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Article *in* Journal of Postgraduate Medicine · February 2018

DOI: 10.4103/jpgm.JPGM_598_16

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Original Article

Effect of Vitamin D therapy on urinary albumin excretion, renal functions, and plasma renin among patients with diabetic nephropathy: A randomized, double-blind clinical trial

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Received : 04-10-2016
Review completed : 24-01-2017
Accepted : 23-05-2017

ABSTRACT

Background: Despite different management strategies, progression of proteinuria occurs in a sizable category of patients with diabetic nephropathy (DN). Increase in serum renin levels induced by the renin–angiotensin system (RAS) may contribute to this. Vitamin D therapy is found to have an inhibitory effect on the RAS. We aimed to study the effects of Vitamin D therapy on renal functions of patients with DN. **Methods:** This was a double-blind, randomized, placebo-controlled study. Patients with DN (urinary albumin [UA] >30 mg/g of creatinine) whose estimated glomerular filtration rate (eGFR) was more than 30 mL/min were selected and their plasma renin, parathyroid hormone, serum Vitamin D, serum calcium, serum creatinine, fasting blood sugar were done as baseline measurements. Subjects were randomized into two groups and treatment group was given Vitamin D, 50000 IU (0.25 ml) intramuscularly (IM) monthly for 6 months; control group received distilled water IM. Investigations were repeated after 6 months of therapy. **Results:** Of 155 patients invited, 85 were randomly assigned to two groups. After 6 months, mean reduction of UA to creatinine ratio in the treatment and control group was 51.8 mg/g (95% confidence interval [CI]; 66.1−−37.5, $P \leq 0.001$); 22.4 mg/g (95% CI; −45.7–0.8, $P = 0.06$), respectively (between group difference $P = 0.001$). Significant increase in the eGFR observed in the treatment group while eGFR remained unchanged in the control group ($P = 0.03$ for the between-group difference). Mean reduction in plasma renin in treatment group and control group was 5.85 pg/mL (95% CI; −6.7−−4.6) ($P < 0.001$) and 0.95 pg/mL (95% CI; −1.4−−0.14, $P = 0.02$), respectively. **Conclusions:** Vitamin D 50000 IU given IM monthly for 6 months reduces urine albumin, serum creatinine, and renin levels in patients with DN.

KEY WORDS: Albuminuria, diabetic nephropathy, renin, Vitamin D

Introduction

Diabetic nephropathy (DN) is the most common cause of end-stage renal disease worldwide.[1,2] Despite the use of conventional therapeutic agents including angiotensin enzyme inhibitors (ACEIs) and angiotensin receptor blockers (ARBs), urine albumin (UA) excretion and renal impairment tend to progress in some patients with diabetes mellitus. Therefore, some novel therapeutic approaches are being tested to combat progression of proteinuria and nephropathy among patients with DN.[3,4]

Vitamin D3 (1, 25$(OH)_2D_3$), the active form of Vitamin D, has an inhibitory effect over renin–angiotensin system (RAS) as it

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Quick Response Code:

Website: www.jpgmonline.com

DOI: 10.4103/jpgm.JPGM_598_16

How to cite this article: Liyanage P, Lekamwasam S, Weerarathna TP, Liyanage C. Effect of Vitamin D therapy on urinary albumin excretion, renal functions, and plasma renin among patients with diabetic nephropathy: A randomized, double-blind clinical trial. J Postgrad Med 0;0:0.

reduces renin synthesis, the first and the rate limiting step in the RAS.[5,6] A preliminary study by Li *et al*. demonstrated that treatment with 1, 25 dihydroxy has favorable effects on reversing the progression of DN.[6]

Methods

Study design

This study was conducted on patients with DN (UA > 30 mg/g of creatinine in two occasions) whose glomerular filtration rate (GFR) was more than 30 mL/min. Patients who had albuminuria in a previous cross-sectional study 6 months back were invited and investigations were repeated. This procedure ensured confirmation of albuminuria at least on two occasions over a period of 6 months. Selected patients were informed about the study and written consent was obtained. Those who had blood pressure >130/80 mmHg during the last two clinic visits, hyper-phosphatemia (serum phosphate >5 mg/dL), hyper- or hypo-calcemia, uncontrolled blood sugar (current hemoglobin A1c >8) and those with liver disease, hyperthyroidism, hyperparathyroidism, or diseases related to calcium or Vitamin D metabolism and congestive heart failure (current) were excluded from the study. All attempts were made to exclude other causes of proteinuria such as ongoing urinary tract infection, urolithiasis, and renal tuberculosis by history, examination, and previous investigations. Patients were recruited and randomized into two groups using a random number table. Concealed envelopes containing the treatment assignment were given to research assistants who assigned participants to treatment and control groups. Treatment group received monthly dose of 50,000 IU of Vitamin D3 intramuscularly and the control group was given an equal volume of distilled water (0.25 mL) in the same manner. Participants, principal investigator, clinicians, and those assessing the outcomes were blinded to the group assignment. Research assistants who gave the injections were not blinded regarding the group assignment.

Blood samples were collected for the measurement of serum Ca, phosphate, creatinine, parathyroid hormone (PTH), renin, and Vitamin D level at the baseline. Serum or plasma samples were frozen at −80°C until they were analyzed. Morning urine samples were collected and urine dipstick test was done to exclude ongoing urine infections. Urine collection was postponed if a patient had fever, urinary symptoms, or menstruation. Only samples which were negative for nitrates were stored at −80°C for UA analysis. After 2 weeks, second sample of urine was collected. Same procedure was followed as for the first urine sample. If the UA excretion of the second sample was inconsistent with the first sample, a third urine sample was checked. The presence of microalbuminuria was confirmed only if two consecutive or two out of three samples were positive for UA >30 mg albumin/g of creatinine. In the same manner, macroalbuminuria was confirmed when UA excretion was >300 mg/g of creatinine.

After the baseline assessment, patients were given either Vitamin D or placebo injection on the 2nd day. A safety visit was scheduled 1 week after starting the treatment to monitor the serum Ca and phosphorus concentrations and to elicit any adverse event. The protocol permitted withdrawal from the trial if serum Ca exceeded 11 mg/dL. During monthly visits, patients were inquired about side effects according to a checklist in the data collection sheet. At 3 months and 6 months, serum Ca and phosphate levels were measured. Patients received monthly injections during the next 6 months. Blood samples were collected for serum creatinine and urine was taken for the assessment of urine microalbumin to creatinine ratio at 3 months. At the end of 6 months, all the baseline measurements were repeated.

In this study, UA was measured by turbid metric method while urinary creatinine concentration was measured using an end point spectrophotometric method with an alkaline-picrate solution.

Biochemical assays were performed using commercial kits. Serum creatinine was measured by auto-creatinine calibrated with autocal which is traceable to reference material. GFR was estimated by chronic kidney disease (CKD)-epidemiology collaboration equation.[7]

Intact PTH (Immunotech, Immuno radiometric assay [IRMA] PTH) and renin (Beckman Coulter, IRMA active renin) by radioimmunoassay and 25-hydroxy Vitamin D were measured using immunochemiluminometric (VITROS immunodiagnostic) assays. Serum creatinine was measured by spectrophotometric method with an alkaline-picrate solution.

Statistical analysis

The baseline characteristics between the two groups were compared by either unpaired *t*-test or Chi-square test. Changes in UA, renal functions, Vitamin D, renin, and PTH during the trial period were analyzed by the repeated-measures ANOVA. *P* value was adjusted for multiple comparisons by the Bonferroni method.

Ethics approval and consent to participate

"All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the Declaration of Helsinki 1964 and its later amendments or comparable ethical standards."

Ethical approval was gained from the Ethical Review Committee Faculty of Medicine, University of Ruhuna. Informed written consent was obtained from all the participants included in the study. Cost of investigations and treatment were met by the research grant.

Results

A total of 157 patients were invited for the study and 72 were excluded due to the presence of one or more exclusion criteria. Remaining 85 were randomly assigned to two groups and 82 subjects completed the study; 41 patients from each group completed the study [Figure 1].

Baseline characteristics of the patients are listed in Table 1. No significant differences were found with regard to the baseline characteristics between the treatment and control groups. All

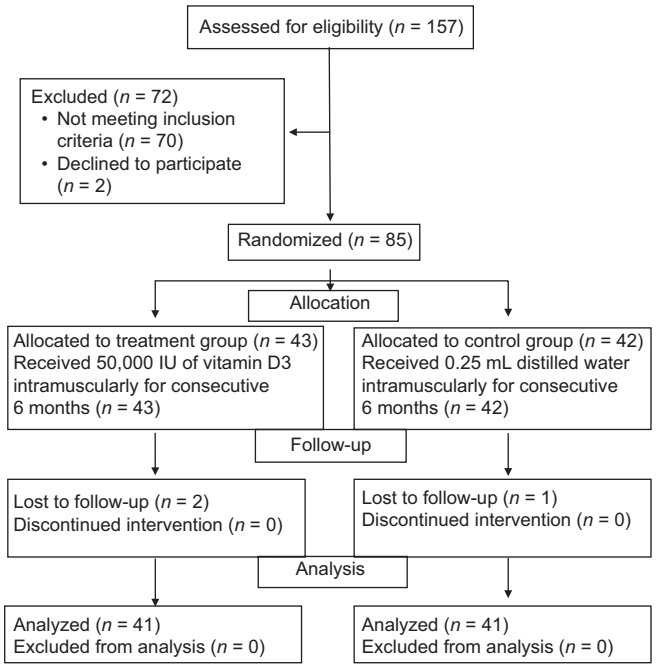

Figure 1: Flow diagram of the recruitment of the patients for the study

patients received either an ARB or ACEI at the baseline. During the study period, oral hypoglycemic drugs were increased in nine patients (six in the treatment group). Losartan was increased in three patients (two in the control group). Blood pressure number and doses of antihypertensive drugs were quite stable during the clinical trial.

Table 2 shows the changes of the urine microalbumin to creatinine ratio, systolic blood pressure (SBP), diastolic blood pressure (DBP), fasting blood sugar (FBS), serum creatinine, estimated GFR (eGFR), PTH, renin, and Vitamin D levels after three and 6 months of treatment in the treatment and control groups. After 6 months, mean reduction of UA to creatinine ratio was 51.8 mg/g (95% confidence interval [CI]; 66.1–−37.5, $P \leq 0.001$) in the treatment group, 22.4 mg/g (95% CI; −45.7 − 0.8, $P = 0.06$) in the control group, and this difference was statistically significant ($P = 0.001$). Significant increase in the GFR was observed in the treatment group while in the control group, GFR remained unchanged ($P = 0.03$ for the between-group difference). There was a significant reduction of serum creatinine in the treatment group but not in the control group. However, the change was not significant between groups.

A significant increase of SBP was seen in the control group whereas SBP remained unchanged in the treatment group and the difference was not statistically significant. Significant trends in the DBP was seen in both groups during the study period, but the difference between the two groups was not statistically significant ($P = 0.17$). Significant reduction of FBS was seen only in the control group and the difference between groups was not statistically significant ($P = 0.23$).

Significant reduction of PTH was observed in both treatment and control groups. However, the change between two groups

Table 1: Baseline characteristics of subjects in the two groups

Variable	Control group (n=43)	Treatment group (n=42)	95% CI
Age (years)	59 (8)	56 (10)	−0.62-7.4
Number of males (%)	42.9	48.8	−0.16-0.28
SBP (mmHg)	121 (7)	120 (8)	−2.0-4.4
DBP (mmHg)	70 (5.9)	71 (5.9)	−4.0-1.1
HbA1c (%)	7.1 (0.5)	6.9 (0.5)	−0.03-0.39
Calcium (mg/dL)	8.9 (0.7)	8.8 (0.6)	−0.2-0.4
Phosphorus (mg/dL)	3. 8 (0.6)	3. 9 (0.5)	−0.3-0.1
PTH (pg/mL)	42.5 (19.0)	38.2 (11.3)	−2.4-11.1
Plasma renin (pg/mL)	15.14 (4.82)	14.64 (5.62)	−1.8-2.8
25(OH)D (nmol/L)	50.0 (16.5)	55. 9 (12.3)	−12.1-0.4
FBS (mg/dL)	130 (12.5)	128 (13.3)	−3.7-7.4
Duration of diabetes (years)	7 (4)	8 (5)	−3.2-1.4
Urine creatinine (mg/dL)	63.6 (10.9)	61.7 (11.9)	−3.0-6.8
Urine albumin (mg/g of creatinine)	184.8 (48.2)	168.9 (33.9)	−2.1-33.7
GFR (mL/min)	83.4 (15.9)	87.0 (14.3)	−10.1-2.9
HDL (mg/dL)	53.5 (10.9)	50.3 (7.5)	−0.9-7.1
TC (mg/dL)	194.6 (32.1)	194.8 (30.1)	−12.2-14.4
LDL (mg/dL)	117.0 (28.1)	119.7 (28.7)	−13.2-11.1
TG (mg/dL)	128.4 (50.8)	122.8 (41.4)	−15.5-24.3
BMI (kg/m^2)	23.2 (4.0)	24.4 (3.4)	−2.8-0.4

SBP: Systolic blood pressure, DBP: Diastolic blood pressure, PTH: Parathyroid hormone, FBS: Fasting blood sugar, HDL: High-density lipoprotein, TC: Total cholesterol, LDL: Low-density lipoprotein, TG: Triglyceride, BMI: Body mass index, HbA1c: Hemoglobin A1c, 25(OH)D: 25-hydroxy Vitamin D, GFR: Glomerular filtration rate, CI: Confidence interval

was not statistically significant ($P = 0.26$). In the treatment group, Vitamin D level increased by 25.64 nmol/L and between the two groups, the change was statistically significant ($P < 0.001$). Mean reduction in plasma renin in the treatment group was 5.85 pg/mL (95% CI; −6.7–−4.6, $P < 0.001$). In the control group, the reduction observed was only 0.95 pg/mL. The difference between the two groups was statistically significant ($P = 0.006$) [Table 2].

A significant inverse correlation was observed in Vitamin D with percentage change in plasma renin level ($\rho = -0.66$, $P < 0.01$) and percentage change in UA levels ($r = -0.47$, $P < 0.01$). Furthermore, percentage changes of renin and UA also showed a significant correlation ($\rho = 0.62$, $P < 0.01$) [Table 3].

According to Table 4, the microalbuminuria suppression is related to the final serum renin level. The suppression of microalbuminuria is highest in the highest tertile of serum renin and it does not vary across the tertiles of serum Vitamin D. The lowest suppression of microalbuminuria is in the lowest tertile of the serum renin. Furthermore, there is a gradient of microalbuminuria suppression across the tertiles of the serum renin concentration.

Serum Ca levels in patients with Vitamin D treated and in the control group were 9.16 (0.61) and 9.045 (0.69) at the end of

Table 2: Changes observed in the treatment and control groups at 3 months and 6 months

Variable	Baseline	At 3 months	At 6 months	Mean difference (95% CI)	*P* within group	*P* between group
SBP (mmHg)						
Control	121 (7)	121 (8)	127 (6)	5.8 (3.2-8.3)	<0.001	0.07
Treatment	120 (8)	120 (8)	121 (7)	0.5 (−1.2-2.2)	0.59	
DBP (mmHg)						
Control	70 (6)	72 (6)	72 (6)	2.7 (1.5-3.8)	<0.001	0.17
Treatment	71 (6)	69 (6)	68 (6)	−2.6 (−3.6-−1.7)	<0.001	
FBS (mg/dL)						
Control	130.2 (12.5)	130.6 (10.1)	127.8 (10.7)	−2.4 (−4.6-−0.2)	0.02	0.23
Treatment	128.3 (13.6)	125.8 (13.4)	125.9 (10.9)	−2.6 (−5.2-0.008)	0.08	
PTH (pg/mL)						
Control	42.5 (19.0)		37.6 (12.6)	−5.2 (−8.7-−1.8)	0.003	0.26
Treatment	38.2 (11.3)		35.7 (7.9)	−3.1 (−4.8-−1.3)	0.001	
25(OH)D (nmol/L)						
Control	49.64 (16.46)		45.67 (17.20)	−3.9 (−6.6-−1.4)	0.004	< 0.001
Treatment	56.11 (12.95)		81.75 (15.03)	25.6 (23.5-27.7)	<0.001	
Plasma renin (pg/mL)						
Control	15.14 (4.82)		14.19 (4.6)	−0.77 (−1.4-−0.14)	0.02	0.006
Treatment	14.64 (5.62)		8.83 (4.81)	−5.7 (−6.7-−4.6)	<0.001	
Urine albumin (mg/g)						
Control	185.8 (50.6)	160.9 (63.4)	163.4 (56.2)	−22.4 (−45.7-0.8)	0.06	0.001
Treatment	169.4 (35.8)	122.1 (54.4)	117.6 (45.2)	−51.8 (−66.1-−37.5)	<0.001	
Serum creatinine (mg/dL)						
Control	0.87 (0.22)	0.87 (0.20)	0.87 (0.20)	−0.005 (−0.04-0.03)	0.84	0.10
Treatment	0.86 (0.13)	0.80 (0.12)	0.77 (0.11)	−0.08 (−0.1-−0.06)	<0.001	
GFR (mL/min)						
Control	83.2 (16.1)	83.4 (15.6)	83.9 (14.9)	0.7 (−2.1-3.5)	0.74	0.03
Treatment	86.7 (14.6)	90.7 (14.8)	93.7 (14.1)	7.1 (4.5-9.7)	<0.001	

SBP: Systolic blood pressure, DBP: Diastolic blood pressure, PTH: Parathyroid hormone, FBS: Fasting blood sugar, GFR: Glomerular filtration rate, 25(OH)D: 25-hydroxy Vitamin D, CI: Confidence interval

Table 3: Correlations (Spearman *r*) between the percentage change in Vitamin D, urine albumin, plasma renin, and parathyroid hormone

Percentage change	Urine albumin	Renin	PTH
Vitamin D	−0.47**	−0.66**	−0.02
Urine albumin		0.62**	−0.08
Renin			−0.02

**Correlations are significant at 0.01 level. PTH: Parathyroid hormone

Table 4: Percentage change in urinary albumin excretion in relation to of Vitamin D and renin

Vitamin D	Renin		
	Low	Middle	High
Low	32.9 (18.3)	11.9 (7.4)	5.7 (9.1)
Middle	45.0 (8.1)	10.0 (11.6)	2.9 (8.6)
High	33.5 (6.9)	12.6 (11.6)	5.8 (8.6)

Values are given mean (SE). SE: Standard error

trial, respectively. The difference was not statistically significant. No adverse events, particularly hypercalcemia, was reported during the study period.

Discussion

Most striking outcome of this randomized double-blind placebo controlled clinical trial conducted among patients with DN was the significant reduction of urine microalbumin after monthly injection of Vitamin D for 6 months. In addition, there was a significant reduction of serum creatinine and improvement of GFR among patients who received Vitamin D. These results are supportive of the renoprotective effects of high dose Vitamin D in diabetic patients with nephropathy who are on optimum medical therapy.

As expected, serum Vitamin D level in the treatment group increased substantially and this in turn reduced PTH and renin levels among them.

Both groups were comparable at the baseline and they remained so at the end of the study. The trends in blood pressures and blood sugar were of same direction and magnitude and the observed changes in the renal indices cannot be explained by them.

However, the significant reduction of Vitamin D (4 nmol/L) and PTH (4 pg/L) among subjects in the control group

was an unexpected finding. This could partly be due to the measurement errors (coefficient of variations) associated with these serum estimations.

There is growing evidence that Vitamin D given in high doses can be renoprotective. This has been confirmed in three studies involving patients with CKD who were not on renal replacement therapy. In a randomized control trial involving paricalcitol, Agarwal *et al*. demonstrated a reduction of urine protein in patients who had stage 3 or 4 CKD with secondary hyperparathyroidism. In the above study, reduction of proteinuria was observed in 51% subjects in the treatment group and 25% in the placebo group ($P = 0.004$).[8] Odds for the reduction of proteinuria was 3.2 times greater in the treatment group compared to controls. This study recruited not only patients with DN but also patients with CKD related to other etiologies. Use of dipstick method to assess proteinuria is an obvious limitation of this study.

In a double-blind randomized trial, Alborzi *et al*. observed a 50% reduction of albuminuria with paricalcitol in 1 month. At the end of the study period, placebo group had 1.35 times greater albuminuria values compared to the baseline whereas paricalcitol 1 μg group and 2 μg group had 0.52 and 0.54 times the baseline UA level, respectively.[9] However, small sample size (only eight subjects in each group) and prevalent Vitamin D deficiency detected at the baseline are the major drawbacks of this study.

A trial in which calcitriol was given 0.5 μg twice a week for 12 weeks for 10 patients with biopsy-proven IgA nephropathy showed a significant reduction in UA to creatinine ratio (1.98–1.48 g/g, $P = 0.007$). However, this trial did not have a placebo arm and the sample size was greatly limited.[10]

The studies mentioned above recruited patients in whom the etiology of CKD was not limited to diabetes, and mostly, they were in advanced renal failure. Agarwal *et al*. recruited predialysis CKD patients who had secondary hyperparathyroidism,[8] and all of them were not patients with diabetes. Hence, these studies do not testify for the ability of Vitamin D high doses to reverse albuminuria in diabetics with established but early renal disease independent of the conventional treatment strategies.

In a prospective study, Mao *et al*. showed that calcitriol supplementation reduce protein in patients with type I diabetes. However, in this study, calcitriol treatment was given to patients who had Vitamin D deficiency ($n = 9$) and Vitamin D insufficiency ($n = 14$), keeping 7 Vitamin D normal patients as a control group. Therefore, it did not show protective therapeutic effect of Vitamin D on DN even those who have normal Vitamin D levels.[11]

In a randomized cross-sectional trial found that 12 weeks of paricalcitol at sufficient doses which can suppress elevated plasma PTH significantly reduce UA in patients with diabetes.[12] However, in our study, we showed that Vitamin D levels reduce UA independently of PTH and Ca which is proven in experimental studies before. Further the patients who were recruited had early DN than the above study.

In our sample, we recruited patients in the early stages of renal disease (eGFR >30 mL/min) and majority of them were not Vitamin D deficient. Therefore, we were able to increase their Vitamin D levels to above physiological limits to examine for the nonclassic benefits of Vitamin D. These benefits were independent of the conventional treatment offered for these patients according to the current treatment guidelines. The current treatment modalities in these patients included tight control of blood sugar, blood pressure, and coprescribing of either ACEI or ARBs. Our patients had satisfactory blood sugar and blood pressure control throughout the study period and all the subjects in the study were on either ACEI or ARBs. Both groups were comparable with regard to the degree of glycemic and BP control and the use of ACEI/ARBs. Although clinicians who were blinded to the type of treatment offered to their patients and were free to optimize the treatment goals of their patients, no major changes in the drug treatment were made to these patients during the trial period.

According to a recent review, the advantages of Vitamin D in relation to multiple health benefits starts when 25-hydroxy Vitamin D [25(OH)D] level is at 75 nmol/L.[13] Mean 25(OH) D levels of the treatment group in the present study reached 81.75 nmol/L and this may explain the observed treatment benefits. Further, the current study used a quantitative method of measuring albuminuria and this may have helped observe subtle changes in the outcome measures.

Role of Vitamin D on RAS has been extensively studied using mice. These experiments found that the renin levels were increased in the presence of Vitamin D deficiency or in Vitamin D receptor mutant mice.[6,14] Furthermore, animal studies proved that renal protection might be independent of PTH concentrations. Randomized clinical trials regarding Vitamin D therapy on renin are very limited. In a trial which recruited patients with chronic heart failure showed a significant reduction of renin concentration after giving 2000 IU of oral Vitamin D for 6 weeks duration ($P = 0.02$).[15] The mean reduction observed in the treatment group was 8 ng/mL. In contrast to other inhibitors of RAS such as ACEI or ARBS, Vitamin D does not increase the plasma renin concentration. In our study, we observed a significant reduction of plasma renin levels compared to control group.

In previous studies, an inverse relationship has been observed between blood pressure and Vitamin D concentrations. In a randomized controlled trial, Vitamin D and calcium administered group showed a significant reduction of SBP compared to calcium alone (13.1 mmHg, 5.7 mmHg).[16] Animal studies also proved this as Vitamin D receptor null mice showed 20 mmHg reduction in SBP compared to Vitamin D receptor positive mice.[6] However, in our study, no clinically significant blood pressure reduction was observed between the two groups. This could be explained as blood pressure of our patients was well under control throughout the study period. Hence, the observed reduction of albuminuria cannot be explained by blood pressure change.

Based on the results of the present study, we claim that 50,000 IU of monthly Vitamin D treatment reduces albuminuria and

improves renal functions of patients with adult onset diabetes complicated with albuminuria and mild renal impairment. Despite having normal Vitamin D levels at the baseline, it did not cause any toxicity or serious adverse effects. However, more clinical trials should be done to prove the findings and to recommend Vitamin D to all patients with diabetes.

The current study was conducted as a randomized double-blind control study and all aspects addressed in the CONSORT statement were observed to improve the validity of the results. Randomization was achieved by an accepted method and all parties who could influence the outcome of the study were blinded. Furthermore, there was a minimal dropout rate and all patients randomized and received the first dose of Vitamin D were included in the final analysis (intention-to-treat analysis). Since we administered parenteral Vitamin D, the problems related to compliance and bioavailability did not arise.

There were several limitations of this clinical trial. Short treatment period and follow-up period did not allow us to determine the long-term effect of Vitamin D on DN, especially on GFR. GFR was not obtained by the gold standard method since our main objective was to estimate the effect of Vitamin D on albuminuria. Disparity in blood pressure control in the two groups too may have influenced the outcome of this study. Further, not including any marker of inflammation or method of endothelial function that could have been an explanation for reduction of albuminuria was a limitation of this study.

Conclusions

This randomized double-blind placebo-controlled clinical trial conducted among patients with early DN showed a significant reduction of UA excretion with Vitamin D treatment. We also observed an improvement in the GFR which requires to be confirmed in further studies with longer duration of follow-up and using directly measured GFR in addition to estimated GFR.

Acknowledgments

We thank all the participants and health-care professionals at Teaching Hospital Galle, Sri Lanka. Heads and staff members of Departments of Medicine, Pharmacology, and Nuclear Medicine Unit of Faculty of Medicine, University of Ruhuna, Sri Lanka, are gratefully acknowledged.

Financial support and sponsorship

The study was funded by the University Grants Commission, Sri Lanka.

Conflicts of interest

There are no conflicts of interest.

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