Antioxidant and antihyperglycaemic effects of naringenin arrest the progression of diabetic nephropathy in diabetic rats

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Background
Long-standing diabetes declines the kidney function and is responsible for diabetic nephropathy (DN). The plant and the phytoconstituents have a promising therapeutic potential in the management of diabetes and diabetes complications.
Aim
This study aimed to evaluate the protective effect of naringenin in diabetic-induced nephropathy in experimental rats.
Materials and methods
Diabetes was induced in Sprague-Dawley rats fed a high-fat diet and injected with streptozotocin (STZ) (35 mg/kg body weight, intraperitoneal). At 48 h after injection, hyperglycaemia was confirmed by estimating the blood glucose levels and the rats were left untreated for 4 weeks. The diabetic rats were orally treated with different doses of naringenin 25 and 50 mg/kg body weight, for the next 4 weeks. At the end of the treatment body weight and kidney weight were recorded, serum and urine were used for various biochemical estimations. Oxidative stress levels and histopathological studies were performed on isolated kidneys. The efficacy of treatment was statistically analysed with diabetic control rats.
Results
Increased blood glucose, lipid levels and abnormal kidney functions were noticed in diabetic rats. Moreover, increased levels of oxidative stress markers and altered histological structure were noted in the kidney of diabetic rats, which mimic DN. Naringenin treatments (25 and 50 mg/kg) in diabetic rats significantly restored their kidney functions and reversed hyperglycaemia and lipids level. Hyperfiltration and increased microalbuminuria, urinary albumin excretion and creatinine clearance were effectively attenuated within 4 weeks of naringenin treatments. Increased levels of oxidative stress and histological alteration were restored towards normal.
Conclusion
Naringenin treatments in diabetic rats arrest the progression of DN due to its multivariate actions such as antihyperglycaemic and antioxidant.

Keywords:
antioxidant, diabetic nephropathy, high-fat diet, naringenin, streptozotocin

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Introduction

Chronic diabetes results in diabetic nephropathy (DN), a very common microvascular complication with characteristic structural and functional kidney abnormalities. The International Diabetes Federation estimates that 366 million people had diabetes worldwide, and this number is expected to rise to 552 million by 2030 [1]. Alterations in glomerular filtration rate (GFR) is the key pathophysiological feature of DN which produces end-stage renal disorder (ESRD) through hyperfiltration, increased albumin excretion, microalbuminuria followed by proteinuria and nodular and diffuse glomerulosclerosis [2]. The chronic hyperglycaemia induced structural metamorphosis such as kidney hypertrophy, glomerular basement membrane thickening, tubular atrophy and interstitial fibrosis. Advanced glycation end product formation, protein kinase C and polyol pathways activation [3–5] as well as increased endothelin activity and renin angiotensin aldosterone system are also responsible for the initiation and propagation to DN [6]. Furthermore, the increased systemic and intraglomerular pressure is responsible for the overproduction of reactive oxygen species, release of cytokines (tumor necrosis factor-α, interleukin-6, interleukin-18) and growth factors (transforming growth factor-β, vascular endothelial growth factor) that often lead to mesangial expansion and glomerulosclerosis [6].

Nephropathy has been associated with diabetes and very few options are available to treat like ACE inhibitors and

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AT1 receptor blockers [7]. However, recently some traditional medicines, mainly from the plant source were found to be effective in the management of diabetes. Recently, in-vitro and in-vivo preclinical studies reported positive effects of flavonoids, an active phytoconstituent with benzopyrone nucleus, in halting the progression and/or ameliorating diabetic kidney diseases [8,9]. There are more than 4000 different flavonoids that have been described and categorized under different classes. Among them naringenin [5,7-Dihydroxy-2-(4-hydroxyphenyl)chroman-4-one] is a polyphenolic flavanone and is abundantly present in citrus fruits such as grapefruits, oranges and tomatoes [10]. Both in-vivo and in-vitro scientific studies have shown its wide range of pharmacological effects such as antidiabetic [9], antiatherogenic [11,12], antidepressant [13], immunomodulatory [14], antitumor [15], anti-inflammatory [9], DNA protective [16], hypo-lipidaemic [17], antioxidant [18], anticancer [19], neuroprotective [20] and peroxisome proliferator-activated receptors activator [21]. However, it remains unclear, whether oral treatment of naringenin can hasten the progression of nephropathy in diabetic rats. Therefore, the present study investigates the effect of naringenin on DN in diabetic rats.

Materials and methods

Drugs and chemicals

Naringenin was obtained from Sisco Research Laboratory (Mumbai, India); streptozotocin was purchased from Enzo Life Sciences (Exeter, UK); captopril was obtained from Wockhardt Ltd (Aurangabad, India) and commercial diagnostic kits were obtained from Biolab, Span and Tulip Diagnostic Pvt Ltd (Mumbai, India). All the reagents and chemicals used were of analytical grade and purchased from the local suppliers of Pune.

Experimental animals

Sprague-Dawley rats of either sex (150–200 g) were procured from the National Institute of Biosciences, Pune. The rats were maintained under standard laboratory conditions at a temperature of 23±2°C with a relative humidity of 55±10% in 12 h light and 12 h dark cycle throughout the experiment. The animals had free access to water and standard laboratory feed ad-libitum prior to the dietary manipulation. Ethical guidelines were strictly followed during all the experimental procedure and they were reviewed and approved (IAEC/2011-12/14) by the Institutional Animal Ethics Committee (IAEC) of Sinhgad College of Pharmacy, Pune, constituted under the Committee for the Purpose of Control and Supervision of Experiments on Animals by the Ministry of Environment and Forests, Government of India.

Induction of experimental diabetes

One week after acclimatization, diabetes was rendered in Sprague Dawley rats [22,23]. Initially for 2 weeks, the rats were fed with a combination of high-fat emulsion (HFE) and high-fat diet (HFD), whereas, age-matched control animals were received normal pellet diet only. HFE and HFD were prepared in such a way that the total calories obtained from fat were of 60%. After 2 weeks, the development of insulin resistance was checked by oral glucose tolerance test. The rats that fail to compensate glucose load were then rendered diabetic, by a single intraperitoneal injection of freshly prepared streptozotocin (STZ) (35 mg/kg body weight). STZ was prepared in cold citrate buffer (pH 4.4, 0.1 mol/l). The control rats were injected with cold citrate buffer (pH 4.4, 0.1 mol/l) only. Diabetes was confirmed by measuring the fasting blood glucose level using a commercial glucometer (Contour TS; Bayer Healthcare, Mumbai, India) after 48 h of STZ injection, rats with a blood glucose level of at least 200 mg/dl were considered as diabetic and left untreated for 4 weeks. During the experimental period, normal rats were fed with a normal pellet diet, whereas the diabetic rats were fed with HFD only.

Experimental design

Diabetic rats and age-matched normal rats were divided into six groups (n=6): normal control (NC), naringenin (50 mg/kg body weight), diabetic control (DC) and diabetic rats treated with naringenin 25 and 50 mg/kg body weight or captopril 100 mg/kg body weight. Naringenin or captopril were suspended/dissolved in 1% w/v carboxymethylcellulose prepared in distilled water and was daily administered orally for 4 weeks. Blood was withdrawn from the retro-orbital plexus from overnight fasted rats, centrifuged at 3000 rpm for 15 min and the serum was separated for estimations of biochemical parameter. A 24 h urine sample was collected by placing the animals individually in a metabolic cage.

Oral glucose tolerance test

Oral glucose tolerance test was performed in overnight fasted rats against an oral glucose load at a dose of 2 g/kg body weight. The blood glucose level was estimated at 0 min (before) and 30, 60, 90 and 120 min after glucose load using a commercial glucometer (Contour TS; Bayer Healthcare).

Body weight, kidney weight and relative kidney weight

Body weight and weight of the left kidney at sacrifice were measured gravimetrically using a digital electronic

balance. The ratio of the kidney weight to the body weight was calculated to determine the relative kidney weight:

$$\text{Relative kidney weight}\,(\%) = \frac{\text{Absolute kidney weight}}{\text{Body weight at sacrifice}} \times 100.$$

Biochemical estimations

At the end of the treatment, fasting glycaemia and serum levels of total cholesterol (TC), triglycerides (TG), blood urea nitrogen (BUN), creatinine, albumin and total protein were estimated using commercial diagnostic kits. Urine was investigated for creatinine and albumin estimation, whereas creatinine clearance (Ccr) and urinary albumin excretion rate were calculated by the following equations:

$$\begin{aligned}\text{Ccr}\,(\text{ml/min/kg body weight}) &= [\text{urinary Cr}\,(\text{mg/dl}) \times \text{urinary volume}\,(\text{ml}) \\ &\quad /\text{serum Cr}(\text{mg/dl})] \times [1000/\text{body weight}\,(\text{g})] \\ &\quad \times [1/1440(\text{min})],\end{aligned}$$

$$\text{UAER}\,(\mu\text{g/24 h}) = \text{urinary albumin}\,(\mu\text{g/ml}) \times \text{24 h urine volume}\,(\text{ml}).$$

Estimation of oxidative stress markers

Rats were killed and the kidneys were quickly excised, washed in ice-cold physiological saline (0.9% NaCl), dried and weighed. The right kidney was taken for histopathological examination and the left kidney was homogenized in chilled 50 mmol/l PBS (pH 7.4). The homogenates were centrifuged at 10 000 rpm for 15 min at 4°C. The supernatant was used to estimate the oxidative stress parameters.

Malondialdehyde, a product of lipid peroxidation, was measured by a standard method [24]. Nitric oxide level in the mitochondrial supernatant was measured by the method of Green *et al.* [25] Reduced glutathione was estimated according to the Ellman methods [26] and the Superoxide dismutase (SOD) activity was determined by the standard method of Sun *et al.* [27].

Histopathological examination

A thin section of renal tissue (2 μm) was stained with haematoxylin and eosin and observed under light microscopy for histopathological changes.

Statistical analysis

All the values were expressed as mean±SEM and were analysed by one-way analysis of variance followed by Tukey's multiple comparison tests or two-way analysis of variance followed by Bonferroni post-test. *P* value up to 0.05 was set as minimum levels of significance.

Results

Oral glucose tolerance test

In comparison with normal rats, HFE and HFD fed rats showed a significant rise in blood glucose levels after 30, 60, 120 and 180 min of oral glucose load ($P<0.001$), whereas normal rats showed normal blood glucose levels after 120 min (Fig. 1).

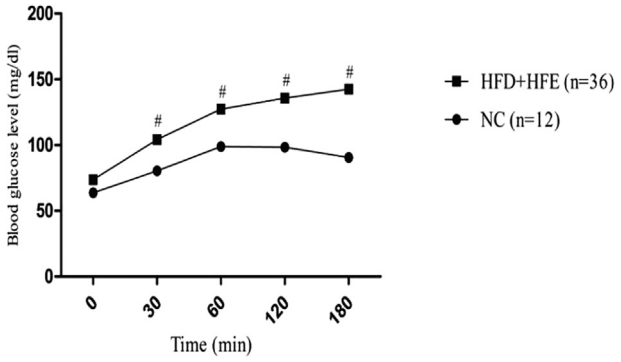
Body weight, kidney weight and relative kidney weight

As shown in Table 1, a significant decrease in body weight accompanied by an increase in kidney weight and relative kidney weight was observed in diabetic rats when compared with normal rats ($P<0.001$). These changes were significantly restored only in a high dose of naringenin (50 mg/kg) treated diabetic rats ($P<0.001$ and <0.01, respectively).

Biochemical estimations

Figure 2 shows a significantly high blood glucose levels in diabetic rats ($P<0.001$), which was attenuated in a

Fig. 1



Oral glucose tolerance curves of normal rats and two weeks of HFE and HFD fed rats $^{\#}P < 0.001$ compared with NC rats. (Two-way ANOVA followed by Bonferroni post-test $P \leq 0.05$).

Table 1 Effect of naringenin on body weight, kidney weight and on relative kidney weight

	Body weight (g)	Kidney weight (g)	Relative kidney weight (%)
NC	219.3±1.20	0.81±0.038	0.373±0.019
NG (50)	236.8±3.47	0.80±0.015	0.341±0.011
DC	199.0±2.17[a]	1.71±0.10[a]	0.866±0.054[a]
D+NG (25)	213.3±4.46	1.30±0.07[@]	0.614±0.040[#]
D+NG (50)	247.2±4.54[#]	0.90±0.05[#]	0.368±0.022[#]
D+CAP (100)	227.7±5.66[@]	0.88±0.04[#]	0.389±0.023[#]

CAP, captopril; DC, diabetic control; NC, normal control; NG, naringenin. [a]$P<0.001$ compared with NC rats; [@]$P<0.01$; [#]$P<0.001$ compared with diabetic control rats.

dose dependent manner after 4 weeks of naringenin treatment at both dose levels, that is, 25 and 50 mg/kg ($P<0.001$). Further our findings indicate the elevated serum levels of TC, TG, BUN and creatinine in diabetic rats as compared with normal rats. However, serum albumin and total protein levels were significantly reduced. Treatment with naringenin at a dose of 25 and 50 mg/kg significantly reduced the levels of serum TC, TG, BUN and creatinine, but significantly increased the levels of albumin and total protein was observed with naringenin 50 mg/kg treated diabetic rats. No statistical significance was observed in normal rats treated with Naringenin (NG) (50 mg/kg) as compared with normal rats (Table 2).

Moreover, in the present investigation, we have observed a significant rise in urine volume, urine creatinine and urine albumin levels as well as Ccr and urinary albumin excretion rate (UAER) in DC rats as compared with normal rats ($P<0.001$). Treatment with naringenin at a dose of 25 and 50 mg/kg body weight in diabetic rats significantly reduced the urine volume ($P<0.001$) and creatinine ($P<0.01$ and <0.001, respectively), whereas decreased levels of Ccr and UAER ($P<0.01$ and <0.001, respectively) were observed only with naringenin 50 mg/kg or captopril 100 mg/kg dose levels (Figs. 2–4).

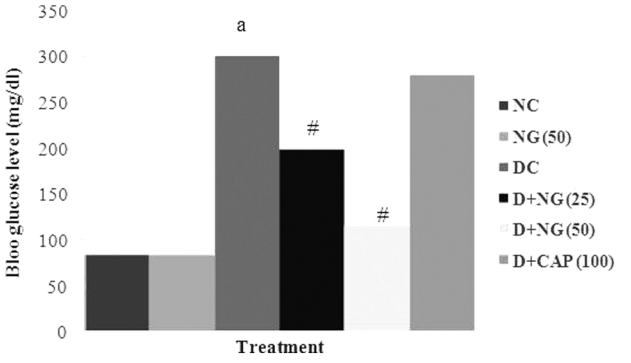
Estimation of oxidative stress

As shown in Fig. 5, significantly increased levels of renal malondialdehyde (MDA), nitric oxide (NO) and decreased levels of glutathione (GSH) as well as SOD activity was observed in diabetic rats ($P<0.001$) as compared with NC rats. Treatment with naringenin at a dose of 25 and 50 mg/kg, body weight for 4 weeks in diabetic rats significantly decreased the MDA levels ($P<0.05$ and <0.001, respectively) as well as increased renal GSH ($P<0.01$ and <0.001) and SOD activity ($P<0.01$ and <0.01). However, a significant reduction in NO levels ($P<0.001$) was observed only in the kidney of diabetic animals treated with naringenin 50 mg/kg.

Histopathological examination

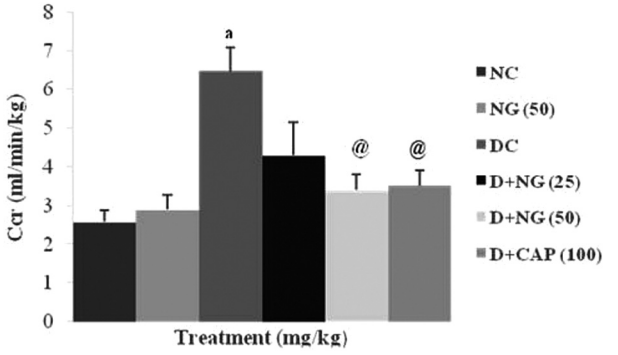
The haematoxylin and eosin stained sections of the kidney of diabetic rats showed glomerular basement membrane thickening and mesangial expansion, while NC and nondiabetic rats treated with naringenin 50 mg/kg body weight showed no abnormalities. However, 4 weeks of treatment with naringenin (25

Fig. 2



Effect of four weeks treatment of naringenin on blood glucose level $^{a}P < 0.001$ compared with NC rats, $^{\#}P < 0.001$compared with DC rats.

Fig. 3



Effect of four weeks treatment of naringenin on creatinine clearance $^{a}P < 0.001$ compared with NC rats, $^{@}P < 0.01$compared with DC rats.

Table 2 Effect of 4 weeks treatment of naringenin on biochemical estimations of diabetic rats

	NC	NG (50)	DC	D+NG (25)	D+NG (50)	D+CAP (100)
TC (mg/dl)	58.12±2.9	52.97±3.9	192.7±4.9^{a}	130.5±3.7$^{\#}$	63.59±4.2$^{\#}$	173.6±6.4
TG (mg/dl)	35.51±1.0	34.08±1.4	185.6±3.1^{a}	127.2±2.7$^{\#}$	43.28±1.8$^{\#}$	156.4±4.4†
BUN (mg/dl)	27.09±2.1	28.10±1.4	68.25±4.7^{a}	51.04±3.4$^{@}$	44.40±2.8$^{\#}$	50.03±2.8$^{@}$
Serum creatinine (mg/dl)	0.54±0.05	0.52±0.06	1.47±0.12^{a}	1.04±0.09$^{@}$	0.65±0.05$^{\#}$	0.73±0.05$^{\#}$
Albumin (g/dl)	3.87±0.16	3.88±0.15	1.58±0.03^{a}	2.17±0.06	3.17±0.20$^{\#}$	3.25±0.16$^{\#}$
Total protein (g/dl)	11.11±0.54	11.78±0.45	6.99±0.35^{a}	8.69±0.21	10.60±0.28$^{\#}$	9.33±0.28$^{@}$
Urine volume (ml)	6.31±0.39	6.73±0.49	23.57±0.80^{a}	13.67±1.53$^{\#}$	9.30±0.56$^{\#}$	11.17±1.01$^{\#}$
Urine creatinine (mg/dl)	64.98±2.71	73.38±3.25	111.7±2.77^{a}	94.06±3.18$^{@}$	81.71±1.68$^{\#}$	74.81±3.99$^{\#}$
Urine albumin (mg/dl)	1.38±0.18	1.37±0.15	2.86±0.15^{a}	2.23±0.15	1.96±0.15$^{@}$	1.89±0.10$^{\#}$

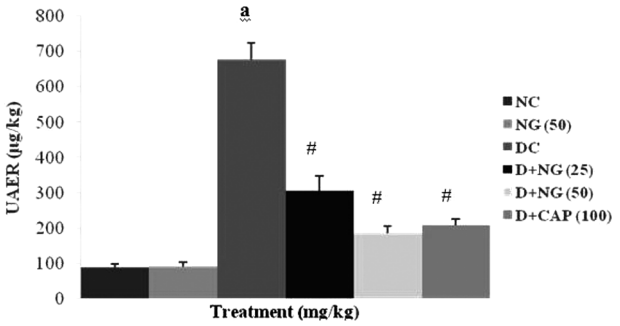
BUN, blood urea nitrogen; CAP, captopril, DC, diabetic control; NC, normal control; NG, naringenin; TC, total cholesterol; TG, triglycerides; $^{a}P<0.001$ compared with NC rats; $^{\dagger}P<0.05$; $^{@}P<0.01$; $^{\#}P<0.001$ compared with DC rats.

and 50 mg/kg) in diabetic rats dose dependently attenuate these progressions (Fig. 6).

Discussion

In parallel with the increase in diabetes, the prevalence of DN in the elderly people accounts for not less than 46% of chronic kidney disease, characterised by reduced glomerular filtration rate, increased urinary albumin excretion, or both, and is an increasing public health issue [28]. Prevalence varies considerably, 8–16% worldwide depending on the distinction between community and regions.

Fig. 4



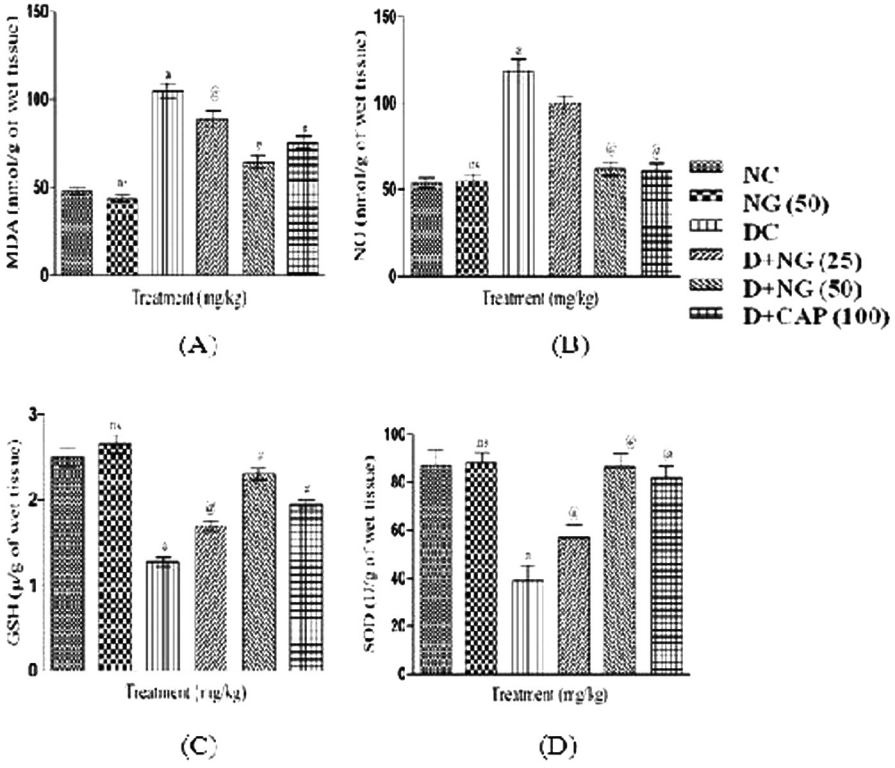
Effect of four weeks treatment of naringenin on UAER $^{a}P < 0.001$ compared with NC rats $^{\#}P < 0.001$ compared with DC rats.

Flavonoids have been reported to possess myriad pharmacological activity with a great margin of safety; therefore, they are used in the treatment of diabetes and long-standing diabetic complications. Here in the present study we investigated whether naringenin can hasten the development of DN and/or slow or stop its progression in type 2 diabetic rat model.

The reported evidences indicate that rat fed with HFD and injected with a low dose of STZ induces type-2 like diabetes, and precipitates an early DN [29]. STZ facilitates preferential uptake of glucose into the pancreatic β-cells through GLUT2 receptor, and activates poly-ADP ribosylation and NO release that hastens the pancreatic β-cell necrosis by alkylating free radicals and limits the rate of synthesis of insulin [30]. Although earlier studies have shown that insulin treatment is effective to prevent renal hypertrophy and subsequent increase in urinary protein excretion [31,32], naringenin exerted an antihyperglycaemic effect in diabetic animals by reducing the blood glucose level.

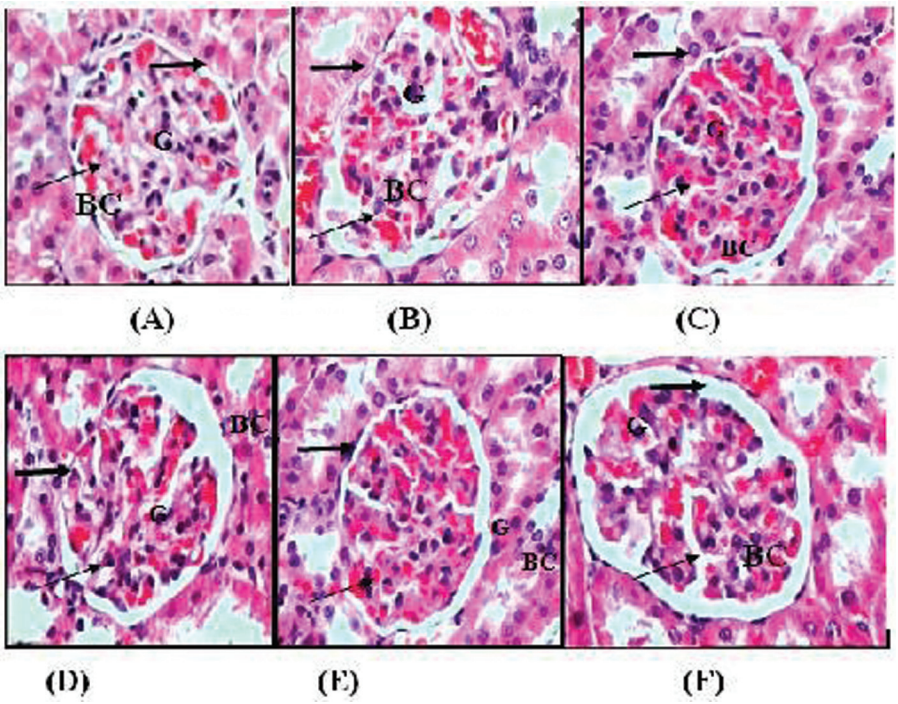
A long-standing hyperglycaemia shifts the metabolism and increased muscle wasting and loss of tissue proteins, resulting in a reduction in body weight [33]. Further,

Fig. 5



Effect of four weeks treatment of naringenin on oxidative stress parameters A) MDA, B) NO, C) GSH & D) SOD activity $^{a}P < 0.001$ compared with NC rats, $^{@}P < 0.01$, $^{\#}P < 0.001$ compared with DC rats.

Fig. 6



Photomicrographs of the glomeruli stained with H&E (A) Normal control rats showing no abnormalities, (B) Diabetic control rats showing GBM thickening and mesangial expansion, (C) D + naringenin (25 mg/kg), (D) D + naringenin (50 mg/kg) showing dose dependent features of healing towards normal basement membrane and mesangial expansion. () Mesangial expansion and () GBM thickening. (E) NG (50) showing no abnormalities. (F) D+CAP (100) showing healing action.

DN is characterized by increased accumulation of extracellular matrix proteins and mesangial expansion which may be responsible for hypertrophy of the kidney and thereby increasing the kidney weight and relative kidney weight [33,34]. In the present investigation, we have observed a significant decrease in body weight and an increase in kidney weight in DC rats, which is in agreement with the previous reports. Treatment with naringenin for 4 weeks significantly restored the body weight and kidney weight and thereby relative kidney weight. The restoration of body weight and kidney weight by naringenin may be linked with its antihyperglycaemic effect.

HMG-coA reductase, a key rate-limiting enzyme which is responsible for the metabolism of cholesterol-rich low-density lipoprotein particles, is inhibited by insulin and therefore insulin deficiency or insulin resistance leads to dyslipidemia [35]. Moreover, it was reported that the progression of DN accelerates with elevated levels of lipids. It was hypothesized that increased intracellular concentration of fatty acids induces glomerular and tubular dysfunction in diabetes [36,37]. In the present investigation, we have noticed a significant rise in serum lipids, that is, TG and TC in rats fed with HFD and in STZ induced diabetic rats. Treatment with naringenin effectively reduced these elevated lipid levels and contributed to its beneficial effects in DN. Earlier reports have also shown the preventive effect of naringenin in dyslipidaemias and other metabolic disorders [38]. Serum creatinine and BUN levels are important parameters to examine kidney function [39]. Elevated levels of serum creatinine and BUN are associated with interstitial atrophy, epithelial necrosis as well as atrophic changes in the glomeruli and thus DN [40,41]. In this study, diabetic rats have shown a significant increase in serum creatinine and BUN, but 4 weeks of naringenin treatment significantly reduces these levels indicating its protective role in halting the progression of DN.

Microalbuminuria and increase in UAER is an indicator of pathophysiological alterations in the kidney such as lesions in glomerular basement membrane and estimation of these is considered key to diagnose nephropathy in diabetic patients [37]. Long-standing diabetes in diabetic rats results in increased urinary excretion of albumin indicating the progression of DN, whereas, 4 weeks of naringenin treatment significantly reduced both microalbuminuria and UAER, thus exerting ameliorative effect on diabetic kidneys. Further captopril treatment in diabetic rats resulted in a significant reduction in urinary albumin excretion which is consistent with earlier reports.

In the early stage of DN, the increased GFR was found to be associated with overproduction of prostaglandin E2 and oxidative stress due to chronic hyperglycaemia [42]. In the present study, significant increase in urine volume and Ccr due to long-standing diabetes was found in diabetic rats compared with normal rats, which indicates early stage of DN. However, 4 weeks of naringenin or captopril treatment showed a reduction in urine volume and Ccr showing recovery towards normal.

Chronic hyperglycaemia results in oxidative stress; MDA level, an index of endogenous lipid peroxidation, was found to be increased significantly in the kidney tissue of diabetic rats reflecting increased oxidative stress [38]. Moreover, enhanced NO synthesis in afferent arterioles and glomerular endothelial cells in hyperglycaemic state could cause preferential dilatation of afferent arterioles, which ultimately induces glomerular enlargement and glomerular hyperfiltration which is a characteristic feature of early stage of DN [41,43]. Various antioxidants were found to be effective in the treatment and/or the prevention of diabetic complications including DN [44,45]. This suggests that the generation of free radicals has a key role in the oxidative stress and thus in the pathogenesis of DN. In present study, we have noticed a significant increase in the oxidative markers such as MDA and NO while a decrease in the activity of endogenous antioxidants, that is, SOD and GSH in diabetic kidney. Treatment with naringenin in diabetic rats significantly restored the antioxidant defence system and contributed towards halting the progression of DN.

Histopathological findings have shown marked alterations in the normal renal architecture. Glomerular basement membrane (GBM) thickening and marked mesangial expansion were observed in DC rats. DN is known to be associated with increased synthesis and/or accumulation of extracellular matrix due to decreased degradation of matrix proteins. Increased oxidative stress, stimulation of renin angiotensin system and expression of growth factors and cytokines in kidney are all responsible for mesangial expansion [35]. We have observed mesangial hypercellularity and capillary basement membrane thickening in the kidney of DC rats. The treatment with naringenin substantially decreased these renal changes; however, more significant changes have been observed with a high dose of naringenin treatment.

Conclusion

Four weeks of naringenin treatment in diabetic rats arrests the progression of early DN and improves the renal function, which may be due to its multivariate actions such as antihyperglycaemic, antioxidant and antihyperlipidaemic activity.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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