5. DISCUSSION

High fat, energy-dense diets have been extensively used by researchers to induce insulin resistance and MS in rodents. HFFD-fed rodents form a good model since the animals present most of the phenotypic characteristics of human MS like obesity, insulin resistance, hyperinsulinemia, dyslipidemia, and hypertension. Pharmaceutical and nutraceutical preparations have been tested for the treatment of diet-induced human MS in this HFFD model. Such studies have helped to not only to understand the disease pathology but also to identify compounds with therapeutic action and determine their mechanism of action. Studies have shown that animals fed energy rich diet also develop cardiac abnormalities (Panchal et al., 2011) and this model was chosen for the present study.

HFFD intake induced insulin resistance, hypertension, dyslipidemia and glucose intolerance. Impaired insulin signaling, dysregulation of lipid metabolism, oxidative stress, cardiac structural and functional abnormalities (hypertrophy and fibrosis) and alterations in cardiac mitochondrial function (respiratory chain complexes, mitochondrial ROS production, decrease in UCPs, mitochondrial biogenesis and dynamics and MPTP induction) were observed in these animals. Since TX has been reviewed in the literature to have antioxidant and anti-inflammatory activities as well as safety and tolerability profile, the present study was aimed to evaluate the cardiac benefits of TX in this model.

5.1. Detrimental effects of HFFD feeding

5.1.1. HFFD induces the components of MS

Significant increase in plasma glucose at 0 and 120 min after glucose load in HFFD-fed mice reflects insulin resistance and the inability of insulin to induce glucose disposal in cardiac tissue. It has
been shown previously that HFFD feeding impairs insulin signaling and ultimately glucose uptake and utilization as early as 15 days in liver and skeletal muscle (Bhuvaneswari et al., 2010; Arunkumar et al., 2012). Hyperglycemia is a compensatory response but in presence of hyperglycemia, it is suggestive of inefficient insulin action. HOMA-IR and QUICKI values serve as indices of insulin resistance and the values reflect insulin insensitivity. Calculating ISI_{0.120} min is considered to be an accurate and easy method as compared to the other two (Gutt et al., 2000). Lower ISI_{0.120} values as compared to control animals indicate impaired insulin sensitivity. HFFD-induced impaired insulin homeostasis is an important predictor for T2D (Huang et al., 2013). Other investigators who have worked on high calorie diet feeding have also reported similar changes in glucose and insulin sensitivity (Wada et al., 2010; Suwannaphet et al., 2010).

Dyslipidemia (elevated levels of TC, TG and FFA, LDL-C and VLDL-C and decreased HDL-C) and lipid accumulation in heart was noted in HFFD-fed mice. Dyslipidemia is considered to be an important risk factor for CVD (Mooradian, 2009). Due to cellular insensitivity to insulin action, glucose uptake and metabolism are diminished and fats tend to be oxidized. Altered myocardial substrate metabolism can lead to lipid accumulation and ultimately cardiac dysfunction (Qin et al., 2010). Our findings are consistent those of McGavock et al., 2006 and Kim et al., 2008.

Elevated blood pressure (hypertension) is closely associated with obesity and commonly occurs in insulin-resistant subjects (Saad et al., 2004). Studies have evidenced that high-fructose diet induces hypertension that is believed to result from several mechanisms like hyperinsulinemia, sympathetic overactivation, oxidative stress and endothelial dysfunction, activated renin-angiotensin system and increased inflammatory mediators (Rault-Nania 2008; Singh et al., 2008).
A rise in BP was observed in HFFD-fed mice as compared to control mice that can lead to changes in the myocardial structure, coronary vasculature, and conduction system of the heart. These changes in turn can lead to the development of LVH, coronary artery disease, conduction system diseases and systolic and diastolic dysfunction of the myocardium and their clinical manifestations including arrhythmias and heart failure.

Body weight is influenced by an interaction between genetic, environmental and psychosocial factors acting through the physiological mediators of energy intake and expenditure. High calorie diet is believed to be one of the environmental factors associated with weight gain. Studies in animals show that diets high in fructose (30%) and fat (60%) increase weight gain and also induce syndrome of insulin resistance (Wada et al., 2010; Sun et al., 2012; Dissard et al., 2013). HFFD used in this study derives 39% calories from fructose and 40% calories from fat source. HFFD caused an increase in Lee’s index compared to the control group suggesting the development of obesity. It can be suggested that ATP depletion induced by high fructose metabolism may provoke the animals to consume enormous food resulting in increased food intake and gain in body weight (Mei et al., 2010). Food intake of the experimental animals did not vary significantly between the groups. However, notable weight gain was observed in HFFD-fed animals which might be result from increase intake of calories and ineffective metabolism of fuels.

5.1.2. HFFD induces dysregulation in FA uptake and metabolism

Flux of long chain fatty acids (LCFA) across the plasma membrane in heart occurs largely via a protein-mediated mechanism (Bonen et al., 2007). A specific 88-kDa FAT protein called CD36 is abundantly expressed in cardiac muscle and appears to be largely
responsible for FA transport in the heart (Van der Vusse et al., 2000). CD36 partially regulates the rate of myocardial FA uptake in humans. Interestingly, CD36 is sensitive to insulin. Bonen and co-workers (2004) suggested that due to hyperinsulinemia, obesity and T2D are associated with an increased translocation of CD36 to the plasma membrane. It was also reported that chronic intake of high-fat diet can induce permanent relocation of CD36 to the plasma membrane leading to increased FA uptake, contractile dysfunction (Ouwen et al., 2007; Carley et al., 2007) and reduced activation of AMPK (Ko et al., 2009). Additionally, transgenic overexpression of FATP-1 in the heart caused lipotoxic cardiomyopathy, suggesting that increase in FA supply to the heart adversely affects cardiac contractile function (Chiu et al., 2005).

Consistent with the literature, increased mRNA expression of CD36 and FATP-1 and lower phosphorylation status of Thr172 AMPK in HFFD-fed mice were noted.

PPAR-α is a ligand-activated transcription factor essential for mediating beta oxidation of FAs. Upon activation, PPAR-α regulates number of genes involved in mitochondrial and peroxisomal β-oxidation in heart (Mandard et al., 2004). PPAR-α targets CPT1, a key enzyme in the outer membrane of mitochondria necessary for the transport of FA into the mitochondria for oxidation. It has been shown that combination of hyperglycemia and hyperinsulinemia increases malonyl-CoA, inhibits CPT-1 activity and directs LCFA away from oxidation and towards storage (Rasmussen et al., 2002). Lowered mitochondrial transport through CPT-1 may contribute to the reduced fat oxidation in the muscle of obese T2D subjects (Blaak, 2004).

Chronic HFFD feeding decreased the expression of PPAR-α and CPT-1b in heart in the study. The decreased expression is responsible for impaired beta oxidation which results in reduced lipid oxidation and increased lipid accumulation in the cells.
AMPK is a phylogenetically conserved fuel-sensing enzyme that plays a central role in nutrient sensing and insulin sensitivity. Dysregulation (defined as decreased activity or impaired activation) of AMPK contributes to the pathogenesis of insulin resistance and MS. The precise mechanism for the suppressed activity of AMPK in insulin resistance is unknown. However, prolonged exposure to excess nutrients (glucose, branched chain amino acids, and FA) has been shown to cause diminished AMPK activity (Saha et al., 2010). Many factors contribute to this decrease, such as 1) reduced phosphorylation of αAMPK Thr172 either by reduced activity of upstream kinases or increased phosphatase activity, 2) increase in phosphorylation of αAMPK ser485/491, a site that is presumed to be inhibitory, 3) changes in adenine nucleotide levels (decreased AMP/ATP ratio) and 4) a change in redox state (Saha et al., 2011).

Animals with a MS phenotype have decreased AMPK activity in muscle and liver (Karegan et al., 2006; Liang and Milson, 2013). Loss of AMPK is reported after metabolic challenge, such as diet-induced insulin resistance and obesity (Zhang et al., 2012), calorie restriction (Chen et al., 2013a), and exercise (Kröller-Schön et al., 2012). In the present study, continuous intake of HFFD reduced the phosphorylation of AMPK resulting in reduced activity of AMPK which contributes to intracellular TG accumulation and then to the development of insulin resistance.

SREBP-1c is a master regulator of de novo lipogenesis (Seo et al., 2013). Studies show that activation of AMPK suppresses SREBP-1c expression through inhibition of LXRα (Lee-Yang et al., 2009) and by direct ser373 phosphorylation of SREBP-1c (Yu et al., 2011). During overnutrition, SREBP-1c gets activated resulting in increased FA and TG synthesis which causes lipid accumulation (Kim et al., 1993). In the present study, mRNA expression of SREBP-1c was found to be
Discussion

upregulated in the heart during HFFD feeding. Insulin stimulates SREBP1c through the MAP kinase pathway. ERK1/2 has been shown to activate the SREBP-1c isoform by phosphorylating ser117 (Roth et al., 2000). The increase in cardiac levels of SREBP-1c may result from reduced AMPK or hyperinsulinemia, a potent stimulator of heart adiposity (Marfella et al., 2009). Rise in SREBP-1c suggests that FAs are directed to lipogenesis rather than oxidation resulting in the accumulation of TG in HFFD-fed mice.

5.1.3. HFFD induces cardiac hypertrophy, cardiac damage and dysfunction

Cardiac hypertrophy is defined as an increase in the size of the entire heart or of a specific cardiac LV chamber relative to body size. LVH is a compensatory mechanism for the heart to work more effectively in response to mechanical or oxidized stress and overactive sympathetic drive, or as a consequence of genetic abnormalities. LVH is an important pathologic hallmark of the disorder of the myocardium. During prolonged stress, cardiac contractility cannot be preserved and may proceed to chamber dilation and impaired cardiac output with an increase in mechanical stress of the ventricular wall and subsequent slippage of the myofibrils (Gradman and Alfayoumi, 2006; Hori and Nishida, 2009). This elicits an increase in collagen synthesis by the ECM which makes the ventricular wall stiffer and thus increases left ventricular diastolic pressure (Hori and Nishida, 2009).

HFFD-fed mice presented increased heart weight/tibia length ratio, LV weight/tibia length and LV weight to right ventricular weight, which is an indicator of LVH and increased cardiac size. LVH may have been produced in response to hypertension caused by high fructose and excess ROS. ROS has been reported as a product of excessive FA oxidation in diabetic cardiomyocytes. As second messenger, ROS can
mediate hypertrophic signals by regulating various intracellular signal transduction cascades and the activity of various transcription factors, such as NF-κB and activator protein-1 (Hirotani et al., 2002), and MAPKs (Molkentin et al., 2004).

Hypertension is a major determinant of LVH that can alter wall stress in the left ventricle. Experimental studies highlighted the important role of renin-angiotensin-aldosterone system (RAAS) in mediating LVH (Schlaich et al., 2000; Milan et al., 2010). Ang-II induces hypertrophy and hyperplasia in myocytes and vascular smooth muscle cells and may regulate collagen synthesis. Antihypertensive treatment with drugs like β-blockers and ACE inhibitors interfere with the actions of the RAAS, thereby lessen the LV mass development.

HFFD-fed mice also showed increased plasma levels of cTnI and cTnT (sarcomeric proteins of the contractile complex) indicating the entry of these proteins from tissue to blood stream due to cell membrane damage and increased permeability.

ECG data can give information on LVH and it is useful to understand cardiac dysfunction and heart disease (Somaratne et al., 2011). ECG observations revealed R-wave enlargement, development of Q-waves, prolonged QT interval and ST-segment depression in HFFD-fed mice. These findings suggest LVH, ventricular dysfunction and decreased heart function in these animals. An increase in RR cycle length induces a threefold increase in QT duration. This change in QT-RR relation suggests cardiac arrhythmia, a risk factor for CVD. In fact, HFFD mice showed increased heart rate.

Inflammation and fatty plaque formation observed in the heart tissue of HFFD-fed mice indicate the severity of pathological events triggered by lipid accumulation and oxidative stress. Initially, the inflammatory infiltration occur perivascularly then it spreads interstitially similar to collagen deposition.
5.1.4. HFFD induces cardiac fibrosis

Cardiac fibrosis is major feature of hypertrophic cardiomyopathy and it is the most frequently occurring pathological response to conditions such as hyperglycemia, dyslipidemia and hypertension (Roever et al., 2014). Interstitial fibrosis has been considered to be a causative factor in the impairment of functional integrity of the heart and is one of the major determinants of morbidity and mortality from CVD (Assayag et al., 1997 González-Vilchez et al., 2005). Impaired contractility and chamber dilation are shown to occur due to: increased ECM turnover and fibrosis formation and myocyte apoptosis.

Altered substrate utilization by heart results in structural and functional impairment of cardiac myocytes, which pave way for cardiac fibrosis ultimately leading to impaired contractile performance and HF (Aronson, 2003). Extended cardiac fibrosis results in increased myocardial stiffness, causing ventricular dysfunction and, ultimately HF. Therefore, reversal of fibrosis may improve organ function and survival.

Fibrosis results from increased activation of growth factors (cytokines), one among which is TGF-β1 (a profibrotic cytokine) and an imbalance in the ECM modulators MMPs and TIMPs (Sun, 2009; Fan et al., 2012). Fibrosis is accompanied by increased expression of α-SMA, a marker for cells having a fibrotic phenotype.

TGF-β1 is a crucial factor that induces transdifferentiation of fibroblasts to myofibroblasts. Alterations in cell morphology, enlargement of cell volume and increase in matrix protein content are accompanied during this differentiation. TGF-β1 is rapidly expressed at the site of injury and involved in the repair process. Studies evidenced that TGF-β1 is the most powerful accelerative factor (Deten et al., 2001) that stimulates the deposition of ECM proteins to heal the
damage. During repeated injury, the continuous production of TGF-β1 leads to matrix deposition thereby promotes expression of surplus ECM proteins causing hypertrophy. Increased level of TGF-β1 has been reported in human and experimental cardiac hypertrophy and fibrosis (Rosenkranz, 2004; Luedde et al., 2006).

Increase in TGF-β1 can be attributed to overproduction of ROS and the prevailing hyperglycemia. One study reported that addition of SOD abolished TGF-β1-induced cardiac myofibroblasts differentiation (Challa et al., 2012) while another showed that glucose induces TGF-β1 in h9c2 culture (Twum et al., 2014).

α-SMA is the actin isoform found in vascular smooth muscle cell and is actively expressed in cells engaged in fibrogenesis. In healing wounds myofibroblasts are required for tissue repair; however in pathologic conditions activated myofibroblasts fail to work, leading to persistence of the myofibroblasts, and consequently expansion of the ECM proteins (fibrosis) and contraction. The myofibroblast phenotype is characterized by the expression of contractile proteins, such as α-SMA and consequently displays a markedly enhanced ability to contract ECM (Porter and Turner, 2009). Increased levels of TGF-β1 and α-SMA in the myocardium suggest fibrosis in HFFD-fed mice which was confirmed by the occurrence of perivascular and interstitial fibrosis in histological observation.

MMP activation is a part of the pathological cardiac fibrosis and remodeling process (Siwik et al., 2001). MMP-TIMP homeostasis plays a key role in the turnover of collagen and matrix remodeling (Fan et al., 2012). LVH might result from an imbalance in extracellular remodeling that in turn would result from an imbalance of ECM turnover and fibrosis formation. Increased protein levels and gelatinase activities of MMPs-9 and-2 and decreased action of TIMPs-1 and-2 were observed in
HFFD-fed mice. An increased ratio of MMP-to-TIMP is associated with greater turnover of ECM representing greater degree of ventricular remodeling process. TGF-β1 can upregulate both MMP-9 and -2 activities (Spinale, 2007), that may be responsible for the rise in MMPs in HFFD-fed mice.

ROS stimulate the production of inflammatory cytokines and inversely, inflammatory cytokines stimulate ROS formation. In chronic stage, ROS and inflammatory cytokines activate the MMP and collagen deposition which contribute to the structural changes and tissue repair of injured myocardium. Activation of MMP elicits degradation of collagens which may cause a slippage in myofibrillar alignment causing LV dilatation.

Collagen is an important protein of the ECM and is a sensitive marker for ventricular remodeling (Cheng et al., 2003). Collagen accumulation was evaluated by histology and hydroxyproline assay in the present study. Masson’s trichrome staining of cardiac sections revealed marked collagen accumulation in HFFD-fed mice with increased cardiac collagen content. The rise in collagen in relation with increase in MMP activity explains fibrosis in HFFD-fed mice.

Thus inflammation and fibrosis in heart played an important role in ventricular remodeling in HFFD-fed mice.

5.1.5. HFFD suppresses activation of insulin signaling molecules

The macronutrient composition of a diet is an important environmental determinant of the quality of insulin action (Bessesen et al., 2001; Axen et al., 2003). HFFD consumption led to insulin resistance which was confirmed after 15 days and at the end of the study period. Tyrosine phosphorylation of IRβ, IRS-1, IRS1-PI3K association and Akt (ser phosphorylation) in HFFD-fed mice were significantly reduced when compared with control mice under insulin-stimulated conditions.
Phosphorylation status of ser/thr residues is one important mechanism that has antagonist action towards IR and IRS proteins, Tyr phosphorylation. Interestingly, high-fat diet–induced insulin resistance is ameliorated when specific ser/thr kinases are genetically ablated or pharmacologically inhibited (Yuan et al., 2001; Kim et al., 2004). Different signals can induce this phosphorylation. These include FFA, DAG, glucose and insulin. Evidence also suggests that ROS can have a significant inhibitory effect on insulin signaling in both in vivo and in cell culture systems (Gardner et al., 2003; Dokken et al., 2008). Studies describe that lipid accumulation in skeletal muscle is associated with ser/thr phosphorylation on IRS-1 and -2 proteins which uncouples them from the receptor and inhibits binding and activation of PI3K and downstream signals and finally stops the insulin signal transduction (Krssak et al., 1999; Peterson and Shulman, 2006). Reduction in tyr phosphorylation of IRS family members has been observed in insulin-resistant animal models and human subjects, including those who are obese or made insulin resistant by lipid infusion (Goodyear et al., 1995; De Fronzo et al., 2000). These data suggest that defects in lipid metabolism leading to impairment of insulin signaling seem to be a major mechanism for insulin resistance during HFFD feeding.

Insulin resistance is a risk factor of left ventricular dysfunction and HF due to impaired delivery of glucose to the cardiomyocyte (Doehner et al., 2005). Impaired myocardial insulin-stimulated glucose uptake has been described in human and animal models of obesity and insulin resistance (Abel, 2005; Abel et al., 2008). Reduced glucose uptake in turn accounts for increased FA utilization. Carroll et al. (2005) demonstrated that glucose uptake is reduced as a consequence of reduced GLUT-4 protein and impaired insulin signaling in the myocardium of db/db mouse. GLUT-4 translocation is reduced in human cardiac muscle biopsies through activation of the IκB kinase (IKK) and
other serine kinases (Nie et al., 2012). One study has documented decrease in GLUT-4 mRNA in the myocardium of fructose-fed animals (Qin et al., 2010). Li et al. (2008) observed both a decrease in GLUT-4 mRNA and insulin-stimulated GLUT-4 translocation to the plasma membrane in rat skeletal muscle. Reduced translocation of GLUT-4 was observed in HFFD-fed mice indicating diminished cardiac glucose uptake.

5.1.6. HFFD induces oxidative stress

Oxidative stress is a state resulting from an imbalance between the systemic manifestation of ROS and the ability of cells to get rid of ROS and the toxic products. Hyperglycemia and high rates of FA oxidation could lead to an increase in ROS production (Francini et al., 2010) causing oxidative stress. It is reported that ROS can cause cardiac dysfunction and failure via cellular damage by oxidation of nucleic acids, lipids, and proteins (Seddon et al., 2007). Studies reported that ROS may play an important role in setting insulin resistance in the cardiovascular tissue (Ritchie et al., 2006).

ROS in the heart can act as second messenger, initiating hypertrophic signaling. It is reported that ROS /oxidants can activate MAPK pathways leading to promotion of cellular growth and stimulation of hypertrophic response (Molkentin, 2004). Studies in rodents and in vitro models reveal that administration of ROS scavengers, antioxidants or NADPH oxidase inhibitors can inhibit the activation of MAPK pathways and the resulting myocardial growth (Elmedal et al., 2004; Ritchie et al., 2007). The role of ROS in cell damage and apoptotic cell death is well documented and its involvement in the progression of cardiac remodeling is found to be associated with diabetic heart disease (Boudina and Abel, 2007).
ROS production in the heart is mediated by β-oxidation and peroxisomal oxidation of FAs. Generally, peroxisomal production of H$_2$O$_2$ serves to detoxify cellular toxins but a small fraction can escape to participate in other oxidative reactions. Heightened catabolism of FFAs by the peroxisomes results in the overproduction of ROS (Singh, 1997) in the heart. It is reported that this pathway is upregulated during insulin resistance, and may play a role in the induction of oxidative stress (Huggins et al., 2008).

Another very important source of ROS in the heart is NADPH oxidase. This enzyme catalyzes the one-electron reduction of O$_2$ to O$_2^-$ using NADPH as the electron donor (Selemidis et al., 2008). Since this enzyme is located in the surface membrane it makes a significant contribution to ROS production, especially in chronic pathologic states like insulin resistance and T2D (Selemidis et al., 2008). Evidences suggest that NADPH oxidase-derived ROS play important roles in the processes underlying cardiac hypertrophy, interstitial cardiac fibrosis and LV remodeling (Paravivini and Toyuz, 2008; Looi et al., 2008). ROS generation in HFFD-fed mice can be linked to increased formation of O$_2^-$ by the overexpression of p22phox, a low molecular weight subunit of NADPH oxidase, essential for the activation of the enzyme.

High calorie diet feeding was found to be associated with increase in ROS production in the aorta and heart, associated with enhanced markers of oxidative stress (Al-Awwadi et al., 2004). Oxidative stress and damage to the myocardium is evident in HFFD-fed mice by the rise in the levels of lipid and protein oxidation products (TBARS, LHP, PC and AOPP). This can be attributed to the prevailing hyperglycemia and increased FFA levels. AOPP are formed by reaction between plasma proteins (especially albumin) and chlorinated oxidants during oxidative stress and are considered as a marker of protein damage (Liu et al., 2006). Several studies have reported reduction in antioxidant potential
(both enzymatic and non-enzymatic) during fat or fructose intake (Thirunavukkarusu et al., 2004; Francini et al., 2010). In this study, reduced levels of enzymatic and non-enzymatic antioxidants were observed in HFFD-fed mice.

5.1.7. HFFD induces mitochondrial alterations

Multiple approaches were used in the present study to investigate the integrity of cardiac mitochondria in experimental animals. The findings reveal that mitochondria from HFFD-fed mice heart are severely damaged have reduced oxidative capacity and show impaired biogenesis thereby increasing susceptibility to apoptosis. The findings were characterized by elevated ROS generation, oxidative damage to lipid and mtDNA, depletion of cellular antioxidants, fall in UCPs -2 and -3, fission- fusion imbalance, rise in mitochondrial calcium, membrane potential generation and caspase-mediated apoptosis.

Mitochondrial injury is reflected by mtDNA damage as well as by a decline in the mitochondrial RNA (mtRNA) transcripts, protein synthesis, and mitochondrial function. mtDNA is more susceptible to oxidative attack than nuclear DNA possibly because of its proximity to the respiratory chain within the inner mitochondrial membrane. Hence, under conditions of oxidative stress, mtDNA gets damaged. The mtDNA content/ copy number serves as a key index for oxidative mitochondrial damage and it has been reported in rats fed high fat diet (Dong et al., 2007). HFFD-fed mice show decreased mitochondrial content of DNA, which was expressed relative to nuclear DNA and confirmed with decreased mRNA expression of TFAM.

Consistent with reduction in mtDNA content and transcript levels, oxidative damage to mtDNA can result in a decrease in ETC complex activities. Multitudes of studies have suggested a possible link between mtDNA damage, increased lipid peroxidation, and decrease in
complex enzyme activities (Dong et al., 2007; Yuzefovych et al., 2013) and our results are consistent with this. The activities of complexes I, III, and IV were significantly lower in HFFD-fed mice hearts as they are encoded by mtDNA whereas the enzymatic activity of complex II encoded by nuclear DNA was not significantly modified. Diminished complex enzyme activities can occur due to decrease in mtDNA content.

5.1.7.1. HFFD impairs mitochondrial biogenesis

The content and composition of mitochondria in a cell is determined by the process of mitochondrial biogenesis that involves the growth and division of pre-existing mitochondria. Mitochondrial biogenesis requires the coordinate expression of nuclear and mitochondrial genes involved in mitochondrial structure, metabolism, and proliferation. The number and size of mitochondria are correlated with mitochondrial oxidative capacity (Ritz and Berrut, 2005) and therefore impaired biogenesis is a major feature of mitochondrial dysfunction. Mitochondrial biogenesis is influenced by factors/conditions stress such as exercise, caloric restriction, low temperature, oxidative stress, cell division and renewal and differentiation.

PGC-1α, and its downstream targets, such as NRF-1, NRF-2 and TFAM, is known to be the main regulators of the mitochondrial biogenesis pathway and that ultimately regulate mitochondrial function. PGC-1α seems to be essential for normal heart function as PGC-1α knockout or deficiency result in diminished cardiac function and HF (Handschin and Spiegelman 2006; Arany et al., 2006). Decreased PGC-1α expression is maladaptive in heart disease.

During pathological growth of the heart, downregulation of PPARα and PGC-1α causes decreased expression of FA oxidation genes (Zoll et al., 2006). Several studies have observed reduction in PGC-1α and its downstream targets in cardiac tissue that could contribute to FA
oxidation and energy production. PGC-1α and its downstream effectors, NRF2 and TFAM have been reported to be reduced in rats with myocardial infarction (Sun et al., 2007), spontaneously hypertensive rats, and in a mouse model of hypertrophic cardiomyopathy (Watson et al., 2007). Mitochondrial biogenesis and the number of mitochondria are reduced in insulin resistant subjects owing to decreased expression PGC-1α and NRF-1 (Patti et al., 2003). Downregulated mRNA expression of PGC-1α and its downstream targets NRF-1 and -2 were noted in HFFD-fed mice.

5.1.7.2. HFFD alters mitochondrial dynamics

Mitochondria are highly dynamic organelles that constantly change shape and number in response to different stimuli and to changes in metabolic demands of the cell (Chen and Chan, 2005; Chan, 2006). Defects in mitochondrial organization and the presence of small and fragmented/disorganized mitochondria are associated with a decrease in OPA1 levels in cardiac myocytes and are correlated with a rise in apoptosis in post-myocardial infarction-induced HF (Chen et al., 2009).

MFN-2 protein is a dynamin-related protein with GTPase activity anchored in the external mitochondrial membrane. The central role of MFN-2 in mitochondrial metabolism is well-established (Honda et al., 2005). MFN-2 activity is crucial in the maintenance of mitochondrial tubules in cultured muscle cells (Pich et al., 2005). Overexpression of MFN-2 in HeLa cells causes perinuclear aggregation of mitochondria, a marked enhancement of mitochondrial membrane potential and increased glucose oxidation (Pich et al., 2005). A study reported that feeding high-fat diet induces insulin resistance and MFN-2 down-regulation in rat muscle (Sebastián et al., 2012). MFN-2 expression and insulin sensitivity were reduced by a high-fat diet with concomitant accumulation of lipid intermediates in the muscle.
Growing evidence suggests that reduction in mitochondrial fusion is an important etiological factor in development of obesity and insulin resistance since inhibition of fission and/or activation of fusion proteins is found to counteract many of the disease phenotypes related to insulin resistance and diabetes (Bach et al., 2003; Civitarese, 2008). A study reported the downregulated mRNA expression of fusion genes (Zhang et al., 2013) and increased expression after exercise and weight loss in humans and animals (Bach et al., 2005; Cartoni et al., 2005; Mingrone et al., 2005).

In the present study, excess energy intake through HFFD caused a shift in the balance between fission/fusion processes thereby altering the mitochondrial number and size. Decreased mRNA expression of OPA1, MFN-1 and -2 and increased mRNA levels of fission proteins such as DRP1 and FIS1 indicate imbalance favouring smaller and fragmented mitochondria. Ultrastructural studies showed reduced mitochondrial density and muddled and smaller mitochondria in hearts of HFFD-fed mice. Decreased mRNA levels of fusion genes and mitochondrial functions in insulin resistant animals fed high calorie diet have been reported (Zhang et al., 2013; Lionetti et al., 2014).

5.1.7.3. HFFD diminishes uncoupling reactions

UCPs are proton translocases that play a protective role by regulating both heat and ROS generation during oxidative phosphorylation. UCP genes have PPAR response elements in their promoter regions (Tu et al., 1999; Acin et al., 1999) and the levels are regulated via PPAR-α and PGC-1α (Puigserver et al., 1998; Young et al., 2001). Reduction in the expression of PPAR-α in HFFD-fed mice may be responsible for the reduction in the UCP genes. Rise in mitochondrial ROS could be due to UCP reduction since UCP has been shown to minimize mitochondrial ROS generation (Mailloux and Harper, 2011).
It is reported that decreased mRNA and protein levels of UCPs-2 and-3 result in increased ROS generation in prediabetic and diabetic subjects (Patti et al., 2003; Arsenijevic et al., 2006), whereas their overexpression offers cytoprotection by limiting oxidative injury (Teshima et al., 2003; Vincent et al., 2004).

5.1.7.4. HFFD impairs calcium handling

Mitochondrial ROS generation can cause impaired intracellular calcium homeostasis increasing calcium influx. ROS can induce intracellular Ca$^{2+}$ overload through the extracellular Ca$^{2+}$ influx mediated by increased membrane lipid peroxidation and the opening up of calcium channels. The predominant alteration in Ca$^{2+}$ homeostasis is decreased uptake of Ca$^{2+}$ into the sarcoplasmic reticulum which results in calcium accumulation in the cardiomyocyte (Belke et al., 2004; Periasamy et al., 2008). Supporting these lines of evidence the study observed decreased mRNA expression of SERCA2a and the NCX in the heart of HFFD-fed mice. This suggests impaired calcium handling in the heart and enhanced Ca$^{2+}$ flux in HFFD-fed mice. In the present study, myocardial calcification in HFFD-fed mice was assessed with hematoxylin and eosin and confirmed with alizarin red S stain.

Mitochondrial swelling is a significant index of mitochondrial dysfunction and suggestive of MPTP opening. Mitochondrial swelling in HFFD-fed mice reveals membrane damage that might contribute to high intramitochondrial Ca$^{2+}$, oxidative stress and increased MPTP. Ultrastructural studies also confirmed the presence of mitochondrial swelling in hearts of HFFD-fed mice. Permanent MPTP opening causes rupture of the mitochondrial membrane, cytochrome $c$ release and apoptosis in HFFD-fed mice.
5.1.8. HFFD induces peroxidation of cardiolipin and apoptosis

Cardiolipin, a highly specialized phospholipid of the inner mitochondrial membrane is attached to cytochrome c and is reported to be the primary target during oxidative stress. Increased lipid peroxidation decreases the levels of cardiolipin in the myocardium of ob/ob mice and STZ-induced diabetic mice (Han et al., 2005). HPLC analysis showed loss of cardiolipin content in the heart of HFFD-fed mice. This loss may be due to its susceptibility to ROS attack either because of its high content of unsaturated FAs or because of its close location to the site of ROS production. The data suggest that HFFD-induced oxidative stress is a pathologic state in which reduction in cardiolipin content contributes to apoptosis through promotion of cytochrome c release into cytosol.

Mitochondria are the gatekeepers of cardiomyocyte death or survival, particularly in heart disease. The opening of the MPTP is considered to be the ‘point of no return’, after which the myocyte is irreversibly committed to necrotic or apoptotic death pathways (Halestrap, 2009). Studies in animal models have proved that feeding energy-dense diet over a month results in lipid accumulation, oxidative stress and depletion of antioxidants and prolonged feeding would culminate in myocardial apoptosis which largely reflect adaptations and maladaptations in the mitochondria (Ballal et al., 2010). Studies have shown that ROS production is the proximate cause of myocardial apoptosis (Li et al., 2009; Zhang et al., 2012). Cellular apoptosis occur after a series of events like cytochrome c release into the cytosol, up-regulation of caspases-9 and-3 activation, BAX stimulation and BCL-2 suppression (Ballal et al., 2010, Hua et al., 2013). HFFD-fed mice showed increase in BAX/BCL-2 ratio that favors apoptosis.
Increased ROS, oxidative damage to cardiolipin, cytochrome c entry into the cytosol, high intracellular calcium, MPTP opening and mitochondrial fusion/fission balance are responsible for the execution of cardiac cell apoptosis observed in HFFD-fed mice.

5.2. Effects of TX supplementation in HFFD-fed insulin resistant mice

Insulin-sensitivity effect and antioxidant replenishing potential of several naturally occurring substances have been studied by many investigators. For example, intervention with compounds such as rutin (Panchal et al., 2011), quercetin (Kannapan et al., 2009) resveratrol (Chen et al., 2011) and berberine (Wang et al., 2011) is shown to promote insulin sensitivity, improve the antioxidant potential and reduce oxidative stress in high calorie diet-fed rats. However, whether administration of TX generates any beneficial effect in HFFD-fed mice has not been explored. The results of the present study are the first evidence to demonstrate that TX effectively suppresses HFFD-induced metabolic changes, promotes insulin sensitivity, activates the antioxidant system, improves mitochondrial biogenesis and downregulates the apoptotic pathway in the myocardium.

Insulin sensitizing, antifibrotic and antiapoptotic effects of TX are the major outcome of the study. Among the four different doses tested, 150 mg/kg and 200 mg/kg effectively reduced body weight gain, plasma glucose and insulin levels. A dose-dependent effect at a concentration of 50 and 100 mg/kg was observed. At 200 mg/kg, a similar effect was observed as that of 150 mg/kg. Thus, the optimal dose was fixed as 150 mg/kg bw for the study.

5.2.1. Effects of TX on the features of MS

In the present study, TX restored the changes in plasma chemistry and alterations in physiology parameters induced by HFFD feeding. Body weight gain and Lee’s obesity were reduced by TX.
Reducing energy intake and increasing energy expenditure are essential to the treatment of obesity. The results obtained with TX suggest that in spite of continued intake of HFFD, TX is able to prevent weight gain by increasing energy expenditure possibly by promoting lipid metabolism. Lu et al. (2011) showed that TX supplementation prevented obesity and restored abnormal glucose, FA, and cholesterol induced by high-cholesterol and high fat diet. In this study, administration of TX to HFFD mice resulted in significant reduction in plasma levels of glucose and insulin, glycated hemoglobin and improvement in glucose tolerance. Improved insulin action in presence of TX explains its beneficial effect. A recent study showed that TX has antihyperlipidemic activity and prevents steatosis in high-fat diet-treated mice (Zhang et al., 2014). Consistent with this, the observed data in our study confirms the antihyperlipidemic activity of TX. TX supplementation significantly reduced the levels of TC, TG, FFA and lipoproteins in heart of HFFD-fed mice. TX decreases circulatory levels of FA and by switching myocardial substrate metabolism towards glucose. Improved insulin signaling by TX would cause increased uptake and cellular availability of glucose and reduce FA oxidation. This suggests that insulin resistance-induced impairment of substrate flexibility is nullified in HFFD-fed mice in presence of TX. TX administration significantly attenuated the rise in BP in HFFD-fed mice that may be attributed to normalization of insulin levels. High BP has been shown to be correlated with oxidative stress and subsequently reduced NO bioavailability (Kobayasi et al., 2010). TX, by scavenging free radicals, should increase the bioavailability of NO and thereby reduce systolic blood pressure.

5.2.2. Insulin sensitizing action of TX

The enhancement of insulin signaling by TX is associated with the activation of IRS1–PI3K–Akt pathway. This leads to efficient glucose uptake which in turn is responsible for the restoration of glucose levels.
Discussion

IR/IRS-1 tyr phosphorylation is the early event in the insulin signaling pathway. Recent studies confirm that lipid intermediates such as long chain fatty acyl CoA species (LCCoAs), DAG, and ceramides disrupt one or more of the early steps in insulin signal transduction (Liu et al., 2007; Schenk and Horowitz, 2007). The negative modulators of insulin signaling primarily act by decreasing tyr phosphorylation and activation of ser phosphorylation of these molecules (IR and IRS-1).

Mice fed high fat diet suffer from cardiac insulin resistance as evidenced by increase in DAG, resulting in activation of PKC which in turn increases ser phosphorylation of IRS-1 with decreased insulin-stimulated IRS-1/2 tyr phosphorylation and downstream insulin signaling (Park et al., 2005; Zhang et al., 2010). In addition, high fructose diet can activate serine kinases like JNK-1, inhibitor of nuclear factor kappa B kinase (IkB) complex and extracellular signal-regulated kinases that inhibit ser 307 phosphorylation of IRS-1 contributing to insulin resistance (Kim et al., 2008). Genetic (Kerouz et al., 1997) and induced (Folli et al., 1993) rodent models of obesity and insulin resistance also present reduced tyrosine phosphorylation of IRS proteins.

TX-treated HFFD-fed mice showed an improved tyrosine phosphorylation of IRβ, IRS-1 and IRS-1/PI3K association, Akt phosphorylation and GLUT-4 translocation. The favourable effect of TX on insulin signaling can be attributed to its antioxidant, anti-inflammatory and lipid lowering activities. These actions of TX may down-regulate the serine kinases. These results corroborate with other investigators who reported that TX being an antioxidant, improves insulin signaling in the hippocampus of mice-fed high cholesterol diet (Lu et al., 2011) and in gastrocnemius muscle of high fat plus sucrose-induced type-2 diabetic adult male rat (Sambath and Karundevi, 2014).
5.2.3. Effect of TX on lipid metabolism

Overconsumption of fat is a significant contributor to the development of obesity and insulin resistance. The study observed disturbances in lipid metabolism after HFFD feeding and accumulation of fat in the myocardium.

To provide a mechanism for the lipid-lowering effect of TX, the expression of genes involved in FA uptake and metabolism and phosphorylation status of AMPK were analyzed. In the pathophysiological context of MS, induction of PPAR-α might induce FA catabolism and prevent fat deposition. Upregulation of PPAR-α reduces hyperlipidemia and improves insulin sensitivity (Ye et al., 2001; Aasum et al., 2008). Agonists of PPAR-α possess the property of reducing lipid dystrophy and obesity (Nagai et al., 2001). Several experimental studies using animals and in vitro models found that PPAR-α activation prevents lipid accumulation in heart by increasing the rate of FA catabolism (Jiang et al., 2004; Haemmerle et al., 2011; He et al., 2012). TX exerted lipid-lowering effect through upregulation of PPAR-α resulting in SREBP-1c reduction thereby promoting beta oxidation.

Supplementation of TX increases AMPK phosphorylation, which, in turn, facilitates FA oxidation. TX may activate PPAR-α either directly or indirectly through AMPK phosphorylation since activated AMPK can target PPAR-α (Kondo et al., 2009) and can inhibit SREBP-1c transcriptional activity (Zhou et al., 2001).

TX simultaneously reduced the gene expression of CD36 and FATP-1 responsible for FA transport. Lipid intermediates such as DAG, ceramide, fatty acyl CoA can result in lipid-induced muscle insulin resistance (Zhang et al., 2013). Acceleration of FA oxidation may lower these intermediates and may lessen the potential for insulin resistance.
The upregulation of CPT-1b by TX, suggest that the redundant lipids in the cytosol could be transported into the mitochondria for β-oxidation, thus relieving the damage to insulin signaling resulting from the accumulated lipid intermediates.

From this data, it can be concluded that TX might improve insulin sensitivity by reducing TG and FFA levels. The expression of transcription factors (PPAR-α and SREBP-1c) and target enzymes may be modified favorably by TX resulting in lipid homeostasis.

5.2.4. Effect of TX on cardiac structure and function

The present data demonstrate that cardiac hypertrophy, damage to the myocardium and cardiac dysfunction are induced in insulin resistant mice. Cardiac hypertrophy is attributed to hyperinsulinemia and defects in insulin signaling. It has been shown that chronic hyperinsulinemia stimulates the Ang-II signaling that is involved in pathological hypertrophy (Samuelsson et al., 2006). Insulin resistance appears first in skeletal muscle and adipocytes inducing elevated plasma insulin levels. This hyperinsulinemia probably facilitates hypertrophy by overactivating the mitogenic effects of insulin. Pathological hypertrophy is characterized by a remodeling of substrate utilization with a shift to carbohydrate usage (Ritchie and Delbridge, 2006; Sharma et al., 2007). It is increasingly evident that this metabolic shift participates in the establishment of hypertrophy (Ritchie and Delbridge, 2006). Since TX has the potential to improve insulin signaling, it is suggested that TX may significantly reduce heart weight, LV weight and blunt the LVH in HFFD-fed mice. TX by allowing efficient FA metabolism, changes this energy shift back to increased FA oxidation so that the elevated energy demands of the hypertensive heart can be met. This potential of TX to increase FA oxidation in the myocytes while reducing oxidative stress by decreasing mitochondrial superoxide generation provides a likely explanation for the attenuation of cardiac remodeling observed in the current study.
The results demonstrate that TX administration effectively improved ECG pattern, with a parallel decrease in cTnI and cTnT levels in plasma. ECG observations revealed normalization of ST segment depression, R wave enlargement and RR interval in TX-treated mice representing a cardioprotective effect of TX against HFFD-induced pathological changes.

5.2.5. Effect of TX on cardiac fibrosis

Protein levels of fibrotic markers and the histopathological data demonstrate the development of fibrosis in heart by HFFD feeding. Metabolic disturbances, increased oxidative stress, inflammation, TGF-β1 expression, MMP activation and collagen accumulation are ascribed to a fibrotic phenotype of the heart. It can be suggested that TX might regulate one or more stages involved in the fibrogenic process.

Many investigators suggested that antioxidant and anti-inflammatory treatment may be a promising approach for reverse remodeling. In the transgenic mice, addition of oxidant scavenger SOD markedly reduced the infarct size following ischemia/reperfusion (Chen et al., 1998; Hori and Nishida, 2009). The antioxidant agents, mercaptopropionyl glycine (Date et al., 2002) and edaravone (Tsujimoto et al., 2005) also inhibit cardiac hypertrophy induced by pressure-overload in mice. Plenty of evidence suggests that antioxidant agents attenuate the LV remodeling following myocardial infarction. Probucol, a potent antioxidant exerts a favourable effect on post-infarction HF in rats (Sia et al., 2002), adriamycin-induced HF (Siveski-Iliskovic et al., 1994) and tachycardia-induced HF in mongrel dogs (Nakamura et al., 2002).

In the present study, TX markedly suppressed the inflammatory and fibrogenic changes of the cardiac tissue that are attributed to cardiac remodeling. Studies show that TX has downregulating effects on the growth factors. For example, TX attenuates oedema, neovascularization
and has supportive activities on capillary permeability and transudation through downregulation of vascular endothelial growth factor (VEGF) and nerve growth factor (NGF) (Chung et al., 2005). These findings may imply that TX would attenuate myocardial fibrosis and thereby improve cardiac function. Thus, TX could be a promising therapeutic target for reversing ROS-induced TGF-β-mediated myocardial fibrosis.

5.2.6. Antioxidant action of TX

The major sources of ROS in the ischemic-reperfused myocardium are mitochondria, xanthine oxidase, and phagocyte NADPH oxidase. ROS directly injure the tissue as key molecules for inducing cell death. Detrimental effects of ROS are clearly demonstrated by the findings that in the transgenic mice in which an antioxidant protein, SOD is overexpressed, infarct size is markedly reduced.

Flavonoid antioxidants confer their radical-scavenging ability by inhibiting the lipid peroxidation in the biological system/endogenous system. The flavonoid derivative TX has been shown to scavenge oxygen-derived free radicals in vitro at various concentrations (Blasig et al., 1987; Blasig et al., 1988; Wenisch and Biffignandi 2001; Kessler et al., 2002). The antioxidant activity of TX is bestowed by the presence of three more hydroxyethyl groups than rutin. TX has been shown to be a potent inhibitor of superoxide, hydroxyl and peroxyl nitrite and an effective protector against oxidative stress-mediated tissue damage (Kessler et al., 2002). Studies report that TX protects mouse kidney cells from D-galactose-induced injury and oxidative DNA damage through its anti-inflammatory and antioxidant effects (Fan et al., 2009; Liu et al., 2010). Recently, the anti-oxidative effect of TX has been demonstrated against ultraviolet B radiation in human HaCaT keratinocyte cells (Singh et al., 2008). These observations account for its potent antioxidative and ROS-scavenging activities.
In the present study, marked reduction in lipid peroxidation markers, AOPP and protein carbonyl levels and a rise in antioxidant levels and FRAP were observed following TX treatment indicating antioxidant power and cytoprotective effects of TX. TX treatment effectively suppressed the levels of p22phox thereby reduced NADPH oxidase-mediated increase of $O_2^\cdot$ production and insulin resistance in heart of HFFD-fed mice. TX-treated mice showed upregulation of UCPs-2 and -3 proteins accounting for improved mitochondrial oxidative phosphorylation and decreased oxidative injury.

Flavonoids display beneficial effects on vessel endothelium and cardiac muscle also because of the fact that they inhibit xanthine oxidase in these structures. This results in the decrease of superoxide and hydroxide free radicals generations during ischemia (Yao et al., 2004). TX, as a flavonoid antioxidant may inhibit the activity of xanthine oxidase and the resultant oxidative stress. These findings confirm that the antioxidant potency of TX would protect the myocardium against oxidative damage.

5.2.7. Effect of TX on mitochondrial function and population quality

Mitochondria are the primary cellular sites for FA oxidation and utilization and therefore defects in mitochondrial number, structure and function may have detrimental consequences in the heart. There is evidence that insulin-resistant obese individuals with T2D have approximately 30% fewer mitochondria in their skeletal muscle than age-matched healthy controls (Ritov et al., 2005).

The HFFD-induced myocardial structural, functional and mitochondrial aberrations were significantly attenuated by TX. The study therefore explored mitochondrial function a possible mechanism for the improvement in myocardial function. TX protected the myocardium by increasing the mitochondrial respiratory capacity, reducing the membrane potential generation and decreasing the calcium retention capacity by upregulating the mRNA expressions of SERCA2a and NCX.
More importantly HFFD-induced depression in mtDNA content/copy number, PGC-1α and its downstream nuclear factors NRF-1 and-2 and TFAM were all restored by TX. TX treatment reduced HFFD-induced elevation in DRP1 and FIS1 mRNA expression with concomitant increase in OPA1, MFN-1 and -2 thereby improving the quality of the mitochondrial population. These findings confirm that mitochondrial defects play a major role in HFFD-induced cardiac damage and that antioxidant addition offers cardioprotection.

Interestingly, TX had a “metabolic effect” on glucose and insulin levels and body weight gain. This suggests that the antioxidant-mediated effects results from cardiac specific effects of TX, including improvement of mitochondrial biogenesis.

TX through its antioxidant property significantly scavenged free radicals generation and thereby protected mtDNA and respiratory complex enzymes in HFFD-fed mice that may be attributed to normalization of mitochondrial function. Our data strongly indicate that this effect is due to enhanced mitochondrial biogenesis through the PGC-1α signaling pathway. Bioactive compounds like resveratrol, rutin and quercetin have specific effects on mitochondria such as increase in mitochondrial biogenesis and energetics (Dorta et al., 2005; Lagouge et al., 2006; Trumbeckaite et al., 2006; Rasbacg and Schnellmann 2008). Antioxidant TX upregulated the PGC-1α-induced nuclear factors and this observation suggests a role for oxidative stress in the regulation of PGC-1α signaling. A study reported that rutin increases PGC-1α activation in a forced swimming mouse model (Su et al., 2014). Since TX is a derivative of rutin, it is envisaged that it can improve mitochondrial biogenesis through PGC-1α activation. Since TX has structural similarities with rutin and additionally possesses three more hydroxyl groups than rutin, TX might well exert beneficial actions on the mitochondria.
The data suggest that high energy intake culminates in a pathologic state of the myocardium in which mitochondrial function and integrity are severely altered. Changes in the cardiac mitochondria in a mouse model of MS may explain the pathogenesis of aberrant myocardial function under obesity, insulin resistance, and diabetes. Increasing mitochondrial biogenesis via PGC-1α activation and reducing ROS may constitute an important approach in preventing mitochondrial abnormalities associated with cardiac disease. It is inferred that TX could be a promising candidate for treating cardiac dysfunction in the MS patients.

5.2.8. Effect of TX on mitochondria-mediated apoptosis

TX treatment effectively reduced peroxidation of cardiolipin and subsequent cytochrome c release from the mitochondria. TX suppressed the activation of BAX but upregulated the expression of BCL-2 thereby decreasing the BAX/BCL-2 ratio. These changes were accompanied by downregulation of APAF-1, caspases -9 and -3. Thus, TX inhibits the apoptosis by attenuating mitochondrial ROS production. Recent studies show that TX has cell-survival properties and inhibits apoptotic rate in both in vitro and in vivo models (Maurya et al., 2005; Ping et al., 2011; Lu et al., 2011; Lee et al., 2014). TX administration significantly improves cell survival by increasing antioxidant potential and by improving mitochondrial function. These two roles of TX are essential in the antiapoptotic action of TX.

Substantial data from literature suggest that using natural antioxidant compounds such as vitamins, flavonoids and polyphenols reduce the metabolic abnormalities as well as cardiac dysfunction in patients and animals with the MS. The antioxidant TX effectively attenuated cardiac apoptosis and exerted a protective role against the
development of cardiac dysfunction in MS condition. Thus, TX could be a promising therapeutic candidate for treating cardiac disease in MS patients.

5.3. Future directions

While the antioxidant property of TX may well explain the reduction in ROS level and improved mitochondrial respiratory capacity, the effects on mitochondrial biogenesis and dynamics remains elusive. It is imperative to explore the mechanism of regulation of AMPK and PGC-1α and mitochondrial biogenesis during antioxidant therapy. The pharmacologic inhibition of the RAAS attenuates oxidative stress and LV remodeling. Further studies to identify the effects of TX on RAAS in hypertrophied cardiac remodeling in vitro using cardiac fibroblasts can be undertaken. In addition, investigation of the effects of TX in H9c2 cells using glucose and/or palmitate as inducers of insulin resistance will provide information on whether TX has a direct effect on the myocytes or it acts indirectly by attenuating insulin resistance. TX is already in use in several countries like China, Europe, USA and Vietnam for the treatment of chronic venous insufficiency. Pharmacokinetic studies in TX may be undertaken before moving on to clinical trials to investigate the health benefits of TX in humans with MS-associated CVD.