High calcium diet alleviates 5/6 nephrectomy-induced bone deteriorations of lumbar vertebrae in mice

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Abstract. Dietary calcium (Ca) supplementation has beneficial effects on bone health. However, it is not clear whether a high calcium diet (HCD) following 5/6 nephrectomy (5/6 Nx) is beneficial to bone health. The aim of the present study was to examine the effects of an HCD on bone metabolism using a chronic kidney disease (CKD) mouse model. Male C57BL/6J mice were divided into three groups: Sham group, 5/6 Nx group and 5/6 Nx + HCD group. Mice were sacrificed 12 weeks post-surgery. Calcium (Ca) and creatinine (Cr) were measured using standard colorimetric methods and picric acid methods, respectively. Bone metabolism-associated markers, FGF-23, PTH, ALP-b and TRAP-5b were measured using ELISA kits. Lumbar vertebrae histomorphological analysis was performed using hematoxylin and eosin staining. The expression of osteoprotegerin (OPG) and receptor activator of nuclear factor κB ligand (RANKL) mRNA was detected using reverse transcription-quantitative polymerase chain reaction. Impaired renal function and histopathological damage was indicated in 5/6 Nx mice. However, HCD had no significant effects on these changes in 5/6 Nx mice. Notably, mineral metabolism disorder and histopathological damage to lumbar vertebrae were markedly improved in HCD-treated 5/6 Nx mice. Compared with 5/6 Nx mice, HCD supplementation significantly elevated the ratio of OPG/RANKL and inhibited RANKL mRNA expression in lumbar vertebrae. To conclude, the present findings indicated that increased Ca intake is effective in increasing bone mineral content of the lumbar vertebrae in 5/6 Nx mice. These results may provide a basis for the clinical use of dietary Ca supplementation as a therapeutic approach to treat CKD-induced disturbance of mineral metabolism and bone loss.

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Introduction

Mineral and bone disorder (MBD) is a common complication associated with chronic kidney disease (CKD) (1). CKD-MBD is characterized by severe renal injury-induced mineral and hormone metabolic disorders accompanied by bone deteriorations (1). In the progression of CKD, low glomerular filtration rate (GFR) is associated with excessive phosphorus (P) levels, which accelerate osteocyte-derived fibroblast growth factor-23 (FGF-23) and parathyroid hormone (PTH) secretion and adversely affects bone remodeling and resorption, eventually resulting in osteoporotic bone complications (2). Previous clinical studies have demonstrated that the severity of CKD is associated with fracture rate (3,4). Dysregulated mineral homeostasis is a typical feature of CKD (3,4). Therefore, improving bone mineralization and microstructure in patients with CKD is necessary to reduce the risk of fracture.

Calcium (Ca) is important for bone health. Low Ca intake is associated with an increased risk of osteopenia and bone fractures, whereas high Ca intake has been indicated to protect against osteoporosis (5). Epidemiological studies have demonstrated that Ca supplementation has beneficial effects on bone mineral content and bone mineral density (BMD) (6,7). In rats, Ca supplementation prevented the bone loss and decline in kidney function induced by a high-P diet (8). Furthermore, a high calcium diet (HCD) increases bone mineral (Ca and P) content in high-fat diet-induced obese mice (9). However, the effects of an HCD on CKD-induced bone have not previously been reported.

Previous studies have employed an 5/6 nephrectomy (5/6 Nx) animal model to evaluate CKD-MBD (10,11). At 16 weeks following 5/6 Nx, diminished bone microarchitecture was observed in the tibial trabecular bone of cluster of differentiation 1 mice (11), which is a well-established mouse model to produce moderate CKD with high-turnover bone injury. In the present study, a CKD-MBD animal model was established in C57BL/6J mice and the effects of an HCD on mineral homeostasis and trabecular bone properties were investigated.

Materials and methods

Animal model. The present study was approved by the Ethics Committee of Shandong Wendeng Orthopedic Hospital

(Wendeng, China) and was performed in accordance with institutional guidelines. A total of 36 8-week-old male C57BL/6J mice (body weight, 20±2 g) were obtained from Shanghai Laboratory Animal Center (Shanghai, China) and acclimated to the environment for 1 week. Normal dietary Ca (TD.04200, containing 0.6% calcium carbonate and 0.4% phosphate by weight) and high dietary Ca (TD.96348, containing 2% calcium carbonate and 1.25% phosphate by weight) were purchased from Harlan Teklad (Madison, WI, USA). The mice individually caged in a temperature-controlled environment (23±2˚C; humidity, 60±5%) with an artificial 12-h light/dark cycle and were provided with free access to food and tap water. The 5/6 Nx mouse model of CKD was established without angiotensin II infusion according to the method published by Souza *et al* (12) and upregulation of blood urea nitrogen and serum creatinine verified successful establishment of the animal model (12). The mice were randomly divided into three groups (n=12 in each group): Sham group, sham-operated mice (without removal of kidneys) treated with normal dietary Ca following sham surgery for 12 weeks; 5/6 Nx group, 5/6 Nx mice treated with normal dietary Ca following Nx surgery for 12 weeks; and 5/6 Nx+HCD group, 5/6 Nx mice treated with HCD following Nx surgery for 12 weeks. All mice were sacrificed at week 12 via an intraperitoneal injection of sodium pentobarbital (200 mg/kg; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Blood was harvested from the heart and centrifuged at 1,500 x g for 15 min at 4˚C to obtain serum. Serum (~800 μl), urine (~2 ml) and lumbar vertebrae (L3-L5) tissues were immediately collected and maintained at -80˚C for further analysis.

Biochemical markers in serum and urine. The concentrations of Ca (cat. no. C004-3; Nanjing Jiancheng Biology Engineering Institute, Nanjing, China) and creatinine (Cr; cat. no. C011-1; Nanjing Jiancheng Biology Engineering Institute) from the serum and urine were measured using standard colorimetric methods (13) and picric acid methods (14), respectively. The level of urinary Ca (UCa) was corrected according to the concentration of urinary Cr (UCr). Serum Cr (SCr; cat. no. C011-1; Nanjing Jiancheng Biology Engineering Institute) and blood urea nitrogen (BUN; cat. no. C013-2; Nanjing Jiancheng Biology Engineering Institute) levels were measured using an autoanalyzer and an enzymatic kinetic method with commercial kits following the manufacturer's protocol. Serum levels of FGF-23 (cat. no. 60-6800; Immutopics, Inc., San Clemente, CA, USA), PTH (cat. no. E-EL-M0709c; Elabscience Biotechnology Co., Ltd., Wuhan, China), tartrate resistant acid phosphatase-5b (TRAP-5b; cat. no. SB-TR201A; Immunodiagnostic Systems, Scottsdale, AZ, USA) and alkaline phosphatase (ALP; cat. no. E-EL-M0200c; Elabscience Biotechnology Co., Ltd.) were detected using murine ELISA assays with a SpectraMax M5 ELISA plate reader (Molecular Devices, LLC, Sunnyvale, CA, USA).

Bone Ca. The lumbar vertebrae were incinerated using a muffle furnace (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 800˚C for 12 h and 10 mg of bone ash was dissolved in 1 ml of 37% HCl diluted with Milli-Q® water. The calcium content was determined using a kit (cat. no. C004-3; Nanjing Jiancheng Biology Engineering Institute).

Histomorphology. Kidney tissues were collected immediately following sacrifice and fixed with 4% formalin at room temperature for 24 h and paraffin-embedded. Tissues were cut into ~5 μm-thick sections, which were stained with Masson's trichrome (cat. no. SBJ-0290; Nanjing SenBeiJia Biological Technology Co., Ltd., Nanjing, China) as previously described (15) and visualized under a microscope (magnification, x200; Leica DM 2500; Leica Microsystems GmbH, Wetzlar, Germany). Renal injury was assessed using a previously described 0-4 scale (16) as follows: 0, none; 1, <10; 2, 10-25; 3, 25-75; or 4, >75%.

The lumbar vertebrae were collected immediately following sacrifice and fixed with 4% formalin at room temperature for 24 h, and then decalcified in 0.5 M EDTA (pH=8.0) and embedded in paraffin according to standard histological procedures. Sections of ~5 μm were cut and stained with hematoxylin and eosin (H&E; cat. no. C0105; Beyotime Institute of Biotechnology,) prior to being visualized under a microscope (magnification, x200; Leica DM 2500).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from lumbar vertebrae was extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. cDNA was synthesized by RT reactions with 2 μg of total RNA using moloney murine leukemia virus reverse transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.) following 37˚C for 50 min and 70˚C for 15 min. A total of 1 μl cDNA was used for PCR using a DNA Engine (ABI) with SYBR Green PCR Master Mix (Invitrogen; Thermo Fisher Scientific, Inc.). The following thermocycling conditions were applied: 95˚C for 10 min followed by 40 cycles of 95˚C for 15 sec, 58˚C for 30 sec and 72˚C for 30 sec. The primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) as follows: osteoprotegerin (OPG), forward 5'-GCATTATGACCC AGAAACT-3' and reverse 5'-ACCTGAGAAGAACCCATC-3'; receptor activator of nuclear factor κB ligand (RANKL), forward 5'-AACCAAGATGGCTTCTATTACC-3' and reverse 5'-AAGGGTTGGACACCTGAATG-3'; and GAPDH, forward 5'-TCACTGCCACCCAGAAGA-3' and reverse 5'-AAGTCG CAGGAGACAACC-3'. Signals were assessed using Quantity One® software version 4.5 (Bio Rad Laboratories, Inc., Hercules, CA, USA) and normalized to the GAPDH. Analysis of relative gene expression data used real-time quantitative PCR and the quantitation cycle (Cq) method (17).

Statistical analysis. Data are presented as the mean + or ± standard deviation as indicated for each group. All statistical analyses were performed using GraphPad Prism software version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Inter-group differences were analyzed using one-way analysis of variance with a post hoc Tukey test for multiple comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

HCD supplementation has no significant effect on renal dysfunction in 5/6 Nx mice. To evaluate the role of HCD

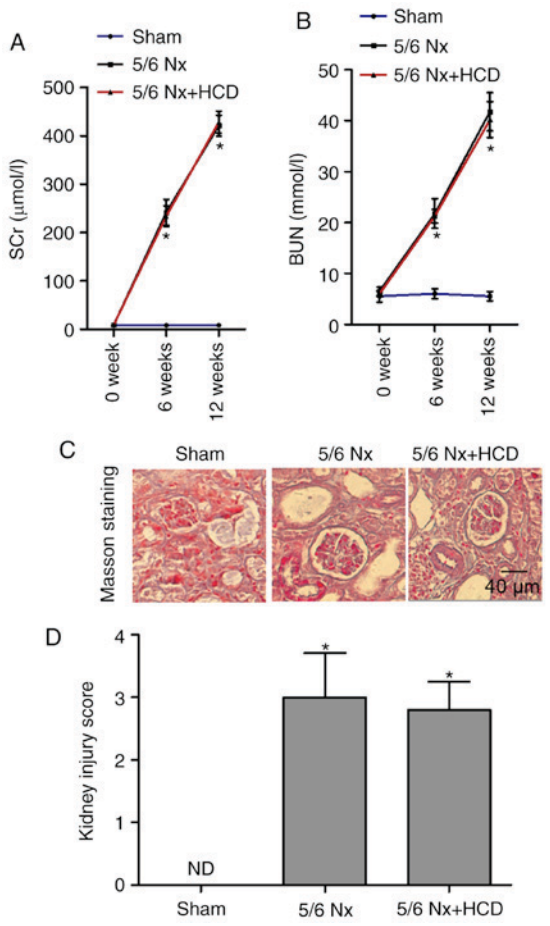

Figure 1. Changes in renal function in 5/6 Nx mice. (A) SCr and (B) BUN levels in 5/6 Nx mice with or without HCD supplementation were measured. Data are presented as the mean ± standard deviation. (C) Masson's trichrome staining was performed in 5/6 Nx mice with or without HCD supplementation to evaluate renal injury (magnification, x200). (D) Kidney injury score for each group. Data are presented as the mean ± standard deviation. *P<0.05 vs. Sham group. 5/6 Nx, 5/6 nephrectomy; SCr, serum creatinine; BUN, blood urea nitrogen; HCD, high calcium diet; ND, not detected.

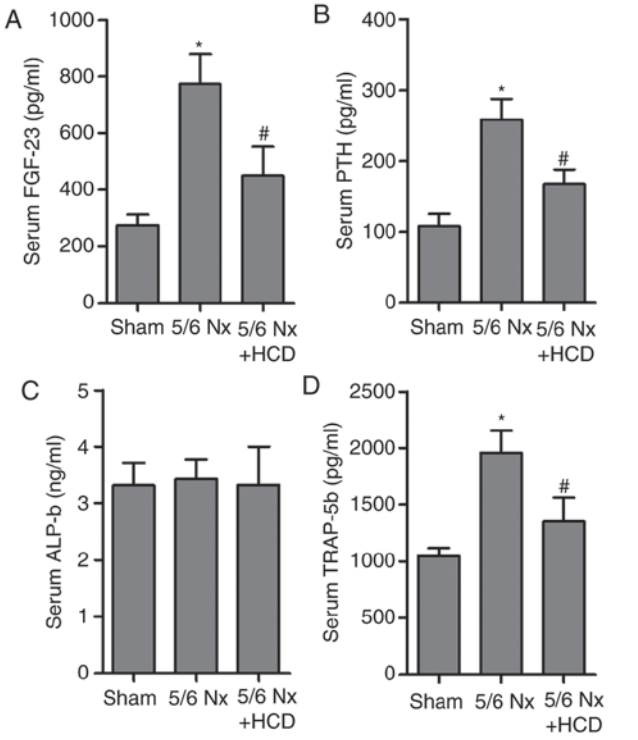

Figure 2. HCD supplementation regulates bone metabolism-associated biomarkers. Serum levels of (A) FGF-23, (B) PTH, (C) ALP and (D) TRAP-5b mice were measured in 5/6 Nx with or without HCD supplementation. Data are presented as the mean ± standard deviation. *P<0.05 vs. Sham group; #P<0.05 vs. 5/6 Nx group. 5/6 Nx, 5/6 nephrectomy; HCD, high calcium diet; FGF-23, fibroblast growth factor-23; PTH, parathyroid hormone; ALP, alkaline phosphatase; TRAP-5b, tartrate resistant acid phosphatase-5b.

supplementation in 5/6 Nx-induced renal dysfunction, serum levels of SCr and BUN were compared across groups. The levels of SCr and BUN were significantly increased in the 5/6 Nx group compared with the sham-operated group at week 6 and week 12 (P<0.05; Fig. 1A and B); however, HCD supplementation in 5/6 Nx mice did not significantly improve these parameters (Fig. 1A and B). Masson staining was performed to observe the extent of renal interstitial fibrosis. As indicated in Fig. 1C and D, increased matrix deposition, interstitial fibrosis and renal lesions were observed in the kidneys from 5/6 Nx mice compared with sham mice, and injury scores were significantly increased (P<0.05; Fig. 1D). Furthermore, HCD supplementation had no marked effect on renal pathological changes in 5/6 Nx mice. These data suggest that the CKD mice models were successfully established and that HCD did not improve renal function in 5/6 Nx mice.

HCD supplementation regulates bone metabolism-associated biomarkers. Acute kidney injury (AKI) or CKD-induced GFR regression predisposes to bone metabolism disturbances via upregulating serum P (18), which accelerates FGF-23 and PTH secretion. Previous studies have demonstrated that increased FGF-23 is associated with CKD or AKI severity (19,20). FGF-23 is a peptide released from osteocytes and osteoblasts, whilst PTH is associated with Ca, P and FGF-23 levels in CKD (21). To evaluate the effect of an HCD in CKD-MBD, the levels of FGF-23 and PTH in the serum of 5/6 Nx mice were measured. Results indicated that serum FGF-23 and PTH levels were significantly increased in the 5/6 Nx group compared with the sham group (P<0.05; Fig. 2A and B). Notably, HCD significantly reversed CKD-induced upregulation of serum FGF-23 and PTH levels in 5/6 Nx mice (P<0.05; Fig. 2A and B). In addition, the effect of 5/6 Nx on markers of bone resorption and formation, ALP and TRAP-5b (22,23), in the serum of 5/6 Nx mice was assessed (Fig. 2C and D). 5/6 Nx induced a significant increase in serum TRAP-5b concentration compared with the sham group; however, HCD supplementation significantly decreased TRAP-5b levels in CKD mice (P<0.05). No significant difference in ALP concentration was observed in any of the experimental groups.

HCD supplementation regulates Ca metabolism and bone remodeling. HCD has an important role in the bone-kidney axis and the regulation of calcium homeostasis in CKD (24,25). The Ca content in serum, urine and bone was measured in CKD mice. Results indicated that 5/6 Nx mice exhibited decreased SCa levels and an ~3-fold increase in UCa excretion compared with the sham group, whereas HCD supplementation significantly increased SCa content and decreased UCa excretion in CKD mice (P<0.05; Fig. 3A and B). At week 12, comparison of the results of bone Ca content between sham and 5/6 Nx groups

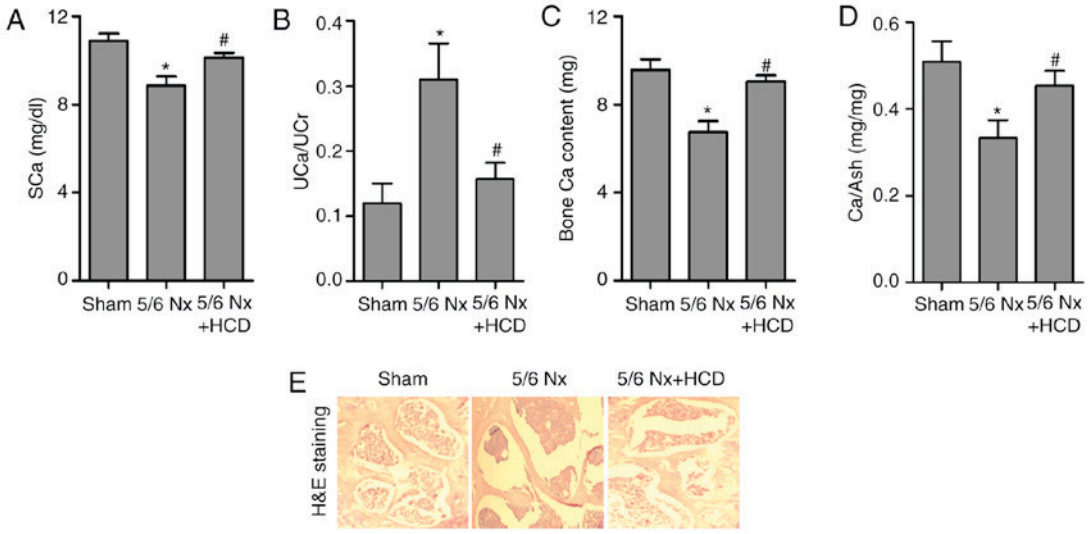

Figure 3. HCD supplementation regulates Ca metabolism and bone remodeling. Concentrations of (A) SCa (B) UCa, (C) bone Ca and (D) Ca/Ash were measured in 5/6 Nx mice with or without HCD supplementation. (E) H&E staining (magnification, x200) was performed in 5/6 Nx mice with or without HCD supplementation to evaluate trabecular bone injury. Data are presented as the mean ± standard deviation. *P<0.05 vs. Sham group; #P<0.05 vs. 5/6 Nx group. 5/6 Nx, 5/6 nephrectomy; Ca, calcium; SCa, serum calcium; UCa, urinary Ca; HCD, high calcium diet; H&E, hematoxylin & eosin.

indicated 5/6 Nx significantly decreased bone Ca content; however, the bone calcium content was significantly increased in 5/6 Nx mice with HCD supplementation (both P<0.05; Fig. 3C). A similar result was obtained for Ca content in the ash of lumbar vertebrae (Fig. 3D). Furthermore, H&E staining was conducted to observe the trabecular bone microstructure of lumbar vertebrae. As indicated in Fig. 3E, a loss of network connection in the trabecular bone was observed in the 5/6 Nx group compared with the sham group; however, the increased disconnections and separation among trabecular bone network were improved by HCD supplementation in CKD mice.

HCD supplementation regulates the OPG/RANKL ratio. The maturation and formation of osteoclasts is primarily regulated by the balance of extracellular OPG and RANKL (26). OPG and RANKL cytokines affect the activity of osteoblast cells and osteoclastogenesis (26). OPG cytokine binds to RANKL and prevents RANKL from binding to the RANK receptor on osteoclast cells, subsequently inhibiting bone resorption and osteoclastogenesis (27). Thus, the ratio of OPG/RANKL expression in lumbar vertebrae was determined in the present study (Fig. 4). RT-qPCR results revealed a significant decrease in the OPG mRNA expression levels and ratio of OPG/RANKL compared with the sham group (P<0.05; Fig. 4A, B and D). Furthermore, RANKL mRNA expression levels were significantly increased in mice treated with 5/6 Nx compared with the sham group (P<0.05; Fig. 4A and C). HCD supplementation significantly reversed the effects of 5/6 Nx on OPG/RANKL and RANKL mRNA expression (P<0.05; Fig. 4C and D). However, no significant difference was observed between the mRNA expression levels of OPG in the 5/6 Nx + HCD groups.

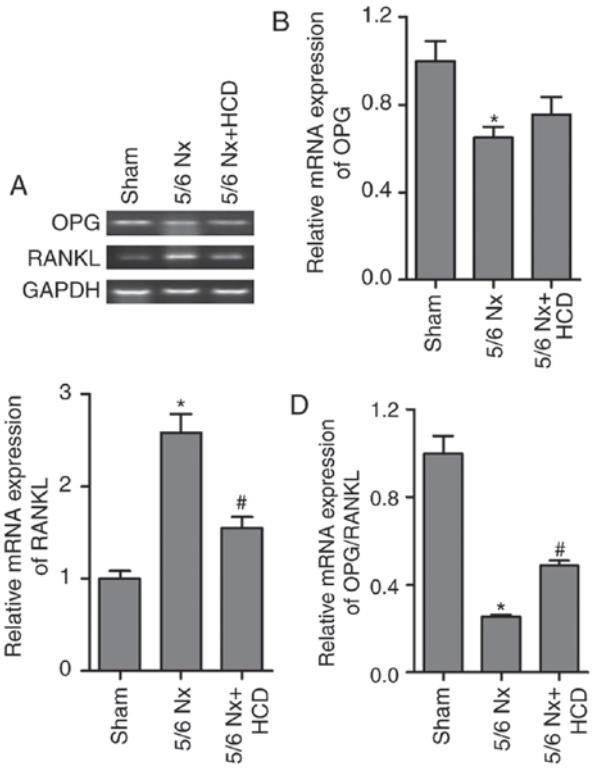

Figure 4. HCD supplementation regulated OPG/RANKL ratio. (A) mRNA expression levels of OPG and RANKL were determined. Quantified results of the mRNA expression levels of (B) OPG and (C) RANKL and (D) the ratio of OPG/RANKL were demonstrated in 5/6 Nx mice with or without HCD supplementation. Data are presented as the mean ± standard deviation. *P<0.05 vs. Sham group; #P<0.05 vs. 5/6 Nx group. OPG/RANKL; osteoprotegerin/receptor activator of nuclear factor kappa B ligand; 5/6 Nx, 5/6 nephrectomy; HCD, high calcium diet.

Discussion

Patients with CKD exhibit marked disruptions in bone and mineral metabolism accompanied with biochemical alterations, including elevated FGF-23 and PTH, decreased 1, 25-dihydroxyvitamin D3 (vitamin D3), elevated serum P and decreased SCa (28). Oral Ca carbonate affects the positive Ca balance in stage 3 and 4 CKD (29). Ca (1,200 mg) combined with vitamin D3 (800 IU) supplementation protects against

BMD loss in women with moderate CKD (30). Furthermore, dietary Ca supplementation has been indicated to prevent bone loss in several animal models, notably ovariectomized and renal injury rodents (31,32). These findings suggest that dietary Ca supplementation may serve as an adjunctive therapy for CKD-induced bone loss.

The lumbar vertebra is a hypersensitive site that is susceptible to bone injury (33). In clinical practice, kidney transplant recipients have a lower lumbar spine trabecular bone score, which is associated with an increased incidence of fractures (34). This suggests that Nx is associated with lumbar vertebrae injury. In the present study, 5/6 Nx significantly decreased SCa content and increased serum PTH and FGF-23 concentration. It is known that PTH stimulates the expression of RANKL, a mediator of osteoclastogenesis and bone resorption (35). In the present study, it was observed that RANKL mRNA expression was significantly increased and OPG mRNA expression was decreased in the lumbar vertebrae of 5/6 Nx mice compared with the sham group. These findings indicate that bone resorption and bone loss are enhanced in mice following 5/6 Nx. However, HCD supplementation significantly increased SCa, bone Ca content and Ca/Ash, whilst bone loss of lumbar vertebrae in 5/6 Nx mice was ameliorated. A previous study indicated that dietary Ca supplementation inhibits RANKL-mediated osteoclastic bone resorption via the suppression of PTH (8). Thus, these findings suggest that HCD supplementation may increase bone Ca content and bone remodeling via inhibiting RANKL-induced bone resorption in 5/6 Nx mice. Piri *et al* (36) assessed the levels of OPG and RANKL associated with dietary supplements of Ca, vitamin D and estrogen, revealing that Ca intake led to an increase in OPG and reduction in RANKL, which ultimately caused an increase in BMD. According to the results of the present study, OPG/RANKL signaling is associated with 5/6 Nx-induced bone deterioration of lumbar vertebrae in mice, which suggests that OPG/RANKL may be an important target for the treatment of CKD-induced bone injury.

FGF-23 is a member of the fibroblast growth factor family and is associated with P homeostasis (37). A recent study has revealed that Ca regulates bone FGF-23 expression and that HCD increases serum FGF-23 concentration in $Cyp27b1^{-/-}$ and $Gcm2^{-/-}$ mice (38). In parathyroidectomized rats, dietary Ca supplementation increased SCa and FGF-23 concentration (39). However, Ca has no effect on FGF-23 promoter activity in cultured osteoblasts (40). Furthermore, it has been reported that serum FGF-23 concentration is positively correlated with serum P levels (41). In the present study, 5/6 Nx mice had high serum P levels, which was associated with high serum FGF-23 despite hypocalcemia. These data suggest that Ca regulation of FGF-23 expression is also controversial. In the present study, it was identified that dietary Ca supplementation significantly decreased serum P and FGF-23 in CKD mice.

Ca and P are substrates for bone mineralization that are tightly regulated by several hormones, including FGF-23, PTH and vitamin D3 (42). Dysregulation of one hormone in this system results in the dysregulation of Ca and P homeostasis; particularly, active vitamin D synthesis is stimulated by hypophosphatemia in the kidney (42). FGF-23 is increased in the primary stage of CKD; however, renal tubular injury accelerates FGF23 secretion and results in a decrease in serum vitamin D3 levels, which in turn increases serum phosphate and decreases ionized Ca (43). In a clinical setting, foods rich in Ca and low in phosphate are permitted in patients with CKD (44). In the present study of 5/6 Nx mice with CKD, serum FGF-23 and PTH were significantly decreased, whereas serum Ca and bone Ca were increased in response to dietary Ca supplementation, ameliorating CKD-induced network connection loss in trabecular bone.

In conclusion, these findings provide a novel insight into the pathophysiological and nutritional role of an HCD in the regulation of Ca homeostasis and bone metabolism-associated hormones in 5/6 Nx mice. In addition, HCD has an anabolic potential to enhance novel bone formation and suppress bone resorption in 5/6 Nx mice. The present study provides the theory base for dietary Ca supplementation beneficial for the balance of bone metabolism in patients with CKD. However, cross-sectional and longitudinal studies are required to elaborate the standards, rules and alimental interaction in routine clinical practice.

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