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Basic nutritional investigation

Attenuation of diabetic nephropathy by dietary fenugreek (*Trigonella foenum-graecum*) seeds and onion (*Allium cepa*) via suppression of glucose transporters and renin-angiotensin system

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ABSTRACT

Objectives: The aim of this study was to determine the effects of dietary fenugreek (*Trigonella foenum-graecum*) seeds and onion on the hyperglycemia-stimulated glucose transporters and activation of renin–angiotensin system-mediated cascade of events leading to renal lesions in diabetic animals.
Methods: The mechanistic aspects of nephroprotective influence of dietary fenugreek seeds (10%) and onion (3%) on diabetic renal lesions was investigated in streptozotocin diabetic rats. Renal damage was assessed by measuring proteinuria, enzymuria, expression of glucose transporters, renin–angiotensin system, and activities of polyol pathway enzymes.
Results: Diabetes resulted in an upregulation of glucose transporters in kidney tissue, which was countered by these dietary interventions. The upregulation of renal angiotensin-converting enzyme and its receptor was also countered by these dietary interventions. Dietary fenugreek and onion significantly reduced metabolites of polyol pathway, nitric oxide, and *N*-acetyl-β-D-glucosaminidase activity. Markers of podocyte damage in kidney (nephrin, podocin, and podocalyxin) and their urinary excretion were normalized along with downregulation of the expression of kidney injury molecule-1 by these dietary interventions. Dietary fenugreek and onion effectively countered the diabetes-induced structural abnormalities of renal tissue.
Conclusion: Feeding fiber-rich fenugreek seeds and sulfur compounds-rich onion produced a blockade in glucose translocation and renin–angiotensin system in the early stage of diabetic nephropathy. This involved a downregulation of the expression of polyol pathway enzymes, partial restoration of the podocyte damage, revival of renal architecture and functional abnormality. The present study also suggested that these two dietary interventions offer a higher renoprotective influence when consumed together.

Introduction

Diabetic nephropathy (DN) is a microvascular complication recognized as a primary cause of end-stage renal disease (ESRD), usually attributed to metabolic consequences of abnormal glucose regulation. Research has suggested that poor glycemic control undoubtedly plays a significant role, whereas metabolic events responsible for triggering DN are not well elucidated. There has been a renewed interest in understanding the possibility of persistent proteinuria, cell damage, and decline in function contributing to the pathogenesis of progressive renal disease. Hyperglycemia influences facilitative glucose transporter to mediate mesangial cell glucose flux, which leads to the activation of a series of signaling cascades favoring glomerulosclerosis [1]. There are several hyperglycemia-mediated metabolic mechanisms that lead to DN through oxidative stress, polyol pathway, advanced glycation end products (AGEs), activated protein kinase C (PKC), hexosamine pathway, xanthine oxidase activity, mitochondrial respiratory chain deficiency, NAD(P)H oxidase, and nitric oxide synthase (NOS), and so on [2].

Apart from this, hyperglycemia-mediated hemodynamic stimuli also leads to DN involving increased systemic and intraglomerular pressure and activation of various vasoactive hormones, which includes the intrarenal renin–angiotensin system (RAS), NO, vascular endothelial growth factor, endothelin, urotensin II, and the kallikrein–kinin system [3]. These hemodynamic changes play an important role in contributing to mesangial matrix

All authors made substantial contributions to the conception and design, animal experiment and acquisition of data, interpretation of the data, and drafting of the manuscript. SRP was responsible for the animal study and analytical work involved in this investigation. KS was responsible for planning and supervision of the experiment and writing the manuscript.

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expansion, podocyte injury, and nephron loss by increasing albumin passage across glomerular capillaries [4].

We recently reported amelioration of hyperglycemia and its associated metabolic abnormalities [5], alleviation of oxidative stress–mediated nephropathy [6], and cardiac damage [7] by a combination of fenugreek seeds and onion in experimental diabetes, the beneficial effect being higher than that of their individual interventions. In the present investigation, mechanistic aspects of the nephroprotective influence of dietary fiber-rich fenugreek seeds and the antioxidant-rich onion on diabetic renal lesions was further investigated in rats. The main aim was to examine the synergistic/additive effects if any, among dietary fenugreek seeds (*Trigonella foenum-graecum*) and onion (*Allium cepa*), as they exert their antidiabetic action by different mechanisms. The effects of these two food ingredients particularly on the hyperglycemia-stimulated glucose transporters and activation of the RAS-mediated cascade of events leading to renal lesions in diabetic animals were studied here.

Materials and methods

Chemicals and materials

Complete details of chemicals and materials used in this study are described in the Supplementary Material.

Animals and experimental design

This animal study was carried out by taking all appropriate measures to minimize pain or discomfort with due approval from the Institutional Animal Ethics Committee (CSIR – CFTRI, Mysore), regulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. Eighty male Wistar rats (140–150 g body mass) raised in the Experimental Animal Production Facility of this institute were housed in individual stainless steel cages under standard laboratory conditions with a 12 h/12 h light/dark cycle. The animals had ad libitum access to food and water. Experimental diabetes was induced with intraperitoneal streptozotocin (STZ; 45 mg/kg) as reported previously [5]. Animals with fasting blood glucose >250 mg/dL (determined by glucose oxidase method) [8] were recruited as diabetic. The dietary interventions were made for a duration of 6 wk. The eight groups of rats among diabetic (12 rats in each group) and non-diabetic (8 rats in each group) category were:

1. Normal control (C),
2. C + fenugreek (10%),
3. C + onion (3%),
4. C + fenugreek (10%) + onion (3%),
5. Diabetic control (D),
6. D + fenugreek (10%),
7. D + onion (3%), and
8. D + fenugreek (10%) + onion (3%).

Details of experimental diets are described in the Supplementary Material.

Urine samples were collected in ice-cold bottles for 24 h, toward the end of the feeding schedule; clarified by filtration, and used for various enzymatic analyses. Collection and extraction of RNA from urine were carried out according to the procedure of Sato et al. [9]. At the end week 6, the animals were sacrificed under euthanasia by exsanguination from the heart and the kidney tissue was quickly excised, weighed, and processed for various analysis.

Renal and urinary enzymes

Established procedures were followed for the assay of various enzymes in kidney or urine samples. Activities of fructose-1,6-bisphosphatase [10], glucose-6-phosphate dehydrogenase (G6 PD) [11], glucose-6-phosphatase (G6 Pase) [12], hexokinase [13], and lactate dehydrogenase (LDH) [14] in renal tissue were assayed according to standard procedures. Renal and urinary alanine aminotransferase (ALT) [15], aspartate aminotransferase (AST) [16], alkaline and acid phosphatise [17], urinary *N*-acetyl-β-D-glucosaminidase (NAG) [18], renal microsomal Na^+,K^+-ATPase [19] and Mg^{2+},Ca^{2+}-ATPase activity [20] were measured independently by adopting previously described methods. Activities of polyol pathway enzymes—aldose reductase (AR) [21], sorbitol dehydrogenase (SDH) [22], and angiotensin-converting enzyme (ACE) [23]—were measured in the kidney tissue.

Nitric oxide and polyol pathway metabolites

Renal and urinary nitric oxide (NO) levels were estimated using a commercial kit (Cat. # K262, Biovision, Mountain View, CA, USA). Sorbitol [24], fructose [25], and glucose [8] were estimated using established procedures.

Renal membrane fluidity

Renal microsomal membrane fluidity was measured by employing 1,6-diphenyl-1,3,5-hexatriene according to the method of Levin et al. [26] and the polarization of fluorescence was expressed in terms of the fluorescence anisotropy.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of urinary proteins

Proteins in urine samples were precipitated using 3% trichloroacetic acid. Urinary proteins were analyzed on 12% polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS) under reducing conditions [27]. The protein bands were visualized in a G-Box Chemi XT4 gel doc system (Syngene Synoptic Ltd., Cambridge, UK).

Western blot analysis

Western blot analyses were performed as previously described [6]. Information regarding antibodies used in this study are mentioned in the Supplementary Material.

Real-time polymerase chain reaction analysis

Total RNA extraction and mRNA expression analysis of kidney and urine samples were performed as reported previously [6]. Primers for the polymerase chain reaction amplifications used were synthesized using supplies from Sigma-Aldrich Chemical Co. (Supplementary Table 1).

Histopathologic, immunohistologic, and confocal laser scanning microscopic analysis

Renal tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Renal sections (5 μm thickness) were stained with hematoxylin and eosin (H&E) for histopathologic observation, 0.5% periodic acid and Schiff solution (PAS) staining for glycogen, and Masson's trichrome (MT) staining for collagen fibers deposition, respectively. Details of immunohistologic and confocal microscopic analysis were described previously [6]. The images were analyzed using a microscope (Model: Olympus BX-5, Japan; ProgRes C-5 software). In confocal microscopic studies (Carl Zeiss, LSM 700, Jena, Germany; ZEN 2009 software), the fluorescence of protein spectra emitted was analyzed by T-PMT detector with spectral increment 1 a.u., with a 30.5-μm pinhole size, and a scanning time of 11.3s. The images were captured at pixels of 512 × 512, with a pixel size of 1.25 μm, and a pixel dwell time of 25.6 μs.

Statistical analysis

All results are expressed as mean ± SEM of eight rats. Statistical analysis was performed using GraphPad Instat statistical software (GraphPad Software, Inc., La Jolla, CA, USA). To evaluate differences between normal (non-diabetic) and diabetic groups over dietary interventions (none, fenugreek, onion, and fenugreek + onion), data were analyzed by two-way analysis of variance. For this analysis, the two dependent variables were dietary intervention and group (normal versus diabetic). Results were evaluated using Tukey's multiple comparison test, and considered significantly different at $P < 0.05$.

Results

Influence on glucose translocation

Diabetic rats were characterized by stimulated glucose transporter isoforms, GLUT1 (189%) and GLUT2 (95%) due to increased intracellular glucose concentration as compared with normal rats (Fig. 1A, B). Feeding diabetic rats with fenugreek, onion, and fenugreek + onion demonstrated a significant decrease in the expression of GLUT1 (63%, 43%, and 68%, respectively) and GLUT2 proteins (70%, 25%, and 75%, respectively) compared with the

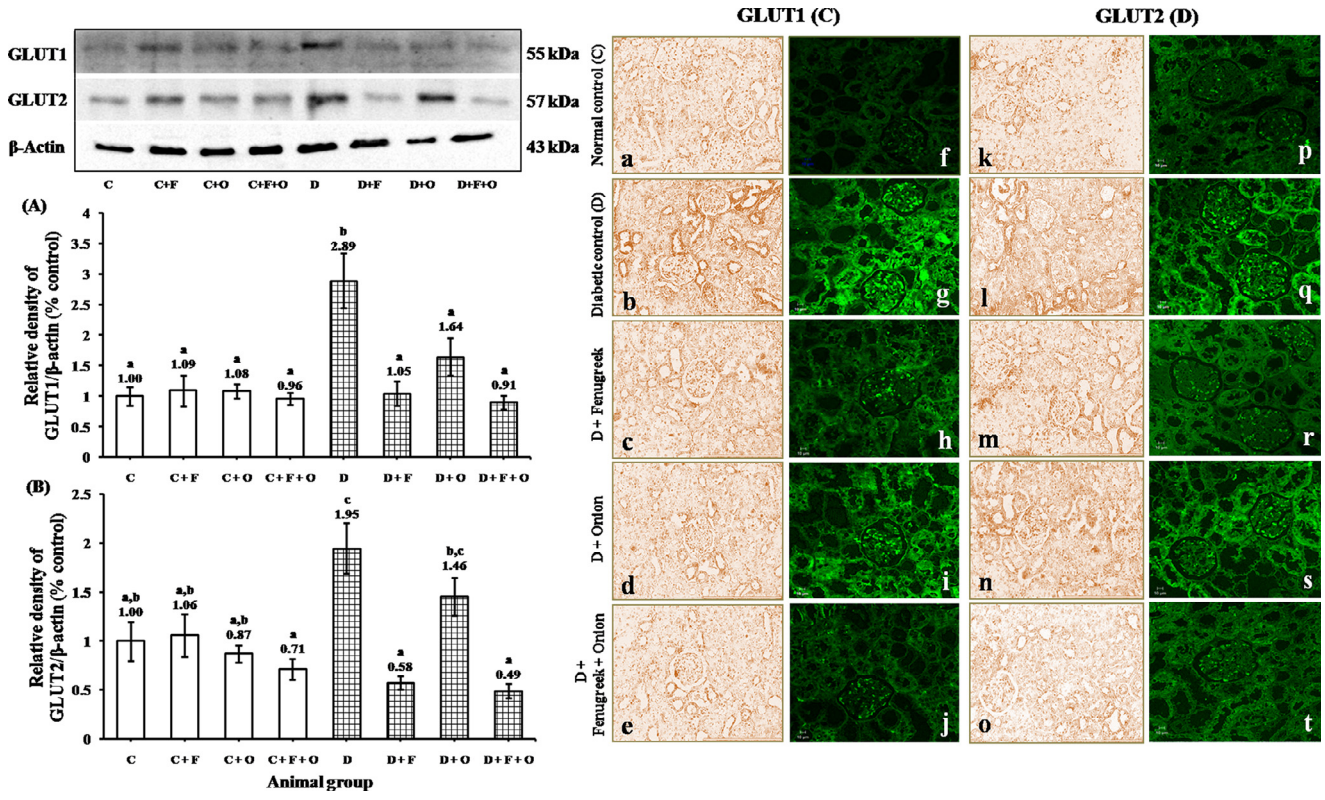

Fig. 1. Influence of dietary fenugreek seeds and onion on renal protein expression of glucose transporters (**A**) GLUT1 and (**B**) GLUT2. Immunohistochemistry (20 × objective) and confocal (20 × objective) image analysis of (**C**) GLUT1 (a–j) and (**D**) GLUT2 (k–t) in diabetic rats. Values are mean ± SEM of eight animals in each group. Each bar carrying different letters (a, b, c) are significantly different ($P < 0.05$). C, normal control; D, diabetic control; F, fenugreek; O, onion.

diabetic control group. Consistent with this, immunohistochemical and confocal image study analysis of the kidney showed that the protein expression of glucose transporters was predominantly increased in diabetes, whereas this was partially reversed by the three dietary interventions (Fig. 1C, D).

Influence on renal enzymes of carbohydrate metabolism, marker enzymes, and membrane fluidity

A hyperglycemic condition resulted in increased activity of renal fructose 1,6-diphosphatase (2.5-fold), G6 Pase (64%), and LDH (38%; Table 1). On the other hand, activities of hexokinase (33%) and G6 PD (48%) were decreased compared with normal control. Diabetic rats maintained on any of the three dietary interventions showed a significant reversal in the activities of these enzymes compared with the diabetic control group. A higher beneficial effect was seen with the combination in this regard. Diabetic rats exhibited increased activity of renal aminotransferases and phosphatases compared with normal control animals (Table 2). AST and ALT were 60% and 2.1-fold higher, respectively, whereas alkaline and acid phosphatases were 25% and 38% higher, respectively, in diabetic animals. Dietary fenugreek and onion significantly countered the activities of aminotransferases by 12% to 18% in diabetic rats. Fenugreek, onion, and fenugreek + onion feeding also significantly reversed the activities of alkaline and acid phosphatases (by 10–19%) in the kidney of diabetic animals.

Diabetic rats showed increased activities of total Na^+,K^+- (41%), ouabain-sensitive (42%), Mg^{2+}-ATPase (65%), and Ca^{2+}-ATPase (31%) in the renal tissue (Table 3). This alteration of membrane-bound enzyme activities as a result of diabetes was countered by dietary fenugreek (by 23%, 22%, 17%, and 16%, respectively) and onion (by 14%, 13%, 16%, and 20%, respectively), which is

Table 1
Influence of dietary fenugreek seeds and onion on renal enzymes of carbohydrate metabolism in diabetic rats

Animal group	Hexokinase*	Fructose-1,6-bisphosphatase†	Glucose-6-P dehydrogenase*	Glucose-6-Phosphatase†	Lactate dehydrogenase*
C	$31.1^{a} \pm 1.60$	$2.98^{a} \pm 0.56$	$0.077^{a} \pm 0.002$	$21.0^{a} \pm 2.60$	$1.59^{a} \pm 0.03$
C + F	$32.0^{a} \pm 1.62$	$3.88^{a,c} \pm 0.21$	$0.067^{a,d} \pm 0.003$	$22.3^{a} \pm 0.57$	$1.58^{a} \pm 0.01$
C + O	$32.6^{a} \pm 1.79$	$2.70^{a} \pm 0.18$	$0.076^{a} \pm 0.002$	$22.5^{a} \pm 0.20$	$1.55^{a} \pm 0.02$
C + F + O	$32.6^{a} \pm 1.29$	$3.33^{a} \pm 0.05$	$0.080^{a} \pm 0.003$	$21.9^{a} \pm 1.30$	$1.39^{b} \pm 0.02$
D	$20.9^{b} \pm 2.53$	$7.47^{b} \pm 0.39$	$0.040^{b} \pm 0.002$	$34.5^{b} \pm 0.40$	$2.19^{c} \pm 0.05$
D + F	$25.5^{a,b} \pm 1.73$	$5.23^{c} \pm 0.74$	$0.054^{c} \pm 0.002$	$28.7^{c} \pm 0.41$	$1.78^{d} \pm 0.04$
D + O	$27.2^{a,b} \pm 1.54$	$6.05^{b} \pm 0.31$	$0.050^{c} \pm 0.002$	$30.1^{c} \pm 0.57$	$1.63^{a} \pm 0.07$
D + F + O	$28.3^{a} \pm 2.11$	$4.93^{c} \pm 0.35$	$0.061^{d} \pm 0.003$	$25.9^{a} \pm 1.03$	$1.93^{d} \pm 0.05$

C, normal control; D, diabetic control; F, fenugreek; O, onion.
Values are mean ± SEM of eight animals in each group.
Means within the same column (in each parameter) carrying different superscripts (a, b, c, d) are significantly different ($P < 0.05$).
*Specific activity unit: μmol of product formed·min·mg^{-1} protein.
†Specific activity unit: μmol Pi released min mg^{-1} protein.

Table 2
Influence of dietary fenugreek seeds and onion on renal aminotransferases and phosphatases in diabetic rats

Animal group	Aspartate aminotransferase*	Alanine aminotransferase*	Alkaline phosphatase†	Acid phosphatase†
C	$0.505^{a} \pm 0.006$	$7.67^{a} \pm 0.06$	$0.319^{a} \pm 0.012$	$0.204^{a} \pm 0.009$
C + F	$0.500^{a} \pm 0.005$	$7.44^{a} \pm 0.19$	$0.322^{a} \pm 0.027$	$0.226^{a} \pm 0.010$
C + O	$0.514^{a} \pm 0.012$	$7.28^{a} \pm 0.08$	$0.336^{a} \pm 0.004$	$0.181^{a} \pm 0.015$
C + F + O	$0.515^{a} \pm 0.012$	$7.54^{a} \pm 0.09$	$0.328^{a} \pm 0.037$	$0.223^{a} \pm 0.014$
D	$0.809^{b} \pm 0.037$	$16.26^{b} \pm 0.13$	$0.399^{b} \pm 0.020$	$0.283^{b} \pm 0.017$
D + F	$0.708^{b,c} \pm 0.028^{c}$	$13.47^{c} \pm 0.28$	$0.330^{a} \pm 0.008$	$0.256^{a,b} \pm 0.010$
D + O	$0.691^{c} \pm 0.028$	$13.24^{c} \pm 0.34$	$0.324^{a} \pm 0.010$	$0.231^{a} \pm 0.007$
D + F + O	$0.665^{c} \pm 0.037$	$12.83^{c} \pm 0.42$	$0.323^{a} \pm 0.018$	$0.256^{a,b} \pm 0.009$

C, normal control; D, diabetic control; F, fenugreek; O, onion.
Values are mean ± SEM of 8 animals in each group.
Means within the same column (in each parameter) carrying different superscripts (a, b, c) are significantly different ($P < 0.05$).
*Specific activity unit: μg pyruvate released·min·mg^{-1} protein.
†Specific activity unit: μmol P-nitrophenol formed·min·mg^{-1} protein.

Table 3
Influence of dietary fenugreek seeds and onion on renal ATPase activities and on renal membrane fluidity in diabetic rats

	Na^{+}, K^{+}-ATPase					
Animal group	Ouabain sensitive	Total	Mg^{2+}-ATPase	Ca^{2+}-ATPase	Anisotropy (r_{DPH})	Anisotropy parameter $[(r_0/r)\text{-}1]^{-1}$
C	$0.321^{a} \pm 0.021$	$0.353^{a} \pm 0.022$	$0.202^{a} \pm 0.001$	$0.367^{a} \pm 0.024$	$0.173^{a} \pm 0.020$	$0.925^{a} \pm 0.045$
C + F	$0.338^{a} \pm 0.010$	$0.366^{a} \pm 0.015$	$0.193^{a} \pm 0.010$	$0.335^{a} \pm 0.014$	$0.195^{a} \pm 0.005$	$1.168^{a} \pm 0.036$
C + O	$0.322^{a} \pm 0.016$	$0.353^{a} \pm 0.063$	$0.201^{a} \pm 0.009$	$0.374^{a} \pm 0.020$	$0.208^{a} \pm 0.06$	$1.351^{a,c} \pm 0.121$
C + F + O	$0.347^{a} \pm 0.021$	$0.379^{a} \pm 0.026$	$0.202^{a} \pm 0.011$	$0.361^{a} \pm 0.013$	$0.189^{a} \pm 0.004$	$1.092^{a} \pm 0.042$
D	$0.458^{b} \pm 0.014$	$0.497^{b} \pm 0.021$	$0.333^{b} \pm 0.021$	$0.480^{b} \pm 0.009$	$0.314^{b} \pm 0.017$	$6.542^{b} \pm 0.354$
D + F	$0.354^{a} \pm 0.009$	$0.382^{a,b} \pm 0.013$	$0.276^{c} \pm 0.014$	$0.402^{a} \pm 0.010$	$0.221^{a,c} \pm 0.010$	$1.567^{c} \pm 0.169$
D + O	$0.398^{a,b} \pm 0.025$	$0.427^{a,b} \pm 0.027$	$0.279^{c} \pm 0.005$	$0.386^{a} \pm 0.011$	$0.275^{b,c} \pm 0.006$	$3.161^{d} \pm 0.147$
D + F + O	$0.340^{a} \pm 0.013$	$0.363^{a} \pm 0.018$	$0.180^{a} \pm 0.012$	$0.357^{a} \pm 0.003$	$0.184^{a} \pm 0.025$	$1.034^{a} \pm 0.112$

C, normal control; D, diabetic control; F, fenugreek; O, onion.
Values (μmol Pi liberated·h·mg^{-1} protein) are mean ± SEM of eight animals in each group.
Means within the same column (in each parameter) carrying different superscripts (a, b, c) are significantly different ($P < 0.05$).

considered as resulting from restoring of renal integrity. Dietary fenugreek + onion restored ouabain-sensitive (26%), total Na^{+}/K^{+}-ATPase (27%), Mg^{2+}-ATPase (46%), and Ca^{2+}-ATPase (25%) activities compared with diabetic control. Fluidity parameters such as anisotropy (82%) and anisotropy parameter (sevenfold) were higher in diabetic animals, indicating decreased fluidity of renal membrane (Table 3). Treatment of diabetic animals with fenugreek seeds (29% and 76%), onion (12% and 52%), and the combination of the two (41% and 84%) significantly reversed the same.

Influence on urinary protein and enzymes

Diabetic rats excreted large amounts of proteins with molecular weight of around 66 kDa and proteins of molecular weight still higher as seen separated by SDS-polyacrylamide gel electrophoresis (PAGE; Supplementary Fig. 1). A lower intensity band of molecular weight 18 kDa was not expressed in diabetic rat urine. The three dietary interventions reduced the excretion of 66 kDa protein and higher molecular mass proteins, whereas 18 kDa protein excretion was restored, the effect being highest with combination intervention.

The diabetic condition resulted in a significantly increased excretion of enzymes (NAG, alkaline phosphatase, and ALT) from proximal tubular origin (7-, 2.7-, and 7.5-fold) compared with normal animals (Table 4). Diabetic animals maintained on these dietary interventions showed a distinct tendency to excrete lesser amounts of these tubular enzymes in the urine, the extent of decrease being 29%, 27%, and 37% in fenugreek; 28%, 35%, and 53% in onion; and 44%, 57%, and 56% in fenugreek + onion groups. Diabetic rats also exhibited increased urinary excretion of enzymes of distal tubular origin such as LDH (7.6-fold), AST (4.8-fold), and acid phosphatase (3.7-fold), respectively (Table 4). Leaching of these enzymes was comparatively less in diabetic animals maintained on fenugreek (45%, 7.4%, and 20% less), onion (50, 12%, and 35% less), and fenugreek + onion (64%, 47%, and 43% less) compared with diabetic control animals.

Influence on polyol pathway

Diabetic rats were characterized by an elevated level of renal polyol pathway enzyme activity, their expression, and metabolite formation compared with normal rats (Table 5 and Fig. 2). The activities of AR and SDH were significantly increased by 62% and 22%, protein by 2.4- and 4.3-fold, and mRNA expression by 2.7- and 3.1-fold, respectively, compared with the normal control group. Dietary fenugreek, onion, and fenugreek + onion significantly reduced AR activity (19%, 13%, and 22%, respectively), protein expression (10%, 24%, and 52%, respectively), and mRNA expression (56%, 51%, and 59%, respectively) in diabetic animals. Increased activity of SDH (34%, 27%, and 45%, respectively), protein expression (48%, 46%, and 51%, respectively), and mRNA expression (57%, 59%, and 73%, respectively) were significantly countered, the effect being higher in the case of combination. Immunohistochemical and confocal image analysis of renal tissue evidenced similar results with respect to protein expression in this tissue.

As expected, increased activity of polyol pathway enzymes in diabetes resulted in increased glucose metabolism (3.7-fold) and metabolite formation (5.6- and 2.4-fold) in this tissue (Table 5). Formation of these metabolites (glucose, sorbitol, and fructose) was significantly reduced by dietary fenugreek (23%, 43%, and 33%,

Table 4
Leaching of renal tubular enzymes (proximal and distal region) in diabetic rats maintained on dietary fenugreek seeds and onion

	Proximal region			Distal region		
Animal group	Alanine aminotransferase*	Alkaline phosphatase†	N-Acetyl-β-D-glucosaminidase†	Aspartate aminotransferase‡	Acid phosphatase§	Lactate dehydrogenase‖
C	$5.69^{a} \pm 0.30$	$0.063^{a} \pm 0.023$	$0.229^{a} \pm 0.052$	$0.479^{a} \pm 0.03$	$0.034^{a} \pm 0.004$	$0.033^{a} \pm 0.001$
C + F	$5.39^{a} \pm 0.72$	$0.064^{a} \pm 0.006$	$0.181^{a} \pm 0.024$	$0.441^{a} \pm 0.06$	$0.045^{a} \pm 0.006$	$0.029^{a} \pm 0.002$
C + O	$5.47^{a} \pm 1.05$	$0.045^{a} \pm 0.006$	$0.215^{a} \pm 0.086$	$0.446^{a} \pm 0.02$	$0.034^{a} \pm 0.000$	$0.027^{a} \pm 0.005$
C + F + O	$4.78^{a} \pm 0.75$	$0.056^{a} \pm 0.010$	$0.205^{a} \pm 0.039$	$0.529^{a} \pm 0.06$	$0.036^{a} \pm 0.005$	$0.029^{a} \pm 0.002$
D	$42.8^{b} \pm 8.24$	$0.172^{b} \pm 0.002$	$1.610^{b} \pm 0.399$	$17.90^{b} \pm 1.99$	$0.164^{b} \pm 0.017$	$0.253^{b} \pm 0.003$
D + F	$31.1^{b,c} \pm 2.72$	$0.108^{c} \pm 0.012$	$1.145^{b,c} \pm 0.082$	$16.57^{b} \pm 0.38$	$0.131^{b,c} \pm 0.008$	$0.139^{c} \pm 0.028$
D + O	$27.9^{b,c} \pm 2.36$	$0.081^{a} \pm 0.002$	$1.157^{b,c} \pm 0.175$	$15.77^{b} \pm 1.49$	$0.107^{c,d} \pm 0.006$	$0.125^{c} \pm 0.009$
D + F + O	$18.4^{d} \pm 1.88$	$0.076^{a} \pm 0.013$	$0.903^{c} \pm 0.033$	$9.43^{c} \pm 0.79$	$0.094^{d} \pm 0.009$	$0.092^{d} \pm 0.006$

C, normal control; D, diabetic control; F, fenugreek; O, onion.
Values are mean ± SEM of eight animals in each group.
Means within the same column (in each parameter) carrying different superscripts (a, b, c) are significantly different ($P < 0.05$).
*Specific activity unit: μg pyruvate released·min·mg^{-1} creatinine.
†Specific activity unit: μmol *P*-nitrophenol formed·min·mg^{-1} creatinine.
‡Specific activity unit: μg pyruvate released·min·mg^{-1} creatinine.
§Specific activity unit: μmol P-nitrophenol formed·min·mg^{-1} creatinine.
‖Specific activity unit: μmol products formed·min·mg^{-1} creatinine.

Table 5
Influence of dietary fenugreek seeds and onion on polyol pathway enzymes and metabolites in diabetic rats

	Renal tissue					
Animal group	Aldose reductase*	Sorbitol dehydrogenase†	Glucose‡	Sorbitol§	Fructose§	Urinary sorbitol‖
C	$12.4^{a} \pm 0.17$	$264.6^{a} \pm 17.90$	$2.17^{a} \pm 0.11$	$87.6^{a} \pm 9.04$	$147.6^{a} \pm 8.30$	$1.23^{a} \pm 0.05$
C + F	$13.2^{a} \pm 0.30$	$264.2^{a} \pm 15.65$	$1.93^{a} \pm 0.12$	$74.7^{a} \pm 5.00$	$131.4^{a} \pm 12.8$	$1.26^{a} \pm 0.42$
C + O	$12.9^{a} \pm 0.24$	$277.6^{a} \pm 12.52$	$2.19^{a} \pm 0.14$	$88.7^{a} \pm 9.34$	$147.9^{a} \pm 14.0$	$1.33^{a} \pm 0.03$
C + F + O	$13.2^{a} \pm 0.18$	$249.0^{a} \pm 13.45$	$1.79^{a} \pm 0.09$	$74.7^{a} \pm 2.78$	$128.4^{a} \pm 6.32$	$1.15^{a} \pm 0.19$
D	$20.1^{b} \pm 0.64$	$321.9^{b} \pm 12.12$	$8.15^{b} \pm 0.30$	$495.2^{b} \pm 67.6$	$359.5^{b} \pm 40.4$	$64.1^{b} \pm 10.9$
D + F	$16.3^{c} \pm 0.39$	$210.7^{c} \pm 14.02$	$6.26^{c} \pm 0.50$	$279.6^{c,d} \pm 25.1$	$242.3^{c} \pm 12.7$	$32.2^{c} \pm 4.46$
D + O	$17.5^{c} \pm 0.28$	$234.6^{a,c} \pm 13.30$	$6.41^{b,c} \pm 0.69$	$331.0^{c} \pm 18.6$	$281.0^{b,c} \pm 23.8$	$34.4^{c} \pm 6.41$
D + F + O	$15.6^{a,c} \pm 0.27$	$177.9^{d} \pm 15.38$	$5.66^{c} \pm 0.40$	$229.2^{d} \pm 14.8$	$198.5^{a,c} \pm 16.4$	$23.6^{c} \pm 4.24$

C, normal control; D, diabetic control; F, fenugreek; O, onion.
Values are mean ± SEM of 8 animals in each group.
Means within the same column (in each parameter) carrying different superscripts (a, b, c, d) are significantly different ($P < 0.05$).
*Unit: μmol of NADPH oxidized·h·100 mg^{-1} protein.
†Unit: μmol of NADH oxidized·h·100 mg^{-1} protein.
‡Unit: μmol/g tissue.
§Unit: nmol/g tissue.
‖Unit: μmol/24 h.

respectively), onion (21%, 33%, and 22%, respectively), and fenugreek + onion (30%, 54%, and 45%, respectively) compared with diabetic control. Increased accumulation of sorbitol in renal tissue can be corroborated with its increased urinary excretion in diabetic rats (52-fold). Dietary intervention with fenugreek (50%), onion (46%), and fenugreek + onion (63%) significantly suppressed sorbitol excretion, the effect being highest in the case of combination.

Influence on RAS

Increased activity of renal ACE (2.3-fold), protein and mRNA expression of ACE (2.9- and 3-fold) and AT1 receptor (7- and 1.8-fold) was evidenced under the diabetic condition (Fig. 3). There was a significant ($P < 0.05$) decrease in the activity of ACE in the fenugreek-, onion-, and fenugreek + onion–fed diabetic groups compared with diabetic control rats. Dietary fenugreek and onion partially countered the expression of ACE and AT_1 in renal tissue, whereas feeding fenugreek + onion to diabetic rats maximally downregulated the protein (by 85% and 71%, respectively) and mRNA expression (by 64% and 49%, respectively).

Influence on renal and urinary NO

Diabetic animals exhibited increased renal iNOS protein (2.2-fold), and mRNA (71%) expression, with simultaneous increase in renal (48%) and urinary NO metabolites excretion (3.9-fold) compared with the normal group (Fig. 4). Dietary fenugreek (by 45%, 23%, and 50%, respectively) or fenugreek + onion (by 58%, 62%, and 66%, respectively) significantly countered protein, mRNA, and urinary NO excretion in diabetic animals.

Influence on podocyte damage

The range of podocyte injury was assessed by measuring the degree of fibrosis (collagen I accumulation), altered mRNA expression of renal structural proteins (nephrin, podocin, podocalyxin, and Kim-1) and their subsequent excretion in urinary sediments of diabetic rats (Figs. 5 and 6). The diabetic condition resulted in downregulated mRNA expression of nephrin (65%), podocin (46%), and podocalyxin (56%) and upregulated Kim-1 (35-fold) in the renal tissue, which was associated with increased urinary excretion of the same. Feeding diabetic animals with fenugreek, onion, and or a combination of the two ingredients significantly reversed

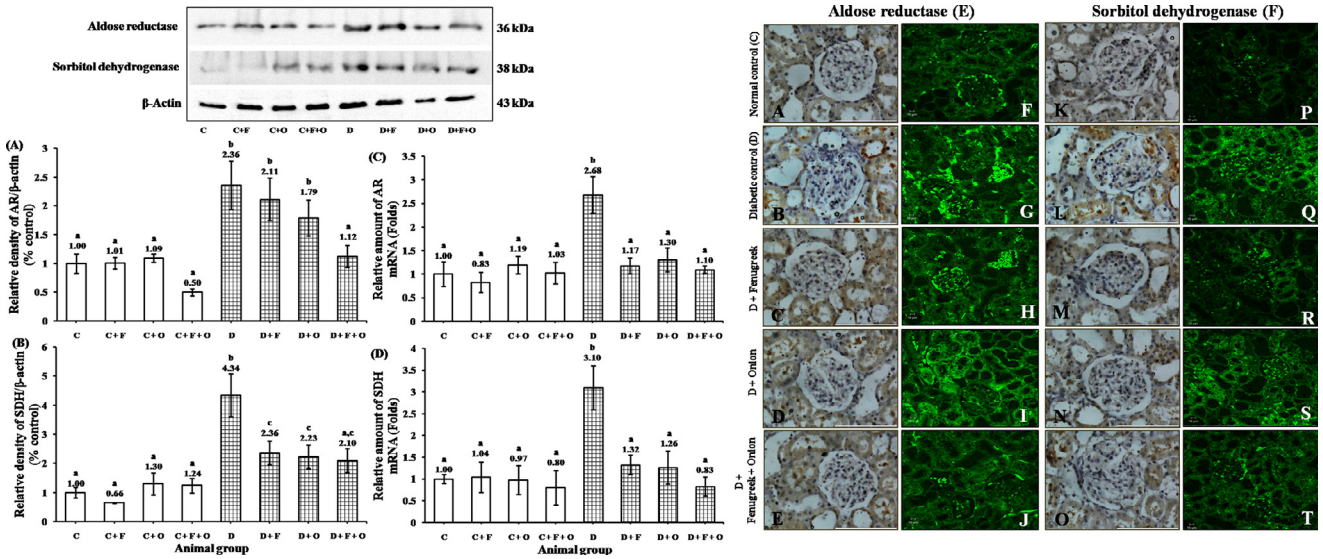

Fig. 2. Influence of dietary fenugreek seeds and onion on renal AR and SDH. (**A** & **B**) Protein; (**C** & **D**) mRNA expression and (**E** & **F**) Immunohistochemistry (40 × objective) and confocal (20 × objective) image analysis of aldose reductase (A–J) and sorbitol dehydrogenase (K–T) in diabetic rats. Values are mean ± SEM of eight animals in each group. Each bar carrying different letters (a, b, c) are significantly different ($P < 0.05$). AR, aldose reductase; C, normal control; D, diabetic control; F, fenugreek; O, onion; SDH, sorbitol dehydrogenase.

the mRNA expression pattern with reduced fibrosis (43%, 31%, and 78%, respectively) compared with diabetic control animals. Excretion of mRNA of the same in the urine of diabetic animals was significantly ($P < 0.05$) countered by all three dietary interventions, with the highest effect shown by the combination amounting to an additive or even a synergistic effect (Fig. 6).

Influence on renal histopathology

A histopathogic observation of kidney section revealed shrunken glomeruli with mesangial matrix expansion, inflammatory cell infiltration, mucopolysaccharide deposition, and higher degree of tubular clarifications or vacuolations in the diabetic control group (Fig. 7B). Nearly normal glomerular and tubular structures, reduced mesangium expansion, and absence of mucopolysaccharide depositions were observed in the kidney sections of the diabetic rats with dietary interventions (Fig. 7C–E). Similarly, PAS- and MT-stained kidney sections evidenced increased purple plaque materials indicating glycogen deposition and blue-colored collagen fibers accumulation in the glomeruli of diabetic rats (Fig. 7G, L), these being promisingly less in diabetic animals provided with fenugreek, onion, or their combination (Fig. 7 H–J, M–O). In the case of normoglycemic animals, these dietary interventions did not make any significant difference in this respect (data not shown).

Discussion

STZ-induced experimental diabetes represents the polymorphism of type 1 metabolic syndrome, which is valued in the elucidation of casual relationships related to human diabetes mellitus. Wistar rats were used to induce experimental diabetes as they develop a persistent disease state [28] characterized by severe hyperglycemia with major clinical signs of diabetes mellitus and can therefore be an useful tool for the pharmacologic evaluation of antidiabetic foods and drugs. DN is the leading cause of ESRD, characterized by a progressive decline in renal function [29]. Because diet plays a major role in combating the same, the present study examined the beneficial potential of two spices with hypoglycemic, insulinotropic, and antioxidant properties in this context. The beneficial influence of fenugreek seeds and onion on the progression of DN was evaluated with a focus on the possible suppression of glucose translocation and RAS.

In acute and long- term studies on individuals with either type 1 or type 2 diabetes, 100 g/d of debittered fenugreek seeds) or 15 g/d of fenugreek seeds effectively reduced the area under the glucose curve [30]. The dietary level of fenugreek used in the present study corresponds to this effective dose. These dietary interventions were recently shown to exert significant beneficial effect on diabetes-induced oxidative stress in the renal tissue [6]. Diabetes-driven higher glucose flux results in the upregulation of glucose transporter isoforms GLUT1 and GLUT2 [31]. Dietary fenugreek seeds and onion, brought about a significant decrease in the expression of GLUT1 and GLUT2 transporters, possibly resulting from hypoglycemic influence evidenced previously [5,32], thereby reducing the transporters for high glucose flux.

Diabetic animals exhibited decreased activity of renal hexokinase, G6 PD (thus lesser glucose oxidation) and increased activity of fructose-1,6-bisphosphatase, G6 Pase, and LDH (responsible for higher gluconeogenesis). The observed increase in the activity of hexokinase and G6 PD by dietary fenugreek and onion would mean increased glycolysis to facilitate utilization of glucose for energy production. Reduced activity of gluconeogenic enzymes may have resulted from increased insulin production and reduced hyperglycemia as previously reported [5]. Fenugreek + onion showed a higher beneficial effect than the individual effects in this context.

ATPase is sensitive to changes in membrane fluidity similar to the activity of most other membrane-bound enzymes that are regulated by the physiochemical state of their lipid environment [33]. Diabetes resulted in an increase in renal Na^+,K^+-ATPase and Ca^{2+}-/Mg^{2+}-ATPase activity. As the majority of filtered ions are reabsorbed in the proximal tubule, alterations in the ATPase activity may play a key role in the development of impaired electrolyte management. Therefore, changes in membrane fluidity in the renal tissue cannot be responsible for the increased activity of ATPases. The physiologic relevance of increased ATPase activities in the kidney may represent an adaptation of nephrons to maintain

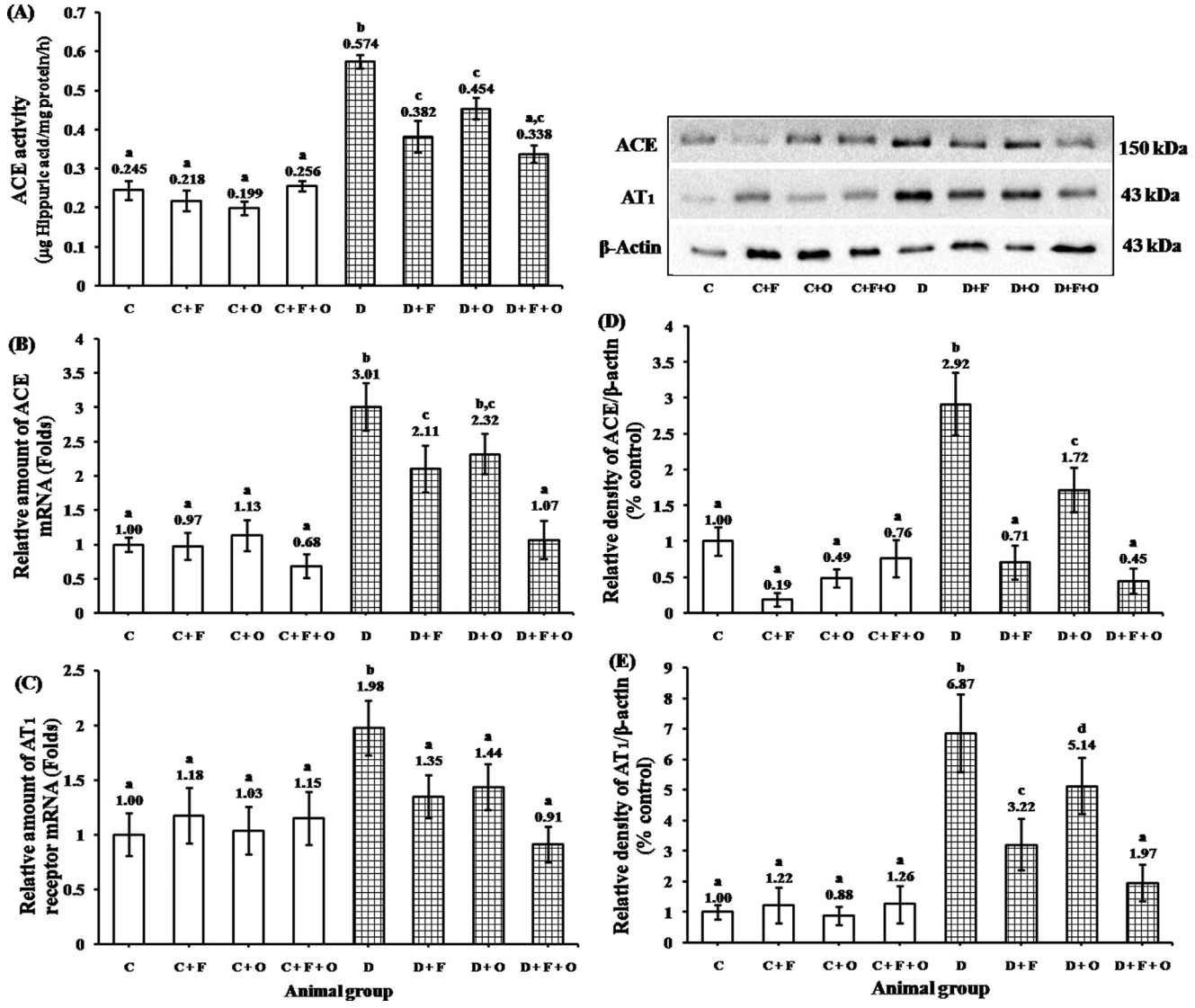

Fig. 3. Influence of dietary fenugreek seeds and onion on renal (**A**) ACE activity; (**B** & **C**) ACE and AT_1 mRNA expression; (**D** & **E**) ACE and AT_1 protein expression in diabetic rats. Values are mean ± SEM of eight animals in each group. Each bar carrying different letters (a, b, c, d) are significantly different ($P < 0.05$). ACE, angiotensin-converting enzyme; C, normal control; D, diabetic control; F, fenugreek; O, onion.

electrolyte homeostasis in diabetes in the face of an increased glomerular filtration rate and osmotic dieresis [33]. Dietary fenugreek and onion significantly regulated the altered activities of ATPases, which may be due to the moderation of blood glucose and lipid peroxidation in the renal tissue as observed previously [6]. In agreement with an earlier report [33] on diabetic animals treated with fenugreek seeds (5% for 21 d), altered renal ATPase activity was significantly countered by dietary fenugreek. There are reports of onion supplementation significantly altering the activity of ATPase in STZ-induced diabetic rats [26].

A hallmark feature of DN is enhanced urinary excretion of protein. This increased loss of macromolecules initiates a self-perpetuating process of progressive glomerulosclerosis, tubulointerstitial inflammation, and scarring, thereby contributing to a progressive loss of renal function [34]. In the present study, protein excretion was increased in diabetic rats resulting from an increased permeability of the glomerular basement membrane. Proteins appear in the urine as a consequence of normal process of renal cell turnover and metabolism [35]. In the present study, a higher excretion of 66 kDa albumin was noticed in diabetic rats, whereas alpha 2u-globulin (20 kDa), the principal urinary protein synthesized and secreted by the hepatic parenchymal cells, disappeared. Insulin deficiency is known to cause a drastic reduction in the urinary excretion of alpha 2u-globulin [36]. All three dietary interventions decreased the urinary protein excretion in diabetic rats.

Increased renal enzymuria could be a surrogate marker of early renal injury preceding diabetic nephropathy. Excessive filtration of albumin, followed by its reabsorption in proximal tubular region, results in lysosomal swelling and rupture causing the liberation of NAG (a lysosomal enzyme) into the tubular lumen [37]. Leaching out of these marker enzymes from both proximal (NAG, ALT, and alkaline phosphatase) and distal (LDH, AST, and acid phosphatase) renal tubular origin into the urine were significantly decreased in diabetic rats maintained on fenugreek, onion, and the combination of the two as reported for onion-fed diabetic rats [26]. The effect of dietary fenugreek on the urinary excretion of enzyme proteins is a novel observation. Hyperglycemia promotes metabolism of glucose via stimulated polyol pathway, leading to an accumulation of

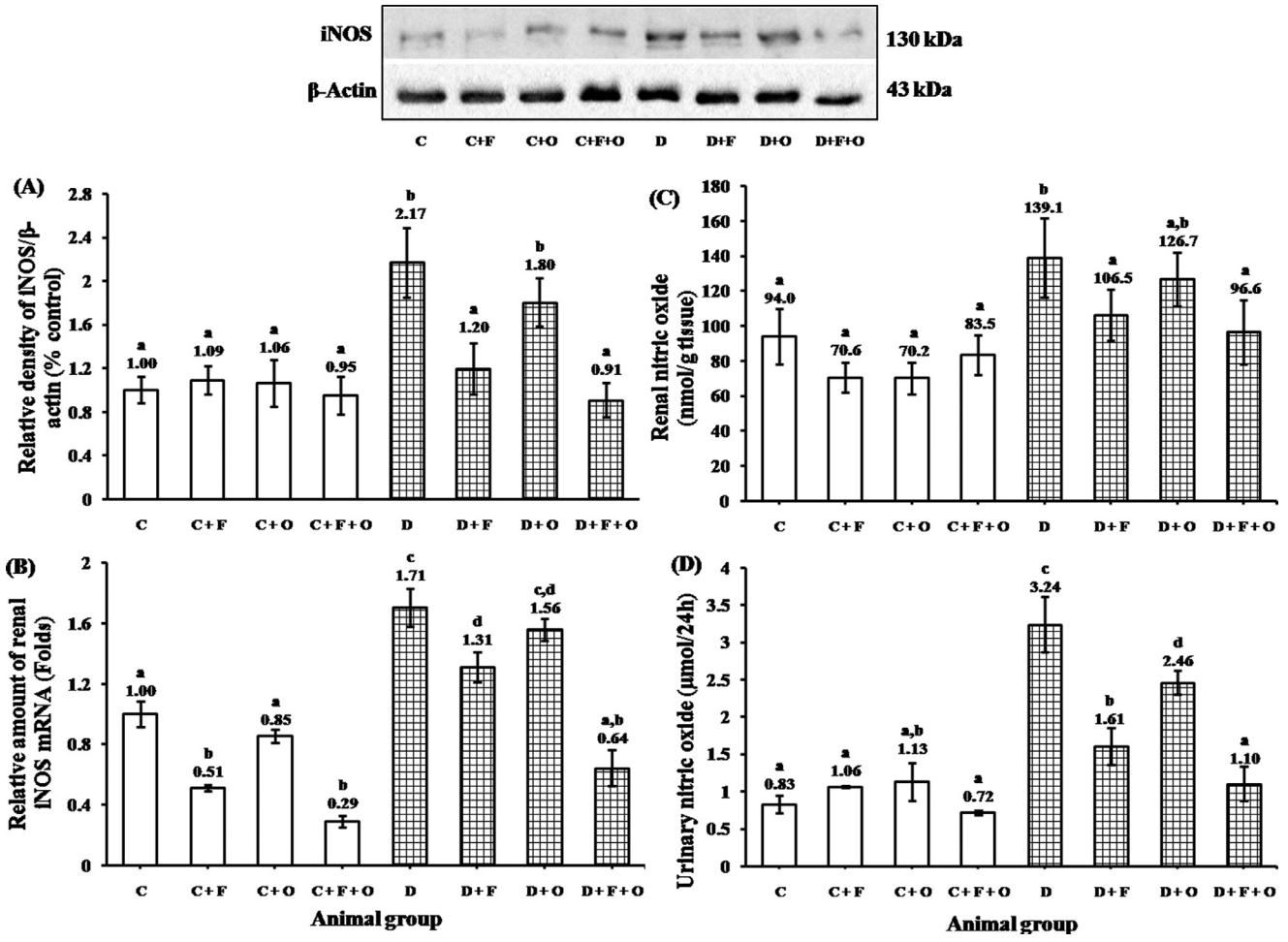

Fig. 4. Influence of dietary fenugreek seeds and onion on renal (**A**) iNOS protein and (**B**) iNOS mRNA expression; (**C**) renal NO and (**D**) urinary NO levels in diabetic rats. Values are mean ± SEM of eight animals in each group. Each bar carrying different letters (a, b, c, d) are significantly different ($P < 0.05$). C, normal control; D, diabetic control; F, fenugreek; iNOS, inducoble nitric oxide synthase; NO, nitric oxide; O, onion.

sorbitol and fructose in tissues [38]. Urinary excretion of sorbitol reflects the extent of polyol pathway activation by hyperglycemia [39]. To our knowledge, this is the first report on dietary fenugreek seeds and onion beneficially countering the stimulation of the concerned enzymes, which is corroborated with a reduced level of fructose and sorbitol in the kidney and urine.

RAS is a multienzyme cascade having a regulatory role in regulating blood pressure and fluid homeostasis. A consistent feature of RAS is increased glomerular pressure, which is associated with the development of proteinuria and glomerulosclerosis [40]. A blockade of the RAS could serve as first-line therapy in the treatment of DN [41]. An increase in renal ACE and AT_1 receptor expression was seen in the kidney of diabetic rats, and this status of RAS in diabetic rat kidney is consistent with previous reports [42–44]. Dietary fenugreek and onion downregulated the renal ACE and AT_1 expression in diabetic rats. Feeding fenugreek essential oil was earlier reported to reduce plasma ACE activity and hypertension in diabetic rats [45]. This study has revealed that there is an increase in renal NO production and excretion and upregulation of iNOS expression during the progression of DN. Because NO plays a crucial role in the regulation of renal hemodynamics, this observation suggests a higher production of NO during the development of glomerular hyperfiltration in STZ-induced diabetes [6]. Dietary intervention with fenugreek and onion to diabetic rats resulted in reduced NO production and excretion and expression of iNOS in kidney, with higher modulatory effect by their combination. Reduced NO in the blood of diabetic rats treated with fenugreek [46] and onion essential oil [47] has been reported.

Podocytes, which constitute the glomerular filtration barrier, are damaged in diabetes resulting in proteinuria and DN [48]. Podocytes contain proteins such as nephrin, podocin, CD2 AP, podocalyxin, TRPC6, etc., involved in maintaining the structure, function and integrity of the glomerular filtration membrane [49]. The present study evidenced markedly downregulated mRNA expression of nephrin, podocin, and podocalyxin with upregulated Kim-1, whose increased urinary leakage is related to the severity of renal damage [50]. Urinary excretion of these proteins or of corresponding mRNA can be used as sensitive and specific markers of renal injury. The present study has evidenced for the first time that dietary fenugreek and onion partially reduced the excretion of urinary mRNA of these markers by diabetic rats.

Diabetic rats exhibited significant alterations in the components of the glomerular filtration barrier. The present study also revealed increased renal glycogen and type IV collagen content, as the renal tissue is independent of insulin action. Increased type IV collagen is due to high glucose and non-enzymatic protein glycosylation, which can suppress normal collagen IV crosslinking contributing to its stability with minimal degradation or extracellular matrix (ECM) accumulation as a result of AGE-induced cytokines action [51]. These AGEs, which accumulate on collagen

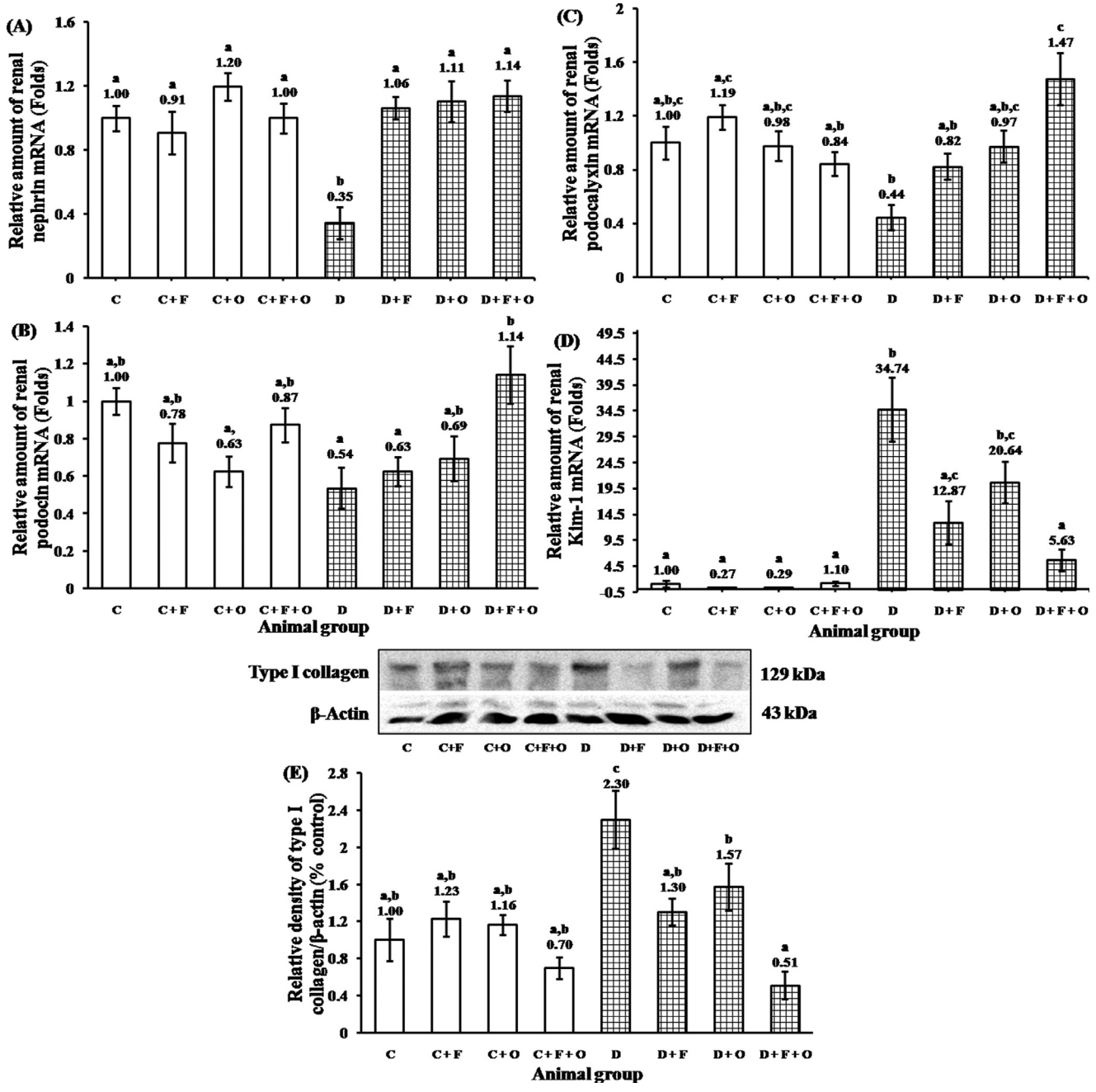

Fig. 5. Influence of dietary fenugreek seeds and onion on renal mRNA expression of (**A**) nephrin; (**B**) podocin; (**C**) podocalyxin; (**D**) Kim-1, and (**E**) type I collagen protein expression in diabetic rats. Values are mean ± SEM of eight animals in each group. Each bar carrying different letters (a, b, c) are significantly different ($P < 0.05$). C, normal control; D, diabetic control; F, fenugreek; O, onion.

fibers in the basement membrane, together with their ability to trap plasma proteins also may contribute to thickening of the basement membrane and altered filtration rate and ultimately loss of glomerular function [52]. Diabetic rats fed with fenugreek and onion partially normalized the renal architecture, whereas fenugreek + onion produced a higher modulation. As reported earlier [53], fenugreek curbed the increase in the renal glycogen in diabetic rats. Trigonelline of fenugreek partially restored the kidney architecture, collagen IV, and fibrosis of kidney in STZ–nicotinamide-induced DN [54]. Essential oil of onion showed antioxidative potential on kidney and glycogen deposition in STZ-induced diabetic rats [47].

Research suggests that the development of DN is associated with the activation of several stress-sensitive signaling pathways that stimulate glomerular hypertrophy, thickening of the glomerular basement membrane, glomerulosclerosis, effacement of podocyte foot processes, increased type I collagen expression, and ECM accumulation. In this context, transforming growth factor (TGF)-β1 is the key regulator of ECM remodeling in the mesangium, leading to mesangial expansion, and of tubular epithelial mesenchymal transition, leading to tubulointerstitial fibrosis. Hyperglycemia-induced oxidative stress increases the AGEs formation, which in turn stimulates the release of TGF-β1 and other cytokines, resulting in the accumulation of ECM and aggravation of apoptosis,

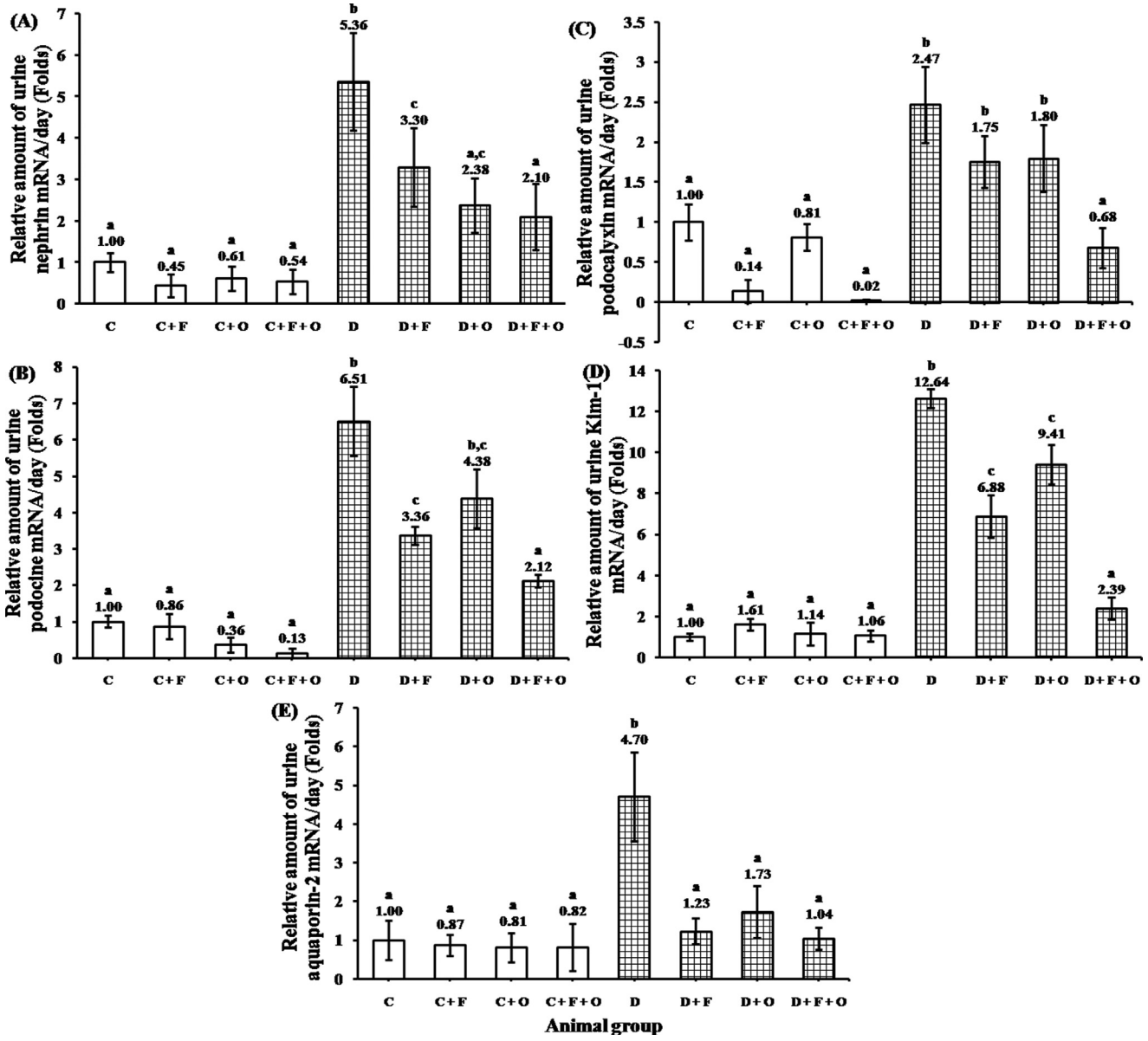

Fig. 6. Influence of dietary fenugreek seeds and onion on urinary mRNA excretion/d of (**A**) nephrin; (**B**) podocin; (**C**) podocalyxin; (**D**) Kim-1, and (**E**) aquaporin-2 in diabetic rats at the end of week 6 urine sample. Values are mean ± SEM of eight animals in each group. Each bar carrying different letters (a, b, c) are significantly different ($P < 0.05$). C, normal control; D, diabetic control; F, fenugreek; O, onion.

inflammation, and proteinuria [55]. Additionally, hyperglycemia-induced angiotensin II directly stimulates TGF-β1 expression and suppresses nephrin expression in the diabetic kidney, with its resultant release in urine [56]. We have previously shown that fenugreek and onion downregulates the overexpression of renal TGF-β1 in diabetic rats [6]. The present study extended this information further on alleviating fibrosis by reducing the accumulation of collagen fibers (types I and IV) in diabetic rat kidney, which is one of the additional mechanism for their renoprotective influence. Therefore, inhibition of the stress-inducing signaling pathway is important in the treatment of DN by blocking the glucose translocation and RAS. This suggests the use of fenugreek + onion as a potential therapeutic intervention for the prevention of DN.

It is likely that dietary fenugreek seeds and onion are acting through a generalized mechanism affecting diabetic adversities throughout the body, with kidneys among others showing these beneficial effects. As reported previously [5–7], it appears likely that some of the benefits may well have been due to generalized hypoglycemic and antioxidant effects of these two food ingredients. Downstream benefits on the kidney could be ascribed to this upstream critical event. The higher than individual beneficial effect in alleviating diabetic nephropathy by the combination of fenugreek and onion nevertheless suggests that these two ingredients act through different yet parallel modes in the body.

In this dietary intervention study, fenugreek seeds were fed to the animals at levels corresponding to about five times the normal consumption of this seed spice among the Indian population [57]. Such high dietary levels are in fact encountered through few specific dishes employing liberal amounts of fenugreek seeds in India. The liberal consumption of the same to derive putative health benefits through its rich fiber content and other bioactive components is proved to be safe. Although the higher additive synergistic

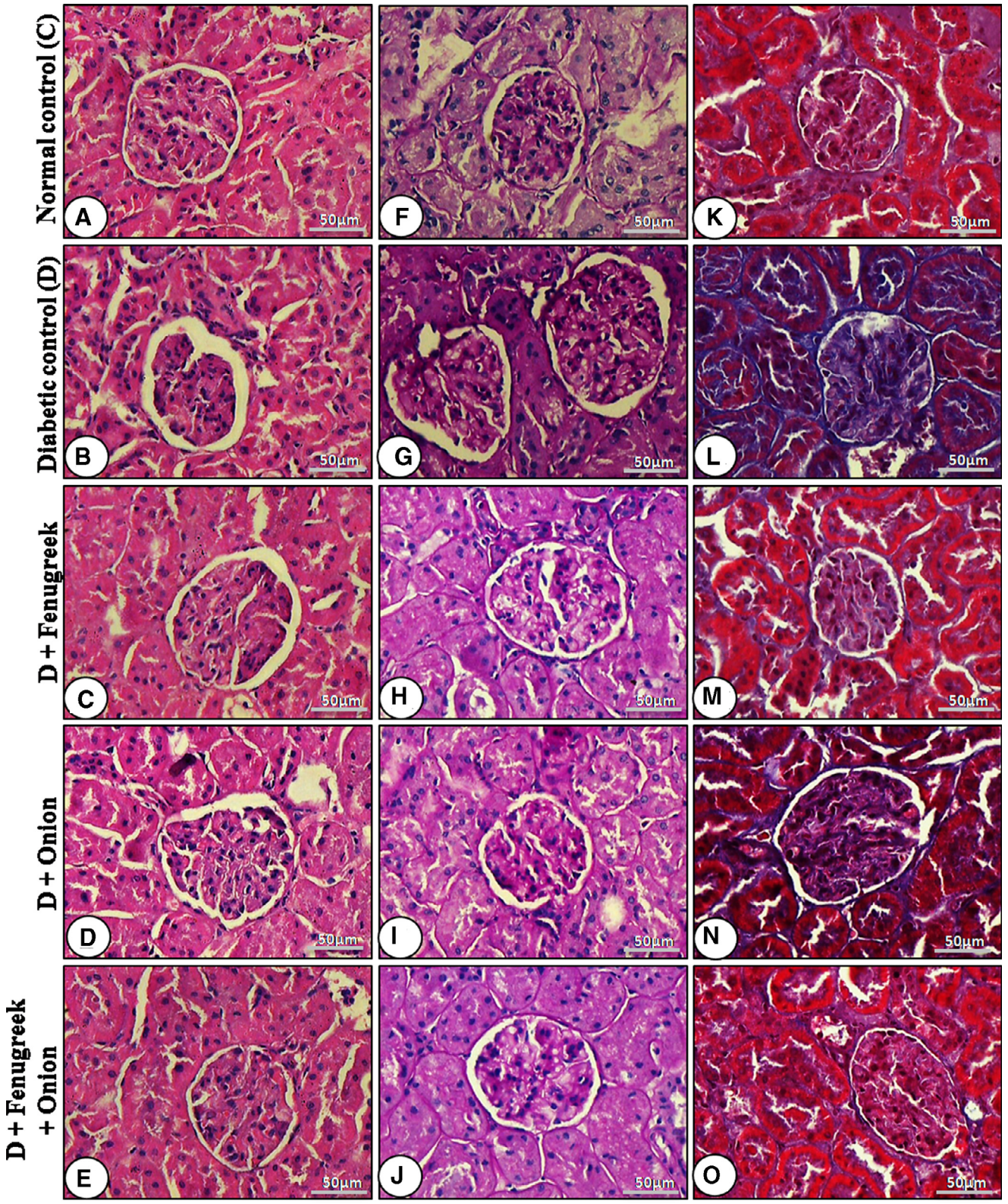

Fig. 7. Influence of dietary fenugreek seeds and onion on renal (**A**–**E**) general histopathological analysis using H&E staining (40 × objective). (**F**–**J**) Representative figures of PAS staining (40 × objective) for glycogen deposition (purple). (**K**–**O**) Representative figures of Masson's trichrome staining (40 × objective) for collagen fibers (blue). PAS, periodic acid and Schiff solution.

influence by the combination of dietary fenugreek seeds and onion in attenuating DN evident in this rodent diabetic model is promising, further trials are warranted to confirm a similar benefit in humans with diabetes.

Conclusion

Dietary intervention with fenugreek seeds and onion was evidenced to bring about a blockade in glucose translocation and the RAS in the early stage of DN. The beneficial influence was also seen in the downregulation of the expression of polyol pathway enzymes. Renal integrity was restored by influencing renal ATPase and leaching of renal tubular enzymes. Podocyte damage was partially restored by upregulating renal expression of nephrin, podocin, podocalyxin along with downegulating Kim-1. Renal architecture and functional abnormality in diabetic rats was revived by these dietary treatments, which is corroborated with reduced glycogen and type I and IV collagen expressions. This study also suggested a higher beneficial influence by the combination of fenugreek seeds and onion, sometimes amounting to an additive or even synergistic effect in attenuating DN.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.nut.2019.06.024.

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