

Ameliorative Effect of Adjunct Therapy of Metformin with Atorvastatin on Streptozotocin-induced Diabetes Mellitus in Rats

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Key words

- diabetes mellitus
- metformin
- atorvastatin
- lipid profiles
- oxidative stress

Abstract

Metformin has been used for the treatment of diabetes, whereas atorvastatin reduces the incidence of atherosclerosis and ischemic heart disease. Therefore, combined treatment with metformin plus atorvastatin may be beneficial in diabetic patients associated with cardiac disease. The present study was designed to evaluate the combination therapy of metformin and atorvastatin on streptozotocin-induced diabetes mellitus in rats. Blood pressure, serum insulin, glucose, lipid profiles and antioxidant enzymes in pancreatic tissues were measured. Histopathological examination of pancreatic tissues was performed. Streptozotocin treated rats showed significant decrease in body weight and body mass index. Streptozotocin-treated rats showed a significant increase in the levels of blood pressure, serum glucose, triglycerides, total cholesterol and thiobarbituric acid reactive substance as well as a significant decrease in the levels of serum insulin, high density lipoprotein and reduced glutathione in pancreatic tissues. Administration of metformin plus atorvastatin for a period of 14 days significantly improved these biochemical parameters near to normal. The protective effect of metformin plus atorvastatin against streptozotocin-induced diabetes was further confirmed by histopathological examination. The results of present study suggest that metformin plus atorvastatin possess antioxidant activity and has a significant protective effect against streptozotocin-induced diabetes mellitus.

Streptozotocin (STZ) a naturally occurring broad spectrum antibiotic and is toxic to the insulin producing pancreatic β -cells. Oxidative stress may be responsible for deterioration of pancreatic β -cells. Thus, oxidative stress play a key role for the development of DM. Studies also reported that administration of STZ led to increase production of ROS resulting from metabolism of excessive glucose and free fatty acids, and decreased expression of antioxidant enzymes. Thus, STZ induced oxidative stress may play a major role for development of DM [2,3]. Metformin, a biguanide, is the most widely prescribed drug for the treatment of hyperglycaemia in type II diabetics [5,6]. It blocks hepatic gluconeogenesis and increases hepatic insulin sensitivity as well as to a lesser extent insulin mediated glucose uptake in fat and muscle tissue [7]. Metformin has a potent antioxidant and anti-inflammatory properties [8]. In recent years, study reported that metformin exerts several pleiotropic effects including beneficial changes in blood rheology, serum lipid profile and putative

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Introduction

Diabetes and its complications are a major health problem in modern societies. Diabetes mellitus (DM) comprises abnormalities of insulin action including insulin deficiency and insulin resistance. It is characterized by chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action or both [1]. It is commonly associated with endothelial dysfunction [2]. Various studies have reported that increased production of reactive oxygen species (ROS) resulting from metabolism of excessive glucose and free fatty acid as well as decreased expression of antioxidant enzymes have been responsible for deterioration of pancreatic β -cells [3]. Hyperglycaemia also enhances the oxidative modification of low density lipoprotein (LDL) which induces production of cellular adhesion molecules (CAM) and increase monocyte adhesion [4].

Streptozotocin (STZ) a naturally occurring broad spectrum antibiotic and is toxic to the insulin producing pancreatic β -cells. Oxidative stress may be responsible for deterioration of pancreatic β -cells. Thus, oxidative stress play a key role for the development of DM. Studies also reported that administration of STZ led to increase production of ROS resulting from metabolism of excessive glucose and free fatty acids, and decreased expression of antioxidant enzymes. Thus, STZ induced oxidative stress may play a major role for development of DM [2,3]. Metformin, a biguanide, is the most widely prescribed drug for the treatment of hyperglycaemia in type II diabetics [5,6]. It blocks hepatic gluconeogenesis and increases hepatic insulin sensitivity as well as to a lesser extent insulin mediated glucose uptake in fat and muscle tissue [7]. Metformin has a potent antioxidant and anti-inflammatory properties [8]. In recent years, study reported that metformin exerts several pleiotropic effects including beneficial changes in blood rheology, serum lipid profile and putative

anti-ischemic effects [9]. Atorvastatin has been reported to reduce the incidence of atherosclerosis, ischemic heart disease associated with diabetes [10] and nephrotoxicity [11]. Since the combined effect of metformin and atorvastatin has not been studied on diabetic rats, thus, the present study aimed to investigate the combined effect of metformin and atorvastatin on STZ-induced diabetes mellitus in rats.

Materials and Methods

Drugs and chemicals

Metformin and atorvastatin calcium were obtained as a gift samples from Alkem Research Center, Navi Mumbai, India. STZ was purchased from Sigma Aldrich (St. Louis, LO, USA). All other chemicals used in this study were of analytical grade.

Animals

Male Wistar rats weighing 180–250 g, were procured from the Animal House facility, KIET School of Pharmacy (Ghaziabad, India). The animals were kept in polypropylene cages under standard laboratory conditions (12 h light and 12 h dark cycle), and had a free access to commercial pellet diet (Pranav Agro Industries, New Delhi) and water *ad libitum*. The animal house temperature was maintained at $25 \pm 2^\circ\text{C}$. The protocol was approved by Institutional Animal Ethics Committee of KIET School of Pharmacy (Registration number 1099/07/CPCSEA).

Experimental design

After acclimatization, all rats were treated with STZ (60 mg/kg in 0.1 M citrate buffer, pH 4.5, intraperitoneally, single dose) except rats of Group I (normal control group). Rats having hyperglycemia (fasting blood glucose level ≥ 200 mg/dl) after 48 h of single injection of STZ were included in this study and divided in following groups of 8 animals: Group I (control group) – rats treated with citrate buffer (0.1 M) solution; Group II (diabetic group) – Animals received streptozotocin (60 mg/kg, intraperitoneally, single dose); Group III (Streptozotocin+metformin): Animals were treated with metformin (200 mg/kg/day) after 48 h of streptozotocin administration, for 14 days. Group IV and V (Streptozotocin+metformin+atorvastatin): Animals were treated with metformin (100 and 200 mg/kg, respectively) and atorvastatin (20 and 40 mg/kg, respectively) after 48 h of streptozotocin administration, for 14 days. Body mass index was calculated.

Measurement of hemodynamic parameters

Hemodynamic parameters (systolic, diastolic, mean arterial blood pressure, and heart rate) were measured by non-invasive blood pressure recorder using rat tail-cuff method (AD Instruments, Australia) on the 15th day.

Measurement of biochemical parameters in serum

Blood was collected from the retro-orbital plexus of overnight fasted rats using microcapillary tubes on 15th day. Serum was separated by centrifugation (4000 rpm for 10 min) and transferred to micro centrifuge tubes. Serum glucose, total cholesterol (TC), triglycerides (TGs) were measured using commercial kits from Span Diagnostics Ltd, Surat, Gujarat, India and high-density lipoprotein-cholesterol (HDL-C) from Reckon Diagnostics Pvt. Ltd., Baroda, Gujarat, India. The concentration of serum

insulin was measured using rat insulin ELISA kit (Alpco Diagnostics, Salem, USA).

Biochemical estimations in pancreatic tissue

Immediately after the sacrifice, the pancreas was excised out, washed in ice cold normal saline and weigh. Subsequently, the weighed quantity of pancreatic tissue from each group homogenized w/v in ice-chilled phosphate buffer (50 mM, pH 7.4) and centrifuge at 10000 g for 20 min. A clear supernatant was used for assaying different biochemical parameter according to the procedure. Lipid peroxidation was determined with spectrophotometric measurement of amount of malondialdehyde equivalents with thiobarbituric acid and was expressed as thiobarbituric acid-reactive substances (nmol malondialdehyde/mg protein) according to method of Ohkawa et al. [12]. Glutathione was measured according to Ellman, [13] method.

Histopathological examination

Pancreas tissue samples were fixed in 10% formalin buffered solution, cut into 5 μm sections and stained with haematoxylin & eosin. The sections were studied for pathological changes.

Statistical analysis

The results are expressed as mean \pm SEM. Comparison between the treatment groups and control groups were performed by analysis of variance (ANOVA) followed by Dunnett's t-test. The criteria for statistical significance was $p < 0.05$.

Results

Assessment of mortality, body weight and body mass index

In the present study, 10% mortality was observed in STZ treated group only. No mortality was found in other groups. Body weight and body mass index were significantly ($P < 0.001$) decreased in STZ treated rats compared to control group. Treatment with metformin along with atorvastatin significantly ($P < 0.05$ and $P < 0.01$) increased the body weight and body mass index as compared to STZ group (Table 1).

Assessment of hemodynamic parameters

There was significant ($P < 0.05$) increase in hemodynamic parameters (systolic, diastolic, mean BP and heart rate) in Group II i.e., diabetic control group as compared to the normal control

Table 1 Influence of metformin and atorvastatin on streptozotocin-induced changes in body weight, body mass index and mortality.

| Groups | BW (gm) | BMI (g/cm ²) | % Mortality |
|----------------------|--------------------------------|-------------------------------|-------------|
| Normal Control | 253.75 \pm 3.24 | 0.67 \pm 0.01 | 0 |
| STZ Control | 142.50 \pm 3.90 ^a | 0.42 \pm 0.021 ^a | 10 |
| STZ + MET 200 | 191.25 \pm 5.85 ^b | 0.54 \pm 0.022 ^b | 0 |
| STZ + MET100 + ATV20 | 193 \pm 6.39 ^b | 0.55 \pm 0.033 ^b | 0 |
| STZ + MET100 + ATV40 | 201.25 \pm 7.58 ^c | 0.58 \pm 0.042 ^c | 0 |

BW indicate body weight; BMI, body mass index; STZ, streptozotocin (60 mg/kg); STZ + MET: streptozotocin (60 mg/kg) + metformin (200 mg/kg); STZ + MET100 + ATV 20: streptozotocin (60 mg/kg) + metformin (100 mg/kg) + atorvastatin (20 mg/kg); STZ + MET200 + ATV40: streptozotocin (60 mg/kg) + metformin (200 mg/kg) + atorvastatin (40 mg/kg)

Data are expressed as mean \pm standard error of mean

^a $P < 0.001$ vs. Normal Control

^b $P < 0.05$ and ^c $P < 0.01$ vs. STZ Control

| Groups | Heart Rate (BPM) | Systolic BP (mmHg) | Diastolic BP (mm Hg) | Mean BP (mmHg) |
|----------------------|---------------------------|--------------------------|--------------------------|--------------------------|
| Normal Control | 392.29±6.5 | 114.56±2.96 | 99.96±1.58 | 107.26±2.39 |
| STZ Control | 568.70±12.34 ^a | 169.07±4.86 ^a | 126.95±2.90 ^a | 147.51±3.17 ^a |
| STZ + MET 200 | 472.09±8.07 ^b | 145.09±4.25 ^b | 115.20±2.71 ^b | 129.45±2.36 ^b |
| STZ + MET100 + ATV20 | 441.09±7.53 ^b | 133.02±3.16 ^b | 106.48±2.22 ^b | 119.25±2.32 ^b |
| STZ + MET100 + ATV40 | 414.21±5.15 ^c | 122.98±3.13 ^c | 102.95±2.13 ^c | 111.47±2.18 ^c |

Data are expressed as mean ± standard error of mean; ^aP<0.001 vs. Normal Control, ^bp<0.05 and ^cp<0.01 vs. STZ Control

Table 2 Influence of metformin and atorvastatin on systolic, diastolic, mean BP and heart rate of Wistar rats.

Table 3 Influence of metformin and atorvastatin on serum Total cholesterol, triglycerides, HDL-C, glucose and insulin levels of Wistar rats.

| Groups | Total Cholesterol (mg/dl) | Triglycerides (mg/dl) | HDL-C (mg/dl) | Glucose (mg/dl) | Insulin (ng/ml) |
|----------------------|---------------------------|--------------------------|-------------------------|--------------------------|-------------------------|
| Normal Control | 148.69±2.00 | 138.72±2.19 | 45.62±1.01 | 97.14±2.16 | 0.268±0.02 |
| STZ Control | 257.06±4.74 ^a | 206±5.63 ^a | 24.28±0.94 ^a | 328.21±4.65 ^a | 0.110±0.35 ^a |
| STZ + MET200 | 198.29±2.71 ^b | 197.59±2.42 | 36.63±0.64 ^b | 156.66±1.24 ^c | 0.156±0.02 ^b |
| STZ + MET100 + ATV20 | 175.08±3.30 ^c | 153.62±3.43 ^c | 40.46±0.79 ^b | 128.25±2.32 ^c | 0.188±0.02 ^c |
| STZ + MET100 + ATV40 | 163.29±2.71 ^c | 144.59±2.42 ^c | 42.86±0.64 ^c | 116.47±2.18 ^c | 0.196±0.01 ^c |

Data are expressed as mean ± standard error of mean; ^aP<0.001 vs. Normal Control, ^bp<0.05 and ^cp<0.01 vs. STZ Control

group (i.e., Group I) while the treatment with metformin along with atorvastatin significantly ($P<0.05$ and $P<0.01$) reduced the blood pressure and heart rate in dose dependent manner as compared to the Group II (○ Table 2).

Assessment of serum glucose

A significant ($P<0.001$) rise in the serum glucose level was observed in STZ treated rats. While the treatment with metformin plus atorvastatin significantly ($P<0.001$) decreased serum glucose as compared to STZ treated rats in a dose dependent manner (○ Table 3).

Assessment of serum high density lipoprotein-cholesterol (HDL-C) and insulin

There was a significant ($P<0.001$) decrease in the serum HDL-C and insulin levels in STZ group compared to control group. Metformin and atorvastatin treatment significantly ($P<0.05$ and $P<0.01$) increased the serum HDL-C and insulin levels in dose dependent manner (○ Table 3).

Assessment of serum total cholesterol and triglycerides

Compared to control group, serum total cholesterol (TC) and triglycerides (TGs) level were significantly ($P<0.01$) increased in STZ treated rats. Treatment of metformin and atorvastatin significantly ($P<0.05$ and $P<0.01$) decreased the TC and TGs level (○ Table 3).

Assessment of myocardial TBARS and antioxidant enzymes

The thiobarbituric acid reactive substance (TBARS) level was significantly ($P<0.001$) increased in STZ treated rats. TBARS level was significantly ($P<0.01$ and $P<0.001$) decreased on treatment with metformin and atorvastatin.

STZ treated group showed a significant ($P<0.001$) decreased in the level of reduced glutathione (GSH) as compared to normal control group. Treatment with metformin and atorvastatin the reduced GSH level was significantly ($P<0.01$ and $P<0.001$) increased in dose dependant manner (○ Table 4).

Histopathological analysis

Histopathological examination of the pancreatic tissue of normal control animals showed clear integrity of pancreatic cell membrane (○ Fig. 1a). No inflammatory cell infiltration was observed. STZ treated pancreatic tissue showed disorientation of

Table 4 Influence of metformin and atorvastatin on lipid peroxides (TBARS) and reduced glutathione (GSH) levels in pancreatic tissue of Wistar rats.

| Groups | TBARS (nmol MDA/mg protein) | GSH (μmol/mg protein) |
|----------------------|-----------------------------|-------------------------|
| Normal Control Group | 0.64±0.13 | 1.014±0.06 |
| STZ Control Group | 3.94±0.26 ^a | 0.365±0.06 ^a |
| STZ + MET 200 | 2.48±0.18 ^b | 0.686±0.07 ^b |
| STZ + MET100 + ATV20 | 1.96±0.33 ^b | 0.816±0.08 ^b |
| STZ + MET100 + ATV40 | 1.24±0.12 ^c | 0.952±0.08 ^c |

Data are expressed as mean ± standard error of mean; ^aP<0.001 vs. Normal Control, ^bp<0.05 and ^cp<0.01 vs. STZ Control

muscle fibers, widespread myocardial structure with moderate infiltration of lymphocytes and macrophages (○ Fig. 1b) as compared to control. Treatment with metformin (100 and 200 mg/kg, respectively) plus atorvastatin (20 and 40 mg/kg, respectively) showed marked improvement in the disorientation of muscle fibers, subendocardial necrosis and reduction in the inflammatory cells (○ Fig. 1c–e).

Discussion

Diabetes is increasing at an alarming rate worldwide, which mainly be attributed to the sedentary life style and calorie-rich diet. Oxidative stress and alteration in lipid profile is often associated with diabetes. In the present study, the combined effect of metformin and atorvastatin attenuated blood pressure, heart rate, serum glucose levels, lipid profile and lipid peroxidation as well as augment of serum insulin and antioxidant activities.

In the present study, there was a significant decrease in body weight and BMI in STZ treated rats which were markedly increased on treatment with metformin and atorvastatin combination. Alterations in body weight and BMI in STZ treated rats could be due to consecutive loss of protein [14].

In diabetes, hyperglycemia is associated with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action or both. There was significant ($P<0.001$) rise in the serum glucose level and decrease in serum insulin levels in STZ treated rats. Treatment with metformin and atorvastatin significantly ($P<0.001$) altered the serum insulin and glucose levels. Such effect may be due to decrease in the rate

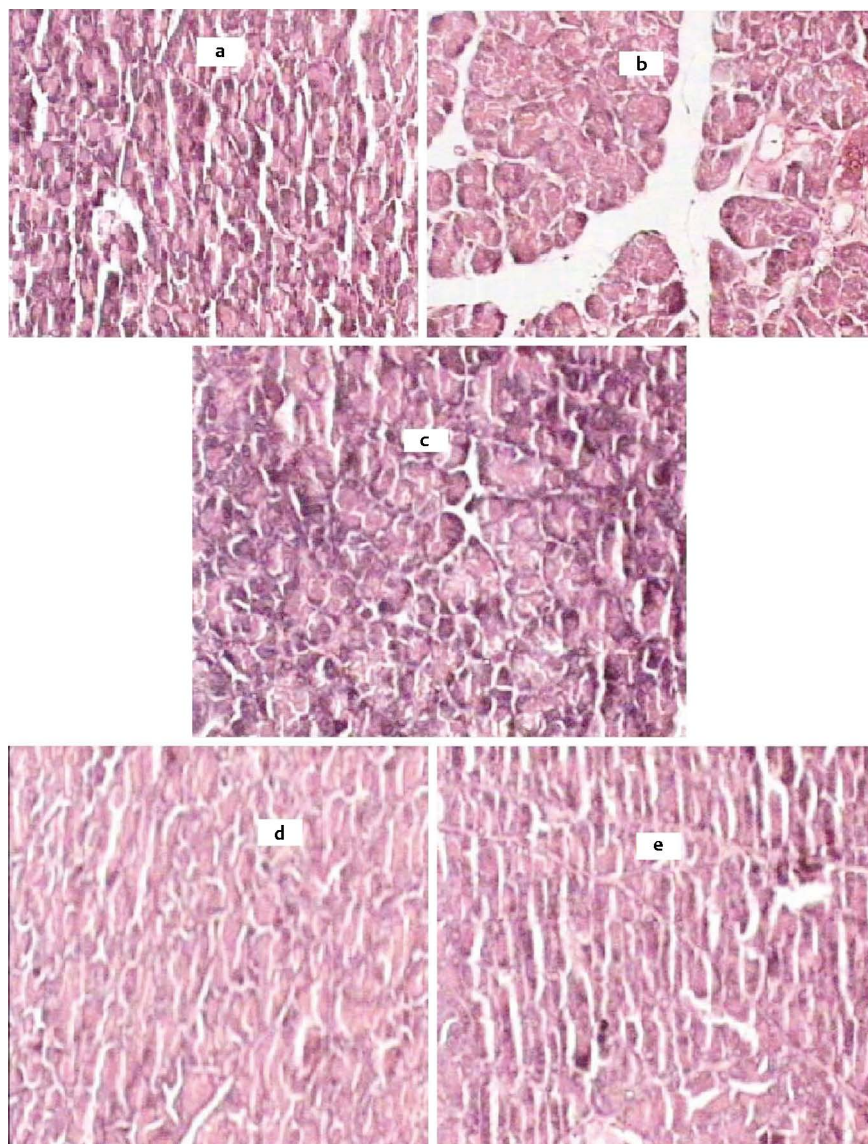


Fig. 1 Effect of metformin and atorvastatin on histopathology of pancreatic tissue. **a** Normal Control treated pancreatic tissue showing normal architecture **b** STZ treated pancreatic tissue showing damaged β -cell & edema **c** STZ + MET200 treated pancreatic tissue showing improvement of damage β -cell and reduces edema formation **d** STZ + MET100 + ATV20 treated pancreatic tissue showing improvement of damage β -cell and reduces edema formation **e** STZ + MET200 + ATV40 treated pancreatic tissue showing damage β -cells near to normal. (Colour figure available online only).

of intestinal glucose absorption or an increase in peripheral glucose utilization [15].

Hyperglycemia is also accompanied with dyslipidemia [16] which causes increased lipolysis in adipose tissues and increased entry of free fatty acids (FFA) to the liver [2, 3]. Increased FFA and insulin independent glucose uptake in the liver is reported to stimulate triglyceride (TG) synthesis [17] that tends to reduce protein content of lipoproteins such as LDL and increases the TG content [18]. Dyslipidemia is an impending atherogenic risk factor in diabetes [18]. In the present study, STZ significantly increased serum total cholesterol (TC) and TG levels as well as significantly decreased serum HDL-C levels which were aligned with previous studies [19]. Administration of combined metformin and atorvastatin significantly attenuated serum TC and TG as well as significantly augment serum HDL-C levels. These finding demonstrated that combination of these drugs ameliorate dyslipidemia associated with diabetes.

Lipid peroxidation is an indication of the severity of STZ-induced diabetes. The increased levels of TBARS may contribute to increased generation of free radicals and/or decreased activities of antioxidant enzymes [20]. The results of the present study showed that combined treatment with metformin and atorvastatin significantly decreased STZ-induced elevation of TBARS

level probably due to restoration of antioxidant enzymes that neutralized and/or scavenged the free radicals.

Hyperglycemia is a main cause for elevated free radical levels, followed by production of ROS, which can lead to altered antioxidant defense, insulin resistance and further impair glucose metabolism in biological system [21]. The antioxidant enzymes such as GSH, SOD and CAT are regarded as the first line of the antioxidant defense system against ROS generated during oxidative stress. In the present study, STZ treated rat's significantly decreased GSH level which goes fine with previous study [22]. Treatment with both metformin and atorvastatin augment GSH level, suggesting that it could boost the cellular antioxidative defense against oxidative stress. Atorvastatin possesses significant antioxidant and antihyperlipidemic activity due to reduction of lipid peroxidation. Therefore, treatment with both metformin and atorvastatin could be useful intervention to the diabetic patients.

Conclusion

The present study provides experimental evidence that combination of metformin and atorvastatin has a potent pleiotropic

effects including beneficial antioxidant activity, changes in blood rheology, serum lipid profile and putative anti-ischemic effects. Hence the combination therapy of metformin and atorvastatin could be useful intervention for the treatment of STZ-induced diabetes mellitus in rats.

Conflict of Interest

Authors have no conflict of interest.

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