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Journal of the Chinese Medical Association 81 (2018) 691−698

www.jcma-online.com

Original Article

Protective effect of calcitriol on podocytes in spontaneously hypertensive rat

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Received July 12, 2017; accepted January 29, 2018

Abstract

Background: Hypertension is a major global public health issue. Uncontrolled hypertension leads to organ damage, especially renal damage. Calcitriol is used to treat osteoporosis, promote bone formation, and increase bone mass. Previous studies have demonstrated that 1,25(OH)2D3, in addition to its classic role, also has multiple immune regulation and renoprotective functions and inhibits the activity of the renin-angiotensin-aldosterone system (RASS). The aim of the current study was to investigate the renoprotective effects of calcitriol in a spontaneously hypertensive rat (SHR) model.
Methods: A total of 18 SHRs and 8 age-matched normal Wistar rats were enrolled. SHRs were randomly divided into a hypertensive nephropathy group (H), a hypertensive nephropathy treated with calcitriol group (D) and a control group (NS). The rats were sacrificed after 16 weeks of treatment. The blood pressure (BP) of rats were measured one time every 4 weeks. The levels of serum albumin, serum creatinine, blood calcium, serum Vitamin D and 24-h urinary protein were measured after 16 weeks treatment. The protein level of WT1, nephrin and vitamin D receptor (VDR) was examined by Western blotting and immunohistochemical staining.
Results: There were no notable changes in blood pressure or serum creatinine in group H and D compared with group NS. The albumin, calcium and vitamin D serum levels in group H were significantly decreased compared with group NS and significantly increased in group D compared with group H. The level of 24-h urine protein significantly increased in group H compared with group NS and significantly decreased in group D compared with group H. The expression of VDR, WT1 and nephrin in the kidney were all significantly decreased in group H compared with group NS and significantly increased in group D compared with group H.
Conclusion: The present results indicated that there was injury of podocytes in hypertensive nephropathy, which can be ameliorated by calcitriol in SHR, but there was no significant anti-hypertensive effect. Vitamin D/VDR decreased proteinuria perhaps by increasing expression of nephrin and WT1 protein in podocyte of SHRs.

Keywords: Calcitriol; Hypertension; Hypertensive nephropathy; Podocyte

1. Introduction

Hypertension is a major global public health issue. Uncontrolled hypertension leads to organ damage, especially renal damage, that can finally lead to end-stage renal disease (ESRD).[1] Proteinuria is one of the sensitive indicators of early renal damage in patients with hypertension. Proteinuria is regarded as an important factor influencing the prognosis of hypertension; it is not only one of the characteristics of hypertensive renal impairment but is also an important risk factor for the progression of ESRD.[2] The current clinical treatment for hypertensive nephropathy mainly consists of the application of antihypertensive drugs, mainly angiotensin converting

Conflicts of interest: The authors declare that they have no conflicts of interest related to the subject matter or materials discussed in this article.

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https://doi.org/10.1016/j.jcma.2018.01.010

enzyme inhibitors and angiotensin receptor blockers. The main mechanism of antihypertensive drugs may be related to decreasing oxidative stress, correction of chronic hypoxia, and inhibition of advanced glycation end-product formation and of abnormal iron deposition.[3−6]

The incidence of chronic kidney disease (CKD) is increasing year by year and has become one of the major public health issues of global concern, suggesting that current treatments are not effective.[7] At the same time, emerging clinical and animal studies have demonstrated that active vitamin D and its analogues can attenuate glomerular damage and tubular interstitial fibrosis.[8] Treatment with 1,25-dihydroxy-vitamin D (1,25(OH)2D3 or calcitriol) or activated vitamin D analogues reduced albuminuria and prevented podocyte injury in a subtotal nephrectomy model,[9,10] puromycin-induced nephropathy,[11,12] adriamycin-induced nephropathy,[11,13] diabetic nephropathy,[3,14,15] immunoglobulin A nephropathy,[16,17] and in CKD patients.[18,19] However, the mechanism of the antiproteinuric effect of vitamin D remains unknown.

Previous studies have demonstrated that 1,25(OH)2D3, in addition to its classic role of regulating calcium and phosphorus metabolism to maintain normal function of the skeletal system, also has multiple immune regulation and renoprotective functions, inhibits the activity of the renin-angiotensin-aldosterone system (RASS), and helps regulate insulin secretion and the function of the nervous system.[19,20] The pleiotropic biological activities of 1,25(OH)2D3 are mediated by the vitamin D receptor (VDR), a member of the nuclear hormone receptor superfamily, including the membrane vitamin D receptor (mVDR) and nuclear receptor (nVDR). mVDR is mainly involved in the maintenance of calcium and phosphorus balance, and nVDR regulates the synthesis of protein by affecting gene expression. We mainly studied the expression of VDR in the glomerular podocytes.[20] VDR is expressed in numerous types of tissues and cells in the body, including cells not involved in calcium and phosphate metabolism. This is the basis for the broad biological function of VDR beyond the regulation of calcium and phosphorus.

The aforementioned research suggests that 1,25(OH)2D3 or activated vitamin D analogues have renoprotective functions. However, it is unclear if vitamin D modulates blood pressure (BP). Observational studies have found associations between vitamin D, increased BP and the risk of developing hypertension.[21,22] In contrast, recent data from randomized trials are mixed.[23] The present study aimed to investigate the protective effect of active vitamin D (calcitriol) on hypertensive nephropathy and to identify its protective mechanism. These data may be useful in developing a novel approach for the treatment of hypertensive nephropathy.

2. Methods

2.1. Reagents

Calcitriol was purchased from Sigma−Aldrich (Merck KGaA, Darmstadt, Germany). Primary antibodies used in this study were rabbit anti-nephrin (Abcam Ltd. Shanghai, China, ab136894), rabbit anti-VDR (Santa Cruz Biotechnology, Inc. sc1008), mouse anti-WT1 (Santa Cruz Biotechnology, Inc. sc393498), rabbit anti-GAPDH (Abcam Ltd. Hong Kong, China, ab9485). Anti-mouse secondary antibodies (115-035-003) and anti-rabbit secondary antibodies (111-035-003) were purchased from Jackson ImmunoResearch Laboratories Inc (West Grove, PA, USA).

2.2. Animals

A total of 18 adult male (purchased from Charles River) 10-week-old SHRs (SHR/NCrl, 121, RT1k), weighing 200 ± 10 g, and 8 adult male, 10-week-old Wistar rats (Crl:WI,102), weighing 200 ± 10 g, were enrolled. Rats were housed separately in cages with a 12-h dark/light cycle and 40%−70% relative humidity, at 18°C-22 °C temperature. Food and water were available ad libitum. All experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

2.3. Animal treatment

Animals were randomly divided into 3 groups: a hypertensive nephropathy group (group H), a hypertensive nephropathy treated with calcitriol group (group D; intragastric administration of calcitriol 3 ng/100 g body weight per day) and a normal control group (group NS). The rats were sacrificed after 16 weeks of treatment. During the treatment period, rat BP was measured by tail artery manometry once every 4 weeks. Before they were sacrificed, the rats were weighed, and blood samples were taken. From these samples, the serum was separated by centrifugation (12,000 × g at 4 °C for 20 min) and stored at −80 °C before analysis. After the rats were sacrificed, both kidneys were immediately excised and weighed, and then each kidney was cut in half along the coronal plane. Two sections of each excised kidney were stored at −80 °C for western blot analysis. The remaining sections were fixed in 4% buffered paraformaldehyde for 72 h at 4 °C, then transferred into 0.5% buffered paraformaldehyde and embedded in paraffin for histopathological observation and immunohistochemical analysis.

2.4. Assessments of renal function and serum changes

The levels of serum albumin, creatinine and calcium were measured in a Cobas® 8000 modular analyser (Roche Diagnostics, Basel, Switzerland). The level of serum Vitamin D was measured in a Cobas® 6000 modular analyser (Roche Diagnostics).

2.5. Histopathological observation

The pathological changes in the kidney were examined by periodic acid-Schiff (PAS) staining. One-fourth of each kidney was immersion-fixed in 0.5% buffered paraformaldehyde and embedded in paraffin to be further examined under a light

microscope. Two 4-μm sections were taken from each animal, at an interval of 100 μm, and stained with PAS reagent.

2.6. Immunohistochemical study

For immunohistochemical analysis, tissue slices were microwaved for 10−15 min in EDTA (ST006, Beyotime, pH 9.0) to allow antigen retrieval. The tissue slices were cooled at room temperature or in iced water, then washed with PBS 3 times. The tissue slices were immersed in 0.1% Triton X-100 for 15 min. To block endogenous peroxidase activity, the tissue slices were incubated at room temperature (25−28 °C) with 3% hydrogen peroxide for 10 min in the dark. The tissue slices were then incubated with 10% goat serum for 60 min at 37 °C, followed by primary antibodies at 4 °C overnight (anti-VDR 1:100, anti-nephrin 1:150, anti-WT1 1:100), while the sections serving as negative controls were incubated with PBS instead of the primary antibody. All of the sections were incubated with secondary antibodies (M 1:100 R 1:100) for 60 min at 37 °C and stained with 3,3′-diaminobenzidine and haematoxylin. Briefly, 10 high-power fields (magnification, × 400 per section) were randomly selected, and two sections per kidney were examined in each experiment. Specimens were scored according to the intensity of the dye colour and the percentage of positively stained areas. Brown areas were considered to be positive. The intensity of the dye colour was graded as 0 (no colour); 1 (light yellow); 2 (light brown) and 3 (brown), and the percentage of positively stained areas was graded as 0 (<5%); 1 (5−25%); 2 (25−50%); 3 (51−75%) and 4 (>75%). The two grades were added to give a final score of expression for each tested protein. Ten high-power fields (× 400) per section were randomly selected and examined in each experiment, taking the average [24]

2.7. Protein sample preparation

The tissue and cell samples were homogenized in TRIzol (Invitrogen, Thermo Fisher Scientific, Inc. Waltham, MA, USA). Following the addition of chloroform (0.1 ml/1 ml TRIzol) (Guangcheng Company, China), the homogenates were centrifuged at 12,000 × *g* for 15 min at 4 °C. The supernatant was discarded, and isopropanol (0.5 ml/1 ml TRIzol) was added to the lower phase, followed by centrifugation at 12,000 × *g* for 15 min at 4 °C. The pellets were washed with 0.3 M guanidine hydrochloride 3 times, washed with ethanol once, then dissolved in 1% SDS at 50 °C for 30 min. Protein concentrations were determined using the Pierce Bicinchoninic Acid Protein Assay kit (Thermo Fisher Scientific, Inc.).

2.8. Western blot analysis

Proteins (50 μg) were subjected to 12% SDS-polyacrylamide gel electrophoresis and then transferred to cellulose acetate membranes. The membranes were blocked with 5% skim milk at room temperature for 1 h, then incubated with primary antibodies (anti-VDR 1:500, anti-nephrin 1:1,000, anti-WT1 1:250, or anti-GAPDH 1:3000) at 4 °C overnight. After 1 h incubation with secondary antibodies (R 1:10000 M 1:5000) at room temperature, the membranes were immersed in enhanced chemiluminescence reagent (Thermo Fisher) and exposed to an X-ray film. Quantification of the luminosity of each protein band was performed using Adobe Photoshop 7.0 software (Adobe Systems, Inc. San Jose, CA, USA). VDR, nephrin and WT1 relative quantities were expressed as a ratio of luminosity relative to the NS group.

2.9. Statistical analysis

Data are presented as the mean ± standard deviation. Differences between groups were evaluated using Student's t-test by SPSS; $p < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. Effect of calcitriol on BP and renal function of SHRs

The diagnostic criteria of hypertension are systolic BP ≥ 150 mmHg and/or diastolic BP ≥ 100 mmHg in rats [25] In the present study, BP was measured by tail artery manometry once every 4 weeks. As shown in Fig. 1, the BP of SHRs (groups H and D) was higher compared with group NS throughout the treatment period. The BP of each group did not change notably during the treatment period. These results suggested that calcitriol had no effect on the BP of SHRs. Furthermore, there was no significant difference in the serum creatinine levels in group H or group D, compared with group NS (Table I).

3.2. Calcitriol protects against hypertensive nephropathy

As shown in Table I, the levels of serum albumin, calcium and vitamin D in group H were significantly decreased compared with group NS ($p < 0.05$). Treatment with calcitriol (group D) significantly increased the levels of serum albumin, calcium and vitamin D compared with group H ($p < 0.05$). Furthermore, the level of 24-h urine protein significantly increased in group H compared with group NS ($p < 0.05$) and

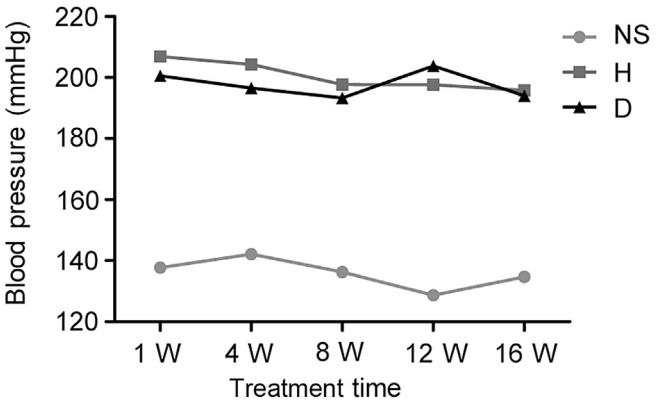

Fig. 1. The BP of all the rats. The control group (NS), the hypertensive nephropathy group (H), hypertensive nephropathy treated with calcitriol group (D). NS(◆), H(■), D(▲) the BP has no obvious change in each group.

Table 1
Animal groups and related biochemical parameters.

Group	No.	VitaminD (ng/ml)	Scr (μmol/l)	Ca2+ (mmol/l)	Alb (g/l)	24 h-urinar proteins(g)	Body weight(g) the initial	Body weight(g) at sacrifice
NS	8	24.87 ± 1.44	44.75 ± 6.56	2.62 ± 0.062	41.19 ± 1.1	9.05 ± 2.35	241.8 ± 10.2	330.65 ± 11.9
H	9	11.03 ± 1.31[b]	44.14 ± 5.61	2.41 ± 0.086[b]	36.47 ± 1.7b	24.71 ± 1.99[b]	247.4 ± 2.79	332.36 ± 7.64
D	9	20.68 ± 1.45[a]	45.63 ± 6.39	2.62 ± 0.076[a]	41.18 ± 1.5[a]	18.39 ± 2.48[a]	240.2 ± 8.07	320.34 ± 10.1

Table 1. Animal groups and related biochemical parameters: No. number of mice in the group; Vitamin D = serum Vitamin D level, Scr = serum creatinine level; Ca^{2+} = serum calcium; Alb = serum albumin; NS = normal control group, H = hypertensive nephropathy; D = hypertensive nephropathy treated with calcitriol group; the Scr has no statistically significant difference between three groups; the body weight has no statistically significant difference between three groups neither the initial nor at sacrifice; the levels of Vitamin D,Ca^{2+} and Alb significant increase in D group compared with H group; the 24 h-urinary proteins' level significant decrease in D group compared with H group ($p < 0.05$).
Data are presented as means ± SD.
[a] statistically significant difference compared to the H group ($p < 0.05$).
[b] statistically significant difference compared to the NS group ($p < 0.05$).

significantly decreased following treatment with calcitriol (group D) compared with group H ($p < 0.05$).

Histopathological observation identified capsular synechiae, focal segmental glomerulosclerosis and proliferation of the mesangial in group H, while in group D, the kidneys maintained a normal structure (Fig. 2). Compared to group H, glomerulus damage was reduced in group D (Fig. 2).

3.3. Calcitriol protects against podocyte damage in the kidneys of SHRs

To assess whether calcitriol protects against podocyte injury induced by hypertension, the present study examined the expression levels of VDR, WT1 and nephrin using western blot analysis. The levels of VDR, WT1 and nephrin were significantly reduced in group H compared with the control ($p < 0.05$; Figs. 3−5), and significantly increased after treatment with calcitriol in group D compared with group H ($p < 0.05$).

4. Discussion

The kidney serves a key function in regulating BP and is also one of the target organs damaged by hypertension. Previous studies have shown that hypertension is an independent risk factor for ESRD. In the early stages of hypertension, microalbuminuria is a marker of renal damage. The mechanism of microalbuminuria is related to changes in renal haemodynamics caused by long-term hypertension, inflammation, oxidative stress and podocyte damage.[10]

As an active form of vitamin D, $1,25(OH)_2D_3$ exhibits a number of biological activities mediated by VDR. VDR is not only present in classic target tissues, such as the small intestine, bone, kidney and parathyroid, but it is also present in the retina, pituitary gland, pancreas, ovary, spleen, skin and some nerve tissue.[19] Previous studies have shown that $1,25(OH)_2D_3$ and its analogues have multiple functions in addition to their classic roles of regulating calcium and phosphorus metabolism to maintain the normal function of the skeletal system. These include immune regulation, RAAS inhibition, insulin secretion regulation, and regulation of muscle and nervous system function.[26,27] There is some evidence that activated vitamin D could be used in the treatment of cardiovascular and kidney diseases.[18,19] The mechanism of $1,25(OH)_2D_3$ may be mediated via inhibiting the activity of RAAS and enhancing the function of endothelial cells in animals and humans.[27,28]

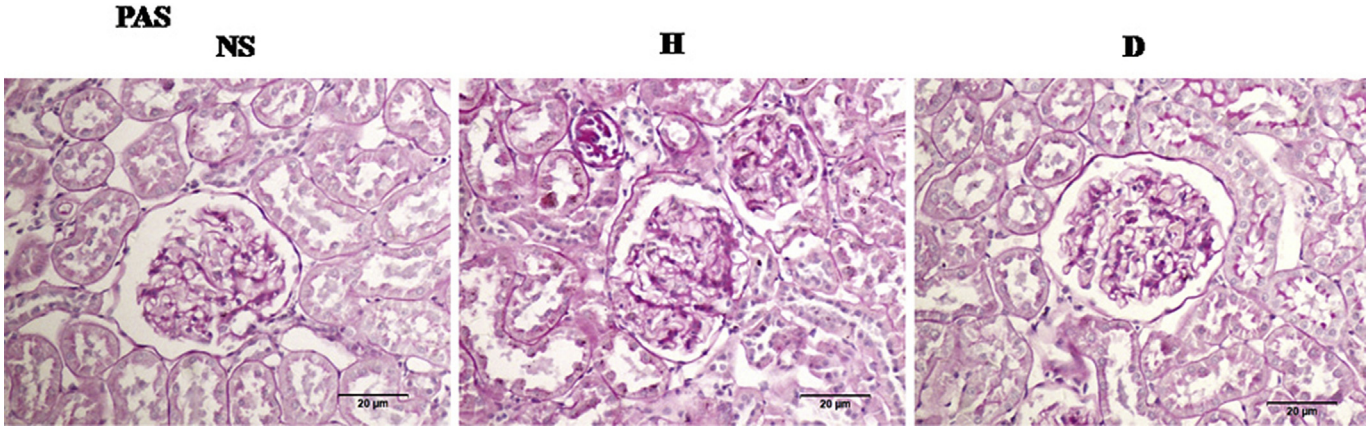

Fig. 2. Microscopic examination of sections stained with.The structure of the kidney was normal in the NS group. The capsular synechia, focal segmental glomerulo sclerosis and the proliferation of the mesangial were found in the H group. Compared to the H group, glomerulus damage was greatly improved in the Group D (×400 magnification).

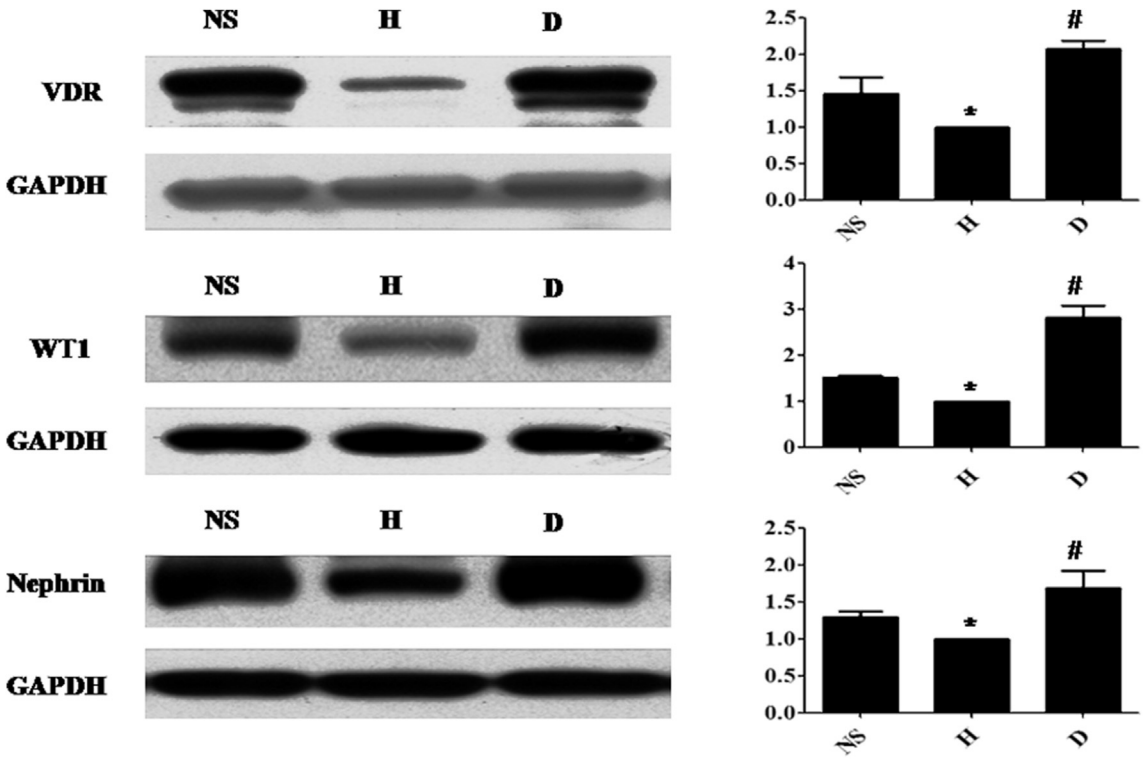

Fig. 3. Western blot assay of expressions of WT1, Nephrin and VDR in different groups.Animals were divided into three groups: the control group (NS), the hypertensive nephropathy group (H), hypertensive nephropathy treated with calcitriol group (D).compared with NS group, the level of VDR, WT1 and nephrin are significantly reduced in the H group, their levels were significantly increased after treated with calcitriol in the Group D. Data are expressed as mean ± SD levels relative to GAPDH.* statistically significant decreased compared to the NS group ($p < 0.05$); #:statistically significant increased compared to the H group ($p < 0.05$).

Previous research reported that VDR-activating agonists blocked intrarenal renin and angiotensin II accumulation induced by hyperglycaemia.[3] Another study demonstrated that VDR-activating agonists attenuated proteinuria, podocyte injury, infiltration by macrophages, activation of markers of oxidative stress, inflammation and fibrosis.[29] The same effects were observed in the treatment of diabetic mice by losartan or paricalcitol alone, which can moderately alleviate kidney injury, the activity of RAAS and proteinuria. In the same study, reduced albuminuria, restored glomerular filtration barrier structure and markedly reduced glomerulosclerosis were observed following combined losartan and paricalcitol treatment.[30,31,32] These effects were produced via increased expression of fibronectin, transforming growth factor β and p-ERK1/2, and reversal of reduced levels of the slit diaphragm protein, nephrin. These data demonstrated that inhibition of RAAS with a combination of vitamin D analogues and RAAS inhibitors effectively prevents renal injury in diabetic nephropathy.[30,31,32]

The present study indicated that the level of proteinuria significantly increased in SHRs compared with control rats but decreased in SHRs treated with calcitriol compared with untreated SHRs. There was no visible decline in BP of SHRs after treatment with calcitriol in this study, suggesting that calcitriol did not have a significant antihypertensive effect. Previous data have suggested that $1,25(OH)_2D_3$ and its analogues have the potential to affect BP by several biologic pathways.[33,34,35] However, the results of randomized trials have suggested that $1,25(OH)_2D_3$ and its analogues cannot decrease BP,[23,36,37] and BP can only be evaluated as a secondary outcome.[38] This would suggest that the renoprotective effects of calcitriol are independent of an antihypertensive effect.

The common characteristics of kidney disease are podocyte damage and dysfunction, which causes proteinuria.[39] Podocytes are key components of the glomerular filtration barrier, which prevents large molecules (>4 kDa) from leaking out of the glomerulus. Proteins expressed in podocytes are key components of the glomerular basement membrane, and podocyte foot processes are components of the major size- and charge-selective barrier.[40] Therefore, podocytes serve a key function in the regulation of glomerular filtration.

Previous reports have suggested that podocyte impairment serves a critical function in the aetiology of diabetic nephropathy, with or without hypertension.[41–43] However, a number of studies have reported that podocyte dysfunction is relevant to proteinuria in hypertensive nephropathy. Kretzler et al. reported ultrastructural alteration of podocytes observed by electron microscopy in deoxycorticosterone-hypertensive rats, and podocyte damage, rather than mesangial expansion, triggered the subsequent glomerular sclerosis.[45] It was found that podocyte injury appeared at 6 weeks, and the renal tissue manifested advanced glomerular lesions in rats.[45] Nagase et al. demonstrated podocyte damage in hypertensive Dahl salt-sensitive rats and reported that mitigation of podocyte damage by eplerenone prevented the development of proteinuria

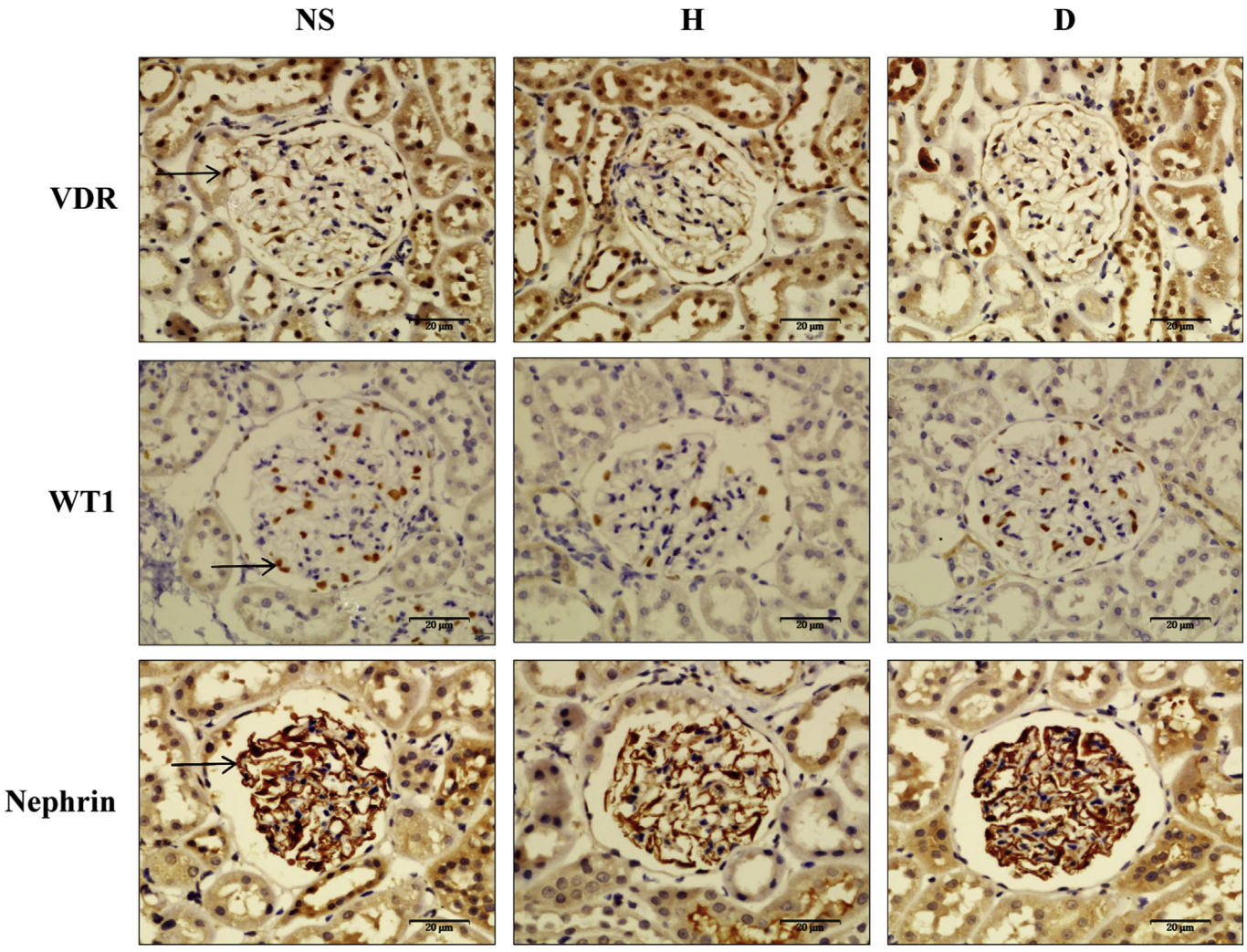

Fig. 4. Immunohistochemical staining of WT1(→), Nephrin (→)and VDR(→). Animals were divided into four groups: the control group (NS), the hypertensive nephropathy group (H), the calcitriol group (D) The brown granules represent positively-stained cells. Magnification, ×400.

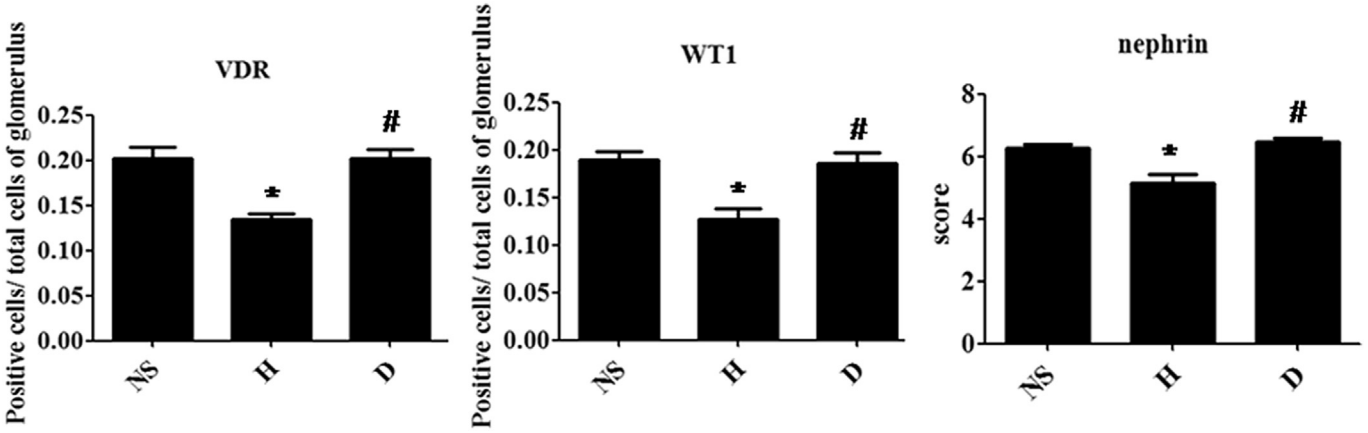

Fig. 5. Semi-quantitative analysis of immunohistochemical staining of WT1, Nephrin and VDR.The expression of WT1, Nephrin and VDR was significantly decreasedin the H group compared to the NS group and significantly increased in the Group D compared to the H group.*: statistically significant decreased compared to the NS group ($p < 0.05$). #:statistically significant increased compared to the H group ($p < 0.05$).

and glomerulosclerosis.[2] These studies suggest that podocyte damage plays a key role in the occurrence and development of hypertensive nephropathy.

The present study demonstrated that expression of nephrin and WT1 protein in podocytes was significantly decreased in hypertensive nephropathy, leading to dysfunction and a reduction in the number of podocytes, consistent with previous research.[2] Expression of nephrin and WT1 significantly increased following treatment with calcitriol compared with the untreated group (Figs. 3−5). Therefore, calcitriol may have a renoprotective effect in hypertensive nephropathy via protection of podocyte function.

Previous studies have found that $1,25(OH)_2D_3$ and activated vitamin D analogues can reduce proteinuria, increase the expression of nephrin and podocin proteins in podocytes, reduce apoptosis of podocytes, and reduce the expression of proliferating cell nuclear antigen, p27 and desmin in adriamycin nephropathy,[41] drug-induced renal injury,[42] diabetic nephropathy[43] and a subtotal nephrectomy rat model.[43,44]

A previous study reported that podocytes express the $1,25(OH)_2D_3$ receptor (VDR) and VDR-null diabetic mice developed more severe albuminuria and renal damage than wild-type mice.[46] Furthermore, $1,25(OH)_2D_3$ and activated vitamin D analogues were reported to block the production of fibronectin in mesangial cells and increase expression of nephrin in podocytes, both stimulated by high glucose.[46] In the present study, the results indicated that expression of VDR decreased significantly in hypertensive nephropathy, but the reduction was reversed by calcitriol treatment. This suggests that calcitriol may have renal protective effects in SHRs by increasing VDR expression in podocytes.

In conclusion, the present results indicated that podocyte injury was present in hypertensive nephropathy and that this could be ameliorated by calcitriol treatment in SHRs. However, calcitriol treatment did not result in a significant antihypertensive effect. Vitamin D/VDR decreased proteinuria, perhaps by increasing expression of nephrin and WT1 protein in the podocytes of SHRs. These results suggest that podocytes may be a key therapeutic target in vitamin D therapy for hypertensive nephropathy. The findings of the present study were limited by the small sample size and the lack of an antihypertensive drug treatment group. Further research is required to investigate the specific molecular mechanisms for the protection of $1,25(OH)_2D_3$ and whether the therapeutic effect of calcitriol depends on the dose administered. In addition, we plan to perform some cell experiments to study this in other ways. We did corresponding research on the protective mechanism of $1,25(OH)_2D_3$, and the results of the present study indicated that the apoptosis in group D was less than that of group H. A previous study reported that calcitriol caused a marked activation of the ERK1/2 pathway with subsequent attenuation of neuronal apoptosis.[47] The renoprotective effects of vitamin D analogues were significantly associated with upregulation of pro-autophagy gene expression and downregulation of the expression of both pro-apoptotic and G1-cell cycle arrest genes and attenuation of serum IL-6, renal TLR-4 and IFN-g gene expression.[48] The present study provided novel clues for understanding the effects of $1,25(OH)_2D_3$ on podocytes in the spontaneously hypertensive rat. We plan to do further research on the downstream mechanism.

In conclusion the present results indicated that there was injury of podocytes in hypertensive nephropathy, which can be ameliorated by calcitriol in SHR, but there was no significant anti-hypertensive effect. Vitamin D/VDR decreased proteinuria perhaps by increasing expression of nephrin and WT1 protein in podocyte of spontaneously hypertensive rats. These results suggest that podocytes might be a key therapeutic target in vitamin D therapy of hypertensive nephropathy.

Acknowledgments

Special thanks to all authors participate in the present study. Thanks to the support from Ministry of Education Key Laboratory of Experimental Teratology and Institute of Molecular Medicine and Genetics, Shandong University School of Medicine. This study was supported by the Natural Science Foundation of Shandong Province (No. ZR2013HM106), Natural Science Foundation of China (No. 81200529), the Natural Science Foundation of Shandong Province (No. ZR2012HQ001), Traditional Chinese Medicine Science and Technology Development Program of Shandong Province (No. 2011-198), Science and Technology Development Program of Shandong Province (No. 2014GSF121005), Science and Technology Development Program of Jinan (No. 201401241), The Outstanding Young Scientist Research Award Fund Project of Shandong Province, No.BS2013YY042.

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