

Evaluation of Simvastatin and/or Ezetimibe Treatment on Some Diabetic Complications in Streptozotocin Induced Diabetes in rats

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Abstract: Statins are hypolipidemic agents that are prescribed extensively in hyperlipidemia and hyperlipidemia associated diseases. They are directly involved in restoring or improving endothelial function, attenuating vascular remodeling, inhibiting vascular inflammatory response and perhaps, stabilizing atherosclerotic plaques. Ezetimibe specifically blocks the intestinal absorption of cholesterol and other related phytosterols. Consequently, the present work was designed to evaluate the effects of simvastatin and/or ezetimibe treatment on some diabetic complications in streptozotocin (STZ) induced diabetes in rats. To this end, fifty adult local strain male albino rats were divided into five equal groups; control, diabetic, diabetic plus simvastatin (SIM), diabetic plus ezetimibe (EZE) and diabetic plus SIM and EZE groups. Blood samples were obtained for determination of serum glucose, total cholesterol, triglycerides, HDL cholesterol, LDL cholesterol, creatinine and SOD levels. It was noted that induction of diabetes mellitus was associated with metabolic and renal dysfunctions associated with significant reduction in serum SOD activity. Treatment with simvastatin or ezetimibe was found to be effective in ameliorating lipid profile, serum SOD activity and serum creatinine. Addition of ezetimibe to simvastatin enhanced or potentiated the ameliorative effect of simvastatin on metabolic and renal dysfunctions associated with STZ-induced diabetes in rats. In conclusion, these findings underscore the importance of ezetimibe to be added to simvastatin to ameliorate metabolic and renal dysfunction. However, further studies are required to clarify possible interactive mechanisms by which ezetimibe enhancing the metabolic ameliorative effects of statins.

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1. Introduction

Diabetes mellitus is a growing metabolic disorder at a fast rate throughout the world and it is the 16th leading cause of global mortality. Although its etiology is not well-defined, viral infection, autoimmune disease and environmental factors have been implicated (King *et al.*, 1998). There are a considerable amounts of data indicating that the chronic elevation of plasma glucose causes many of the major complications of diabetes including nephropathy, retinopathy, neuropathy, macro- and microvascular damages (Brownlee, 2001).

A causative role for elevated free fatty acid (FFA) levels in the development of microvascular complications remains to be established. However increased levels of FFAs are positively correlated with insulin resistance and β -cell dysfunction in the context of concomitant hyperglycemia which may result from oxidative stress (McGarry, 2002).

Oxidative stress in diabetes plays an important role in the progression of diabetic complications (Brownlee, 2001). Oxidative stress is determined by the relationship between reactive oxygen species (ROS) and the antioxidant defense system including

antioxidant enzymes (Wild *et al.*, 2004). ROS leads to lipid peroxidation, which amplifies oxidative injury and causes damage to macromolecules such as protein and nucleic acid (Sheetz and King, 2002).

Many mechanisms are involved in the production of ROS including glucose auto-oxidation, non-enzymatic protein glycation, generation of advanced glycation end products (AGE), activation of protein kinase C (PKC) and NADPH oxidase (Wautier *et al.*, 2001).

Hyperlipidemia and premature atherosclerosis are common features of diabetes mellitus in humans. In rats, streptozotocin induced insulin deficiency leads to an increased cholesterol content which may be attributed to decreased clearance of lipoproteins. In addition, it has been suggested that intestine may contribute to hyperlipidemia in diabetic rats due to increased absorption of cholesterol (Muruges *et al.*, 2006).

Statins, including simvastatin is an HMG-CoA reductase inhibitors has been shown to be an effective lipid-lowering agents (Jialal *et al.*, 2001).

Ezetimibe is a selective cholesterol absorption inhibitor that blocks a yet-unidentified sterol

transporter that moves cholesterol into the wall of the small intestine. Ezetimibe blocks the intestinal absorption of dietary and biliary cholesterol and decreases the cholesterol content of chylomicrons, which in turn reduces the amount of cholesterol delivered to the liver resulting in a compensatory increase in LDL-receptor expression and enhanced clearance of LDL particles (Neal and Jones, 2003).

The present work was designed to evaluate the effects of simvastatin and/or ezetimibe treatment on some diabetic complications in streptozotocin induced diabetes in rats.

2. Materials and Methods

Animals and Experimental Design:

The experimental protocol and animal handling were approved and performed according to guidelines of animal use of the Ethical committee of Al-Azhar University. In this study, 50 healthy adult local strain male albino rats, weighing 180-200 gm were utilized. They were left for two weeks in the laboratory room before any experimental interference for acclimatization with free access to standard rat laboratory diet and tap water. Following that, rats were divided into five equal groups:

Group I (control): received no treatment and served as control group.

Group II (DM + V): were received equal volume of distilled water (vehicle) by oral gavage daily and subjected to induction of diabetes.

Group III were subjected to induction of diabetes and received simvastatin (SIM) in a dose of 2 mg/kg/day for four weeks.

Group IV were subjected to induction of diabetes and received ezetimibe (EZE) in a dose of 5 mg/kg/day for four weeks.

Group V were subjected to induction of diabetes and received simvastatin plus ezetimibe (SIM + EZE) for four weeks.

* Preparation and induction of diabetes:

Rats of the experimental groups (groups II, III, IV and V) were subjected to induction of diabetes by intraperitoneal injection of 70 mg/kg of freshly prepared solution of streptozotocin (STZ) dissolved in 2 ml of 0.05 M citrate buffer (Kandasamy *et al.*, 2006).

* Simvastatin supplementation and dose preparation:

Simvastatin was purchased as white powder and dissolved in distilled water. It was given by gastric tube as an oral dose of 2 mg/kg body weight/day for four weeks (Gianella *et al.*, 2007).

* Ezetimibe supplementation and dose preparation:

Ezetimibe was purchased as white powder and dissolved in distilled water. It was given by gastric

tube as an oral dose of 5 mg/kg body weight/day for four weeks (Sudhop *et al.*, 2002).

Blood sampling:

Five days after STZ injection, rats were fasted overnight and blood samples were obtained from the retro-orbital plexus for determination of blood glucose levels. Rats having blood glucose level below 180 mg/dl were considered non-diabetic and excluded from the study.

At the end of the experimental period (33 days), rats were fasted overnight and under light ether anesthesia, blood samples were withdrawn from the retro-orbital plexus into test tubes. Samples were centrifuged for 15 min. serum was separated for determination of total cholesterol, triglycerides, HDL cholesterol, LDL cholesterol, creatinine and super oxide dismutase (SOD) levels. Samples were stored frozen (-80°C) until assayed. Rats were carefully monitored every day and weighted every week.

Biochemical assay:

Serum glucose levels were estimated by oxidase method (Barham and Trinder, 1972). Serum total cholesterol levels were determined by enzymatic colorimetric test according to Allain *et al.* (1974). Serum triglycerides levels were determined according to Fossati and Prencipe (1982). Serum HDL cholesterol levels were determined according to Fruchart (1982) while serum LDL cholesterol was measured according to Friedewald *et al.* (1972) formula in mg/dl where $LDL = Total\ cholesterol - (HDL + Triglyceride/5)$. Serum creatinine were determined photometrically according to Henry, (1974).

SOD levels were determined kinetically according to the Pyrogallol autooxidation method of Minami and Yoshikawa (1979). 0.1 ml of Triton x-100, 0.25 ml of nitro blue tetrazolium solution and 0.25 ml of blood hemolysate containing SOD were mixed and incubated for 5 minutes at 37 °C. The reaction was started by adding 0.1 ml of pyrogallol and stopped after 5 minutes by adding 0.3 ml of stopper solution. The percentage inhibition of pyrogallol reflected the activity of SOD according to a standard curve.

Statistical analysis:

Data input and analysis were done using SPSS computer program. All results were expressed as the mean \pm standard error. Mean values of the different groups were compared using a one way analysis of variance (ANOVA). Least significant difference (LSD) post hoc analysis was used to identify significantly different mean values. *P* value < 0.05 was accepted to denote a significant difference.

3.Results

Changes in body weight and blood glucose in control and experimental groups (Table 1 & Fig. 1):

Table (1) and figure 1 show insignificant changes between initial and final body weight in the

control group. However, significant final weight loss was observed in the diabetic groups compared to their initial weights. Streptozotocin (STZ) injection significantly ($p < 0.0001$) increase the blood glucose level in the experimental groups as compared to the control group.

Table (1): changes in weight and glucose levels in the experimental groups.

Groups/Parameters	Initial weight (gm)	Final weight (gm)	Blood glucose (mg/dl)
Control group	190.5 ± 5.1	192.8 ± 3.28	102.1 ± 4.21
DM + V group	192.1 ± 3.88	169.4 ± 2.41**	267.81 ± 6.18 ###
DM + SIM group	194.1 ± 2.73	168.8 ± 2.4 ***	258.59 ± 5.6 ###
DM + EZE group	194.6 ± 4.34	168.5 ± 5.42 *	261.23 ± 4.98 ###
DM + SIM + EZE group	191.7 ± 6.22	167.3 ± 4.83 *	263.74 ± 5.12 ###

Data are mean ± SE. (n=10 rats per each group) One way ANOVA followed by Tukey's Multiple Comparison Test: As compared with the initial weight, * $p < 0.005$, ** $p < 0.001$, *** $p < 0.0001$. as compared with the control group, ### $p < 0.0001$.

Effects of diabetes, simvastatin and/or ezetimibe treatment on serum lipid profile (Table 2 & Fig. 2):

Table (2) and figure 2 show that induction of diabetes significantly ($p < 0.001$) increase the level of total serum cholesterol, triglyceride (TG) and LDL levels associated with significant ($p < 0.001$) decrease in the HDL level.

Treatment with simvastatin as a monotherapy or in combination with ezetimibe significantly ($p < 0.001$) decrease the total serum cholesterol and TG level with significant decrease in the level of LDL ($p < 0.01$), ($p < 0.001$) respectively. On the other hand, there was significant ($p < 0.001$) increase in the

level of HDL compared to the diabetic un-treated (vehicle) group.

Treatment with ezetimibe as a monotherapy showed significant decrease in the total serum cholesterol, TG ($p < 0.001$) and LDL ($p < 0.05$) levels with significant ($p < 0.001$) increase in the HDL level compared to the diabetic un-treated (vehicle) group.

Statistical analysis shows that treatment with simvastatin in combination with ezetimibe significantly ($p < 0.001$) decrease the total serum cholesterol and TG levels with significant ($p < 0.001$) increase in the level of HDL compared to the diabetic SIM- treated group. However, there was insignificant difference in the level of LDL compared to the diabetic SIM- treated group.

Table (2): Effects of diabetes, simvastatin and/or ezetimibe treatment on serum lipid profile.

Groups/Parameters	Total cholesterol (mg/dl)	LDL (mg/dl)	TG (mg/dl)	HDL (mg/dl)
Control group	81.00 ± 2.000	22.00 ± 1.000	73.00 ± 3.000	44.33 ± 1.528
DM + V group	185.0 ± 8.185 ###	135.3 ± 5.686 ###	174.0 ± 8.185 ###	15.00 ± 1.000###
DM + SIM group	125.0 ± 1.732 ***	75.67 ± 3.786 **	114.3 ± 1.155 ***	28.33*** ±1.155
DM + EZE group	144.3 ± 4.041 ***	99.00 ± 3.464 *	134.0 ± 3.464 ***	20.33 ± 0.577***
DM + SIM + EZE group	97.00 ± 3.000 *** †††	59.33 ± 29.16 ***	87.00 ± 2.646 ***†††	38.33 ± 0.577 ***†††

Data are mean ± SE. (n=10 rats per each group) One way ANOVA followed by Tukey's Multiple Comparison Test: ### $p < 0.001$ compared with the control group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the diabetic un-treated (vehicle) group. ††† $p < 0.001$ compared with the diabetic SIM-treated group.

Effects of diabetes, simvastatin and/or ezetimibe treatment on serum creatinine (Table 3 & Fig. 3):

Table (3) and figure 3 show that induction of diabetes produced significant ($p < 0.001$) increase in serum creatinine level from 0.4467 ± 0.135 ng/ml in the control group to 0.7467 ± 0.092 ng/ml in the diabetic un-treated (vehicle) group.

Treatment with simvastatin either as monotherapy or in combination with ezetimibe produced significant ($p < 0.01$) decrease in serum creatinine compared to the diabetic un-treated (vehicle) group.

Treatment with ezetimibe alone produced significant ($p < 0.05$) decrease in serum creatinine in comparison to the diabetic un-treated (vehicle) group.

Statistical analysis shows that treatment with simvastatin in combination with ezetimibe significantly ($p < 0.001$) decreased the serum

creatinine level compared to the diabetic SIM- treated group.

Table (3): Effect of simvastatin and/or ezetimibe treatment on serum creatinine.

Groups/Parameters	Serum creatinine (ng/ml)
Control group	0.4467 ± 0.135
DM + V group	0.7467 ± 0.092 ###
DM + SIM group	0.4833 ± 0.111 **
DM + EZE group	0.5383 ± 0.039 *
DM + SIM + EZE group	0.4883 ± 0.108 **

Data are mean ± SE. (n=10 rats per each group). One way ANOVA followed by Tukey's Multiple Comparison Test: ### $p < 0.001$ compared with the control group. * $p < 0.05$, ** $p < 0.01$, compared with the diabetic un-treated (vehicle) group.

Effects of diabetes, simvastatin and/or ezetimibe treatment on SOD activity (Table 4 & Fig. 4):

Table (4) and figure 4 show that induction of diabetes produced significant ($p < 0.001$) decrease in the serum SOD activity in comparison to the control group.

Treatment with simvastatin either alone or in combination with ezetimibe produced significant increase in the serum SOD activity ($p < 0.01$) and (p

< 0.001) respectively compared to the diabetic un-treated (vehicle) group.

Also, treatment with ezetimibe alone produced significant ($p < 0.05$) increase in the serum SOD activity in comparison to the diabetic un-treated (vehicle) group.

Statistical analysis showed that treatment with simvastatin in combination with ezetimibe significantly ($p < 0.001$) increased serum SOD activity compared to the diabetic SIM- treated group.

Table (4): Effect of simvastatin and/or ezetimibe treatment on SOD activity.

Groups/Parameters	SOD activity (U/L)
Control group	8.067 ± 0.252
DM + V group	2.200 ± 0.183 ###
DM + SIM group	4.700 ± 0.100 **
DM + EZE group	4.067 ± 1.422 *
DM + SIM + EZE group	6.767 ± 0.208 *** †††

Data are mean ± SE. (n=10 rats per each group). One way ANOVA followed by Tukey's Multiple Comparison Test: ### $p < 0.001$ compared with the control group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the diabetic un-treated (vehicle) group. ††† $p < 0.001$ compared with the diabetic SIM-treated group.

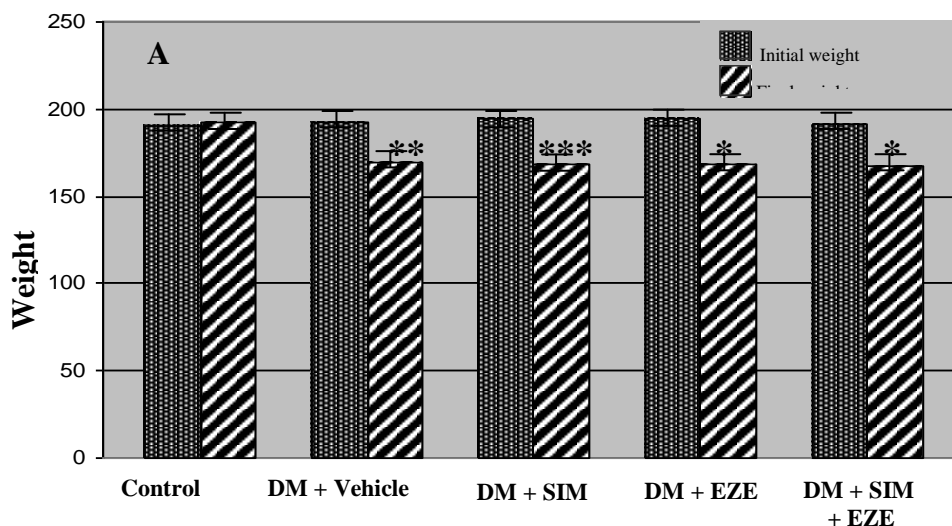


Fig. 1A: Mean changes in the body weight in the experimental groups. One way ANOVA followed by Tukey's Multiple Comparison Test: As compared with the initial weight, * $p < 0.005$, ** $p < 0.001$, *** $p < 0.0001$.

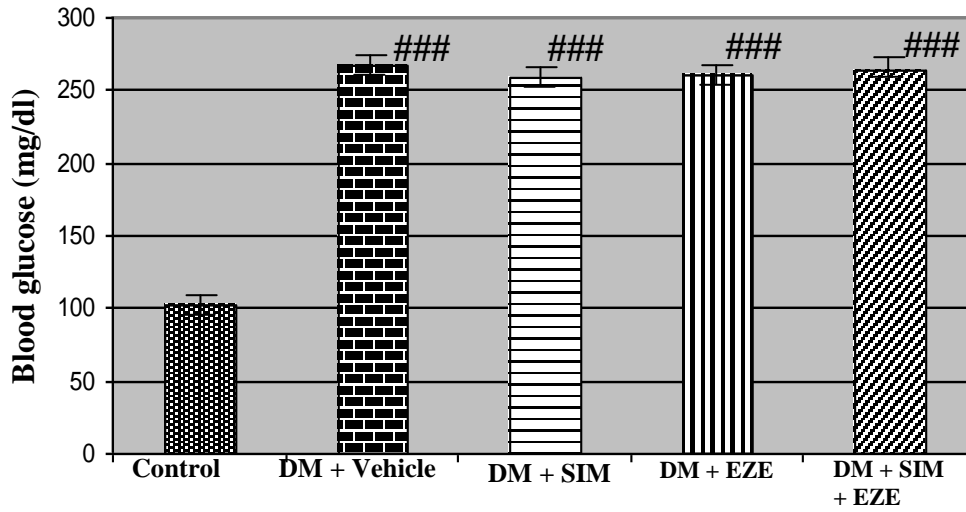


Fig. 1B: Mean changes in blood glucose levels in the experimental groups. One way ANOVA followed by Tukey's Multiple Comparison Test: As compared with the control group, ### $p < 0.0001$.

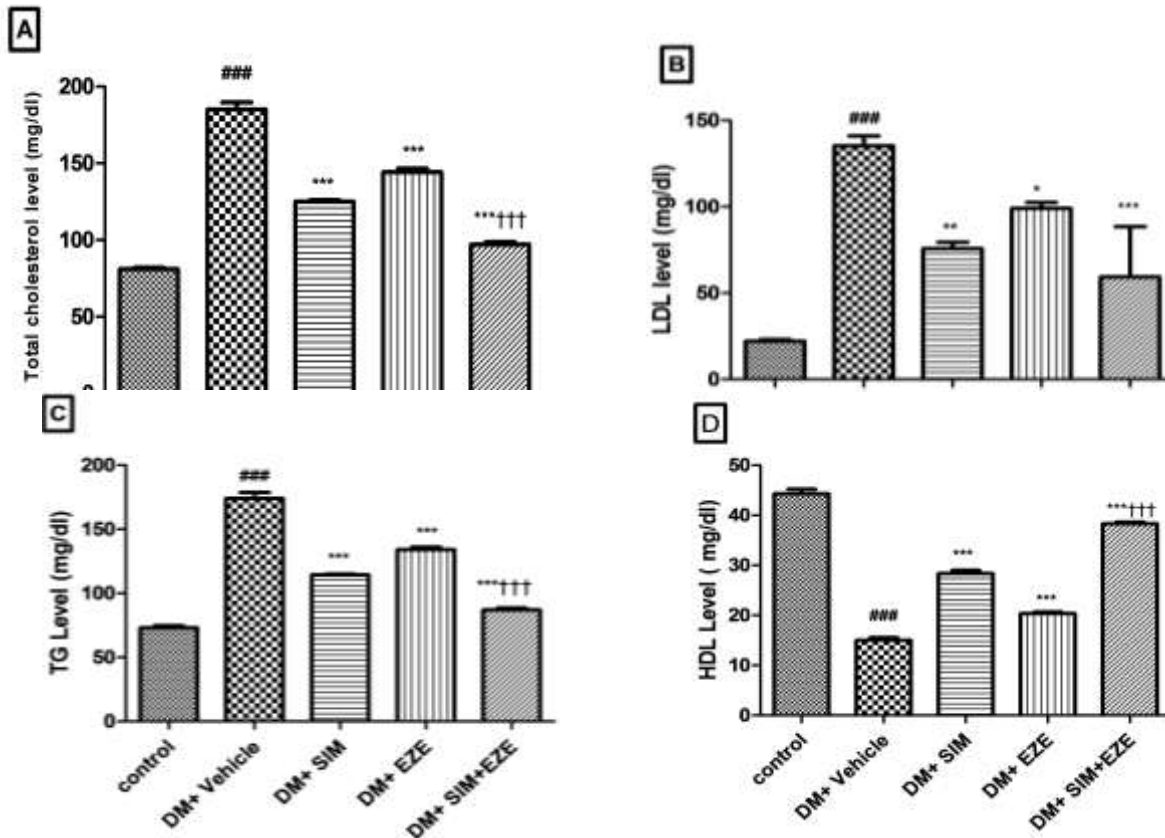


Fig. 2: Mean changes in serum total cholesterol (A), LDL (B), triglycerides (C) and HDL (D) in the experimental groups. One way ANOVA followed by Tukey's Multiple Comparison Test: ### $p < 0.001$ compared with the control group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the diabetic un-treated (vehicle) group. ††† $p < 0.001$ compared with the diabetic SIM-treated group.

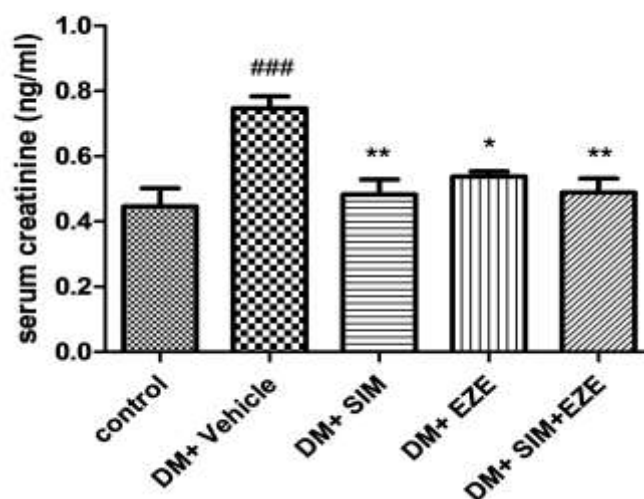


Fig. 3: Mean changes in serum creatinine (ng/ml) in the experimental groups. One way ANOVA followed by Tukey's Multiple Comparison Test: ### $p < 0.001$ compared with the control group. * $p < 0.05$, ** $p < 0.01$, compared with the diabetic un-treated (vehicle) group.

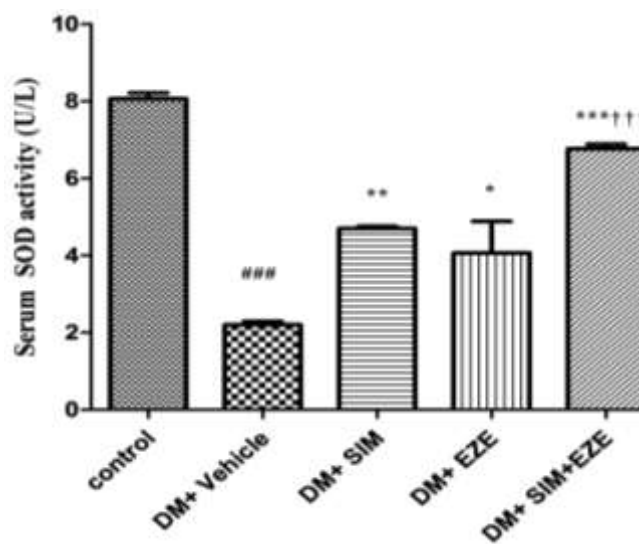


Fig. 4: Mean changes in serum SOD activity (U/L) in the experimental groups. One way ANOVA followed by Tukey's Multiple Comparison Test: ### $p < 0.001$ compared with the control group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the diabetic un-treated (vehicle) group. ††† $p < 0.001$ compared with the diabetic SIM-treated group.

4. Discussion

Diabetes mellitus (DM) is one of the most common human metabolic diseases. Derangements in lipid metabolism in DM are often important determinants of the course and status of the disease (Fumelli *et al.*, 1996).

Much of the pathophysiological changes linking diabetes and dyslipidemia has been elucidated. Diabetic dyslipidemia is one of the common events

responsible for accelerated macro and microvascular diseases in diabetic patients (Goldberg, 2001).

Statins are the cornerstone in the management of dyslipidemia. It has been demonstrated that statin therapy either for primary or secondary prevention of cardiovascular events is associated with a significant reduction of cardiovascular morbidity and mortality (Baigent *et al.*, 2005).

The present work was designed to evaluate the effects of simvastatin and/or ezetimibe treatment on some diabetic complications in streptozotocin induced diabetes in rats.

Results of the present work showed that induction of diabetes by STZ led to significant weight loss, significant elevation of serum glucose, total cholesterol, triglycerides, LDL, serum creatinine levels associated with significant decrease in serum HDL level and serum SOD activity in diabetic rats.

Results of the present work were concordant with **Eidi et al. (2007)** who reported that STZ induced DM led to significant final weight loss in diabetic rats associated with significant elevation of serum glucose, total cholesterol, triglycerides, LDL and serum creatinine levels in addition to significant decrease in serum HDL level. **Zhu et al. (2005)** has reported that plasma SOD levels were decreased in STZ-induced DM.

These effects may be due to metabolic disturbances in diabetes reflected in high activities of xanthine oxidase, lipid peroxidation and increased triacylglycerol and cholesterol levels (**Madinov et al., 2000**). Moreover, protein glycation in diabetes may lead to muscle wasting and increased release of purine as well as increased activity of xanthine oxidase (**Anwar and Meki, 2003**). Elevation of serum creatinine as significant renal function marker, is related to renal dysfunction in diabetic hyperglycemia (**Almadal and Vilstrup, 1988**).

Statins are lipid-lowering agents that competitively and reversibly inhibit HMG-CoA reductase enzyme which catalyzes the conversion of HMG-CoA to mevalonic acid, the rate-limiting step in cholesterol formation (**Maron et al., 2000**).

Ezetimibe is a class of lipid lowering compounds that selectively inhibits the intestinal absorption of cholesterol (**Neal and Jones, 2003**).

Our data showed that treatment with either simvastatin or ezetimibe significantly decrease total serum cholesterol, TG, LDL and serum creatinine levels. On the other hand, there was significant increase in serum HDL and SOD activity levels. However, a better outcome was observed on addition of ezetimibe to simvastatin which enhanced the ameliorative effect of simvastatin on metabolic and renal dysfunctions.

Results of the present study are consistent with previous works demonstrated that addition of ezetimibe to statins is more efficient in lowering lipid profile and enhancing metabolic outcome in cholesterol-fed hamster mice, dogs and rats (**Davis et al., 2001 & Van et al., 2001**). Also, **Al-Shaer et al. (2004)**, showed that, the addition of ezetimibe to statins produced further lowering of LDL-C of around 20% and had a more favorable effect on

HDL-C and triglycerides than statin therapy alone, even after doubling the dose of statins.

Addition of ezetimibe to simvastatin in type II diabetic patients demonstrated greater improvements in the lipid profile and a higher proportion of patients achieving LDL-C targets compared with doubling the dose of either simvastatin or atorvastatin (**Davis et al., 2001**).

Oxidative stress is determined by the relationship between reactive oxygen species (ROS) and the antioxidant defense system including antioxidant enzymes. ROS has been implicated in the pathogenesis of diabetes mellitus where increased ROS leads to lipid peroxidation which amplifies oxidative injury and causes damage to biomacromolecules as protein and nucleic acid (**Brownlee, 2001**).

In consistence with the present study, **Zhu et al. (2005)** has reported that in STZ-induced diabetic rats, plasma SOD was decreased and treatment with simvastatin showed a significant increase in the plasma level of SOD.

Statins have been proven to have antioxidative effects through elimination of free radicals directly, inhibit HMG-CoA reductase and promote synthesis of nuclear factor (**Zhu et al., 2005**).

In agreement with the present study ezetimibe at the dose of 3 mg/kg orally in rats produced a significant increase in SOD activity suggesting that ezetimibe reduces oxidative stress, thereby prevents the generation of free radicals and inhibits the development of atherosclerosis (**Pandya et al., 2006**).

It has demonstrated that administration of ezetimibe (10 mg/day) for three months prolongs the lag time to LDL oxidation and adding to simvastatin induces further prolongation of LDL oxidation compared to simvastatin monotherapy in hypercholesterolemic patients. Also, it has been reported that ezetimibe treatment attenuated vascular functions as endothelial dysfunction, oxidative stress and inflammation in high-fat fed apoE-deficient mice (**Nakagami et al., 2009**).

Our data showed that induction of diabetes by STZ produced significant increase in serum creatinine compared to the control group.

Lipids have been shown to play a role in renal inflammation and fibrosis, both of which lead to kidney injury. Experimental and clinical evidence suggest that lipid-lowering agents may preserve renal function in patients with chronic kidney disease (**Campese and Park, 2007**).

It has been reported that treatment with statins reduced the rate of decline in glomerular filtration rate (GFR) with a possible trend toward reduction of proteinuria (**Fried et al., 2001**). In addition, it has been reported that lowering the elevated serum lipids

has been associated with prevention of the decline in renal functions in patients with chronic renal disease (Shepherd *et al.*, 2008).

A study carried out by Zhang *et al.* (2011) supports the effectiveness of short-term high-dose statin pretreatment for both decreasing the level of serum creatinine and reducing the rate of contrast-induced nephropathy in patients undergoing diagnostic and interventional procedures requiring contrast media. Nakamura *et al.* (2009) proved that ezetimibe for six months reduced urinary excretion levels of protein in patients with non-diabetic chronic kidney disease and dyslipidemia.

These effects could be related to immunomodulatory effects, attenuation of endothelial dysfunction, enhances renal perfusion and reduction of abnormal permeability to plasma proteins in addition to reduction of blood pressure (Milionis *et al.*, 2007 & Feldstein, 2010). In addition, Usui *et al.* (2003) has reported that, statin treatment of STZ-induced diabetes in rats resulted in amelioration of albuminuria and glomerular hypertrophy.

From the results of the present study, it could be concluded that in DM, treatment with simvastatin or ezetimibe was found to be effective in ameliorating lipid profile, level of serum SOD activity and serum creatinine. However, addition of ezetimibe to simvastatin result in enhanced ameliorative effects of simvastatin on metabolic and renal dysfunctions in STZ-induced diabetes in rats.

Further studies are still necessary to clarify the possible underlying mechanism(s) of drug interaction of lipid lowering agents for better outcome of diabetic complications.

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