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Ameliorative effect of combination of benfotiamine and fenofibrate in diabetes-induced vascular endothelial dysfunction and nephropathy in the rat

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Abstract The study has been designed to investigate the effect of benfotiamine and fenofibrate in diabetes-induced experimental vascular endothelial dysfunction (VED) and nephropathy. The single administration of streptozotocin (STZ) (50 mg/kg, i.p.) produced diabetes, which was noted to develop VED and nephropathy in 8 weeks. The diabetes produced VED by attenuating acetylcholine-induced endothelium dependent relaxation, impairing the integrity of vascular endothelium, decreasing serum nitrite/nitrate concentration and increasing serum TBARS and aortic superoxide anion generation. Further, diabetes altered the lipid profile by increasing the serum cholesterol, triglycerides and decreasing the high density lipoprotein. The nephropathy was noted to be developed in the diabetic rat that was assessed in terms of increase in serum creatinine, blood urea, proteinuria, and glomerular damage. The benfotiamine (70 mg/kg, p.o.) and fenofibrate (32 mg/kg, p.o.) or lisinopril (1 mg/kg, p.o., a standard agent) treatments were started in diabetic rats after 1 week of STZ administration and continued for 7 weeks. The treatment with benfotiamine and fenofibrate either alone or in combination attenuated diabetes-induced VED and nephropathy. In addition, the combination of benfotiamine and fenofibrate was noted to be more effective in attenuating the diabetes-induced VED and nephropathy when compared to treatment with either drug alone or lisinopril. Treatment with fenofibrate normalizes the altered lipid profile in diabetic rats, whereas benfotiamine treatment has no effect on lipid alteration in diabetic rats. It may be concluded that diabetes-induced oxidative stress, lipids alteration, and consequent development of VED may be responsible for the induction of nephropathy in diabetic rats. Concurrent administration of benfotiamine and fenofibrate may provide synergistic benefits in preventing the development of diabetes-induced nephropathy by reducing the oxidative stress and lipid alteration, preventing the VED and subsequently improving the renal function.

Keywords Diabetes · Oxidative stress · Vascular endothelial dysfunction · Nephropathy · Benfotiamine · Fenofibrate

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Introduction

Diabetes is a metabolic disorder characterized by hyperglycemia followed by micro and macrovascular complications [1]. Diabetic nephropathy is a leading cause of morbidity and mortality in developing countries and its prevalence is continuously increasing. Vascular endothelium is an interior lining of blood vessels and lies between circulating blood and the vascular smooth muscle cells [2]. Endothelium regulates vascular tone and maintains free flow of blood in vessels [3, 4]. VED has been characterized by reduced activation of endothelial nitric oxide synthase (eNOS), reduced generation and bioavailability of nitric oxide (NO) and increased production of reactive oxygen species (ROS) [5–8]. VED has been associated in the pathogenesis of diabetes mellitus [9–11], nephropathy [12, 13], hypertension [7], coronary artery disease [5], and atherosclerosis [14]. The basic and clinical studies have demonstrated the strong correlation between diabetes and VED [9–11, 13, 15, 16]. The increased serum concentration of advanced glycation end products (AGEs) in patients with diabetes has been associated with VED [10]. High

concentration of glucose has been shown to induce endothelial apoptotic cell death by activating bax-caspase proteases pathway [14, 17]. It has been demonstrated that hyperglycemia scavenges NO and induces VED, which ultimately results in nephropathy [12, 16, 18]. VED increases the deposition of extra cellular matrix that leads to glomerulosclerosis and progressive decline in glomerular filtration rate to produce nephropathy [19]. Diabetes upregulates Rho-kinase, which is a negative regulator of eNOS and thus implicated in the pathogenesis of VED [20]. Further, inhibition of Rho-kinase by fasudil produced renoprotective effects in diabetic rats [21]. These results reveal the pathological relationship between VED and diabetic nephropathy. In diabetes, high cytosolic glucose concentration in renal endothelial cells activates protein kinase-Cβ, hexosamine, and polyol pathways that lead to oxidative stress and accumulation of AGE products [22]. The activation of transketolase converts glucose substrates directly in to pentose phosphate pathway, thus transketolase activation blocks endogenous AGE formation [23]. Benfotiamine, a lipophilic derivative of thiamine, has been shown to activate transketolase and thus improve the function of endothelium and diabetic kidney by preventing the accumulation of AGE products [23, 24]. The pleiotropic actions of benfotiamine in improving the function of vascular endothelium have been related to the activation of Akt, stimulation of eNOS, and inhibition of ROS formation [23, 25]. The experimental evidences suggest that hyperlipidemia is a major risk factor involved in diabetic nephropathy by increasing the expression of sterol regulatory element-binding protein (SREBP), which synthesizes triglycerides and low density lipoprotein (LDL) in diabetic kidney to induce glomerulosclerosis [26, 27]. Activation of PPAR-α has been well known to produce hypolipidemic action [28–30]. Treatment with fenofibrate, a PPAR-α agonist improved the function of endothelium by increasing NO bioavailability and reducing the level of LDL and downregulating the expression of proinflammatory cytokine like tumor necrosis factor-α (TNF-α) [29, 30]. Further, fenofibrate has been shown to reduce glomerular hypertrophy, mesangial matrix expansion and suppress the expression of plasminogen activator inhibitor-1 (PAI-1) and transforming growth factor-β (TGF-β) [31]. Therefore, the present study has been undertaken to investigate the combined effect of benfotiamine and fenofibrate in diabetes-induced experimental VED and nephropathy.

Materials and methods

The experimental protocol used in the present study was approved by the Institutional Animal Ethical Committee. Age matched young Wistar rats weighing about 200–250 g were employed in the present study. Rats were fed on standard chow diet and water ad libitum. They were acclimatized in animal house and were exposed to normal cycle of light and dark.

Assessment of diabetes and lipid profile

At the end of the experimental protocol, the blood samples were collected and serum was separated. The serum samples were frozen until analyzing the biochemical parameters. The serum glucose concentration was estimated by glucose oxidase peroxidase (GOD-POD) method [32] using the commercially available kit (Vital diagnostics, Mumbai, India). The serum total cholesterol was estimated by cholesterol oxidase peroxidase (CHOD-POD) method [33] using the commercially available kit (Medsource Ozone Biomedicals Pvt. Ltd., Faridabad, India). The serum triglyceride was estimated by glycerophosphate oxidase peroxidase (GPO-POD) method [34] using the commercially available kit (Kamineni Life Sciences Pvt. Ltd., Hyderabad, India). The serum high density lipoprotein (HDL) was estimated by cholesterol oxidase peroxidase (CHOD-POD) method [34] using the commercially available kit (Medsource Ozone Biomedicals Pvt. Ltd., Faridabad, India).

Assessment of vascular endothelial dysfunction

Isolated rat aortic ring preparation

The rat was decapitated, thoracic aorta was removed, cut into a ring of 3–4 mm in length and mounted in an organ bath containing Krebs–Henseleit solution (NaCl, 119 mM; KCl, 4.7 mM; $NaHCO_3$, 25 mM; $MgSO_4$, 1.0 mM; glucose, 11.1 mM; KH_2PO_4, 1.2 mM; and $CaCl_2$, 2.5 mM) of pH 7.4, bubbled with carbonated oxygen (95% O_2 and 5% CO_2) and maintained at 37°C. The preparation was allowed to equilibrate for 90 min under 1.5 g tension. The isometric contractions were recorded with a force-transducer (Ft-2040) connected to a physiograph (INCO, Ambala, India). The aortic ring preparation was primed with 80 mM KCl to check its functional integrity and to improve its contractility. The cumulative dose responses of acetylcholine (Ach; 10^{-8} to 10^{-4} M) or sodium nitroprusside (SNP; 10^{-8} to 10^{-4} M) were recorded in phenylephrine (3×10^{-6} M) precontracted preparation with intact or denuded endothelium, respectively [20, 35].

Scanning electron microscopic study

The scanning electron microscopic study was performed to examine the integrity of vascular endothelium [36, 37]. A total of 3–4 mm longitudinal strips of thoracic aorta were fixed in 3% glutaraldehyde phosphate buffer (pH 7.4) and subsequently dehydrated in a series of alcohol and

acetone solution (50% for 20 min, 70% for 20 min, 80% for 20 min, 90% for 20 min, and 100% for 50 min) and isoamylacetate (100%): acetone (100%) solution (1:1) for 20 min followed by isoamylacetate (100%) for 20 min. Arterial segments were dried using four flush of liquid CO_2 with 100 psi pressure in critical point drier. The dried tissues from each arterial segment were mounted on aluminum stubs and coated with gold palladium (JFC-1100) and were viewed using JOEL JSM 6100 scanning electron microscope to observe the integrity of vascular endothelium (800×).

Estimation of serum nitrite/nitrate concentration

A total of 400 µl of carbonate buffer (pH 9.0) was added to 100 µl of serum sample followed by the addition of small amount (~0.15 g) of copper–cadmium alloy. The tubes were incubated at room temperature for 1 h to reduce nitrate to nitrite. The reaction was stopped by adding 100 µl of 0.35 M sodium hydroxide. Following this, 400 µl of zinc sulfate solution (120 mM) was added to deproteinate the serum samples. The samples were allowed to stand for 10 min and then centrifuged at 4,000*g* for 10 min. Greiss reagent (250 µl of 1.0% sulfanilamide and 250 µl of 0.1% *N*-naphthylethylenediamine) was added to aliquots (500 µl) of clear supernatant and serum nitrite/nitrate was measured spectrophotometrically at 545 nm. The standard curve of sodium nitrite (1–40 µM) was plotted to calculate concentration of serum nitrite/nitrate [35].

Assessment of oxidative stress

The oxidative stress was assessed by estimating serum thiobarbituric acid reactive substances (TBARS) and aortic superoxide anion generation.

Estimation of TBARS

A total of 1 ml of 20% trichloroacetic acid was added to 100 µl of serum and 1% thiobarbituric acid (TBA) reagent (1.0 ml), which were mixed and incubated at 100°C for 30 min. After cooling on ice, samples were centrifuged at 1,000*g* for 20 min. Serum concentration of TBARS was measured spectrophotometrically at 532 nm. A standard curve using 1,1,3,3-tertramethoxyopropane (1–10 µM) was plotted to calculate the concentration of TBARS [35].

Estimation of superoxide anion

Aorta was cut into transverse rings of 6 mm in length and placed in 5 ml of Krebs–Henseleit solution containing 100 µM of nitroblutetrazolium (NBT) and incubated at 37°C for 1.5 h. The NBT reduction was stopped by adding 5 ml of 0.5 N HCl. The rings were minced and homogenized in a mixture of 0.1 N NaOH and 0.1% sodium dodecoyl sulphate (SDS) in water containing 40 mg/l of diethylene triamine pentaacetic acid (DTPA). The mixture was centrifuged at 20,000*g* for 20 min and the resultant pellets were resuspended in 1.5 ml of pyridine and kept at 80°C for 1.5 h to extract formazan. The mixture was centrifuged at 10,000*g* for 10 min and the absorbance of formazan was determined spectrophotometrically at 540 nm. The amount of reduced NBT was calculated using the following formula:

$$\text{Amount of reduced NBT} = \frac{A \times V}{(T \times \text{Wt} \times \varepsilon \times l)}$$

where A is absorbance, V is volume of solution (1.5 ml), T is time for which aortic rings were incubated with NBT (90 min), Wt is blotted wet weight of aortic rings, ε is extinction coefficient (0.72 l mM^{-1} mm^{-1}), and l is length of light path (10 mm). The result was expressed as reduced NBT in picomoles per min per mg of wet tissue [35].

Assessment of nephropathy

The serum creatinine concentration was estimated by alkaline picrate kinetic method [38] using the commercially available kit (Vital diagnostics, Mumbai, India). Further, the blood urea was estimated by Berthelot method [39] using the commercially available kit (Kamineni Life Sciences Pvt. Ltd., Hyderabad, India). Moreover, urinary protein concentration was estimated by pyrogallol red method [40] using the commercially available kit (Kamineni Life Sciences Pvt. Ltd., Hyderabad, India).

Histopathological study

The early diabetic changes in glomeruli were assessed histologically as previously described [41, 42]. The kidney was excised and immediately immersed in 10% formalin. The kidney was dehydrated in graded concentrations of alcohol, immersed in xylene and then embedded in paraffin. From the paraffin blocks, sections of 5 µm in thickness were made and stained with hematoxylin and eosin to assess the pathological changes occur in glomeruli using light microscopy (400×).

Experimental protocol

Eight groups were employed in the present study and each group comprised of 12–14 rats. The benfotiamine and fenofibrate were dissolved in 1% w/v of carboxy methyl cellulose (CMC). Group I (Normal Control), rats were maintained on standard food and water and no treatment was given. Group II (Diabetic Control), rats were

administered STZ (50 mg/kg, i.p. once) dissolved in citrate buffer of pH 4.5. Group III (Benfotiamine per se), normal rats were administered benfotiamine (70 mg/kg, p.o.) dissolved in 1% w/v of CMC for 7 weeks. Group IV (Fenofibrate per se), normal rats were administered fenofibrate (32 mg/kg, p.o.) dissolved in 1% w/v CMC for 7 weeks. Group V (Benfotiamine Treated Diabetic Group), the diabetic rats after 1 week of STZ administration were treated with benfotiamine (70 mg/kg, p.o.) for 7 weeks. Group VI (Fenofibrate Treated Diabetic Group), the diabetic rats after 1 week of STZ administration were treated with fenofibrate (32 mg/kg, p.o.) for 7 weeks. Group VII (Benfotiamine + Fenofibrate Treated Diabetic Group), the diabetic rats after 1 week of STZ administration were treated with the combination of benfotiamine (70 mg/kg, p.o.) and fenofibrate (32 mg/kg, p.o.) for 7 weeks. Group VIII (Lisinopril Treated Diabetic Group), the diabetic rats after 1 week of STZ administration were treated with lisinopril (1 mg/kg, p.o.) for 7 weeks.

Statistical analysis

All values were expressed as mean ± SEM. Data for isolated aortic ring preparation were statistically analyzed using repeated measures of ANOVA followed by Newman Keuls test. The data for serum levels of nitrite/nitrate, TBARS and aortic superoxide anion generation, serum creatinine, blood urea, proteinuria serum glucose, and lipid profile were statistically analyzed using one way ANOVA followed by Tukey's multiple range test. A P value <0.05 was considered to be statistically significant and P values were of two-tailed.

Drugs and chemicals

Streptozotocin and acetylcholine hydrochloride were obtained from Sigma-Aldrich Ltd., St. Louis, USA. Diethylene triamine pentaacetic acid (DTPA) and nitroblue tetrazolium (NBT) were purchased from Sanjay Biologicals, Amritsar, India. 1,1,3,3-tetra methoxypropane and carboxymethyl cellulose were purchased from V. K. Chemicals and Instruments, Ambala, India. Benfotiamine was obtained from Orchid Health Care Ltd., Chennai. Fenofibrate was obtained from Ranbaxy Laboratory Ltd., Gurgaon, India. Lisinopril was obtained from Dr. Reddy's Laboratory Ltd., Hyderabad, India. All other chemicals used in the present study were of analytical grade.

Results

The administration of benfotiamine (70 mg/kg, p.o., 7 weeks) or fenofibrate (32 mg/kg, p.o., 7 weeks) or lisinopril (1 mg/kg, p.o., 7 weeks) to normal rats did not produce any significant per se effects on various parameters assessed in the present study. Administration of streptozotocin (50 mg/kg, i.p., once) produced hyperglycemia after 72 h (serum glucose > 180 mg/dL). After 7 days of STZ administration, the rats showed blood glucose level >220 mg/dL were selected and were named as diabetic rats. Benfotiamine, fenofibrate, and lisinopril were administered to diabetic rats after 7 days of single injection of STZ and their treatments were continued for 7 weeks. All parameters were assessed at the end of 7 weeks in normal and diabetic rats with or without drug treatments. The diabetes for 7 weeks (8 weeks after STZ single injection) was observed to produce less than 10% mortality in rats with or without drug treatments.

Effect of pharmacological interventions on serum glucose

The marked increase in serum concentration of glucose was noted in diabetic rats when compared with normal rats. Treatment with benfotiamine (70 mg/kg, p.o., 7 weeks) or fenofibrate (32 mg/kg, p.o., 7 weeks) did not affect the serum glucose concentration in diabetic rats. However, treatment with lisinopril (1 mg/kg, p.o., 7 weeks) slightly reduced the glucose level in diabetic rats; but the result was not statistically significant (Table 1).

Effect of pharmacological interventions on lipid profile

The increase in serum concentration of total cholesterol and triglycerides and decrease in HDL were noted in diabetic rats when compared with normal rats. Treatment with benfotiamine (70 mg/kg, p.o., 7 weeks) did not affect the lipid alterations in diabetic rats. However, treatment with fenofibrate (32 mg/kg, p.o., 7 weeks) either alone or in combination with benfotiamine (70 mg/kg, p.o., 7 weeks) significantly attenuated diabetes-induced alterations in serum lipids. In addition, treatment with lisinopril partially prevented diabetes-induced increase in total cholesterol and triglycerides and decrease in HDL level (Table 1).

Effect of pharmacological interventions on endothelium dependent and independent relaxation

Acetylcholine (Ach) and sodium nitroprusside (SNP) were noted to produce endothelium dependent and independent relaxation, respectively, in phenylephrine (3×10^{-6} M) precontracted isolated normal rat aortic ring preparation in a dose–dependent manner. In diabetic rat aortic ring preparation, Ach-induced endothelium dependent relaxation was

Table 1 Effect of benfotiamine (benfo), fenofibrate (feno), and lisinopril on serum glucose and lipid profile

Assessment	Normal control	Diabetic control	Benfo per se	Feno per se	Benfo treated diabetic group	Feno treated diabetic group	Benfo + feno treated diabetic group	Lisinopril treated diabetic group
Serum glucose (mg/dl)	110.5 ± 7.6	329.4 ± 11.4[a]	109.2 ± 6.9	114.6 ± 8.3	320.6 ± 11.1	324.7 ± 9.5	318.6 ± 10.6	294.3 ± 9.8
Serum cholesterol (mg/dl)	73.5 ± 6.6	168.1 ± 10.1[a]	73.6 ± 5.9	73.1 ± 6.4	161.2 ± 11.1	95.4 ± 7.2[b]	83.6 ± 6.3[b,c]	106.7 ± 8.1[b]
Triglycerides (mg/dl)	80.2 ± 5.4	145.2 ± 9.3[a]	80.5 ± 6.1	79.1 ± 5.4	140.9 ± 10.3	95.8 ± 7.9[b]	90.3 ± 8.2[b,c]	107.8 ± 8.6[b]
High density lipoprotein (mg/dl)	38.6 ± 1.2	31.4 ± 1.3[a]	38.4 ± 0.9	39.9 ± 1.2	32.7 ± 0.8	37.8 ± 0.9[b]	40.4 ± 1.3[b]	36.2 ± 0.7[b]

All values are represented as mean ± SEM

[a] $P < 0.05$ versus normal control

[b] $P < 0.05$ versus diabetic control

[c] $P < 0.05$ versus lisinopril treated diabetic group

noted to be significantly attenuated, which was prevented in aortic rings of diabetic rats treated with benfotiamine (70 mg/kg, p.o., 7 weeks) or fenofibrate (32 mg/kg, p.o., 7 weeks) or lisinopril (1 mg/kg, p.o., 7 weeks). Moreover, marked and significant attenuation of diabetes mediated reduction in Ach-induced endothelium dependent relaxation was noted in aortic rings of diabetic rats concurrently treated with both benfotiamine (70 mg/kg, p.o., 7 weeks) and fenofibrate (32 mg/kg, p.o., 7 weeks) than treatment with either drug alone or lisinopril (Figs. 1 and 2).

Effect of pharmacological interventions on integrity of vascular endothelium

The integrity of vascular endothelium was noted to be impaired in thoracic aorta of diabetic rats. However, the treatment with benfotiamine (70 mg/kg, p.o., 7 weeks) or fenofibrate (32 mg/kg, p.o., 7 weeks) or lisinopril (1 mg/kg, p.o., 7 weeks) improved the integrity of vascular endothelium in diabetic rats. Moreover, the combination of benfotiamine (70 mg/kg, p.o., 7 weeks) and fenofibrate

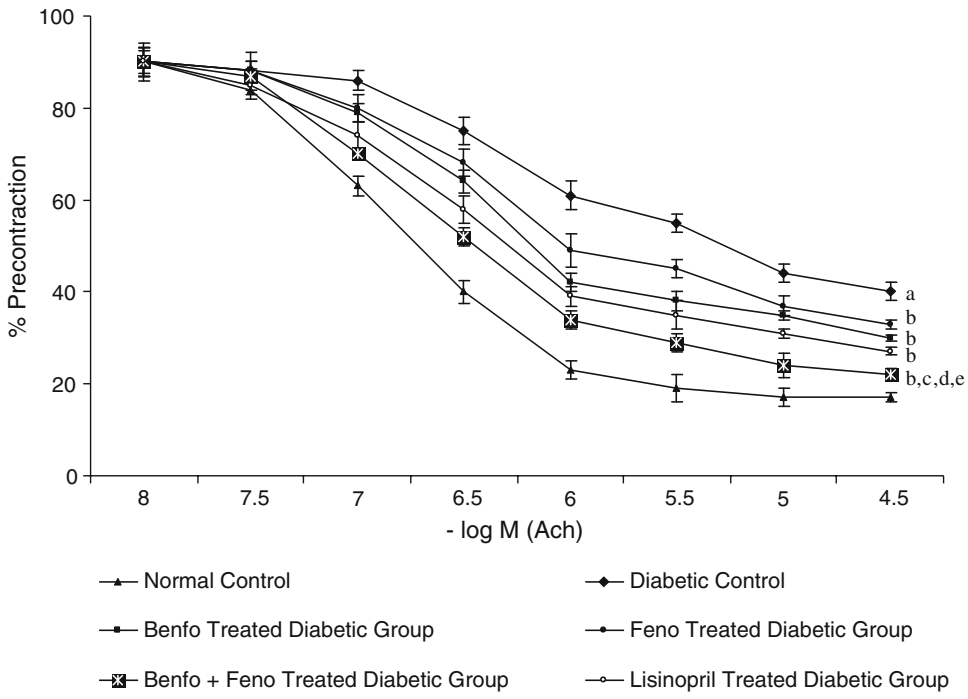

Fig. 1 Effect of benfotiamine (benfo) and fenofibrate (feno) on acetylcholine (Ach)-induced endothelium dependent relaxation. Responses were expressed as percentage of maximum contraction induced by phenylepherine (3×10^{-6} M). All values are represented as mean ± SEM. a = $P < 0.05$ versus normal control; b = $P < 0.05$ versus diabetic control; c = $P < 0.05$ versus benfo treated diabetic group; d = $P < 0.05$ versus feno treated diabetic group; e = $P < 0.05$ versus lisinopril treated diabetic group

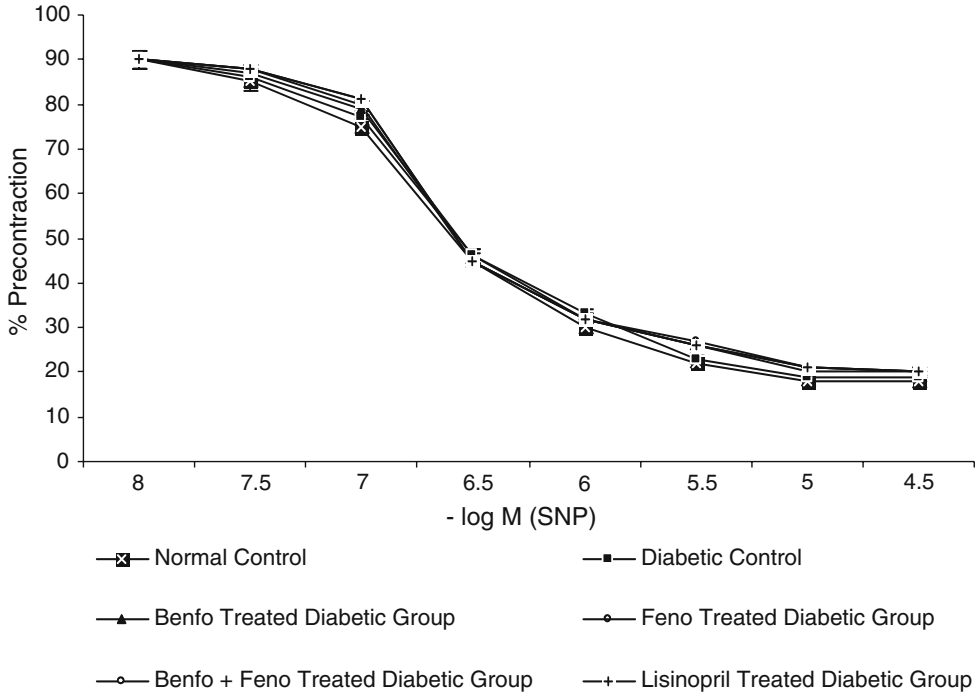

Fig. 2 Effect of benfotiamine (benfo) and fenofibrate (feno) on sodium nitroprusside (SNP)-induced endothelium independent relaxation. Responses were expressed as percentage of maximum contraction induced by phenylephrine (3×10^{-6} M). All values are represented as mean ± SEM

(32 mg/kg, p.o., 7 weeks) markedly prevented the diabetes-induced impairment in integrity of endothelium as compared to treatment with either drug alone or lisinopril (Fig. 3).

Effect of pharmacological interventions on serum nitrite/nitrate concentration

The serum concentration of nitrite/nitrate was noted to be reduced in diabetic rats when compared with normal rats. However, treatment with benfotiamine (70 mg/kg, p.o., 7 weeks) or fenofibrate (32 mg/kg, p.o., 7 weeks) or lisinopril (1 mg/kg, p.o., 7 weeks) significantly attenuated diabetes-induced reduction in serum nitrite/nitrate concentration. Moreover, concurrent administration of benfotiamine (70 mg/kg, p.o., 7 weeks) and fenofibrate (32 mg/kg, p.o., 7 weeks) markedly restored the reduced serum concentration of nitrite/nitrate in diabetic rats when compared with treatment of either drug alone or lisinopril (Fig. 4).

Effect of pharmacological interventions on serum TBARS and aortic superoxide generation

The increase in serum TBARS concentration and aortic superoxide anion generation was noted in diabetic rats when compared with normal rats. However, treatment with benfotiamine (70 mg/kg, p.o., 7 weeks) or fenofibrate (32 mg/kg, p.o., 7 weeks) or lisinopril (1 mg/kg, p.o., 7 weeks) significantly attenuated diabetes-induced increase in serum TBARS and aortic superoxide anion generation. In addition, the marked reduction in serum TBARS and aortic superoxide generation was noted in diabetic rats treated with combination of benfotiamine (70 mg/kg, p.o., 7 weeks) and fenofibrate (32 mg/kg, p.o., 7 weeks) as compared to treatment with either drug alone or lisinopril (Figs. 5 and 6).

Effect of pharmacological interventions on serum creatinine, blood urea, and proteinuria

The concentrations of serum creatinine, blood urea, and proteinuria were noted to be markedly increased in diabetic rats after 7 weeks when compared with normal rats. Treatment with benfotiamine (70 mg/kg, p.o., 7 weeks) or fenofibrate (32 mg/kg, p.o., 7 weeks) or lisinopril (1 mg/kg, p.o., 7 weeks) partially attenuated diabetes-induced increase in serum creatinine, blood urea, and proteinuria. However, the concurrent administration of benfotiamine (70 mg/kg, p.o., 7 weeks) and fenofibrate (32 mg/kg, p.o., 7 weeks) markedly reduced the high serum concentrations of creatinine, blood urea, and elevated urinary protein in diabetic rats as compared to treatment with either drug alone or lisinopril (Figs. 7–9).

Effect of pharmacological interventions on histopathological study on kidney

The diabetes was noted to develop pathological changes in the glomeruli such as reduced capillary size and extracellular mesangial expansion in diabetic rats after 7 weeks when compared with normal rats. Treatment with

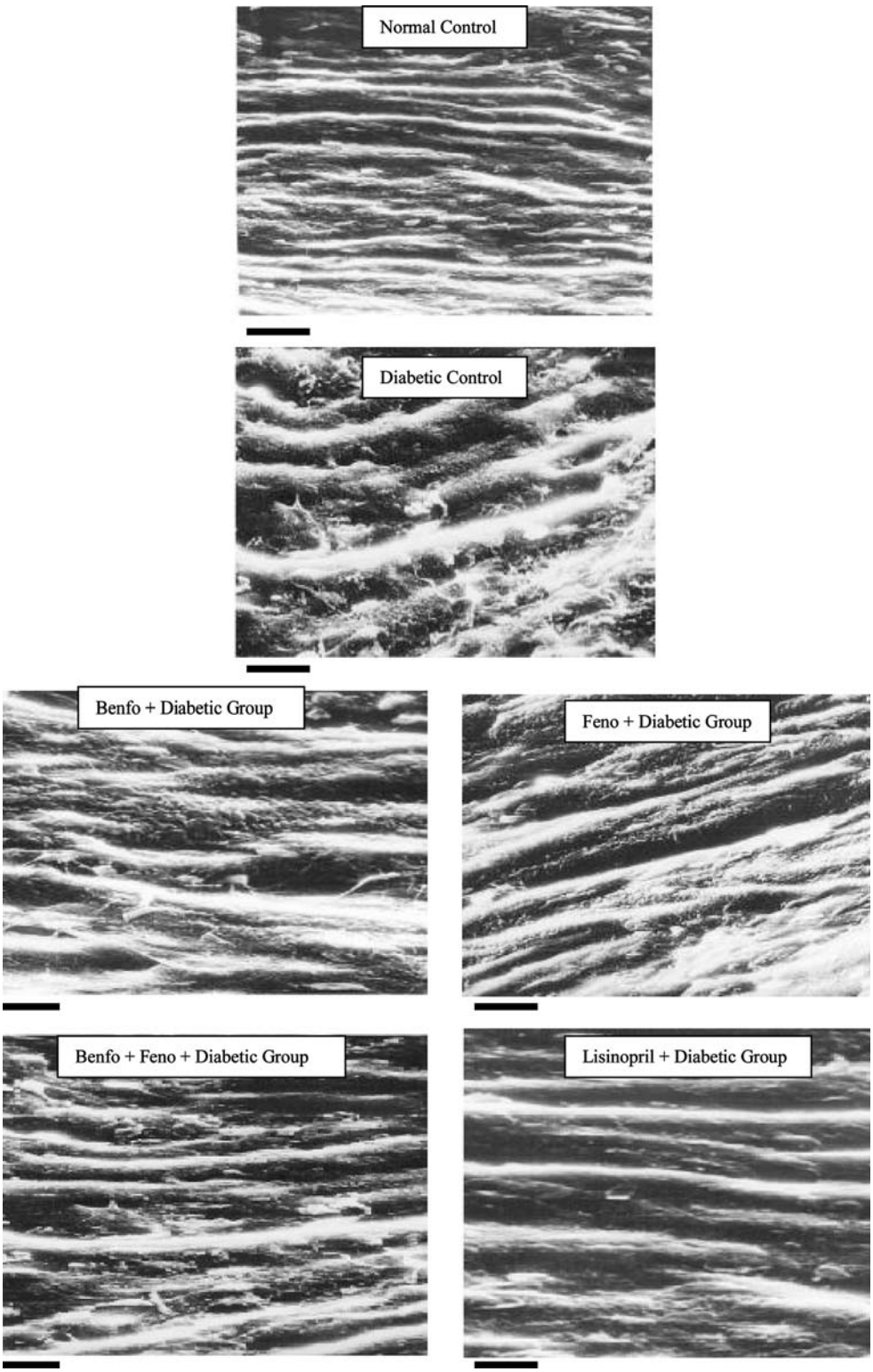

Fig. 3 Effect of benfotiamine (benfo), fenofibrate (feno), and lisinopril on integrity of vascular endothelium. The scanning electron microscopic study was performed to examine the integrity of vascular endothelium using JOEL JSM 6100 scanning electron microscope (800×). The impairment in integrity of vascular endothelium was noted in thoracic aorta of diabetic rats. The combination of benfotiamine and fenofibrate markedly prevented the diabetes-induced impairment in integrity of endothelium as compared to treatment with either drug alone or lisinopril (bars = 100 μm)

benfotiamine (70 mg/kg, p.o., 7 weeks) or fenofibrate (32 mg/kg, p.o., 7 weeks) or lisinopril (1 mg/kg, p.o., 7 weeks) partially attenuated diabetes-induced pathological changes in glomeruli. Moreover, the concurrent administration of benfotiamine (70 mg/kg, p.o., 7 weeks) and fenofibrate (32 mg/kg, p.o., 7 weeks) markedly reduced the pathological changes in glomeruli by improving the glomerular capillary size and reducing the mesangial expansion as compared to treatment with either drug alone or lisinopril (Fig. 10).

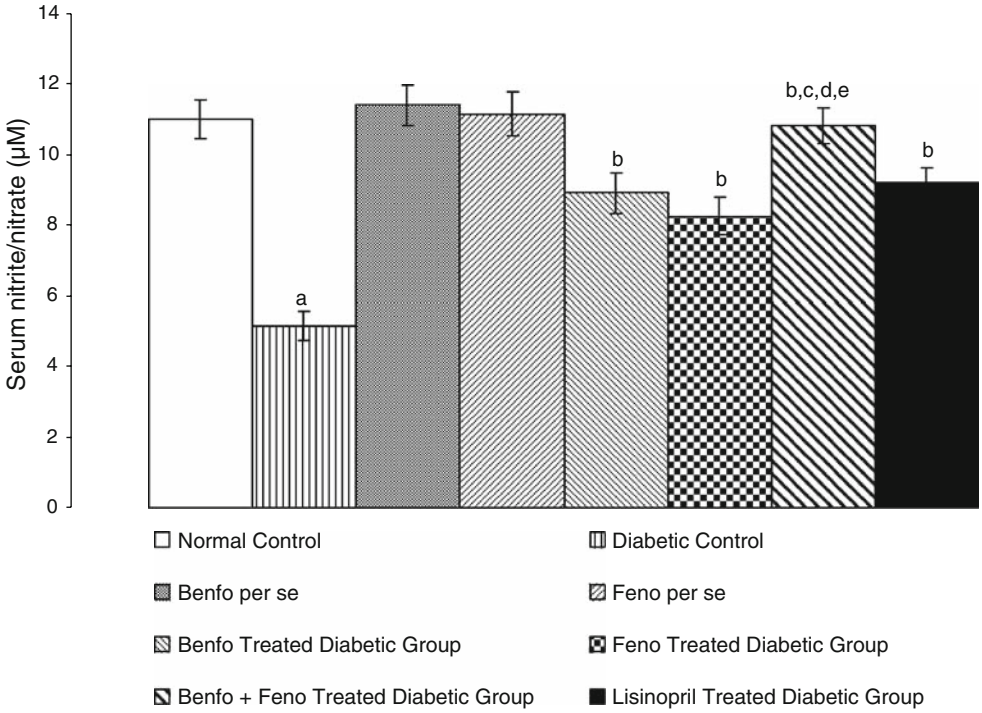

Fig. 4 Effect of benfotiamine (benfo) and fenofibrate (feno) on serum concentration of nitrite/nitrate. All values were represented as mean ± SEM. a = $P < 0.05$ versus normal control; b = $P < 0.05$ versus diabetic control; c = $P < 0.05$ versus benfo treated diabetic group; d = $P < 0.05$ versus feno treated diabetic group; e = $P < 0.05$ versus lisinopril treated diabetic group

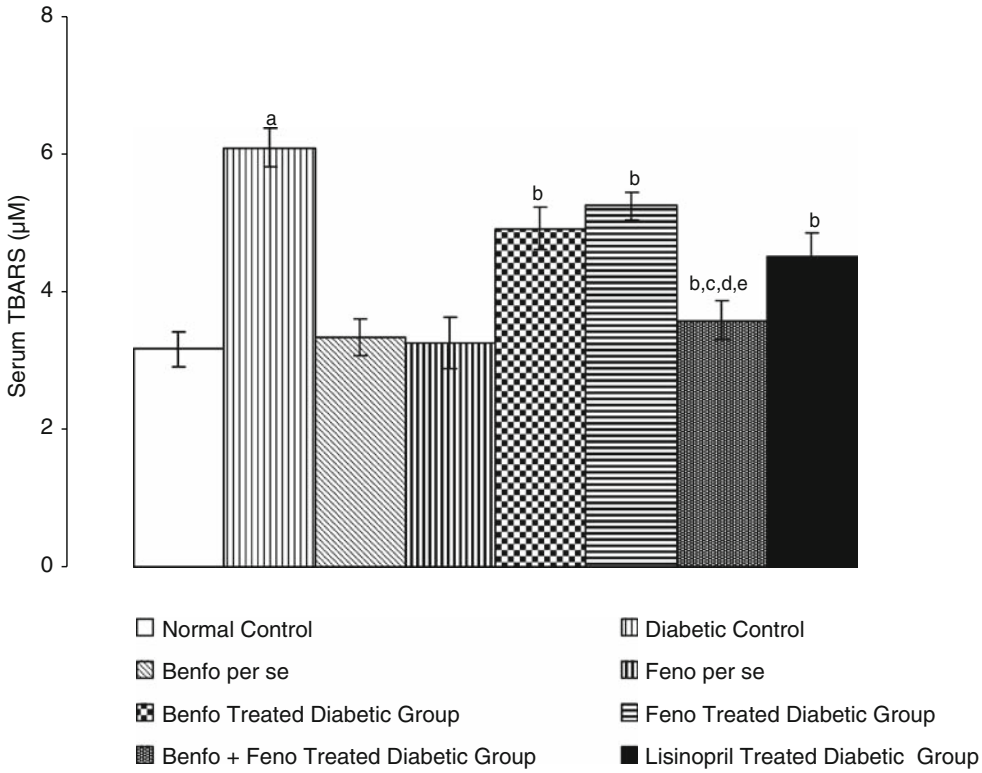

Fig. 5 Effect of benfotiamine (benfo) and fenofibrate (feno) on serum concentration of thiobarbituric acid reactive substances (TBARS). All values were represented as mean ± SEM. a = $P < 0.05$ versus normal control; b = $P < 0.05$ versus diabetic control; c = $P < 0.05$ versus benfo treated diabetic group; d = $P < 0.05$ versus feno treated diabetic group; e = $P < 0.05$ versus lisinopril treated diabetic group

Discussion

The impairment in the integrity of vascular endothelium, decrease in serum nitrite/nitrate concentration, and reduction in Ach-induced endothelium dependent relaxation have been documented to be an index of experimental VED [20, 35]. In the present study, the diabetes has been noted to impair the integrity of vascular endothelium, decrease the serum concentration of nitrite/nitrate and consequently reduce the Ach-induced endothelium dependent relaxation.

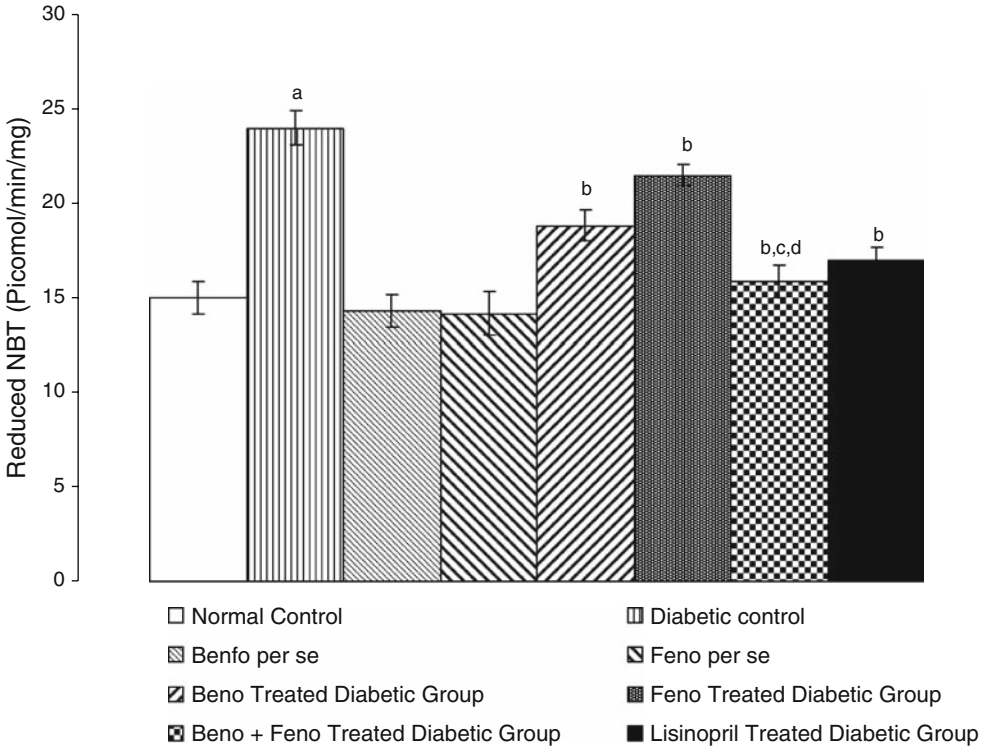

Fig. 6 Effect of benfotiamine (benfo) and fenofibrate (feno) on superoxide anion generation assessed by estimating reduced nitrobluetetrazolium (NBT). All values were represented as mean ± SEM. a = $P < 0.05$ versus normal control; b = $P < 0.05$ versus diabetic control; c = $P < 0.05$ versus benfo treated diabetic group; d = $P < 0.05$ versus feno treated diabetic group

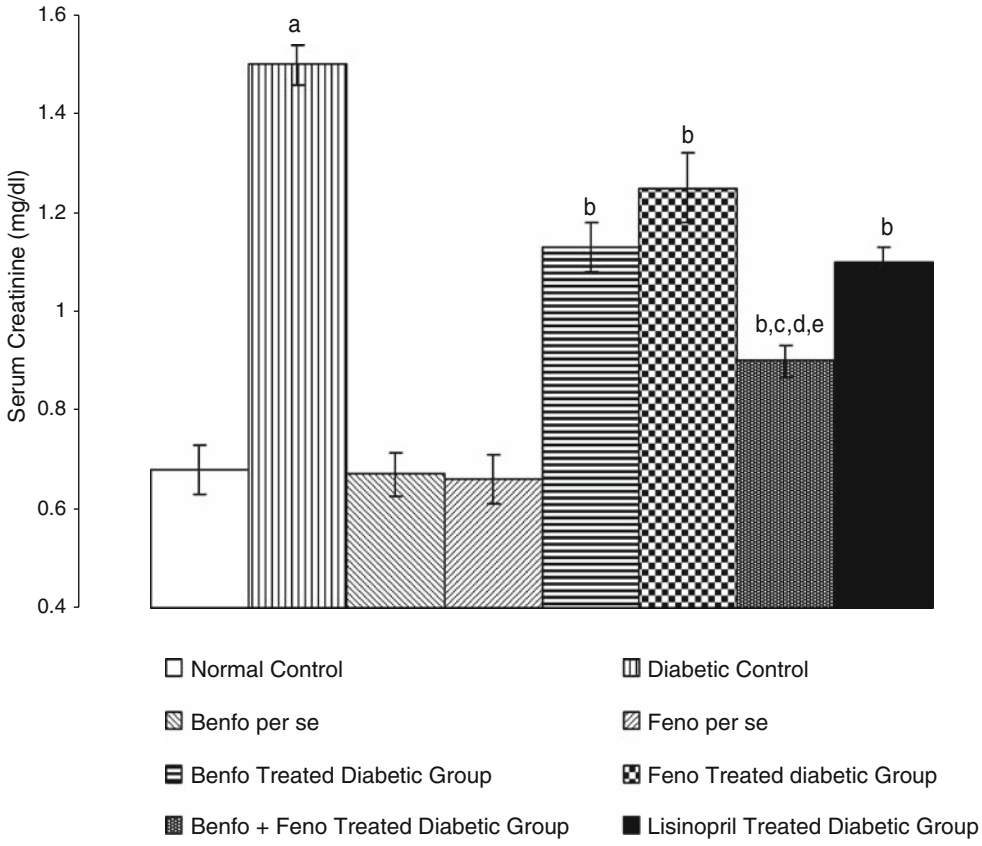

Fig. 7 Effect of benfotiamine (benfo) and fenofibrate (feno) on serum creatinine. All values were represented as mean ± SEM. a = $P < 0.05$ versus normal control; b = $P < 0.05$ versus diabetic control; c = $P < 0.05$ versus benfo treated diabetic group; d = $P < 0.05$ versus feno treated diabetic group; e = $P < 0.05$ versus lisinopril treated diabetic group

It suggests the development of VED, which is consistent with a recent report [20]. The increase in serum TBARS level and aortic superoxide generation are regarded as an index of development of oxidative stress [20, 35]. In the present study, diabetes has been noted to increase the serum TBARS and aortic superoxide anion generation. It

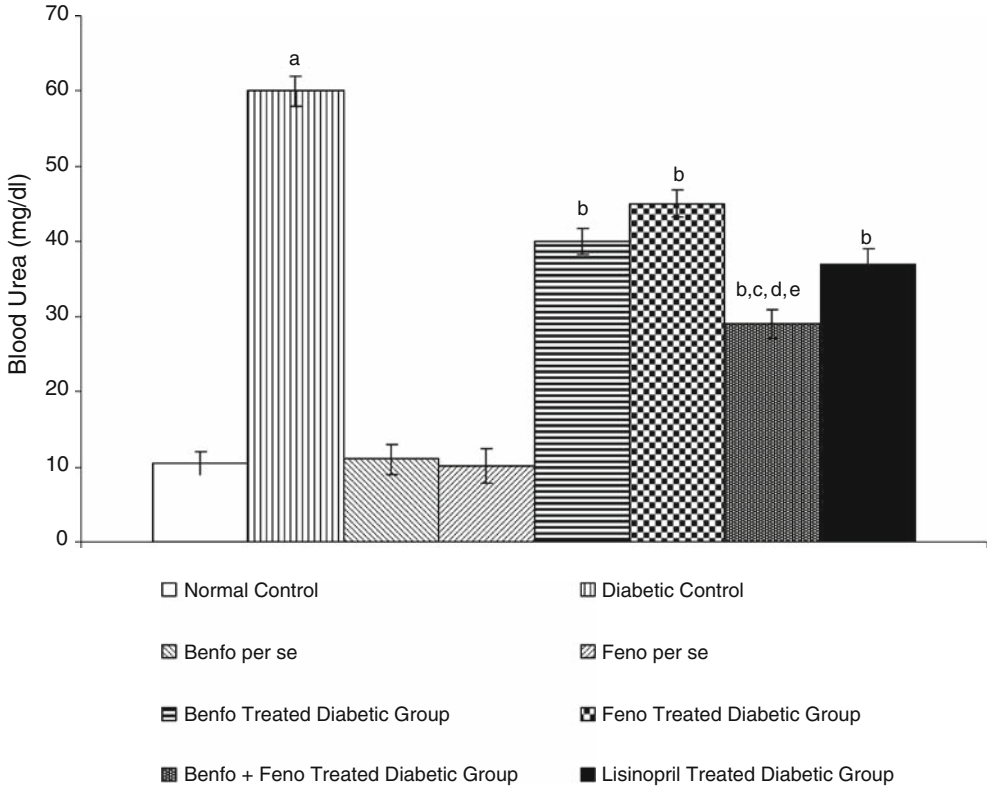

Fig. 8 Effect of benfotiamine (benfo) and fenofibrate (feno) on blood urea. All values were represented as mean ± SEM. a = $P < 0.05$ versus normal control; b = $P < 0.05$ versus diabetic control; c = $P < 0.05$ versus benfo treated diabetic group; d = $P < 0.05$ versus feno treated diabetic group; e = $P < 0.05$ versus lisinopril treated diabetic group

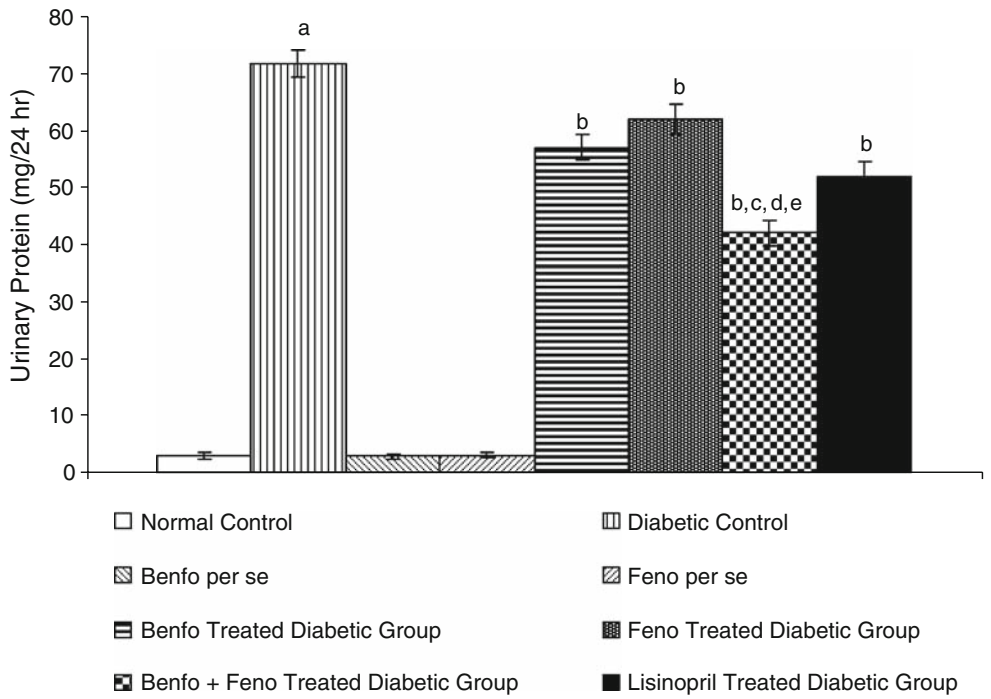

Fig. 9 Effect of benfotiamine (benfo) and fenofibrate (feno) on urinary protein. All values were represented as mean ± SEM. a = $P < 0.05$ versus normal control; b = $P < 0.05$ versus diabetic control; c = $P < 0.05$ versus benfo treated diabetic group; d = $P < 0.05$ versus feno treated diabetic group; e = $P < 0.05$ versus lisinopril treated diabetic group

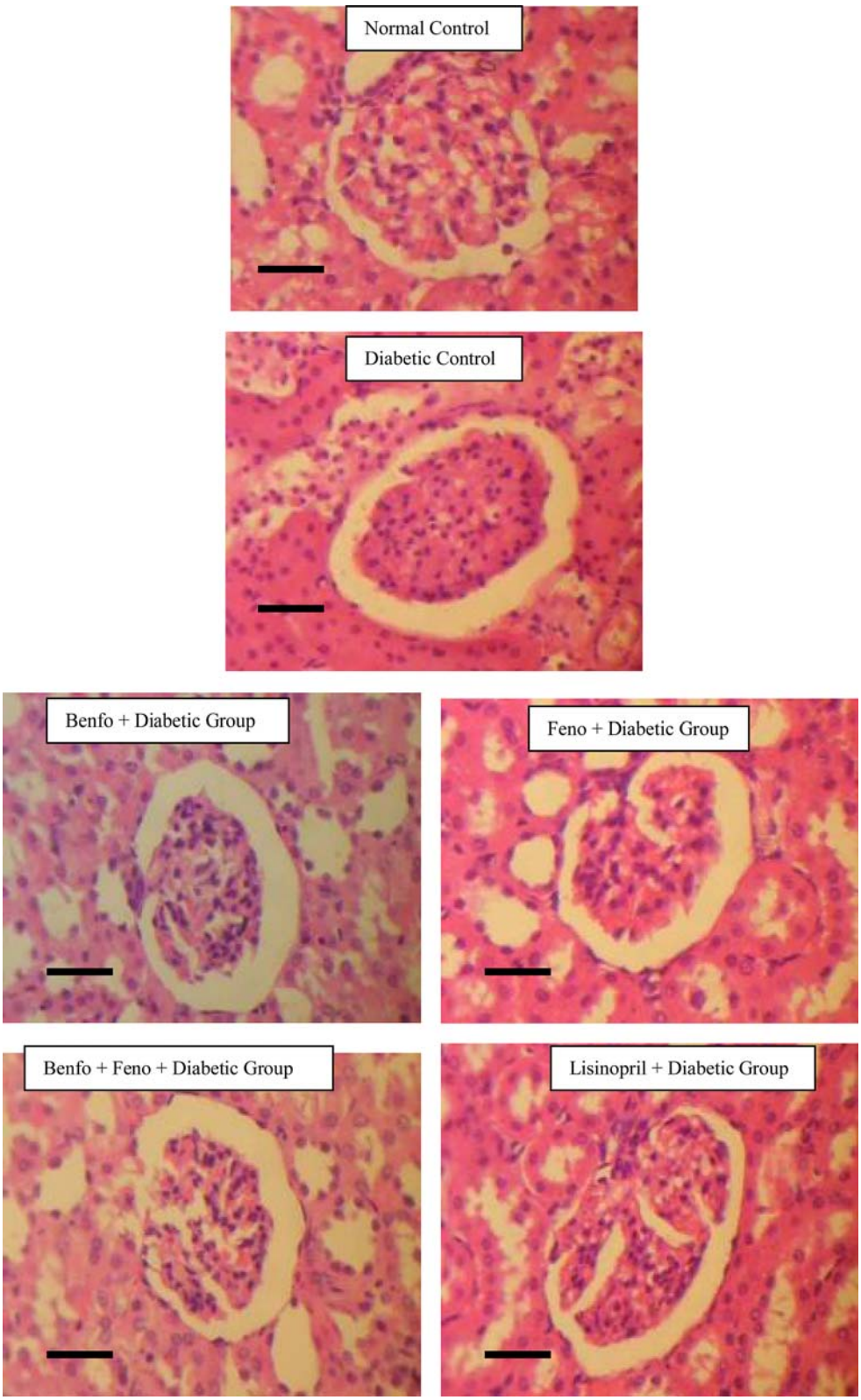

Fig. 10 Effect of benfotiamine (benfo), fenofibrate (feno), and lisinopril on pathological changes in glomeruli. The section of 5 μM in thickness were made and stained with hematoxylin and eosin to assess the pathological change occurs in glomeruli using the light microscopy (400×). The glomerular capillary size reduction and extracellular mesangial expansion were developed in kidney of diabetic control rats as compared to normal control rats. The concurrent administration of benfotiamine and fenofibrate markedly reduced the pathological changes in glomeruli by improving the glomerular capillary size and reducing the mesangial expansion as compared to treatment with either drug alone or lisinopril (bars = 100 μm)

suggests the development of diabetes-induced oxidative stress. The oxidative stress has been documented to play a major role in the progression of VED and nephropathy [13, 43]. The increase in serum creatinine, blood urea, and proteinuria and pathological changes in glomeruli have been documented to be index of nephropathy [13, 21].

In the present study, the serum creatinine, blood urea, and proteinuria were noted to be increased in diabetic rats as compared to normal rats. Moreover, diabetic rats developed pathological changes in glomeruli in 7 weeks. These results suggest the development of diabetes-induced nephropathy. The strong correlations between VED and diabetic nephropathy have been reported [12, 13, 16, 18]. It has been recently demonstrated that Rho-kinase plays a pivotal role in the pathogenesis of VED [44]. Interestingly, the pathological role of Rho-kinase has been implicated in diabetic nephropathy and inhibition of Rho-kinase using fasudil, markedly prevented diabetes-induced nephropathy [21]. It has been shown that diabetes downregulates and inactivates eNOS, increases the production of ROS and consequently reduces the synthesis and bioavailability of NO, which result in nephropathy [12, 13, 16, 18, 31]. Thus, it may be suggested that diabetes-induced development of oxidative stress, down regulation of eNOS and reduction in generation and bioavailability of NO may produce VED, which consequently may develop nephropathy in diabetic rats. This contention is supported by the results obtained in the present study that increase in serum TBARS and aortic superoxide anion generation and impairment in integrity of vascular endothelium, reduction in serum nitrite/nitrate concentration and subsequent increase in serum creatinine, blood urea, and proteinuria were noted in diabetic rats. However, pharmacological treatment with benfotiamine or fenofibrate prevented diabetes-induced VED and nephropathy by improving the integrity of vascular endothelium, increasing the serum nitrite/nitrate level, enhancing the Ach-induced endothelium dependent relaxation, decreasing the serum TBARS, aortic superoxide generation, serum creatinine, blood urea, and proteinuria and reducing the pathological changes in glomeruli.

Benfotiamine has been shown to be an activator of transketolase [23, 24]. Fenofibrate is a well-known activator of PPAR-α [29]. The concurrent administration of both benfotiamine and fenofibrate to diabetic rats markedly attenuated the development of VED and diabetic nephropathy as compared to treatment with either drug alone. Benfotiamine has been reported to activate Akt and eNOS that consequently increase the generation of NO to prevent VED [23–25, 45], which supports the results obtained in the present study. Further, benfotiamine activates transketolase, which converts glucose substrates directly into pentose phosphate pathway and thus reduces the endogenous AGE formation to prevent diabetes-induced nephropathy [23, 24]. In addition, benfotiamine has been reported to prevent diabetic nephropathy by decreasing proteinuria and reducing oxidative stress [23]. Thus, the observed beneficial effect of benfotiamine in preventing diabetes-induced VED and nephropathy may be due to activation of eNOS through Akt/Protein kinase B, reduction of ROS generation and consequent upregulation of synthesis and bioavailability of NO. Fenofibrate has been noted to improve the function of endothelium by increasing the bioavailability of NO and reducing the oxidative stress [46]. Asymmetric dimethylarginine (ADMA) is an endogenous negative regulator of eNOS [47]. Fenofibrate has been shown to reduce the serum level of ADMA and thus improve the function of vascular endothelium [29, 30]. These results support the fenofibrate mediated vascular protective effects obtained in the present study. The TGF-β is a well known potent fibrogenic cytokine implicated in the pathogenesis of diabetic nephropathy [31]. The overexpression of TGF-β is involved in glomerulosclerosis [48]. Plasminogen activator inhibitor-1 (PAI-1) has been shown to regulate the expression of TGF-β by binding to urokinase plasminogen activator receptor and activating the extracellular-regulated signal kinase/mitogen-activated protein kinase (ERK/MAPK) pathway [49]. PAI-1 has not been noted to be expressed in the normal kidney; but it is overexpressed in diabetic kidney [50]. Overexpression of PAI-1 has been shown to play a pathological role in diabetic nephropathy [51, 52]. Activation of PPAR-α by fenofibrate has been reported to produce renoprotective effect by downregulating the expression of TGF-β and PAI-1 [31]. These studies explain the possible mechanisms involved in fenofibrate mediated renoprotective effect noted in the present study.

Lipoprotein lipase (LPL) located in the vascular endothelium involves in the breakdown of triglycerides into free fatty acids. LPL gets downregulated during insulin deficiency [53]. Further, insulin has an inhibitory action on 3-hydroxy-3-methyl-glutaryl-Co-A (HMG-COA) reductase, a key rate limiting enzyme involved in the synthesis of cholesterol. Hypoinsulinemia during diabetes activates HMG-COA reductase to stimulate the synthesis of high cholesterol [54]. Moreover, the occurrence of proteinuria has been suggested to upregulate HMG-COA reductase to produce hypercholesterolemia [55, 56]. Thus, STZ-induced diabetes is often associated with hypercholesterolemia and hypertriglyceridaemia. This contention is supported by the results obtained in the present study that the total cholesterol and triglycerides levels were noted to be markedly increased and HDL level gets decreased in diabetic rats. The diabetes-induced alteration in lipid profile such as hypercholesterolemia and hypertriglyceridaemia affect the function of glomerulus and produce glomerulosclerosis, which ultimately leads to diabetic nephropathy [26]. Fenofibrate has been well reported to reduce triglycerides and cholesterol through activation of PPAR-α [28–30, 46]. Hence, in the present study, the fenofibrate mediated reduction in high total cholesterol and triglycerides levels may be due to activation of PPAR-α. Benfotiamine treatment did not modulate the lipid profile in diabetic rats.

Taken together, the overall observed beneficial effect of combination of benfotiamine and fenofibrate in preventing diabetic nephropathy may be due to reduction of oxidative stress, prevention of VED and inhibition of high circulating lipids such as triglycerides and total cholesterol. Lisinopril has been noted to improve the function of endothelium by markedly reducing the oxidative stress [57]. Further, the renoprotective effect of lisinopril has been well reported in basic and clinical studies [58, 59]. Therefore, lisinopril has been employed as a standard drug in the present study. The beneficial effect of either benfotiamine or fenofibrate in preventing the diabetic nephropathy has been observed to be almost similar to the effect produced by lisinopril. However, the concurrent administration of both benfotiamine and fenofibrate markedly prevented diabetic nephropathy as compared to treatment with either drug alone or lisinopril.

On the basis of above discussion, it may be concluded that diabetes-induced oxidative stress, lipid alterations and consequent induction of vascular endothelial dysfunction may be responsible for the development and progression of nephropathy in diabetic rats. Concurrent administration of benfotiamine and fenofibrate may provide synergistic benefits in preventing the development of diabetes-induced nephropathy by reducing the oxidative stress and lipid alteration, preventing the vascular endothelial dysfunction and subsequently improving the renal function.

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