"EVALUATION OF ROLE OF MAGNESIUM VALPROATE ON TYPE 2 DIABETES INDUCED CARDIO-RENAL COMPLICATIONS"

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IN

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BY

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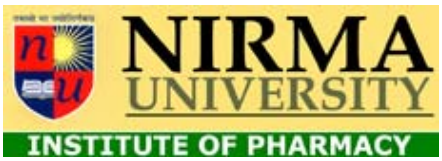

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CERTIFICATE

This is to certify that the dissertation work entitled "Evaluation of role of Magnesium Valproate on type 2 diabetes induced cardio-renal complications" submitted by Ms. Urvashi Porwal with Regn. No. (10MPH209) in partial fulfillment for the award of Master of Pharmacy in "Pharmacology" is a bonafide research work carried out by the candidate at the Department of Pharmacology, Institute of Pharmacy, Nirma University under our guidance. This work is original and has not been submitted in part or full for any other degree or diploma to this or any other university or institution.

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DECLARATION

I hereby declare that the dissertation entitled "Evaluation of role of Magnesium Valproate on type 2 diabetes induced cardio-renal complications" is based on the original work carried out by me under the guidance of Dr. Bhoomika M. Patel, Assistant professor, Mrs. Shraddha Bhadada, Assistant professor, Department of Pharmacology, Institute of Pharmacy, Nirma University. I also affirm that this work is original and has not been submitted in part or full for any other degree or diploma to this or any other university or institution.

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"God is like a big circle, whose centre is everywhere but circumference is nowhere"

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1. ABSTRACT

Background and Objective- Cardiomyopathy and Nephropathy is the leading cause of morbidity and mortality in patients associated with type 2 diabetes. Histone hyperacetylation is known to exhibit cardioprotective and renoprotective role. Valproic acid has been shown to inhibit histone deacetylase thus causing hyperacetylation of histone proteins We have studied the effect of 8 weeks treatment with MgV on cardiovascular and renal complications associated with streptozotocin (STZ) induced diabetes in neonatal rats.

Material and methods - Sprague Dawley rats of 2 day age were made diabetic with STZ (90 mg kg^{-1}, ip). Various biochemical, cardiac, hemodynamic and urine parameters were measured at the end of 8 weeks of treatment with MgV.

Results- STZ treated diabetic rats showed increase in food and water intake and decrease in body weight. Treatment with MgV produced significant decrease in food and water intake and significantly increased the body weight. STZ produced significant hyperglycaemia and dyslipidaemia which includes increased serum level of glucose ,LDL, total cholersterol ,VLDL ,triglyceride and significantly decreased HDL level and logTG/HDL ratio. Treatment with MgV significantly decreased hyperglycaemia, total cholesterol, LDL, VLDL and triglyceride levels while produced significant increase in HDL level and logTG/HDLratio.There was significant increase in the cardiac biomarker level in STZ treated diabetic rat which includes increased level of LDH,CK-MB and CRP.Chronic treatment with MgV exhibited significant reduction in cardiac biomarker level.STZ treated rats was associated with haemodynamic change accompanying hypertension, bradycardia, reduced rate of pressure development and decay. MgV improved haemodynamic function by controlling hypertension and bradycardia and significantly increase the rate of pressure development and decay. Diabetic rats also showed significantly increased cardiac hypertrophy index(CHI), left ventricular(LV) hypertrophy index, LV/RV ratio, LV collagen deposition and LV cardiomyocyte diameter . Chronic treatment with MgV significantly reduced CHI, LV hypertrophy index, LV/RV ratio, LV collagen deposition and LV cardiomyocyte size.STZ treated rat showed imbalance between pro-oxidant and antioxidant compounds and exhibited increased LV prooxidant levels like nitrite and malondialdehyde and significantly reduced LV

antioxidant enzyme activity like superoxide dismutase(SOD) and reduced glutathione(GSH) level . STZ also significant reduced LV Na^+/K^+ ATPase enzyme activity. Chronic treatment with MgV significantly decreased LV nitrite and malondialdehyde levels while significantly increased SOD activity, GSH levels as well as Na^+/K^+ ATPase enzyme activity.Histological studies revealed that the diabetic rats exhibited fibrosis,distortion,clustering of nuclei etc .Treatment with MgV significantly regress the pathological alteration of LV.

STZ treated rats showed significantly high serum levels of alkaline phosphatase(ALP), creatinine, uric acid, blood urea nitrogen(BUN), BUN:Creatinine ratio and significantly low level of serum albumin. Treatment with MgV significantly decreased serum levels of ALP, creatinine, uric acid, BUN, BUN:Creatinine ratio and significantly increased serum albumin level. Furthermore, diabetic rats showed significantly reduced uric acid, creatinine and urinary sodium and chloride excretion and creatinine clearance and significantly increased urinary albumin, urinary albumin:creatinine ratio and total protein levels. Treatment with MgV significantly increased uric acid, creatinine and urinary electrolytes excretion and creatinine clearance and significantly decreased urinary albumin, urinary albumin:creatinine ratio and total protein levels. STZ treated rat showed increased LV prooxidant levels like nitrite and malondialdehyde and significantly reduced LV antioxidant enzyme activity like superoxide dismutase(SOD) and reduced glutathione(GSH) level. Diabetic rats also showed significantly increased renal tissue collagen deposition and treatment with MgV significantly reduced renal tissue collagen deposition. Histological analysis showed increased glomerular lesions, mesangial expansion and tubular atrophy. Chronic treatment with MgV produced regression in pathological alterations of kidney.

Magnesium valproate showed no CNS side effect in diabetic treated rats which was evident from locomotor activity and forced swimming test.

Conclusions- Our data suggests that MgV has beneficial effect on cardiovascular complications associated with streptozotocin (STZ) induced diabetes in neonatal rats as depicted by prevention of hyperglycemia, hyperinsulinaemia, dyslipidemia, hypertension, bradycardia, cardiac and left ventricular hypertrophy, oxidative stress, reduction in cardiac biomarker levels and preserving structural integrity of the myocardium. In

addition, it also prevents renal complications by controlling urinary electrolytes, preserving kidney function decreasing microalbuminuria and regressing pathological alteration of kidney.Thus MgV could be considered as an “add on” therapy for management of cardio-renal complication of diabetes.

2. INTRODUCTION

Diabetes, a chronic metabolic disorder that afflicts 150 million people around the world, is set to rise to 300 million by 2025 (Vats et.al.,2005). India leads the world with largest number of diabetic subjects earning the dubious distinction of being termed as “diabetes capital of the world”. According to the Diabetes Atlas (2006) published by the International Diabetes Federation, the number of people with diabetes in India is currently around 40.9 million and is expected to rise to 69.9 million by 2025.(Mohan et.al.,2007). Diabetes is not only an endocrine but also a vascular disease(Ergul,2011)and is associated with both microvascular and macrovacular complications such as diabetic neuropathy, retinopathy, nephropathy, microangiopathy, heart disease and stroke (Ergul,2011; Balakumar et.al.,2009).

Among the vast array of secondary problems associated with diabetes, diabetic patients are prone to significant perturbations at the cellular level causing functional and structural abnormalities in the myocardium, leading to ‘diabetic cardiomyopathy’. (Hayat et.al.,2004). Nearly 80% of the deaths associated with diabetes are due to cardiac complications (Consensus statement/American Diabetes Association, 1993; Hayat et al., 2004;Farhangkhoee et.al.,2006). The development of diabetic cardiomyopathy is a multifactorial process (Buijs et.al.,2005)associated with biochemical anomalies of diabetes showing cross-interaction and complex interplay culminating in the activation of several intracellular signaling molecules(Farhangkhoee et.al.,2006)such as increased non-enzymatic glycation, sorbitol–myoinositol-mediated changes, redox potential alterations, and protein kinase C (PKC) activation. The metabolic abnormalities may include defective glucose transport, increased myocyte fatty acid uptake, and dysmetabolism. These biochemical changes manifest as hemodynamic alterations resulting in structural changes and functional changes that include capillary basement membrane thickening, interstitial fibrosis, myocyte hypertrophy and necrosis, reduced or low-normal diastolic function and left ventricular hypertrophy (Farhangkhoee et.al.,2006)

Diabetic nephropathy is the most common cause of end-stage renal disease. The incidence of diabetic nephropathy has increased substantially over the past few years.Around 20% and 40% of patients with diabetes ultimately develop nephropathy (Dronavalli ,2008).

The failure in the glycemic control with chronic diabetes mellitus produces marked structural changes in the kidney such as thickening of the glomerular basement membrane, glomerular hypertrophy, glomerulosclerosis, mesangial cell expansion, podocyte loss, tubular atrophy, and tubulointerstitial fibrosis (Arya et.al.,2010) which all collectively account for the development of renal functional abnormalities such as progressive albuminuria, reduction in glomerular filtration rate, elevation of arterial blood pressure and fluid retention (Balakumar et.al.,2009).

Hyperglycemia plays a central part in a cascade of damaging effects mediated by cytokines and growth factors that produces oxidative stress, abnormal glycosylation, lipid peroxidation, and the production of further inflammatory elements (Dronavalli,2008). Chronic hyperglycaemia may orchestrate several structural and functional abnormalities during cardio-renal complications and the various mediators, receptors and enzymes responsible include MAP kinase, protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3K), tumor necrosis fctor α, reactive oxygen species, Histone deacetylase(HDAC) and estrogen receptors (Poornima,2006; Bush,2010). Alarming estimates indicate that the rate of diabetes, metabolic syndrome, and associated complications are rapidly increasing, and therefore additional strategies to curb these trends are needed (Villeneuve and Natarajan 2010**).** The Diabetic Control and Complication Trial clearly shown that improved glycaemic control reduces cardiac and renal complications (Veldman et al., 2009). However in addition to glucose control more intensive therapy, with stringent target level is required for preventing diabetic complications.

Acetylation of histone and nonhistone proteins provides a key mechanism for controlling signalling and gene expression in heart and kidney .Histone deacetylases (HDACs) are a group of enzymes that exert epigenetic effects by altering the acetylation status of histone and nonhistone proteins(Advani, 2011). Acetylation of the ε-amino groups of lysine residues in nucleosomal histone tails by Histone acetyltransferase (HATs) is thought to relax chromatin structure by weakening the interaction of the positively charged histone tails with the negatively charged phosphate backbone of DNA, allowing access of transcriptional activators and gene induction.Deacetylation of histones by HDACs alters

the electrostatic properties of chromatin in a manner that favors gene repression. Pharmacological inhibition of protein deacetylation with histone deacetylase (HDAC) inhibitors has shown promise in preclinical models of cardiovascular and renal disease. HDAC inhibitors have pleiotropic salutary actions on a variety of cell types and pathophysiological processes, including myocyte hypertrophy, fibrosis, inflammation and epithelial-to mesenchymal transition. It has been reported that Trichostatin(TSA), a histone deacetylase inhibitor, significantly reduces the fibrosis in renal tubular cells as well as the epenchymal to mesenchymal transition(EMT) and significantly reverts the cardiac fibrosis in rats (Pang & Zhuang., 2010). Restoration of protein acetylation with HDACis holds promise as a completely novel therapeutic approach for diseases of the cardiorenal axis (Bush and McKinsey 2010).

Estrogen has pleiotropic effects on the cardiorenal complications. It induces protective effects on the renal and cardiovascular system by altering smooth muscle cells, glomerular mesangial cells, or endothelial cell biology(Dubey and Jackson ,2001) 17 β estradiol suppresses collagen synthesis in the glomerular mesangial cells (GMCs) by modulating mitogen activated protein (MAP) kinase activity and the expression of the transcription factor AP-1 ,which suggest that estradiol may limit the progression of glomerulosclerosis. In cultured mesangial cells, it also inhibits apoptosis and transforming growth factor (TGF)-β activity and expression including reduction in synthesis of ECM proteins(Mankhey, Bhatti and Maric, 2004). In cardiac complications estradiol stimulated brain natriuretic peptide protein expression and substantially prevented ventricular interstitial cardiac fibrosis (collagen deposition). 17 β estradiol acting mainly through ERβ mitigates the important signaling by Angiotensin II that produces cardiac hypertrophy and fibrosis (Wang et.al, 2009 ; Dubey and Jackson ,2001).

Valproic acid(VPA) is one of the most widely used anticonvulsants today, has several new approved indications including the treatment of bipolar disorders, neuropathic pain, and as migraine prophylaxis (Kramer et al., 2003). Valproic acid is also currently undergoing clinical trials for cancer (Bialer and Yagen,2007). Valproic acid and its analogues were shown to inhibit class I HDACs (HDACs 1–3) and class II HDACs (HDACs 4, 5 and 7).Histone deacetylase inhibitors (HDIs) are valuable drugs in breast

cancer where estrogen receptor alpha (ER α) can be silenced by epigenetic modifications(Fortunati et.al.,2010). The effect of the clinically available HDI, valproic acid (VPA), on ER α expression and function in ER-negative breast cancer cells has been reported (Fortunati et.al.,2010). HDAC inhibitors such trichostatin and valproic acid can function to attenuate cardiac hypertrophic changes induced by diverse stimuli including receptor-mediated agonists and pressure overload .It has been found that when isolated myocytes in primary culture are treated with chemical HDAC inhibitors, gene expression characteristics of hypertrophic signaling is paradoxically inhibited.(Kee et.al.,2006). In streptozotocin (STZ)-induced diabetic kidneys and TGF-β1-treated normal rat kidney tubular epithelial cells (NRK52-E), it has been found that trichostatin A,decreased mRNA and protein expressions of ECM components and prevented endothelial to mesengial transition. Valproic acid and class I-selective HDAC inhibitor also showed similar effects (Noh et.al.,2009).

Magnesium valproate is newly introduced salt of valproic acid and not much information is available other than its use in epilepsy. Magnesium valproate dissociates in the gastrointestinal tract and is absorbed into the circulation as magnesium ions and valproic acid ions and it exhibits a slower and more regular absorption rate (Fagundes ,2008).

Previous studies from our laboratory have shown that Magnesium Valproate is beneficial in preventing cardiovascular and renal complication in the STZ induced type 1 diabetes. Thus the same study is extended in type-2 diabetes.Considering the above mentioned facts, the objectives of the present study are

1) To study the effect of magnesium valproate on cardiac complications associated with type 2 diabetes in rats.
2) To study the effect of magnesium valproate on renal complications associated with type 2 diabetes in rats.
3) To develop therapeutic strategy for management of cardiorenal complications of diabetes.

3. REVIEW OF LITERATURE

3.1 PREVALENCE

Diabetes is characterized by chronic hyperglycaemia and the development of diabetes-specific microvascular pathology in the retina, renal glomerulus and peripheral nerve. As a consequence of its microvascular pathology, diabetes is a leading cause of blindness, end stage renal disease and a variety of debilitating neuropathies. Diabetes is also associated with accelerated atherosclerotic macrovascular disease affecting arteries that supply the heart, brain and lower extremities. As a result, patients with diabetes have a much higher risk of myocardial infarction, stroke and limb amputation (Brownlee, 2001). Sixteen million individuals in the United States with type 2 diabetes mellitus and an additional 30–40 million with impaired glucose tolerance result in health care costs exceeding 100 billion dollars annually (Mahler and Adler, 1998). Vascular disease is a frequent cause of morbidity and mortality among patients with diabetes. (Nuzum and Merz 2009). Diabetes mellitus affect cardiac structure and function in the absence of changes in blood pressure and coronary artery disease, a condition called diabetic cardiomyopathy. This term was introduced 30 years ago by Rubler et al, who described 4 diabetic patients with congestive heart failure and normal coronary arteries. Since then, diabetic cardiomyopathy has been defined as ventricular dysfunction that occurs independently of coronary artery disease and hypertension. Diabetes has a prevalence of 30% in patients with cardiac failure and may be up to four times as prevalent in patients with newly diagnosed HF.Diabetes is also a powerful predictor of cardiovascular morbidity and mortality, and is an independent risk factor for death in patients with established HF. Diabetic patients are also more likely than non-diabetic patients to develop HF following MI (myocardial infarction), despite comparable infarct sizes (Mak et.al.,1997). The Framingham Heart Study reported a 2.4-fold increase in the incidence of HF in diabetic men and a 5.1-fold increase in diabetic women, when compared with age-matched controls (Asghar,2009). Diabetic cardiomyopathy may be characterized by diastolic dysfunction, which becomes more apparent in the presence of hypertension or myocardial ischemia (Boudina , 2007).

Diabetes is the leading cause of ESRD because diabetic nephropathy develops in 30 to 40% of patients. It is a clinical syndrome characterized by the occurrence of persistent microalbuminuria in concomitance with insulin- or non–insulindependent diabetes and produces marked structural changes in the kidney such as thickening of the glomerular

basement membrane, glomerular hypertrophy, glomerulosclerosis, mesangial cell expansion, podocyte loss, tubular atrophy, and tubulointerstitial fibrosis (Arya et.al., 2011 ; Schena and Gesualdo, 2005).

3.2 MECHANISM OF DIABETIC CARDIOMYOPATHY

3.2.1. HYPERGLYCAEMIA

Hyperglycemia, a consequence of decreased glucose clearance and augmented hepatic gluconeogenesis, plays a central role in the pathogenesis of diabetic cardiomyopathy. In patients with T2DM, endogenous glucose production is accelerated. Chronic hyperglycemia promotes the overproduction of reactive oxygen species (ROS) through the electron transport chain which can induce apoptosis and activate poly (ADP-ribose) polymerase-1 (PARP). This enzyme mediates the direct ribosylation and inhibition of glyceraldehyde phosphate dehydrogenase (GAPDH), diverting glucose from the glycolytic pathway toward alternative biochemical cascades that participate in hyperglycemia-induced cellular injury. These include increases in advanced glycation end products (AGEs) and the activation of the hexosamine pathway, the polyol pathway, and protein kinase C. Hyperglycemia-induced apoptosis is stimulated by ROS, PARP, AGEs and aldose reductase. Hyperglycemia also contributes to altered cardiac structure and function through post-translational modification of extracellular matrix components and altered expression and function of both the ryanodine receptor (RyR) and sarco(endo)plasmic reticulum Ca2+- ATPase (SERCA), which in aggregate contribute to decreased systolic and diastolic function(Battiprolu et.al., 2010;. Hayat et.al, 2004).

3.2.2 FATTY ACIDS

Enhanced lipid synthesis in hepatocytes and increased lipolysis in adipocytes together lead to increases in circulating FAs and triglycerides (TGs) in patients with diabetes. Also, insulin stimulates FA transport into cardiomyocytes. Thus, elevated circulating lipids and hyperinsulinemia together increase FA delivery to cardiac cells which rapidly adapt by promoting FA utilization (Battiprolu et.al, 2010) .Thus there is increased β-oxidation and mitochondrial accumulation of long-chain acyl carnitines, leading to uncoupling of oxidative phosphorylation . Enhanced fatty acid oxidation decreases glucose and pyruvate utilization by inhibiting pyruvate dehydrogenase. Pyruvate oxidation is reduced further by pdk4 and activated by PPAR (peroxisome-proliferator-activated receptor). The net result is an excess of glycolytic intermediates and increased synthesis of ceramide leading to apoptosis, thus impaired glycolysis, pyruvate oxidation, lactate uptake and a greater dependence on fatty acids as a source of acetyl CoA leads to a perturbation of myocardial bioenergetics and contraction/relaxation coupling (Hayat et.al, 2004).

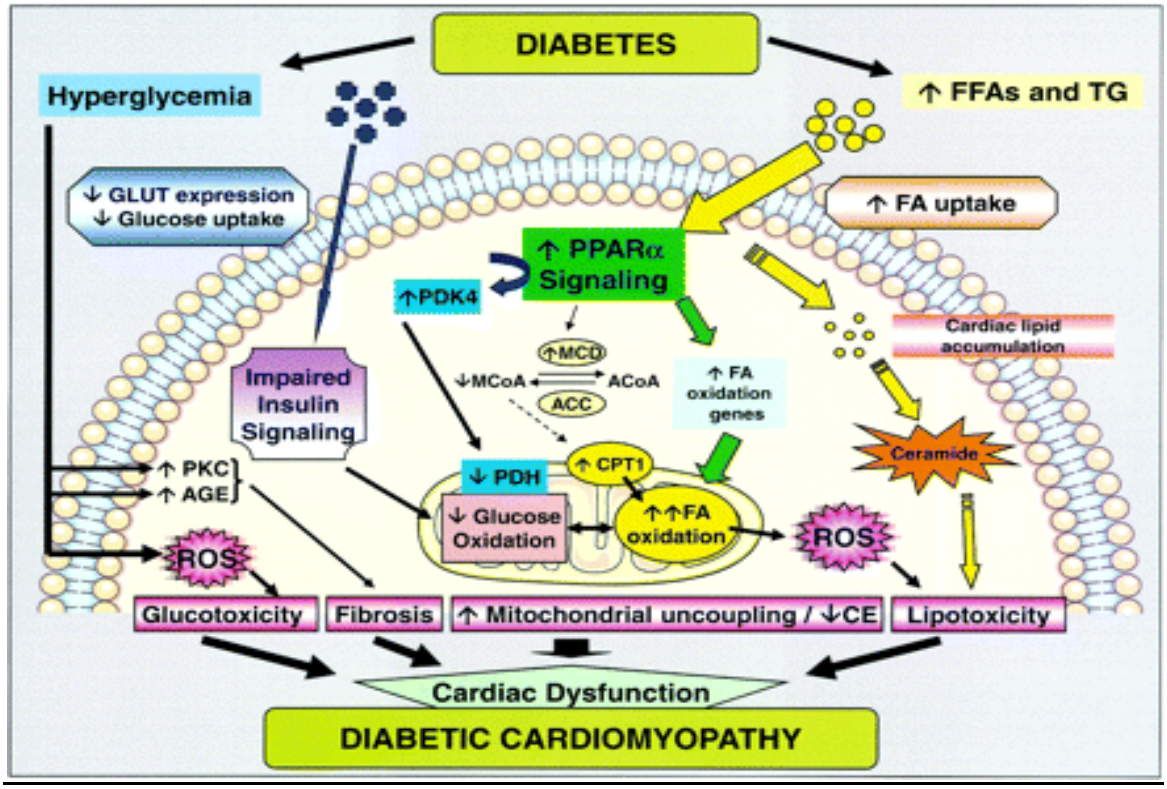


FIG.3.2.2 -Potential contributors to the development of diabetic cardiomyopathy (Adapted from Boudina et.al., 2007)

3.2.3. PKC (PROTEIN KINASE C)

Increased activation of the DAG (diacylglycerol)-activated PKC signal transduction pathway has been identified in vascular tissues from diabetic animals and in vascular cells exposed to elevated glucose. This has been shown to induce many of the changes in diabetic cardiomyopathy which include a reduction in tissue blood flow, enhanced extracellular matrix deposition, capillary basement membrane thickening and increased vascular permeability with alterations in neovascularization.(Hayat et.al., 2004)

3.2.4 ACTIVATION OF THE RENIN-ANGIOTENSIN SYSTEM

The role of activation of the renin-angiotensin system in the development of diabetic cardiomyopathy is well recognized. Angiotensin II receptor density and mRNA expression are elevated in the diabetic heart. Activation of the renin-angiotensin system during diabetes mellitus has been shown to be associated with increased oxidative damage and cardiomyocyte and endothelial cell apoptosis and necrosis in diabetic hearts, which

contributes to the increased interstitial fibrosis. Blockade of the renin-angiotensin system in streptozotocin-treated rats attenuated cardiac dysfunction partially through restoration of sarcoplasmic calcium handling (Boudina and Dale Abel , 2007).

3.2.5 ENDOTHELIAL DYSFUNCTION

Sustained hyperglycemia causes vascular endothelial cell dysfunction, resulting in increased permeability, reduced blood flow, and subsequently tissue ischemia. In response to tissue ischemia,endothelial cells release growth factors that increase basement membrane (BM) thickening and extracellular matrix (ECM) deposition. Growth factor and cytokine elaboration may also contribute to myocyte dysfunction and loss. In long-standing diabetes, this response is sustained and exacerbated, ultimately leading to diabetic cardiomyopathy and heart failure (Farhangkhoee et.al., 2006)

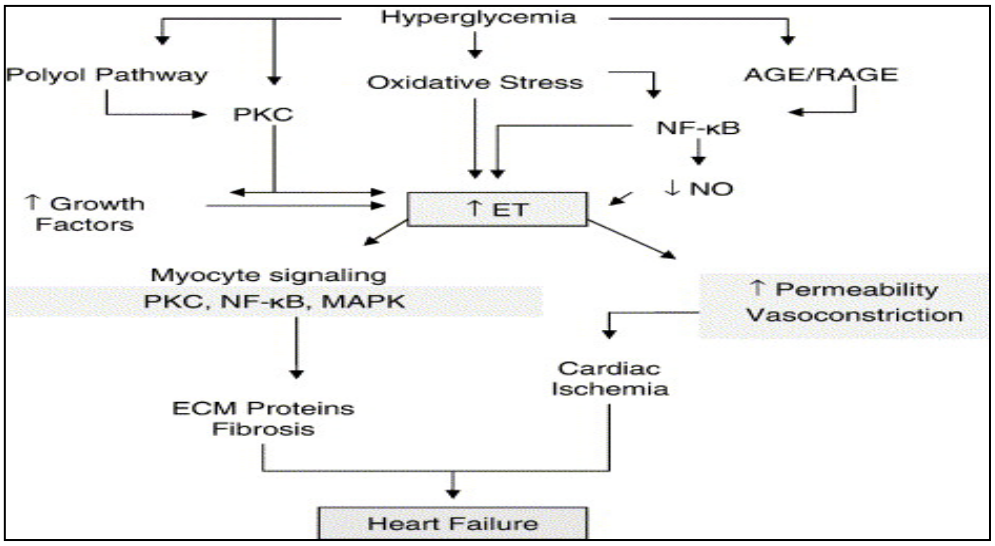


FIG. 3.2.5- Proposed mechanisms of endothelin (ET) alteration and functional consequences in cardiac endothelial cells and myocytes (Farhangkhoee et.al.,2006)

3.2.6. VEGF (VASCULAR ENDOTHELIAL GROWTH FACTOR)

VEGF may play an important role in the response to cardiac injury, in diabetes, the expression of mRNA and protein for VEGF and its receptors, VEGF-R1 and VEGF-R2, has been shown to be decreased significantly (40–70 %) in the myocardium of both diabetic and insulin-resistant non-diabetic rats, together with a 2-fold reduction in VEGF and VEGF-R2 in ventricles from diabetic patients compared with non-diabetic donors . This suggests that, in

diabetic patients, the normal molecular processes which regulate angiogenesis are impaired (Hayat et.al, 2004)

3.2.7. IMPAIRED CALCIUM HOMEOSTASIS

Intracellular calcium (Ca^{2+}) is a major regulator of cardiac contractility. In the cardiomyocyte, Ca^{2+} influx induced by activation of voltage-dependent L-type Ca^{2+}channels on membrane depolarization triggers the release of Ca^{2+}via Ca^{2+} release channels (ryanodine receptors) of sarcoplasmic reticulum (SR) through a Ca^{2+}induced Ca^{2+} release mechanism. Ca^{2+} then diffuses through the cytosolic space to reach contractile proteins, binding to troponin C and resulting in the release of the inhibition induced by troponin I. By binding to troponin C, the Ca^{2+} triggers the sliding of thin and thick filaments, which results in cardiac force development and/or contraction. [Ca^{2+}] then returns to diastolic levels mainly by activation of the SR Ca^{2+} pump (SERCA2a), the sarcolemmal Na^{+}-Ca^{2+} exchanger, and the sarcolemmal Ca^{2+} ATPase. It has long been appreciated that calcium and other ion homeostasis is altered in diabetic cardiomyocytes. The mechanisms by which disturbed calcium homeostasis alters cardiac function in diabetes include reduced activity of ATPases, decreased ability of the SR to take up calcium, and reduced activities of other exchangers such as Na^{+}-Ca^{2+} and the sarcolemmal Ca^{2+} ATPase. Indeed, the SR Ca^{2+} store and rates of Ca^{2+} release and reuptake into SR were depressed in type 1diabetic rat myocytes (Hayat et.al, 2004).

3.3 STRUCTURAL CHANGES DURING CARDIOMYOPATHY

3.3.1 LEFT VENTRICULAR HYPERTROPHY

Increased left ventricular mass is an independent marker of cardiovascular risk that often occurs independently of arterial blood pressure in type 2 diabetes. Thus diabetes is an independent contributor to left ventricular hypertrophy (LVH) and myocardial stiffness. The presence of LVH has been linked with increased markers of systemic inflammation [fibrinogen and CRP (C-reactive protein) and microalbuminuria and, in a study of 1299 Type II diabetic patients, increased albuminuria was a marker of endothelial damage and increased atherothrombotic risk (Hayat et.al 2004) .The Framingham study investigators used echocardiography and reported a significant increase in left ventricular wall thickness in women with diabetes(Boudina ,2009).

3.3.2 INTERSTITIAL FIBROSIS

Diabetic cardiomyopathy is characterized by interstitial fibrosis, mainly composed of collagen, and perivascular fibrosis. Regan et al. found a significant increase in deposition of collagen around the vessel and between the myofibers in heart biopsies from diabetic patients. In addition, a significant increase in collagen type III, but not type I or VI, was found in endomyocardial biopsies obtained from patients with type 2 diabetes, free of CAD and hypertension. Furthermore, diastolic dysfunction detected in a population of patients with uncomplicated type 2 diabetes correlated with pro-collagen type I carboxy-terminal peptide , suggesting a mechanistic involvement of myocardial fibrosis in the myocardial dysfunction that occurs in diabetes (Boudina, 2009).

3.3.3 INCREASED CELL DEATH AND OXIDATIVE STRESS

Diabetes is associated with myocyte cell death; however, it is unclear whether diabetes can directly activate cell death or, rather, it activates pathways known to induce this process. Indeed, activation of the renin–angiotensin system (RAS) was associated with increased oxidative stress and cardiomyocyte and endothelial cell death in hearts of patients with diabetes. Both forms of cell death (necrosis and apoptosis) were identified in myocardium biopsies of patients with diabetes, apoptosis was maximally induced in the diabetic myocardium,whereas necrosis was exaggerated by hypertension. ROS can also interact with nitric oxide to form nitrotyrosine, which was found to be increased in myocardial biopsies of humans with type 2 diabetes (Boudina, 2009).

3.3.4 MYOCARDIAL LIPOTOXICITY

Diabetic myocardium is also characterized by increased deposition of intramyocardial lipids, which can contribute to cell death and thus to cardiac dysfunction. Regan et al. identified deposits of lipofuscin, which are brown pigment granules composed of lipid-containing residues, in left ventricular transmural biopsies obtained from diabetic patients. Furthermore,they measured myocardial triglyceride and cholesterol content in these biopsies and found a significant increase. Similarly, Oil Red O staining of heart sections of non ischemic failing hearts revealed an increased deposition of lipid that was exacerbated by diabetes. More importantly, increased myocardial triglyceride in patients with type 2 diabetes was associated with diastolic, but not systolic, dysfunction (Boudina 2009).

3.4 FUNCTIONAL CHANGES DURING CARDIOMYOPATHY

3.4.1 DIASTOLIC DYSFUNCTION

In patients with well controlled type 2 diabetes revealed a prevalence of diastolic dysfunction in upto 30%. Diastolic function of the left ventricle is determined by its passive elastic properties, coupled with the process of active relaxation(Boudina ,2009).Diastolic dysfunction is characterized by impairment of relaxation and passive filling of the left ventricle, and diastolic HF is said to exist when diastolic dysfunction is associated with an elevated end diastolic pressure.(Asghar et.al., 2009).As diastolic function worsens, early diastolic LV filling (E wave) is reduced, and the patient demonstrates a delayed relaxation pattern. However, as left atrial pressure increases, the E wave returns to normal, producing a mitral pattern indistinguishable from normal (pseudonormal), until the development of a restrictive filling pattern, which reflects a high left atrial pressure, usually to the extent that symptoms of left heart failure appear(Fang et.al., 2004).

3.4.2 SYSTOLIC DYSFUNCTION

Systolic dysfunction is impairment in the ability of the heart to eject blood; this is different from systolic HF where symptoms and signs of HF are developed secondary to systolic dysfunction. Although the principle hallmark of systolic dysfunction is a depressed LV ejection fraction. In the context of diabetic cardiomyopathy, systolic dysfunction occurs late, often when patients have already developed significant diastolic dysfunction. The prognosis in patients with depressed systolic dysfunctionis poor with an annual mortality of 15–20 % (Hayat et.al.,2004).Friedman *et al.* demonstrated that diabetic patients had an increased end-systolic diameter and volume, a diminished ejection fraction, and a decreased minor axis shortening and velocity of circumferential fiber shortening. In a similar study of 40 type II normotensive diabetic patients, 22 (55%) patients had systolic dysfunction, but only three (7.5%) had electrocardiographic changes compatible with cardiac ischemia; 16 (40%) patients were also found to have LV hypertrophy.

3.4.3 IMPAIRED CONTRACTILE RESERVE

Diabetic cardiomyopathy involves several stages of disease, including a period in which symptoms are not present, and resting left ventricular dimension and function are still normal. In this early phase, left ventricular dysfunction can be characterized by exercise. Indeed,

impaired augmentation of LVEF occurs in as many as 40% of patients with diabetes .Recent reports in both type 1 and type 2 diabetes showed that longitudinal functional reserve indicated by reduced mitral annular systolic and early diastolic velocities and left ventricular contractility reserve indicated by depressed peak exercise left ventricular stroke index, cardiac index, and LVEF were reduced after exercise, whereas no change in these parameters was observed at rest. Thus cardiac performance after exercise could be a tool with which to detect early contractile dysfunction in diabetes(Boudina, 2009).

3.4.4 MITOCHONDRIAL DYSFUNCTION

Reagan et al. first reported increased numbers of mitochondria with pleomorphism, but no swelling or evident distortion of cristae in the myocardium of patients with diabetes. Using phosphorus-31 nuclear resonance spectroscopy, Clarke's group provided evidence for decreased cardiac energetics in patients with type 2 diabetic who were free from CAD. Finally, studies related to the response of the diabetic heart to ischemic preconditioning have suggested that a defect in the mitochondrial ATP-sensitive potassium channel could explain the inability of the diabetic myocardium to respond to ischemic preconditioning and increase the risk for myocardial infarction(Boudina , 2009).

3.5 MECHANISM OF DIABETIC NEPHROPATHY

3.5.1. HYPERGLYCEMIA

Hyperglycemia play major role in the kidney damage due its effects on glomerular and mesangial cells. Mesangial cells are important for the maintenance of glomerular capillary structure and for the glomerular filtration. Hyperglycemia cause mesangial cell proliferation and hypertrophy as well as increase matrix production, basement membrane thickening, increase mesangial cell and mesangial cell apoptosis . In diabetes mellitus the expression of growth factors in podocytes gets increased and markedly increases the vascular permeability. At early stage kidney damage may be due to hyperglycemia. Nonenzymatic glycosylation that generates advanced glycosylation end products, activation of PKC, and acceleration of the aldose reductase pathway are the three major mechanisms found in hyperglycemia induced tissue damage (Arya et.al, 2010)

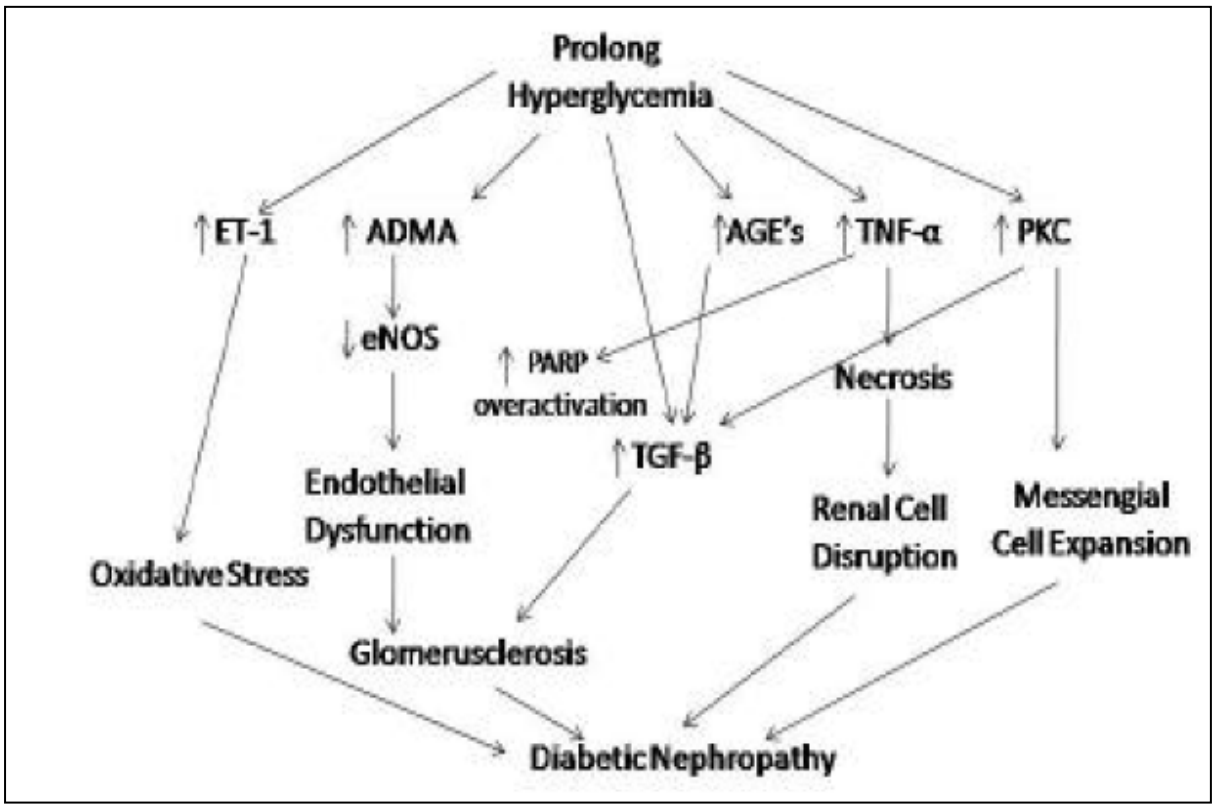


FIG.3.5.1-Pathologic mechanisms in diabetic nephropathy (Arya et.al, 2010)

3.5.2. GLYCOSYLATION

Glycosylation of tissue proteins contributes to the development of diabetic nephropathy and other microvascular complications. In chronic hyperglycemia, some of the excess glucose combines with free amino acids on circulating or tissue proteins. This nonenzymatic process affects the glomerular basement membrane and other matrix components in the glomerulus and initially leads to formation of reversible early glycosylation end products and, later, irreversible advanced glycosylation end products. These advanced products can be involved in the pathogenesis of diabetic nephropathy by altering signal transduction via alteration in the level of soluble signals, such as cytokines, hormones and free radicals. Circulating levels of advanced glycosylation end products are raised in people with diabetes, particularly those with renal insufficiency, since they are normally excreted in the urine. The net effect is tissue accumulation of advanced glycosylation end products inpart by cross-linking with collagen that contributes to the associated renal and microvascular complications. Moreover, advanced glycosylation end products (AGE) interact with the AGE receptor, and nitric oxide concentrations are reduced in a dose-dependent manner (Dronavalli et.al.,2008).

3.5.3 ADVANCED GLYCOSYLATION END PRODUCTS

Reactions like glycoxidation or lipoxidation between sugars and the free amino groups on proteins, lipids and nucleic acids result in AGE formation. Mesangial cell expansion and increased glomerular basement membranes thickness have been noted in early diabetic nephropathy due to accumulation of various AGE products. AGE products show their damaging effect binding with their receptor.Interaction of AGEs with their receptor lead to oxidative stress and activation of nuclear factor-kB (NF-kB) and other proinflammatory mediators. AGE products stimulate growth factors like insulin-like growth factor-I, II, PDGF and TGF-ß, which results in collagen type IV generation. Type IV collagen generation reduce their ability to interact with proteoglycans and increase albumin glomerular permeability (Atul Arya et.al.). In the initial study by Soulis-Liparota et al. aminoguanidine, an inhibitor of AGE formation, which acts by scavenging intermediates in the advanced glycation pathway attenuated the rise in albuminuria observed in diabetic rodents, while preventing increases in collagen related fluorescence in isolated glomeruli and renal tubules (Arya et.al. 2010).

3.5.4 REACTIVE OXYGEN SPECIES

Diabetic nephropathy is characterized by excessive deposition of extracellular matrix (ECM) in the kidney, leading to glomerular mesangial expansion and tubulointerstitial fibrosis. Clinical studies,have demonstrated that high blood glucose is the main cause of initiation and progression of diabetic vascular complications including nephropathy. High reactive oxygen species (ROS) induced by glucose and upregulates TGF-β1 and extra cellular matrix protein (ECM) expression in the glomerular mesangial cells .Hyperglycemia induced ROS generation and ROS-induced activation of signal transduction cascade and transcription factors and overexpression of genes and proteins in glomerular mesangial and tubular epithelial cells leading to ECM accumulation in diabetic kidney (Arya et.al 2010)

3.5.5. CYTOKINES

Activation of cytokines, profibrotic elements, inflammation, and vascular growth factors such as VEGF might be involved in the matrix accumulation that arises in diabetic nephropathy. High glucose levels, TGF-β1, and angiotensin II stimulate VEGF expression, which leads to the synthesis of endothelial nitric oxide. This action promotes vasodilatation and hyperfiltration, which are the early processes in diabetic nephropathy. VEGF also stimulates the production of the α3 chain of collagen IV, an important component of the glomerular basement membrane. Indirect evidence suggests that increased production of this collagen chain contributes to the thickening of the glomerular basement membrane observed in diabetic nephropathy. In animal studies, administration of an antibody to VEGF decreased urinary albumin excretion compared with that in untreated diabetic controls. Hyperglycemia also increases the expression of TGF-β1 in the glomeruli and of matrix proteins specifically stimulated by this cytokine. In the glomeruli of rats with streptozotocininduced diabetes, TGF-β1 levels are increased, and use of a neutralizing antibody to TGF-β1. prevents renal changes of diabetic nephropathy in these animals. In addition, connective tissue growth factor and heat shock proteins, which are encoded by TGF-β1-inducible genes, have fibrogenic effects on the kidneys of patients with diabetes. TGF-β1 contributes to the cellular hypertrophy and increased synthesis of collagen, both of which occur in diabetic nephropathy. Inflammatory cytokines also contribute to the development and progression of diabetic nephropathy, specifically interleukin 1 (IL‑1), IL-6 and IL-18 and tumor necrosis factor. Concentrations of all these cytokines were increased in models of diabetic nephropathy and seemed to affect the disease via multiple mechanisms. In addition, raised

levels of several of these cytokines in serum and urine correlate with progression of nephropathy, indicated by increased urinary albumin excretion(Dronavalli et.al., 2008) .

3.5.6. PRORENIN

The prorenin receptor in the kidney is located in the mesangium and podocytes, and its blockade has a beneficial effect on kidneys in animal models of diabetes. This effect is mediated by intracellular signals that are both dependent on and independent of the renin–angiotensin system. Prorenin binds to a specific tissue receptor that promotes activation of p44/p42 MAPK. Possible pathogenic role for prorenin in the development of diabetic nephropathy was noted in an experimental model of diabetes—mice with streptozotocin-induced diabetes. Sustained prorenin-receptor blockade abolished MAPK activation and prevented the development of nephropathy despite an unaltered increase in angiotensin II activity (Dronavalli et.al., 2008)

3.5.7. LIPID MEDIATORS

Small lipids derived from arachidonic acid have been implicated in the pathogenesis of diabetic nephropathy. Cyclo-oxygenase 2 breaks down arachidonic acid into several different prostanoids. In a rat model of streptozotocin-induced diabetes, levels of inflammatory prostanoids, such as prostaglandins E2 and I2, were raised. Furthermore, increased expression of cyclooxygenase 2 has been reported in animal studies of diabetes and in the macula densa of kidneys from humans with diabetes. In diabetic rats, inhibition of cyclo-oxygenase 2 is associated with decreased glomerular hyperfiltration(Dronavalli et.al., 2008).

3.5.8. ANGIOTENSIN CONVERTING ENZYME 2 (ACE2)

ACE2 is part of the enzymatic cascade of the RAS. Specifically, it seems to act as a negative regulator of the RAS, counterbalancing the function of ACE, thus promoting vasodilation. Indeed, it is implicated in the conversion of Ang I to Ang and in the degradation of Ang II to Ang, a peptide that has been postulated to counteract the potentially detrimental actions of Ang II via the AT1R,resulting in vasodilator, natriuretic, and antiproliferative effects. ACE2 is part of the enzymatic cascade of the RAS. Specifically, it seems to act as a negative regulator of the RAS, counterbalancing the function of ACE, thus promoting vasodilation. Indeed, it is implicated in the conversion of Ang I to Ang and in the degradation of Ang II to Ang , a peptide that has been postulated to counteract the potentially detrimental actions of

Ang II via the AT1R, resulting in vasodilator, natriuretic, and antiproliferative effects. In the kidney, ACE2 has a distribution similar to ACE with majorlocalization in renal tubules. Both ACE2 gene and protein expression are downregulated in the kidneys in experimental models of hypertension and in streptozotocin-induced diabetic Sprague–Dawley rats after 24 weeks of diabetes. it has been postulated that renal ACE2 deficiency as occurs in long-term diabetes leads to a local increase in tubular Ang II, which, in turn, may promote tubulointerstitial fibrosis.Interestingly, ACE inhibition seems to prevent the diabetes associated decreases in renal ACE2 expression, suggesting that ACE inhibition may confer some of its renoprotective effects via modulation of ACE2 expression(Giunti et.al., 2006).

3.6 STRUCTURAL CHANGES DURING NEPHROPATHY

3.6.1 MESANGIAL EXPANSION

Mesangial expansion is defined as an increase in extracellular material in the mesangium such that the width of the interspace exceeds two mesangial cell nuclei in at least two glomerular lobules. The difference between mild and severe mesangial expansion is based on whether the expanded mesangial area is smaller or larger than the mean area of a capillary lumen. Expansion of cellular and matrix components in the mesangium is a hallmark of type 1 and type 2 DM. It can be detected in some patients within a few years after the onset of type 1 diabetes. When the mesangium expands, it restricts and distorts glomerular capillaries and diminishes the capillary filtration surface (Vestra et.al 2003).

3.6.2 GLOMERULAR BASEMENT MEMBRANE THICKENING(GBM)

GBM thickening is a consequence of extracellular matrix accumulation, with increased deposition of normal extracellular matrix components such as collagen types IV and VI, laminin, and fibronectin. Such accumulations result from increased production of these proteins, their decreased degradation, or a combination of the two. GBM thickening may already be present in type 1 diabetes patients who are normoalbuminuric. GBM thickening has even been described as a “prediabetic” lesion: In patients with proteinuria and isolated GBM thickening but without overt diabetes, 20% were positive on a blood test for diabetes at the time of biopsy, whereas 44% were diagnosed with diabetes at 6 months, and 70% at 2 years after the biopsy was taken. Long-term glucose control and urinary albumin excretion (UAE) correlate strongly with basement membrane thickness.
(Vestra et.al 2003)

3.6.3 GLOMERULOSCLEROSIS

Glomerulosclerosis in DN is the end point of multifactorial mechanisms that lead to excessive accumulation of extracellular matrix proteins such as collagen types I, III, and IV and fibronectin in the mesangial space, which through stages of mesangial expansion and development of Kimmelstiel–Wilson lesions finally result in glomerulosclerosis. The clustering of sclerotic lesions in columns perpendicular to the kidney surface suggests that vascular factors relating to the interlobular arteries also contribute(Vestra And Fioretto, 2003).

3.6.4 TUBULOINTERSTITIAL INJURRY AND FIBROSIS

Proteinuria is an important predictor of progression of renal disease and persistent protein load in the tubular milieu as a result of impaired absorption may lead to injury and scarring of tubular lumen. This may lead to increased oxidative stress and stimulate activation of proinfammatory cytokine in tubular cell (Singh And Farrington, 2010)

3.6.5 PODOCYTE DAMAGE

In recent years, podocyte alterations have been considered as potential contributors to the pathogenesis of diabetic nephropathy .Podocytes are an integral part of the filtration barrier, and changes in their structure have been observed in a broad range of proteinuric glomerular diseases, including diabetes. At variance with other glomerular cells, podocytes are considered to have limited capacity to replicate, at least postnatally. Thus, the loss of podocytes would necessarily require the residual cells to cover a larger area of GBM. This could cause foot process widening and reduce the ability of the podocytes to remain attached to GBM, with consequent areas of bare GBM, which are potential starting points for glomerulosclerosis. The numerical density of podocytes was significantly decreased in all type 2 diabetic groups. A decrease in numerical density can be consequent to enlargement in glomerular volume, decrease in the absolute number/glomerulus, or a combination of both factors (Vestra et.al 2003).

3.7 FUNCTIONAL CHANGES DURING NEPHROPATHY

3.7.1 PROTEINURIA

Proteinuria is a clinical hallmark of diabetic nephropathy, albumin has been detected in yhe urine of newly diagnosed type I and II diabetes with poor metabolic control. The mechanism responsible for proteinuria in diabetes is not clearly understood. Hyperglycaemia, hormones like rennin, glucagon, growth hormones , alterations in GBM compossitin have been implicated (Reddi And Davalos,1990).

3.7.2GLOMERULAR FILTRATION RATE (GFR)

Increased GFR seem to be due to increased renal plasma flow caused by greater reduction in affrent than efferent arteriolar resistance and increased glomerilar transcappillary hydraulic preesure gradient .With advanced diabetic nephropathy,GFR decreases to a significant extent (Reddi And Davalos,1990).

3.8 ROLE OF HDAC IN CARDIORENAL COMPLICATION

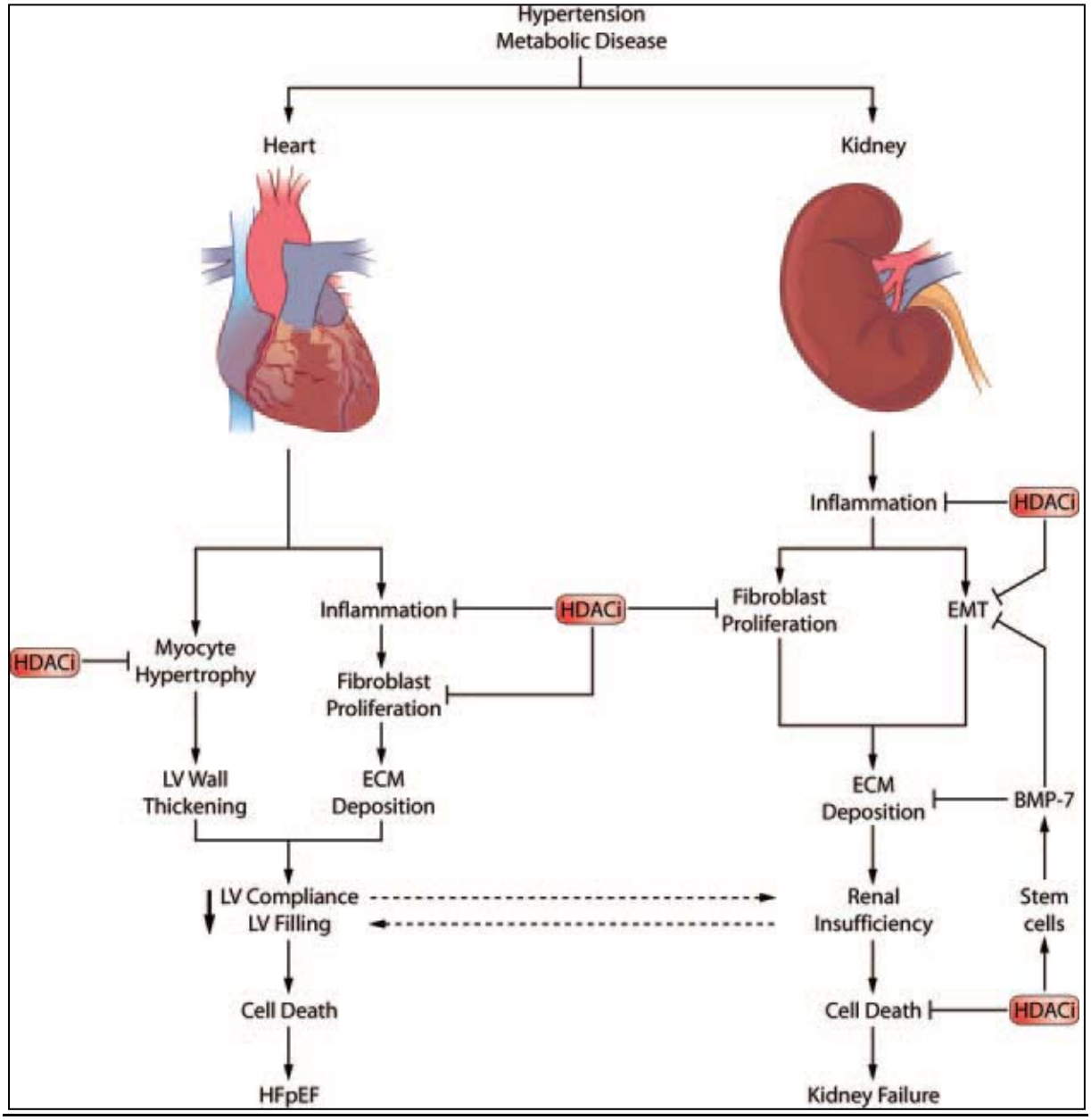


FIG 3.8-HDACis target multiple pathological mechanisms of chronic cardiac and renal disease (Bush and McKinsey,2010).

3.8.1 CARDIAC COMPLICATION

Histone acetyltransferases (HATs) and HDACs act in an opposing manner to control the acetylation state of proteins.The most well characterized role for protein acetylation is in the control of gene transcription. Acetylation of the ε amino groups of lysine residues in nucleosomal histone tails by HATs is thought to relax chromatin structure by weakening the interaction of the positively charged histone tails with the negatively charged phosphate backbone of DNA, allowing access of transcriptional activators and gene induction. Deacetylation of histones by HDACs alters the electrostatic properties of chromatin in a manner that favours gene repression. A role for HDACs in the regulation of cardiac growth was originally revealed by the discovery that class IIa HDACs function as signal-responsive repressors of pathological cardiac hypertrophy. In response to stress signals,class IIa HDACs are shuttled out of cardiomyocyte nuclei and are thus unable to repress genes that promote myocyte growth.

The serendipitous discovery of the antihypertrophic action of HDACis suggested novel therapeutic strategies for heart failure. In support of this, follow-up in vivo studies in mouse and rat models of pathological hypertrophy and heart failure have clearly demonstrated that nonselective, pan-HDACis can effectively halt, or even reverse, the disease process. Treatment with TSA or valproic acid for 2 weeks blocked the development of cardiac hypertrophy in transgenic mice that overexpress an HDAC2-dependent serum response factor inhibitor, Hop. Similar 2-week regimens of pan-HDACi treatment also effectively suppressed cardiac hypertrophy induced by continuous infusion of isoproterenol or angiotensin II, as well as pressure-overload imposed by aortic constriction. Importantly, TSA treatment was also shown to regress established cardiac hypertrophy in mice subjected aortic constriction. Pan-HDACis have also been shown to reduce maladaptive ventricular remodeling and improve cardiac performance in rats subjected to coronary artery ligation, a model of post–myocardial infarction remodeling and systolic heart failure.

Furthermore, pan-HDACis have proven to be cardioprotective in the setting of myocardial ischemia-reperfusion injury, significantly reducing infarct size and improving cardiac function. The cardioprotective effects of HDACis may, in part, derive from their ability to upregulate expression of endogenous antioxidant enzymes. In addition to promoting expression of protective genes, HDACis also appear to directly block expression of pathological genes, which is paradoxical because HDAC action is typically associated with gene repression.

Two recent studies shed light on the mechanism by which HDACis repress genes in the heart. Expression of the gene encoding B-type natriuretic peptide (BNP) is dramatically enhanced in ventricular myocytes during pathological cardiac hypertrophy .Using cultured neonatal rat cardiac myocytes, Gardner and colleagues demonstrated that upregulation of BNP expression in response to endothelin signaling is dependent on association of HDAC2 with the yin-yang (YY)1 transcription factor on the *BNP* gene promoter. YY1 was shown to be acetylated in cardiac myocytes, and deacetylation of the transcription factor by HDAC2 enhanced its ability to stimulate *BNP* gene transcription. TSA treatment disrupted YY1:HDAC2 complexes and suppressed endothelin-induced BNP expression. (Bush, McKinsey 2010).

HATs and HDACs can also modulate NF-κB transcriptional activity , resulting in changes in downstream inflammatory gene expression levels. Interestingly, high glucose treatment of cultured monocytes increased recruitment of the HATs CPB and p/CAF, leading to increased histone lysine acetylation at the cyclooxygenase-2 (COX-2) and TNF-α inflammatory gene promoters, with a corresponding increase in gene expression (Villeneuve, and Natarajan 2010.)

Class II HDACs also inhibit the expression of genes containing MEF2-binding sites in their regulatory regions by binding to MEF2. These targets include immediate early genes, such as c-jun and nur77, and fetal cardiac genes, including a-skeletal actin, all of which are potently activated in response to hypertrophic cues . Importantly, MEF2 also binds to p300 and binding of HAT or HDAC to MEF2 is mutually exclusive because the proteins target an overlapping site on the transcription factor. Thus, MEF2 provides a connection among HATs, HDACs and downstream genes that control a hypertrophic response and has equally important roles as a positive and a negative regulator of pathological cardiac gene expression. it is predicted that HDAC inhibitors will increase myofibrillar ATPase activity and improve contractility in the failing heart (McKinsey, Olson 2004).

3.8.2 RENAL COMPLICATIONS

Diabetes damages glomerular cells, resulting in cytokine release and inflammation. Elevated levels of transforming growth factor (TGF)-β activate resident mesangial cells and fibroblasts, stimulating production of ECM proteins. TGF-β also promotes pathological dedifferentiation of renal epithelial cells into matrix-producing mesenchymal cells via a process known as epithelial-to-mesenchymal transition (EMT). Organ function declines with progressive fibrosis and loss of epithelial integrity, and resulting proteinuria is a contributing

factor to further renal injury. Diabetes and TGF-β1 activate HDAC-2 in the kidneys, which may be involved in the subsequent accumulation of ECM and EMT. In addition ROS can directly increase HDAC-2 activity and mediate TGF-β1-induced HDAC-2 activation (Bush, McKinsey 2010).

Initial observations in cultured human renal proximal tubular epithelial cells demonstrated that TSA effectively blocked TGF-β–driven EMT, reducing profibrotic ECM expression and preserving expression of E-cadherin, a key functional marker of epithelial identity. (Noh et.al. 2009) In this study, TSA also increased expression of the renoprotective factor, bone morphogenetic protein (BMP)-7, an endogenous inhibitor of TGF-β signaling that is known to suppress EMT and reverse renal fibrosis. More recently, TSA was also shown to reduce fibrosis, suppress EMT and improve renal function in a rat model of streptozotocin-induced diabetic nephropathy. A key role for class I HDACs in renal EMT was suggested, because small interfering RNA knockdown of HDAC2 inhibited TGF-β- induced EMT in cultured proximal tubule epithelial cells. Class I HDACs appear to play a key role in repression of the E-cadherin gene promoter as components of a repression complex containing the SMAD3 transcription factor and the Snail and ZEB corepressors.

Finally, it should be mentioned that HDACi therapy has shown promise as a treatment for autoimmune nephropathies like lupus nephritis. TSA and low doses of SAHA effectively reduced pathological cytokine expression and reverse glomerulonephritis (Bush , Mc Kinesy.,2010) .

3.9 ROLE OF ESTROGEN RECEPTORS IN CARDIORENAL AXIS

3.9.1 ESTROGEN RECEPTOR IN CARDIAC AXIS

Estrogen exerts complex biological effects through the two isoforms of estrogen receptors (ERs): ERα and ERβ. Whether through alteration of gene expression or rapid, plasma membrane-localized signaling to non-transcriptional actions, estrogen-activated ERs have significant implications in cardiovascular physiology. 17-β-estradiol (E2) generally has a protective property on the vasculature. Estrogen treatment is anti-atherogenic, protecting injured endothelial surfaces and lowering LDL oxidation in animal models. Increased NO production stimulated by E2 results in vasodilation of the coronary vascular bed, and involves rapid activation of phosphotidylinositol-3 kinase (PI3K)/Akt signaling to eNOS in carotid and femoral arteries. Both isoforms of ERs impact various vascular functions, modulating ion channel integrity, mitigating the response to arterial injury, inducing vasodilation, and preventing development of hypertension in animal models. In addition to reducing afterload by vasodilation, ERs have a direct antihypertrophic effect on the myocardium. E2-activated ERs (E2/ER) antagonize the hypertrophic pathway induced by vasoactive peptides such as angiotensin II by activating PI3K, subsequent MICIP gene expression, leading to the inhibition of calcineurin activity and the induction of hypertrophic genes. In models of ischemia-reperfusion, E2/ER is antiapoptotic for cardiomyocytes, exerting the protective actions via PI3K and p38 MAP kinases and suppressing the generation of reactive oxygen species. In sum, E2-activated ERs consistently and positively modulate multiple aspects of the cardiovascular system(Kim and Levin 2006).

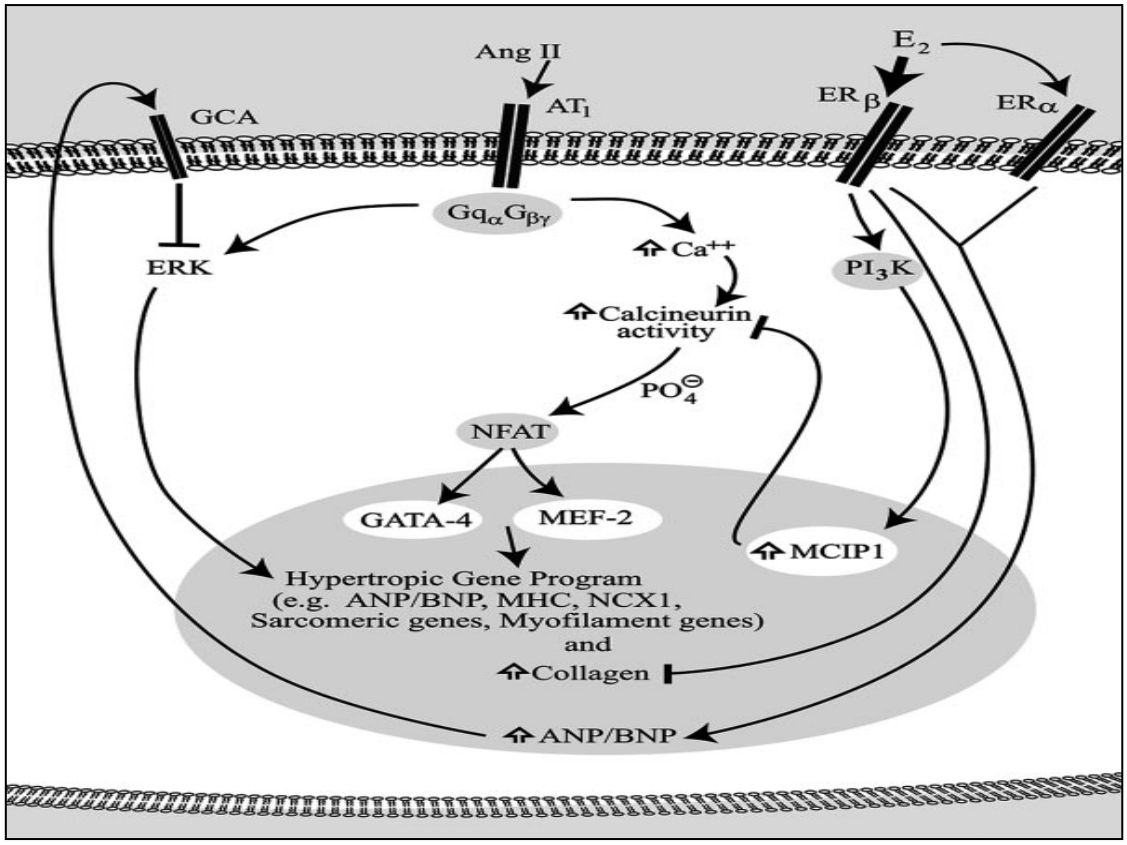


FIG:3.9.1- E2 and ERβ inhibit AngII-induced cardiac hypertrophy (Pedram et.al 2008)

3.9.2 ESTROGEN RECEPTOR IN RENAL AXIS

The potential role of estradiol in regulating renal function is evident from the observation that the kidney expresses both the classic ER-α and the newly discovered ER-β. In human fetal kidneys, ER-β is the prominent renal ER expressed, whereas ER-α is only marginally expressed. Estradiol suppresses collagen synthesis in glomerular mesangial cell (GMCs), suggesting that estradiol may limit the progression of glomerulosclerosis by reducing matrix accumulation after glomerular injury. The inhibitory effects of estradiol on collagen synthesis in GMCs are mediated via activation of MAP kinase and upregulation of transcription factor AP-1, specifically the c-fos component.In addition to inhibiting serum-induced collagen synthesis, estradiol also inhibits collagen synthesis induced by ANG II and TGF-β , growth factors implicated in the pathophysiology of progressive renal injury in various experimental models for kidney disease . TGF-β is known to mediate the mitogenic effects of ANG II as well as ET-1 on SMC and GMC growth Moreover, estradiol antagonizes the effects of TGF-β on collagen synthesis in mesangial cells because ET-1 and ANG II induce their mitogenic effects on mesangial cells via TGF-β , it is likely that estradiol also attenuates the deleterious effects of ET-1 and ANG II on the kidney.In addition growth within the glomerulus is also

stimulated by free radicals .Free radicals induce GMC cell growth and contribute to the process of glomerulosclerosis in various renal diseases. The mechanism by which free oxygen radicals induce their mitogenic effects on GMCs include induction of ET-1 synthesis, oxidation of LDL to oxidized LDL, oxidation of lipoproteins, and activation of the MAP kinase pathway . Because estradiol is a potent antioxidant that scavenges free radicals, estradiol may protect GMCs against the growth effects of free radicals (Dubey, Jackson, 2001.)

3.10 VALPROIC ACID

3.10.1 INTRODUCTION

Valproic acid (VPA) is a short-chained fatty acid widely used in humans as an anticonvulsant, a mood stabilizer and has recently been shown to inhibit histone deacetylases (HDACs)(Phiel et.al. 2001).

3.10.2 MECHANISM

Effects of VPA appear mediated by the following mechanisms:(Fagundes.2008)

- Prolonged recovery of inactivated sodium channel
- Enhanced potassium conductance, producing hyperpolarized, inhibited neurons
- Reduction of low-threshold (T-type) calcium currents which mediate repetitive neuronal firing
- Increased brain concentrations of gamma-aminobutyric acid by inhibiting degradation and/or enhancing production of the neurotransmitter.

3.10.3 PHARMACOKINETICS (Stephen, Linda ,2003)

PARAMETERS	RESULT
Absorption	Rapidly absorbed orally
Metabolism	More than 95% of valproate is broken down in iver by CYP2C9,Glucuronylated and β oxidation
Distribution	0.16 L/kg
Half life	14 hrs
Elimination	5% excreted unchanged in urine
Protein binding	95%
Peak plasma concentration	1-3 hrs

Table 3.10-Pharmacokinetics of valproic acid

3.10.4 USE AND DOSE (Fagundes 2008)

- Mania:60 mg/kg/day
- Migraine:1000 mg/day
- Seizures:30-60 mg/kg/day

3.10.5 ADVERSE EFFECT

Common side effect includes dyspepsia,weight gain, dysphoria, fatigue, dizziness, drowsiness,hair loss, headache, nausea, sedation and tremor.Valproic acid can impair liver function, cause thrombocytopoenia, and prolongate the blood coagulation times (Kostrouchová et.al., 2007)

4. MATERIAL AND METHODS

4.1 PROTOCOL

The protocol of the experiment was approved by institutional animal ethics committee as per the guidance of committee for the purpose of control and Supervision of experiments on animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. (IPS/PCOL/MPH 11-12/2002, 10th August 2011).

4.2 INDUCTION OF TYPE II DIABETES MELLITUS

Healthy adult Sprague-Dawley rats of both sex of about 6-8 weeks of age were choosen and were kept for mating in the ratio of 2:1 (i.e 2 female for every one male) for the study and maintained under well-controlled conditions of temperature (22 ± 2°C), humidity (55 ± 5%) and 12h/12h light-dark cycle. Standard laboratory rat chew and UV-filtered water was provided *ad libitum*.

The 2 days old neonates were injected intravenously (i.v.) with 90 mg/kg STZ (Sigma Ltd., USA) dissolved in citrate buffer (0.1M, pH- 4.5). Six weeks after the injection of STZ, animals were checked for blood glucose levels with the help of available diagnostic kit . The animals showing blood glucose levels >250 mg/dl were considered as diabetic.

4.3 TREATMENT PROTOCOL

The rats were then randomly divided into four groups as follows:

CON – Normal Control

COV – Control treated with Magnesium Valproate (210 mg/kg/day, p.o).

DIC – Diabetic control

DIV – Diabetic treated with Magnesium Valproate (210 mg/kg/day, p.o)

Magnesium valproate was dissolved in distilled water and was administered orally at a dose of 210 mg/kg/day for 60 days in control treated and disease treated groups. Animals were maintained with free access to conventional dietary feed and water *ad libitum* through out experimental period. All animals were monitored regularly for changes in body weight and mortality throughout the course of study.

4.4 BLOOD SAMPLE AND TISSUE COLLECTION AND SERUM ANALYSIS:

Blood samples were collected in clean dry centrifuge tubes as the end of experimental period from the retro orbital plexuses under light ether anesthesia and were allowed to clot for 30 min at room temperature. Serum was separated by centrifugation at 5000rpm for 20 min and stored at -20^0C until the analysis was carried out. Serum samples were analyzed for Total cholesterol, HDL- cholesterol, Triglycerides, LDL- cholesterol. C-Reactive Protein (CRP), Lactate De-Hydrogenase (LDH), Creatinine Kinase (CK), Alkaline phosphatase(ALP), Creatinine, Blood urea, Albumin and Uric acid spectrophotometrically (Shimadzu UV-1601, Japan) using available biochemical diagnostic kits (Labcare Diagnostics Pvt. Ltd., India). For the measurement of urine parameters, animals were placed in metabolic cages with free access to water and food and urine was collected for 24 hours in a clean, dry beaker and filterd to remove turbidity. Urine samples were analysed for Creatinine, Albumin, Uric acid and Chloride spectrophotometrically (Shimadzu UV-1601, Japan) using biochemical diagnostic kits (Labcare Diagnostics Pvt. Ltd., India) while Sodium and Potassium in urine were measured using flame photometer. Hemodynamic parameter viz. blood pressure, heart rate, rate of pressure development and decay were recorded by carotid artery cannulation using (Labscrib System Inc., IWORX, USA, Version 118). After withdrawal of blood samples from retro-orbital plexus and recording hemodynamic parameters, animals were sacrificed, hearts and kidneys and were excised, extraneous tissues were separated and wet weight of the entire heart and both kidneys, left ventricle, right ventricle and femur length was noted down.

4.5 Serum/Urine Biochemical Estimations

4.5.1. Estimation of glucose

Principle-

Glucose is determined after enzymatic oxidation in the presence of glucose oxidase. The hydrogen peroxide formed reacts, under catalysis of peroxidase, with phenol and 4-aminophenazone to form a red-violet quinoeimine dye as indicator. Glucose is a major source of energy for most cells of the body; insulin facilitates glucose entry into the cells. Diabetes is a disease manifested by hyperglycemia; patients with diabetes demonstrate an inability to produce insulin.

Procedure-

Reagents and samples were pipetted out as shown below:

	Blank	Standard	Serum sample
Sample	-	-	10 µl
Standard	-	10 µl	-
Reagent	1000 µl	1000 µl	1000 µl

The contents were mixed and incubated for 30 min. at R.T. and absorbance of sample (AT) and standard (AS) against reagent blank was measured at 505 nm.

Calculations:

Total Glucose (mg/dl) = AT/AS x conc. Standard

Concentration of Std. = 100 mg/dl

4.5.2 LIPID PROFILE

- **Estimation of Total cholesterol:**

Principle

Cholesterol esters are hydrolyzed by Cholesterol esterase to produce cholesterol. Hydrogen Peroxide is then produced from oxidation of cholesterol by cholesterol oxidase. The indicator quinoneimine is formed from hydrogen peroxide and 4 - aminoantipyrine in the presence of phenol and peroxide. The absorption of the red quinoneimine dye is proportional to the concentration of cholesterol in the sample.

Procedure:

Reagents and samples were pipetted out as shown below:

	Blank	Standard	Serum sample
Sample	-	-	10 µl
Standard	-	10 µl	-
Reagent	1000 µl	1000 µl	1000 µl

The contents were mixed and incubated for 30 min. at R.T. and absorbance of sample (AT) and standard (AS) against reagent blank was measured at 505 nm.

Calculations:

Total Cholesterol (mg/dl) = Abs. of TC / Abs. of Std. X 200 mg/dl

- **Estimation of Triglycerides:**

Principle:

Triglycerides are determined after enzymatic hydrolysis with lipases. The quinonemine indicator is formed from hydrogen peroxide, 4-aminophenazone, and 4-chlorophenol under the catalytic influence of peroxidase.

Procedure:

Reagents and samples were pipetted out as shown below:

	Blank	Standard	Serum sample
Sample	-	-	10 µl
Standard	-	10 µl	-
Reagent	1000 µl	1000 µl	1000 µl

The contents were mixed and incubated for 30 min. at R.T. and absorbance of sample (AT) and standard (AS) against reagent blank was measured at 505 nm.

Calculations:

Triglycerides (mg/dl) = Abs. of TG / Abs. of Std. X 200 mg/dl

- **Estimation of LDL Cholesterol:**

Principle:

Direct determination of serum LDL-C (low density lipoprotein cholesterol) levels without the need for any pre-treatment of centrifugation steps. The assay takes place in two steps.

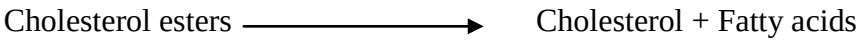
-1^{o} elimination of lipoprotein non-LDL

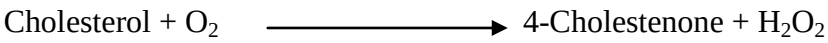
$$\text{Cholesterol esters} \xrightarrow{\text{CHE}} \text{Cholesterol + Fatty acids}$$

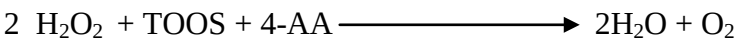
$$\text{Cholesterol + } O_2 \xrightarrow{\text{CHOD}} \text{4-Cholestenone + } H_2O_2$$

$$2\ H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

-2^{o} measurement of LDL-C







The intensity of color formed is proportional to the LDL-C concentration in the sample.

Procedure:

Reagents and samples were pipetted out as shown below:

	Blank	Standard	Serum sample
R1 (µl)	375	375	375
Standard (µl)	-	5	-
Sample (µl)	-	-	5

Mixed and incubated for 5 mins at 37°C

R2 (µl)	125	125	125

The contents were mixed and incubated for 30 min. at R.T. and absorbance of sample (AT) and standard (AS) against reagent blank was measured at 546 nm.

Calculations:

LDL-C (mg/dl) = Abs. of sample / Abs. of Calibrator X Calibrator conc.

- **Estimation of VLDL:**

  VLDL level was estimated using Friedewald formula as below:

  -Triglyceride(mg/dl) / 5

- **Estimation of HDL Cholesterol:**

Principle:

Direct determination of serum HDL-C (High Density Lipoprotein Cholesterol) levels without the need for any pre-treatment or centrifugation of the sample. The method depends on the properties of a detergent which solubilizes only the HDL so that HDL-C is released to react with the cholesterol esterase, cholesterol oxidase and chromogens to give colour. The non HDL lipoprotein LDL, VLDL and chylomicrons are inhibited from reacting with the enzymes due to abruption of the detergents on their surfaces. The intensity of the color formed is proportional to the HDL-C concentration in the sample.

Procedure:

Reagents and samples were pipetted out as shown below:

	Blank	Standard	Serum sample
R1 (µl)	750	750	750
Standard (µl)	-	10	-
Sample (µl)	-	-	10

Mixed and incubated for 5 mins at 37°C. Read absorbance A1.

R2 (µl)	250	250	250

The contents were mixed and incubated for 30 min. at R.T. and absorbance of sample (AT) and standard (AS) against reagent blank was measured at 505 nm.

Calculations:

HDL-C (mg/dl) = ΔA sample / ΔA Calibrator X Calibrator conc.

4.5.3 CARDIAC BIOMARKERS:

- **Estimation of Lactate dehydrogenase:**

Principle-

Lactate is oxidised to Pyruvate in the presence of NAD by the action of lactate dehydrogenase. The rate of formation of NADH is directly proportional to LDH concentration.

Procedure

Reagents and samples were pipetted out as shown below:

	Blank	Serum sample
Rw (µl)	1000	1000
Sample (µl)	-	25

The contents were mixed and incubated for 1 min. at R.T. and measured the change in absorbance per min. (Δ A/min.) for next 2 minutes at 340 nm.

Calculations:

Activity (U/L) = Δ A/min x 6592

- **Estimation of creatinine kinase:**

Principle-

This procedure involves measurement of Creatine kinase (CK) activity in the presence of an antibody to CK-M monomer. This antibody completely inhibits the activity of CK-MM and half of CKMB while not affecting the B subunit of CK-MB and CK-BB. Than the CK method is used to quantitatively determine CK-BB activity. CK catalyses the reaction between creatine phosphate and ADP, giving creatine and ATP. ATP and glucose in the presence of G6PDH oxidises, and reduces NAD to NADH. The rate of NADH formation is determined photometrically at 340 nm & is directly proportional to CK-BB activity. The CK-MB activity is calculated by multiplying CKBB x 2.

Procedure

Reagents and samples were pipetted out as shown below:

	Blank	Serum sample
Rw (µl)	1000	1000
Sample (µl)	-	50

The contents were mixed and incubated for 10 min. at R.T. and measured the change in absorbance every minute for next 5 minutes at 340 nm.

Calculation-

Δ A/min. x 3376 = U/l CKBB

CKMB = CKBB x 2

- **Estimation of CRP**

Principle-

The CRP-Turbilatex is a quantitative turbidimetric test for the measurement of C-reactive protein (CRP) in human serum or plasma. Latex particles coated with specific anti-human CRP are agglutinated when mixed with samples containing CRP. The agglutination cause an absorbance change, dependent upon the CRP content of the patient sample that can be quantified by comparison from a calibrator of known CRP concentration. CRP is an acute-phase protein present in normal serum, which increases significantly after most forms of tissue, bacterial and virus infections, inflammation and malignant neoplasia. During tissue necrosis and inflammation resulting from microbial infections, the CRP concentration can rise up to 300 mg/L in 12-24 hours.

Procedure-

Reagents and samples were pipetted out as shown below:

	Blank	Calibrator	Serum sample
Rw (µl)	1000	1000	1000
Calibrator (µl)	-	5	-
Sample (µl)	-	-	5

The contents were mixed and measured the absorbance at 540 nm after 10 Seconds (A1) and after 2 minutes (A2) of the sample addition.

Calculation-

(A2-A1) sample __ x Calibrator concentration =mg/L CRP
(A2-A1) calibrator

4.5.4 RENAL MARKERS

- **Estimation of Alkaline phosphatase**

Principle-

p-Nitrophenyl phosphate is converted to p-nitrophenol and phosphate by alkaline phosphatase. The increase of absorption at 405 nm is proportinal to the alkaline phosphatase concentration in the sample.

Procedure-

Reagents and samples were pipetted out as shown below:

	Blank	Serum sample
Rw (µl)	1000	1000
Sample (µl)	-	20

The contents were mixed and incubated for 1 min. at R.T. and measured the absorbance increased every 30 secs for 2 minutes at 405 nm.

Calculation-
A/min. x 2720 = U/l Alkaline Phosphatase

- **Estimation of Creatinine**

Principle-

Creatinine in alkaline solution reacts with picrate to form a coloured complex which absorbs at 500 - 520 nm. The amount of complex formed is directly proportional to the creatinine concentration

Procedure-

Reagents and samples were pipetted out as shown below:

	Blank	Standard	Serum/Urine Sample
Rw (µl)	1000	1000	1000
Standard (µl)	-	100	-
Sample (µl)	-	-	100

The contents were mixed and incubated for 30 sec. at R.T. and absorbance of sample (AT) and standard (AS) against reagent blank was measured at 520 nm.

Calculation-

Δ Ac / Δ As x Conc. of std. = mg Creatinine/dl Serum

Δ Ac / Δ As X C X 20 = mg Creatinine/dl Urine

- **Estimation of Blood Urea**

Principle

Urea is hydrolysed in the presence of water and urease to produce ammonia and carbon dioxide. The ammonia produced combines with alfa-oxoglutarate and NADH in the presence of glutamate dehydrogenase to yield glutamate and NAD.

Procedure

Reagents and samples were pipetted out as shown below

	Blank	Standard	Serum/Urine Sample
Rw (µl)	1000	1000	1000
Standard (µl)	-	10	-
Sample (µl)	-	-	10

The contents were mixed and incubated for 30 sec. at R.T. and absorbance of sample (AT) and standard (AS) against reagent blank was measured at 578 nm.

Calculation

Urea (mg/dl) = AT / AS x Conc. of Std.

- **Estimation of Albumin**

Principle-

The measurement of serum albumin is based on its quantitative binding to the indicator 3, 3', 5, 5' - tetrabromo-m-cresol sulphophthalein (bromocresol green, BCG). The albumin-BCG complex absorbs maximally at 578 nm.

Procedure-

Reagents and samples were pipetted out as shown below:

	Blank	Standard	Serum/Urine Sample
Rw (µl)	1000	1000	1000
Standard (µl)	-	5	-
Sample (µl)	-	-	5

The contents were mixed and incubated for 10 min. at R.T. and absorbance of sample (AT) and standard (AS) against reagent blank was measured at 578 nm.

Calculation-

Ac/As x Conc. of Std. = g/dl Albumin in serum

Ac/As x Conc. of Std. x 5 = g/dl Albumin in urine

- **Estimation of Uric acid**

Principle-

Uric acid is converted by uricase to allantoin and hydrogen peroxide, which under the catalytic influence of peroxidase, oxidises 3, 5 - dichloro - 2 - hydroxybenzenesulfonic acid and 4- aminophenazone to form a red-violet quinoneimine compound.

Procedure-

Reagents and samples were pipetted out as shown below:

	Blank	Standard	Serum/Urine Sample
Rw (µl)	1000	1000	1000
Standard (µl)	-	25	-
Sample (µl)	-	-	25

The contents were mixed and incubated for 10 min. at R.T. and absorbance of sample (AT) and standard (AS) against reagent blank was measured at 520 nm.

Calculation-

Ac/As x Conc. of std. = mg/dl Uric Acid in Serum

Ac/As x Conc. of std. x 10 = mg/dl Uric Acid in Urine

- **Estimation of Total protein**

Principle-

Cupric ions, in an alkaline medium, interact with protein peptide bonds resulting in the formation of a colored complex.

Procedure-

Reagents and samples were pipetted out as shown below:

	Blank	Standard	Urine sample
Rw (µl)	1000	1000	1000
Standard (µl)	-	20	-
Sample (µl)	-	-	20

The contents were mixed and incubated for 20 min. at R.T. and absorbance of sample (AT) and standard (AS) against reagent blank was measured at 540 nm.

Calculation-

Ac/As x Conc. Standard = g/dl protein

- **Esimation of Chloride**

Principle-

Chloride ions react with mercurous thiocyanate to form mercury perchlorate and thiocyanate. Thiocyanate forms a red complex with ferric ions in the presence of nitric acid.

Procedure-

Reagents and samples were pipetted out as shown below:

	Blank	Standard	Urine sample
Rw (µl)	1000	1000	1000

Standard (µl)	-	10	-
Sample (µl)	-	-	10

The contents were mixed and incubated for 30 min. at R.T. and absorbance of sample (AT) and standard (AS) against reagent blank was measured at 480 nm.

Calculation-

Ac/As x Conc. of std. = mEq/l Chloride in Urine

- **Estimation of Sodium and Potassium**

Procedure

Estimation of Sodium and Potassium were done using flame photometer.

Calculation

Sodium and Potassium content were estimated by extrapolation method from standard curve of sodium and potassium plotted in a range of 10-180 mmol/l.

4.6 HEMODYNAMIC PARAMETERS

The animals were anaesthetized by Urethane (1.5 mg/kg, i.p) and Diazepam(4mg/kg, i.m). The carotid artery behind the trachea was exposed and cannulated for the measurement of hemodynamic parameters using a transducer (BP 100) and Labscribe System (IWORX, New Hampshire, USA). The hemodynamic parameters observed were systolic blood pressure(SBP), diastolic blood pressure (DBP), mean arterial blood pressure(MABP), heart rate, rate of pressure development (dp/dt_{max}) and rate of pressure decay(dp/dt_{min}). All the data were analyzed using Labscribe software (Version 118).

4.7 HYPERTROPHIC PARAMETERS

After measurement of hemodynamic parameters, animals were sacrificed. The skin was quickly incised at the midline over the sternum and the hearts were exposed by cutting the pericardium. Hearts were isolated from the body, blotted with filter paper to remove excess of blood, remaining extraneous tissues were removed and weight of the heart, left ventricular weight, right ventricular weight and femur length was noted down. Index of cardiac hypertrophy was calculated as wet heart weight to femur length ratio and left ventricular hypertrophy index was calculated as wet left ventricle weight to wet heart weight ratio. Also left ventricular weight to right ventricular weight ratio was estimated. left ventricular wall thickness was measured using micrometer.

4.8 OXIDATIVE STRESS PARAMETERS

Heart and kidney tissues were minced and homogenized in phosphate buffer saline, pH 7.4. The homogenate was used to measure Malondialdehyde level and clear supernatant was used for other prooxidant and antioxidant enzymes assays.

1. Total Protein estimation:-

Total Protein was estimated by the method of **Lowry et al, 1951.**

Principle :-

The phenolic group of tyrosine and trytophan residues (amino acid) in a protein will produce a blue purple color complex , with maximum absorption in the region of 660 nm wavelength, with Folin- Ciocalteau reagent which consists of sodium tungstate molybdate and phosphate. Thus the intensity of color depends on the amount of these aromatic amino acids present and will thus vary for different proteins.

Reagents :-

1. Reagent A :-2 % sodium carbonate in 0.1 N NaOH.

2. Reagent B:-0.5 % copper sulphate ($CuSO_4$. 5 H_2O) in 1 % potassium sodium tartrate.

3. Reagent C :-Alkaline copper solution (Mix 50 ml of solution A & 1 ml of solution B prior to use)

4.Reagent D :- Folin- Ciocalteau reagent.

Procedure :-

Blank	**Test**
0.2 ml of D.W.	0.2 ml of supernatent
Diluted upto 1 ml with Tris HCL	Diluted upto 1 ml with Tris HCL

5 ml Reagent C	5 ml Reagent C
Allowed it for 10 minutes	
0.5 ml Reagent D	0.5 ml Reagent D

All reagents were mixed well and kept at room temperature for 30 min. in dark place and absorbance was read against blank at 600 nm. The protein level was calculated using standard curve which was plotted using standard albumin(Sigma chemicals, USA).

2. Lipid peroxidation: (MDA) :-

Malondialdehyde formation (MDA) was estimated by the method of **Ohkawa et al., 1979.**

Principle:-

The method estimates Malondialdehyde (MDA), a product of lipid peroxidation process. One molecule of MDA reacts with two molecules of thiobarbituric acid (TBA) under mildly acidic conditions to form a pink coloured chromogen, whose intensity was measured colorimetrically at 535 nm.

Reagents:-

1. **Sodium lauryl Sulphate (SLS) (8%) :-** 8 gm of SLS in 100 ml of distilled water.

2. **Acetic acid (20 %) :-** Prepared in 0.27 M hydrochloric acid (2.29 ml HCL in 100 ml water)

3. **Thiobarbituric acid(TBA) (1% in Tris hydrochloride, pH 7): (Freshly prepared):-** 1 gm of thiobarbituric acid in 100 ml of Tris hydrochloride buffer pH 7.

Procedure:-

Blank	**Test**
0.2 ml of D.W.	0.2 ml of Homogenate
0.2 ml of SLS	0.2 ml of SLS
1.5 ml acetic acid in HCl	1.5 ml acetic acid in HCl
1.5 ml TBA	1.5 ml TBA
0.6 ml DW	0.6 ml DW
Heated for 45 min in water bath at 95^0C and cool	
5 ml mixture of n-butanol:pyridine(15:1)	5 ml mixture of n-butanol:pyridine(15:1)

All reagents were mixed well and pink colour developed in upper organic layer, the absorbance of which was read against blank at 532 nm. Malondialdehyde level was calculated using molar extinction coefficient of malondialdehyde.

3. Nitrite

Determination of nitrite level in left ventricle was performed according to method described by Dohare et. al., 2008.

Principle:-

The measurement of nitrite content was done by the Griess reaction, by adding 100 µl of Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride in 2% H3PO4) to 100 µl of samples. Griess reagent converts nitrite into a deep purple azo compound. Photometric measurement of the absorbance at 550 nm due to this azo chromophore accurately determines NO2- concentration. Nitrite concentration was calculated by comparison with absorbance at 550 nm of standard nitrite solution. Results are expressed as µmol/mg protein.

Reagents:-

1. **Griess reagent:-** 1% sulfanilamide and 0.1% naphthylethylene diamine dihydrochloride in 2% H3PO4

Procedure:-

Blank	**Test**
0.5 ml of D.W.	0.5 ml of supernatent
0.5 ml of Griess reagent	0.5 ml of Griess reagent
Incubate for 20 min. at 2-5 C	

All reagents were mixed well and absorbance was read against blank at 550 nm. The nitrite level was calculated using standard curve which was plotted using sodium nitrite.

4. <u>Reduced Gluathione :-</u>

Reduced of glutathione (GSH) was estimated by the method of **Moran et. al., 1979.**

Principle: -

Glutathione present in RBC consist of sulfhydryl groups. 5,5 dithiobis 2- nitro benzoic acid (DTNB), A disulphide compound, gets readily attacked by these sulfhydryl groups and forms a yellow coloured anion which measured colorimetrically at 412 nm.

Reagents :-

1. Trichloroacetic acid (TCA) (10%) :- 10 gm of TCA in 100 ml of distilled water.

2. 0.3 M Na_2HPO_4 :- 4.26 gm of Na_2HPO_4 in distilled water.

3. DTNB (Dithiobis nitro benzoic acid) (Fresh) :- 40 mg in 100 ml of 1% Sodium citrate & cover with aluminium foil.

Procedure:-

Blank	**Test**
0.2 ml of D.W.	0.2 ml of supernatent
1 ml of TCA(10%)	1 ml of TCA (10%)
Keep in ice bath for 30 min & Centrifuge for 10 min at 4˚C at 3000 RPM, take 0.5 ml of supernatant	
0.5 ml of Supernatant	0.5 ml of Supernatant
2 ml di-sodium hydrogen phosphate	2 ml di- sodium hydrogen phosphate
0.25 ml DTNB(Cover with aluminium foil)	0.25 ml DTNB(Cover with aluminium foil)

All reagents were mixed well absorbance was read against blank at 412 nm. The GSH level was calculated using standard curve which was plotted using standard GSH(Sigma Chemicals, USA).

5. SOD

SOD was estimated by the method of **Mishra and Fridovich, 1972**.

Principle:

The O_2^{-}, substrate for SOD is generated indirectly in the oxidation of epinephrine at alkaline pH by theaction of oxygen on epinephrine. As O_2^{-} builds in the solution, the formation of adrenochrome accelerates because O_2^{-} also reacts with epinephrine to form adrenochrome . Toward the end of the reaction, when the epinephrine is consumed, the adrenochrome formation slows down. If observed for long times, the adrenochrome disappears and brown, insoluble products form in the solution.

SOD reacts with the O_2^- formed during the epinephrine oxidation and therefore slows down the rate of formation of the adrenochrome as well as the amount that is formed. Because of this slowing process, SOD is said to inhibit the oxidation of epinephrine.

Reagent:

1. EDTA 0.0001 M (9.3 mg/250 ml)
2. Carbonate buffer pH 9.7 (8.4 gm $NaHCO_3$ + 10.6 gm Na_2CO_3 in 500 ml)
3. Epinephrine – 0.003 M (50 mg/100 ml in 2 pH HCL and cover with aluminium foil.)

Procedure:

Blank	**Test**
0.2 ml of D.W.	0.2 ml of supernatent
0.1 ml EDTA	0.1 ml EDTA
0.5 ml Carbonate buffer	0.5 ml Carbonate buffer
1 ml epinephrine	1 ml epinephrine

All reagents were mixed well and absorbance was read against blank at an interval of 30 sec.for 3 min. at 480 nm. The SOD level was calculated using standard curve which was plotted using standard SOD(MP Bio, USA)

4.9 Estimation of Na^+ K^+ ATPase

This activity was performed according to method described by Tsimaratos et al., 2001.

Preparation of sample and solution:-

The tissue for Na+K+-ATPase activity was homogenized in 0.01M Tris–HCl buffer (PH 7.4) and 4% w/v left ventriclular homogenate was utilised for further estimation.

Reagents

184mM Tris–HCl buffer with PH 7.4,

600mM NaCl,

50mM KCl,

1mM sodium EDTA and

80mM ATP

1-amino-2-napthol-4-sulphonic acid (ANSA) (1 mg in 40 ml distil water)

2.5% w/v Ammonium molybdate

Procedure:-

(Sovoboda and Mossinger, 1981; Rao and Deshpande, 2005; Babua and Ramanathan, 2011)

Blank	**Test**
250 µl of Tris–HCl	250 µl of Tris–HCl
50µl of 600 mM NaCl	50µl of 600 mM NaCl
50µl of 50 mM KCl	50µl of 50 mM KCl
50µl of 1mM sodium EDTA	50µl of 1mM sodium EDTA
50µl of 80mM ATP	50µl of 80mM ATP
Incubated for 10 min at 37^0C	
25µl of D.W	25µl of 4% homogenate
Incubated for 1 hr. at 37^0C	
10% TCA	10% TCA
Centrifuged at 3500 rpm for 10min	

50 µl of the supernatant	50 µl of the supernatant
925 µl of distilled water	925 µl of distilled water
125 µl of ammonium molybdate	125 µl of ammonium molybdate
50 µl of ANSA	50 µl of ANSA
Incubated for 10 min at 37 ◦C.	

All reagents were mixed well and kept at room temperature for 30 min. in dark place and absorbance was read against blank at 640 nm. The phosphorus liberated was calculated using standard curve which was plotted using standard potassium dihydrogen phosphate(CDH Lab, India).

4.10 Estimation of Collagen

This estmation was done according to method described by Drobnik et al., 2009.

Preparation of hydrolysate of sample

The tissue for collagen measurement was hydrolyzed with 6 N Hydrochloric acid at 110º C for 12 hrs. And after 12 hrs hydrolysate obtain was used for estimation.

Reagents:

1. 6 N Hydrochloric acid
2. Citrate buffer : For 10 ml, mixture of 8.2 ml of sodium citrate (294.1 mg/ 10ml) and 1.8 ml of Citric acid (210.14mg/10ml)
3. Chloramin T: 1.4% in citrate buffer
4. Ehrlich Reagent: 2.4 g para dimethy aminobenzaldehyde in 8 ml of Ethanol (95%) + 0.54 ml Concentrated H_2SO_4.

Procedure:-

Blank	**Sample(LV/Kidney)**
200µl of Distilled water	200µl of tissue hydrolysate
200µl of Ethanol (95%)	200µl of Ethanol (95%)
200µl of Chloramine T	200µl of Chloramine T
Oxidised for 20 min at RT	
400µl of Ehrlich reagent	400µl of Ehrlich reagent
Incubate for 15 min at 60º C	

After incubation, absorbance was read against blank at 573 nm and calculation was done using standard curve of hydroxyproline.

4.11 Fixation And Processing Of Tissues For Histological Studies

Heart and kidneys were collected after the rats were sacrificed. After blotting free of blood and tissue fluids, were fixed in 10% formalin solution. After 24 hours the tissues were washed thouroughly in repeated changes of 70% alcohol and then dehydrated in ascending grades of alcohol (70-100%). Dehydration in absolute alcohol was followed by treatment of tissues with toluene: xylene (50:50) followed by 10%, 50%, 70%, 90% paraffin wax in toluene and finally 2 changes in 100% wax (paraffin wax, 60-62ºC) followed by embedding of tissue in wax.

5-15µm thick section were serially cut on a leitz microtome in horizontal plane and mounted in glass slide with the help of egg albumin in glycerine solution (50% v/v). The sections were deparafinned in xylene and downgraded through 100%, 90%, 70%, 50%, & 30% alcohol and finally in water. They were then stained with 10% hematoxylin for 3-5 minutes and the staining was intensified by placing in running water. The hematoxylin stained sections were stained with 10% eosin for 2 minutes and were then quickly passed through ascending grades of alcohol and finally treated with xylene followed by mounting in DPX.

The sections were observed and desired areas were photographed in an Olympus photomicroscope for morphometric studies of different cells. The sections were viewed under 40X and 100X magnification and cell measurements were carried out using Image analyzer(IMAGE J, Ver. 1.45).

4.12 CNS Activity Estimation

4.12.1 FORCED SWIM TEST

Rats were placed individually into glass cylinders (height 25 cm, diameter 10 cm) containing 10 cm of water, maintained at 23–25°C. The animals were left in the cylinder for 6 min. After the first 2 min the total duration of immobility was measured during a 4-min test. The mouse was judged to be immobile when it remained floating passively, performing slow motion to keep head above the water (Poleszak et.al., 2006).

4.12.2 EFFECT ON LOCOMOTOR ACTIVITY

Rats were placed in the digital photoactometer , which consists of a cage which is 30 cm long and 30 cm deep with a wire mesh at the bottom. A continuous beam of light from about six lights was made to fall on corresponding photoelectric cells; the photoelectric cell got activated when an animal crossed the beam of light and thereby cuts off the rays of light falling on it. These cutoffs were counted for a period of 10 min and the figure was taken as a measure of the locomotor activity of the animal (Shalam et.al.,2007).

4.13 STASTICAL ANALYSIS:

All the values are expressed as mean ± S.E.M. Statistical differences between groups were applied using SPSS software version 17.0(USA). Data were considered to be statistically significant at p value < 0.05.

5. RESULTS

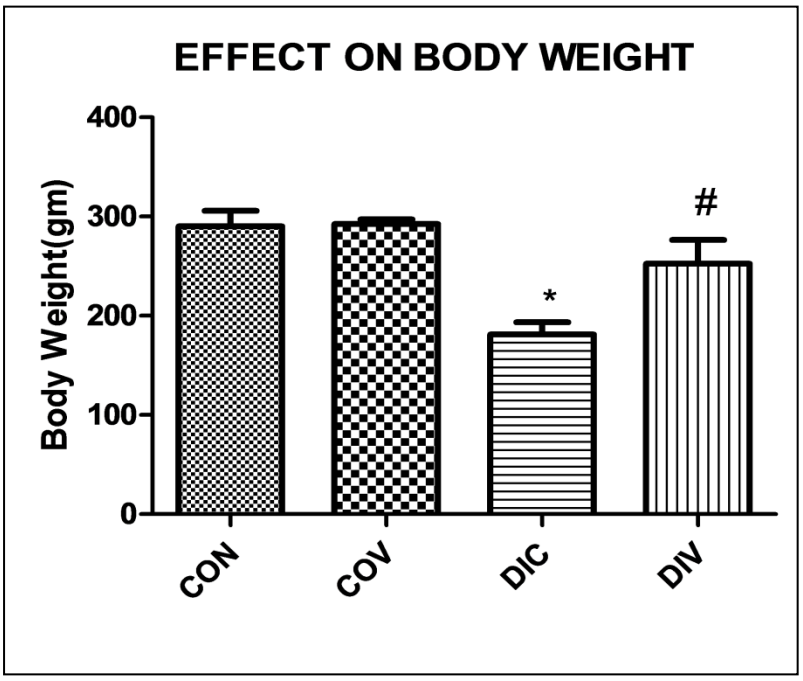
A. RESULTS OF DIABETIC CARDIOMYOPATHY

5.1 General Parameter

Effect of Magnesium Valproate (MgV) on Body weight, Food intake, Water intake

At the end of 8 weeks of treatment, STZ produced a significant ($p < 0.05$) decrease in body weight and significant ($p < 0.05$) increase in food and water intake in diabetic rats as compared to control rats. Treatment with MgV (210mg/kg/day, p.o) produced a significant ($p < 0.05$) increase in body weight and significantly ($p < 0.05$) decreased food and water intake. The reduction in food and water intake appeared after 4 weeks of treatment and continued till the end of 8 weeks. Treatment with MgV did not produce any significant change in body weight, food intake and water intake in control rats. (Fig. 5.1a.5.1b, 5.1c)

Fig. 5.1a Effect of MgV on body weight



*significantly different from control group ($p<0.05$)
Significantly different from diabetic control group ($p<0.05$)

Each bar represents Mean ± SEM of 6 animals
CON – Normal Control
COV – Control treated with MgV (210 mg/kg/day, p.o)
DIC – Disease Control
DIV – Disease Control treated with MgV (210 mg/kg/day, p.o)

Figure 5.1b: Effect of MgV on Food intake

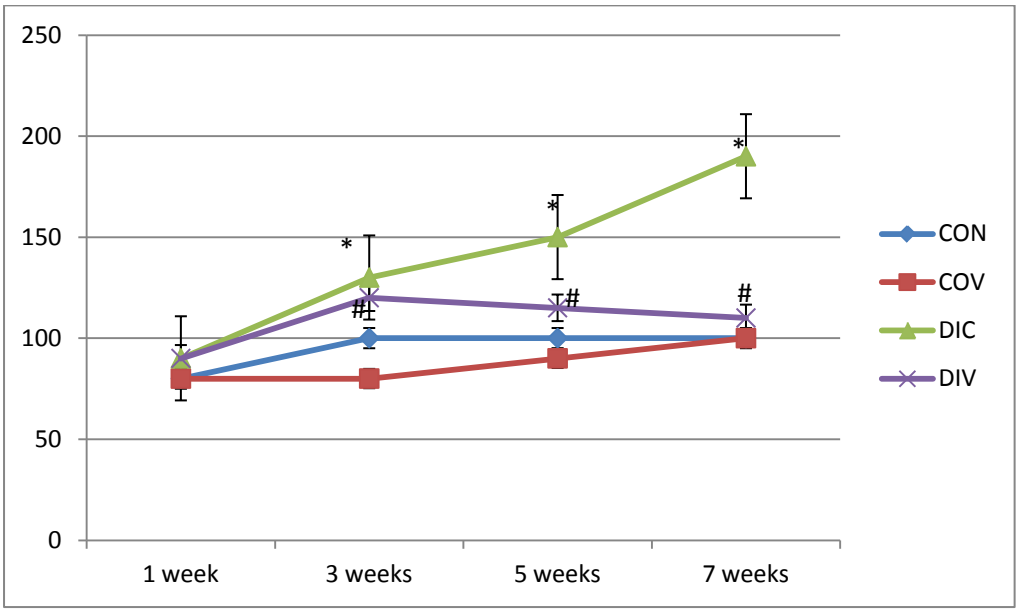
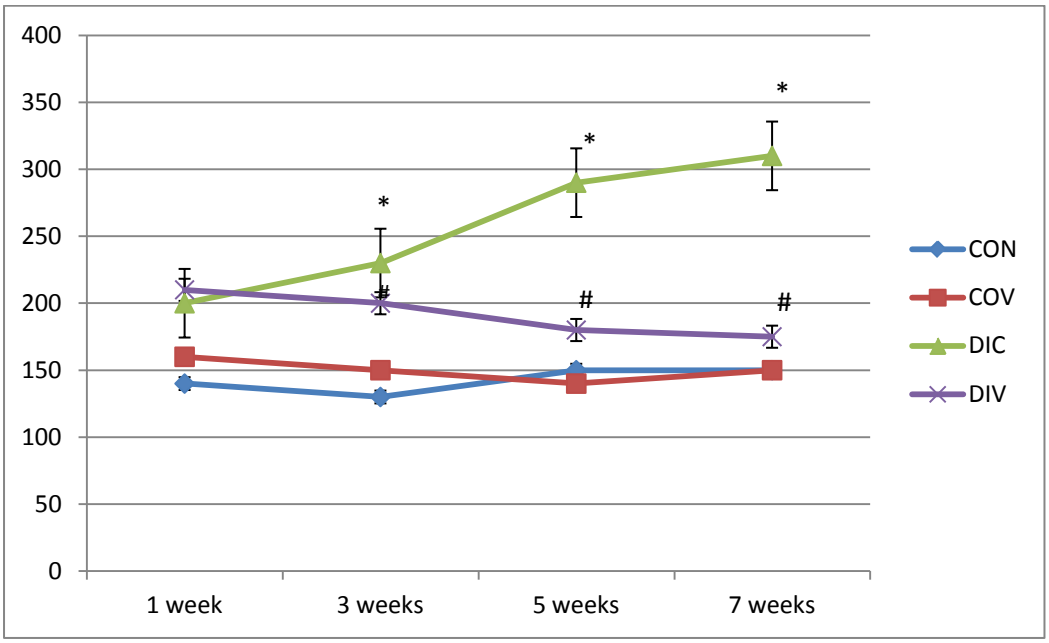


Figure 5.1c: Effect of MgV on Water intake



* significantly different from control group ($p<0.05$)
\# significantly different from diabetic control group ($p<0.05$)

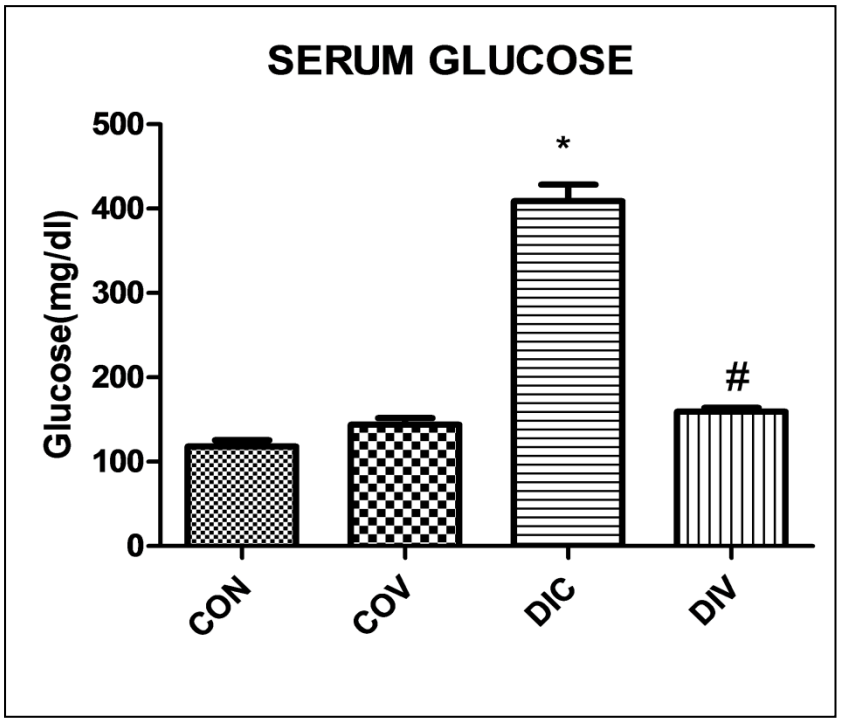
Each point represents Mean ± SEM of 6 animals.
CON – Normal Control
COV – Control treated with Magnesium valproate (210 mg/kg/day, p.o)
DIC – Diabetic Control
DIV – Diabetic Control treated with Magnesium valproate (210 mg/kg/day, p.o)

5.2 SERUM BIOCHEMICAL PARAMETERS

5.2.1 EFFECT OF GLUCOSE

Streptozotocin-diabetic rats, at the end of 8 week were found to exhibit significant (p<0.05) hyperglycemia as compared to control rats. However chronic treatment with MgV (210mg/kg/day, p.o) showed significant(p < 0.05) decrease in serum glucose level. Treatment with MgV did not alter glucose level in control rats.(Fig. 5.2 a)

Fig 5.2 a Effect of MgV on serum glucose level



* significantly different from control group ($p<0.05$)
\# significantly different from diabetic control group ($p<0.05$)

Each bar represents Mean ± SEM of 6 animals
CON – Normal Control
COV – Control treated with MgV (210 mg/kg/day, p.o)
DIC – Disease Control
DIV – Disease Control treated with MgV (210 mg/kg/day, p.o)

5.2.2 LIPID PROFILE

There was a significant (p<0.05) increase in serum cholesterol, LDL, VLDL, triglycerides levels (TG), and logTG/HDL ratio while there was significant (p<0.05) decrease in high density lipoprotein (HDL)-cholesterol levels in STZ diabetic rats as compared to control rats. Administration of MgV showed significant(p < 0.05) reduction in serum total cholesterol, LDL, VLDL, and triglyceride level but do not show any reduction in logTG/HDL ratio Also, a remarkable increase in HDL-C levels was seen with the chronically treated animals. Further, treatment with MgV did not produce any significant change in serum total cholesterol, LDL, VLDL, triglyceride and HDL levels and logTG/HDL ratio in control rats.(Table 5.2; Fig. 5.2b, 5.2c, 5.2d, 5.2e, 5.2f, 5.2g)

TABLE 5.2: Effect of MgV on lipid profile

PARAMETERS	CON	COV	DIC	DIV
SERUM CHOLESTEROL (mg/dl)	48.14 ±4.86	42.69 ±2.09	137.0 ±18.42*	51.29 ±4.16#
SERUM LDL (mg/dl)	13.86 ±1.28	14.77 ±0.93	33.08 ±1.30*	14.73 ±0.58#
SERUM VLDL (mg/dl)	12.46 ±1.970	10.20 ±2.101	24.76 ±3.024*	14.88 ±0.87#
SERUM TRIGLYCERIDE (mg/dl)	62.30 ±9.85	58.93 ±5.03	123.8 ±15.12*	74.40 ±4.40#
SERUM HDL (mg/dl)	60.67 ±6.82	57.52 ±2.45	31.52 ±2.40*	56.73 ±6.83#
LogTG/HDL RATIO	0.26 ±0.02	0.03 ±0.02	0.51 ±0.06*	0.10 ±0.01#

* significantly different from control group ($p<0.05$)
\# significantly different from diabetic control group ($p<0.05$)

Values are expressed as Mean ± SEM of 6 animals
CON – Normal Control
COV – Control treated with MgV (210 mg/kg/day, p.o)
DIC – Diabetic Control
DIV – Diabetic Control treated with MgV (210 mg/kg/day, p.o)

Figure 5.2b: Effect of MgV on Total Cholesterol level

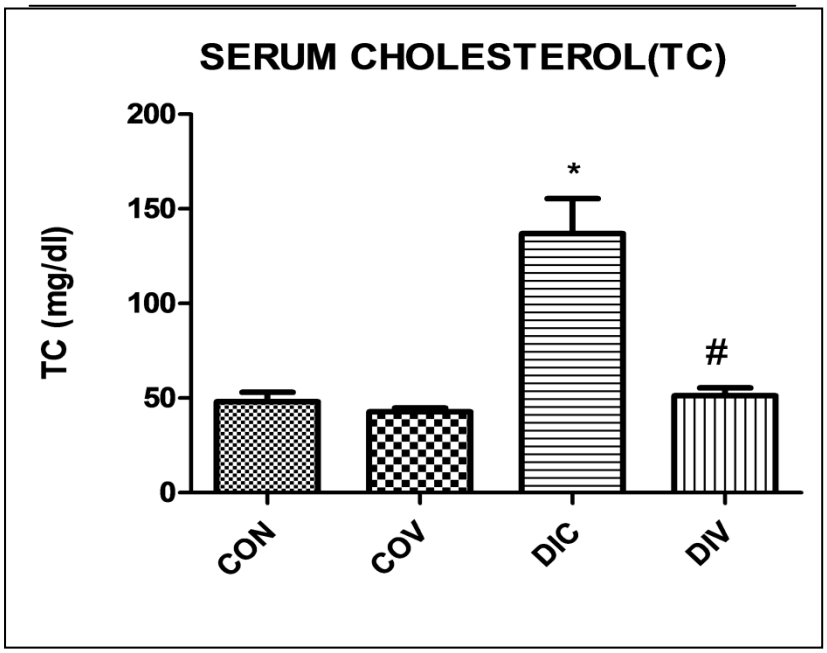
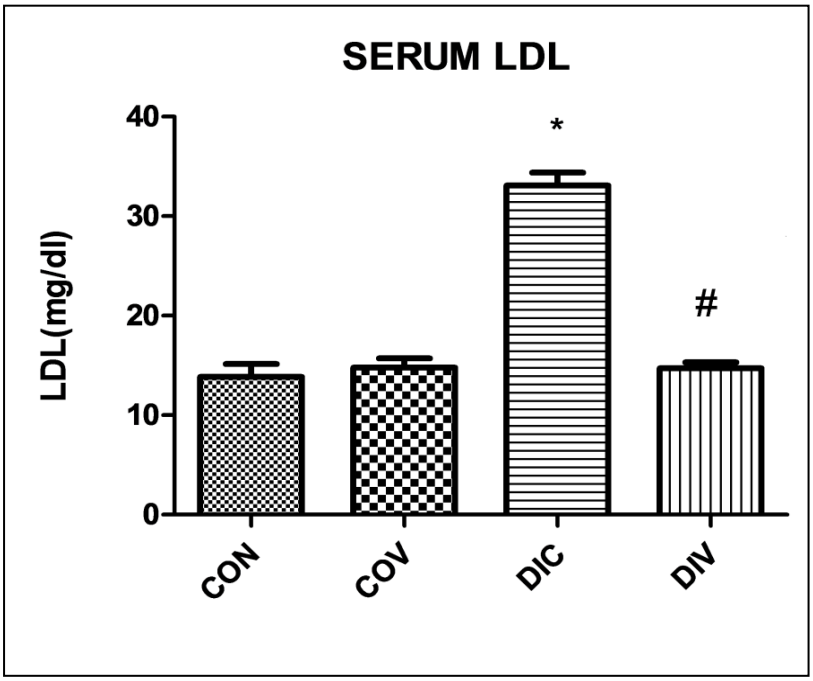


Figure 5.2c: Effect of MgV on serum LDL level



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# significantly different from diabetic control group ($p<0.05$)
.
Each bar represents Mean ± SEM of 6 animals
CON – Normal Control
COV – Control treated with MgV (210 mg/kg/day, p.o)
DIC – Diabetic Control
DIV – Diabetic Control treated with MgV (210 mg/kg/day, p.o)

Figure 5.2d: Effect of MgV on serum VLDL level

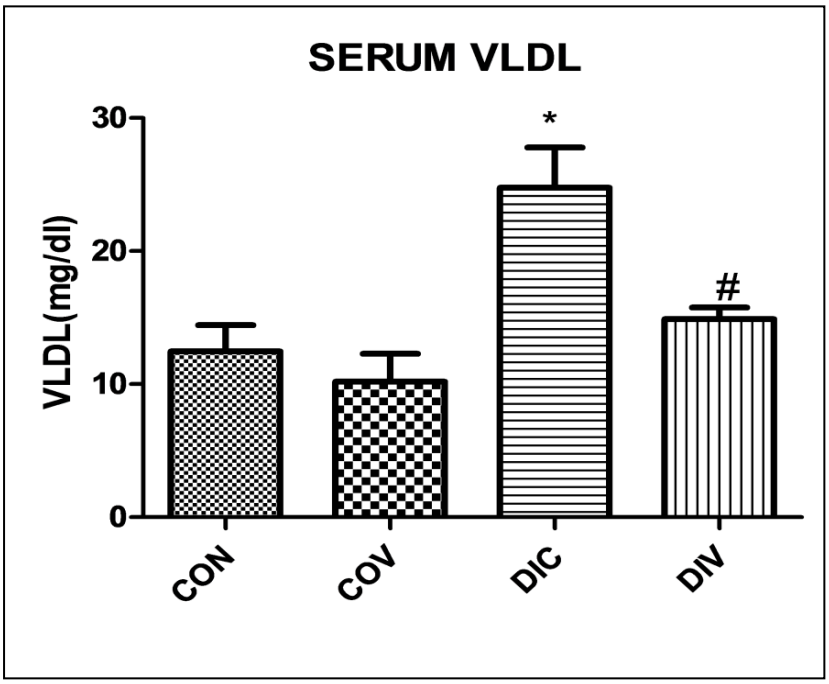
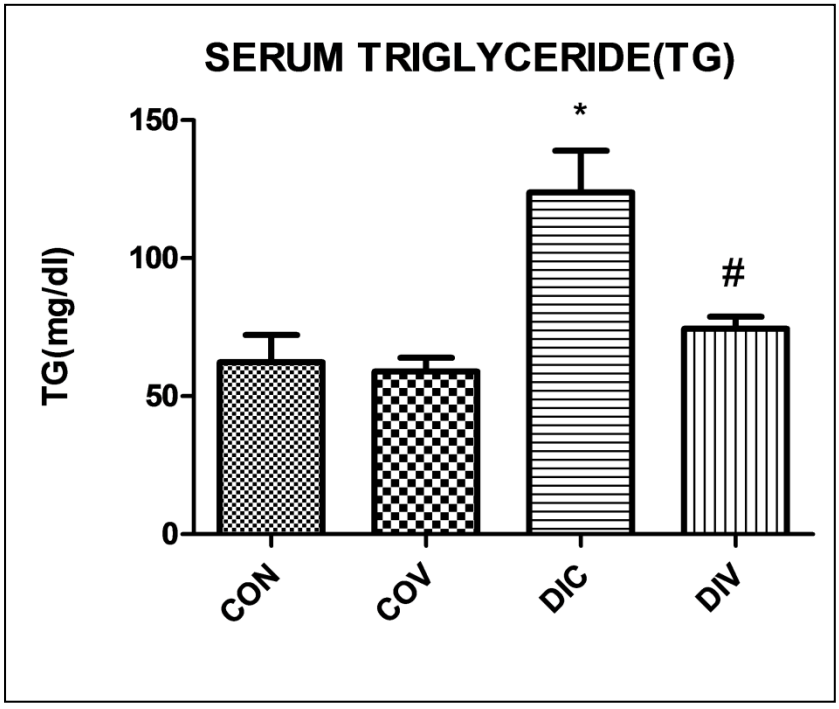


Figure 5.2e: Effect of MgV on serum Triglyceride level



* significantly different from control group ($p<0.05$)
# significantly different from diabetic control group ($p<0.05$)
Each bar represents Mean ± SEM of 6 animals
CON – Normal Control
COV – Control treated with MgV (210 mg/kg/day, p.o)
DIC – Diabetic Control
DIV – Diabetic Control treated with MgV (210 mg/kg/day, p.o)

Figure 5.2f: Effect of MgV on serum HDL level

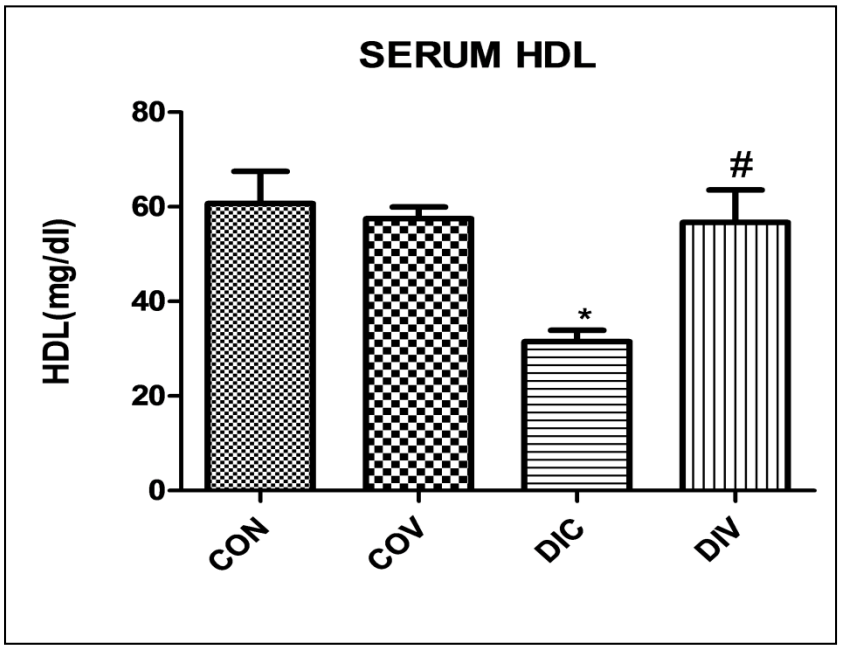
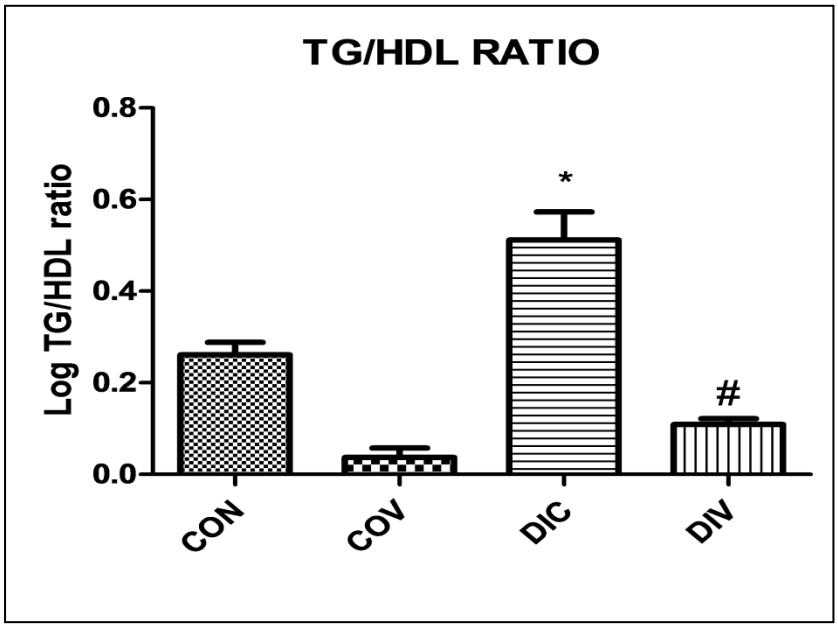


Figure 5.2g: Effect of MgV on logTG/HDL ratio



* significantly different from control group ($p<0.05$)
\# significantly different from diabetic control group ($p<0.05$)
.
Each bar represents Mean ± SEM of 6 animals
CON – Normal Control
COV – Control treated with MgV (210 mg/kg/day, p.o)
DIC – Diabetic Control
DIV – Diabetic Control treated with MgV (210 mg/kg/day, p.o)

5.3 SERUM CARDIAC MARKERS

Effect of MgV on serum Creatinine Kinase-MB(CK-MB),Lactate dehydrogenase(LDH) and C reactive protein(CRP) level.

Streptozotocin-diabetes produced a significant (p<0.05) increase in serum LDH and CK levels as compared to control rats. Chronic treatment with MgV significantly (p<0.05) changes the elevated serum LDH levels and CK levels of diabetic rats. .(Table 5.3A, Fig. 5.3a, 5.3b)

Streptozotocin-diabetes also produced a significant (p<0.05) increase in serum CRP level as compared to control rats. Chronic treatment MgV significantly (p<0.05) reduced the elevated CRP levels of diabetic rats. The drug did not produce any change in CRP levels of control rats(Table 5.3A, Fig. 5.3c)

TABLE 5.3A: Effect of MgV on serum Cardiac markers

PARAMETERS	CON	COV	DIC	DIV
LDH (U/l)	789.4 ±30.77	626.2 ±35.14	1485 ±79.90*	880.8 ±53.39#
CK-MB (U/l)	71.74 ±8.97	82.71 ±11.07	261.8 ±24.62*	92.42 ±7.46#
CRP (mg/l)	2.187 ±0.72	1.379 ±0.51	19.21 ±1.83*	6.752 ±1.09#

* significantly different from control group ($p<0.05$)
\# significantly different from diabetic control group ($p<0.05$)

Values are expressed as Mean ± SEM of 6 animals
CON – Normal Control
COV – Control treated with MgV (210 mg/kg/day, p.o)
DIC – Disease Control
DIV – Disease Control treated with MgV (210 mg/kg/day,

Figure 5.3a: Effect of MgV on serum LDH level

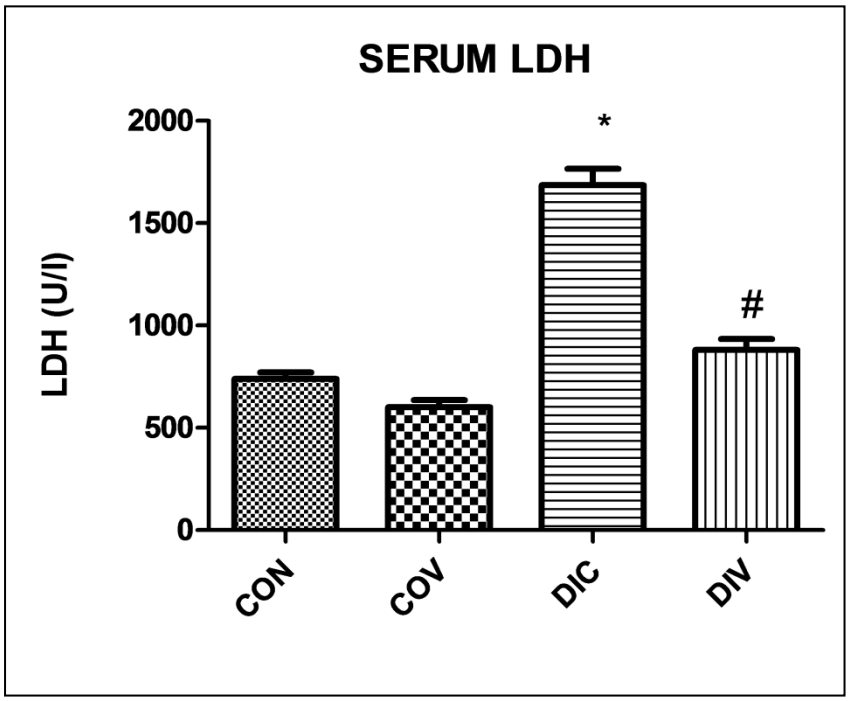
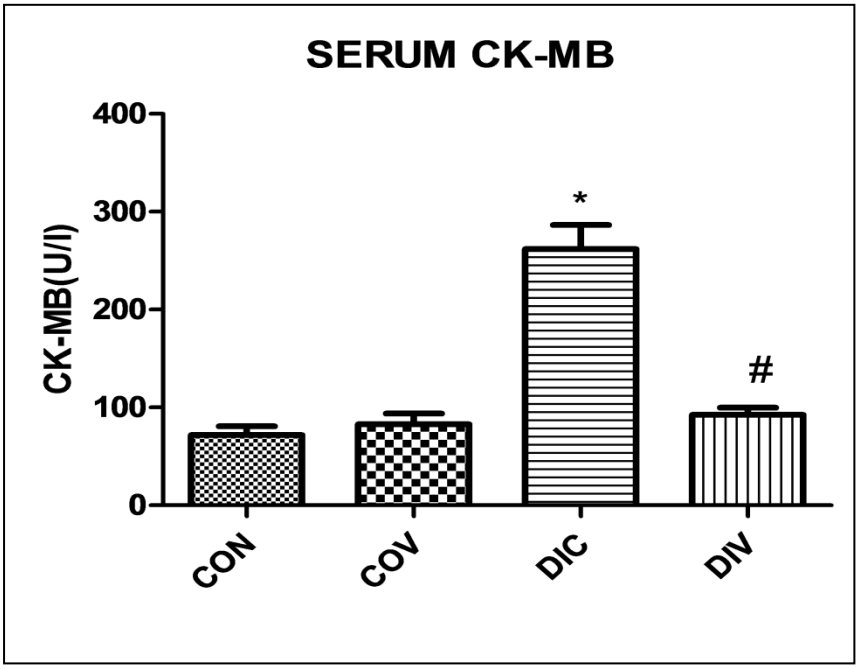


Figure 5.3b: Effect of MgV on serum CK-MB level



* significantly different from control group ($p<0.05$)
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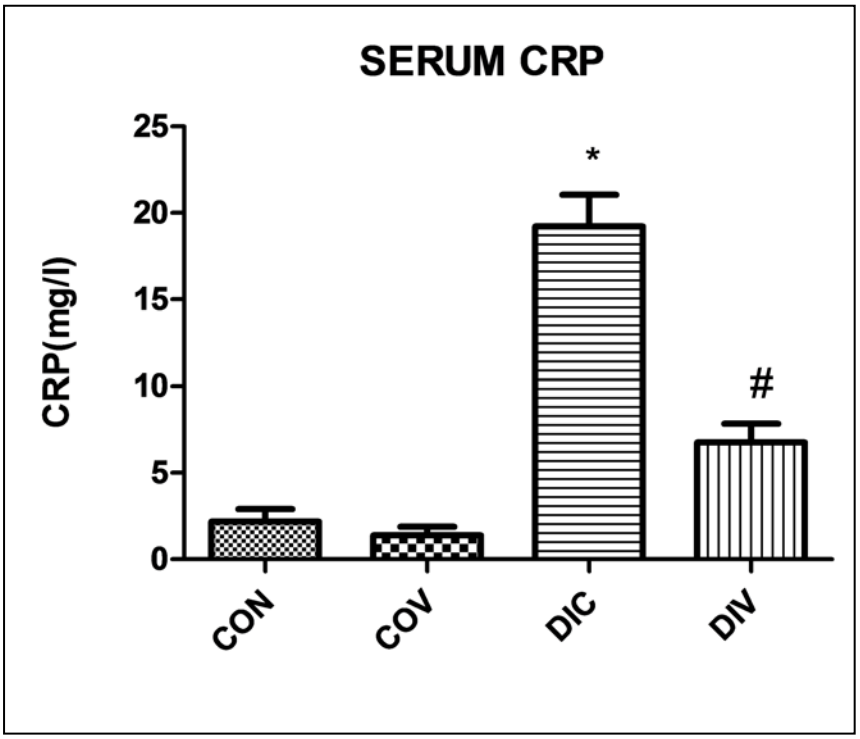
Each bar represents Mean ± SEM of 6 animals
CON – Normal Control
COV – Control treated with MgV (210 mg/kg/day, p.o)
DIC – Disease Control DIV – Disease Control treated with MgV (210 mg/kg/day,
DIV – Disease Control treated with MgV (210 mg/kg/day,

Figure 5.3c: Effect of MgV on serum C Reactive Protein level



* significantly different from control group ($p<0.05$)
\# significantly different from diabetic control group ($p<0.05$)

Values are expressed as Mean ± SEM of 6 animals
CON – Normal Control
COV – Control treated with MgV (210 mg/kg/day, p.o)
DIC – Disease Control
DIV – Disease Control treated with MgV (210 mg/kg/day, p.o)

5.4 HYPERTROPHIC PARAMETERS

Effect of MgV on Cardiac hypertrophic index, Left ventricular hypertrophic index,

Diabetic rats exhibited reduced wet heart weight as compared to nondiabetic rats. However, the ratio of heart weight to femur length which is a measure of cardiac hypertrophic index was significantly (p<0.05) higher diabetics as compared to those of control rats. Further, left ventricular hypertrophy index, left ventricle/right ventricle ratio, left ventricular wall thickness and cardiomyocyte diameter was also significantly (p<0.05) high in diabetic control animals as compared to control animals. Treatment MgV significantly (p<0.05) decreased the elevated cardiac hypertrophic index, left ventricular hypertrophic index, left ventricle/right ventricle ratio, left ventricular wall thickness and cardiomyocyte diameter of diabetic rats. Chronic treatment with MgV did not alter cardiac hypertrophic index, left ventricular hypertrophic index, left ventricle/right ventricle ratio, left ventricular wall thickness and cardiomyocyte diameter in control rats.(Table 5.4A, Fig. 5.4a, 5.4b, 5.4c, 5.4d, 5,4e).

TABLE 5.4A: Effect of MgV on Hypertrophic parameter

PARAMETERS	CON	COV	DIC	DIV
Cardiac hypertrophy index (mg/mm)	20.47 ±0.47	24.94 ±1.05	29.38 ±1.29*	21.77 ±0.52#
Left ventricular hypertrophy index (mg/mg)	0.65 ±0.007	0.62 ±0.008	0.72 ±0.007*	0.68 ±0.007#

* significantly different from control group ($p<0.05$)
\# significantly different from diabetic control group ($p<0.05$)
.
Values are expressed as Mean ± SEM of 6 animals
CON – Normal Control
COV – Control treated with MgV (210 mg/kg/day, p.o)
DIC – Disease Control
DIV – Disease Control treated with MgV (210 mg/kg/day, p.o)

Figure 5.4a: Effect of MgV on Cardiac hypertrophic index

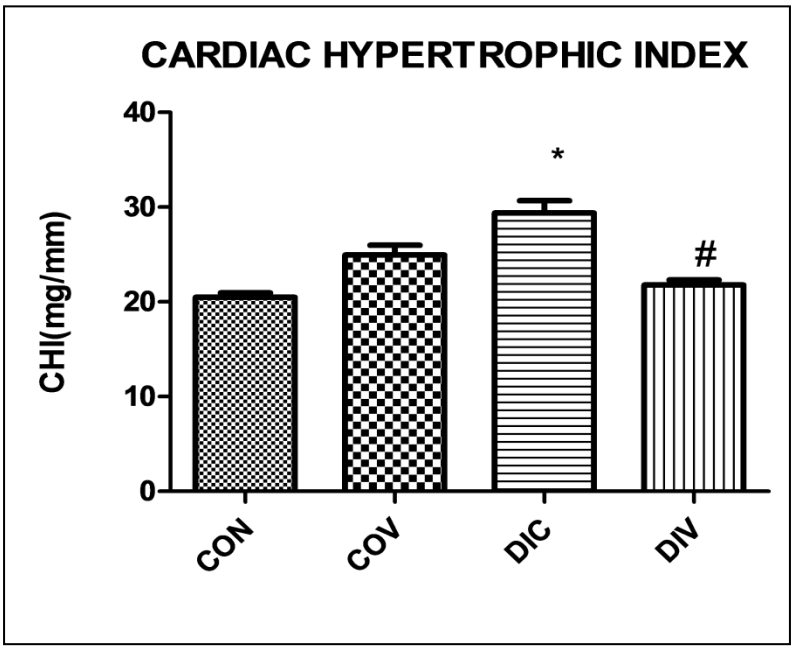
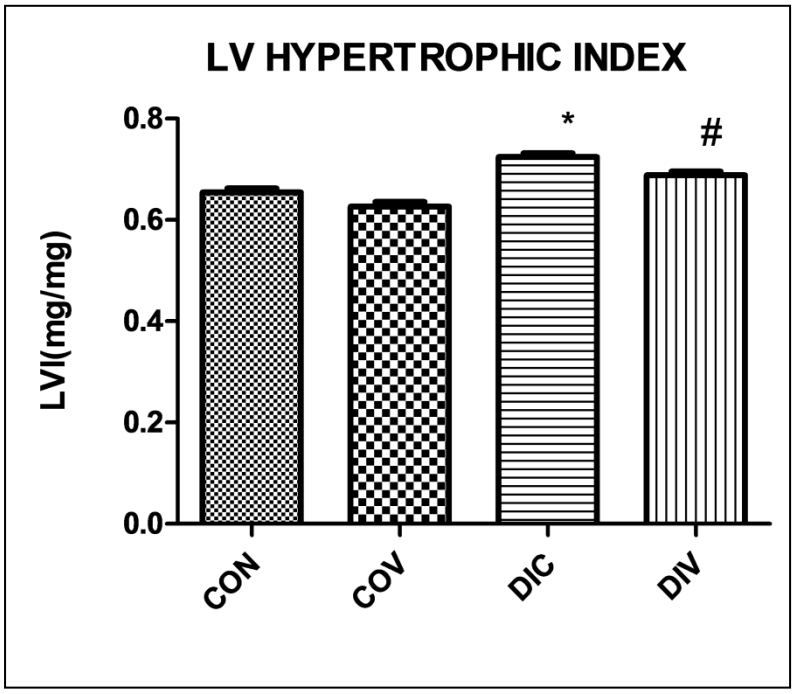


Figure 5.4b: Effect of MgV LV hypertrophy index



* significantly different from control group ($p<0.05$)
\# significantly different from diabetic control group ($p<0.05$)

Each bar represents Mean ± SEM of 6 animals
CON – Normal Control
COV – Control treated with MgV (210 mg/kg/day, p.o)
DIC – Disease Control
DIV – Disease Control treated with MgV (210 mg/kg/day, p.o)

5.5 HEMODYNAMIC PARAMETERS

Effect of MgV on Blood pressure, Heart rate, Rate of pressure development and decay.

At the end of 8 weeks of treatment, streptozotocin(STZ) treated rat exhibited significant($p < 0.05$) increase in blood pressure and significant($p < 0.05$) decrease in heart rate, rate of pressure development and decay as compared to control rats. Treatment with MgV (210 mg/kg/day, p.o) produce significant($p < 0.05$) reduction in blood pressure but did not produce significant($p < 0.05$) increase in heart rate .

Chronic treatment with the drug produced significant($p < 0.05$) increase in rate of pressure development but did not exhibited any alteration in rate of pressure decay as compared to diabetic control rats. Further, treatment with MgV did not produce any significant change in blood pressure, heart rate, rate of pressure development and rate of pressure decay.(Table 5.5A, Fig. 5.5a, 5.5b, 5.5c, 5.5d)

TABLE 5.5A: Effect of MgV on Blood pressure, Heart rate, Rate of pressure development and decay.

PARAMETERS	CON	COV	DIC	DIV
BLOOD PRESSURE (mmHg)	118.8 ±1.30	119.2 ±1.06	150.3 ±4.63*	129.0 ±6.65#
HEART RATE (beats per minute)	311.0 ±9.64	294.7 ±4.60	250.7 ±10.48*	271.6 ±7.35#
RATE OF PRESSURE DEVELOPMENT (mmHg/sec)	1064 ±59.29	1206 ±27.71	692.6 ±44.58*	1089 ±69.09#
RATE OF PRESSURE DECAY (mmHg/sec)	790.3 ±11.88	762.7 ±19.51	462.0 ±17.30*	644.2 ±18.27#

* significantly different from control group ($p<0.05$)
\# significantly different from diabetic control group ($p<0.05$)

Values are expressed as Mean ± SEM of 6 animals
CON – Normal Control
COV – Control treated with MgV (210 mg/kg/day, p.o)
DIC – Disease Control
DIV – Disease Control treated with MgV (210 mg/kg/day, p.o)

Figure 5.5a: Effect of MgV on Blood pressure

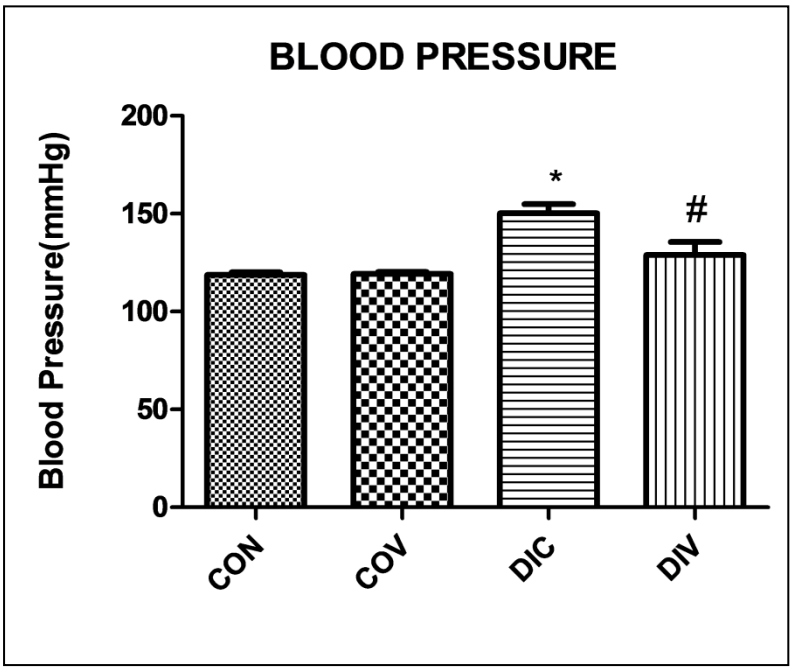
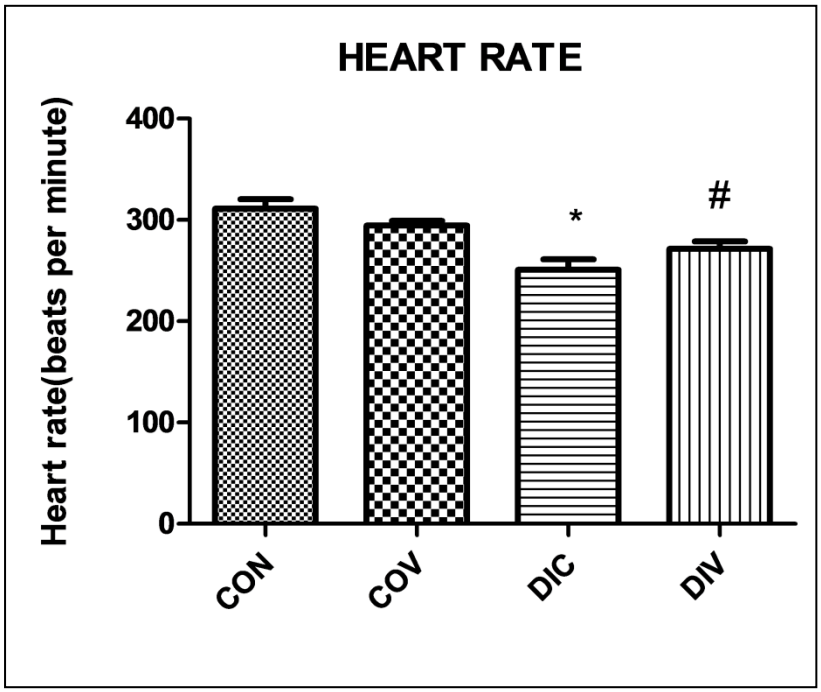


Figure 5.5b: Effect of MgV on Heart Rate



* significantly different from control group ($p<0.05$)
\# significantly different from diabetic control group ($p<0.05$)

Each bar represents Mean ± SEM of 6 animals
CON – Normal Control
COV – Control treated with MgV (210 mg/kg/day, p.o)
DIC – Disease Control
DIV – Disease Control treated with MgV (210 mg/kg/day, p.o)

Figure 5.5c: Effect of MgV on Rate of Pressure development

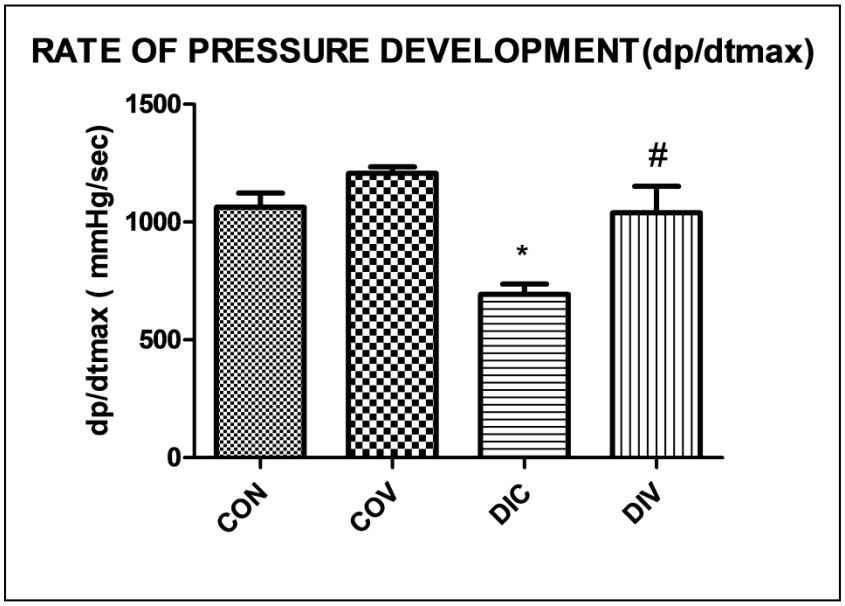
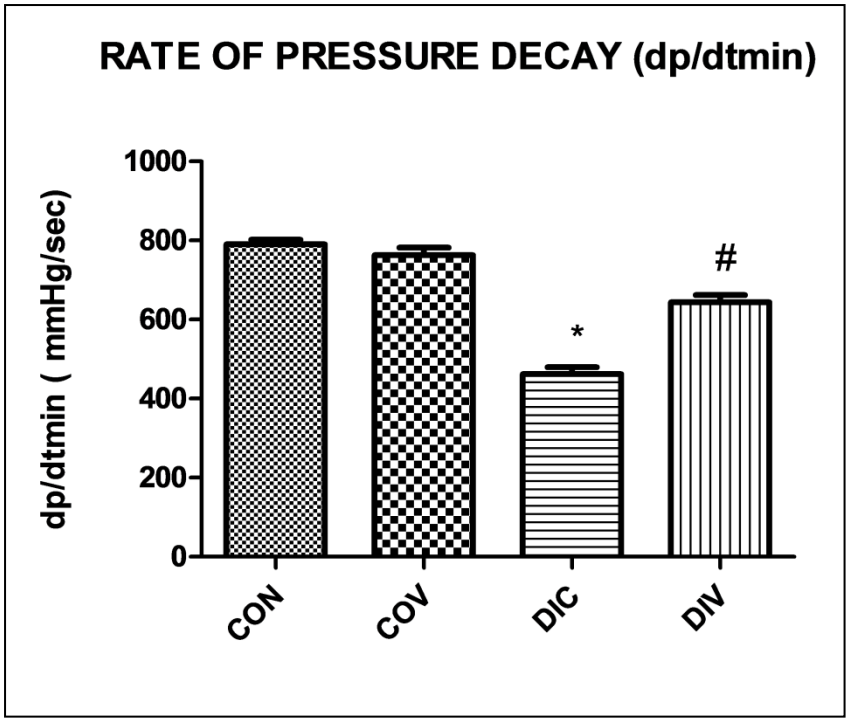


Figure 5.5d: Effect of MgV on Rate of Pressure decay



* significantly different from control group ($p<0.05$)
\# significantly different from diabetic control group ($p<0.05$)
.
Each bar represents Mean ± SEM of 6 animals
CON – Normal Control
COV – Control treated with MgV (210 mg/kg/day, p.o)
DIC – Disease Control
DIV – Disease Control treated with MgV (210 mg/kg/day, p.o)

5.7 LV OXIDATIVE STRESS PARAMETERS

Effect of MgV on left ventricular(LV) protein, LV malondialdehyde(MDA), LV nitrite, LV Superoxide Dismutase (SOD) and LV reduced glutathione (GSH) levels

STZ treated diabetic rat exhibited significantly($p < 0.05$) increased levels of LV protein, LV MDA and LV nitrite and significantly($p < 0.05$) decreased levels of LV SOD and LV GSH levels as compared to control rats. Treatment with MgV (210 mg/kg/day, p.o) significantly ($p < 0.05$) changed the elevated level of LV protein, LV MDA and LV nitrite and significantly($p < 0.05$) produced increased levels of LV SOD and LV GSH levels as compared to diabetic control rats. Further, treatment with MgV did not produce any significant change in LV protein, LV MDA, LV nitrite, LV SOD and LV GSH in control rats.(Table 5.7A, Fig. 5.7a, 5.7b, 5.7c, 5.7d, 5.7e)

TABLE 5.7A: Effect of MgV on LV SOD, Glutathione, Catalase, Protein, MDA levels

PARAMETERS	CON	COV	DIC	DIV
TOTAL PROTEIN LEVEL (µg/mg tissue)	435.6 ±9.03	431.6 ±9.23	748.6 ±23.66*	566.1 ±35.87#
MDA LEVEL (nmoles/mg protein)	0.3241 ±0.009	0.3561 ± 0.02	0.9609 ±0.009*	0.4478 ±0.02#
NITRITE LEVEL (µmol/mg protein)	13.34 ±0.98	12.23 ±1.10	5.37 ±0.55*	10.10 ±1.11#
SOD LEVEL (U/mg protein)	3.10 ±0.10	2.72 ±0.35	0.91 ±0.05*	2.21 ±0.03#
GSH LEVEL (µg/mg protein)	2.33 ±0.09	2.41 ±0.16	0.97 ±0.08*	1.90 ±0.07#

* significantly different from control group ($p<0.05$)
\# significantly different from diabetic control group ($p<0.05$)
.
Values are expressed as Mean ± SEM of 6 animals
CON – Normal Control
COV – Control treated with MgV (210 mg/kg/day, p.o)
DIC – Disease Control
DIV – Disease Control treated with MgV (210 mg/kg/day, p.o)

Figure 5.7a: Effect of MgV on LV Protein levels

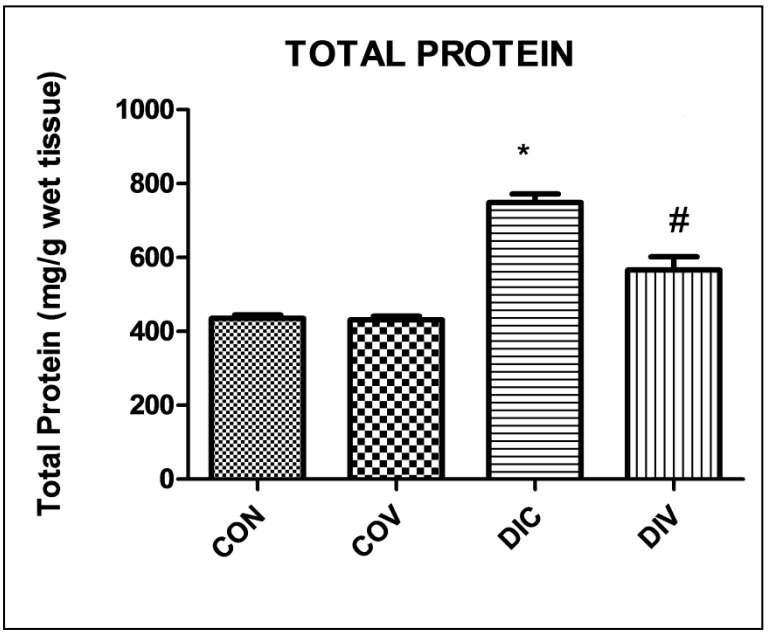
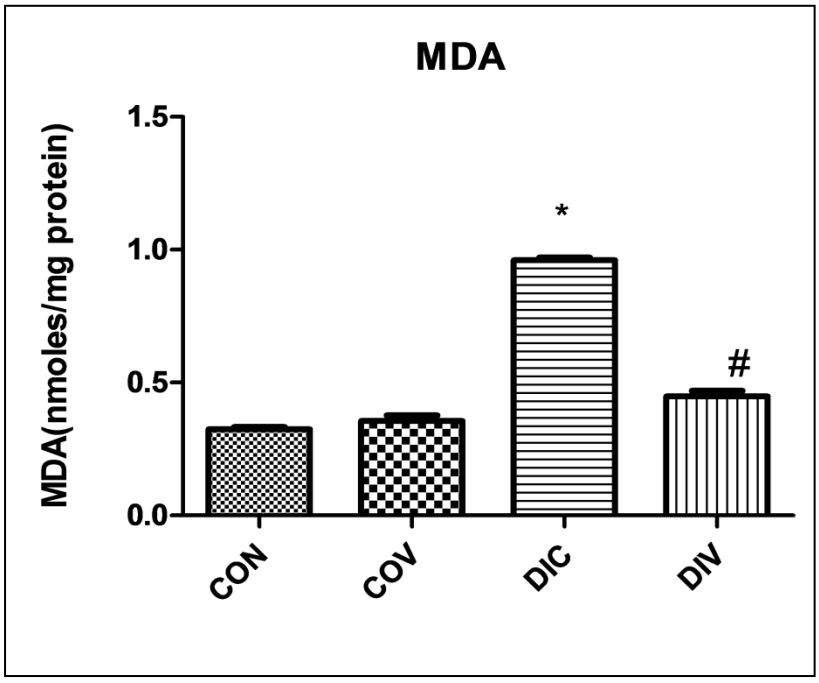


Figure 5.7b: Effect of MgV on LV MDA Levels



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.
Each bar represents Mean ± SEM of 6 animals
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COV – Control treated with MgV (210 mg/kg/day, p.o)
DIC – Disease Control
DIV – Disease Control treated with MgV (210 mg/kg/day, p.o

Figure 5.7c: Effect of MgV on LV Nitrite level

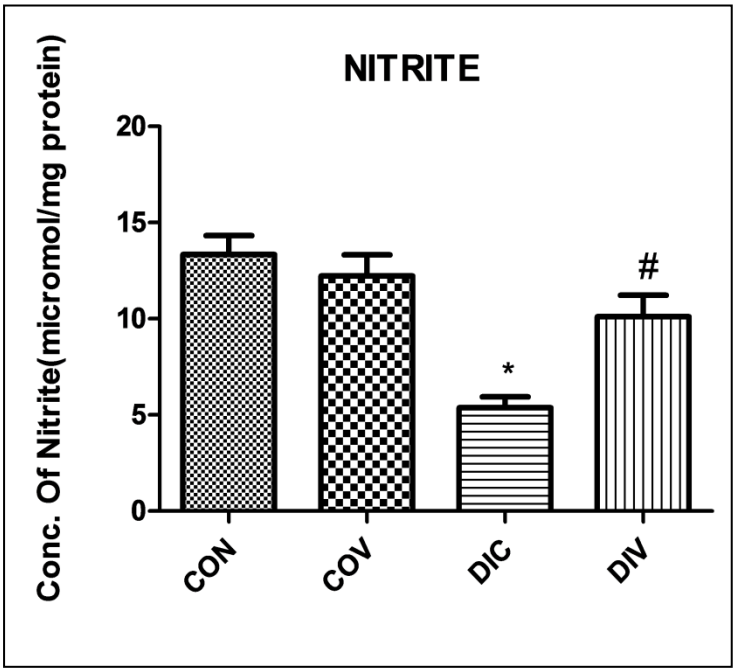
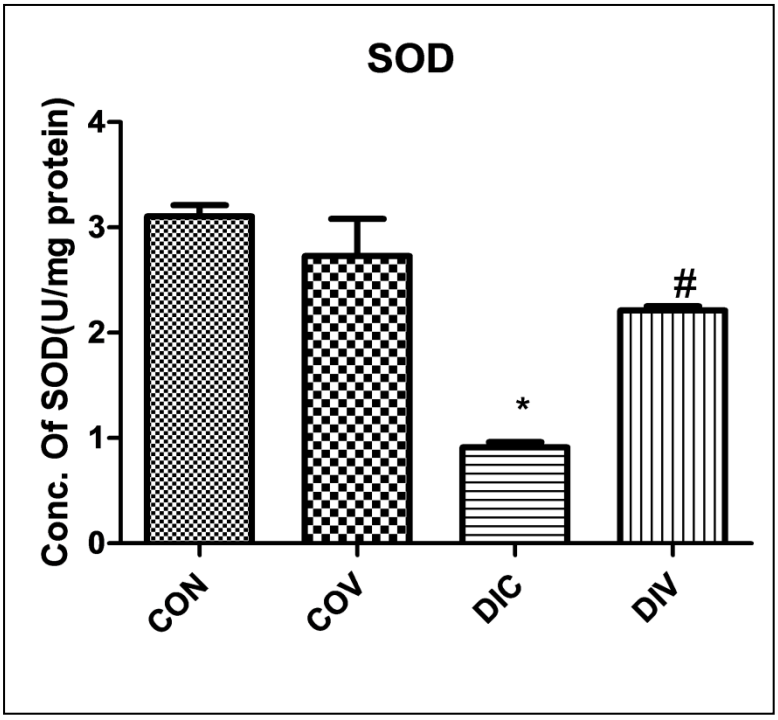


Figure 5.7d: Effect of MgV on LV SOD activity



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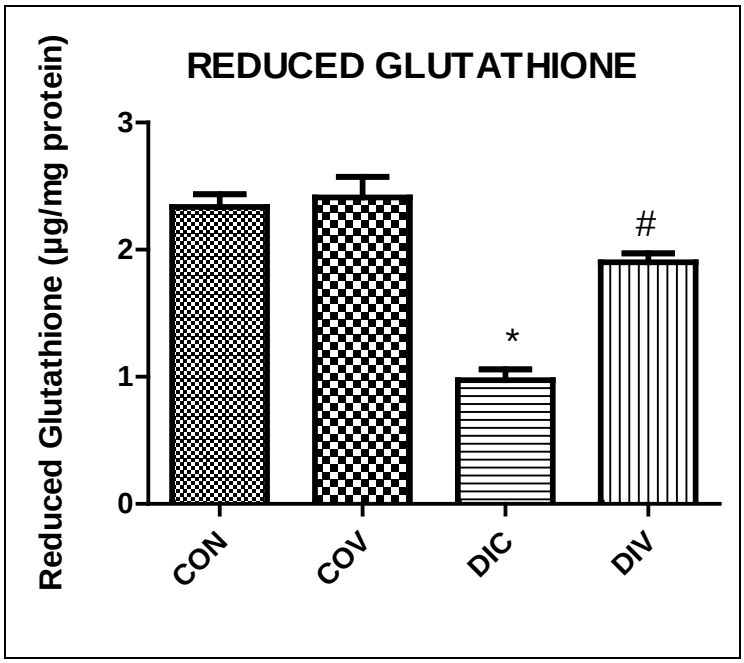
DIC – Disease Control
DIV – Disease Control treated with MgV (210 mg/kg/day, p.o

Figure 5.7e: Effect of MgV on LV Glutathione levels



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Each value represents Mean ± SEM of 6 animals
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COV –f Control treated with MgV (210 mg/kg/day, p.o)
DIC – Disease Control
DIV – Disease Control treated with MgV (210 mg/kg/day, p.o)

5.8 HISTOPATHOLOGICAL STUDY OF LEFT VENTRICLE

Microscopically, control rats showed no pathological changes in the left ventricular(LV) myocardial cells.(Fig. 5.8a)

The control treated LV myocardial cells (Fig. 5.8b) did not show any histological alterations.

Histopathological examinations of the sections of LV cells from diabetic rats (Fig. 5.8c) showed marked microscopic chaages like reduction in extracellular space, increase in cardiomyocyte diameter, intense fibrosis and cell disarray.

The MgV treated sections showed less reduction in extracellular space (Fig. 5.8d) and less increased in cardiomyocyte diameter. The extent of fibrosis and cardiac fibre disarray was also reduced.

Fig. 5.8a: Control

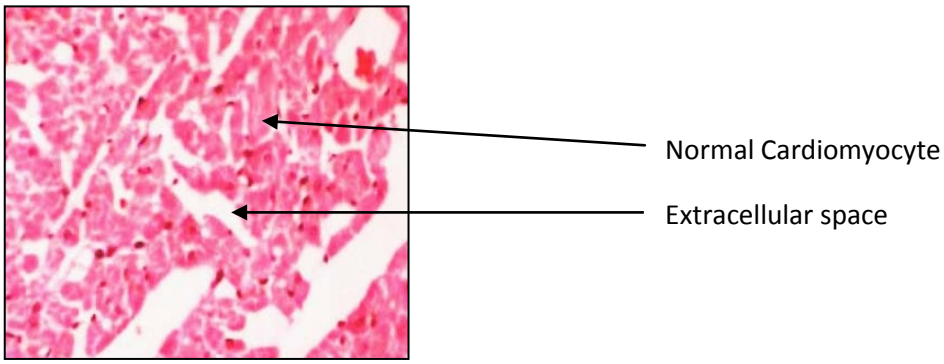


Fig. 5.8b: Control Treated

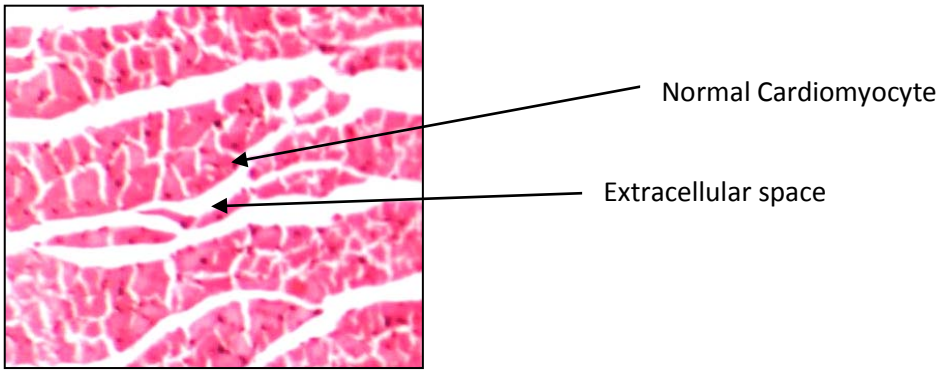


Fig. 5.8c: Diabetic Control

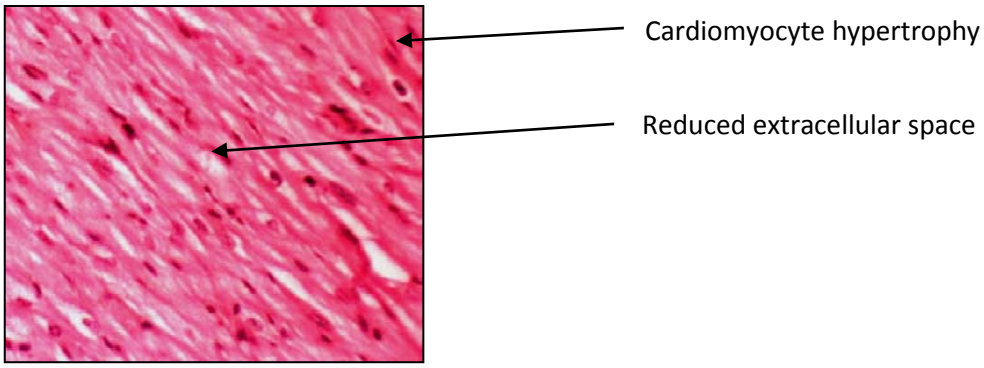
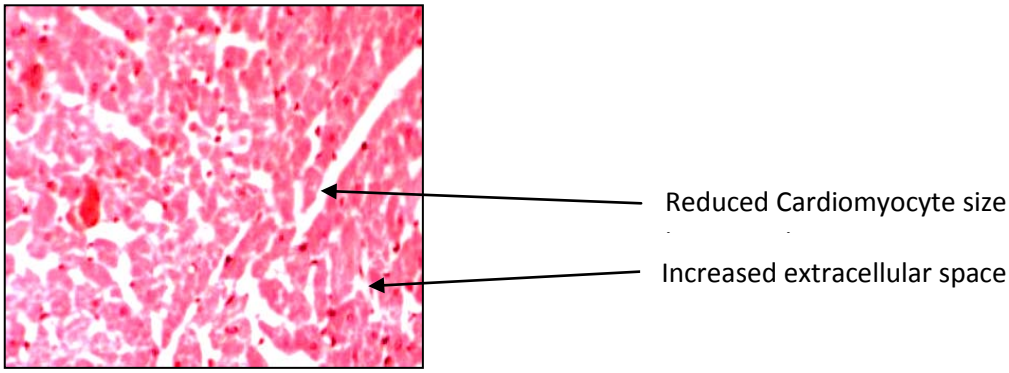


Fig. 5.8d: Diabetic Treated



B. RESULTS OF DIABETIC NEPHROPATHY

5.9 SERUM MARKERS FOR NEPHROPATHY

Effect of Magnesium valproate on Serum Alkaline phosphatase(ALP), Albumin, Uric acid, Blood urea nitrogen(BUN), Creatinine and BUN:Creatinine ratio

STZ treated rat exhibited significantly($p < 0.05$) increased levels of ALP, uric acid, BUN, creatinine and significantly($p < 0.05$) decreased levels of albumin as compared to control rats. Treatment with MgV (210 mg/kg/day, p.o) produced significant($p < 0.05$) reduction in serum ALP, uric acid, BUN and creatinine and significant($p < 0.05$) increase in albumin levels. Further, treatment with MgV did not produce any change in serum ALP, uric acid, BUN, creatinine, and albumin of control rats.(Table 5.9A, Fig. 5.9a, 5.9b, 5.9c, 5.9d, 5.9e,)

TABLE 5.9A: Effect of MgV on Serum Alkaline phosphatase(ALP), Albumin, Uric acid, Blood urea nitrogen(BUN),Creatinine

PARAMETERS	CON	COV	DIC	DIV
ALKALINE PHOSPHATASE (U/l)	29.92 ±6.37	38.08 ±5.66	155.9 ±40.96*	68.00 ±15.90#
ALBUMIN (g/dl)	3.30 ±0.30	3.22 ±0.27	1.49 ±0.11*	2.94 ±0.16#
URIC ACID (mg/dl)	0.53 ±0.10	0.53 ±0.06	1.86 ±0.25*	1.013 ±0.18#
BLOOD UREA NITROGEN (mg/dl)	48.75 ±6.95	25.18 ±6.95	151.2 ±6.95*	94.11 ±6.95#
CREATININE (mg/dl)	0.08 ±0.01	0.05 ±0.02	0.29 ±0.03*	0.10 ±0.04#

* indicates significantly different from normal group ($p<0.05$)
# indicates significantly different from Disease control group ($p<0.05$)

Values are expressed as Mean ± SEM.
CON – Normal Control
COV – Control treated with MgV (210 mg/kg/day, PO).
DIC – Disease Control
DIV – Disease Control treated with MgV (210 mg/kg/day, PO)

Figure 5.9a: Effect of MgV on Serum Alkaline phosphatise

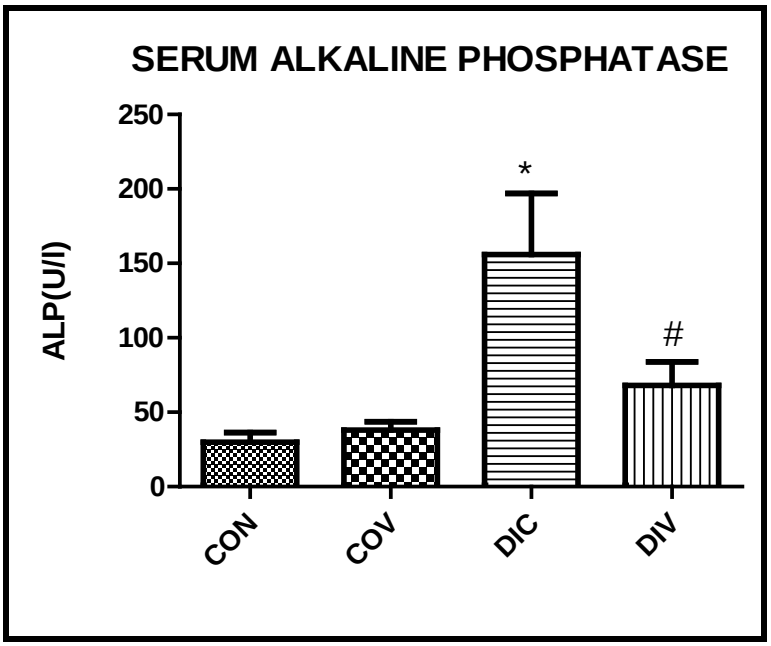
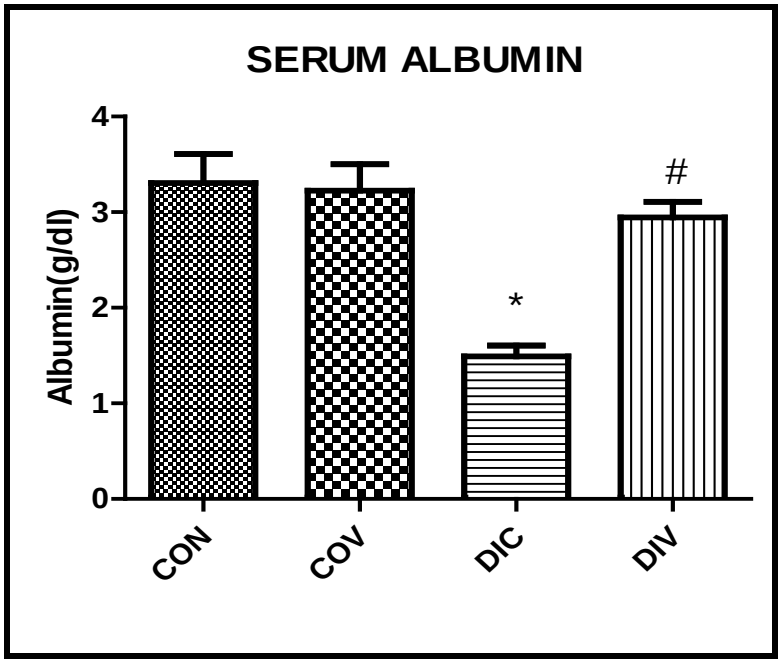


Figure 5.9b: Effect of MgV on Serum Albumin



* significantly different from control group ($p<0.05$)
\# significantly different from diabetic control group ($p<0.05$)

Each bar represents Mean ± SEM of 6 animals
CON – Normal Control
COV – Control treated with MgV (210 mg/kg/day, p.o)
DIC – Disease Control
DIV – Disease Control treated with MgV (210 mg/kg/day, p.o)

Figure 5.9c: Effect of MgV on Serum Uric acid

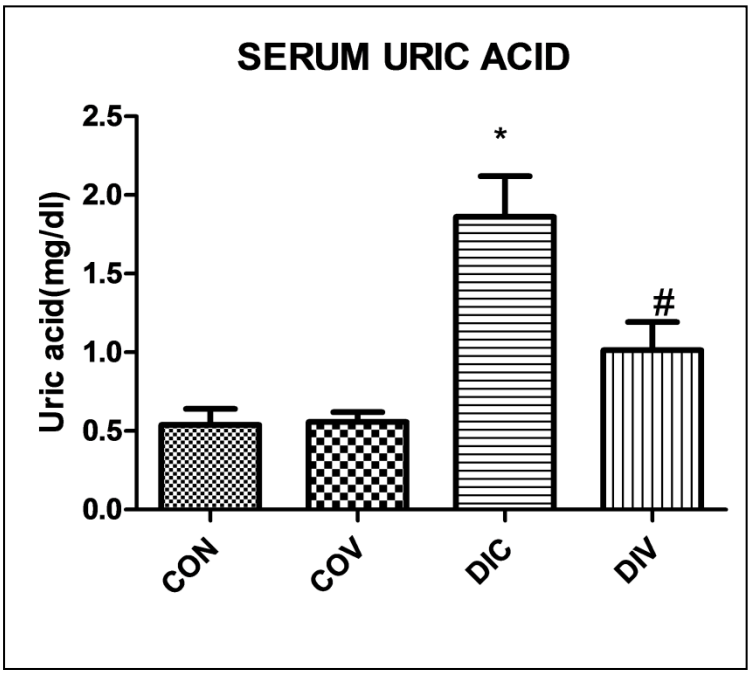
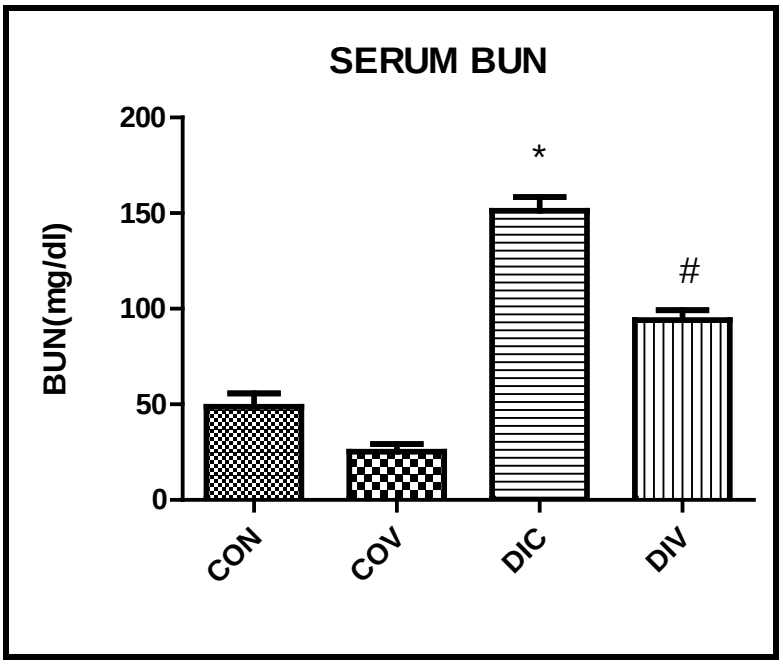


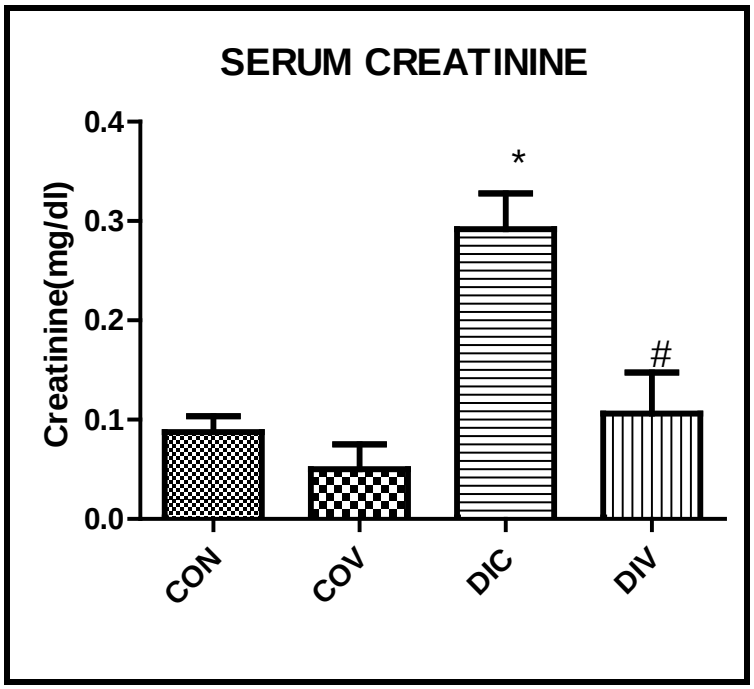
Figure 5.9d: Effect of MgV on Serum Blood Urea Nitrogen



* significantly different from control group ($p<0.05$)
\# significantly different from diabetic control group ($p<0.05$)

Each bar represesnts Mean ± SEM of 6 animals
CON – Normal Control
COV – Control treated with MgV (210 mg/kg/day, p.o)
DIC – Disease Control
DIV – Disease Control treated with MgV (210 mg/kg/day, p.o)

Figure 5.9e: Effect of MgV on Serum Creatinine



* significantly different from control group ($p<0.05$)
\# significantly different from diabetic control group ($p<0.05$)

Each bar represesnts Mean ± SEM of 6 animals
CON – Normal Control
COV – Control treated with MgV (210 mg/kg/day, p.o)
DIC – Disease Control
DIV – Disease Control treated with MgV (210 mg/kg/day, p.o)

5.10 URINARY MARKERS FOR NEPHROPATHY

Effect of MgV on Urine and Electrolyte excretion

Streptozotocin treated rats showed significant($p < 0.05$) increase in urine excretion and decreased levels of sodium and chloride in urine as compared to control rats. Treatment with MgV(210 mg/kg/day, p.o) significantly($p < 0.05$) reduced urine excretion and increase the levels of both sodium and choride in urine as compared to diabetic control rats.. Further, MgV did not produce any change in urine excretion and urine levels of sodium and chloride in control rats. (Table 5.10A, Fig. 5.10a, 5.10b, 5.10c, 5.10d)

TABLE 5.10A: Effect of MgV on Urine and Electrolyte excretion

PARAMETERS	CON	COV	DIC	DIV
URINE EXCRETION (ml/day)	5.88 ±0.47	5.53 ±0.32	76.82 ±3.65*	47.65 ±6.57#
SODIUM (mmol/l)	121.5 ±3.100	124.1 ±4.430	71.25 ±6.020*	112.0 ±6.66#
CHLORIDE (mmol/l)	185.4 ±8.36	173.4 ±12.11	105.9 ±1.85*	168.6 ±8.36#

* significantly different from control group ($p<0.05$)
\# significantly different from diabetic control group ($p<0.05$)

Values are expressed as Mean ± SEM of 6 animals
CON – Normal Control
COV – Control treated with MgV (210 mg/kg/day, p.o)
DIC – Disease Control
DIV – Disease Control treated with MgV (210 mg/kg/day, p.o)

Figure 5.10a: Effect of MgV on Urine excretion

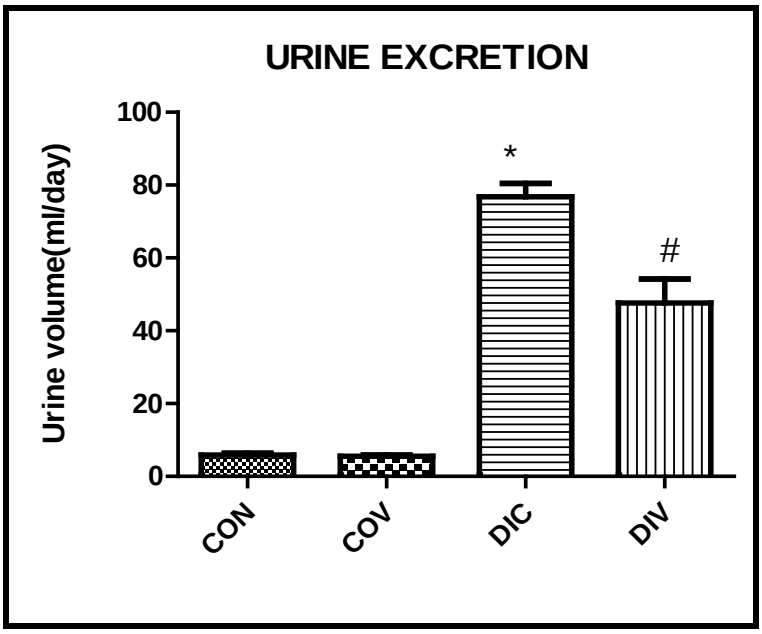
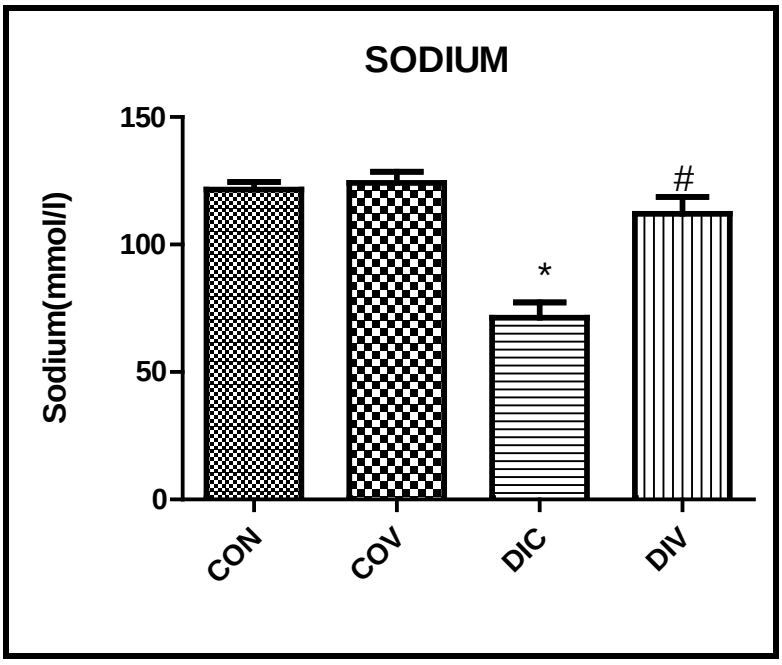


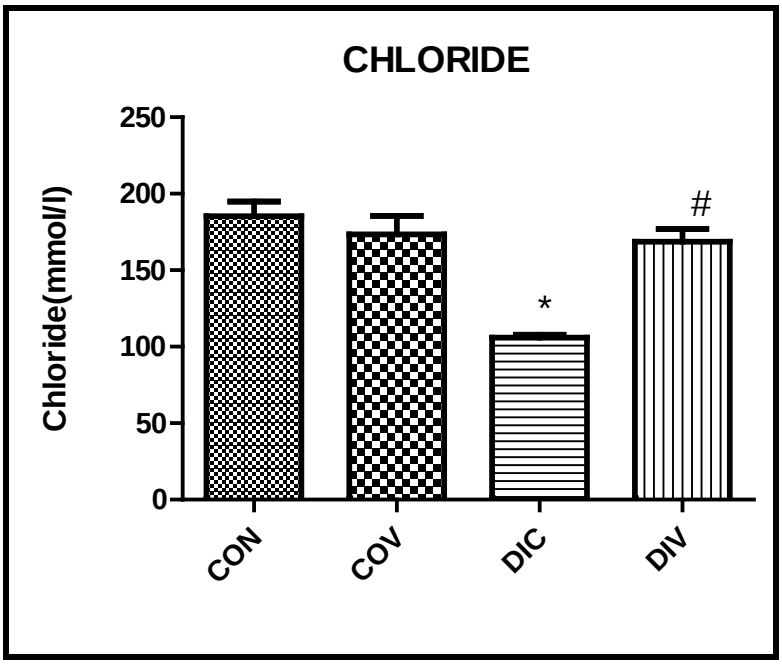
Figure 5.10b: Effect of MgV on Sodium excretion



* significantly different from control group ($p<0.05$)
\# significantly different from diabetic control group ($p<0.05$)

Each bar represents Mean ± SEM of 6 animals
CON – Normal Control
COV – Control treated with MgV (210 mg/kg/day, p.o)
DIC – Disease Control
DIV – Disease Control treated with MgV (210 mg/kg/day, p.o)

Figure 5.10c: Effect of MgV on Chloride excretion



* significantly different from control group ($p<0.05$)
\# significantly different from diabetic control group ($p<0.05$)
.
Each bar represents Mean ± SEM of 6 animals
CON – Normal Control
COV – Control treated with MgV (210 mg/kg/day, p.o)
DIC – Disease Control
DIV – Disease Control treated with MgV (210 mg/kg/day, p.o)

Effect of MgV on excretion of Uric acid, Total protein, Albumin Creatinine and Urinary Albumin:Creatinine(UAC) ratio and Creatinine clearance

After 8 weeks of treatment, streptozotocin(STZ) treated rat exhibited significantly decreased levels of uric acid and creatinine in urine and also significant($p < 0.05$) decrease in creatinine clearance as compared to control rats. Treatment with MgV(210 mg/kg/day, p.o) showed significant($p < 0.05$) increase in urine levels of uric acid and creatinine and also significant($p < 0.05$) increase in creatinine clearance as compared to diabetic control rats. Further, treatment with MgV did not produce any significant change in excretion of uric acid, creatinine, and creatinine clearance in control rats.(Table 5.10B, Fig. 5.10d, 5.10e, 5.10f)

STZ treated rats exhibited increased levels of total protein and albumin in urine compared to control rats. Treatment with MgV(210 mg/kg/day, p.o) showed significant($p < 0.05$) reduction in urine levels of total protein and albumin and UAC ratio as compared to diabetic control rats.But didi not show any changes in UAC ratio. Further, treatment with MgV did not produce any significant change in urinary total protein, albumin and UAC ratio. (Table 5.10B, Fig. 5.10g, 5.10h, 5.10i)

TABLE 5.10B: Effect of MgV on Urinary Creatinine, Uric acid Creatinine Clearance, Total protein, Albumin and UAC ratio

PARAMETERS	CON	COV	DIC	DIV
CREATININE (mg/dl)	46.00 ±3.00	41.45 ±5.05	12.00 ±3.75*	35.95 ±1.10#
URIC ACID (mg/dl)	14.66 ±3.12	10.10 ±0.96	2.38 ±0.552*	7.26 ±1.15#
CREATININE CLEARANCE (ml/24hrs)	128.1 ±5.09	117.4 ±1.61	66.91 ±4.15	95.97 ±4.51
TOTAL PROTEIN (g/dl)	-	-	3.23 ±0.16*	1.22 ±0.20#
ALBUMIN (g/dl)	-	-	0.45 ±0.03*	0.23 ±0.01#
UAC RATIO (g/mg)	0.001 ±0.0004	0.002 ±0.001	0.018 ±0.008	0.007 ±0.001

* indicates significantly different from normal group ($p<0.05$)

# indicates significantly different from Disease control group ($p<0.05$)

Values are expressed as Mean ± SEM of 6 animals
CON – Normal Control
COV – Control treated with MgV (210 mg/kg/day, PO).
DIC – Disease Control
DIV – Disease Control treated with MgV (210 mg/kg/day, PO)

Figure 5.10d: Effect of MgV on Creatinine excretion

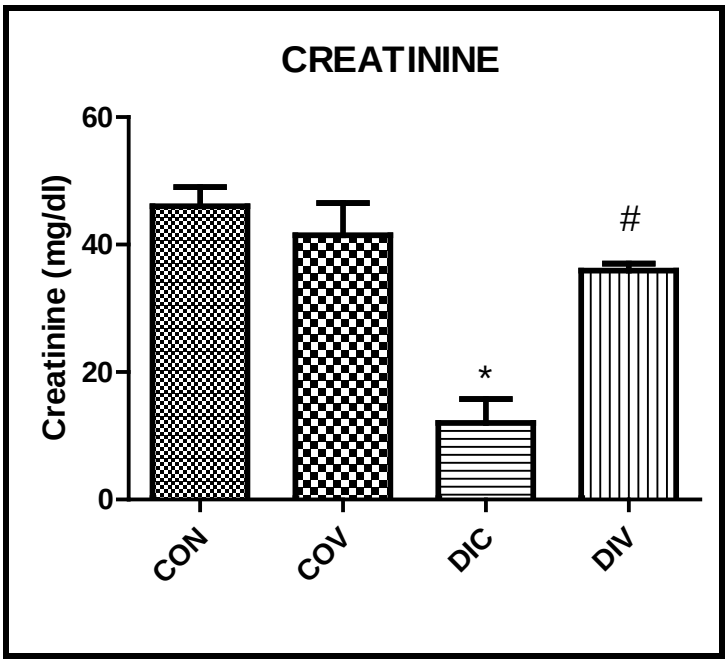


Figure 5.10e: Effect of MgV on Uric acid excretion

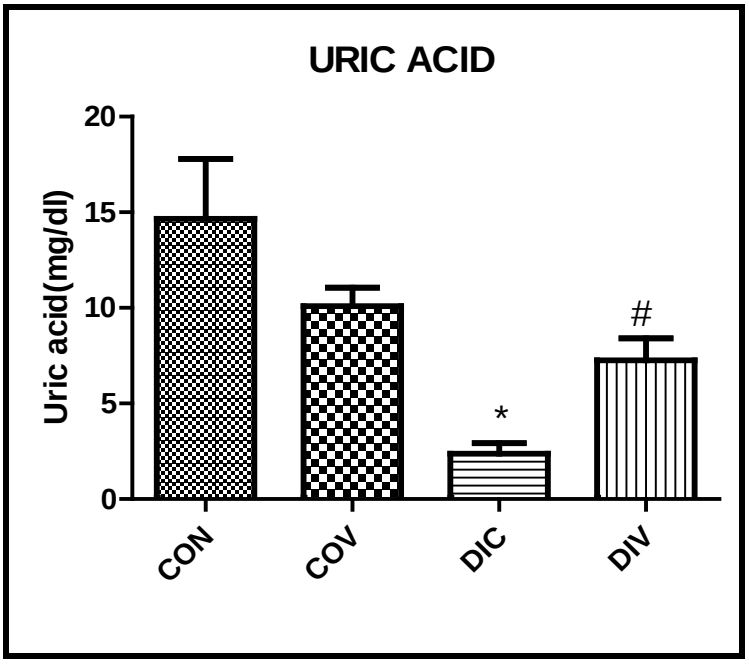


Figure 5.10f: Effect of MgV on Creatinine clearance

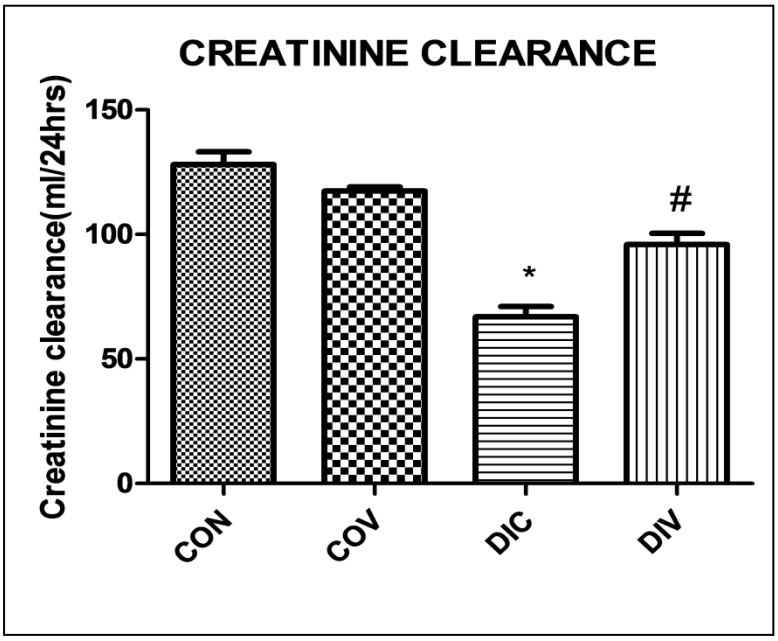
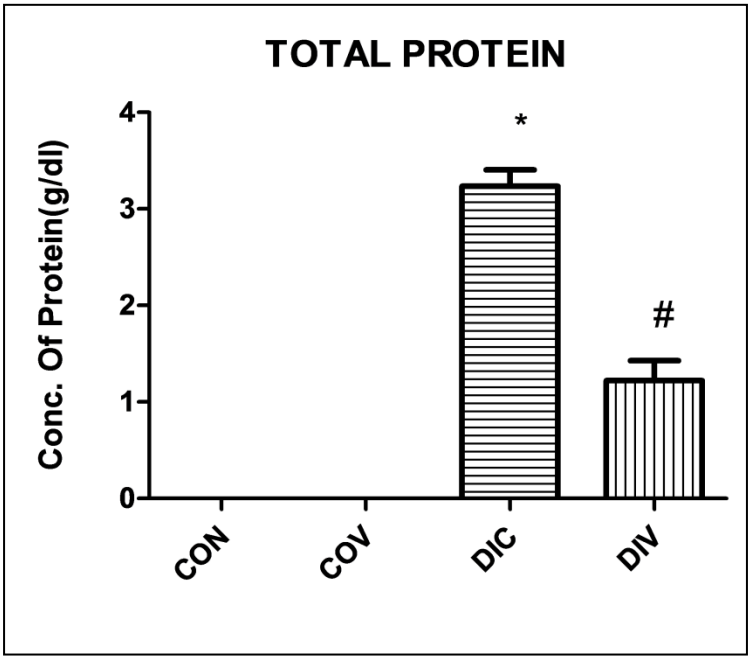


Figure 5.10g: Effect of MgV on Total protein excretion



* significantly different from control group ($p<0.05$)
\# significantly different from diabetic control group ($p<0.05$)

Each bar represesnts Mean ± SEM of 6 animals
CON – Normal Control
COV – Control treated with MgV (210 mg/kg/day, p.o)
DIC – Disease Control
DIV – Disease Control treated with MgV (210 mg/kg/day, p.o)

Figure 5.10h: Effect of MgV on Albumin excretion

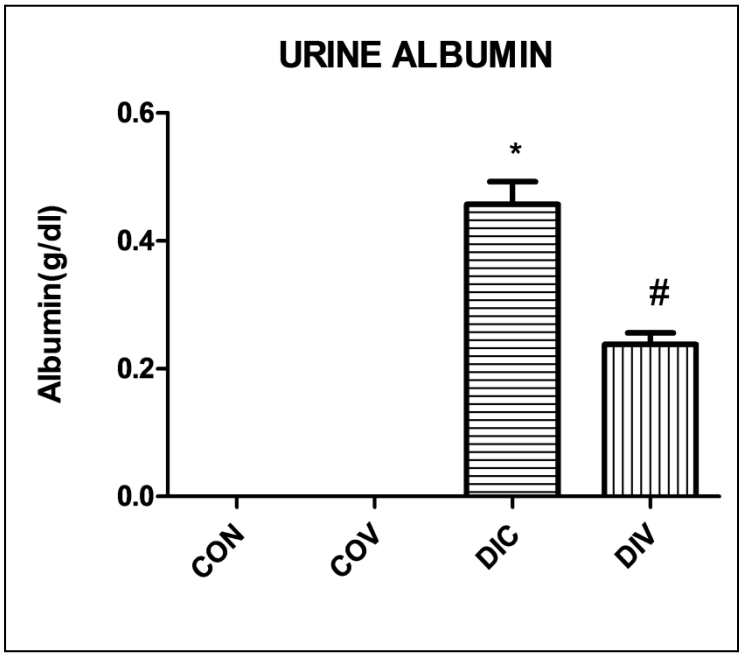
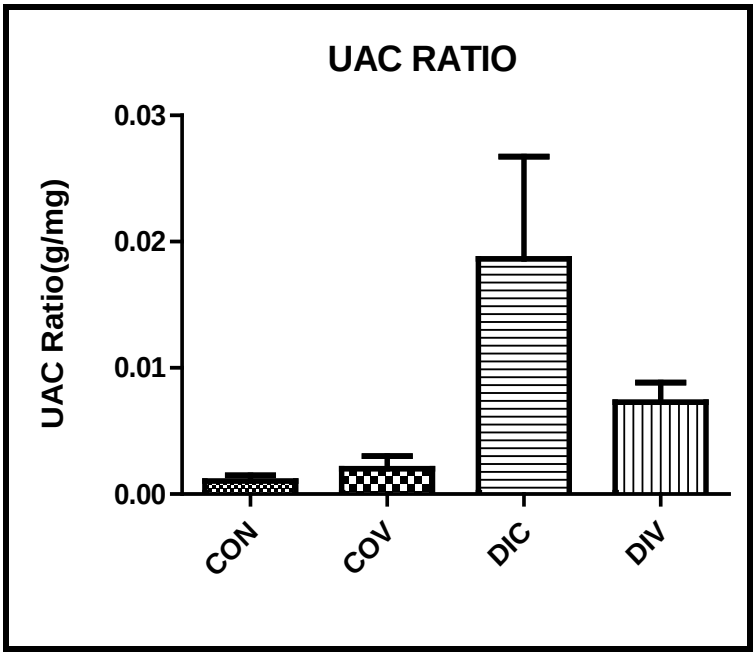


Figure 5.10i: Effect of MgV on UAC Ratio



* significantly different from control group ($p<0.05$)
\# significantly different from diabetic control group ($p<0.05$)

Each bar represesnts Mean ± SEM of 6 animals
CON – Normal Control
COV – Control treated with MgV (210 mg/kg/day, p.o)
DIC – Disease Control
DIV – Disease Control treated with MgV (210 mg/kg/day, p.o)

5.12 RENAL OXIDATIVE STRESS PARAMETERS

Effect of MgV on renal protein, renal malondialdehyde(MDA), renal nitrite, renal superoxide Dismutase (SOD) and renal reduced glutathione (GSH) levels

STZ treated rat exhibited significantly($p < 0.05$) increased levels of renal protein, renal MDA and renal nitrite and significantly($p < 0.05$) decreased levels of renal SOD and renal GSH as compared to control rats. Treatment with MgV (210 mg/kg/day, p.o) produced significant($p < 0.05$) reduction in renal protein, renal MDA and renal nitrite and significantly($p < 0.05$) increased levels of renal SOD and renal GSH as compared to diabetic control rats. Further, treatment with MgV did alter any significant change in renal protein, renal MDA, renal nitrite, renal SOD and renal GSH in control rats.(Table 5.12A, Fig. 5.12a,5.12b, 5.12c, 5.12d, 5.12e)

TABLE 5.12A: Effect of MgV on Renal SOD, Glutathione, Catalase, Protein, MDA levels

PARAMETERS	CON	COV	DIC	DIV
RENAL PROTEIN LEVEL (mcg/mg tissue)	415.5 ±23.55	438.7 ±5.28	721.2 ±9.68*	566.1 ±35.87#
RENAL MDA LEVEL (nmoles/mg protein)	0.22 ±0.03	0.27 ±0.02	0.57 ±0.050*	0.28 ±0.019#
RENAL NITRITE LEVEL (µmoles/mg protein)	9.627 ±0.27	12.56 ±2.14	20.53 ±0.73*	11.92 ±0.05#
RENAL SOD LEVEL (U/mg protein)	1.89 ±0.31	1.50 ±0.34	0.51 ±0.05*	0.91 ±0.53#
RENAL GLUTATHION LEVEL (mcg/mg protein)	1.62 ±0.005	1.68 ± 0.10	0.64 ±0.01*	0.99 ±0.04#

* indicates significantly different from normal group ($p<0.05$)
# indicates significantly different from Disease control group ($p<0.05$)

Values are expressed as Mean ± SEM of 6 animals
CON – Normal Control
COV – Control treated with MgV (210 mg/kg/day, PO).
DIC – Disease Control
DIV- Disease treated with MgV (210 mg/kg/day, PO)

Figure 5.12a: Effect of MgV on Renal protein level

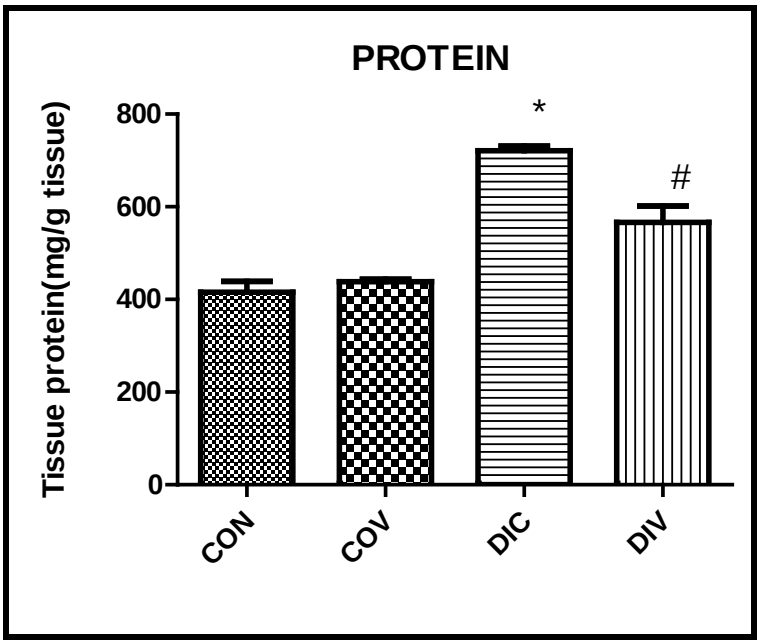
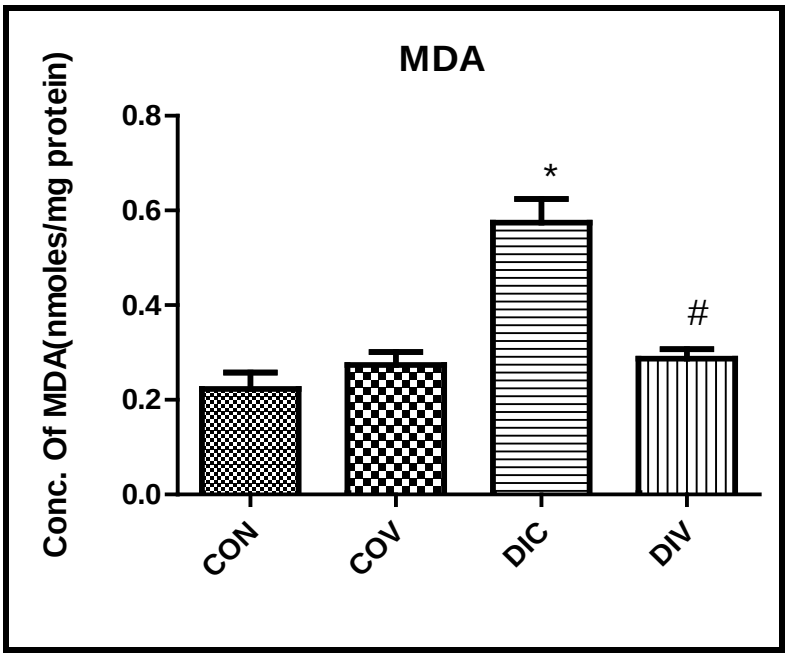


Figure 5.12b: Effect of MgV on Renal MDA level



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\# significantly different from diabetic control group ($p<0.05$)

Each value represent Mean ± SEM of 6 animals
CON – Normal Control
COV – Control treated with MgV (210 mg/kg/day, p.o)
DIC – Disease Control
DIV – Disease Control treated with MgV (210 mg/kg/day, p.o)

Figure 5.12c: Effect of MgV on Renal Nitrite level

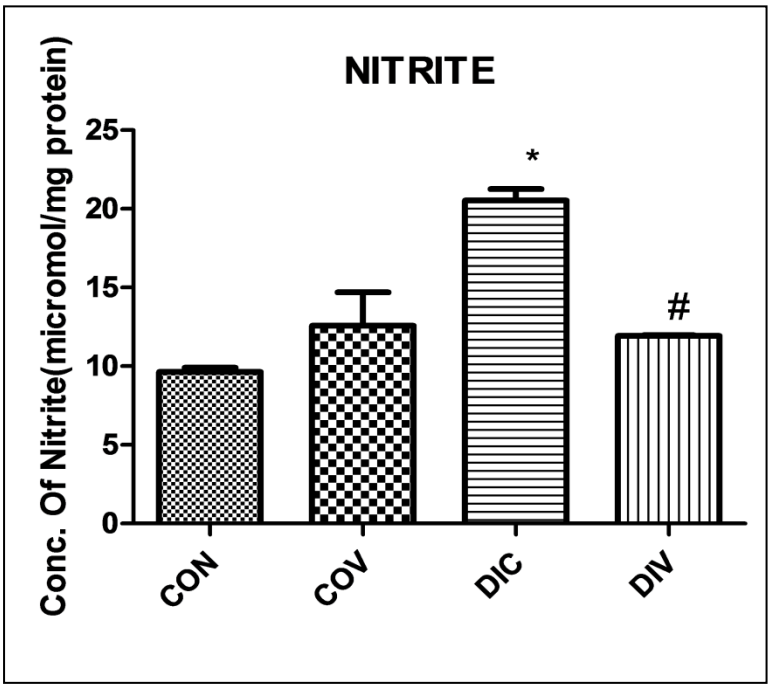
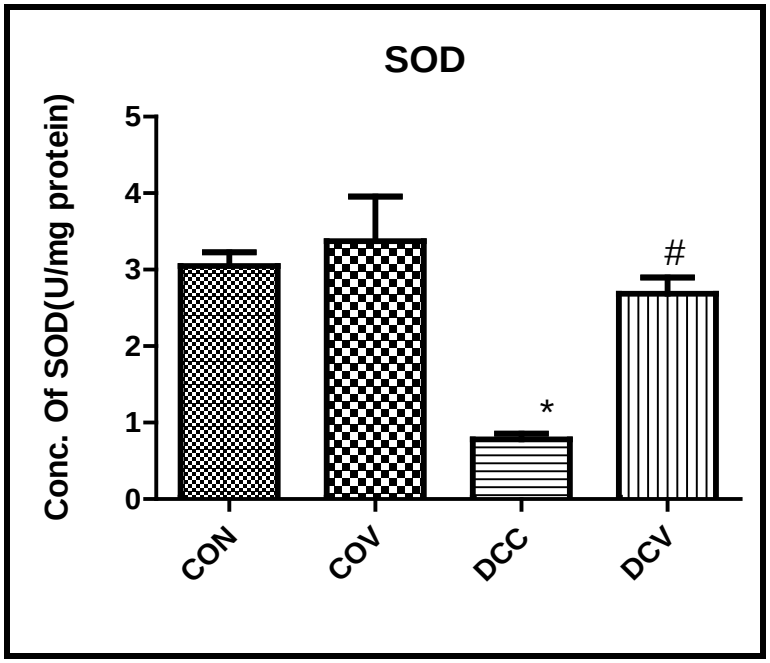


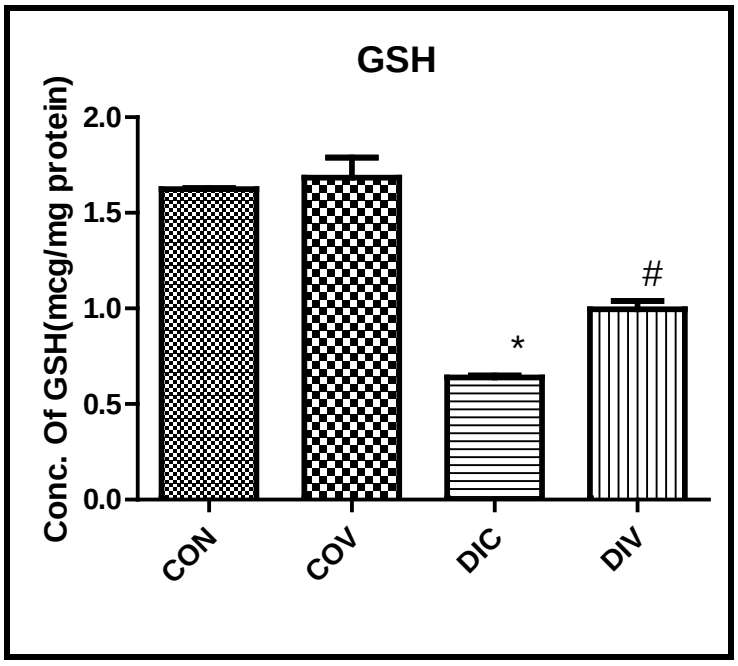
Figure 5.12d: Effect of MgV on Renal SOD level



* significantly different from control group ($p<0.05$)
\# significantly different from diabetic control group ($p<0.05$)

Each value represent Mean ± SEM of 6 animals
CON – Normal Control
COV – Control treated with MgV (210 mg/kg/day, p.o)
DIC – Disease Control
DIV – Disease Control treated with MgV (210 mg/kg/day, p.o)

Figure 5.12e: Effect of MgV on Renal Glutathione level



* significantly different from control group ($p<0.05$)
\# significantly different from diabetic control group ($p<0.05$)

Each value represent Mean ± SEM of 6 animals
CON – Normal Control
COV – Control treated with MgV (210 mg/kg/day, p.o)
DIC – Disease Control
DIV – Disease Control treated with MgV (210 mg/kg/day, p.o

5.13 HISTOPATHOLOGICAL STUDY OF KIDNEY TISSUE

The histological findings as depicted by the transverse sections of renal tissue revealed that there was increase in glomerular lesions and mesangial expansion(Fig. 5.13c) as compared to control rats(Fig. 5.13a).Treatment with MgV(210mg/kg/day, p.o) for 8 weeks showed decrease in glomerular lesions and mesangial expansion (Fig. 5.13d) as compared to diabetic control rats. Further, treatment with MgV did not produce any change in glomerular lesions, mesangial expansion in control rats(Fig. 5.13b).

Fig. 5.13a: Control

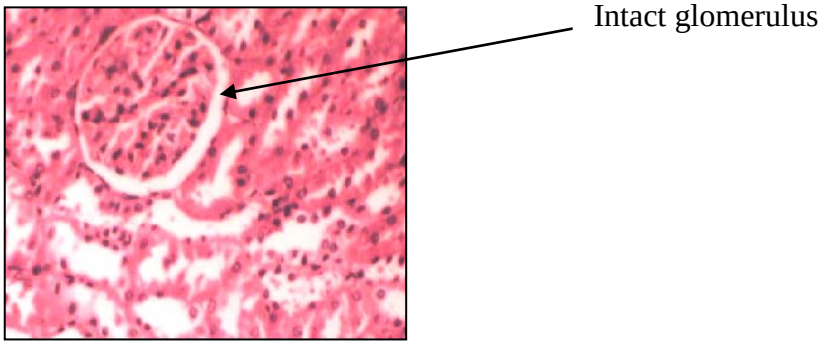


Fig. 5.13b: Control Treated

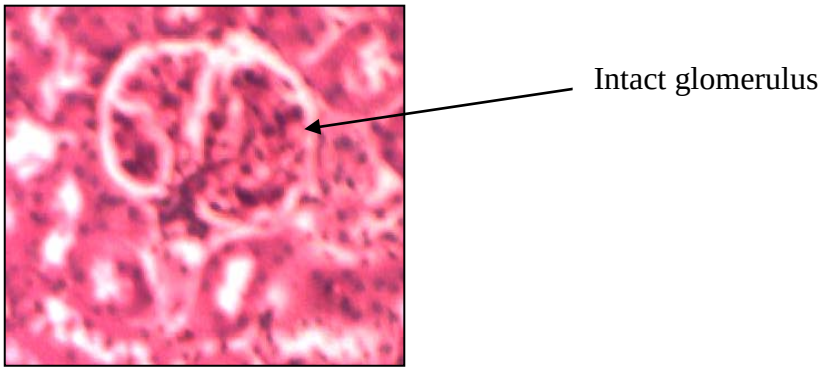


Fig. 5.13c: Diabetic Control

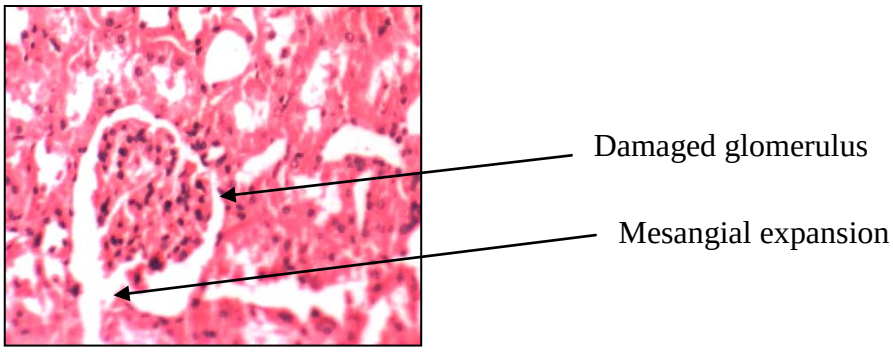
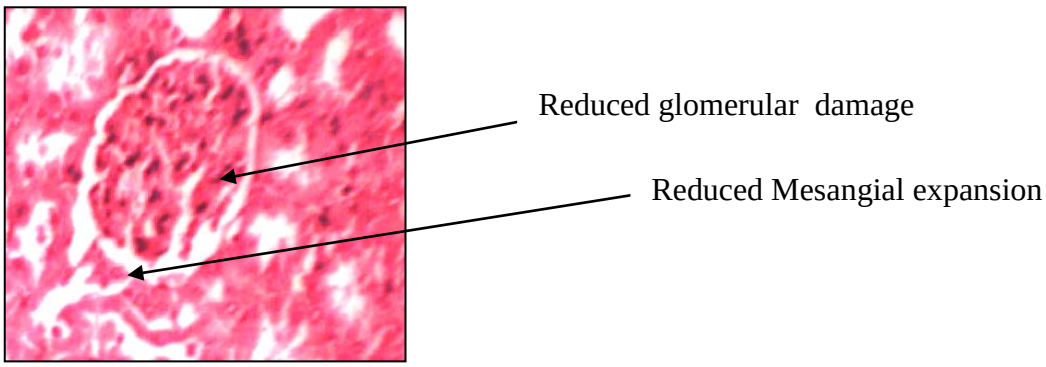


Fig. 5.13d: Diabetic Treated



6. DISCUSSION

6.1 DIABETIC CARDIOMYOPATHY

Type 2 or non-insulin-dependent diabetes mellitus is a multifactorial disease which is characterized by insulin resistance associated not only with hyperinsulinaemia and hyperglycaemia but also with atherosclerosis, hypertension and abnormal lipid profile (vats et.al,2005) The pathophysiology of STZ-induced diabetes includes a cardiomyopathy that is frequently associated with contractile dysfunction and heart rhythm disturbances(Arora et.al.,2009) including an increased stiffness of the left ventricular wall associated with accumulation of connective tissue and insoluble collagen.(An and Rodrigues, 2006).

Treatment with streptozotocin (STZ) produces a diabetic state that is characterized by loss of weight, polydipsia, polyuria, glucosuria, polyphagia, hypoinsulinaemia and hyperglycaemia (Hakim et al. 1997). In present study we found that STZ diabetic rats presented a progressive worsening of general parameters which includes body weight, glycemia, and food water consumption indicating the increasing severity of the diabetic state .Direct measurements of food and water consumption ,diuresis, and glycosuria confirmed the classic signs of polyphagia ,polydipsia, polyuria, and glycosuria .However treatment with MgV significantly increase the body weight and decrease the food and water intake indicating that chronic treatment with MgV may reverse the cardinal signs of type 2 diabetes.

The diabetogenic agent streptozotocin (STZ) is a glucose analog (2-deoxy-2-[3-methyl-e-nitrosourido]-D-glucopyranose) It possesses a diabetogenic effect due to its specific damaging action to pancreatic beta cell (Takada et.al, 2006). Neonatal rats treated with STZ at birth exhibit insulindeficient acute diabetes 3–5 days after birth characterized by a rapid spontaneous remission .This remission is accompanied by an incomplete spontaneous b-cell regeneration and a progressive increase in the pancreatic insulin stores starting from 3–5 days after birth. during this favored regeneration, replication from preexisting differentiated b-cells that survived the toxic effect of STZ represents an important but not exclusive mechanism of regeneration, and obviously many of the b-cells found 3–5 days after birth may originate from undifferentiated duct cells. Adult rats

that were treated with STZ at birth exhibited type 2 diabetes with specific failure of insulin release in response to glucose.(Tourrel et.al.,2001)

In present study STZ treated diabetic rats exhibited increased serum glucose level.Chronic treatment with MgV produced significant reduction in serum glucose level in diabetic rats. Our results are in consistent with the idea that in response to the fasting hormone glucagon, Class IIa HDACs are rapidly dephosphorylated and translocated to the nucleus where they associate with the promoters of gluconeogenic enzymes such as Glucose 6-phospahtase . In turn, HDAC4/5 recruit HDAC3, which results in the acute transcriptional induction of these genes via deacetylation and activation of Foxo family transcription factors. Loss of Class IIa HDACs in murine liver results in inhibition of FOXO target genes and lowers blood glucose, resulting in increased glycogen storage. Thus suggesting that suppression of Class IIa HDACs in mouse models of Type 2 Diabetes ameliorates hyperglycemia(Mihaylova et.al.,2011) .In one of the study it has been reported that in addition to improving insulin resistance and preventing β-cell inflammatory damage, HDAC inhibitors (HDACi) also promote β-cell development, proliferation, differentiation and function (Christensen et.al., 2011).Thus this may suggest the possible mechanism by which MgV may improve the hyperglycaemic condition.

Type 2 diabetes and insulin resistance are associated with a complex change of metabolism of plasma lipoproteins that conveys a substantial part of increased cardiovascular (CV) risk of the diabetics. While quantity of lipoprotein particles (lipid concentrations) remain close to normal, their quality (composition) changes markedly. Lipoprotein metabolism in type 2 diabetes differs from normal at three levels. First, the production of very low-density lipoproteins (VLDLs) in the liver is elevated because of increased free fatty influx from enlarged intra abdominal adipose tissue mass. This leads to changes HDL metabolism as well as production of small dense LDL particles. Second, catabolism of lipoproteins decreases due to lower activity of lipolytic enzymes and receptor changes. Third, insulin resistance is associated with profound changes of lipoprotein assembly and trafficking in the enterocyte and, thus, postprandial lipoprotein metabolism (Vrablik, 2010). In Diabetes, cholesterol synthesis (Nestel et al. 1973) and turnover (Nestel et al. 1969) are markedly enhanced and the cholesterol absorption

efficiency is decreased .In present study STZ treated diabetic rats exhibited elevated levels of total cholesterol, LDL, VLDL, triglycerides and significantly reduced levels of HDL .Chronic treatment with MgV exhibited significant reduction in levels of total cholesterol, LDL, VLDL, triglycerides and significantly produced increase in levels of HDL. It has been found that Trichostatin,an HDAC inhibitor, treatment significantly affects the cholesterol biosynthesis in F9 EC cells, most specifically at those steps involved in the synthesis of low density lipoprotein. Several genes encoding essential enzymes in the LDL synthesis pathway are down regulated by this treatment (Chittur et.al., 2008**).** Acetyl-CoA is used for fatty acid synthesis and produced by β-oxidation of fatty acids, amino acid catabolism, and/or glycolytic flux and it has been reported that HDACI-induced changes in Acetyl CoA dynamic may contribute to changes in fatty acid metabolism (Wardell et.al.,2009**).** This may serve as the probable mechanism of MgV, in control of dyslipidaemia during type 2 diabetes**.**

The creatine kinase-MB fraction (CK-MB) is part of total CK and more specific for cardiac muscle that other striated muscle. It tends to increase within 3 to 4 hours of myocardial necrosis, then peak in a day and return to normal within 36 hours (Saenger and Jaffe, 2007) (Kumar and Cannon, 2009). The CK-MB is also useful for diagnosis of reinfarction or extent of an myocardial infarction because it begins to fall after day, so subsequent elevations are indicative of another event (Chattington et al, 1994). Several of the adenosinetriphosphatase enzymes that are responsible for cardiac muscle contraction rely on high-energy phosphates supplied by the creatine kinase (CK) system. These enzymes normally exist in cellular compartment and leak out into the plasma during myocardial injury due to disintegration of contractile elements and sarcoplasmic reticulum (Pasupathi ,2009) thus elevated levels of creatine kinase MB (CK-MB) have been regarded as biochemical markers of myocyte necrosis(Yilmaz et.al., 2006).In present study STZ treated diabetic rats exhibited increased level of CK-MB.Chronic treatment with MgV produced decreased serum level of CK-MB. In one of the study it has been reported that the increase in CK-MB levels after severe scald on heart tissue of rats was lowered in the valproic acid (VPA)treated group,which suggested heart protection(Hui-ying et.al.,2011). Thus, Similarly Increased serum LDH levels in diabetic rats indicate cardiac muscle damage which in turn signifies myocardial inury. (Amin and Naggy,2009) .Further diabetes mellitus has been shown to cause a decrease in the

maximal contractile pesrformance of the heart and this decrease in contractile performance may be explained in part by a decrease in the enzyme activity(Popovich et.al,1989**).** In one of the study it has been shown that IL-1a involves redistribution of lactate dehydrogenase (LDH) isoforms and particularly an increase in the expression and activity of LDH A, which is known to favor the conversion of pyruvate into lactate(Nehar et.al.,1998). In present sudy STZ treated diabetic rats produced increase in serum LDH level. Treatment with MgV produced significant reduction in the serum LDH level . VPA is reported to suppressTNF-α and IL production (Ichiyama et.al., 2000).This may serve as possible mechanism behind reduction in LDH level on chronic treatment with MgV in diabetic rats.

High-sensitivity C-Reactive Protein (hs-CRP) is an acute-phase response protein produced within the liver and atherosclerotic arteries. CRP is an independent risk marker for coronary events in individuals without overt hyperlipidemia, thus adding prognostic information to cardiovascular risk assessment (Ridker.,2003). Insulin resistance appears to be the major determinant of hs-CRP levels (Fiesta et.al.,2003) Elevated hs-CRP predicts the development of type 2 diabetes (Freeman et.al.,2000). Elevated markers of inflammation, in particular CRP, are associated with an increased risk of future cardiovascular events in healthy subjects, in patients with stable or unstable coronary artery disease and acute myocardial infarction (Buffon et al., 2002; Zairis et al., 2002). CRP has been reported to be elevated during acute myocardial infarction (Zebrack et al., 2002). C-reactive protein is a principle downstream mediator of acute phase response and is primarily derived via IL-6 dependent hepatic biosynthesis. In rodent model of glucose metabolism the *invivo* infusion of recombinant IL-6 has been shown to induce gluconeogenesis, subsequent hyperglycaemia and compensatory hyperinsulinaemia (Pradhan et.al.,2001). Recent results have indicated that HDAC inhibitors can reduce the cytokine and NO production that contribute to various inflammatory diseases (Blanchard,2005).These agent inhibits the production of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6 and interferon-gamma. Inhibition of HDAC activity efficiently blocks the production of IL-6, and modulation of IL-6 mRNA stability which may serve as a potential mechanism for lowering CRP (Grabeic et.al,2011).Theses evidences are in support of our study in which STZ treated diabetic rats exihibited significant increase in the levels of CRP and the chronic treatment

with MgV produced significant reduction in the levels of CRP .In one of the study it has been reported that VPA significantly inhibited LPS-induced production of TNF-α and IL-6 by THP-1 cells in glioma cells (Ichiyama et.al.,2000). Thus this might suggest the possible mechanism behind reduction in level of CRP in MgV treated diabetic rats.

Cardiac hypertrophy is defined as an enlargement of the heart with the increase in volume of cardiomyocyte cell (Chang et.al 2011). Diabetes is an independent contributor to left ventricular hypertrophy (LVH) and myocardial stiffness. The Framingham study investigators used echocardiography and reported significant increase in left ventricular wall thickness in women with diabetes. In contrast, the Strong Heart Study,conducted in a population of American Indians, found that both men and women with diabetes had greater left ventricular mass and wall thickness. It was shown that increased left ventricular mass can be seen only in patients with diabetes, as compared to patients with impaired or normal fasting glucose concentrations (Rerkpattanapipat et.al, 2009), suggesting that alterations in the geometry of the heart in diabetic individuals are not an early defect but, rather, a consequence of changes associated with diabetes such as hyperglycemia or obesity (Boudina,2009). In the present study, cardiac hypertrophy index, LVH index . Treatment with MgV significantly decreased these hypertrophic parameter. It has been reported that Ang II is known to induce cardiac hypertrophy by activating receptor-mediated signaling cascades, including those involving mitogen-activated protein kinases, phosphatidylinositol 3-kinase, Akt, and janus-activating kinases. Recently, it has been shown that cardiac hypertrophy is also induced by transgenic overexpression of the homeodomain only protein, HOP, a transcriptional corepressor in the developing heart. A recent report implicated the class I HDAC member HDAC2 as a positive regulator of hypertrophy and showed that this prohypertrophic activity is acetylase-dependent and possibly involves release of repressors of Akt signaling by interaction with an atypical homeodomain protein, HOP (Vondriska and Wang, 2008). Recent studies, including those examined in the article by Kee et al,suggest an alternative mechanism for the participation of the class I HDAC2 in hypertrophy in which hypertrophic stressors like AngII induce association of HDAC2 with Hsp70, leading to repression of antihypertrophic genes(Vondriska and Wang, 2008). In one of the study it has been reported that cardiac hypertrophy induced by transgenic overexpression

of HOP or by chronic infusion of Ang II is prevented or significantly attenuated by concomitant treatment with trichostatin (TSA) and VLA (Kee et.al.,2006).Thus our reports are in consistence with other reports stating MgV attenuates cardiac hypertrophy. However, to best of our knowledge, ours is the first report in diabetic animals.

Type 2 diabetes is associated with structural and functional changes in large arteries that lead to increased stiffness, abnormal pulse wave travel and systolic hypertension. Structural changes result mainly from glycation of wall components. Diabetic cardiomyopathy refers to a disease process which affects the myocardium in diabetic patients causing a wide range of structural abnormalities eventually leading to LVH and diastolic and systolic dysfunction or a combination of these.(hayat et.al). In patients with type 2 diabetes the risk of diabetic complications was strongly associated with raised blood pressure and reduced heart rate (Adler et.al, 2000; Bhagyalakshmi et.al., 2007). Abnormal heart rate variability in diabetes represents an increased risk for ventricular arrhythmias, as well as total cardiovascular morbidity and mortality(Bhagyalakshmi et.al,2007).Maximal rate of pressure development to the instantaneous pressure are used as indices of systolic function. The left ventricular dysfunction has been associated with decrease in rate of pressure development and decay. In present study, we found changes in haemodynamics accompanying hypertension, bradycardia and decline in rate of pressure development and decay. Treatment with MgV significantly increased the rate of pressure development,reduced B.P and improved bradycardia . It has been recently demonstrated that hypertensive drive is partially controlled through the overexpression of proinflammatory cytokines, especially TNF, along with downstream alterations in NF-κB, ROS, and RAS components, as regulated through AT1R activation .It has been suggested that HDACi with VPA reduced inflammation, ROS, and AT1R, thereby attenuating hypertension and its secondary consequences in SHR rats(Cardinale et. al.,2010).Moreover it has been found that VLA treatment reduces hypertension in animal model of hypertension (Cardinale et. al.,2010).In one of the study treatment with VLA reduced myocardial remodelling and increase the rate of pressure development and decay which was declined in infracted rats(Lee,et.al.,2006).This suggest MgV is beneficial in improving the haemodynamics in diabetic animals.

Implication of oxidative stress in the pathogenesis of diabetes is suggested, not only by oxygen free-radical generation, but also due to nonenzymatic protein glycosylation, auto-oxidation of glucose, impaired glutathione metabolism, alteration in antioxidant enzymes, lipid peroxides formation and decreased ascorbic acid levels. The metabolic abnormalities of diabetes cause mitochondrial superoxide overproduction. This increased superoxide production causes the activation of 5 major pathways involved in the pathogenesis of complications: polyol pathway flux, increased formation of AGEs (advanced glycation end products), increased expression of the receptor for AGEs and its activating ligands, activation of protein kinase C isoforms(with effects ranging from vascular occlusion to expression of proinflammatory genes), and overactivity of the hexosamine pathway(mediating increased transcription of genes for inflammatory cytokines). It also directly inactivates 2 critical antiatherosclerotic enzymes, endothelial nitric oxide synthase and prostacyclin synthase. Increased intracellular reactive oxygen species (ROS) also activate a number of proinflammatory pathways, and cause long-lasting epigenetic changes that drive persistent expression of proinflammatory genes (Giacco, Brownlee, 2010). During long standing diabetes, the physiological response to combat oxidative stress is overwhelmed, resulting in an imbalance between prooxidtive and anti-oxidative compounds. In present study , there was significant increase in pro-oxidant malondealdehyde and NO level in LV and decrease in antioxidant enzyme activity such as SOD,GSH levels in LV of diabetic rats. Treatment with MgV showed significant decrease in prooxidant and increase in antioxidant enzyme level in diabetic LV. Long-term inhibition of histone deacetylase with valproic acid might attenuate hypertrophic and hypertensive responses by modulating reactive oxygen species and proinflammatory cytokines in SHR rats. A recent study showed that HDACi can deactivate Akt, a potential mediator of cardiac hypertrophy and oxidative stress, via dephosphorylation by HDAC-protein phosphatase 1 complexes (Cardinale et.al.,2010). This might serve as possible mechanism in reducing the oxidative stress by MgV.

6.2 DIABETIC NEPHROPATHY

Diabetic nephropathy is associated with increased mortality in diabetic patients and is a major cause of end stage renal disease (Arya et. al., 2010)**.** Clinical hallmarks of diabetic nephropathy include a progressive increase in urinary albumin excretion and a decline in glomerular filtration rate (GFR), which occur in association with an increase in blood pressure, ultimately leading to endstage renal failure. These renal functional changes develop as a consequence of structural abnormalities, including glomerular basement membrane thickening, mesangial expansion with extracellular matrix accumulation and changes in glomerular epithelial cells (podocytes), including a decrease in number and/or density, podocyte foot process broadening and effacement, glomerulosclerosis, and tubulointerstitial fibrosis(Giunti et.al., 2006).

Sodium retention and intracellular potassium depletion occurs as a characteristic alteration in type 1 as well as type 2 diabetes mellitus which was consistently found in various stages of progression of diabetic nephropathy(Shahid et.al.,2005). Urinary tumor necrosis factor (TNF) is an important mediator of diabetic nephropathy (Dipetrillo et.al,2003). It has been previously reported that TNF promotes distal tubule sodium retention and renal hypertrophy during diabetes (Dipetrillo et.al, 2003).In the present study STZ treated diabetic rats exhibited reduced concentration of sodium and chloride level in urine indicating sodium and chloride retension.Chronic treatment with MgV produced significant increase in the concentration of sodium and chloride level in urine.This is consistent with the idea that VPA treatment robustly suppresses lipopolysaccharide induced secretion of proinflammatory TNF-α and production of NO (Peng et al., 2005 ; Kim et.al.,2007**)** .Thus ,MgV possibly suppress TNF-α production which in turn inhibits TNF mediated distal tubule sodium retension during diabetes.

In Type 2 diabetes, hyperuricemia seems to be associated with the insulin-resistance syndrome, impaired glucose tolerance, and an early onset of nephropathy. It has been shown that patients with insulin resistance or impaired glucose tolerance have reduced values of urinary uric acid clearance and chronically increased extracellular adenosine concentrations, thereby contributing to increasing uric acid synthesis(Lippi et.al.,2007). Mild hyperuricemia causes hypertension and renal injury in the rat via a crystal-

independent mechanism, with stimulation of the renin-angiotensin system and inhibition of neuronal NO synthase(Mazzali et.al.,2001).It is suggested that increased serum level of uric acid is an injurious factor for kidneys, as it is shown that hyperuricemia-induced endothelial dysfunction, glomerular hypertension, and renal hypertrophy decrease renal perfusion via stimulation of the afferent arteriolar vascular smooth muscle cell proliferation (Bonakdaran et.al.,2011).In the present study STZ treated diabetic rat showed increase in serum uric acid level. Treatment with MgV significantly reduced serum uic acid in diabetic rats. MgV showed to reduce the hyperuricaemia and so it may prevent the kidney damage associated with hyperuricaemia.

Serum creatinine and Blood urea nitrogen (BUN) tests can reveal the patients renal function. If the kidneys are failing serum creatinine levels increase indicating the derangement of kidney function. The serum creatinine concentration is widely interpreted as a measure of the glomerular filtration rate (GFR) and is used as an index of renal function(Wagle .,2010). Long term hyperglycaemia caused increase in muscle damage and release of creatinine in to blood (Capse et.al.,1949) .In our study, STZ treated diabetic rats produced increase in serum creatinine level and treatment with MgV significantly reduced the increased serum creatinine level which may be due to reduction in serum glucose as mentioned earlier in the present study suggesting that the MgV may reduce the hyperglycaemic risk associated with kidney function**.**

Microalbuminuria is considered to be an early stage of diabetic nephropathy.Excretion of albumin in urine can be regarded as a sign of kidney involvement and can reflect generalized vessel damage throughout the body(Manaviat et.al,2004). Elevated insulin levels that occur with insulin resistance increase glomerular hemodynamic pressures that increase albumin excretion (Lane , 2004). High glucose causes dysregulation of mediators including TNFα and enhanced production of ROS, which directly damage the glomerular endothelial glycocalyx leading to microalbuminuria (Satchell and Tooke ,2008).In present study STZ treated diabetic rat showed significant albuminuria and treatment with MgV significantly reverse the condition. The major underlying factors associated with microalbuminuria is the up-regulation and action of growth factors such as transforming growth factor-beta (TGF-β) and cytokines produced in response to changes in systemic factors, particularly blood pressure or hyperglycemia. One of the study has identified the

relationship of elevated levels of TGF-β to increased levels of intact albumin in the urine and is directly linked to the effect of TGF-β on albumin uptake and the lysosomal breakdown of filtered albumin by proximal tubular cells prior to excretion (Russo ET.AL.,2004). It was shown that trichostatin A (TSA), an HDAC inhibitor, prevented TGF-β1–expression in cultured human renal proximal tubular epithelial cells (Yoshikawa et.al., 2007). This might suggest the possible mechanism behind reducing the excretion of albumin in MgV treated rats. Proteinuria, a marker and potential contributor to renal injury, accompanies diabetic nephropathy. Increased glomerular permeability will allow plasma proteins to escape into the urine. Some of these proteins will be taken up by the proximal tubular cells, which can initiate an inflammatory response that contributes to interstitial scarring eventually leading to fibrosis. Our study have shown significant proteinuria and treatment with MgV showed decrease in proetinuria condition. In one the study it has been reported that Administration of valproic acid before kidney injury prevented the development of proteinuria and the onset of glomerulosclerosis(Beneden et.al.,2011).

Hyperglycemia induces renal damage directly or through hemodynamic modifications. It induces activation of protein kinase C, increased production of advanced glycosylation end products, diacylglycerol synthesis and hemodynamic alterations such as glomerular hyperfiltration, shear stress, and microalbuminuria. These alterations contribute to an abnormal stimulation of resident renal cells that produce more TGF-β1. TGF-β1 causes augmented extracellular matrix protein deposition i.e. collagen types I, IV, V, and VI; fibronectin, and laminin at the glomerular level, thus inducing mesangial expansion and glomerular basement membrane thickening (Schena and Gesualdo.,2005). In our present study STZ treated rats showed increased level of renal collagen levels indicating the risk of fibrosis. Treatment with MgV significantly decreased LV collagen level.(Schena and Gesualdo.,2005).In one of the sudy it has been reported that VPA prevented the expression of collagen type 1 α1 and TGF-β1 in activated LI90 cells at the mRNA and protein levels (Watanabe et.al.,2011).

High glucose level stimulated reactive oxygen species (ROS) production via a PKC-dependent activation of NAD(P)H oxidase in cultured aortic endothelial cells, smooth muscle cells, and renal mesangial cells. In addition, expression of NAD(P)H oxidase

components were shown to be upregulated in vascular tissues and kidney from animal models of diabetes. (Inoguchi et.al.,2003 Furthermore, several agents that were expected to block the mechanism of a PKC dependent activation of NAD(P)H oxidase clearly inhibited the increased oxidative stress in diabetic animals.These findings strongly suggest that the PKC-dependent activation of NAD(P)H oxidase may be an essential mechanism responsible for increased oxidative stress in diabetes (Inoguchi et.al.,2003). In present study , there was significant increase in pro-oxidant malondealdehyde and NO level in kidneys and decrease in antioxidant enzyme activity such as SOD,GSH levels in kidney of diabetic rats. Treatment with MgV showed significant decrease in prooxidant and increase in antioxidant enzyme level in diabetic kidney .In one of the study chronic exposure of rat C6 glioma cells to "therapeutic" concentrations of VPA resulted in decreased PKC activity in both membrane and cytosolic fractions and increased the cytosol/membrane ratio of PKC activity (Chen et.al.,1994).This might suggest the possible mechanism by which MgV reduced the oxidative stress in diabetic rats.

Tubulointerstitial fibrosis is seen in advanced stages of diabetic nephropathy and is a better predictor of renal failure than glomerular sclerosis. Hyperglycemia, angiotensin II, TGF-β, and likely proteinuria itself all play roles in stimulating this fibrosis. There is an epithelial-mesenchymal transition that takes place in the tubules, with proximal tubular cell conversion to fibroblast-like cells. These cells can then migrate into the interstitium and produce collagen and fibronectin (Butt, 2008).In present investigation histological studies revealed increased glomerular lesion, mesangial expansion and tubular atrophy in STZ treated diabtetic rat and treatment with MgV showed reduction in the pathological condition in treated rats.These results are consistent with the study report suggesting that histone deacetylase inhibitors (HDACis) exert antifibrogenic effects in several organs. In particular, transforming growth factor-β1 (TGF-β1) is considered as a key factor in accelerating fibrosis . TSA completely prevented TGF-β1–induced morphologic changes and significantly prevented TGF-β1–induced downregulation of E-cadherin and upregulation of collagen type I (Yoshikawa et.al.,2007). Thus prevention of renal tissue alterations by MgV is justified.

From ongoing discussion, it appears that MgV is beneficial in prevention of cardiorenal complication of diabetes. However it has been known that large amount of valproic acid may result in CNS depression; ranging from drowsiness to coma (Manoguerra et.al.,2006)..Hence, we thought it worthwhile to see the effect of MgV on CNS to explore its associated side effect. In lieu of this we evaluated locomotor and immobility activity test .The results depict that MgV treated diabetic rats showed no significant difference in thier locomotor activity and immobility as compared with control group rats.This indicates that the present dose of 210mg/kg/day,MgV is safe and not associated with major side effects. Also we would like to mention that the current dose of 210 mg/kg/day is the converted clinical dose to animal dose considering the body surface area (Gosh.,2007)

In conclusion our data suggests that MgV has beneficial effect on cardiovascular complications associated with streptozotocin (STZ) induced diabetes in neonatal rats as depicted by prevention of hyperglycemia, hyperinsulinaemia, dyslipidemia, hypertension, bradycardia, cardiac and left ventricular hypertrophy, oxidative stress, reduction in cardiac biomarker levels and preserving structural integrity of the myocardium. In addition, it also prevents renal complications by controlling urinary electrolytes, preserving kidney function decreasing microalbuminuria and regressing pathological alteration of kidney.Thus MgV could be considered as an “add on” therapy for management of cardio-renal complication of diabetes.

7. SUMMARY AND CONCLUSIONS

In summary, our data suggests that chronic treatment with MgV

-produced a significant decrease in serum glucose levels.

- Significantly reduced serum total cholesterol, LDL, VLDL and triglyceride levels and significantly increase the HDL levels .

-produced a significant decrease in serum cardiac markers viz. CK-MB, LDH and significantly decreased the inflammatory marker, CRP.

-significantly reduced LV and renal collagen level and significantly increased LV Na^+-K^+ ATPase activity.

-significantly decreased hypertrophic parameters like cardiac hypertrophic index, LV hypertrophic index, LVW/RVW ratio, LV wall thickness and cardiomyocyte diameter.

-produced significant reduction in blood pressure and increase in heart rate and significantly increased the rate of pressure development and decay.

-significantly reduced prooxidant levels like nitrite and malondialdehyde in LV and significantly increased antioxidant enzyme activity like SOD and GSH levels in heart.

-significantly reduced urine excretion as well as sodium and chloride excretion.

-significantly reduced serum ALP, uric acid, BUN and creatinine and significantly increased serum albumin level.

-significantly reduced urinary albumin, total protein excretion and UAC ratio and significantly increased uric acid and creatinine excretion and creatinine clearance.

-significantly reduced prooxidant levels like nitrite and malondialdehyde in kidney and significantly increased antioxidant enzyme activity like SOD and GSH levels in kidney.

In conclusion, Our data suggests that MgV has beneficial effect on cardiovascular complications associated with streptozotocin (STZ) induced diabetes in neonatal rats as depicted by prevention of hyperglycemia, hyperinsulinaemia, dyslipidemia, hypertension, bradycardia, cardiac and left ventricular hypertrophy, oxidative stress, reduction in

cardiac biomarker levels and preserving structural integrity of the myocardium. In addition, it also prevents renal complications by controlling urinary electrolytes, preserving kidney function decreasing microalbuminuria and regressing pathological alteration of kidney.Thus MgV could be considered as an “add on” therapy for management of cardio-renal complication of diabetes.

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