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ORIGINAL ARTICLE

Vitamin D protection from rat diabetic nephropathy is partly mediated through Klotho expression and renin–angiotensin inhibition

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ABSTRACT
Objective: We hypothesised that vitamin D has a beneficial renal protective effect from diabetic nephropathy (DN).

Methods: Four rat groups were included: normal control (control), type 2 diabetes for eight weeks (DM), treated group with angiotensin receptor blocker losartan (DM + L), and vitamin D-treated group started from the onset of diabetes (DM + Vit D).

Results: In the both treated groups, we found a significant (p < .05) reduction in the renal pro-inflammatory and profibrotic markers induced by diabetes. Vitamin D caused more reduction in monocyte chemoattractant protein-1 (MCP-1), transforming growth factor (TGF–β1), and renin–angiotensin levels that gave better kidney function compared to the DM + L group.

Conclusion: Vitamin D may have a valuable role in the renal protective effect from DN, this may occur via expression of its VDR, Klotho and blocking renin–angiotensin activation, so vitamin D should be considered as a target in renal prophylactic measures against DN.

INTRODUCTION

Diabetes mellitus (DM) is a widely spread chronic disease that can lead to multiple health complications. Diabetic nephropathy (DN) is one of the most important diabetic complications and a cause of mortality in patients with diabetes. The critical factors in the pathogenesis of DN are renal inflammation, fibrosis causing glomerulosclerosis, and tubulointerstitial fibrosis, which may be the cause of reduced kidney excretory function (Cheng et al. 2013).

Low vitamin D is found in patients with diabetes associated with higher fasting blood glucose and glycated haemoglobin levels (Kositsawat et al. 2010). There is an evidence supporting that nuclear vitamin D receptor (VDR) is involved in the prognosis of glomerular injury, and mice lacking the VDR was reported to be more susceptible to hyperglycaemia-induced renal injury (Sanchez-Niño et al. 2012).

Kuro-o et al. discovered an aging suppressor gene called Klotho (Kuro-o et al. 1997). The choice of the name was referred to a Greek goddess Klotho who was thought to prolong the lifespan. Interestingly, CKD is associated with marked decrease in renal Klotho expression (Koh et al. 2001). Furthermore, the overexpressed Klotho gene caused attenuation of diabetic renal injury in mice. Thus, Klotho expression may have a renoprotective effect in diabetic nephropathy, as the increased Klotho gene expression by vitamin D was reported to be a factor in the suppression of diabetes-induced renal hypertrophy (Kadoya et al. 2016).

The hyperglycaemia and oxidative stress activate renin–angiotensin system (RAS). RAS is considered as a major mediator in the development and progression of DN (Zhang et al. 2008). Although RAS inhibitors are widely used in the therapy of renal diseases, the major problem of these drugs is the compensatory renin rise. The increase in renin activity stimulates the conversion of Angl and ultimately AngII, which limits the efficacy of RAS inhibition (Zhang et al. 2008).

There have been multiple studies in the protective effect of vitamin D but little is known about the role mediated by both Klotho and RAS.

These observations justify the need for a pre-clinical study to test whether there is an impact of vitamin D supplementation on RAS system and to test the influence on Klotho expression in modifying the pathophysiology of rat DN model.

MATERIALS AND METHODS

Animals and experimental design

Forty adult male Wistar albino rats (weighing 150–170 g) were included in the study, purchased from the laboratory animal house unit of Faculty of Medicine, Cairo University. All experimental procedures had been approved by the animal care and use committee of Cairo University (CU-IACUC) number (CU/III/S/58/17). The animals were housed 7 days before the experiments in a 12 h light/dark cycle with free access to...
water and standard rat chow in the institutional animal care unit. Rats were randomly designated into (1): control group (10 rats), and type 2 diabetes (T2DM) was induced in the remaining 30 rats. Diabetes was induced by feeding high-fat diet (40% fat, 41% carbohydrate, and 18% protein) as a percentage of total kcal for a period of 2 weeks, followed by injection of a single streptozotocin (STZ) dose of freshly prepared (50 mg/kg/i.v. in 0.01 mol/l citrate buffer; Sigma, St. Louis, MO) (Reed et al. 2000). Fasting blood glucose was measured 1 week after STZ injection to confirm diabetes using Accu Check meter (Roche Diagnostics, Mannheim, Germany), rats expressed blood glucose more than 13.5 mmol/l were included in the study. The rats with induced diabetes were randomly divided into the following three groups (2): diabetic untreated group (DM), with only intraperitoneal corn oil injection in equal volume to that of treated rats (as a vehicle for vitamin D), (3); treated group with angiotensin receptor blocker (losartan at 30 mg/kg/day, dissolved in drinking water) (Kong et al. 2010), and (4); treated group with vitamin D (DM + Vit D) received vitamin D (doxercalciferol) supplementation immediately after establishment of diabetes in a dose of (0.5 μg/kg i.p., three times weekly for 8 weeks) (Lokeshwar et al. 1999).

After the eight weeks of treatment, all animal groups were exposed to a 24 h urine collection in metabolic cages for assessment of protein and the body weight was taken.

**Blood sample collection and measured parameters**

Following urine collection, animals were anesthetised using ketamine (50 mg/kg/i.p.) (Sigma, Cairo, Egypt), blood samples were drawn from the rat tail vein for biochemical measurement of serum creatinine and BUN (directly by Quantichrom™ creatinine Assay Kit (Corporate Place, Hayward, MO)). Serum concentrations of calcium were measured on a Hitachi 902 biochemistry analyser by commercial colorimetric kits. Triglyceride was measured using quantification kit (BioVision Research, CA). Measurement of fasting plasma glucose kits are supplied by Diamond Diagnostics (Egypt). Plasma insulin levels were analysed using enzyme-linked immunosorbent assay (Dako, Carpinteria, CA) according to the manufacturer’s instruction. Homeostatic model assessment of insulin resistance (HOMA-IR) is a mathematical method based on basal glucose and insulin. It was calculated as fasting glucose (mmol/l) × fasting insulin (μU/ml)/22.5. Results more than 4.0 were considered as insulin resistance (Salgado et al. 2010). All animals were humanely euthanised by decapitation under anaesthesia (50 mg/kg pentobarbital i.p.); their kidneys were rapidly excised, washed with saline, the right kidneys were used in the biochemical analysis and the left kidneys for histopathological examination.

**Measurement of parathyroid hormone (PTH), 25-hydroxyvitamin D, fibroblast growth factor 23 (FGF23), renin & angiotensin II (AngII)**

These parameters were measured in the serum by rat ELISA kit supplied by MyBiosource (USA) according to the manufacturer’s instructions.

**Determination of renal TGFβ1 and MCP-1**

Renal TGF beta and MCP-1 were measured by using ELISA kits supplied by Quantikine (R&D Systems, MN) according to the manufacturer’s instructions.

**Detection of renal VDR, Klotho, heme oxygenase-1 (HO-1) and connective tissue growth factor (CTGF) gene expression by real-time PCR**

Real-time PCR for gene expression measurement involves total RNA extraction from tissues using TRizol method. In brief, RNA was extracted by homogenisation in TRizol reagent (Invitrogen, Life Technologies, USA). Complementary cDNA synthesis was done from 1 μg RNA using SuperScript III First-Strand Synthesis System as described in the manufacturer’s protocol (Invitrogen, Life Technologies). Real-time quantitative PCR; the relative abundance of mRNA species was assessed using the SYBR Green method on an ABI prism 7500 sequence detector system (Applied Biosystems, Foster City, CA). PCR primers (Table 1) were designed with Gene Runner Software (Hasting Software, Inc., Hasting, NY) from RNA sequences from GenBank. Relative expression of studied gene mRNA was calculated using the comparative Ct method. All values were normalised to the beta-actin gene and reported as fold change over background levels detected in diseases group (Livak and Schmittgen 2001).

**Detection of renal P38 mitogen-activated protein kinase (P38MAPK) by Western blot technique**

Briefly, the protein concentration for each sample was determined using Bradford assay. Proteins were incubated overnight with anti-beta actin antibody (1:1000 dilution; Thermoscientific, Thermo Fisher, MA), MAPK antibody (1:2000 dilution; Abcam, Cambridge, MA) incubated with a mouse anti-rabbit secondary monoclonal antibody conjugated to horseradish peroxidase at room temperature for 2h. Chemiluminescence detection was performed with the BioRad detection kit according to the manufacturer’s protocols. The amount of studied protein was quantified by ChemiDoc™ imaging system with Image Lab™ software version 5.1 (Bio-Rad Laboratories Inc., Hercules, CA). Results were expressed as arbitrary units after normalisation for β-actin.

**Table 1. Primers sequence for studied genes.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>VDR</td>
<td>F: 5’-GGCCCTCTATAAGGTCCAGGTG-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’-GGATACGGGGTTCCCTGCTGGAATGG-3’</td>
</tr>
<tr>
<td>Klotho</td>
<td>F: 5’-GGATACGGGGTTCCCTGCTGGAAGGC-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’-GAGGGTCATAGGCGGATATGC-3’</td>
</tr>
<tr>
<td>CTGF</td>
<td>F: 5’-AT CTCGGCAACCAAG 3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’-CAACTG CCT TGGAAAGCTCCG-3’</td>
</tr>
<tr>
<td>HO-1</td>
<td>F: 5’-GAGGCCCAACAGCTCGACAG-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’-CTGTCGACAGCAGCACCACAC-3’</td>
</tr>
<tr>
<td>Beta actin</td>
<td>F: 5’-TGCTGCTCCTGATCTCCCTCT-3’</td>
</tr>
<tr>
<td></td>
<td>R: 3’-TAATGTCGACAGCAGTATCC-5’</td>
</tr>
</tbody>
</table>

VDR: vitamin D receptor; CTGF: connective tissue growth factor; HO-1: heme oxygenase-1.
Histopathology

The left kidneys were weighed, then formalin-fixed; dehydrated in ethanol, cleared in xylene, and embedded in a paraffin block, sections were prepared from serial cuts (3 μ thick); stained with haematoxylin/eosin. All stained slides were examined under light microscope to evaluate the presence of the following: (a) glomerular changes (mesangial matrix expansion, mesangial hypercellularity, and capillary wall thickening) and (b) tubular changes (atrophy and casts “if present”).

Statistical analysis

The results are given as mean ± standard deviation (SD). Results were analysed using the Statistical Package for Social Sciences software (IBM SPSS version 20, Chicago, IL). Normally distributed quantitative variables were analysed by analysis of variance (ANOVA) with multiple comparisons. Tukey post hoc test was used for comparisons between groups. Difference <.05 was considered to be significant.

Results

Glucose and lipid homeostatic parameters

In comparison to the control group, induced diabetes in the DM group showed a significant increase (p < .001) in glucose homeostatic parameters [glucose (mmol/ml), insulin (mic IU/l), and HOMA-IR] and triglycerides (mg/dl). Both the losartan- and vitamin D-treated groups showed significant (p < .05) improvement in glucose homeostatic parameters and triglycerides (mg/dl) (p < .05) (Table 2).

The effects on kidney functions

There was significant renal function deterioration (p < .001) in the DM group relative to the control in BUN (mg/dl), creatinine (mg/dl), and proteinuria (mg/24 h). The losartan administration group expressed significant improvement (p < .05) in the renal function parameters. Further improvement was observed in the vitamin D group (p < .001), especially in proteinuria and creatinine level when compared to the DM group (Table 2).

The histopathological examination of the HE-stained slides of the DM groups did not reveal significant glomerulosclerosis or interstitial nephropathic changes. However, intratubular proteinaceous casts were pronounced in the untreated DM group reflecting a proteinuric state which was subsided in both the treated group (Figure 1). In addition, no significant change was observed in KW/BW% between the groups (Table 2).

Renin and AngII levels

In the DM group, we noticed elevated (p < .05) renin and AngII levels. A further increase was observed in the losartan-treated group (p < .05) in both renin and AngII levels compared to the DM groups. Interestingly, vitamin D was associated with renin and angiotensin II reversal (p < .001) compared to both the losartan and DM groups (Table 3).

Assessment of 25-hydroxyvitamin D, PTH, and FGF23 and calcium circulating levels

Table 3 describes the effect of diabetes and the treatment with losartan or vitamin D on the serum levels of vitamin D, PTH, and FGF23. In the DM group, there was a significant (p < .001) decrease in vitamin D associated with an elevation in PTH and FGF23 levels. Losartan showed no effect on the circulating vitamin D level, but a significant (p < .001) elevation was seen in the vitamin D-treated group compared to the DM group. The PTH and FGF23 level showed a significant reduction (p < .05) in both the treated groups. Calcium level was reduced (p = .026) in the DM groups compared to the controls, then losartan or vitamin D administration significantly (p < .05) returned this reduction with normalisation of its level.

Vitamin D receptor (VDR) and Klotho expression in the renal tissue

Type 2 diabetes group showed a significant decrease (p < .001) in both VDR expression and Klotho compared to the control. Losartan caused a non-significant increase in VDR or Klotho expression. However, the treatment with vitamin D induced a significant increase (p < .001) in VDR expression and Klotho in comparison to the DM group (Figure 2(A,B)).

Modulation of renal tissue markers

The pro-inflammatory markers (p38MAPK, MCP-1) levels were significantly increased (p < .001) in the kidney tissue of the

Table 2. Glucose homeostasis and renal function detection in the studied groups.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>DM</th>
<th>DM + L</th>
<th>DM + Vit D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mmol/ml)</td>
<td>5.07 ± 0.62</td>
<td>14.75 ± 1.28*</td>
<td>11.75 ± 1.77#</td>
<td>9.99 ± 1.06#</td>
</tr>
<tr>
<td>Insulin (mic IU/l)</td>
<td>8.19 ± 1.41</td>
<td>16.78 ± 1.73*</td>
<td>12.8 ± 1.3#</td>
<td>12.28 ± 1.6#</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>92.32 ± 8.5</td>
<td>145.15 ± 15.3*</td>
<td>117.15 ± 1.32#</td>
<td>119.88 ± 5.7#</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>1.85 ± 0.37</td>
<td>10.26 ± 1.5*</td>
<td>6.69 ± 1.56#</td>
<td>5.48 ± 1.16#</td>
</tr>
<tr>
<td>KW/BW %</td>
<td>0.42 ± 0.05</td>
<td>0.36 ± 0.06</td>
<td>0.40 ± 0.04</td>
<td>0.42 ± 0.04</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>36.15 ± 9.22</td>
<td>97.53 ± 11.50*</td>
<td>70.17 ± 6.16#</td>
<td>66.68 ± 7.19#</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.97 ± 0.18</td>
<td>6.10 ± 0.85*</td>
<td>4.1 ± 0.99#</td>
<td>2.83 ± 0.96#</td>
</tr>
<tr>
<td>Proteinuria (mg/24h)</td>
<td>0.72 ± 0.15</td>
<td>9.3 ± 1.5*</td>
<td>4.9275 ± 0.62#</td>
<td>3.1 ± 0.7*#</td>
</tr>
</tbody>
</table>

All Values are expressed as mean ± SD. Diabetic group (DM), losartan treated (DM + L), vitamin D treated diabetic rats (DM + Vit D), blood urea nitrogen (BUN), and kidney weight/body weight % (KW/BW %).  
*Significant p as compared to control (p < .05).  
#Significant p as compared to DM (p < .05).  
@Significant p as compared to DM + L group (p < .05).
DM group in comparison to the control group. Losartan and vitamin D supplementation resulted in a significant reduction ($p < .05$) in these pro-inflammatory markers. Furthermore, vitamin D expressed more reduction ($p < .05$) in MCP-1 compared to the losartan-treated group (Figure 3(A–C)).

Analysis of the renal profibrotic markers (TGFβ-1 and CTGF) revealed significant increase ($p < .05$) in the DM group compared to the control group. Losartan and vitamin D led to significant reversal ($p < .001$) in these profibrotic markers (TGFβ-1 and CTGF) induced in DM (Figure 4(A,B)), and further elevation of HO-1 ($p < .001$) (Figure 3(D)). Administration of vitamin D resulted in more reduction ($p < .05$) in the TGFβ-1 level compared to the losartan-treated group.

**Table 3.** Serum vitamin D, PTH, FGF23, calcium renin and angiotensin detection.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>DM</th>
<th>DM + L</th>
<th>DM + Vit D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin D level (ng/ml)</td>
<td>52.03 ± 2.76</td>
<td>21.2 ± 3.96*</td>
<td>22.05 ± 2.85*</td>
<td>46.15 ± 2.96#(ⅱ)</td>
</tr>
<tr>
<td>PTH (pg/ml)</td>
<td>23.8 ± 4.66</td>
<td>89.8 ± 6.14*</td>
<td>53.4 ± 10.03*#</td>
<td>54.7 ± 8.97#</td>
</tr>
<tr>
<td>FGF23 (pg/ml)</td>
<td>45 ± 4.96</td>
<td>119.2 ± 17.23*</td>
<td>77.15 ± 14.35*#</td>
<td>69.2 ± 15.87*#</td>
</tr>
<tr>
<td>Ca $^{2+}$ (mg/dl)</td>
<td>9.8 ± 0.92</td>
<td>8.2 ± 1.06*</td>
<td>9.5 ± 0.74</td>
<td>9.7 ± 0.94</td>
</tr>
<tr>
<td>Renin (uIU/ml)</td>
<td>19.02 ± 2.95</td>
<td>99.05 ± 19.97*</td>
<td>118.8 ± 11.57*</td>
<td>44.65 ± 12.6*#(ⅱ)</td>
</tr>
<tr>
<td>Angiotensin II (pg/ml)</td>
<td>2.21 ± 0.4</td>
<td>11.15 ± 1.57*</td>
<td>14.92 ± 2.38*#</td>
<td>4.75 ± 0.97*#(ⅱ)</td>
</tr>
</tbody>
</table>

All values are expressed as mean ± SD. Diabetic group (DM), losartan treated (DM + L), vitamin D treated diabetic rats (DM + Vit D), parathyroid hormone (PTH), fibroblast growth factor 23 (FGF23).

*Significant $p$ as compared to control ($p < .05$).
#Significant $p$ as compared to DM ($p < .05$).
ⅱSignificant $p$ as compared to DM + L group ($p < .05$).

**Figure 2.** (A) Vitamin D receptor (VDR) and (B) Klotho relative gene expression. Diabetic group (DM), losartan treated (DM + L), vitamin D treated diabetic rats (DM + Vit D). *Significant $p$ as compared to control. #Significant $p$ as compared to DM. ⅱSignificant $p$ as compared to DM + L group ($p < .05$).
Discussion

This study reveals that vitamin D supplementation to type 2 induced diabetes in rats three times weekly is better than oral losartan in a dose of 30 mg/kg/day for 8 weeks started from the onset of diabetes verification. We reported kidney protection in the form of reduction of proinflammatory, profibrotic, and increased antioxidative mediators in the kidney tissue. This protective effect is partly mediated through Klotho expression and RAS inhibition.

The STZ has been widely used in the induction of both type 1 and type 2 diabetes (T2DM). The animal model obtained depends on its dose, the number of doses, the time interval between doses, the route of administration, the rat strain, and the feeding state upon which STZ is administered (Skovsø 2014). STZ could induce T2DM with a state of hyperinsulinemia (beta cell compensation) (Reed et al. 2000, Qian et al. 2015).

In our model, the untreated DM group was progressed to express diabetic nephropathy (DN) manifestations represented by elevated creatinine, BUN, and proteinuria proved by urine analysis and confirmed by tubular protein cast in histology examination.

RAS is one of the most significant contributors in developing DN. Angiotensin (AngII) is known to induce insulin resistance via mechanisms involving ROS degradation of IRS (Ramalingam et al. 2016). Alongside the effects of hyperglycaemia, induced release of AngII (Deng et al. 2015), there is local upregulation of intrarenal RAS (Lavoie and Sigmund 2003) resulted in hemodynamic disturbances including efferent arteriolar vasoconstriction that leads to increased glomerular capillary pressure and permeability (Iinigo et al. 2001). Its local, non-hemodynamic effects are represented in: increased cytokine production, glomerular and tubular cell proliferation, extracellular matrix accumulation, and the generation of reactive oxygen species (ROS) (Gilbert et al. 2003).
This creates a suitable media for the development and progression of diabetic kidney disease.

The current approach in proteinuric DN treatment is RAS inhibitors. However, the breakthrough phenomenon in which renin and aldosterone levels may actually increase with continuous treatment limits the RAS inhibitors usage (Gallagher and Suckling 2016).

Vitamin D insufficiency is defined as serum 25-hydroxyvitamin D levels <50 nmol/l, and it is common in patients with T2DM (Scragg et al. 2004). The relation between vitamin D deficiency and T2DM is not well known. However, there was an association between T2DM and 1 alpha-hydroxylase gene polymorphisms (Kadoya et al. 2016). There is a link between VDR gene polymorphisms and T2DM, that differs from one population to another (Mathieu 2005) and mice lacking the VDR are more susceptible to hyperglycaemia-induced renal injury (Alvarez and Ashraf 2010).

The vitamin D plays a pivotal role in preserving glucose homeostasis in T2DM. 1,25(OH)2D directly activates transcription of the human insulin receptor gene, enhancing glucose transport (Alvarez and Ashraf 2010). In addition to the reduction of triglycerides level, vitamin D could inhibit free fatty acid-induced insulin resistance, via reduction of JNK activation (Zhou et al. 2008). This explains the inverse association between vitamin D status and A1C level in diabetic and prediabetic adults (Kositsawat et al. 2010).

1,25(OH)2D deficiency results in elevated PTH, which in turn disturb calcium metabolism and may inhibit insulin-target cells from glucose transport resulting in insulin resistance (Worrall and Olefsky 2002). Vitamin D supplementation inhibits the synthesis and secretion of PTH controlling the secondary hyperparathyroidism in renal failure (Bikle 2009).

Consequently, an early reduction in Klotho expression was found in DN mice (Tsujikawa et al. 2003) and patients (Liu et al. 2014). The Klotho actions need fibroblast growth factor (FGF23) as a co-receptor. FGF23 is produced primarily by osteoblasts and osteocytes that is stimulated by 1,25(OH)2D3 (Bikle 2009).

Although losartan treatment did not affect the serum vitamin D or VDR and Klotho levels in the renal tissue, losartan itself significantly improved the glucose homeostatic parameters, reduced urinary albumin secretion that ameliorate the renal functions. Losartan could decrease the overexpressed renal PTH-related protein in nephrotoxin-damaged kidney (Lorenzo et al. 2002).

Klotho exerts its renal protection in part by targeted regulation of RAS, it abolishes the induction of multiple RAS proteins, including angiotsinogen, renin, angiotsin-converting enzyme, and angiotsin II type 1 receptor (Zhou et al. 2015).

In the kidney, hyperglycaemia causes increased ROS (Nishikawa et al. 2015). Heme oxygenase-1 (HO-1) is an inducible protective microsomal rate-limiting enzyme. It catalyses pro-oxidant heme metabolism, yielding antioxidant iron, carbon monoxide, and bilirubin guarding against various kidney diseases (Ndisang and Tiwari 2014).

The nuclear factor E2-related factor 2 (Nrf2) is a transcription factor that induces antioxidant enzymes such as HO-1. Vitamin D inhibited the hyperglycaemia-induced down-regulation of Nrf2 and expresses HO-1 in DN rats (Nakai et al. 2014). Klotho also has its own anti-oxidant effect by enhancing HO-1 protein expression levels (Benedetta et al. 2014). Moreover, the crosstalk between RAS and ROS is defined. Losartan prevents mitochondrial uncoupling protein 2 protecting tissues from oxidative damage (Ramalingam et al. 2016).

Actually, we noticed that the vitamin D-treated group had a significant decrease in the inflammatory markers mitogen-activated protein kinases P38 (p38MAPK) and monocyte chemoattractant protein-1 (MCP-1). Vitamin D blocks cytokine genes by down-regulation of nuclear factor-xB (Riachy et al. 2015). The MCP-1 protein was increased in glomeruli of DN rats, then, it was reversed by calcitriol (Sanchez-Niño et al. 2012). Additionally, Klotho has an anti-inflammatory effect that attenuated cultured renal fibroblasts activated p38 kinase (Adhikary et al. 2004). Similarly, Klotho attenuated tumour necrosis factor-α activated p38 kinase in human embryonic kidney cells (Cheng et al. 2013). Furthermore, losartan has several AT1 receptor-independent actions, including its anti-inflammatory and antioxidant properties (Krämer et al. 2002).

The profibrotic factor TGF-β1 has been identified as the most potent growth factor which can induce epithelial to mesenchymal transition. It was reported that TGF-β1 stimulates downstream Smad3-dependent CTGF expression resulting in collagen-I generation (Lu et al. 2015). AngII has been demonstrated to induce TGF-β expression, promotes the generation of ROS, and augment renal inflammation (Zhou et al. 2015). Vitamin D could decrease Smad3 protein level, altering bioactive TGF-β1 signalling proteins in renal tissue (Aschenbrenner et al. 2001). As ERK1/2 and p38 kinase are part of the non-Smad pathways of TGF-β1 signalling, Klotho attenuates high glucose activated ERK1/2 and p38 kinase by decreasing TGF-β1 signalling (Javier and Mart 2015). Because TGF-β1 is up-regulated by AngII, the suppression of these factors by losartan treatment is likely to be expected (Zhang et al. 2008). However, the increased renin can also act through the prorenin/renin receptor, which may cause renal damages independent of Ang II which is a major drawback of losartan treatment (Matavelli et al. 2010).

Vitamin D administration has a renal protective effects from diabetic nephropathy injury indicated by reversal of proinflammatory, profibrotic markers involved in the DN pathogenesis, this may occur through expression of its VDR as well as the Klotho gene, expression and through blocking the compensatory induction of renin–angiotensin II associated with the use of losartan. So it should be considered as an important target in the prophylaxis of diabetic nephropathy.

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Disclosure statement
The authors declare that they have no conflict of interest.

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