null-template controls. Reaction efficiency of both the target genes and the endogenous control was calculated based on the formula,

$E = [10^{21/\text{slope}}] - 1$ . Primers having a slope value between 23.2 and 23.5 only were used. The “22DDCT method” described by Livak and Schmittgen<sup>18</sup> was applied to obtain the RQ values [using the software of Applied Biosystems (SDS 7500, v. 2.0.3)]

and exported in excel sheet to study the fold change in the expression levels between the treatments.

Beta actin was selected as the loading control.

### ***Statistical Analyses***

Numerical data are presented as mean  $\pm$  SD. A minimum of 6 animals was used for each group. One-way analysis of variance was carried out to test the response to treatment in animals of different age groups. The difference between groups was assessed by the 2-tailed Student t-test.  $P < 0.05$  was considered statistically significant

#### **(f) Results:**

##### **AGE AT INITIATION OF CARDIAC REMODELING IN SHR:**

To identify the age at which hypertrophic changes are initiated in SHR, rats of different ages (1, 2, 4, 6, 8 months) were compared with age matched Wistar rats. Morphological and molecular markers of hypertrophy were examined for assessment of cardiac remodeling. Cardiac index, heart weight and body weight of SHR was compared with that of Wistar rats.

The body weight of the two strains were comparable, but a significantly higher heart weight and cardiac index was observed in SHR from 2 months (Table 1).

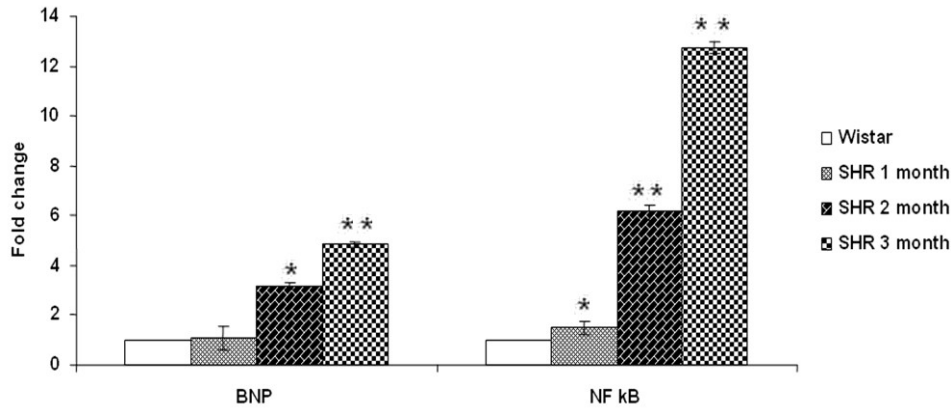
**Table 1: Age dependent variation in the heart weight, body weight and hypertrophy index of Spontaneously Hypertensive Rat (SHR) and Wistar rat**

Age in months	Heart weight(mg)		Body weight(g)		Ventricular mass (mg) / body weight (g)	
	<i>SHR</i>	<i>Wistar</i>	<i>SHR</i>	<i>Wistar</i>	<i>SHR</i>	<i>Wistar</i>
1	0.3 $\pm$ 0.04	0.28 $\pm$ 0.03	80 $\pm$ 14	75.47 $\pm$ 8	3.75 $\pm$ 0.08	3.71 $\pm$ 0.09
2	0.6 $\pm$ 0.04*	0.47 $\pm$ 0.03	185 $\pm$ 12	171.5 $\pm$ 12	3.24 $\pm$ 0.08*	2.74 $\pm$ 0.09
4	1.04 $\pm$ 0.03**	0.6 $\pm$ 0.07	374 $\pm$ 16*	245 $\pm$ 5	2.78 $\pm$ 0.09*	2.45 $\pm$ 0.09
6	1.22 $\pm$ 0.09**	0.69 $\pm$ 0.05	405 $\pm$ 9*	300 $\pm$ 11	3.0 $\pm$ 0.07**	2.3 $\pm$ 0.08
8	1.28 $\pm$ 0.05**	0.71 $\pm$ 0.04	400 $\pm$ 11*	351 $\pm$ 9	3.2 $\pm$ 08**	2.02 $\pm$ 0.06

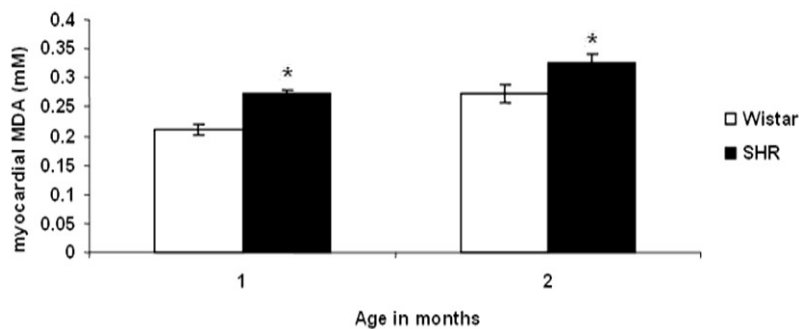
\* $p < 0.05$ , \*\* $p < 0.001$  vs age matched Wistar rat.

Real time PCR analysis for Brain Natriuretic Peptide, the molecular marker of hypertrophy showed no difference in expression in one month old SHR, but a significant

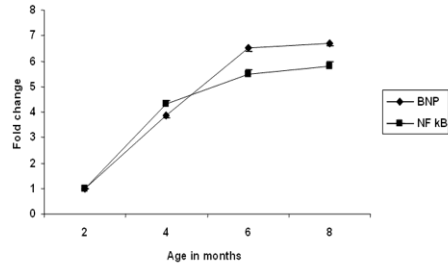
increase at 2 and 3 months, thereby validating the morphological changes (Fig 1). Because oxidative stress is intimately associated with hypertrophy the markers of oxidative stress were also assessed. The expression of NFκB was significantly high in SHR compared to Wistar rats in all 3 age groups (Fig 1). Enhanced NFκB expression prior to increase in BNP expression stipulates the role of inflammation in the pathophysiology of cardiac hypertrophy. This is supported by the observation of enhanced malondialdehyde levels in the cardiac tissue of SHR at 1 months of age (Fig. 2). An age dependant increase was observed for BNP and NFκB. From 2 months to 6 months a steady increase was observed for BNP and NFκB but no significant difference was observed between 6 and 8 months (Fig. 3).



**Fig 1-** Expression of Brain Natriuretic Peptide (BNP) and NF-κB in Spontaneously hypertensive rat (SHR) as a function of the level in Wistar rats. Data are presented as fold change in m-RNA level of SHR with the level in age matched Wistar rats taken as 1. \* p<0.01 and \*\*p<0.001 significantly higher compared to Wistar

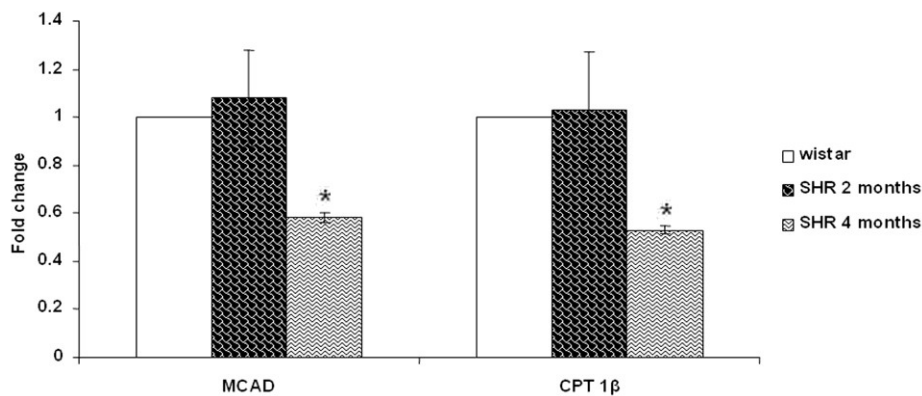


**Fig. 2.** Cardiac tissue malondialdehyde (MDA) level in Spontaneously hypertensive rat (SHR) and Wistar rat \*p<0.01 compared to age matched Wistar rat

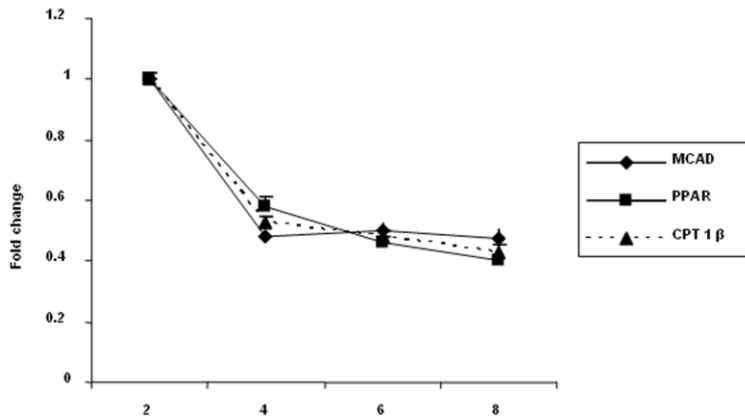


**Fig 3.** Expression of brain natriuretic peptide (BNP) and nuclear factor-kappa B (NF-κB) in Spontaneously Hypertensive Rat of different ages  
 Data are presented as fold change compared to the m-RNA expression in 2-month-old rats  
 \*p<0.01 significantly different compared to 2 months

AGE DEPENDANT VARIATION IN THE EXPRESSION OF METABOLIC MARKERS IN SHR - To identify whether down regulation of fatty acid metabolism precedes or succeeds hypertrophic changes, markers of fatty acid metabolism was screened in SHR of different ages (2, 4, 6 and 8 months). Real time PCR analysis was carried out for the expression of fatty acid transporter protein CD 36 and Medium chain acyl CoA dehydrogenase (MCAD) that is a key enzyme in fatty acid metabolism. The downregulation of the enzymes was seen only at 4 months compared to Wistar rats, indicating that the enzymes are downregulated with progression of hypertrophy. An age dependent decrease in the expression of the enzymes was observed (Fig 4) Expression of CPT 1β and MCAD remained unaffected at 2 months of age, indicating that fatty acid metabolism remains the major source of energy in the heart of SHR and metabolic changes are not apparent at the time of initial manifestation of hypertrophy. At 4 months, the expression of enzymes associated with fatty acid metabolism was significantly lower in SHR compared to age matched Wistar rats (Fig. 5), indicating that a shift in energy metabolism occurs at 4 months of age.



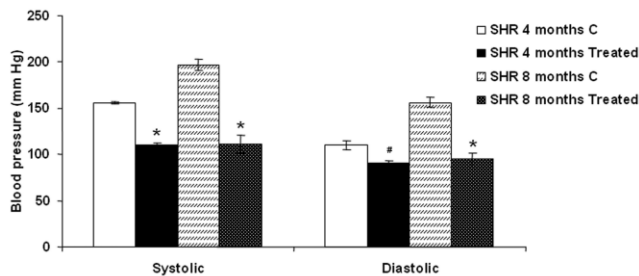
**Fig. 4-** Expression of carnitine palmitoyltransferase 1 $\beta$  (CPT 1 $\beta$ ) and medium chain acyl CoA dehydrogenase (MCAD) in 2 and 4-month-old Spontaneously Hypertensive rat (SHR) as a function of the level in age matched Wistar rats. Data are presented as fold change in m-RNA level of SHR with the level in age matched Wistar rats taken as 1. \*  $p < 0.0001$  significantly lower compared to Wistar



**Fig 5:** Expression of carnitine palmitoyltransferase 1 $\beta$  (CPT 1 $\beta$ ), medium chain acyl CoA dehydrogenase (MCAD) and peroxisome proliferator-activated receptor-alpha (PPAR) in Spontaneously Hypertensive Rat of different ages. Data are presented as fold change compared to the m-RNA expression in 2-month-old rats. \* $p < 0.01$  significantly different compared to 2 months

#### CARDIOVASCULAR RESPONSE TO PEROXISOME PROLIFERATOR- ACTIVATED RECEPTOR ALPHA REACTIVATION

**a. Antihypertensive potential of fenofibrate:** Irrespective of the age of the animal, fenofibrate significantly reduced both systolic and diastolic blood pressure (Fig. 6).



**Fig. 6.** Blood pressure (mm/Hg) of SHR following 60 days of treatment with fenofibrate (100mg/kg/day) (SHR treated) compared with untreated control (SHR C). \*  $p < 0.001$ , # $p < 0.05$  – Significantly different compared to control

**b. Reactivation of PPAR $\alpha$  for prevention and regression of LVH:** Morphological, histological and molecular markers of hypertrophy were compared between treated and untreated animals.

*i) Cardiac morphology:* The mean hypertrophy index of 4-month-old treated SHR was significantly lower than that of age matched untreated control (Fig. 7a) and comparable to Wistar rats (2.45 $\pm$ 0.06). A paradoxical effect was observed in 8-month-old SHR, with a significant increase in hypertrophy index upon treatment (Fig. 7a).

*ii) Cardiomyocyte cross sectional area:* The mean cross sectional area of cardiomyocytes decreased significantly with treatment in young SHR ( $p < 0.01$ ), but showed a significant increase in the older group ( $p < 0.01$ ). (Fig. 7c & Fig 8-I)

*iii) Interstitial fibrosis:* Histomorphometric analysis for interstitial collagen in sirius red stained ventricular sections revealed that the mean percentage stained area in 4 month old treated SHR was significantly lower than that of untreated rats. But, a contradictory effect was observed in 8 month old treated rats. (Fig 7b, 8II) The level of collagen I and III was also higher in the treated 8 month old SHR. (Fig 8-III)

*iv) Expression of molecular indicators of cardiac remodeling:* Expression of BNP was significantly lower ( $p < 0.001$ ) in 4 month old treated SHR (Fig.7d). But, the treatment upregulated the expression of BNP in 8-month-old animals ( $p < 0.001$ ) (Fig. 7e). Similarly, the expression of pro collagen I and III mRNA was downregulated in 4 month old SHR but upregulated in the older group ( $p < 0.01$ ) (Fig. 7 d,e) indicating stimulation of fibrosis upon treatment in the older rat

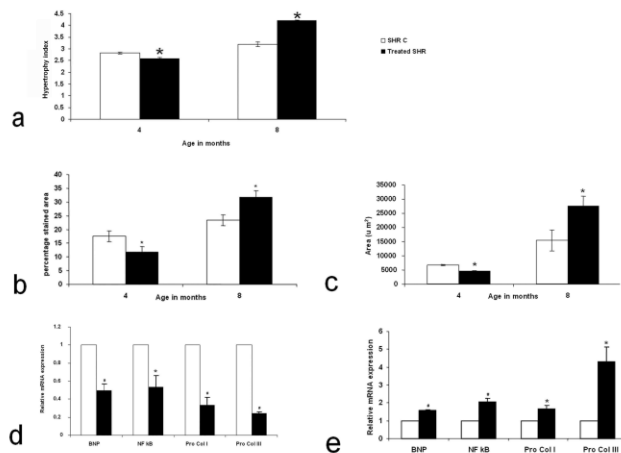


Fig. 7. Cardiac response to PPAR $\alpha$  stimulation in 4 and 8 month old SHR following 60 days of treatment with fenofibrate (100mg/kg/day) (SHR treated) compared with untreated control (SHR C)

- Hypertrophy index (heart weight -mg/ body weight-g)
  - Fibrosis in 4 and 8 month old SHR as indicated by sirius red staining (Stained red)
  - Cross sectional area of LV cardiomyocytes immunostained with anti-dystrophin
  - Relative mRNA expression of BNP, NF- $\kappa$ B, Pro Col I and III of 4 month old SHR
  - Relative mRNA expression of BNP, NF- $\kappa$ B, Pro Col I and III of 8 month old SHR
- \* p<0.01, # p< 0.001 - Significantly different compared to untreated control (SHR C).

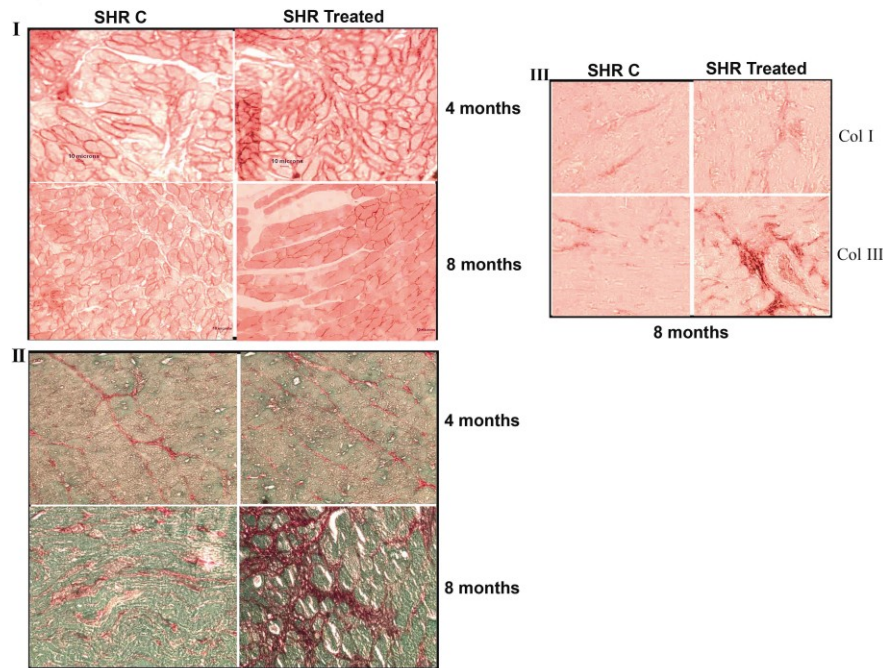


Fig. 8. Sections of left ventricular wall of SHR stained for morphometric analysis of cardiomyocyte area and extent of fibrosis following 60 days of treatment with fenofibrate (100mg/kg/day) (SHR treated) compared with untreated control (SHR C)

I Immunohistochemical staining with anti-dystrophin for detection of cell outline.

II Sirius red staining for detection of areas with fibrosis

III Immunohistochemical staining for detection of collagen I and III

**c. Effect of fenofibrate on regulators of fatty acid metabolism:** Expression of CPT 1 $\beta$  and MCAD were significantly increased in treated SHR of both the age groups (Fig. 9 a,b). Fenofibrate had no significant effect on the expression of PPAR $\alpha$  and CD36 in 4-month-old treated group (Fig.9a). Down regulation of CD36, and upregulation of PPAR $\alpha$  expression at the mRNA level (Fig.9 b) was observed in treated 8 month old SHR. Enhanced expression of mRNA was not accompanied by a statistically significant increase in the protein level of PPAR $\alpha$  (Data not shown). Oil red O staining did not

indicate the presence of lipid accumulation in the myocardium of either treated or untreated SHR (data not given).

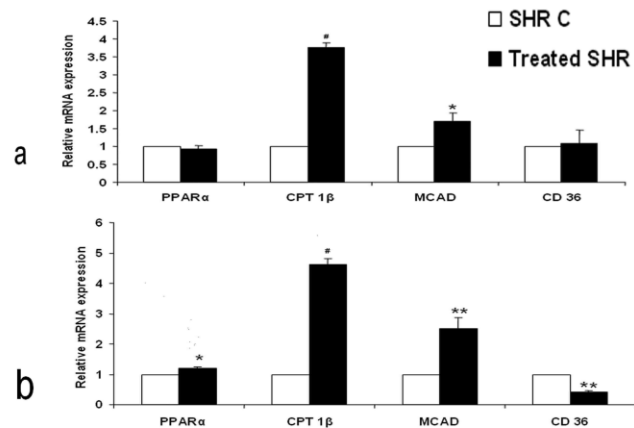


Fig. 9 Relative mRNA expression of PPARα, MCAD, CPT 1β and CD36 in (a) in 4-month old and (b) 8-month old SHR and following 60 days of treatment with fenofibrate (100mg/kg/day)

(SHR treated) compared with untreated control (SHR C)

\*p<0.05, \*\* p< 0.01 ,#p<0.001 Significantly different compared to control

**d. Effect of fenofibrate on oxidative stress:** The treatment reduced the expression of NF-κB in 4-month-old SHR (p<0.01) (Fig. 7d). Serum MDA level was also marginally lower in treated rats (Fig. 10). Enhanced NF-κB expression was accompanied by elevated serum MDA level in 8-month-old treated SHR (Fig. 7e, 10).

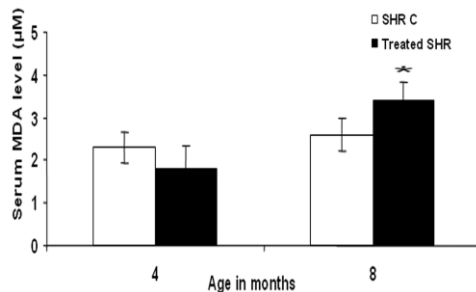


Fig. 10 Serum MDA level in 4 and 8 month old SHR following 60 days of treatment with fenofibrate (100mg/kg/day) (SHR treated) compared with untreated control (SHR C)

\*p<0.01 - Significantly higher compared to control

**f. Serum cholesterol:** Serum cholesterol was measured because fenofibrate is a lipid-lowering drug.. Total cholesterol levels were comparable in treated (66.42±7.16 mg/dl) and untreated (65.51±11.04 mg/dl). animals. The drug is known to reduce cholesterol levels only when there is dislipidemia.

CARDIAC RESPONSE TO SUPPLEMENTATION OF MEDIUM CHAIN TRIGLYCERIDES- Stimulation of fatty acid metabolism using the PPAR $\alpha$  agonist fenofibrate led to increase of oxidative stress and hypertrophy in older SHR. The down regulation of CD36 in older animals despite upregulation of fatty acid metabolism led to the notion that the former change could be the consequence of substrate insufficiency, which inturn may be responsible for the adverse cardiac remodeling. Hence, it was expected that supplementation with medium chain triglycerides (MCT) would be appropriate for stimulation of fatty acid oxidation.

The feed of 2 and 6-month old SHR was supplemented with MCT (5%). Following 4 months of treatment the hypertrophy index, redox status of cardiac tissue, stimulation of fatty acid metabolism and marker of cardiac remodeling was evaluated.

**Effect of supplementation of MCT on fatty acid metabolism-** Expression of Medium chain acyl CoA dehydrogenase (MCAD) was used as a measure of fatty acid metabolism. The expression of MCAD was determined by Western blotting. Bactin was used as te loading control. The level of MCAD increased significantly following supplementation with MCT suggesting stimulation of fatty acid metabolism in both the age groups (Fig. 11).

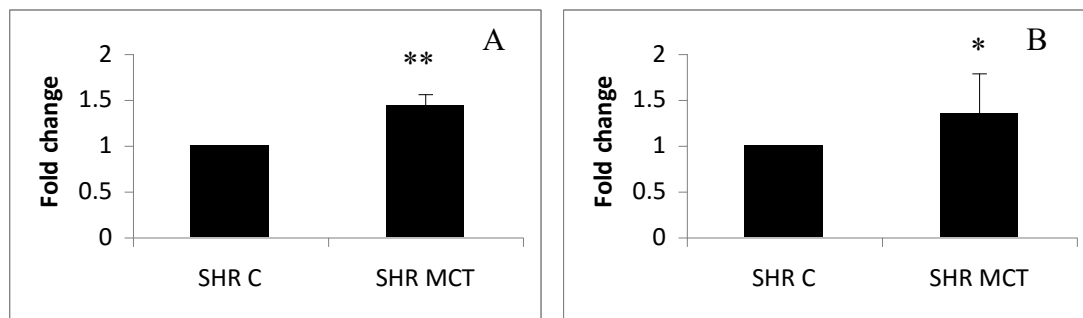
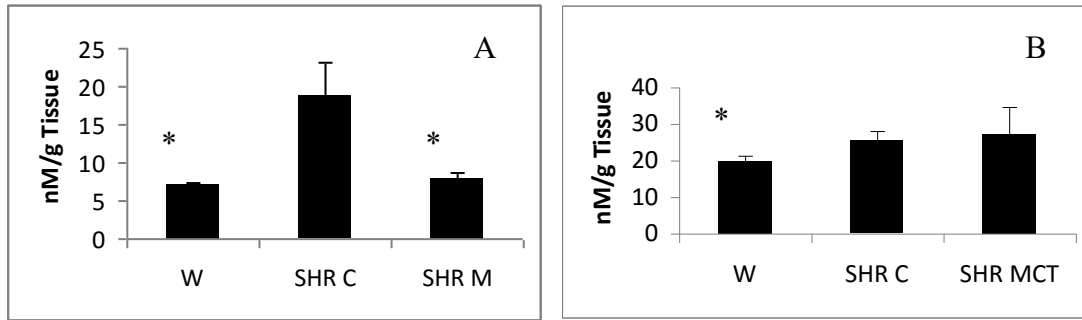


Fig. 11- Cardiac fatty acid metabolism measured as level of MCAD following supplementation of MCT (5%) in the diet of SHR for 4 months. A- 6 month old rat B- 10 month old rat

\*\* p<0.005, \* p< 0.05 compared to untreated SHR

**Effect of supplementation of MCT on oxidative stress-** Lipid peroxidation was used as a measure of oxidative stress. Cardiac tissue MDA levels were estimated colorimetrically. There was significant decrease in oxidative stress the younger rsts (fig. 12). Stimulation of fatty acid oxidation using the PPAR $\alpha$  agonist fenofibrated

exacerbated oxidative stress in older animals. However, the use of MCT did not enhance oxidative stress.

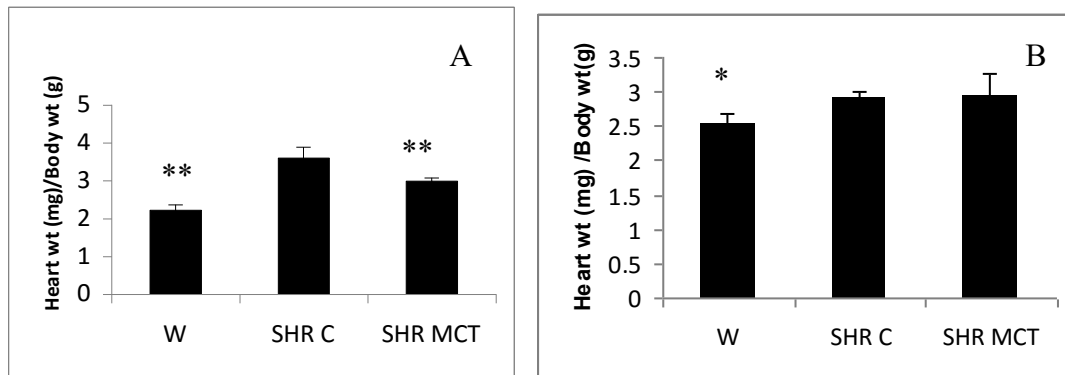


**Fig. 12-** Cardiac oxidative stress measured as extent of lipid peroxidation, following supplementation of MCT (5%) in the diet of SHR for 4 months. A- 6 month old rat B- 10 month old rat

\* p< 0.01 compared to untreated SHR

**Effect of supplementation of MCT on lipid profile-** Total cholesterol HDL cholesterol and triglycerides were unaffected following supplementation of MCT (5%) in the diet.

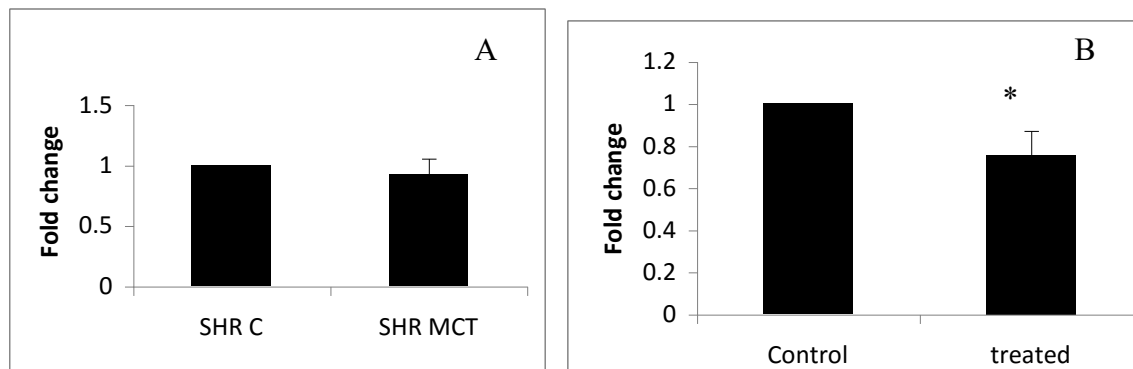
**Effect of supplementation of MCT on blood pressure and cardiac hypertrophy-** The blood pressure was determined by the tail cuff method. Blood pressure was unaffected by the treatment. Hypertrophy index (Ventricular weight (mg)/ Body weight (g)) was measured following the treatment. A significant decrease in hypertrophy index was observed in younger rats. The hypertrophy index was unaffected in the older rats (Fig. 13). This corresponds with the pattern seen for oxidative stress (Fig. 12).



**Fig. 13-** Hypertrophy index following supplementation of MCT (5%) in the diet of SHR for 4 months. A- 6 month old rat B- 10 month old rat

\*\* p< 0.001, \*p< 0.05 compared to untreated SHR

**Effect of supplementation of MCT on cardiac calcineurin-** Enhanced expression of calcineurin is associated with cardiac failure. Expression of calcineurin was assessed by Western blotting. Supplementation of MCT reduced the expression of cardiac calcineurin, but the extent of reduction was significantly more in the older animals (Fig. 14). Increased calcineurin expression is associated with cardiac failure. Reduction of calcineurin following supplementation with MCT underscores the protective effect of the latter in adverse cardiac remodeling.



**Fig. 14-** Expression of calcineurin following supplementation of MCT (5%) in the diet of SHR for 4 months. A- 6 month old rat B- 10 month old rat  
\*p< 0.005 compared to untreated SHR

### (g) Conclusions

#### Salient observations:

#### *Age dependent variation in manifestation hypertrophy, oxidative stress and metabolic shift:*

- Evaluation of morphological and molecular indicators of hypertrophy has shown that SHR develops cardiac hypertrophy as early as two months of age. Evidence of hypertrophic changes as early as 2 months has not been reported earlier.
- The expressions of hypertrophic and inflammatory markers have shown a positive association with age. Inflammatory changes were observed prior to the initiation of hypertrophy suggesting that inflammation can be the trigger for development of hypertrophy in SHR.
- Expression of regulators of fatty acid metabolism has shown an age dependant decrease. Though hypertrophy was seen at 2 months of age, expression of MCAD and

CD 36 decreased at 4 months of age. Down regulation of PPAR  $\alpha$ , a key transcriptional regulator of enzymes in fatty acid metabolism can account for the down-regulation of MCAD and CD 36 during cardiac hypertrophy.

**Inference:** Based on the above sequence in the gene expression of variables associated with hypertrophy, it is concluded that hypertrophy is triggered by inflammation and that metabolic shift occurs following the development of hypertrophy testifying that metabolic remodeling is not the cause, but develops subsequent to cardiac remodeling.

***Effect of stimulation of PPAR $\alpha$  on cardiac remodeling and the stage specific response***

- The treatment up regulated fatty acid oxidation in both the groups as indicated by increased expression of MCAD and CPT 1 $\beta$  signifying activation of PPAR $\alpha$ .
- Irrespective of the age of the animal, fenofibrate treatment significantly reduced both systolic and diastolic blood pressures.
- Morphological, histological and molecular markers of hypertrophy were compared between treated and untreated animals. The mean hypertrophy index and cardiomyocyte cross-sectional area of 4-month-old treated SHR was significantly lower than that of age-matched untreated control and comparable with that of Wistar rats; but showed a significant increase in the older group ( $P < 0.01$ )
- Enhanced oxidative stress as indicated by increase in serum MDA levels and expression of NF- $\kappa$ B was observed in older animals but in younger animals, there was significant reduction of oxidative stress.
- There was significant reduction in the expression of the fatty acid transporter CD 36.

**Inference:** Reactivation of PPAR $\alpha$  for stimulation of fatty acid oxidation can be beneficial in the early stages of cardiac remodeling but can prove detrimental subsequent to metabolic remodeling. This possibly explains the contradictory reports showing both beneficial as well as adverse effects on the heart consequent to PPAR $\alpha$  reactivation. Further, the finding has clinical significance because, fenofibrate is used as a lipid-lowering drug, and it is likely that the duration of illness and the metabolic state determine the cardiac response to the treatment.

It is postulated that the negative response to fenofibrate in older animals is possibly due to substrate deficiency. Hence it is proposed to supplement MCT in the diet and examine whether it is effective in the regression of LVH in older animals.

***Effect of supplementation of Medium Chain triglycerides on cardiac remodeling:***

- Supplementation of MCT in the diet stimulates fatty acid metabolism as indicated by increased expression of MCAD.
- Blood pressure was unaffected by the treatment.
- Unlike the stimulation of fatty acid metabolism with fenofibrate, supplementation of MCT did not enhance oxidative stress in older animals.
- Hypertrophy index was also not affected in older animals by the treatment.
- Supplementation of MCT induced reduction in the expression of calcineurin, a marker of adverse cardiac remodeling.

**Inference:** Reduction in the expression of calcineurin suggests that MCT can prevent adverse cardiac remodeling in chronic pressure overload induced hypertrophy.

**(h) Papers published:**

Sreeja Purushothaman, M.B. Mohamed Sathik, R.Renuka Nair. Reactivation of Peroxisome Proliferator-activated Receptor alpha in spontaneously hypertensive rat: Age associated paradoxical effect on the heart. *J. Cardiovasc. Pharmacol.* . 2011; 58:254-262.

Saifudeen Ismael, Sreeja Purushothaman, V.S. Harikrishnan, R.RenukaNair. Cardiac response to agonist and substrate-mediated stimulation of fatty acid metabolism in Spontaneously Hypertensive Rat. (Manuscript under preparation)

**Papers presented at conferences:**

Sreeja Purushothaman and R.Renuka Nair. Reactivation of PPAR alpha for prevention of cardiac hypertrophy. Paper presented at the Kerala Science Congress. Trivandrum February 2012

Saifudeen Ismael, V.S. Harikrishnan, R. Renuka Nair. Effect of medium chain triglycerides on cardiac oxidative stress in Spontaneously hypertensive rat. Paper presented at the International conference on Biochemical Applications in Therapeutics. Kerala University, Trivandrum, January 2013.

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. Paper presented at the 6th International Conference on Recent Advances in Cardiovascular Sciences held at Delhi Institute of Pharmaceutical Sciences and Research (DIPSAR), New Delhi. January 2014

R. Renuka Nair. Relationship between oxidative stress and energy metabolism in hypertension induced cardiac remodeling. 4<sup>th</sup> International Conference in Clinical and Experimental Cardiology organized by The Omics Group at San Antonio, Texas. April 2014.

**(i) Abstract highlighting the salient features of the project:**

Hypertensive heart disease is associated with metabolic shift, which in course of time can lead to energy depletion. The shift to predominantly glucose oxidation is mediated by down regulation of PPAR $\alpha$ . The study was based on the hypothesis that stimulation of fatty acid metabolism by reactivation of PPAR $\alpha$  can prevent adverse cardiac remodeling. Spontaneously hypertensive rat (SHR) was used as the experimental model. As the response to PPAR $\alpha$  stimulation can depend on the pathophysiological condition, fenofibrate, the ligand-mediated activator of PPAR $\alpha$  was administered to adult SHR by gavage at a dose of 100mg/kg body weight in two different stages of cardiac hypertrophy: (a) at the initial stage (2 months of age) and (b) in established hypertrophy (6 months of age). Following 60 days of treatment, the morphological and molecular markers of hypertrophy, expression of genes associated with metabolism and indicators of oxidative stress were examined.

Fenofibrate upregulated fatty acid oxidation in both the groups as indicated by increased expression of MCAD and CPT 1 $\beta$ . In the young rat hypertrophy index and myocyte cross sectional area as well as expression of BNP, and indicators of fibrosis testified prevention of cardiac remodeling, accompanied by a reduction of oxidative stress. On the contrary treatment of older rats resulted in aggravation of hypertrophy along with augmentation of oxidative stress. In young SHR, where treatment was initiated before the onset of metabolic remodeling, prevention of metabolic shift was accompanied by regression of hypertrophy and reduction of oxidative stress compared to untreated control. In older rats reactivation of PPAR $\alpha$  was associated with enhanced oxidative stress and exacerbation of cardiac hypertrophy. Reactivation of PPAR $\alpha$  after the occurrence of metabolic shift can be detrimental. Whereas expression of CD36 was unaffected in young SHR, the gene was downregulated in older animals. CD36 plays an important role in fuel substrate choice in

the heart and decreased expression of CD36 correlates with reduced fatty acid oxidation and lipid incorporation. Assuming substrate insufficiency as the cause for downregulation of CD36 in older animals, the diet of 2 and 6 month old SHR was supplemented for 4 months with medium chain triglycerides (5%). Fatty acid oxidation was stimulated and blood pressure, hypertrophy index and redox status were not affected in both the age groups. The most significant observation was the reduced expression of calcineurin, a marker of adverse cardiac remodeling. This indicated that supplementation of medium chain triglycerides can prevent adverse cardiac remodeling in pressure overload induced hypertrophy.

**(j) Suggested end use of work:** Though the currently used hemodynamic agents are helpful in the management of hypertension a complementary therapy with metabolic modulation is expected to have a better prognosis. Fibrates are used in therapeutics as a lipid-lowering agent, but the cardiac response has not been evaluated. The observations of the study suggest that use of fenofibrate in the early stages of hypertrophy can be beneficial but may be deleterious in the later stages; where supplementation of medium chain triglycerides can have a positive role. The study has categorically shown that reduction of oxidative stress can prevent adverse cardiac remodeling.

**(k) Likely end users-** Fenofibrate is used in treatment of dislipidemia. Prospective and retrospective analysis of the clinical course of those with hypertensive heart disease shall provide valuable information. The observations of the study highlight the importance of reduction of oxidative stress for prevention of adverse cardiac remodeling in chronic pressure overload.

**(l) Honours, Awards, Patents, if any pertinent to the project-**

Mr. Saifudeen Ismael won the Best Poster Award for his paper entitled, “Reactivation of fatty acid metabolism by medium chain triglycerides modulates oxidative stress and promotes cardiac antiremodeling in spontaneously hypertensive rat” at the 6th International Conference on Recent Advances in Cardiovascular Sciences held at Delhi Institute of Pharmaceutical Sciences and Research (DIPSAR), New Delhi from 31st January to 1st February 2014