5. Discussion

The present investigation demonstrates the renoprotective and neuroprotective effect of coenzyme Q10 alone and its combination with antidiabetic or antihyperlipidemic drug against hyperglycemia-mediated oxidative stress in streptozocin–nicotinamide-induced diabetic nephropathy and neuropathy.

Diabetic nephropathy (DN) is the most common cause of end-stage renal disease and is one of the leading causes of morbidity and mortality worldwide. The pathogenesis of diabetic nephropathy is multifactorial in which chronic hyperglycemia plays a major role. During diabetic milieu, supraphysiological glucose is involved in the construction of AGEs and the mitochondrial production of free radicals and as a result, to cell death and renal dysfunction. Therefore, oxidative stress plays an important role in the progression of diabetic nephropathy, which is characterized by thickening of glomerular basement membranes, expansion of mesangial cells, glomerular hypertrophy and loss of podocytes, expansion of tubular basement membranes, tubular atrophy, interstitial fibrosis, arteriosclerosis which ultimately progress to glomerular structural damage evidenced by proteinuria, microalbuminuria, decreased glomerular filtration rate and end-stage renal failure.

The pathophysiological changes and deteriorated renal functions created by chronic hyperglycemia-mediated oxidative stress in the experimental rats are closely comparable to that of human diabetic nephropathy. In addition, streptozocin–nicotinamide-induced experimental diabetes is demonstrated by moderate and stable hyperglycemia, glucose intolerance and significantly altered glucose-stimulated insulin secretion that contributes number of features similar to human diabetes.

Preventing the progression of DN has been a challenge in biomedical research. Increased levels of serum glucose, creatinine, BUN, uric acid are the indicators for diabetic nephropathy that can be normally seen in this complication. Administration of STZ-nicotinamide in Wistar rats produced marked and sustained increase in glucose level. STZ cause diabetes by the rapid reduction of β-cells of pancreas. In addition, when it was administered along with nicotinamide, it causes minor damage to pancreatic β-cells.
5. Discussion

In the present study co-administration of coenzyme Q10 and metformin has a significant reduction in elevated hemoglobin A1C, urea, creatinine and uric acid as compared to diabetic rats. These results are in accordance with the earlier study in which it was shown that metformin alone produced a beneficial effect on diabetic nephropathy.62,145 In this study metformin when administered along with an antioxidant like coenzyme Q10 produced a synergic effect in reducing the development of DN.

In the current study, co-administration of coenzyme Q10 and sitagliptin has a significant reduction in elevated hemoglobin A1C, urea, creatinine and uric acid as compared to diabetic rats. These results are in accordance with the earlier study in which it was shown that sitagliptin alone produced a beneficial effect on diabetic nephropathy.168 In this study sitagliptin when administered along with an antioxidant like coenzyme Q10 produced a synergic effect in reducing the development of DN.

However, coenzyme Q10 in combination with metformin and sitagliptin treatment group showed more significant reduction in elevated hemoglobin A1C, urea, creatinine and uric acid as compared to diabetic rats treated with coenzyme Q10 and metformin or sitagliptin was given together.

It was previously reported that statins have pleiotropic effects on cardiovascular, cerebrovascular, and microvascular disease independent of their cholesterol-lowering effects in several clinical investigations; evidence of renoprotection in humans has not been presented. Previous reports have shown the pleiotropic effects of statins, such as anti-inflammatory effects and antioxidative stress effects in vitro and in vivo.219,220

Proteinuria is considered as a principal indicator of diabetic nephropathy.111 In the current study, proteins appeared in trace in the urine of STZ-nicotinamide induced diabetic nephropathy. This study was shown that treatment of coenzyme Q10, metformin, sitagliptin and rosuvastatin alone showed a significant decrease in urinary protein. Besides, coenzyme Q10 in combination with metformin or sitagliptin showed more significant decrease in urinary protein than when administered singly. In contrast, treatment with coenzyme Q10 in combination therapy with metformin and sitagliptin showed a better renoprotective effect by virtue of significant reduction of urinary protein. These results are accordance with the earlier reports.190
It was also previously shown that diabetic dyslipidemia plays a pathogenetic role in the development and continuation of diabetic nephropathy (DN). Lipids may lead to renal injury by stimulating transforming growth factor-β, thereby inducing the production of reactive oxygen species (ROS) and causing damage to the glomeruli and glomerular glycocalyx. Triglycerides-rich lipoproteins (TGRLs) can activate monocytes, degrade glycocalyx, and enhance permeability of the glomerular filtration barrier, which may contribute to the development of diabetic nephropathy.\textsuperscript{174}

In the present study, concomitant administration of coenzyme Q10 with rosvastatin in diabetic rats has a significant reduction in elevated hemoglobin A\textsubscript{1C}, urea, creatinine uric acid and urinary protein as compared to diabetic untreated rats. These results are in accordance with the earlier study in which it was shown that statins prevented the microalbuminuria and nephropathy in diabetic.\textsuperscript{221,222} In this study, rosvastatin when administered along with an antioxidant like coenzyme Q10 produced a synergic effect in reducing the development of DN.

The exact mechanisms underlying DN are complex and not well defined. Chronic hyperglycemia, the major determinant of the beginning and propagation of DN, not only generates more reactive oxygen metabolites, but also attenuates anti-oxidative defense mechanisms through non-enzymatic glycation of the scavenging enzymes. Furthermore, hyperglycemia can be toxic either by non-enzymatic reaction of glucose with proteins and consequent construction of advanced glycosylation end products (AGEs) or by increased metabolism leading to increased oxidative stress and activation of protein kinase C, resulting in increased production of proinflammatory cytokines.\textsuperscript{223} Several studies have shown that oxidative stress generated by free radicals such as superoxide and lipid peroxidation product like malondialdehyde plays a major role in the pathogenic pathway of diabetic injuries which can be responsible for cell and tissue damage.\textsuperscript{146,200}

It was earlier reported that antioxidants such as vitamin E, coenzyme Q10 and antiperoxidative such as SOD, CAT, GSH playing a crucial role in protection of the cells and tissue damage against oxidative stress mediated injuries.\textsuperscript{224} It was also reported that systemic administration of antioxidants like vitamin C, vitamin E, free amino acids and the isolated bioactive moieties including curcumin, naringin, quercetin provides the hope for more effective treatments for diabetes mellitus and its complications.\textsuperscript{225,226}
It was also shown in previous study, antioxidant effects via increase of gene expression of antioxidant enzymes and a number of the most important protection mechanisms against free radicals damage containing superoxide dismutase, glutathione peroxidase, and catalase.\textsuperscript{227}

The results of our study showed that in coenzyme Q10 alone and its combination with antidiabetic or antihyperlipidemic drugs treated diabetic rats, body weights were increased significantly probably due to the controlling muscle wasting, ie, reversal of gluconeogenesis.\textsuperscript{228} However, in the diabetic control group, body weights decreased significantly due to increasing muscle wasting.\textsuperscript{229}

The kidney weight of the diabetic rats significantly increased due to renal enlargement, which is one of the key features occurring during initial changes by DM. In earlier stages of diabetic nephropathy, hypertrophy and hyperfunctioning of the kidneys with a typical increase in kidney size and glomerular filtration rate is observed.\textsuperscript{230} The treatment with coenzyme Q10 alone, metformin alone, sitagliptine alone, rosuvastatin alone, coenzyme Q10 in combination with metformin or sitagliptin or rosuvastatin, coenzyme Q10 in combination with metformin and sitagliptin showed a significant reduction in kidneys weight when compared to the diabetic control group. However, coenzyme Q10 in combination with metformin and sitagliptin showed more significant reduction in kidney weight that of mono-therapy or co-administration of both drugs.

Results in the present study also indicate that there is an increase in the oxidative stress after STZ-nicotinamide induced diabetic nephropathy. It has been further shown that lipid peroxidation was significantly reduced after the treatment with coenzyme Q10 or metformin or coenzyme Q10 + metformin. However, co-administration of coenzyme and metformin has more beneficial effect than when administered singly. These results are in accordance with the earlier study in which it was shown that treatment of type II diabetic patients with metformin restores the antioxidant status, enzymatic activity and inflammatory parameters, and hence improves the status of oxidative and nitrosative stress altered in DM.\textsuperscript{231} These results are also in accordance with earlier studies whereas the levels of α-tocopherol and coenzyme Q10 and the activities of pancreatic mitochondrial MnSOD and GSH-Px are increased.\textsuperscript{144}
In present study, there was a significant reduction in lipid peroxidation and increase in SOD, CAT, and GSH levels after the treatment with coenzyme Q10 or sitagliptin or coenzyme Q10 + sitagliptin. In contrast, co-administration of coenzyme and sitagliptin have shown more significant alteration in MDA, SOD, catalase and GSH levels than when administered singly.

Oxygen free radicals exert their cytotoxic effects on membrane phospholipids resulting in the formation of lipid peroxidation product malondialdehyde (MDA). MDA is a highly reactive three carbon dialdehyde produced as a byproduct of polyunsaturated fatty acid peroxidation. The present study showed that the renal concentration of MDA was elevated and that of GSH, SOD decreased in diabetic animals. The present investigation are in line with previous work. However, the treatment with coenzyme Q10 in combination with metformin and sitagliptin showed more beneficial effect in reducing the oxidative stress in terms of significant alteration in MDA, SOD, catalase and GSH levels than when administered conenzyme Q10 in combination with metformin or sitagliptin. Elevated concentration of MDA is due to reactive oxygen species (ROS). Moreover decreased SOD activity and GSH concentration may be due to defense mechanism against oxygen free radicals. These results are in line with previous work in which chronic treatment with concomitant administration of trigonelline + sitagliptin significantly decreased MDA content and increased SOD, GSH concentration. The present results are also accordance with earlier studies in which coenzyme Q10 also may serve as an antioxidant by activating and increasing expression of mitochondrial uncoupling proteins, an effect which is antiapoptotic and leads to a reduction in free radical generation in vivo.

On the other hand, the animals treated with coenzyme Q10 or rosuvastatin or their combination there was a significant reduction in lipid peroxidation and increase in SOD, CAT, and GSH levels. In contrast, there was more significant reduction in the levels of MDA and an increase SOD, catalase activities and GSH levels when coenzyme Q10 and rosuvastatin were given together than administered singly. These results are accordance with the previously reported studies in which simvastatin or rosuvastatin treated animals showed a significant reduction in MDA levels and a increase the SOD, catalase activities and GSH levels in cisplatin induced nephotoxicity. In the present study, it was shown
that treatment of rosuvastatin showed a significant decrease in oxidative stress which was in accordance with previous reports confirming the pleiotropic effects of statins.\textsuperscript{219,220} The previously study showed that coenzyme Q10 has beneficial effects in increasing the reduced serum antioxidant enzymes, GSH and protective effects on lipid peroxidation, glomerular hypertrophy, glomerulosclerosis and leukocyte infiltration in alloxan-induced-diabetic rats. Hence, attenuation of lipid peroxidation and acceleration of antioxidant enzymes can decrease diabetic complication such as nephropathy in diabetic patients.\textsuperscript{146}

Patients with DN often have multiple lipoprotein abnormalities.\textsuperscript{237} They include higher plasma levels of very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), intermediate-density lipoprotein (IDL), and triglycerides but lower concentration of high-density lipoprotein (HDL) than levels observed in patients without diabetes. Findings from basic and clinical studies strongly suggest that excess amount of a variety of lipoproteins and lipids worsens diabetes-associated microvascular and macrovascular diseases, increases glomerular injury, increases tubulointerstitial fibrosis, and accelerates the progression of DN.\textsuperscript{238-241} Lipids may influence renal damage by stimulating the production of reactive oxygen species (ROS) and causing damage to the glomeruli and glomerular glycocalyx, and ultimately end stage renal failure.\textsuperscript{174}

In present study, STZ-nicotinamide injection showed a significant increase in serum total cholesterol, serum triglyceride and a decrease in HDL-C levels. However, the treatment with coenzyme Q10 alone and its combination with metformin or sitagliptin or rosuvastatin showed a significant alteration in serum lipid levels as compared to diabetic control rats. Moreover, the treatment with coenzyme Q10 in combination with metformin and sitagliptin showed more significant improvement on the lipid profile than combination therapy of two drugs, while the sitagliptin or metformin treated rats did not show a significant difference in serum total cholesterol and HDL-C levels as compared to diabetic control rats. These data in present study are line with the previous study in which treatment statins or coenzyme Q10 showed a significant improvement in serum lipid levels.\textsuperscript{242,243}

In the present study, the administration of STZ-nicotinamide to rats induced a model of DN manifested as an increase in tissue TNF-\(\alpha\), tissue TGF-\(\beta\), tissue and MPO
activity with marked histopathological changes manifested by massive glomerulosclerosis.

It was previously demonstrated that TGF-β, a fibrogenic cytokine plays a key role in the development of DN. TGF-β expression was noted to be elevated in the glomeruli of sterptozotocin-induced diabetic rats and diabetic NOD mice. Further, TGF-β mRNA levels were found to be elevated in patients with early DN. Chronic diabetes mellitus activates TGF-β mediated downstream signalling pathways in the renal cortex of experimental diabetic mouse. TGF-β has been shown to upregulate the expression of GLUT-1 in mesangial cells, which increases the concentration of glucose intracellularly and accelerates metabolic abnormalities in the diabetic kidney. Further, TGF-β stimulates the expression of CTGF, which promotes the deposition of extra cellular matrix components like collagen I, collagen IV and fibronectin and thus enhances the disassembly and hypertrophy of mesangial cells. In addition, TGF-β induces the synthesis and accumulation of extra cellular matrix protein. This ultimately leads to the structural and functional features characteristic of DN.

In the present study, administration of coenzyme Q10, metformin, sitagliptin, rosvastatin alone showed a significant decrease in renal TGF-β levels. In addition, coenzyme Q10 in combination with metformin or sitagliptin resulted more significant reduction in renal TGF-β levels than that of mono-therapy. Moreover, treatment with coenzyme Q10 in combination therapy with metformin and sitagliptin showed a better renoprotective effect by virtue of significant reduction of TGF-β levels in renal tissue. These results are accordance with the earlier reports in which resveratrol, curcumin and metformin treated rats showed anti-inflammatory by virtue of their inhibitory effects on TGF-β in renal tissues.

Tumor necrosis factor-α is a potent mediator of immune and inflammatory responses. TNF-α is produced by many activated cell types including monocytes, macrophages, astrocytes, granulocytes, T and B lymphocytes, NK cells, keratinocytes, fibroblasts, and certain tumor cells. TNF-α exerts many regulatory influences on the activation, growth, and differentiation of leukocytes and other cells. For example, TNF-α can co stimulate the proliferation of activated T and B lymphocytes, upregulate the expressed levels of MHC class I and class II molecules by various cell types, as well as
induce the expression of adhesion molecules by endothelial cells. TNF-α is selectively cytotoxic for some transformed cell lines and can exert cytotoxic effects against certain solid tumors. In vivo, TNF-α serves as a primary mediator in protective immune responses against microbial and viral pathogens. However, TNF-α has also been implicated as a central mediator in a number of pathologic responses including diabetic nephropathy, septic shock, cachexia and autoimmune diseases.\textsuperscript{254,255} DN occurs as a result of the effects of both metabolic and hemodynamic insults, which at cellular level lead to the activation of intracellular signaling pathway and transcription factors. This effect is due to the release of TNF-α and myeloperoxidase (MPO).\textsuperscript{256,257}

In current study, treatment of coenzyme Q10, metformin, sitagliptin, rosvastatin alone showed a significant decrease in renal TNF-α level. Besides, coenzyme Q10 in combination with metformin or sitagliptin showed more significant decrease in renal TNF-α level than when administered singly. In contrast, treatment with coenzyme Q10 in combination therapy with metformin and sitagliptin showed a better renoprotective effect by virtue of significant reduction of TNF-α level in renal tissue. These results are similar with the earlier studies which showed that administration of metformin has antioxidant and anti-inflammatory properties which may play a role in the protection against DN.\textsuperscript{63,258} One of the studies demonstrated that curcumin was found to increase the antioxidant status of pancreatic B-cells and inhibit the activation of both TNF-α and NF-kB which have an important role in the development of DN.\textsuperscript{259}

In present study, there was a significantly decreased renal MPO level in coenzyme Q10, metformin, sitagliptin, rosvastatin alone treated rats. Moreover, coenzyme Q10 in combination with metformin or sitagliptin showed more significant decrease in renal MPO levels level than when administered singly. On the contrary, treatment with coenzyme Q10 in combination therapy with metformin and sitagliptin showed a better renoprotective effect by virtue of significant reduction of MPO levels level in renal tissue. These results are similar with the earlier studies which showed that administration of metformin has antioxidant and anti-inflammatory properties which may play a role in the protection against DN.\textsuperscript{63}

Peroxynitrite anions have been generated by the reaction of nitric oxide with superoxide anion. These peroxynitrite anions oxidize biomolecules, which finally leads to
lipid peroxidation and tubular cell damage. Large amounts of nitric oxide can lead to the depletion of cellular ATP which can inactivate enzymes that contain iron-sulfur clusters, such enzymes involved in mitochondrial electron transport. Nitrosylation of sulfhydryl groups or tyrosine residues in proteins may impair the functional properties of these proteins. Nitric oxide damages DNA, and this in turn, stimulates the DNA repair enzyme poly-ADP-ribose synthetase.

In the present study, diabetic rats showed a significant increase in tissue nitrite content as compared to normal control rats, while treatment of coenzyme Q10, metformin, sitagliptin, rosuvastatin alone showed a significant decrease in tissue nitrite content. Besides, coenzyme Q10 in combination with metformin or sitagliptin showed more significant decrease in tissue nitrite content than when administered alone. In contrast, treatment with coenzyme Q10 in combination therapy with metformin and sitagliptin showed a better renoprotective effect by restoring the levels of nitrite content in renal tissue. These results are similar with the earlier studies which showed that the effect of curcumin and its analogues on peroxynitrite anions scavenging activity in vitro using sodium nitroprusside (SNP) generating nitric oxide system.

Diabetic neuropathy is most common long-term complications of diabetes affecting 50% of the patient worldwide. Diabetic nephropathy occurs as a result of damage to the nervous system due to persistent hyperglycemia can affect many parts of the body. The symptoms of diabetic neuropathy include pain, numbness, tingling. Neuropathic pain is usually considered to be one of the most upsetting complications affecting diabetic patients. Current treatment for neuropathic pain includes antidepressant ( duloxetine, citalopram, venlafaxine), anticonvulsants (pregabalin, gabapentin, carbamazepine) and opioid and opioid-like drugs ( tramadol, oxycodone). Pain relief with existing therapy is associated with many side effects. It was previously reported that persistent and chronic hyperglycemia responsible for generation of the reactive oxygen species and yield in the oxidative stress due to depletion of antioxidant defense system and damage to the peripheral neurons.

STZ-nicotinamide induced diabetic neuropathy is well-established and animal model is widely used to investigate the potential of bioactive moieties to treat diabetic peripheral neuropathies. Beside that increased aldose reductase (AR) activity,
accumulation of Advanced Glycation End Products (AGE) and lowed Na+/K+-ATPase activity, alterations in fatty acid metabolism and accumulation of long-chain fatty acid esters in the nerve are other mechanisms involved in the progressive damage to nervous system by STZ.\textsuperscript{279–282}

Diabetic neuropathy is clinically manifested by an array of behavioral and biochemical aberration depicting the pathological role of long term diabetes. Allodynia and hyperalgesia comprise an inevitable due of behavioral markers that can be evaluated by measurement of reduced threshold to painful stimuli.\textsuperscript{283} These symptoms owe their etiology to reduced threshold of peripheral neurons to painful stimuli invoked due to intraperitoneal administration of STZ.\textsuperscript{160,284,285} It was previously stated that generation of oxidative stress after intraperitoneal administration of STZ-nicotinamide causes vascular and neuronal complications of painful neuropathy. Hyperglycemia along with oxidative stress together causes formation of edema, ischaemia and hypoxia which intern caused multifocal axonal degeneration in peripheral nerves.\textsuperscript{93}

The alteration in neural nociception occurred due to peripheral receptors sensitization, ischemic tissue injury and ectopic discharge from sprouting fibers along with changes in dorsal root ganglia cells.\textsuperscript{286–287} Thus, to evaluate the potential of various analgesic drugs on the central pain, mechanical allodynia, mechanical hyperalgesia and thermal hyperalgesia in laboratory animals, tail flick, von-Frey hair, Randall Selitto and Hargreaves test has been widely used as well as established methods.\textsuperscript{288} Development of diabetic neuropathy was evident from alteration in muscle grip strength, nociception (paw withdrawal and tail flick response) and biochemical changes including oxidative stress.

In present study, it was shown that after six weeks of streptozotocin-nicotinamide treatment, muscular grip strength in diabetic rats were found to be reduced as compared to normal control rats. Nociception was evaluated by increased in paw withdrawal response and tail flick response (hyperalgesia).

In present study, there was a significantly increased in muscular grip strength in coenzyme Q10, metformin, sitagliptin, rosuvastatin alone treated rats. Moreover, coenzyme Q10 in combination with metformin or sitagliptin showed more significant increase muscular grip strength than when administered singly. In contrast, treatment with coenzyme Q10 in combination therapy with metformin and sitagliptin showed a
better neuroprotective effect by virtue of significant increase muscular grip strength. These results are in accordance with the earlier study in which it was shown that metformin or resveratrol or α-lipoic acid alone produced a beneficial effect on diabetic nephropathy.\textsuperscript{169,289,290}

In current study, administration of coenzyme Q10, metformin, sitagliptin, rosvastatin alone showed a significant decrease in paw withdrawal response and tail flick response when compared to diabetic control rats. In addition, coenzyme Q10 in combination with metformin or sitagliptin or rosvastatin resulted more significant decrease in paw withdrawal response and tail flick response than that of mono-therapy. Moreover, treatment with coenzyme Q10 in combination therapy with metformin and sitagliptin showed a better neuroprotective effect by virtue of significant decrease paw withdrawal response and tail flick response. These results are similar with the earlier studies which showed that coenzyme Q10 and pyridoxine not only attenuated the hyperglycemia but also showed the significant decrease in neuropathic pain via inhibition of elevated levels of hyperlipidemia, glycated Hb, oxidative–nitrosative stress as well as elevation in the membrane bound inorganic phosphate enzyme that leads to diminution in the allodynia and hyperalgesia.\textsuperscript{147}

In this study, there was a significant increase MDA level and decrease in the level of GSH, an endogenous antioxidant and antiperoxidative enzymes (SOD) in the untreated diabetic rat sciatic nerve. Thus, it was concluded that the elevated level of MDA might be responsible for decrease in enzymatic and non-enzymatic antioxidant of defense systems in diabetic rats. Similarly, in earlier study, it was shown that increased MDA level and depletion of GSH and SOD have been found in sciatic nerve of diabetic rats.\textsuperscript{178,179,291} In our study, it was shown that treatment with coenzyme Q10, metformin, sitagliptin, rosvastatin alone, coenzyme Q10 in combination with metformin or sitagliptin or rosvastatin prevented the increased in the levels of MDA and decreased GSH, SOD in sciatic nerve. In contrast, treatment with coenzyme Q10 in combination therapy with metformin and sitagliptin showed more beneficial neuroprotective effect in terms of reducing the oxidative stress than combination of two drugs. Histopathological study of sciatic nerve of rats in diabetic group showed a significant degeneration of nerve
tissue, while combined treatment of coenzyme Q10 with metformin or sitagliptin or rosvastatin showed regeneration of sciatic nerve tissues.