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Original Article

Newly Developed Rat Model of Chronic Kidney Disease−Mineral Bone Disorder

Kentaro Watanabe[1], Hideki Fujii[1], Shunsuke Goto[1], Kentaro Nakai[1], Keiji Kono[1], Shuhei Watanabe[1], Masami Shinohara[2] and Shinichi Nishi[1]

[1]Division of Nephrology and Kidney Center, Kobe University Graduate School of Medicine, Kobe, Japan
[2]Plannning and Development Section, CLEA Japan, Inc., Tokyo, Japan

***Aim*: Chronic kidney disease−mineral bone disorder (CKD−MBD) is associated with all-cause and cardiovascular morbidity and mortality in patients with CKD. Thus, elucidating its pathophysiological mechanisms is essential for improving the prognosis. We evaluated characteristics of CKD−MBD in a newly developed CKD rat model.**

***Methods*: We used male Sprague−Dawley (SD) rats and spontaneously diabetic Torii (SDT) rats, which are used as models for nonobese type 2 diabetes. CKD was induced by 5/6 nephrectomy (Nx). At 10 weeks, the rats were classified into six groups and administered with a vehicle or a low- or high-dose paricalcitol thrice a week. At 20 weeks, the rats were sacrificed; blood and urinary biochemical analyses and histological analysis of the aorta were performed.**

***Results*: At 20 weeks, hemoglobin A1c (HbA1c) levels, blood pressure, and renal function were not significantly different among the six groups. Serum calcium and phosphate levels tended to be higher in SDT-Nx rats than in SD-Nx rats. The urinary excretion of calcium and phosphate was significantly greater in SDT-Nx rats than in SD-Nx rats. After administering paricalcitol, serum parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF23) levels were significantly higher in SDT-Nx rats than in SD-Nx rats. The degree of aortic calcification was significantly more severe and the aortic calcium content was significantly greater in SDT-Nx rats than in SD-Nx rats.**

***Conclusions*: We suggest that our new CKD rat model using SDT rats represents a useful CKD−MBD model, and this model was greatly influenced by paricalcitol administration. Further studies are needed to clarify the detailed mechanisms underlying this model.**

Key words: Chronic kidney disease−mineral bone disorder, Vascular calcification, Vitamin D

Introduction

Chronic kidney disease−mineral bone disorder (CKD−MBD) is a systemic disorder that includes not only mineral bone disorders but also vascular calcification[1)]. Many previous studies reported that hyperphosphatemia leads to the progression of vascular calcification and that both abnormalities are significantly associated with increased morbidity and mortality in predialysis and dialysis patients with CKD[1-6)]. Therefore, the development of an appropriate treatment for CKD−MBD is very important for these patients to improve their prognosis.

Vitamin D agents and Vitamin D receptor activator (VDRA) are frequently administered to patients with CKD to control CKD−MBD in the clinical setting. Furthermore, vitamin D is known to have several pleiotropic effects on bone and mineral metabolism, immune function, and cardiovascular systems[7)]. Moreover, it has been reported that vitamin D deficiency is associated with mortality in patients with CKD[8)]. Many previous clinical reports showed that the administration of vitamin D agents or VDRA favorably influences the life prognosis of patients with CKD[9-13)]. However, the effects of vitamin D agents or VDRA on the vasculature are complicated[14)], and an excessive administration of vitamin D agents or VDRA can lead

Address for correspondence: Hideki Fujii, Division of Nephrology and Kidney Center, Kobe University Graduate School of Medicine, 7-5-2 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan
E-mail: fhideki@med.kobe-u.ac.jp
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Table 1. Animal characteristics in each group at 20 weeks of age.

	SDT ($n=6$)	SD ($n=6$)	SDT-Nx+V ($n=6$)	SD-Nx+V ($n=6$)	SDT-Nx+LP ($n=7$)	SD-Nx+LP ($n=8$)	SDT-NX+HP ($n=6$)	SD-Nx+HP ($n=6$)
SBP (mmHg)	114.9±3.1	113.6±4.1	131.8±5.8	142.8±6.4	128.6±3.7	134.3±13.2	131.1±5.9	142.6±7.1
FPG (mg/dL)	NA	NA	140.0±6.8	134.7±5.9	143.1±5.5	137.7±8.3	132.1±12.3	138.6±10.3
HbA1c (%)	5.5±0.5	3.2±0.1	3.2±0.1	3.2±0.1	3.3±0.1	3.2±0.1	3.2±0.1	3.3±0.1
Ccr (mL/min)	4.5±0.5	5.2±0.4	2.7±0.3	2.6±0.2	2.3±0.2	2.3±0.3	2.4±0.2	2.3±0.3
BUN (mg/dL)	20.8±0.3	17.7±1.3	33.5±6.0	34.2±1.6	32.5±7.8	36.5±4.9	29.8±3.8	38.2±9.0
Albumin (g/dL)	3.7±0.1	3.7±0.1	3.0±0.3	3.0±0.2	3.1±0.1	3.1±0.2	3.3±0.1	3.2±0.2

Values represent the mean±SEM.
SDT, spontaneously diabetic torii; SD, Sprague Dawley; Nx, 5/6 nephrectomy; V, vehicle; LP, low-dose paricalcitriol; HP, high-dose paricalcitriol; FPG, fasting plasma glucose; HbA1c, hemoglobin A1c; Ccr, creatinine clearance; BUN, blood urea nitrogen; SBP, systolic blood pressure; NA, not available.

to the induction of vascular calcification.

Consequently, it is essential to clarify the detailed pathophysiology of CKD−MBD by experimental and clinical studies and to achieve appropriate management strategies for CKD−MBD in patients with CKD.

We incidentally discovered that 5/6 nephrectomized type 2 diabetic model rats manifest severe vascular calcification without diabetes in a preliminary study (unpublished). In the present study, we performed an experimental investigation to evaluate the characteristics of this new model and the potential influence of VDRA on CKD−MBD compared with 5/6 nephrectomized Sprague−Dawley (SD) rats.

Materials and Methods

Animals

Male spontaneously diabetic Torii (SDT) rats spontaneously develop gradual impairment of insulin secretion and hyperglycemia at around 20 weeks of age with an incidence of 100% until 40 weeks of age[15]. Pathological changes within pancreatic islets such as congestion, capillary expansion, and hemorrhage are sporadically observed at approximately 8 weeks and inflammatory cell infiltration and fibrosis occur in almost all islets at around 20 weeks[16].

Male SDT and SD rats were obtained from CLEA Japan, Inc., Tokyo, Japan. Rats were housed under light- and temperature-controlled environments and were fed a standard diet with water available *ad libitum*.

We created a CKD model by 5/6 nephrectomy (Nx) using male SDT and SD rats. At 8 weeks of age, the rats underwent 2/3 nephrectomy of the left kidney, followed by total nephrectomy of the right kidney one week later. At 10 weeks, the rats were started on a high phosphorus diet (1.0% calcium, 1.2% phosphate), and Nx rats (both SDT and SD) were randomly assigned to the following six study groups: (i) SDT-Nx rats treated with vehicle (SDT-Nx+V, $n=6$), (ii) SD-Nx rats treated with vehicle (SD-Nx+V, $n=6$), (iii) SDT-Nx rats treated with a low-dose paricalcitol (SDT-Nx+LP, $n=7$), (iv) SD-Nx rats treated with a low-dose paricalcitol (SD-Nx+LP, $n=8$), (v) SDT-Nx rats treated with a high-dose paricalcitol (SDT-Nx+HP, $n=6$), and (vi) SD-Nx rats treated with a high-dose paricalcitol (SD-Nx+HP, $n=6$). Paricalcitol was administered intraperitoneally at a low dose of 0.1 μg/kg of body weight or a high dose of 0.3 μg/kg of body weight thrice a week for 10 weeks. Furthermore, as references, we added control SDT and SD rats (SDT, $n=6$; SD, $n=6$).

All animal care and procedures were approved by the Institutional Animal Care and Use Committee guidelines at Kobe University School of Medicine (Permit Number: P130103) and were in strict accordance with the recommendations stipulated by the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Blood Pressure Measurement and Biochemical Analysis

At 20 weeks of age, systolic blood pressure was measured by tail-cuff plethysmography (MK-2000; Muromachi Kikai Co. Ltd., Japan). To reduce the possibility of stress artifacts, the rats were allowed to acclimatize to the environment for at least 15 min; then, the mean of 10 measurements was calculated.

Consecutively, urine was collected from each rat, held in individual metabolic cages, over a 24-h time period (Tecniplast, Exton, PA); the rats were then sacrificed under anesthesia. Blood samples were collected from the left ventricle for serum biochemical analysis. Serum samples were collected following centrifugation for 5 min at 860 G and the samples were stored at

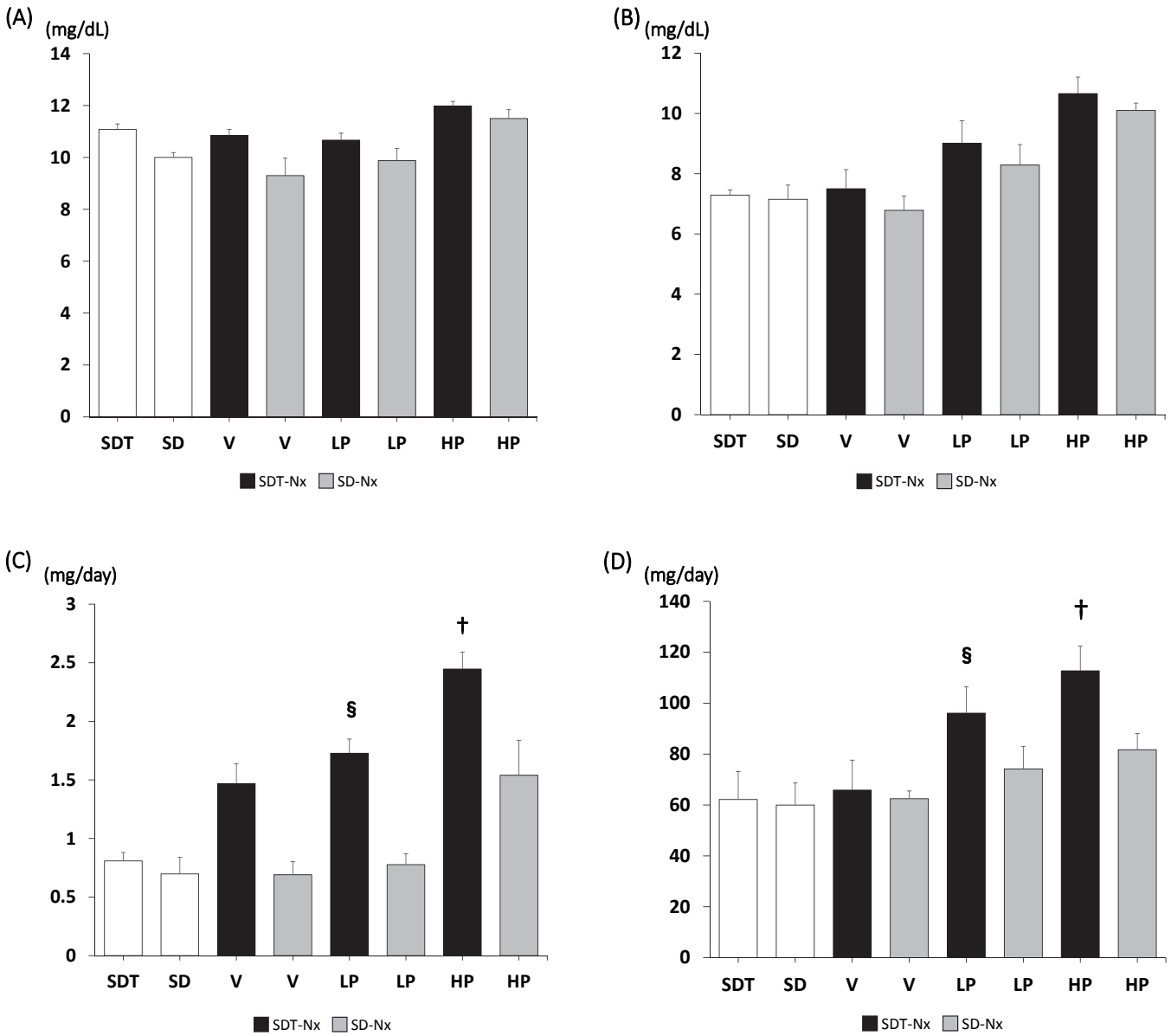

Fig. 1. Serum and urinary calcium and phosphate levels.

(A) Serum calcium levels.
(B) Serum phosphate levels.
(C) 24-h urinary excretion of calcium.
(D) 24-h urinary excretion of phosphate.
V, vehicle; LP, low paricalcitol; HP, high paricalcitol. White bar shows sham-operated SD or SDT rats; solid black bar shows SDT-Nx rats; solid gray bar shows SD-Nx rats. *SDT-Nx vs SD-Nx, $p<0.05$; §SDT-Nx + LP vs SD-Nx + LP, $p<0.05$; †SDT-Nx + HP vs SD-Nx + HP, $p<0.05$.

−80℃ until analysis. Urine samples were also stored at −80℃ for subsequent analysis.

Blood urea nitrogen, serum albumin, fasting plasma glucose, serum creatinine, calcium, and phosphorus levels, along with urine calcium and urine phosphorus levels were measured using a Fuji Dri-Chem 3500 system (FUJIFILM, Tokyo, Japan). Urinary creatinine levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemical Company, Ann Arbor, MI, USA). Serum intact parathyroid hormone (i-PTH) levels were measured using a rat PTH−ELISA kit (Immutopics International, San Clemente, CA, USA), whereas serum intact fibroblast growth factor 23 (i-FGF23) levels were

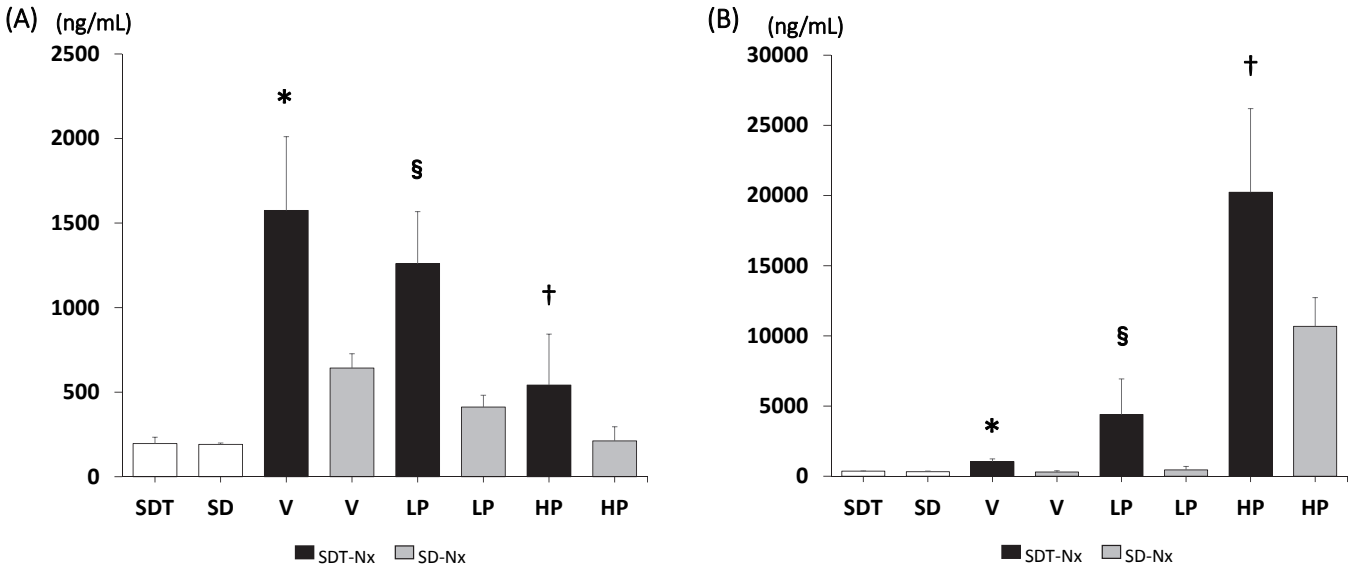

Fig. 2. Changes in CKD−MBD-related hormone levels.

(A) Serum i-PTH levels.
(B) Serum i-FGF23 levels.
V, vehicle; LP, low paricalcitol; HP, high paricalcitol. White bar shows sham-operated SD or SDT rats; solid black bar shows SDT-Nx rats; solid gray bar shows SD-Nx rats. *SDT-Nx vs. SD-Nx, $p<0.05$; §SDT-Nx + LP vs. SD-Nx + LP, $p<0.05$; †SDT-Nx + HP vs. SD-Nx + HP, $p<0.05$.

measured using an FGF23 ELISA kit (KAINOS laboratories Inc., Tokyo, Japan). Finally, hemoglobin A1c (HbA1c) levels were determined using a DCA2000 analyzer (Bayer Medical, Tokyo, Japan).

Calculation of the Positive Areas of von Kossa Staining in the Aorta

The positive areas of von Kossa staining in the aorta and the aortic sectional areas were measured using image analysis software (LUMINA VISION version 3.7.4.2, Mitani, Tokyo, Japan). The percentage of the positive areas of von Kossa staining in the aorta was calculated using the following formula:

von Kossa-positive areas in the aorta (%) = von Kossa-positive areas in the aorta/aortic sectional areas × 100

Quantification of Aortic Calcification

To quantify aortic calcification, the abdominal aorta was freeze-dried and decalcified with 0.6 N HCl at 37℃ for 24 h. Then, the calcium content of the supernatant was determined using the Calcium E-test Wako (Wako, Osaka, Japan). Aortic calcium contents in each sample were corrected for the dry tissue weight and expressed as μg Ca/mg dry weight. For histological analysis, a transverse section of the abdominal aorta was embedded in paraffin, sectioned, and stained with von Kossa. Since some of the rats in the SDT-Nx + LP or SDT-Nx + HP groups exhibited extremely severe aortic calcification with von Kossa staining, these rats were excluded when comparing calcium contents of the aorta among the study groups.

Statistical Analysis

Values are presented as means ± standard error of the mean (SEM). Differences in the data between each corresponding SD-Nx and SDT-Nx groups were analyzed using the Mann−Whitney *U* test. A *p*-value of <0.05 was considered statistically significant. Statistical analyses were performed using IBM SPSS Statistics version 23.0 (Chicago, IL, USA).

Results

Animal Characteristics and Biochemical Data

The characteristics and biochemical data of the rats at 20 weeks of age are shown in **Table 1**. There were no significant differences in systolic blood pressure, HbA1c levels, creatinine clearance, blood urea nitrogen, and serum albumin among the Nx groups. HbA1c levels were slightly higher in SDT rats than in nephrectomized SDT rats and there were no differences in fasting plasma glucose levels among SDT-Nx and SD-Nx rats. Serum calcium and phosphorus levels tended to be higher in SDT-Nx rats than in SD-Nx rats although there were no statistically significant dif-

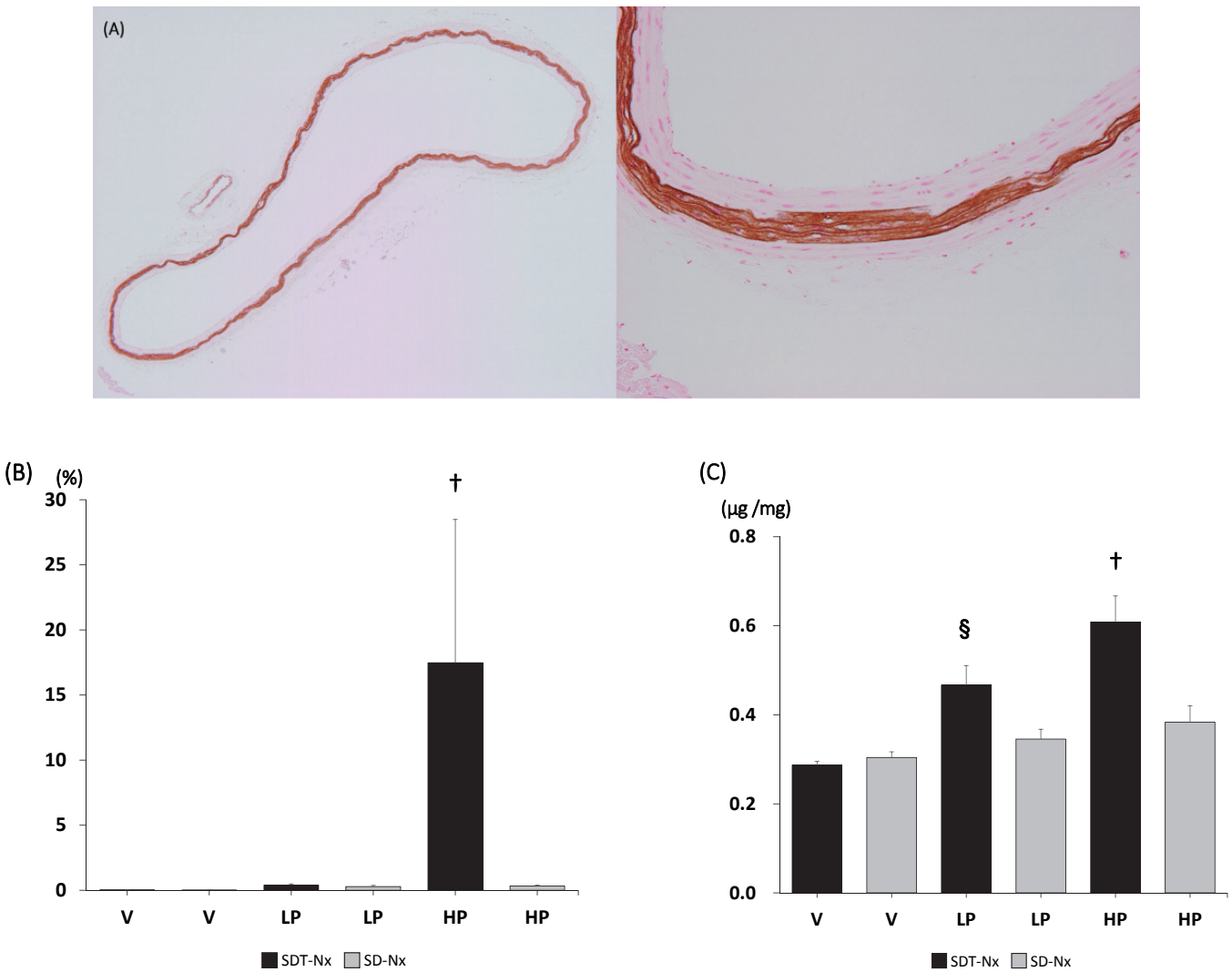

Fig. 3. Evaluation of aortic calcification.

(A) Histological findings of aortic calcification. The aorta of SDT-Nx rats at 20 weeks of age treated with high doses of paricalcitol was stained with von Kossa. Data revealed circumferential medial calcification. A portion of the calcified aortic wall is shown magnified.
(B) The portion of the von Kossa-positive area of the aorta.
(C) Calcium content of the aorta.
V, vehicle; LP, low paricalcitol; HP, high paricalcitol. Solid black bar shows SDT-Nx rats; solid gray bar shows SD-Nx rat. [§] SDT-Nx + LP vs. SD-Nx + LP, $p < 0.05$; [†] SDT-Nx + HP vs. SD-Nx + HP, $p < 0.05$.

ferences between the two groups (**Fig. 1A, B**). The 24-h urinary calcium excretion was significantly greater in SDT-Nx rats than in SD-Nx rats (**Fig. 1C**). The 24-h urinary phosphorus excretion was also significantly higher in SDT-Nx rats than in SD-Nx rats for both doses of paricalcitol (**Fig. 1D**). As shown in **Fig. 2A** and **B**, serum i-PTH and i-FGF23 levels were significantly higher in the SDT-Nx group than in the SD-Nx group for both doses of paricalcitol; paricalcitol administration reduced serum i-PTH levels and increased serum i-FGF23 levels in a dose-dependent manner.

Aortic Calcification Analysis

Evaluation of aortic calcification by histology stained with von Kossa and the calcium content of the aorta are shown in **Fig. 3**. The percentage of the positive areas observed on von Kossa staining in the aorta was significantly greater in the SDT-Nx + HP group than in the SD-Nx + HP group (17.5% ± 11.0% vs. 0.33% ± 0.05%, $p < 0.05$); however, there were no statistically significant differences between SDT-Nx + V and SD-Nx + V groups or SDT-Nx + LP and SD-Nx + LP groups (0.03% ± 0.02% vs. 0.02% ± 0.02%, NS; 0.40% ± 0.09% vs. 0.28% ± 0.09%, NS) (**Fig. 3A, B**).

The calcium content of the aorta was significantly greater in SDT-Nx rats than in SD-Nx rats regardless of the dose of paricalcitol (**Fig. 3C**).

Discussion

In the present study, we created a CKD model using SDT-Nx rats, which compared with SD-Nx rats revealed more drastic changes in CKD−MBD parameters and more severe vascular calcification. In addition, paricalcitol strongly affected CKD−MBD parameters in this novel CKD model in a dose-dependent manner.

There are many CKD models available, and nephrectomized rats are popular as CKD rat models. In 1932, Chauntin and Ferris developed the 5/6 nephrectomized rat model which has been used ever since[17]. However, it is commonly known that it is difficult to induce enough vascular calcification in this model; therefore, researchers have been developing alternative animal models. For example, adenine rat models exhibit severe vascular calcification without operations. Hence, this model is prevalently used for the experimental analysis of vascular calcification in CKD. However, this model has some critical issues in terms of clarifying the pathophysiological mechanisms of CKD−MBD. One of the problems associated with this model is weight loss and malnutrition caused by the adenine diet[17]. In addition, adenine induces systemic inflammation; therefore, arterial calcification can be caused by a simple low-protein diet. Thus, the pathophysiological conditions appear to differ between the adenine-induced CKD models and other CKD models. On the other hand, since our new model exhibited severe vascular calcification without such chemical effects, it is possible that our rat model resembles the relative status of patients with CKD. Han:SPRD rats with autosomal dominant polycystic kidney disease also develops CKD−MBD on a normal phosphorus diet. However, evident arterial calcification is observed around the age of 38 weeks, although this period is too long for an experimental study[18]. In our present study, SDT-Nx rats showed that CKD−MBD parameters changed more drastically, and with more severe vascular calcification, by 20 weeks of age.

The effects of vitamin D agents and VDRA on the vasculature are very complicated[14]. Although a low dose of vitamin D agents and VDRA plays a vasculoprotective role, an excessive dose can be harmful for the vasculature[19]. An excessive dose of vitamin D agents and VDRA can be burden of calcium and phosphorus, and previous studies showed that calcium and phosphorus were associated with arterial stiffness and vascular calcification[20, 21]. In the present study, our new CKD model revealed that VDRA exhibited significant influence on CKD−MBD parameters including calcium and phosphorus metabolism and vascular calcification. It is well-known that paricalcitol is one of the VDRA that has been developed to minimize the adverse effects such as hypercalcemia while treating hyperparathyroidism as effectively as calcitriol. In fact, differing from the findings in SDT-Nx rats, calcium contents of the aorta and the percentage of the aortic areas positively stained by von Kossa were similar between SD-Nx + LP and SD-Nx + HP groups. Our previous study using SDT rats without CKD demonstrated that maxacalcitol, another kind of VDRA, has a vasculoprotective effect[22]. Therefore, we speculated that in the present study the dose of paricalcitol in the HP group was too high to prevent adverse effects and that the dose of vitamin D agents or VDRA is very important for patients with CKD. Although we could not perform further analysis, we considered that it is interesting whether there are any differences in the effects on CKD−MBD among paricalcitol and other vitamin D agents. We think that a further study is needed to elucidate this issue.

A recent study reported that vascular calcification is mediated by local activation of 1-alfa-hydroxylase[23], indicating that excessive local VDR signal enhancement may lead to a harmful influence on the progression of vascular calcification. The new CKD model is more susceptible to the effect of VDRA on the vasculature or mineral metabolism than SD-Nx rats. The reason for this may be associated with VDR signal enhancement although we could not identify the specific underlying mechanism. Additionally, previous studies demonstrated that VDR polymorphism is associated with calcium absorption[24]. Taking these facts into account, we speculate that one explanation for the characteristics of our new CKD model is susceptibility to VDRA due to VDR gene polymorphism. To explore the detailed mechanism of the susceptibility to VDRA in the SDT-Nx rats, we need to examine the expression of VDR and downstream components of VDR signaling or gene polymorphism.

In the present study, serum i-FGF23 levels were higher and vascular calcification was more severe in the paricalcitol-treated groups compared to vehicle-treated groups. However, the association between FGF23 and vascular calcification is the focus of much controversy. In a large cohort of patients with CKD, FGF23 was shown not to be significantly associated with vascular calcification[25]. On the other hand, another clinical study demonstrated that FGF23 was independently correlated with vascular calcification[26]. As for data arising from experimental studies, FGF23

added to cultured VSMC did not increase the calcium content of the cells, even in high-phosphate media[27]. FGF23-null mice developed marked renal vascular calcification[28]; FGF23 has a stimulatory effect on the production of Fetuin A in osteocytes[29]. However, a recent experimental study showed that FGF23 enhanced phosphate-induced calcification of vascular smooth muscle cell via the ERK1/2 pathway[30]. Although it is well-known that FGF23 levels are regulated by VDRA[31] and paricalcitol itself might predominantly have an impact on FGF23 production even in the present study, it remains unclear whether the elevation of serum FGF23 levels was related to the pathogenesis of vascular calcification or represented a reaction to vitamin D agents or VDRA administration. The effects of FGF23 on the vasculature are also complicated and need to be elucidated more clearly in future studies.

Since the data of the present study did not meet the diagnostic criteria for diabetes, we considered that SDT rats did not develop diabetes mellitus following Nx despite representing a model of type 2 diabetes. Although this finding is also very important, unfortunately, we could not clarify this mechanism in the present study. As a potential mechanism, we speculated that impaired renal function might affect reduction in the urinary excretion of insulin and/or preservation of insulin secretion. Although we do not know the mechanism and could not examine the histology of pancreas, uremic status might delay the progression of pancreatic tissue damage. Further experiments, including histological evaluation of the pancreas, insulin excretion from the pancreas, and the tests for glucose intolerance are needed to clarify the detailed mechanisms involved.

Conclusions

Our study indicated that 5/6 nephrectomized SDT rats represent a suitable model to examine the pathophysiology of CKD−MBD and were significantly influenced by VDRA. Further studies are needed to clarify the detailed mechanisms underlying these observations.

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Notice of Grant Support

None.

Conflict of Interest

All authors disclose no conflict of interest.

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