Original Research

Pyridoxamine, an inhibitor of protein glycation, in relation to microalbuminuria and proinflammatory cytokines in experimental diabetic nephropathy

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Abstract

Diabetic nephropathy (DN) is one of the major complications that develop as consequence of chronic and uncontrolled hyperglycaemia. Hyperglycaemia initiates various processes, one of which is protein glycation, leading to the formation of advanced glycation end products. Alteration of intracellular signalling, gene expression, release of proinflammatory molecules and free radicals are examples of such changes and they contribute to the initiation of diabetic complications. In the current manuscript, we studied the effect of pyridoxamine (PM) on protein glycation, oxidative stress, interleukin-1α (IL-1α), IL-6, C-reactive protein (CRP), gene expression of tumour necrosis factor-α (TNF-α) and transforming growth factor-β1 (TGF-β1) in relation to microalbuminuria and kidney functions in a model of alloxan-induced diabetic rats. We have observed that onset of microalbuminuria has preceded the gradual increase of blood sugar level in diabetic rats. In diabetic rats, gene expression of TNF-α and TGF-β1 recorded a gradual increase and marked increase was observed after one and two weeks of alloxan administration, in comparison with normal rats. PM induced significant decrease in kidney malondialdehyde content and the gene expression of TNF-α and TGF-β1, in addition to levels of serum glucose, fructosamine, urea, creatinine, IL-1α, IL-6, CRP and urine microalbumin. Histopathological examination of kidney tissues showed certain improvements as compared with diabetic control. In conclusion, our results may provide a supporting evidence for the therapeutic benefit of PM in DN.

Keywords: Nephropathy, pyridoxamine, malondialdehyde, tumour necrosis factor-α, transforming growth factor-β1, microalbuminuria

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Introduction

Diabetic nephropathy (DN) is a clinical syndrome characterized by decline in glomerular filtration rate and persistent albuminuria and arterial hypertension, in the absence of clinical or laboratory evidences of any other kidney disease.[1] It remains the most common cause of end stage renal disease (ESRD), accounting for more than 40% of patients treated with dialysis, and its treatment delays the onset of ESRD.[2] DN has been traditionally considered a non-immune disease; however, accumulating evidences now indicate that immunological and inflammatory mechanisms may play a significant role in the development and progression of DN.[3] Chronic hyperglycaemia is the major initiator for diabetic vascular complications. Enhanced polyol hexosamine pathways, activation of protein kinase C (PKC), oxidative stress and over-production of advanced glycation end products (AGEs) may collectively contribute to induce such complications.[4] The interaction of AGEs with their RAGE receptors located on many cell types may alter intracellular signalling, gene expression, release of proinflammatory molecules (cytokines) and free radicals, which are mostly responsible for the subsequent diabetic complications.[5] Among the most well known proinflammatory cytokines are interleukins, (IL)-1, IL-6, IL-18), and tumour necrosis factor-alpha (TNF-α)).[6] IL-6, for example, has been associated with the glomerular basement membrane thickening that occurs early in diabetic kidney disease (DKD).

TNF-α is a multifunctional regulating cytokine that is involved in the inflammatory response in diabetes.[7,8] It is highly expressed in adipocyte,[9] inhibits insulin signalling pathway,[10] impairs peripheral glucose uptake and alters the expression of major genes that control glucose and lipid metabolism.[11,12] In addition, TNF-α has been linked to an increase in the permeability of the glomerular wall.[13] Transforming growth factor-β1 (TGF-β1) is also an

important mediator in the pathogenesis of DN.[14,15].It is a prosclerotic cytokine that is responsible for promoting collagen matrix formation and contributes to the progression of DKD. Its concentration also increases as a consequence of hyperglycaemia and AGEs.[16–18] Despite the standard of care and treatment of DM, the prevalence of ESRD in diabetic patients has increased.[19] New molecular entities and even existing medications with new applications are being studied for the treatment of DKD. These agents focus on interrupting the pathogenesis of DKD by inhibiting the formation of AGEs and/or inflammatory cytokines to prevent fibrosis in the glomerulus.[17] Several anti-AGEs agents have been tested in diabetic animal models and proved to be protective.[20] Pyridoxamine (PM), a derivative of the vitamin B family, acts as an inhibitor for AGEs and advanced lipoxidatin end products.[21] In animal models, it delays the development of DN and reduces albuminuria of both type 1 and type 2 DN.[22,23] In diabetic rats, PM was shown to inhibit the progression of retinopathy[22] and attenuated the accumulation of AGEs on aortic collagen.[24] The development of specific interventions of PM against AGEs is in progress and currently under preclinical evaluation, particularly those effects associated with diabetes.[25,26] In preclinical and in some clinical trials, PM showed conflicting results.[2,8,25] In a phase II study of patients with diabetes and overt nephropathy, PM showed a beneficial effect on the progression of renal disease.[25] On the other hand, a trial enrolling patients demonstrated some serious adverse drug events and did not show any benefit in DKD patients.[17]

The multiple activities and promising safety of PM suggest that it can be a candidate as a medication or medicinal food for treating AGEs correlated disorders. This study, therefore, aimed to determine the effects of PM on alloxan diabetic rat model. The changes in oxidative stress, renal function and proinflammatory cytokines in relation to microalbuminuria and histopathological examination of kidney tissues were also evaluated.

Materials and methods

Animals

Forty adult male Wistar albino rats weighing 170 ± 20 g, supplied by the Egyptian Organization for Biological Products and Vaccines (Cairo, Egypt), were used in this study. Rats were housed in stainless steel rodent cages under environmentally controlled conditions and allowed one week for acclimatization at room temperature with a 12-h dark/light cycle before beginning the experimental work. Rats were fed rodent chow (El-Nasr Pharmaceuticals and Chemicals Industry, Egypt) and allowed free access to drinking water. The protocols for animal experimentation and the handling of animals were in accordance with the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals established by Zagazig University, Zagazig, Egypt.

Experimental protocol

Diabetes was induced by administration of a single dose of alloxan intraperitoneally (90 mg/kg body weight) and serum glucose was checked after interval days. Rats that achieved serum glucose level more than 200 mg/dL were expressed as diabetic and enrolled in the study. Three experimental groups of eight animals each were used – normal control (NC) group: normal rats received drug-free vehicle; diabetic control (DC) group: diabetic rats received drug-free vehicle and PM-treated diabetic group: diabetic rats treated with PM 180 mg/kg daily[27] for six weeks using oral gavage. This dose is within the range of safe and effective PM doses (100–400 mg/kg/day) that have been used previously in diabetic models.[23,28–30] Another two diabetic groups of rats ($n = 8$ each) were included to detect the gene expression of TNF-α and TGF-β1 in the kidney after one and two weeks of alloxan treatment.

Blood and urine sampling

Urine samples were collected after 2, 4, 6, 21 and 42 days of alloxan administration, after six weeks of PM treatment and processed for determination of microalbumin content in urine. Blood samples were collected parallel to urine samples at the specified days and processed for blood sugar determination. At the end of the six weeks of treatment, rats were fasted overnight, blood samples were collected via retro-orbital bleeding and centrifuged directly for serum separation. Samples were processed instantly for determination of glucose, creatinine, urea, fructosamine, IL-1α and IL-6.

Tissue collection

Following blood collection, rats were killed by decapitation. Kidneys were removed instantly, rinsed with cold normal saline and dried with filter paper. One specimen was quickly frozen in liquid nitrogen (−170°C) and stored at −20°C for determination of malondialdehyde (MDA) and gene expression of TNF-α and TGF-β1. The other specimen was kept in 10% formalin–saline at 4°C for at least one week (1ry fixation); then the specimens were dehydrated with a series of ascending grade ethanol from 75 to 100%. Tissues were placed thereafter in xylol and embedded in paraffin. Cross-sections of about 2 μm thickness were sliced using a microtome (Leica RM 2155, England) and stained with haematoxylin and eosin[31] for microscopical examination.

Analytical methods

Serum glucose was determined according to Trinder[32] using commercial kits provided by Spinreact Kits, Spain. This method has intra-assay and inter-assay coefficient of variation (CV) values of 0.8 and 1.6%, respectively, and a detection limit of up to 600 mg/dL. Fructosamine was determined according to the method of Schleicher and Vogt[33] using QCA Kits, Spain. This method has intra-assay CV of 1.7% and inter-assay CV of 1.9%, with a detection upper limit of 800 μmol/L (143 mg/dL). Creatinine was determined according to the method described by Henry,[34] using Diamond Kits, Egypt. The intra-assay CV and

inter-assay CV values of creatinine assay are 2.1 and 4%, respectively, with a detection limit of up to 20 mg/dL. Urea was determined according to the method of Fawcett and Scott[35] using Diamond Kits, Egypt (intra-assay CV = 3.2%, inter-assay CV = 4.6%, with a detection upper limit up to 200 mg/dL). Microalbumin content in urine was determined according to the method of Gall et al.[36] using Orgentec ELISA Kits, Germany (intra-assay CV = 3.3% and inter-assay CV = 5.1%, with a detection upper limit of up to 25 μg/L). Serum IL-1α and IL-6 were determined by ELISA using RayBio® Rat IL-1α and RayBio® Rat IL-6 ELISA Kits supplied by Ray Biotech, Inc., Norcross, GA, USA. C-reactive protein (CRP) was determined according to the method of Banerjee *et al.*[37] using BD Biosciences ELISA kits, USA.

Kidney MDA and reduced glutathione levels

Lipid peroxidation was quantified by measuring the formation of thiobarbituric acid reactive substances, expressed in terms of MDA, according to the method of Ohkawa *et al.*,[38] and oxidative stress activity was determined by measuring the level of glutathione (GSH) using Bio-diagnostic Kits, Egypt, following the instructions of the manufacturers.

RNA isolation and reverse transcriptase polymerase chain reaction assay for TGF-β1 and TNF-α genes

For the detection of TGF-β1 and TNF-α by semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR), RNA was extracted using SV Total RNA isolation system (Promega, Madison, WI, USA), reverse transcribed into cDNA and amplified by PCR using RT-PCR kit (Stratagene, USA). The oligonucleotide sequences of forward and reverse primers are as follows: TGF-β1 (Forward primer: 5′-TCACTTGTTTTGGTGGATGC-3′; Reverse primer: 5′-TTCTGTCTCTCAAGTCCCCC-3′); TNF-α (Forward primer: 5′-GGCAGGTCTACTTTGGAG TCATTGC-3′; Reverse primer: 5′-ACATTCGGGGATC CAGTGAGCTCCG-3′) and β-actin (forward primer: 5′-ACTGCCGCATCCTCTTCCTC-3′; reverse primer: 5′-ACTCCTGCTTGCTGATCCACAT-3′).

The semi-quantitative determination of PCR products was performed using the gel documentation system (BioDO, Analyser) supplied by Biometra. According to the following amplification procedure, relative expression of each studied gene (R) was calculated using the formula: R = Densitometrical units of each studied gene/densitometrical units of β-actin.

Statistical analyses

Results are expressed as mean ± SD. Statistical analyses were performed using GraphPAD Prism version 5.0. The statistical significance of differences between groups was determined by one-way and two-way ANOVA tests with Newman–Keuls post hoc test. The significance of relationships between variables was calculated by linear regression analysis. Differences were considered significant at a $P < 0.05$.

Results

Metabolic parameters

Table 1 shows the metabolic parameters in sera, kidney and urine of all groups studied. DC rats demonstrated significant increases in levels of serum glucose, fructosamine, creatinine, urea, CRP, kidney MDA and urine microalbumin, while kidney GSH was significantly reduced. Similar increase was also observed in the production of cytokines IL-α and IL-6, as compared with the normal group ($P < 0.05$). Treatment of diabetic rats with PM for six weeks induced significant decrease of these markers, with the exception of Kidney GSH, which showed a significant increase compared with the DC group ($P < 0.05$).

The onset of microalbuminuria in DC started as early as day 2 of alloxan administration and the flow chart demonstrated further increase after 4, 6, 21 and 42 days following alloxan administration (Figure 1a). Interestingly, the onset of hyperglycaemia was not as quick as that of microalbuminuria. Glucose took, to a certain extent, longer than one week following alloxan administration to show significant rise (Figure 1b).

Table 1 Metabolic parameters measurements in control rats and diabetic rats treated with pyridoxamine

	Parameters	NC ($n = 8$)	DC ($n = 8$)	PM ($n = 8$)
Serum	Glucose (mg/dL)	94.3 ± 11.1	480 ± 13.2*	156.5 ± 2.9**
	Fructosamine (mg/dL)	8.5 ± 1.2	45.0 ± 1.8*	19.1 ± 0.3**
	Creatinine (mg/dL)	0.23 ± 0.04	2.50 ± 0.3*	0.50 ± 0.04**
	Urea (mg/dL)	28.5 ± 2.6	80.3 ± 8.4*	48.0 ± 0.6**
	CRP (ng/mL)	3.39 ± 0.17	12.1 ± 0.6*	4.5 ± 0.14**
	IL-1α (pg/mL)	21.6 ± 3.6	86.1 ± 11.9*	52.6 ± 4.9**
	IL-6 (pg/mL)	6.5 ± 1.6	26.3 ± 4.5*	14.7 ± 1.9**
Kidney	GSH (mmol/g tissue)	4.68 ± 0.68	1.57 ± 0.32*	2.47 ± 0.04**
	MDA (nmol/g. tissue)	9.4 ± 2.6	42.8 ± 2.7*	17.0 ± 3.5**
Urine	Microalbumin (μg/mL)	2.7 ± 0.4	18.0 ± 1.3*	5.5 ± 0.1**

Rats from normal control (NC) and diabetic control (DC) groups were treated with drug-free vehicle, while rats from PM group were treated with pyridoxamine (180 mg/kg daily) for six weeks ($n = 8$ each). Values were presented as mean ± SD. *Significant difference from NC at $P < 0.05$. **Significant difference from DC at $P < 0.05$.

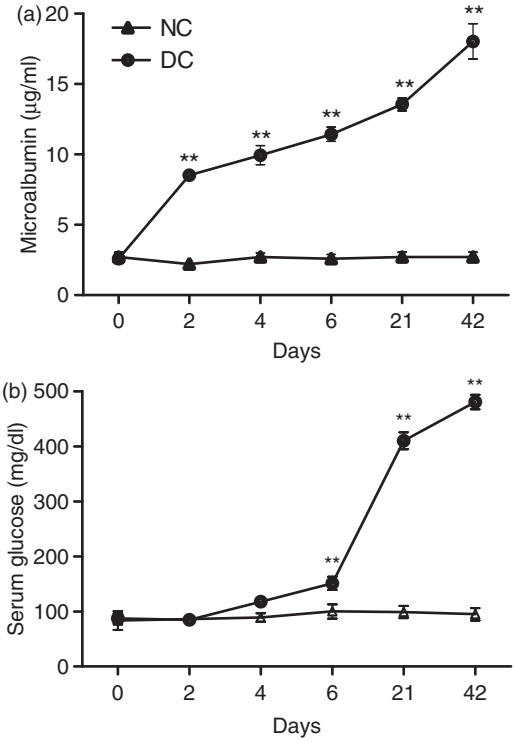

Figure 1 The onset of (a) microalbumin in urine and (b) glucose in the sera of rats at baseline and 2, 4, 6, 21 and 42 days following the administration of a single dose of alloxan intraperitoneally (90 mg/kg body weight). Results were expressed as mean ± SD. *Significant difference from NC at $P < 0.05$

The gene expression of TNF-α and TGF-β1 in the kidney

The diabetic group showed a significant increase in the gene expression of TNF-α and TGF-β1 after one, two and six weeks (Figure 2a and b, respectively). Treatment with PM for six weeks induced a significant decrease in the gene expression of both TNF-α and TGF-β1 (Figure 2c). Both of them demonstrated positive correlation with the serum fructosamine and with urine microalbumin, as shown in Figure 3.

Histopathological pattern

The microscopical examination of the kidney of NC rats (Figure 4a) showed normal pattern of renal parenchyma, tubules and capillaries. Kidneys from diabetic rats (Figure 4b–d) had wedge-shaped necrotic area containing extravasted erythrocytes in the renal cortex (Figure 4b). Glomeruli in general had proliferative glomerular tufts that appeared empty joined with oedema in the vascular wall of renal blood vessels and perivascular tissue (Figure 4c). The blood vessels appeared congested with focal haemorrhages in renal medulla (Figure 4d). The kidney tissue from PM-treated rats (Figure 4e–g) showed thickened basement membrane of glomeruli, congested blood vessels, peritubular capillaries and perivascular oedema in renal cortex (Figure 4e). The majority of renal tubules appeared normal with focal interstitial lymphocytic aggregations (Figure 4f). Mild regenerative attempts and dilated peritubular capillaries could be seen (Figure 4g), as compared with DC rats (Figure 4b–d).

Discussion

The hallmark of treatment of DKD and preservation of kidney function has historically focused on management of hyperglycaemia and proteinuria using hypoglycaemic agents, angiotensin converting enzyme (ACE) inhibitors and angiotensin receptor blockers.[17] New treatment strategies have begun to emerge since 2000, which target the biochemical activity of glucose molecules on kidney tissues. Increased protein glycation and AGEs formation as a consequence of hyperglycaemia are mostly implicated and are responsible for diabetic complications due to their ability to alter enzymatic activity, decrease ligand binding, modify protein half-life and alter immunogenicity.[21] Our studies were designed to evaluate the efficiency of PM as an inhibitor of protein glycation on the development of renal disease in alloxan-diabetic rats. The latter showed hyperglycaemia additionally increased formation of fructosamine. Hyperglycaemia is known to induce shifts in intracellular ratios of redox coenzymes both *in vitro* and *in vivo*.[39] Our results are in agreement with these studies, where a state of redox imbalance in the kidney tissues of diabetic rats was observed. Diabetic rats exhibited characteristic changes in renal function and structure, including increased albuminuria and plasma creatinine associated with renal injury. IL-1α, IL-6, CRP and the gene expression of TNF-α and TGF-β1 were also elevated in diabetic rats. Relating these changes to hyperglycaemia, we can propose an interaction of AGEs, a known outcome of protein glycation (even it is not determined in the present study), with their cellular receptors, specifically RAGE. The latter can be stimulated not only by AGEs but other ligands including S100-calgranulins, which are a group of proinflammatory cytokines, amphoterin and amyloid-β.[40] Expression of RAGE is enhanced in certain cells during diabetes and inflammation[40] Such an effect might be caused by the interaction of AGEs with RAGE on macrophages, which causes oxidative stress and activation of nuclear factor-κB (NF-κB) via activation of P^{21ras} and the mitogen-activated protein kinase signalling pathway.[41] NF-κB modulates gene transcription for generation of proinflammatory cytokines such as interleukine-1α (IL-1α), IL-6 and TNF-α.[41] Distinct from their role as mediators of immunological reactions and inflammatory processes, inflammatory cytokines have been associated with significant renal effect, which plays a certain role in renal injury development in type 2 diabetic patients.[42–44] TNF-α as a candidate is a multifunctional regulatory cytokine involved in the inflammatory response in diabetes and is highly expressed in adipocytes[7–9,45] and can inhibit insulin signalling pathways,[10] impair peripheral glucose uptake[46] and alter the expression of genes that control glucose and lipid metabolism.[11,12] Binding of TNF-α to its receptors (TNF-R1) activates a number of signal transduction pathways, leading to the expression of transcription growth factors, mediators of inflammatory process and acute phase proteins.[47]

Regarding TGF-β1, it is an important mediator for the pathogenesis of DN[14,15,48] and may inhibit matrix

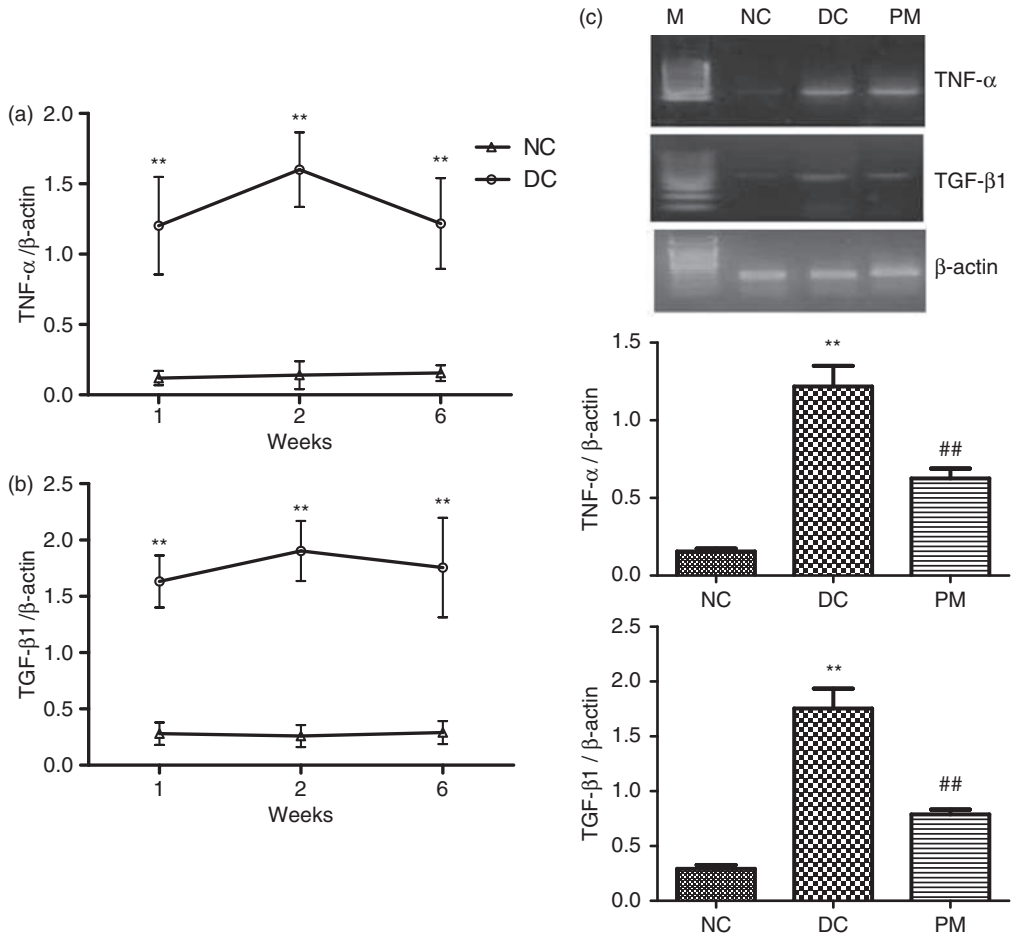

Figure 2 The gene expression of (a) TNF-α and (b) TGF-β in the kidney of rats one, two and six weeks following the administration of a single dose of alloxan intraperitoneally (90 mg/kg body weight). The PCR product was separated on (c) agarose gel and the gene expression was calculated relative to β-actin after six weeks of treatment with PM in diabetic rats. Results were expressed as mean ± SD. *Significant difference from NC at $P < 0.05$ and **significant difference from DC at $P < 0.05$

degradation, upregulate adhesion molecules and enhance chemoattraction. The increase observed in the gene expression of TGF-β1 is mostly attributed to hyperglycaemia.[49] This can activate PKC and hexosamine and enhance the formation of AGEs.[50] TGF-β1 is a powerful stimulator for the synthesis, deposition of collagen and other ECM proteins. It may account even partially for the thickening of the basement membrane in DN.[51] Therefore, the observed increase of gene expression of TNF-α and TGF-β1 in alloxan diabetic rats is mostly due to hyperglycaemia and AGEs and additionally NF -κB effects.

Certain studies indicated that cultured mesangial cells possess AGE receptors and respond to AGEs by increased synthesis of matrix proteins and type IV collagen.[23,52] These circulating AGE-peptides are normally cleared by the kidney; the reverse is true in DN, where their serum concentration rises.[53] Accordingly, its measurement can predict expansion of the mesangial layer and thickening of the basement membrane.[54] Our results demonstrated positive correlations between fructosamine and the gene expression of both TNF-α and TGF-β1. At the dose of PM tested, we observed favourable effects on the biochemical parameters, as illustrated by decreased levels of fructosamine, urea, creatinine and urine microalbumin and decreased gene expression of TNF-α and TGF-β1 and oxidative stress. This suppression in oxidative stress also decreases NF-κB activation, which leads to significant decreases in serum IL-1α and IL-6 and marked improvement in DN. The histopathological pattern of kidney tissue has provided certain support for the biochemical data. In the current study, we were not able to use real-time PCR for gene expression of TNF-α and TGF-β1 due to technical issues but instead reverse transcription PCR conjugated with densitometric analysis of the PCR products was used for semi-quantitative analysis for the expression of these genes relative to β-actin. The quantitation of AGEs was not performed but fructosamine was an indicator for serum protein glycation.

PM has been shown to prevent diabetic complications in animal models by inhibiting the degradation of the protein glycation intermediate, identified as fructosamine to AGEs.[55] Several experimental and clinical studies have been conducted lastly on PM. The first one demonstrated its protective effect on protein backbone against fragmentation induced via different oxidative mechanisms including

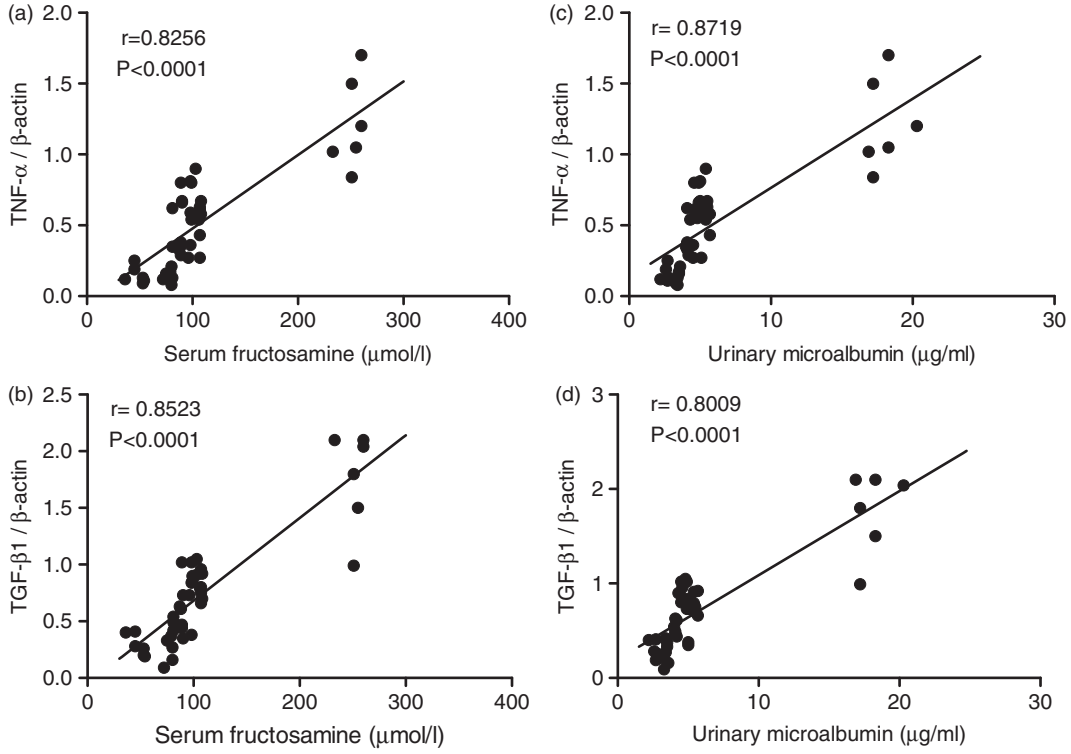

Figure 3 The correlation between protein glycation (fructosamine) and the gene expression of (a) TNF-α and (b) TGF-β and the correlation between microalbumin in urine and the gene expression of (c) TNF-α and (d) TGF-β in all studied groups ($n = 24$)

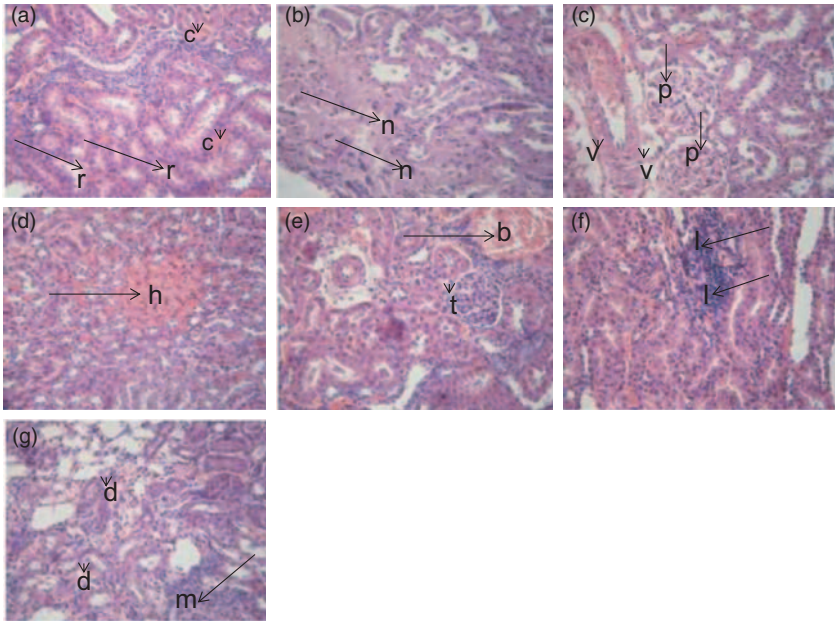

Figure 4 Haematoxylin and eosin-stained kidney tissues from (a) rats from NC group showing normal renal parenchyma, normal renal tubules (arrowheads showing letter r) and normal capillaries (arrowheads showing letter c), (b–d) diabetic rats showing wedge-shaped area co-aggulative necrosis containing extravasted erythrocytes (arrowheads showing letter n), proliferative glomerular tuft (arrowheads showing letter p), oedema in vascular wall and perivascular tissue (arrowheads showing letter v), focal haemorrhages in renal medulla (arrowhead showing letter h) and (e–g) PM-treated rats showing congested blood vessels (arrowhead showing letter b), thickened glomerular basement membrane (arrowheads showing letter t), focal interstitial lymphocytic aggregations (arrowheads showing letter l), mild regenerative attempts (arrowheads showing letter m) and dilated capillaries (arrowheads showing letter d). Haematoxylin and eosin 1200×. (A color version of this figure is available in the online journal)

autoxidation of glucose.[56] This protection was attributed to the hydroxyl radical scavenging properties of PM. A recent study that used chronic renal failure model of rats (subtotal nephrectomy) has referred to significant improvements in the clearance of creatinine, blood urea nitrogen and AGEs after eight weeks of treatment with PM.[20] Currently, a randomized double-blind, placebo-controlled trial is being conducted in 300 patients with DKD. This is to illustrate further the effect of PM in decreasing serum creatinine.[17] Another clinical study done in Indonesia has demonstrated mishandling of thiamine, increased degradation of vitamin B_6 and cytosolic metabolic resistance to vitamin B_{12} in type 2 diabetic patients.[57]

Conclusion

In conclusion, using agents to interrupt the harmful biochemical reactions that occur between glucose and the kidney microvasculature is a novel approach for the treatment of DN. Prospective trials are needed to elucidate a potential role for PM in adjunctive therapy and to confirm the adequate dose for enhanced renal outcomes in DN.

Author contributions: MME designed the idea, supervised and revised the experimental work, manuscript and conclusion. SEE supervised the experimental work and revised the results. NNY collected data from literatures, revised and contributed to writing the manuscript. MSZ performed the experimental work, recorded the results and collected data from the literature.

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