Benfotamine attenuates nicotine and uric acid-induced vascular endothelial dysfunction in the rat

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A B S T R A C T

The study has been designed to investigate the effect of benfotamine, a thiamine derivative, in nicotine and uric acid-induced vascular endothelial dysfunction (VED) in rats. Nicotine (2 mg kg⁻¹ day⁻¹, I.P., 4 weeks) and uric acid (150 mg kg⁻¹ day⁻¹, I.P., 3 weeks) were administered to produce VED in rats. The development of VED was assessed by employing isolated aortic ring preparation and estimating serum and aortic concentration of nitrite/nitrate. Further, the integrity of vascular endothelium was assessed using the scanning electron microscopy (SEM) of thoracic aorta. Moreover, the oxidative stress was assessed by estimating serum thiobarbituric acid reactive substances (TBARS) and aortic superoxide anion generation. The administration of nicotine and uric acid produced VED by impairing the integrity of vascular endothelium and subsequently decreasing serum and aortic concentration of nitrite/nitrate and attenuating acetylcholine-induced endothelium dependent relaxation. Further, nicotine and uric acid produced oxidative stress, which was assessed in terms of increase in serum TBARS and aortic superoxide generation. However, treatment with benfotamine (70 mg kg⁻¹ day⁻¹, p.o.) or atorvastatin (30 mg kg⁻¹ day⁻¹, p.o., a standard agent) markedly prevented nicotine and uric acid-induced VED and oxidative stress by improving the integrity of vascular endothelium, increasing the concentration of serum and aortic nitrite/nitrate, enhancing the acetylcholine-induced endothelium dependent relaxation and decreasing serum TBARS and aortic superoxide anion generation. Thus, it may be concluded that benfotamine reduces the oxidative stress and consequently improves the integrity of vascular endothelium and enhances the generation of nitric oxide to prevent nicotine and uric acid-induced experimental VED.

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1. Introduction

Endothelium is an innermost layer of blood vessels. A healthy endothelium possesses antiatherogenic, antiplatelet and antiproliferative properties to regulate the vascular tone and maintain the free flow of blood in vessels [12]. Nitric oxide (NO), a potent vasodilatory substance, is generated from l-arginine by endothelial nitric oxide synthase (eNOS) in the presence of cofactors such as Ca²⁺-calmodulin, reduced nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD) and tetrahydrobiopterin (BH4) in endothelial cells [3,4]. Vascular endothelial dysfunction (VED) is characterized by suppression of endothelium dependent vasorelaxation caused by reduced generation and bioavailability of nitric oxide (NO) due to reduced activity of eNOS and increased oxidative stress in the vessel wall, which in turn impair the regulation of vascular homeostasis [5–7]. VED has been associated in the pathogenesis of atherosclerosis [8], hypertension [9], diabetes mellitus [2,10], coronary artery diseases [11] and stroke [12]. Various risk factors including hyperuricemia and cigarette smoking are implicated in the vascular pathogenesis [13,14]. Hyperuricemia, a condition of increased serum uric acid, has been implicated in pathogenesis of VED [14,15]. Uric acid is the major product formed from purine metabolism. Gout, an inflammatory disorder characterized by increased uric acid level and has been reported to produce vascular pathogenesis [14–16]. Hyperuricemia has been noted to decrease the vascular generation of NO through inactivation of eNOS [16]. Further, hyperuricemia has been shown to produce reactive oxygen species (ROS) excessively through activation of NADPH oxidase and xanthine oxidase [17–20]. The C-reactive protein (CRP) level was noted to be increased in hyperuricemia-induced VED [21]. Hyperuricemia has been regarded as an independent risk factor for various cardiovascular disorders such as atherosclerosis [22], hypertension [23] and heart failure [24]. Nicotine exposure via cigarette smoking has been implicated in pathogenesis of cardiovascular disorders like atherosclerosis [25] and hypertension [26]. Moreover, nicotine has been noted to produce VED by downregulating the expression of eNOS, increasing the generation of ROS and upregulating asymmet
ric dimethylarginine (ADMA), an endogenous inhibitor of eNOS and thus impair the endothelial production of NO [13,27,28]. Nicotine has been noted to upregulate the expression of various proteins such as basic fibroblast growth factor, tumor necrosis factor-α (TNF-α) and plasminogen activator inhibitor-1 [29]. In addition, nicotine induces mononuclear leukocyte adherence and expression of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule (ICAM) in endothelial cells [30]. No satisfactory therapeutic option is available to treat nicotine and uric acid-induced vascular pathogenesis. Thus, the present study has been aimed to explore the possible therapeutic strategy to prevent nicotine and uric acid-induced dysfunction of vascular endothelium. Benfotiamine, S-acyl thiamine derivative, has been shown to reduce the formation of advanced glycation end products (AGE) by activating transketolase [31,32]. Benfotiamine has been noted to possess clinical efficacy in the treatment of diabetic cardiomyopathy [33], diabetic nephropathy [34] and diabetic neuropathy [35,36]. Moreover, benfotiamine has been shown to reduce the oxidative stress through a mechanism unrelated to AGE formation [37]. Activation of Akt (protein kinase B) has been demonstrated to stimulate eNOS activity, increase the bioavailability of NO and reduce the generation of ROS [38]. Benfotiamine has been reported to improve the function of endothelium by activating Akt and subsequently stimulating eNOS and inhibiting the generation of ROS [39,40]. Thus, the present study has been designed to investigate the effect of benfotiamine in nicotine and uric acid-induced experimental vascular endothelial dysfunction.

2. Materials and methods

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

2.1. Assessment of vascular endothelial dysfunction

2.1.1. 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; NaHCO₃, 25 mM; MgSO₄, 1.0 mM; glucose, 11.1 mM; KH₂PO₄, 1.2 mM and CaCl₂, 2.5 mM) of pH 7.4, bubbled with oxygen (95% O₂ and 5% CO₂) 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 Physiograph (INCO, Ambala, India). The aortic ring preparation was primed with 80 mM of 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 [41]. The intimal layer of the aortic ring was rubbed gently with a moistened filter paper for 30 s to obtain endothelium free preparation [14,42]. Loss of Ach (1 × 10⁻⁶ M)-induced relaxation confirmed the absence of vascular endothelium.

2.1.2. Scanning electron microscopy (SEM)

The scanning electron microscopic study was performed to examine the integrity of vascular endothelium [24,43,44]. 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 acetone solution (50% for 20 min, 70% for 20 min, 80% for 20 min, 90% for 20 min, 100% for 50 min) and isoamylacetate (100%); acetone (100%) solutions (1:1) for 20 min followed by isoamylacetate (100%) for 20 min. Arterial segments were dried further using four flushes of liquid CO₂ with 100 psi pressure in critical point dryer. Then, the segments were mounted on aluminium 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.

2.1.3. Estimation of serum or aortic nitrite/nitrate concentration

The aortic tissue was homogenized in phosphate-buffer saline of pH 7.4 and centrifuged at 10,000 × g for 20 min. The supernatant was used to estimate the aortic concentration of nitrite/nitrate and protein content. 400 µL of carbonate buffer (pH 9.0) was added to 100 µL of serum sample or 100 µL of supernatant from homogenized aortic samples, followed by 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 sulphate solution (120 mM) was added to deproteinate the samples. The samples were allowed to stand for 10 min and then centrifuged at 4000 × g for 10 min. Greiss reagent (250 µL of 1% sulfanilamide and 250 µL of 0.1% N-naphthylethylenediamine) was added to aliquots (500 µL) of clear supernatant and nitrite/nitrate concentration was measured spectrophotometrically (Thermo Double Beam Spectrophotometer, Thermo Electron Corporation, UK) at 545 nm. The standard graph of sodium nitrite (0.5–40 µM) was plotted to calculate concentration of serum nitrite/nitrate (µM) in aortic nitrite/nitrate (µM/mg of protein) [14,45,46]. The protein concentration in homogenized aortic preparation was estimated by Lowry's method [47].

2.2. Assessment of oxidative stress

2.2.1. Estimation of serum thiobarbituric acid reactive substances (TBARS)

1 mL of 20% trichloroacetic acid was added to 100 µL of serum and 1 mL of thioarbituric acid reagent and then mixed and incubated at 100°C for 30 min. After cooling on ice, samples were centrifuged at 1000 × g for 20 min. Serum concentration of TBARS was measured spectrophotometrically at 532 nm. A standard graph using 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49 g/L of standard solution was plotted to calculate the concentration of TBARS [14,48].

2.2.2. Estimation of superoxide anion

Aorta was cut into transverse rings of 6 mm in length and placed in 5 mL of Krebs–Henseleit solution buffer containing 100 µM of nitrobutyltetrazolium (NBT) and incubated at 37°C for 1.5 h. 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 sodium hydroxide (NaOH) and 0.1% sodium dodecyl sulphate (SDS) in water containing 40 mg/L of diethylenetriamine 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 [14,49]. The amount of reduced NBT was calculated using the following formula. Amount of reduced NBT = A × V/(T × Wt × ε × l), where A is the absorbance, V the volume of solution (1.5 mL), T the time for which aortic rings were incubated with NBT (90 min), Wt the blotted wet weight of aortic rings, ε the extinction coefficient (0.72 L mmol⁻¹ mm⁻¹) and l is the length of light path (10 mm).
2.3 Estimation of serum uric acid

Serum uric acid was estimated spectrophotometrically using a commercial available kit (Transia Bio-Medical Ltd., Daman, India). 20 μL of serum sample was added to 1000 μL of working uric acid reagent to prepare test sample. 20 μL of standard uric acid solution (6 mg dL−1) was added to 1000 μL of working uric acid reagent to prepare standard sample. 20 μL of distilled water was added to 1000 μL of working uric acid reagent to prepare blank. The test, standard and blank samples were incubated at 37 °C for 5 min. The absorbances of test and standard samples were measured against blank spectrophotometrically at 510 nm. The amount of serum uric acid level was calculated using the following formula:

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\text{Concentration of serum uric acid (mg/dL)} = \frac{\text{absorbance of test}}{\text{absorbance of standard}} \times \text{Concentration of standard (6 mg/dL)}
\]

2.4 Experimental protocol

Eight groups were employed in the present study and each comprised of 7 rats. The uric acid and atorvastatin were dissolved in 0.5% w/v of carboxy methyl cellulose (CMC). The benfotiamine was dissolved in 1% w/v of CMC. Group I (Normal Control), rats were maintained on standard food and water and no treatment was given. Group II (Nicotine Control), rats were administered nicotine (2 mg kg\(^{-1}\) day\(^{-1}\), i.p.) for 4 weeks. Group III (Uric Acid Control), rats were administered uric acid (150 mg kg\(^{-1}\) day\(^{-1}\), i.p.) for 3 weeks. Group IV (Benfotiamine per se), rats were administered benfotiamine (70 mg kg\(^{-1}\) day\(^{-1}\), p.o.) dissolved in 1% w/v of CMC for 4 weeks. Group V (Benfotiamine Treated Nicotine), rats administered nicotine (2 mg kg\(^{-1}\) day\(^{-1}\), i.p., 4 weeks) were treated with benfotiamine (70 mg kg\(^{-1}\) day\(^{-1}\), p.o.) and the treatment was started 3 days before the administration of nicotine and it was continued for 4 weeks from the day of administration of nicotine. Group VI (Benfotiamine Treated Uric Acid), rats administered uric acid (150 mg kg\(^{-1}\) day\(^{-1}\), i.p., 3 weeks) were treated with benfotiamine (70 mg kg\(^{-1}\) day\(^{-1}\), p.o.) and the treatment was started 3 days before the administration of uric acid and it was continued for 3 weeks from the day of administration of uric acid. Group VII (Atorvastatin Treated Nicotine), rats administered nicotine (2 mg kg\(^{-1}\) day\(^{-1}\), i.p., 4 weeks) were treated with atorvastatin (30 mg kg\(^{-1}\) day\(^{-1}\), p.o.) as mentioned in Group V. Group VIII (Atorvastatin Treated Uric Acid), rats administered uric acid (150 mg kg\(^{-1}\) day\(^{-1}\), i.p., 3 weeks) were treated with atorvastatin (30 mg kg\(^{-1}\) day\(^{-1}\), p.o.) as mentioned in Group VI.

2.5 Statistical analysis

All values were expressed as mean ± S.D. Data for isolated aortic ring preparation were statistically analyzed using repeated measures of ANOVA followed by Newman Keul’s test. The data for serum and aortic levels of nitrite/nitrate, serum TBARS, aortic superoxide anion generation and serum uric acid were statistically analyzed using one-way ANOVA followed by Tukey’s multiple comparison test. The p < 0.05 was considered to be statistically significant.

2.6 Drugs and chemicals

Acetylcholine hydrochloride and L-phenylephrine were purchased from Sigma–Aldrich Ltd., St. Louis, USA. Diethyl trimamine pentaacetic acid, nitroblue tetrazolium and uric acid were purchased from Sanjay Biologicals Amritsar, India. 1,1,3,3 tetramethoxypropane and carboxymethyl cellulose were purchased from V. K Chemicals, India. Nicotine was obtained from Nicosulf India Pvt. Ltd., Dakor, Gujarat, India. Atorvastatin was obtained from Dr. Reddy’s Laboratory Ltd., Hyderabad, India. Benfotiamine was obtained from Orchid Healthcare Ltd., Chennai. The uric acid estimation kit was purchased from Transia Bio-medical Ltd., Daman, India.

3. Results

The treatment with benfotiamine (70 mg kg\(^{-1}\) day\(^{-1}\), p.o.) or atorvastatin (30 mg kg\(^{-1}\) day\(^{-1}\), p.o.) to normal rats did not produce any significant per se effects on various parameters performed in the present study such as endothelium dependent relaxation and independent relaxation, serum and aortic level of nitrite/nitrate, TBARS and aortic superoxide anion generation and serum uric acid level. Less than 5% of mortality rate was observed in nicotine and uric acid administered rats with or without treatments.

3.1 Effect of pharmacological interventions on endothelium dependent and independent relaxation

Ach and SNP were noted to produce endothelium dependent and independent relaxation, respectively in phenylephrine (3 × 10\(^{-6}\) M) precontracted isolated rat aortic ring preparation in a dose dependent manner. Administration of nicotine (2 mg kg\(^{-1}\) day\(^{-1}\), i.p., 4 weeks) and uric acid (150 mg kg\(^{-1}\) day\(^{-1}\), i.p., 3 weeks) significantly attenuated Ach-induced endothelium dependent relaxation; but their administration did not affect SNP-induced endothelium independent relaxation. The treatment with benfotiamine (70 mg kg\(^{-1}\) day\(^{-1}\), p.o.) or atorvastatin (30 mg kg\(^{-1}\) day\(^{-1}\), p.o.) significantly prevented nicotine and uric acid-provoked attenuation of Ach-induced endothelium dependent relaxation. Marked Ach-induced endothelium dependent relaxation was noted in uric acid administered rats treated with atorvastatin as compared with benfotiamine treated rats (Figs. 1 and 2).

3.2 Effect of pharmacological interventions on integrity of vascular endothelium

The integrity of vascular endothelium was noted to be impaired in thoracic aorta of nicotine (2 mg kg\(^{-1}\) day\(^{-1}\), i.p., 4 weeks) and uric acid (150 mg kg\(^{-1}\) day\(^{-1}\), i.p., 3 weeks) administered rats. However, treatment with benfotiamine (70 mg kg\(^{-1}\) day\(^{-1}\), p.o.) or atorvastatin (30 mg kg\(^{-1}\) day\(^{-1}\), p.o.) markedly improved the integrity of vascular endothelium in nicotine and uric acid administered rats (Fig. 3).

3.3 Effect of pharmacological interventions on serum and aortic nitrate/nitrate concentration

The serum and aortic concentration of nitrite/nitrate was noted to be reduced in nicotine (2 mg kg\(^{-1}\) day\(^{-1}\), i.p., 4 weeks) and uric acid (150 mg kg\(^{-1}\) day\(^{-1}\), i.p., 3 weeks) administered rats when compared with normal rats. Moreover, marked reduction in serum and aortic nitrite/nitrate occurs in rats administered uric acid as compared to nicotine-administered rats. However, treatment with benfotiamine (70 mg kg\(^{-1}\) day\(^{-1}\), p.o.) or atorvastatin (30 mg kg\(^{-1}\) day\(^{-1}\), p.o.) significantly attenuated nicotine and uric acid-induced decrease in serum and aortic nitrite/nitrate concentration. Atorvastatin produced marked restoration of decreased serum and tissue nitrite/nitrate concentration in rats administered uric acid when compared with benfotiamine treated rats (Figs. 4 and 5).
3.4. Effect of pharmacological interventions on serum TBARS and aortic superoxide anion generation

The increase in serum TBARS concentration and aortic superoxide anion generation was noted in animals administered with nicotine (2 mg kg\(^{-1}\) day\(^{-1}\), i.p., 4 weeks) and uric acid (150 mg kg\(^{-1}\) day\(^{-1}\), i.p., 3 weeks). Moreover, marked increase in serum TBARS and aortic superoxide anion generation was noted in rats administered uric acid as compared to nicotine administered rats. However, treatment with benfotiamine (70 mg kg\(^{-1}\) day\(^{-1}\), p.o.) or atorvastatin (30 mg kg\(^{-1}\) day\(^{-1}\), p.o.) significantly attenuated nicotine and uric acid-induced increase in serum TBARS concentration and aortic superoxide anion generation (Figs. 6 and 7).

3.5. Effect of pharmacological interventions on serum uric acid concentration

The concentration of serum uric acid was noted to be markedly increased in uric acid (150 mg kg\(^{-1}\) day\(^{-1}\), i.p., 3 weeks) administered rats when compared with normal rats. The treatment with benfotiamine (70 mg kg\(^{-1}\) day\(^{-1}\), p.o.) has no effect on serum uric acid level; but treatment with atorvastatin (30 mg kg\(^{-1}\) day\(^{-1}\), p.o.) markedly reduced the serum concentration of uric acid in rats administered uric acid (150 mg kg\(^{-1}\) day\(^{-1}\), i.p., 3 weeks) (Fig. 8).

4. Discussion

The impairment in integrity of vascular endothelium, decrease in serum and aortic nitrite/nitrate concentration and reduction in Ach-induced endothelium dependent vasodilation have been documented to be an index of experimental VED [14,42,50]. In the present study, administration of nicotine (2 mg/kg) for 4 weeks and uric acid (150 mg/kg) for 3 weeks in rats impaired the integrity of vascular endothelium, decreased the serum and aortic concentration of nitrite/nitrate and consequently reduced the Ach-induced endothelium dependent vasodilation. It suggests that both nicotine and uric acid produced VED, which is consistent with our recent studies and reports by others [14,15,51,52]. Nicotine has been documented to reduce the activity of eNOS and decrease the generation and bioavailability of NO [53,54]. Further, nicotine has been noted to upregulate ADMA, which is an endogenous eNOS inhibitor [28]. Hyperuricemia has been shown to decrease the generation and bioavailability of NO by inactivating eNOS and damaging the integrity of vascular endothelium [14,16]. Thus, the observed nicotine and uric acid-induced VED may be due to downregulation of eNOS and subsequent reduction in generation of NO in the vessel wall. This contention is supported by the results obtained in the present study that administration of nicotine and uric acid significantly reduced the serum and aortic nitrite/nitrate levels.

In the present study, rats administered uric acid produced VED markedly as compared to nicotine administered rats. The oxidative stress has been documented to play a major role in the progression of VED [55,56]. Elevated level of serum TBARS and aortic superoxide generation are regarded as an index of development.
The impairment in integrity of vascular endothelium was noted in thoracic aorta of nicotine or uric acid administered rats. The treatment with benfotiamine or atorvastatin markedly prevented nicotine and uric acid-induced impairment in integrity of vascular endothelium.

Administration of nicotine and uric acid increased serum TBARS and aortic superoxide anion generation, which indicate the development of oxidative stress. Nicotine has been reported to generate ROS through activation of NADPH [59]. Hyperuricemia has been reported to produce ROS excessively by activating NADPH oxidase and xanthine oxidase [18]. Thus, the noted marked induction of VED in uric acid administered rats may be due to the development of high degree of oxidative stress and consequent inactivation of NO. This contention is strongly supported by the fact that in the present study, uric acid administered rats showed high degree of oxidative stress and marked reduction in serum and aortic nitrite/nitrate concentration as compared to nicotine administered rats.

The pharmacological treatment with benfotiamine, a lipophilic thiamine derivative [60] has been noted to prevent nicotine and uric acid-induced VED by improving the integrity of vascular endothelium, increasing the serum and aortic nitrite/nitrate level and enhancing the Ach-induced endothelium dependent relaxation. Benfotiamine has been reported to reduce the generation of ROS through the mechanism unrelated to AGE formation [37] and prevent the process of apoptosis [61]. Benfotiamine has been reported to activate Akt/Protein kinase B [39,40]. Activation of Akt/Protein Kinase B has been noted to further activate eNOS [62] and reduces the oxidative stress [63]. Activation of eNOS increases the generation and bioavailability of NO and thus improves the function of vascular endothelium [62,64]. Thus, the observed beneficial
effect of benfotiamine in preventing nicotine and uric acid-induced experimental VED 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. This contention is supported by the results obtained in the present study that benfotiamine increases the serum and aortic concentration of nitrite/nitrate and reduces the oxidative stress by decreasing serum TBARS and aortic superoxide anion generation. Atorvastatin has been well noted to improve the function of endothelium by markedly reducing the oxidative stress [14,42,65]. Further, atorvastatin has been reported to upregulate eNOS and improve the integrity of vascular endothelium [14,42]. Therefore, in the present study, atorvastatin has been employed as a standard drug to compare the potential of benfotiamine in preventing VED. The beneficial effect of benfotiamine in preventing the nicotine-induced VED has been observed to be almost similar to the effect produced by atorvastatin. But, the vascular protective effect of atorvastatin was noted to be slightly superior in attenuating the uric acid-induced
VED as compared to benfotiamine. Atorvastatin has been reported to have uricosuric effect as it increases the excretion of uric acid [66]. Thus, the additional uricosuric property of atorvastatin may be responsible to markedly attenuate the uric acid-induced VED. This contention is supported by the fact that the serum uric acid level was noted to be markedly reduced by treatment with atorvastatin. On the basis of the above discussion, it may be concluded that benfotiamine reduces the oxidative stress and consequently improves the integrity of vascular endothelium and enhances the generation of nitric oxide to prevent nicotine and uric acid-induced experimental vascular endothelial dysfunction.

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