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The Effect of Vitamin D on Cellular Pathways of Diabetic Nephropathy

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Abstract

Background: Diabetic nephropathy is one of the most important microvascular complications and a major cause of morbidity and mortality in diabetic patients. This study was designed to investigate the effect of vitamin D on the expression of three key genes involved in the development of diabetic nephropathy.

Methods: Twenty-four male Sprague–Dawley rats were randomly divided into three groups. The first group served as control and the other two groups received intraperitoneal injections of 45 mg/kg STZ to develop diabetes. The groups were treated for four weeks either with placebo or two vitamin D injections of 20,000 IU/kg. Serum glucose, insulin, and HbA1c levels, and AGE cellular receptor (*RAGE*), aldose reductase (*AR*) and glutamine: fructose-6-phosphate aminotransferase (*GFAT*) gene expression were assessed in kidney tissue at the end of the experiment.

Results: Vitamin D treatment resulted in a significant increase in insulin concentration, which could improve hyperglycaemia in diabetic rats. Serum HbA1c decreased slightly but insignificantly following the vitamin D injections. In addition, expression of *GFAT*, a key regulatory enzyme in the hexosamine pathway, was significantly reduced following vitamin D administration.

Conclusions: Vitamin D may reduce diabetic nephropathy not only by improving blood glucose and insulin levels, but also by modulating hexosamine pathways in kidney.

Keywords: Diabetes Mellitus, Hexosamine pathway, Nephropathy, Vitamin D.

Introduction

Diabetic nephropathy, one of the most important microvascular complications of diabetes, affects about one-third of diabetics and leads to end-stage renal disease (ESRD) and hemodialysis. Diabetic nephropathy is a clinical syndrome associated with symptoms that include albuminuria, progressive reduction of glomerular filtration rate (GFR), arterial hypertension, and increased cardiovascular mortality (1, 2). The pathogenesis of diabetic nephropathy is multifactorial, with various genetic and environmental factors playing roles. The most important cellular pathways known for diabetes complications include the polyol pathway, advanced glycation end products (AGE) pathway, hexosamine biosynthesis pathway (HBP), protein kinase C pathway, and oxidative stress (3, 4). Currently, the main treatment for diabetic nephropathy is to control hyperglycemia and

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hypertension through lifestyle modifications and medications (5). New research focuses on finding novel agents that can affect nephropathy's cellular pathways and prevent its incidence and progression. One compound that has been discussed in association with nephropathy is vitamin D. Some reports suggested an inverse association between the serum levels of this vitamin-hormone and the microvascular complications of diabetes, such as nephropathy (6, 7). Several experimental and clinical studies have examined the effects of vitamin D and its derivatives on nephropathy, some of which suggest that this vitamin can reduce the complications of diabetes and the mortality associated with chronic kidney diseases (CKDs) (8-10). Our meta-analysis of available data showed that although most cross-sectional studies indicate a relationship between vitamin D deficiency and nephropathy, supplementation with this vitamin does not necessarily improve renal function (11). Considering the controversial information, further studies are required to clarify the precise mechanisms of vitamin D in renal tissue. The present study aimed to evaluate the effect of vitamin D on expression of AGE cellular receptor (*RAGE*), aldose reductase (*AR*), and glutamine: fructose-6-phosphate aminotransferase (*GFAT*), whose products are the main regulators of AGE, polyol, and hexosamine pathways in diabetic rat kidneys.

Materials and methods

Initially, 30 Sprague-Dawley male rats aged 3-4 months with an average weight of 300 ± 40 g were purchased and kept in standard laboratory conditions at 20-25 °C and with 12-h light/dark cycle for 10 days prior to the start of the study. Throughout the study, animals had free access to drinking water and standard laboratory chow containing 0.95 to 1.0% calcium and 0.65 to 0.70% phosphorus. Animals were cared for in accordance with Guide on the Care and Use of Experimental Animals (12). Streptozotocin (STZ) was purchased from Sigma (St Louis, MO, USA) and dissolved in sterile sodium citrate buffer, pH 5-6, immediately before injection.

The rats were randomly divided into three groups and two rats were placed in each cage. At the beginning of the study, the rats in groups 2 and 3 received an intraperitoneal (i.p) injection of STZ (45 mg/kg, ~ 20 µl) and the rats in the control group (group 1) were injected with the same volume of citrate buffer as placebo. One week later, fasting plasma glucose (FPG) of all animals was measured by glucometer (Accu-check, Roche Diagnostic GmbH, Mannheim, Germany) and values greater than 250 mg/dl were considered as an indicator of diabetes. Finally, eight rats that met the inclusion criteria were assigned to each group. Animals in group 3, on the 1st and 14th days of diabetes development, received intramuscular (i.m) injections of 20,000 IU/kg of vitamin D. Sesame oil was used to dilute the vitamin D and injected into the other groups as a placebo. Food intake was checked and weighed per cage on a daily basis. The animals' weights were recorded weekly and on the final day. After four weeks, all the animals were anesthetized and euthanized with i.p injections of 50 mg/kg ketamine and 30 mg/kg xylazine. Blood and kidney samples were immediately collected and stored at -80 °C.

Fasting blood samples were collected between 8:00 and 10:00 a.m. by cardiac puncture and centrifuged immediately to isolate the serum. The sera and kidney samples were immediately frozen in liquid nitrogen and stored at -80 °C until biochemical assays. Serum glucose, HbA1c, and calcium levels were evaluated on a BT-1500 auto-analyzer (Biotecnica Instruments, Italy). Vitamin D and insulin concentrations were measured by commercial enzyme-linked immunosorbent assay (ELISA) kits (IBL international, Hamburg, Germany).

Kidney tissues were homogenized and mRNA was extracted using a Hybrid-R RNA isolation kit (GeneAll, Korea). The amount and purity of the RNA were evaluated on a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, Del., USA) and 260/280 and 260/230 ratios of ~ 2.0 were considered as acceptable values. Then, cDNA was synthesized and *RAGE*, *AR*, and *GFAT* expression assessed by quantitative real-time PCR using SYBR Premix Ex Taq II (Takara Bio Inc., Japan). Primers were designed by using OligoCalc, Primer BLAST, and GeneRunner software (Table 1). Relative mRNA expression was calculated with ΔCt method using glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as the housekeeping gene.

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Data were expressed as means ± SDs and analyzed using Statistical Package for the Social Sciences (SPSS, version 21.0; SPSS Inc., Chicago, Illinois, USA). The graphs were designed by GraphPad Prism (Version 7.03; GraphPad Software, La Jolla California, USA). The Kolmogorov-Smirnov test was used to assess the normality of the data. To remove the effects of confounding factors such as body weight and food intake, analysis of covariance (ANCOVA), followed by the Bonferroni post-hoc test, was used to compare statistical differences between the groups. Furthermore, the non-parametric data were transformed logarithmically for analysis. *P* values less than 0.05 were considered statistically significant.

Table 1. Primer sequences for real-time PCR

Gene	Sequence (5' → 3')	Length	Tm	GC%
RAGE	F: ACAGAAACCGGTGATGAAGGA	21	59.3	47
	R: TCTCCTCGAGTCTGGGTTG	19	58.1	57
AR	F: AGTAGCTGAGGAGTTTCTTCG	21	57.1	47
	R: CATAGGACTGGAGTTCTAAGCA	22	56.9	45
GFAT	F: TTGATTCTGATTGCTTGTGGC	21	57.4	43
	R: ACAGTAGCGAAGACCCATCA	20	58.4	50
GAPDH	F: CATTCTTCCACCTTTGATGCTG	22	57.9	46
	R: TGGTCCAGGGTTTCTTACTCC	21	58.9	52

RAGE, Receptor for advanced glycation end products; AR, aldose reductase; *GFAT*, glutamine: fructose-6-phosphate aminotransferase; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; F, forward; R, reverse.

Results

At the beginning and end of the study, body weight and food intake were compared between groups. No significant differences were observed between the three groups at the beginning; however, the two diabetic groups had significantly lower body weights and less food intake than the control group at the end ($p < 0.001$). Therefore, the ANCOVA test was used to control the possible confounding effect of these factors.

Following four weeks of treatment, the mean FPG level was significantly greater in the diabetic rats (groups 2 and 3) than in the control rats (group 1) ($p < 0.001$). Group 3 had significantly greater cholecalciferol and less hyperglycaemia and hypoinsulinemia than group 2 ($p = 0.005$ and $p = 0.01$, respectively). HbA1c was slightly less in group 3 than in group 2; however, this difference was not significant (Table 2).

Table 2. Biochemical factors in different experimental groups

Groups	FPG (mg/dL)	Insulin (mIU/L)	HbA1c (%)	Vitamin D (ng/mL)
Control	85.87±12.63$^{\#}$	3.37±0.83$^{\#}$	4.65±0.50$^{\#}$	20.93±2.49
Diabetic	479.37±27.90*	2.15±0.79*	8.75±0.48*	21.33±2.44
Diabetic + vitamin D	428.87±37.74$^{*\#}$	3.31±0.65$^{\#}$	8.30±0.55*	35.42±3.96$^{*\#}$

Data are presented as the mean ± SD (n = 8 for all groups). FPG, fasting plasma glucose. Statistical differences were determined using ANCOVA followed by a Bonferroni post-hoc test; *, $p < 0.05$ compared with the control group; #, $p < 0.05$ compared with the diabetic group

Expression of *GFAT*, the gene encoding the key enzyme of the hexosamine pathway, was significantly less in group 3 than in groups 1 or 2 ($p < 0.05$ for both), while *AR* and *RAGE* expression were not significantly different between the groups ($p > 0.05$) (Fig. 1).

Discussion

Vitamin D is recognized as a fat-soluble vitamin as well as a secosteroid hormone. This vitamin is synthesized from cholesterol in skin cells by exposure to ultraviolet radiation (UVB), and then activated by hydroxylase enzymes in the liver and kidney. In addition, it can be obtained in the

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diet from egg yolks, fatty fish, and enriched dairy products (13, 14). However, the prevalence of vitamin D deficiency is high worldwide with a greater risk in the elderly, obese, and black people (15, 16). Previous studies have shown that vitamin D deficiency is associated with type 1 and type 2 diabetes (17, 18). What increases the importance of vitamin D is the presence of its cell receptor in various body tissues, which can affect gene expression, protein function, and various cellular pathways (19). For this reason, we evaluated the effect of vitamin D on the mechanisms of the renal complications of diabetes.

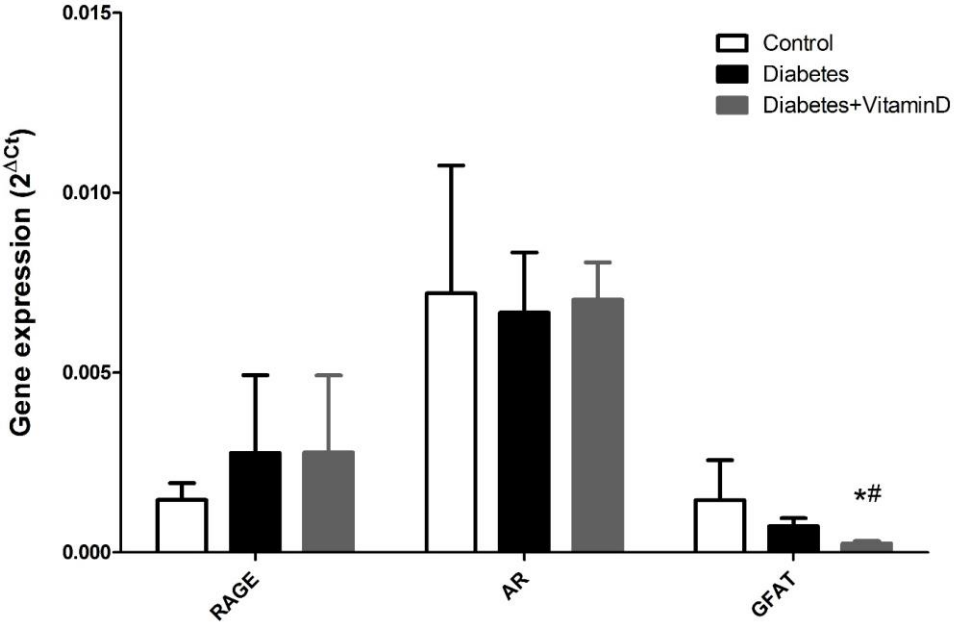

Fig. 1. Gene expression of receptor for advanced glycation end products (*RAGE*), aldose reductase (*AR*) and glutamine: fructose-6-phosphate aminotransferase (*GFAT*) in different experimental groups. *, $p < 0.05$ compared with the control group; #, $p < 0.05$ compared with the diabetic group.

We showed that elevated vitamin D resulted in a significant reduction in fasting blood glucose and a significant increase in insulin levels. Previous studies have reported that the vitamin D receptor is present in pancreatic beta cells, and lack of vitamin D is associated with impaired insulin synthesis and secretion (20). It has also been reported that vitamin D may improve glycemic control by increasing glucose transporter type 4 (*GLUT4*) expression and improving glucagon-like peptide-1 (GLP-1) function (21, 22). Vitamin D deficiency also increases the level of parathyroid hormone, which contributes to increased insulin resistance and increases the risk of hyperglycemia and hypertension (23). Also, in this study, HbA1c levels were lower in group 3 than in group 2, although this difference was not statistically significant. This might be due to the short duration of the intervention, as HbA1c changes usually occur in the long term.

It should be noted that in this study, body weight and food intake were significantly less in the diabetic rats than in healthy controls. For this reason, the possible confounding effect of these variables was considered and eliminated in all statistical analyses.

The expression level of *RAGE* in renal tissues of diabetic rats did not change after receiving vitamin D. Advanced glycation end products are the products of non-enzymatic glycation of proteins and lipids and their accumulation has unfavorable effects on vascular and tissue functions. A large body of evidence suggests that AGE production increases in diabetes. These compounds, by binding to their cellular receptors, activate inflammatory pathways, producing chemokines, cytokines, and adhesion molecules and their resulting microvascular complications (24). To date, few studies have addressed the effect of vitamin D on *RAGE* expression, with conflicting results in different

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conditions and tissues. Therefore, further studies are required in this field.

Also, vitamin D did not affect the expression of *AR*, which encodes the main enzyme of the polyol pathway. Products of this pathway include alcohols such as sorbitol and galactitol, and their accumulation in tissues is known to be responsible for some complications of diabetes. Based on previous studies, increasing *AR* expression or the activity of this enzyme in diabetes leads to retinopathy, neuropathy, nephropathy, and angiopathy (4). Deleting this gene or inhibiting the enzyme in mice helps to prevent the microvascular complications of diabetes (25). Although *AR* expression changes were not observed in the present study, during 4-week period.

The expression of *GFAT*, whose product is the main regulator of the hexosamine pathway, was significantly less in group 3 than in group 1 or 2. The enzymatic glycosylation of proteins in the hexosamine pathway impairs the function of intra- and extra-cellular proteins, and based on existing studies, the use of inhibitors of this enzyme can reduce cell apoptosis and delay the complications of diabetes mellitus (26). Therefore, it seems that reducing *GFAT* expression in renal tissues can play a key role in preventing the development of diabetic nephropathy.

Finally, our study indicates that increased serum vitamin D not only reduces blood glucose levels and increases insulin secretion, but also may help to prevent diabetic nephropathy by reducing the production of GFAT as the key enzyme of the hexosamine pathway in renal tissue.

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