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Involvement of vascular endothelial nitric oxide synthase in development of experimental diabetic nephropathy in rats

Atul Arya · Harlokesh Naryan Yadav · P. L. Sharma

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Abstract Endothelial nitric-oxide synthase (eNOS) acts as a common pathogenic pathway in diabetic nephropathy (DN). However, its functional consequences are still not fully understood. Caveolin, a membrane protein, inhibits the eNOS by making caveolin–eNOS complex, and its expression is upregulated during diabetes mellitus (DM). This study was designed to determine the role of caveolin in eNOS-mediated NO synthesis and release in DN. DM in rat was induced by feeding of high-fat diet (HFD) for 2 weeks, followed by single dose of streptozotocin (STZ) (35 mg/kg, ip) further followed by HFD for further 8 weeks. Serum nitrite/nitrate ratio was measured to determine the plasma level of NO. Diabetic rat, after 6 weeks of STZ, developed elevated level of BUN, protein in urine, urinary output, serum creatinine, serum cholesterol, kidney weight, kidney weight/body weight, and renal cortical collagen content, while serum nitrite/nitrate concentration was significantly decreased as compared to normal control group. Treatment with sodium nitrite (NO donor), L-arginine (NO precursor), daidzein (caveolin inhibitor), and combination of L-arginine and daidzein for 2 weeks markedly attenuated these changes and increased serum nitrite/nitrate ratio. However, treatment with L-NAME, a eNOS inhibitor, significantly attenuated the L-arginine-, daidzein-, or combination of L-arginine and daidzein-induced ameliorative effects in DN. The finding of this study suggests that caveolin plays a vital role in the eNOS-mediated decrease in renal level of NO, which may be responsible for the development of DN in rats.

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Introduction

Diabetes mellitus (DM) is a complex metabolic syndrome characterized by absolute insulin deficiency or development of insulin resistance that leads to hyperglycemia and an altered glucose, fat, and protein metabolism [1]. Long term complications of DM include retinopathy, neuropathy, cardiomyopathy, and nephropathy (DN). DN is a microvascular complication and leading cause of morbidity and mortality [2]. DN is characterized by glomerular hypertrophy, accumulation of extracellular matrix protein, increased basement membrane thickness, mesangial expansion, podocyte loss, and vascular endothelial dysfunction progressively leading to glomerulosclerosis, tubulointerstitial fibrosis, and proteinuria [3]. Further, the blood urea nitrogen (BUN) and serum creatinine level are significantly increased, during DN [4].

The exact pathogenesis of DN is still not clear. However, hyperglycemia is the leading cause of development of DN [5], which is followed by formation of advance glycation end-products (AGEs), reactive oxygen species (ROS), increase in the cellular oxidative stress [6]. Further, DN is associated with activation of various intracellular signaling mechanisms and transcription factors, i.e. protein kinase C (PKC), mitogen activated protein kinase (MAPKs), nuclear factor kappa B (NFκB) [7]. Furthermore, overexpression of various growth factors, i.e. transforming growth factor (TGF-β), vascular endothelial growth factor (VEGF), and cytokines, i.e. tumor necrosis factor α (TNFα), interleukin 1 (IL1), and insulin-like growth factor-1 (IGF-1), stimulates proliferation of mesangial cells,

contributing to glomerulosclerosis and tubulointerstitial fibrosis [8].

Renin–angiotensin–aldosterone system (RAAS) gets overactivated during the DN [9] while the systemic RAAS is generally suppressed. Intrarenal RAAS is overactivated even in early course of DM [10]. RAAS is a coordinated cascade of proteins and peptide hormones, the principle effector of which is octapeptide angiotensin II (Ang II) [11]. Further, increased expression of Ang II has been noted in DN [12]. Ang II hemodynamically leads to hyperfiltration and increased intraglomerular pressure and nonhemodynamically increases gene expression of TGF-β with the consequent increase in expression of collagen, fibronectin, and stimulation of mesangial cell proliferation resulting in increased synthesis of extracellular matrix [13].

Caveolae's are invaginations of 50–100 nm in size at the surface of the plasma membrane [14] and caveolins are transmembrane proteins present in these small invaginations of caveolae [15]. Caveolins are a family of 21–24 kDa integral membrane protein, which have three mammalian isoforms known as caveolin-1, caveolin-2, and caveolin-3 [16]. In DM, an increased expression of caveolin has been observed in endothelial cells [17]. Caveolin inhibits endothelial nitric-oxide synthase (eNOS) in quiescent cells both by impeding the signaling of caveolae-targeted receptors that transduce eNOS-stimulatory signals as well as by sterically blocking the calmodulin binding site in eNOS. Caveolin is noted to interact with eNOS and inhibits the activity of eNOS [18]. While the expression of caveolin is upregulated in DM and the activity of eNOS is diminished [19]. Moreover, in the caveolin-knockout animals, the activity of eNOS gets upregulated [20]. The decreased activity of eNOS in diabetic kidney results in a decrease in the renal nitric oxide [21, 22]. Thus, the vascular endothelial nitric oxide synthase appears to play a crucial role in the development of DN in rat. The present study was therefore, designed to investigate this hypothesis.

Materials and methods

Animals

Male, Wistar rats with an initial body weight of 230–260 g were used in these studies. Animal care and treatment were conducted in conformity with study protocol approved by Institutional Animal Ethical Committee (IAEC). All animals were housed in standard light/dark cycle with free access to standard high-fat diet and tap water ad libitum. The animals were housed in metabolic cages. A 24-h urine collection was obtained from each rat for laboratory investigations.

Induction and assessment of diabetes

DM in rat was induced by feeding of HFD for 2 weeks (powdered normal pellet diet 365 g, lard 310 g, casein 250 g, cholesterol 10, vitamin and mineral mix 60 g, DL-methionine 0.3 g) followed by single dose of streptozotocin (STZ) (35 mg/kg, ip) further followed by HFD for further 8 weeks. Diabetic rats having a blood glucose level of 200 mg/dl at 1 week following STZ injection and HFD were used for the present study. Blood samples were obtained from retro-orbital sinus and serum glucose levels were determined by glucose oxidase–peroxidase (GOD-POD) method [23] using commercially available kit (Coral clinical system, Goa, India).

Estimation of total renal collagen content

The renal cortical collagen content was determined by analysis of hydroxyproline content [24].

Renal function measurement

Blood urea nitrogen (BUN), protein in urine, and creatinine were determined in all blood samples by using standard diagnostic kits (Coral Clinical System, Goa, India).

Estimation of serum nitrite and nitrate levels

Nitrite and nitrate are the primary oxidation products of NO subsequent to reaction with oxygen and, therefore, the nitrite/nitrate concentration in serum was used as an indirect measure of NO synthesis. Quantitation of nitrate and nitrite was based on the Griess reaction, in which a chromophore with a strong absorbance at 550 nm is formed by reaction of nitrite with a mixture of naphthylethylenediamine and sulfanilamide. The nitrate was reduced to nitrite by 30 min incubation with nitrate reductase in the presence of nicotinamide adenine dinucleotide 3-phosphate (NADPH). Total nitrite/nitrate concentration was calculated by using standard sodium nitrate. Results were expressed as micromoles per liter.

Estimation of serum total cholesterol

The total cholesterol was estimated by blood samples collected by retero orbital sinus by cholesterol oxidase peroxidase (CHOD-PAP) method [25] using commercially available kit (Coral clinical system, Goa, India).

Experimental procedure ($n = 6$)

Seventy eight rats were allocated to following experimental groups, each group consisting of six animals.

Group I (*Normal control*): rats were maintained on standard food and water regimen and no treatment was given. *Group II* (*sodium nitrite in normal rats*): rats were administered sodium nitrite, nitric oxide donor (12.5 mM) in drinking water for 2 weeks. *Group III* (*L-arginine in normal rats*): rats were administered L-arginine, nitric oxide precursor (2 g/l) in drinking water for 2 weeks. *Group IV* (*Daidzein in normal rats*): rats were administered daidzein, inhibitor of the expression of caveolin (0.2 mg/kg, sc) for 2 weeks. *Group V* (*Diabetic control*): normal rats were fed on HFD for 2 weeks, followed by single dose of streptozotocin (STZ) (35 mg/kg, ip), further followed by HFD for another 8 weeks. *Group VI* (*DMSO in diabetic rats*): diabetic rats were administered DMSO for 2 weeks, after 6 weeks of streptozotocin administration. *Group VII* (*Sodium nitrite treated diabetic rats*): diabetic rats were administered sodium nitrite (12.5 mM), a nitric oxide donor with drinking water, for 2 weeks, after 6 weeks of STZ administration. *Group VIII* (*L-arginine treated diabetic rats*): diabetic rats were administered nitric oxide precursor, L-arginine (2 g/l), in drinking water for 2 weeks, after 6 weeks of STZ administration. *Group IX* (*Daidzein treated diabetic rats*): diabetics rats were administered with inhibitor of the expression of caveolin, daidzein (0.2 mg/kg, sc) for 2 weeks, after 6 weeks of STZ administration. *Group X* (*L-arginine with daidzein treated diabetic rats*): diabetic rats were administered nitric oxide precursor, L-arginine (2 g/l), in drinking water and caveolin inhibitor daidzein (0.2 mg/kg, sc) for 2 weeks, after 6 weeks of STZ administration. *Group XI* (*L-arginine and L-NAME treated diabetic rats*): diabetic rats were administered nitric oxide precursor, L-arginine (2 g/l) in drinking water and endogenous nitric oxide synthase inhibitor, L-NAME (10 mg/kg, ip) for 2 weeks, after 6 weeks of STZ administration. *Group XII* (*Daidzein and L-NAME treated diabetic rats*): diabetic rats were administered caveolin inhibitor daidzein (0.2 mg/kg, sc) with nitric oxide synthase inhibitor, L-NAME (10 mg/kg, ip) for 2 weeks, after 6 weeks of STZ administration. *Group XIII* (*Daidzein, L-arginine and L-NAME treated diabetic rats*): diabetic rats were administered caveolin inhibitor, daidzein (0.2 mg/kg, sc) with nitric oxide precursor, L-arginine (2 g/l), and nitric oxide synthase inhibitor, L-NAME (10 mg/kg, ip) for 2 weeks after 6 weeks of STZ administration.

Statistical analysis

The data were analyzed using two-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test for comparing means from different treatment groups. The data were expressed as mean ± SD and a value of $P < 0.05$ was considered statistically significant.

Results

Body weight

All animal data are summarized in Tables 1 and 2. Two-week treatment with sodium nitrite, nitric oxide donor (12.5 mmol/l, orally), L-arginine nitric oxide precursor (2 g/l, orally), and daidzein caveolin inhibitor (0.2 mg/kg, sc) did not significantly alter the body weight in diabetic rats as compared to normal rat (Fig. 1).

Serum glucose

Combination of HFD and administration of STZ significantly increased the serum concentration of glucose as compared with normal rats. Two-week treatment with sodium nitrite nitric oxide donor (12.5 mmol/l), L-arginine nitric oxide precursor (2 g/l), and daidzein caveolin inhibitor (0.2 mg/kg, sc) did not significantly alter the serum glucose level in diabetic rats (Fig. 2).

BUN, protein in urine, and urinary output in diabetic rats

The concentration of BUN, protein in urine, and volume of urinary output were noted to be significantly increased ($P < 0.05$) in diabetic rats when compared with normal rats. Treatment with sodium nitrite nitric oxide donor (12.5 mmol/l), L-arginine nitric oxide precursor (2 g/l, orally), and daidzein caveolin inhibitor (0.2 mg/kg, sc) for 2 weeks after 6 weeks of STZ administrations significantly reduced BUN, protein in urine, and urinary output when compared with diabetic rats. Moreover, concurrent treatment of L-arginine, a nitric oxide precursor, in presence of daidzein, a caveolin inhibitor, for 2 weeks after 6 weeks of STZ administrations significantly reduced BUN, protein in urine, and urinary output when compared with diabetic rats.

However, administration of L-NAME, a nitric oxide synthase inhibitor (10 mg/kg, ip), for 2 weeks after 6 weeks of STZ administrations with L-arginine, daidzein, or in combination with daidzein and L-arginine significantly attenuated the L-arginine or combination of L-arginine and daidzein-induced decrease in BUN, protein in urine, and urinary output in diabetic rats (Figs. 3, 4, 5).

Serum creatinine

The concentration of serum creatinine was noted to be significantly increased ($P < 0.05$) in diabetic rats when compared with age matched normal rats. Treatment with sodium nitrite (12.5 mmol/l) in drinking water for 2 weeks after 6 weeks of STZ administration significantly reduced serum creatinine level when compared with diabetic rats.

Table 1 Effect of various pharmacological interventions on serum glucose, urinary output, serum nitrite/nitrate ratio, total renal collagen content, kidney weight/body weight on 56 day

Groups	Serum glucose (mg/dl)	Urine output (ml/24 h)	Serum nitrite/ nitrite (μM)	Total renal collagen content (mg/g)	Kidney weight/body weight %
Normal control	104.7 ± 8.68	9.08 ± 3.13	11.46 ± 0.52	2.748 ± 0.28	0.791 ± 0.06
Sodium nitrite per se	105.2 ± 10.92	9.83 ± 3.58	12.34 ± 0.66	2.648 ± 0.27	0.7750 + 0.08
L-Arginine per se	109.9 ± 10.61	9.50 ± 4.41	11.60 ± 0.71	2.695 ± 0.29	0.803 + 0.05
Daidzein per se	103.2 ± 9.37	9.95 ± 2.42	11.52 ± 0.75	2.872 ± 0.14	0.825 + 0.047
Diabetic control	363.1 ± 18.10[a]	90.00 ± 5.58[a]	2.468 ± 0.48[a]	5.690 ± 0.30[a]	1.147 ± 0.06[a]
Vehicle-treated diabetic group	361.6 + 17.89[a]	89.00 ± 5.41[a]	2.06 ± 0.46[a]	5.59 + 0.28[a]	1.16 + 0.08[a]
Sodium nitrite in diabetic group	343.6 ± 16.22[a]	35.00 ± 3.46[b]	11.03 ± 0.58[b]	3.258 ± 0.22[b]	0.853 ± 0.07[b]
L-Arginine in Diabetic group	344.2 ± 10.88[a]	50.33 ± 2.25[b]	7.032 ± 0.58[b]	3.832 ± 0.29[b]	0.905 ± 0.05[b]
Daidzein in diabetic group	338.7 ± 12.17[a]	63.33 ± 3.26[b]	5.362 ± 0.41[b]	4.087 ± 0.16[b]	0.921 ± 0.06[b]
L-Arginine + daidzein diabetic group	335.6 ± 14.71[a]	43.00 ± 4.38[b]	8.830 ± 0.64[b]	3.580 ± 0.35[b]	0.886 ± 0.04[b]
L-Arginine + L-NAME in diabetic group	338.2 ± 12.50[a]	89.50 ± 6.02[a,c]	3.153 ± 0.54[a,c]	5.362 ± 0.32[a,c]	1.090 ± 0.03[a,c]
Daidzein + L-NAME in diabetic group	336.6 ± 11.38[a]	87.17 ± 2.85[a,d]	3.382 ± 0.31[a,d]	5.352 ± 0.15[a,d]	1.050 ± 0.04[a,d]
Daidzein + L-arginine + L-NAME in Diabetic group	341.0 ± 18.31[a]	85.17 ± 3.43[a,e]	3.605 ± 0.28[a,e]	5.178 ± 0.20[a,e]	1.038 ± 0.04[a,e]

All values are expressed as mean ± SD

[a] $P < 0.05$ versus normal control

[b] $P < 0.05$ versus diabetic control

[c] $P < 0.05$ versus L-arginine in diabetic control

[d] $P < 0.05$ versus daidzein in diabetic control

[e] $P < 0.05$ versus daidzein + L-arginine in diabetic control

Table 2 Effect of various pharmacological interventions on blood urea nitrogen, serum creatinine, protein in urine, protein/creatine ratio, serum cholesterol level after treatment on 56 day

Groups	BUN (mg/dl)	Serum creatinine (mg/dl)	Protein in urine (mg/24 h)	Protein–creatinine Ratio	Serum cholesterol (mg/dl)
Normal control	19.24 ± 6.18	0.62 ± 0.07	5.00 ± 1.66	8.3:1	61.42 ± 5.67
Sodium per se	18.97 ± 5.39	0.63 ± 0.09	4.47 + 1.75	6.9:1	61.53 + 5.81
L-Arginine per se	20.76 ± 3.20	0.62 ± 0.08	4.64 + 1.23	7.6:1	62.89.73 ± 6.72
Daidzein per se	18.81 ± 4.95	0.66 ± 0.08	4.59 + 1.38	7:1	63.28 ± 5.83
Diabetic control	98.21 ± 6.27[a]	1.67 ± 0.05[a]	83.53 ± 4.12[a]	51.8:1	160.31 ± 8.082[a]
Vehicle-treated diabetic group	96.27 ± 5.97[a]	1.64 + 0.04[a]	84.22 ± 4.17[a]	51:1	158.07 ± 5.65[a]
Sodium nitrite in diabetic group	34.57 ± 3.07[b]	0.79 ± 0.04[b]	20.27 ± 1.64[b]	25.5:1	156.89 ± 7.27[a]
L-Arginine in diabetic group	56.28 ± 3.11[b]	1.09 ± 0.06[b]	37.95 ± 3.35[b]	35:1	164.30 ± 11.52[a]
Daidzein in diabetic group	66.93 ± 3.83[b]	1.29 ± 0.05[b]	47.40 ± 5.14[b]	36.6:1	168.12 ± 5.60[a]
L-Arginine + daidzein in diabetic group	45.79 ± 2.567[b]	0.94 ± 0.06[b]	28.90 ± 4.52[b]	31:1	162.80 ± 5.48[a]
L-Arginine + L-NAME in diabetic group	94.29 ± 3.79[a,c]	1.58 ± 0.09[a,c]	81.54 ± 4.94[a,c]	51.6:1	181.1 ± 6.08[a]
Daidzein + L-NAME in diabetic group	91.13 ± 5.04[a,c]	1.55 ± 0.09[a,d]	78.97 ± 5.35[a,d]	51:1	176.0 ± 8.72[a]
Daidzein + L-arginine + L-NAME in diabetic group	89.74 ± 6.21[a,c]	1.55 ± 0.08[a,e]	77.97 ± 5.20[a,e]	50:1	169.20 ± 5.38[a]

All values are expressed as mean ± SD

[a] $P < 0.05$ versus normal control

[b] $P < 0.05$ versus diabetic control

[c] $P < 0.05$ versus L-arginine in diabetic control

[d] $P < 0.05$ versus daidzein in diabetic control

[e] $P < 0.05$ versus daidzein + L-arginine in diabetic control

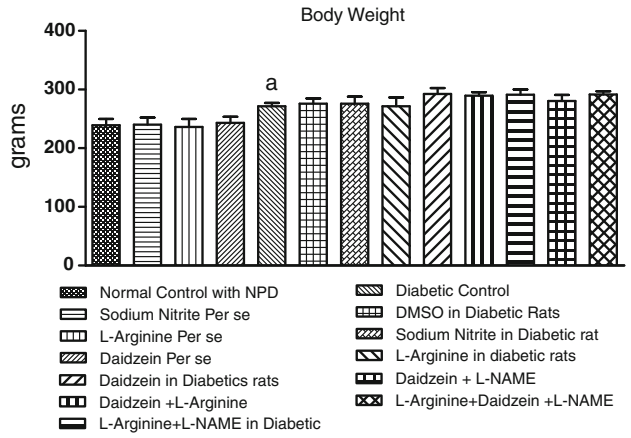

Fig. 1 Effect of various pharmacological interventions on body weight. Results are expressed as mean ± SD. $a = P < 0.05$ versus normal control fed with normal pellets diet

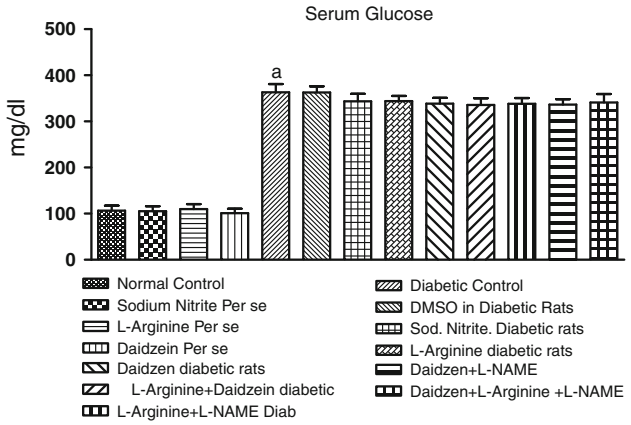

Fig. 2 Effect of various pharmacological interventions on serum glucose. Results are expressed as mean ± SD. $a = P < 0.05$ versus normal control

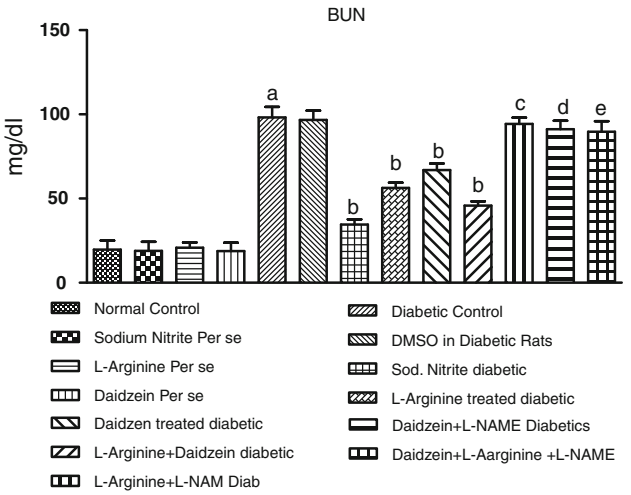

Fig. 3 Effect of various pharmacological interventions on blood urea nitrogen. Values are expressed as mean ± SD. $a = P < 0.05$ versus normal control; $b = P < 0.05$ versus diabetic control; $c = P < 0.05$ versus L-arginine in diabetic control

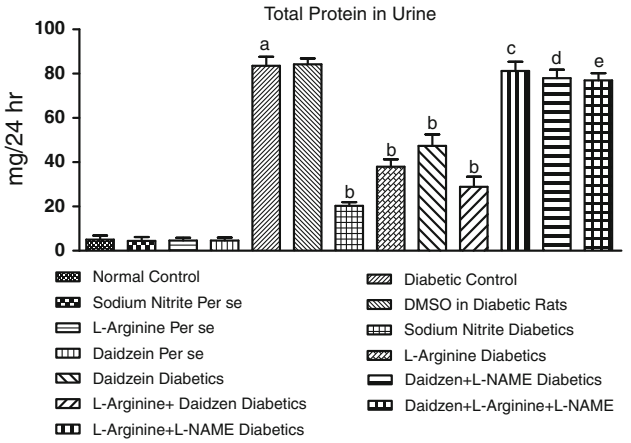

Fig. 4 Effect of various pharmacological interventions on total protein in urine. Values are expressed as mean ± SD. $a = P < 0.05$ versus normal control; $b = P < 0.05$ versus diabetic control; $c = P < 0.05$ versus L-arginine in diabetic control

Moreover, concurrent treatment of L-arginine in presence of daidzein significantly reduced the serum creatinine levels when compared with diabetic rats. Further, treatment with L-arginine partially reduced serum creatinine when compared with sodium nitrite-treated diabetic rats.

However, administration of L-NAME (10 mg/kg, ip) for 2 weeks after 6 weeks of STZ administration with L-arginine, daidzein or in combination with daidzein and L-arginine significantly attenuated the L-arginine or combination of L-arginine and daidzein-induced decrease in serum creatinine in diabetic rats (Fig. 6).

Serum cholesterol

A significant increase ($P < 0.05$) in serum cholesterol was noted in diabetic rats when compared with age matched normal rats. Treatment with sodium nitrite (12.5 mmol/l), L-arginine (2 g/l), and daidzein, (0.2 mg/kg, sc) alone or in combination for 2 weeks after 6 weeks of STZ administration did not significantly decrease the serum cholesterol. Moreover, concurrent treatment of L-arginine with daidzein did not significantly reduce the serum cholesterol levels when compared with diabetic rats.

Also, administration of L-NAME (10 mg/kg, ip) for 2 weeks after 6 weeks of STZ administrations with L-arginine, daidzein, or in combination with daidzein and L-arginine did not significantly reduce the serum cholesterol level in the diabetic rats (Fig. 7).

Serum nitrite/nitrate levels

The serum concentration of nitrite/nitrate was noted to be significantly reduced ($P < 0.05$) in diabetic rats when

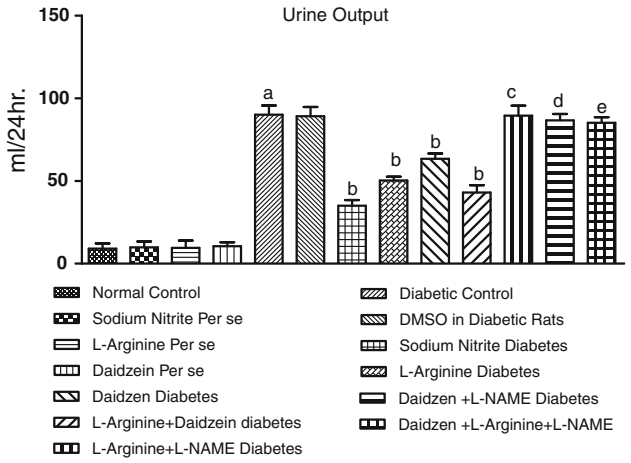

Fig. 5 Effect of various pharmacological interventions on urine output. Values are expressed as mean ± SD. $a = P < 0.05$ versus normal control; $b = P < 0.05$ versus diabetic control; $c = P < 0.05$ versus L-arginine in diabetic control

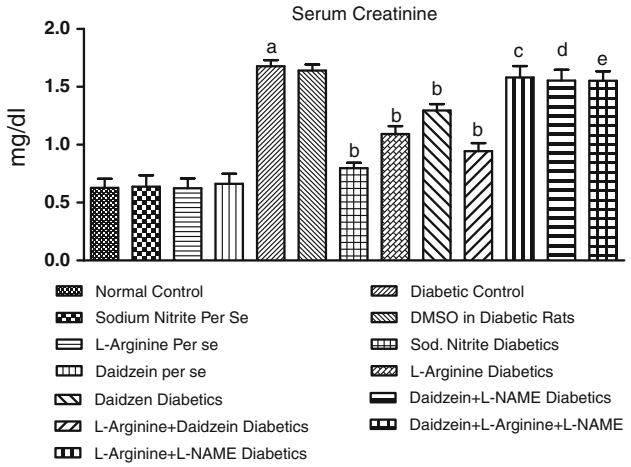

Fig. 6 Effect of various pharmacological interventions on serum creatinine. Values are expressed as mean ± SD. $a = P < 0.05$ versus normal control; $b = P < 0.05$ versus diabetic control; $c = P < 0.05$ versus L-arginine in diabetic control

compared with age matched normal rats. Treatment with sodium nitrite, nitric oxide donor (12.5 mmol/l), L-arginine, nitric oxide precursor (2 g/l), and daidzein, a caveolin inhibitor (0.2 mg/kg, sc), for 2 weeks after 6 weeks of STZ administration significantly increased ($P < 0.05$) the serum concentration of nitrite/nitrate levels when compared with diabetic rats. Moreover, concurrent treatment of L-arginine nitric oxide precursor in presence of daidzein, a caveolin inhibitor, significantly increased the serum nitrite/nitrate level when compared with diabetic rats.

However, administration of L-NAME nitric oxide synthase inhibitor (10 mg/kg, ip) for 2 weeks after 6 weeks of STZ administrations with L-arginine, daidzein or in combination with daidzein and L-arginine significantly

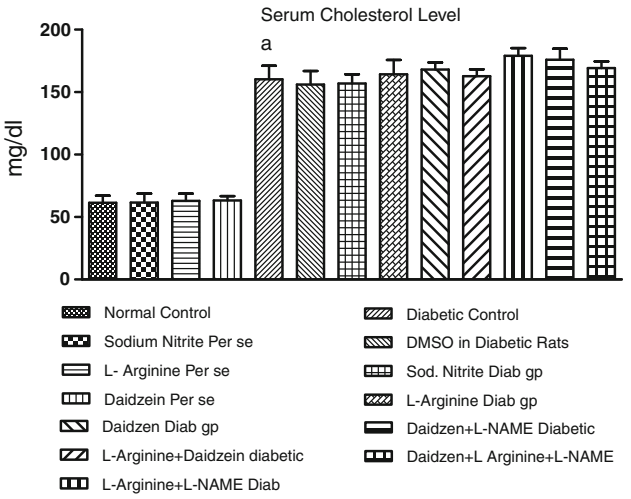

Fig. 7 Effect of various pharmacological interventions on serum cholesterol level. Values are expressed as mean ± SD. $a = P < 0.05$ versus normal control

attenuated the L-arginine or combination of L-arginine and daidzein-induced increase in serum nitrite/nitrate levels in diabetic rats (Fig. 8).

Kidney weight and ratio of kidney weight/body weight

A significant increase in kidney weight and kidney weight/body weight (%) was noted in diabetic rats when compared with normal rats. Treatment with sodium nitrite (12.5 mmol/l), L-arginine (2 g/l), and daidzein (0.2 mg/kg, sc) for 2 weeks after 6 weeks of STZ administration significantly decreased the kidney weight and kidney

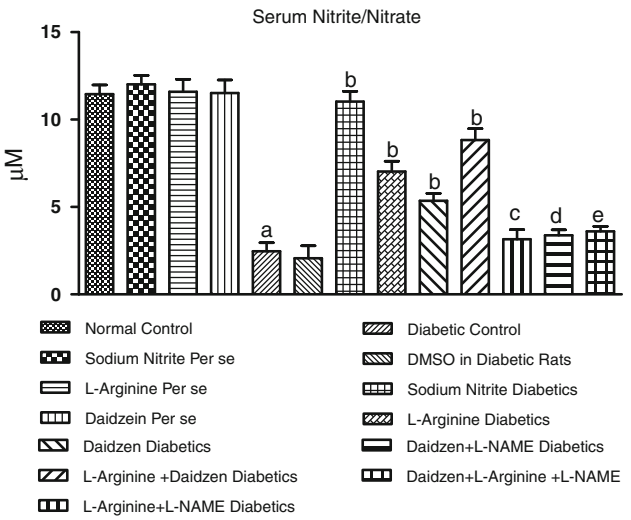

Fig. 8 Effect of various pharmacological interventions on serum nitrite/nitrate ratio. Values are expressed as mean ± SD. $a = P < 0.05$ versus normal control; $b = P < 0.05$ versus diabetic control; $c = P < 0.05$ versus L-arginine in diabetic control

weight/body weight ratio as compared to diabetic rats. Moreover, concurrent treatment of L-arginine, in presence of daidzein, significantly decreased the kidney weight and kidney weight/body weight ratio as compared to diabetic control rats.

Administration of L-NAME (10 mg/kg, ip) for 2 weeks after 6 weeks of STZ administrations with L-arginine, daidzein, or in combination with daidzein and L-arginine significantly attenuated the L-arginine or combination of L-arginine and daidzein-induced decrease in kidney weight and kidney weight/body weight (Figs. 9, 10).

Renal cortical collagen content

A significant increase ($P < 0.05$) in total renal cortical collagen content was noted in diabetic rats when compared with age matched normal rats. Treatment with sodium nitrite (12.5 mmol/l), L-arginine, (2 g/l), and daidzein, a caveolin inhibitor (0.2 mg/kg, sc), for 2 weeks after 6 weeks of STZ administration significantly decreased the renal cortical collagen content as compared to diabetic rats. Moreover, concurrent treatment of L-arginine, a nitric oxide precursor, in presence of daidzein, significantly decreased the renal collagen content as compared to diabetic control rats.

Administration of L-NAME, (10 mg/kg, ip) for 2 weeks after 6 weeks of STZ administrations with L-arginine, daidzein, or in combination with daidzein and L-arginine significantly attenuated the L-arginine or combination of L-arginine and daidzein-induced decrease in renal cortical collagen content (Fig. 11).

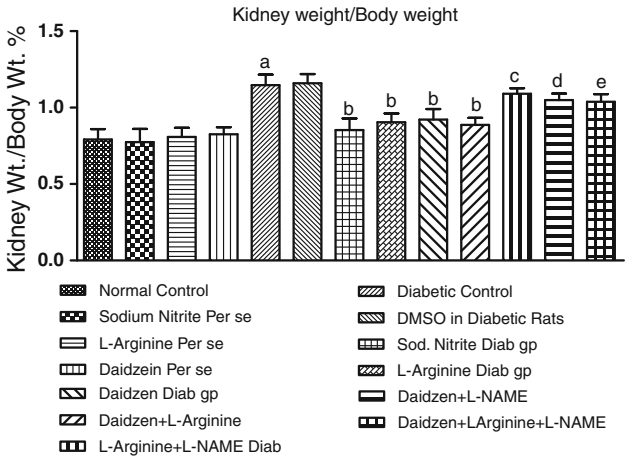

Fig. 10 Effect of various pharmacological interventions on kidney weight/body weight ratio. Values are expressed as mean ± SD. $a = P < 0.05$ versus normal control; $b = P < 0.05$ versus diabetic control; $c = P < 0.05$ versus L-arginine in diabetic control

Discussion

Feeding of HFD for 8 weeks and single dose administration of streptozotocin (35 mg/kg, ip) significantly increased the serum glucose level [26]. The increase in serum creatinine, blood urea nitrogen, and proteinuria has been documented to be index of nephropathy [4]. In the present study, administration of STZ and feeding of HFD significantly increased the serum creatinine, blood urea, and proteinuria. A decrease in serum nitrite/nitrate concentration has been documented to be a marker of vascular endothelial dysfunction (VED) [27, 28], and a strong correlation between VED and DN has been reported [3, 29]. Nephropathy is late complication of DM and renal eNOS

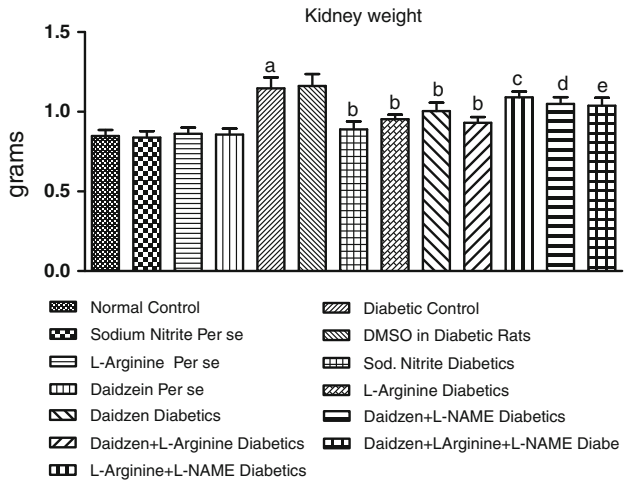

Fig. 9 Effect of various pharmacological interventions on kidney weight. Values are expressed as mean ± SD. $a = P < 0.05$ versus normal control; $b = P < 0.05$ versus diabetic control; $c = P < 0.05$ versus L-arginine in diabetic control

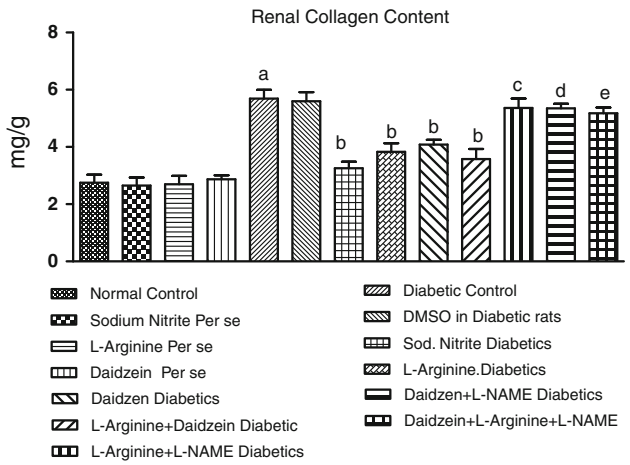

Fig. 11 Effect of various pharmacological interventions on total renal collagen content. Values are expressed as mean ± SD. $a = P < 0.05$ versus normal control; $b = P < 0.05$ versus diabetic control; $c = P < 0.05$ versus L-arginine in diabetic control

production has been reported to be decreased in prolong DM [3]. The serum concentration of nitrite/nitrate, weight of kidney, ratio of kidney weight/body weight and collagen deposition has been reported to be markers of DN [30, 31]. In the present study, the weight of kidney, ratio of kidney weight/body weight, and collagen content were noted to increase while the serum nitrite/nitrate ratio was decreased in diabetic group as compared to normal rats.

Renin–angiotensin–aldosterone system (RAAS) gets overactivated during DN [9]. The principal effect of RAAS is generation of Ang II, which is a potent vasoconstrictor [32]. The increased level of Ang-II further exacerbates the renin release from kidney by decreasing its blood supply, glomerular injury by increasing the intraglomerular pressure and collagen deposition by increasing the expression of the gene of TGF-β, which leads to nephropathy [33]. Nitric oxide acts as a major regulator of release of renin from juxtaglomerular cells [34]. The release of renin is increased by the decreased level of nitric oxide in the renal cortex [35, 36]. In the studies from different laboratories, it has been reported that renal release of nitric oxide is diminished during diabetic mellitus [22, 37]. Moreover, prolonged hyperglycemia is noted to decrease nitric oxide by increasing the metabolism of nitric oxide [38].

In our study, 2-week administration of L-arginine (nitric oxide precursor) did not produce any significant change in normal rats but produced a significant decrease in the blood urea nitrogen, serum creatinine, protein in urine, collagen content of kidney, kidney weight and ratio of kidney weight/body weight, and increased the serum nitrite/nitrate concentration in diabetic rat. Administration of L-NAME (eNOS inhibitor) with L-arginine significantly attenuated the L-arginine-induced decrease in blood urea nitrogen, serum creatinine, protein in urine, collagen content, kidney weight and ratio of kidney weight/body weight, and increase in serum nitrite/nitrate concentration in diabetic rats. Thus, it is possible that the prolonged decrease in renal level of nitric oxide in diabetic rats is responsible for the development of nephropathy. Also, 2 weeks of administration of sodium nitrite in drinking water decreased the blood urea nitrogen, serum creatinine, protein in urine, collagen content of kidney, kidney weight and ratio of kidney weight/body weight, and the increase in serum nitrite/nitrate concentration as compared to the diabetic rats. Our results are in agreement with reports from other laboratories [28, 39].

Caveolae's are invaginations of 50–100 nm in size, at the surface of the plasma membrane protein, consisting of caveolin [14, 16]. It has been reported that caveolin acts as signalosomes and regulate the different signaling pathways including the signaling of G-Protein coupled receptor [40]. Caveolin is a negative regulator of nitric oxide [41]. It has been reported that caveolin interacts with endothelial eNOS by making a caveolin–eNOS complex, which leads to diminished production of NO [18]. The expression of caveolin is upregulated during DM and dyslipidemia [19]. Daidzein, a phytoestrogen, is noted to decrease the expression of caveolin and increase the release of nitric oxide by decreasing the binding of eNOS with caveolin [42]. In present study, only male rats were used to minimize the effect of estrogen (i.e., female rats have a higher level of estrogen compared to males) and 2 weeks of administration of daidzein, a caveolin inhibitor, significantly decreased the blood urea nitrogen, serum creatinine, protein in urine, collagen content, kidney weight, ratio of kidney weight/body weight and increased the level of serum nitrite/nitrate in diabetic rats while it did not produce any significant change in normal rats. It may be suggested that treatment of daidzein may increase the renal nitric oxide level by increasing the activity of eNOS by inhibiting its interaction with caveolin. It has been reported that caveolin inhibits eNOS impeding the signaling of caveolae-targeted receptors that transduce eNOS-stimulatory signals as well as by sterically blocking the calmodulin binding site in eNOS. Moreover, caveolin is noted to interact with eNOS and inhibits the activity of eNOS [18]. The expression of caveolin is upregulated in DM [19, 43]. The decreased activity of eNOS in diabetic kidney results in a decrease in the renal nitric oxide level [22, 37]. Administration of daidzein with L-arginine markedly attenuated the diabetic mellitus induced increased in blood urea nitrogen, serum creatinine, protein in urine, collagen content, kidney weight and ratio of kidney weight/body and decreased serum nitrite/nitrate concentration as compare to diabetic control rats. Thus, both the induction of eNOS by administration of L-arginine and inhibition of caveolin-eNOS complex by diadzein, counteract the changes induced by DM by enhancing the renal levels of NO.

Two-week administration of L-NAME, with daidzein or in combination of daidzein and L-arginine significantly attenuated the daidzein and L-arginine-induced decrease in blood urea nitrogen, serum creatinine, protein in urine, collagen content, kidney weight and ratio of kidney weight/body and increase in serum nitrite/nitrate concentration in diabetic rats.

On the basis of above discussion it may be concluded that a decrease in the renal level of nitric oxide, due to a decrease in the activity of vascular endothelial nitric oxide synthase, may be responsible for the development of nephropathy in HFD and streptozotocin-induced diabetes in rats. Treatment with daidzein, a negative regulator of expression of caveolin, increases the renal levels of NO in diabetic rats, by reducing the interaction of eNOS with caveolin and consequently increasing the activity of eNOS.

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Conflict of interest None.

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