A rat model of SHPT with bone abnormalities in CKD induced by adenine and a high phosphorus diet

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Abstract

The study of parathyroid hyperplasia with bone disease as a critical manifestation of chronic kidney disease-mineral and bone disorders (CKD-MBDs) is challenging due to the lack of a suitable research model. Here, we established a rat model with secondary hyperparathyroidism (SHPT) and bone disease induced by adenine and a high phosphorous diet and analyzed the skeletal characteristics. We performed blood analysis, emission computed tomography (ECT), dual energy X-ray absorptiometry (DEXA), micro-computed tomography (micro-CT), bone histomorphometry, and bone mechanical tests. The CKD rats with SHPT induced by adenine and a high phosphorus diet showed severe abnormalities in calcium and phosphorus metabolism and exhibited parathyroid hyperplasia. The bone mineral density (BMD) of femurs and lumbar vertebrae was significantly lower in the CKD rats than in the control (CTL) rats. The cortical and trabecular bone parameters of femurs showed significant bone loss. In addition, we found decreases in ultimate force, work to failure, stiffness, and elastic modulus in the CKD rats. In conclusion, our findings demonstrated that the CKD rats with SHPT induced by adenine and a high phosphorus diet may serve as a useful model for skeletal analysis in CKD with SHPT.

1. Introduction

Chronic kidney disease (CKD) is a worldwide health burden associated with high morbidity and mortality [1–4]. CKD is often complicated by the development of renal osteopathy due to disturbances in mineral metabolism, which are characterized by increased bone loss and bone fractures [5,6]. Secondary hyperparathyroidism (SHPT) is a common cause of renal osteopathy [7]. However, its detailed pathophysiology remains largely unknown. Consequently, developing a reliable rat model of osteoporosis with SHPT is of vital significance to study the basic aspects of this disease.

Exogenous adenine is immediately metabolized to 2,8-dihydroxyadenine, which precipitates and forms crystals in the microvilli and the apical region of the proximal tubular epithelia only 2 days after administration [8]. Increased crystal production induces degenerative changes in the cells of these tissues and causes renal dysfunction with increased levels of serum creatinine (Scr) and inorganic phosphate and decreased levels of serum calcium [9,10]. Many previous studies have reported that hyperphosphatemia in CKD leads to the increased progression of cardiovascular disease or vascular calcification, peripheral vascular disease, endothelial dysfunction, disorders of mineral and bone metabolism including bone fracture, and a higher mortality rate [11–13]. Recently, rat models of CKD with SHPT established by the administration of adenine and a high phosphorus diet were used to research vascular calcification and renal fibrosis [14–16]. However, a detailed analysis of the skeletal abnormalities in this model was insufficient.

In this study, we examined the skeletal system in an adenine-induced and high phosphorus-fed model of SHPT in CKD rats. Our data suggest that this is a reliable method for inducing severe SHPT with associated bone loss and chronic renal failure, which is more compatible with the clinical findings in kidney dialysis patients.
2. Methods

2.1. Animals

Study protocols were received and approved by the institutional animal care and use committee of Southeast University (Nanjing, China). Eight-week-old male Sprague Dawley rats (Animal Laboratory of Nantong University, China) were randomly assigned to two groups: (1) the control (CTL) group (n = 10) and (2) the CKD group (n = 10). CKD was induced by feeding 0.75% adenine for 4 weeks. After adenine withdrawal, all rats were maintained on a 1.5% phosphorus diet until the time of sacrifice at week 42. All diets were provided by Enuojia Feed Co., Ltd. (Nanjing, China).

2.2. Serum biochemistry

Serum creatine (Scr), blood urea nitrogen (BUN), and total calcium and phosphate concentrations were measured by a semi-automated biochemical analyzer (ECA-2000A, Jilin, China) and UV-5100 spectrophotometer (Shanghai, China). Serum parathyroid hormone (PTH) determinations were performed via ELSIA (MEIMIAN).

2.3. Emission computed tomography (ECT)

Parathyroid glands were scanned by tomography using a Siemens E-CAM SPECT instrument at the Southeast University-affiliated Zhongda Hospital. After using a minimum amount of anesthesia and avoiding artifacts due to animal movements, injection of $^{99m}$TcO$_4$-MIBI (0.37 MBq) was administered to rats. Images of the parathyroid glands were acquired at 15 min, 1 h, 2 h, 3 h, and 4 h.

2.4. Bone histology

For bone histopathology, rat tibias were isolated, and the soft tissue was carefully removed with a scalpel. Bone samples were fixed in 10% neutral buffered formalin and decalcified in 10% (w/v) ethylenediaminetetraacetic acid (EDTA). Bone samples were then embedded in paraffin, sectioned and subjected to hematoxylin and eosin (H&E) stain [17].

2.5. Bone mineral density (BMD) by dual energy X-ray absorptiometry (DEXA)

The BMD of femurs and lumbar vertebrae was measured by DEXA using a bone mineral analyzer (HOLOGIC, America) at the Southeast University-affiliated Zhongda Hospital.

2.6. Micro-computed tomography (micro-CT)

Using micro-CT (SkyScan 1176), the bone volume fraction, calculated as the ratio of the bone volume to tissue volume (BV/TV, %) and the bone architecture (number, spacing, and thickness) were determined from trabecular bone isolated from the metaphysis of the distal femur. The cortical bone geometry (area and thickness) was determined from the femoral midshaft. The metaphyseal scanning region of the intact femur consisted of 100 slices beginning 1 mm proximal to and extending away from the growth plate. The diaphyseal region was located halfway between the femoral head and distal condyles and consisted of 100 slices around the center. A scanning resolution of 18 μm was chosen for all animals, a minimum amount of anesthesia was used and artifacts due to animal movements were avoided. Data were analyzed using CT Analyzer (SkyScan software). Three dimensional (3D) images and animations were created using CtVox (SkyScan software). Micro-CT parameters are reported according to international guidelines [18].

2.7. Bone mechanics

Structural mechanical properties and apparent material properties were assessed by uniaxial compression testing of the L5 vertebra (Instron 5943, America) as previously described. Prior to mechanical testing, the vertebral arch and endplates were removed. Samples were loaded at a rate of 0.5 mm/min, producing a force-displacement curve for each sample, and structural mechanical properties were obtained directly from these curves as previously described [19].

2.8. Statistical analysis

Data are expressed as the mean ± standard deviation (SD). Statistical analyses were conducted via one-way ANOVA by SPSS 21.0 statistical software. A value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Renal function and biochemical parameters

The biochemical data of the rats at 42 weeks of age are shown in Fig. 1. Compared with the CTL rats, rats in the CKD group developed severe renal failure, as evidenced by a significant increase in their Scr levels (462.82 ± 91.29 μmol/l vs 42.75 ± 9.48 μmol/l, $P < 0.05$, Fig. 1d). Serum calcium levels were lower (2.28 ± 0.25 mmol/l vs 2.52 ± 0.26 mmol/l, $P < 0.05$), whereas the levels of serum phosphorus (3.69 ± 0.16 mmol/l vs 2.72 ± 0.16 mmol/l, $P < 0.05$) and PTH (542.94 ± 98.51 pg/ml vs 75.90 ± 12.10 pg/ml, $P < 0.05$) were higher in the uremic rats than in the CTL rats (Fig. 1a–c, P < 0.05).

3.2. Parathyroid hyperplasia

Next, we assessed parathyroid function by ECT (Fig. 2). Normal parathyroid gland functions were observed in the CTL group. However, the radiation aggregation at 3 h indicated parathyroid adenoma with hyperparathyroidism in the CKD group. Elevated serum PTH levels and ECT evidence confirmed the success of the SHPT model in CKD rats.

3.3. Bone histology and histomorphometry

To further describe the bone disorders, we performed H&E staining of rat tibias at the time point of 42 weeks (Fig. 3a). Well-formed, normal, connected and mature bony trabeculae with a benign bone thickness were observed in the CTL group. However, widely separated, thin-walled trabecular bone containing bone marrow elements were observed in the CKD group, suggesting osteoporosis.

BMD measurements by DEXA are shown in Fig. 3b. The BMD of femurs and lumbar vertebrae were significantly reduced in the CKD group compared with that in the CTL group (femur: 0.216 ± 0.016 g/cm$^2$ vs 0.255 ± 0.019 g/cm$^2$, P < 0.05; vertebra: 0.294 ± 0.033 g/cm$^2$ vs 0.362 ± 0.040 g/cm$^2$, P < 0.05).

The trabecular and cortical bone parameters measured by micro-CT are shown in Fig. 3c–j. The CKD animals had lower trabecular and cortical bone parameters than the CTL animals. In the distal femur, the BV/TV (29.387 ± 5.130% vs 44.248 ± 9.892%, P < 0.05), the trabecular thickness (Th.Th, 0.102 ± 0.010 mm vs 0.119 ± 0.015 mm, P < 0.05), and the trabecular number (Th.N, 2.908 ± 0.402 1/mm vs 3.577 ± 0.730 1/mm, P < 0.05) were
Fig. 1. Analysis of (a) serum PTH, (b) Ca, (c) P and (d) Scr concentrations in the study groups. The data are expressed as the means ± SD (n = 10 for each group). *P<0.05 vs the CTL group. PTH, parathyroid hormone; Ca, calcium; P, phosphorus; Scr, serum creatinine; CTL, control group; CKD, chronic kidney disease group.

Fig. 2. ECT images of parathyroid glands. The CTL group: bilateral thyroids shown at 15 min; the 1-hr image clearly shows a thyroid with a regular edge; the thyroid shadow disappeared at 2 h, and a mild radioactive accumulation was shown at 2 h and 3 h, which suggested normal parathyroid glands. The CKD group: bilateral thyroid shown at 15 min; the 1-hr image clearly shows a thyroid with a regular edge; the thyroid shadow disappeared at 2 h, and the left parathyroid image shows radiation aggregation, while the 3-hr image is consistent with the 2-hr image. The persistent radiation aggregation suggests parathyroid hyperplasia. ECT, emission computed tomography.
decreased and the trabecular separation (Tb.Sp, 0.177 ± 0.023 mm vs 0.151 ± 0.030 mm, P<0.05) was increased in CKD rats (Fig. 3c–g). The cortical bone of the femoral midshaft was also negatively affected by CKD. The cortical area (Ct.Ar, 4.637 ± 1.566 mm² vs 6.335 ± 1.460 mm², P<0.05) and the cortical thickness (Ct.Th, 0.455 ± 0.173 mm vs 0.652 ± 0.136 mm, P<0.05) were smaller in the CKD rats than in the normal CTL rats (Fig. 3i–j). Their 3D animations were shown in video still.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.bbrc.2018.03.038.

The vertebral compression testing revealed that the CKD rats had lower values of ultimate force (251.256 ± 17.457 N vs 307.621 ± 55.750 N, P<0.05), work to failure (52.468 ± 10.975 mJ vs 112.398 ± 31.823 mJ, P<0.05), stiffness (502.196 ± 102.715 N/mm vs 962.848 ± 49.378 N/mm, P<0.05) and elastic modulus (337.631 ± 152.499 MPa vs 652.718 ± 103.491 MPa, P<0.05) than the normal CTL rats (Fig. 4a, c, d, h). There were no differences in total displacement, maximum stress, maximum strain, toughness, or elastic modulus (Fig. 4b, e–g).

4. Discussion

Patients with advanced CKD are extremely vulnerable to osteoporosis, which presents high risks for adverse outcomes, such as disability, worsening chronic illnesses and mortality [5,20–22]. SHPT is the most common cause of renal osteopathy in patients with CKD who are undergoing dialysis. Hence, animal models of SHPT are of vital importance for facilitating research on renal osteopathy. In the present study, we examined the feasibility of a rat model of adenine and high phosphorus-induced renal failure and subsequent SHPT pathology and bone disease.

In 1973, Ritz et al. introduced the term “mixed uremic osteodystrophy” [23]. After 33 years, the Kidney Disease: Improving Global Outcomes (KDIGO) introduced the term “CKD-mineral and bone disorder (CKD-MBD)” [24]. Even though more than ten years have passed, our understanding of renal osteopathy is far from satisfactory. Some researchers use subtotal nephrectomy to study SHPT [25]. However, these models require surgical skills and post-operative care, and the mortality rate is terribly high, which prompted us to develop a non-surgical model. There are some common non-surgical options to study CKD including radiation nephropathy and the administration of nephrotoxic drugs such as folic acid [26], cyclosporine [27,28] and cisplatin [29]. Nevertheless, these models have restricted use due to systemic toxicity. Genetic mouse models are also available, but these are restrictive due to the need for breeding and the expensive costs [30]. Considering all of these factors, we applied the adenine model to perform the current study. Hyperphosphatemia stimulates PTH secretion and parathyroid cell proliferation [31,32]. High phosphorus diets in uremic rats significantly increase the levels of serum PTH to over 3 times than that in uremic rats fed normal diets [33–35]. Recently, rats fed adenine and a high phosphorus diet were used as SHPT models in CKD for the evaluation of vascular calcification and renal fibrosis [14–16]. Our study is an important supplement to the existing models of CKD with SHPT for skeletal studies.

To our knowledge, there is no study that compares changes in the skeletal system via multiple methods. In our previous studies, we analyzed the vascular calcification and renal fibrosis in rats with chronic renal failure by feeding high phosphorus and adenine diets [15,16]. However, the skeletal analysis was still lacking. In the present study, we focused on the skeletal system in this CKD and SHPT model. An apparent bone loss was observed in CKD rats via...
H&E staining and BMD analysis (Fig. 3a and b). In addition, we analyzed the skeletal changes via micro-CT (a more precise metric) in the cortex, and the morphometric parameters showed decreased cortical thickness and cortical area in the CKD rats (Fig. 3c–g). These results might represent increased bone resorption due to high levels of serum PTH [36,37]. In the distal femurs, we observed noteworthy trabecular bone loss according to decreases in BV/TV, Tb.Th, and Tb.N and an increase in Tb.Sp (Fig. 3h–j). These observations likely result from hypocalcemia [21,38], the infiltration of bone marrow fat [39,40] and uremic toxins [41]. Some researchers have identified increased trabecular bone in renal failure models [42], which may be a consequence of the anabolism of trabecular bone stimulated by PTH [37]. Blood vessels are prevalent in cancellous bone, and our previous in vivo and in vitro studies demonstrated that elevated PTH can induce an endothelial-to-osteoblast transition via endothelial-mesenchymal transition (EMT) [15]. We might infer that elevated PTH levels could also promote trabecular bone formation via endothelial-to-osteoblast transition. The differential responses to PTH stimuli between cortical and trabecular bone still need further research. Various factors such as elevated serum PTH levels, vitamin D deficiency, hypocalcemia, and accumulation of uremic toxins can influence the skeletal metabolism in CKD-MBD. The net effect on bone tissue depends on the combined actions of CKD and MBDS, which requires further research. In addition, we found that CKD animals exhibited compromised mechanical properties (Fig. 4), which might be a consequence of reduced cortical and trabecular bone mass. Taken together, these data suggest that bone lesion rats fed an adenine and high phosphorus diet might serve as a reliable model to study skeletal disease in end-stage kidney disease with SHPT.

Furthermore, we first prolonged the duration of modeling to 34 weeks, which might better simulate the long course of CKD-MBD. As we known, it takes 2 months for animal models of CKD to display steady disease characteristics after the intervention (e.g., 5/6 nephrectomy or adenine administration), and it takes even longer (4–6 months) to establish SHPT in these models. In addition, it takes at least 5 weeks to complete a bone remodeling cycle in skeletally mature rats (nearly 6 months in humans). Some studies presented obvious bone loss in adenine-fed rats or rats that underwent a 5/6 nephrectomy within 4 or 6 weeks [43–45]. However, the previous course of disease was too short to complete bone remodeling in the CKD condition let alone establish SHPT. The short-term rat models could not serve as reliable models for studying the skeletal changes caused by SHPT. Considering these factors, we prolonged the study duration. In this study, we observed elevated serum PTH levels and parathyroid hyperplasia (Figs. 1a and 2). In addition, our rat model provides the advantage of simulating the human disease condition as much as possible.

In summary, we have established a CKD with SHPT rat model and analyzed the mineral and bone disorders in uremic rats with SHPT fed an adenine and high phosphorus diet. Since these rats showed severe abnormalities in the parathyroid glands and skeletal system, they might serve as useful models for the manifestations of parathyroid hyperplasia and bone disease in CKD with SHPT.

Fig. 4. Tissue-level mechanical properties of the vertebrae in the CTL and CKD rats (n = 5 for each group). The results are presented as the means ± SD; *P < 0.05 vs the CTL group.
Conflicts of interest

The authors report no conflicts of interest.

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