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Moderate chronic kidney disease impairs bone quality in C57Bl/6J mice

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ABSTRACT

Chronic kidney disease (CKD) increases bone fracture risk. While the causes of bone fragility in CKD are not clear, the disrupted mineral homeostasis inherent to CKD may cause material quality changes to bone tissue. In this study, 11-week-old male C57Bl/6J mice underwent either 5/6th nephrectomy (5/6 Nx) or sham surgeries. Mice were fed a normal chow diet and euthanized 11 weeks post-surgery. Moderate CKD with high bone turnover was established in the 5/6 Nx group as determined through serum chemistry and bone gene expression assays. We compared nanoindentation modulus and mineral volume fraction (assessed through quantitative backscattered scanning electron microscopy) at matched sites in arrays placed on the cortical bone of the tibia mid-diaphysis. Trabecular and cortical bone microarchitecture and whole bone strength were also evaluated. We found that moderate CKD minimally affected bone microarchitecture and did not influence whole bone strength. Meanwhile, bone material quality decreased with CKD; a pattern of altered tissue maturation was observed with 5/6 Nx whereby the newest 60 μm of bone tissue adjacent to the periosteal surface had lower indentation modulus and mineral volume fraction than more interior, older bone. The variance of modulus and mineral volume fraction was also altered following 5/6 Nx, implying that tissue-scale heterogeneity may be negatively affected by CKD. The observed lower bone material quality may play a role in the decreased fracture resistance that is clinically associated with human CKD.

1. Introduction

Chronic kidney disease (CKD) is recognized as a global health problem [1]. An estimated 7.2% of adults over age 30 and 23–36% of persons aged 64 and over have at least moderate kidney dysfunction [2]. Bone fragility is an important consequence of CKD. In a three-year study, patients with moderate CKD had a 1.6–2.4-fold increase in fracture prevalence compared with age-matched controls having mild or no disease. With severe CKD, fracture rate increases by 3.1–5.1-fold [3]. Moreover, the presence of moderate CKD increases fracture-related mortality by two-fold in patients over 70 years of age [4]. The mechanisms by which bone fragility develops in CKD are incompletely understood.

Although bone mineral density is used clinically to diagnose fracture risk, bone fragility in CKD cannot be explained by declines in bone mineral density alone [4]. However, a growing body of work across several models of bone disease demonstrates that bone strength and fracture resistance also depend on bone quality. Bone quality is the comprehensive state of tissue microarchitecture and bone material properties, including mechanical response, mineralization, collagen cross-linking, and accumulation of microdamage [5,6]. CKD is a disease of profoundly dysregulated mineral homeostasis; thus, changes in bone mineralization and other parameters of bone quality may be expected [7,8].

Few previous studies have investigated bone quality in CKD. Some of these prior works employed 5/6th nephrectomy (5/6 Nx), which is well-established to produce moderate CKD with high-turnover osteodystrophy that mimics key aspects of human kidney dysfunction [9,10]. Bone quality has been observed to decrease after 5/6 Nx;

Abbreviations: CKD, Chronic Kidney Disease; 5/6 Nx, 5/6th nephrectomy; TPTx, thyroparathyroidectomy; qBSE, quantitative backscattered scanning electron microscopy; BUN, blood urea nitrogen; PTH, parathyroid hormone; BV/TV, trabecular bone volume fraction; Tb.N, trabecular number; Tb.S, trabecular spacing; Th.Th, trabecular thickness; Conn.D, connectivity density; TMD, total mineral density; Ct.Th, cortical thickness; BA, cortical bone area; TA, cortical total area; Ct.Po, cortical porosity; I_{max}, moment of inertia about the anterior–posterior direction; I_{min}, moment of inertia about the medial–lateral axis; C_{min}, distance between the centroid and bone surface in the anterior–posterior direction; *Alpl*, gene for alkaline phosphatase; *Ibsp*, gene for bone sialoprotein; *Bglap*, gene for osteocalcin; *Col1a1*, gene for type I collagen α 1 chain; MAR, mineral apposition rate; PMMA, poly(methyl)methacrylate; minVf, mineral volume fraction; Endo.P, endosteal perimeter; Peri.P, periosteal perimeter; Endo.A, endosteal area; Peri.A, periosteal area; Cort.A, cortical area.

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diminished bone microarchitecture (BV/TV, Tb.N, Conn.D, and increased Tb.Sp) was observed for tibial trabecular bone in Crlj:CD1 mice 16 weeks following 5/6 Nx [11]. Microscale cortical bone material properties have been assessed after 5/6 Nx, but results for these studies are in disagreement about whether bone material quality is diminished or preserved. In a complex model of CKD (5/6 Nx with additional thyroparathyroidectomy (TPTx) in rats), bone developed a low-turnover response while Raman Spectroscopy revealed higher mineral-to-matrix ratio, carbonate-to-phosphate ratio, collagen maturity, and pentosidine-to-amide ratio in cortical bone compared with TPTx alone [12]. In a subsequent study by the same group, rats were given either 5/6 Nx alone or alongside TPTx. 5/6 Nx produced high-turnover bone response as well as increased mineral-to-matrix ratio, while 5/6 Nx and TPTx resulted in low bone turnover and decreased mineral to matrix ratio. Increased pentosidine-to-matrix ratio, and decreased crystallinity and degree of orientation of the c-axis of bone mineral crystals were general to both uremic models [13]. Meanwhile, Kadokawa et al. did not observe changes in FTIR-observed mineral-to-matrix ratio, mineral maturity, collagen maturity, or indentation modulus in mice after 5/6 Nx compared with sham. Critically, these studies all employed microscale materials characterization techniques (*e.g.*, nanoindentation, FTIR, Raman Spectroscopy) within the middle of the cortical thickness [11–13]. Yet new rodent bone is apposited primarily on periosteal and endosteal surfaces [14,15]. It is possible that the inconsistent observation of bone material changes after 5/6 Nx results from surveying bone that predominantly existed prior to nephrectomy; that is, material property assessment regions of interest were not necessarily placed on bone actively forming under the influence of CKD.

Bone material quality has not been studied in rodent cortical bone for tissue apposited after nephrectomy. In this study, we evaluate the influence of moderate CKD established *via* 11 weeks of 5/6 Nx in C57Bl/6J mice, a low bone density, well-characterized inbred mouse strain. We seek to characterize how bone microscale material properties may diminish in bone established during CKD, and, together with microarchitecture and whole bone strength, describe bone quality in a mouse model of moderate CKD with high bone turnover.

2. Materials and methods

2.1. Specimens

Eight-week-old male C57Bl/6J mice (n = 26) were obtained from Jackson Laboratories (Catalog number 000,664, Bar Harbor, ME). Mice were maintained on a 12-h light/12-h dark cycle and housed (n = 5 max) in polycarbonate cages with standard bedding. Mice were fed a normal rodent chow diet with water freely available. We randomly assigned mice to sham (n = 12) or 5/6 Nx (n = 14) groups. The 5/6 Nx group underwent a two-stage nephrectomy procedure; at 10 weeks of age, 2/3 of the right kidney was ablated, followed by complete ablation of the left kidney one week later. The control group received sham operations with the same timeframe. Mice were anesthetized using 1.5% isoflurane during procedures. A post-operative dose of buprenorphine (0.5 mg/kg) was administered after surgeries and prior to recovery and every 12 h for the following 2 days. Calcein (10 mg/kg) and tetracycline (20 mg/kg) were administered *via* intraperitoneal injection two weeks and two days before euthanasia, respectively. Mice were euthanized at 11 weeks following the second procedure by exsanguination and midline thoracotomy. Following euthanasia, femurs, tibiae, and humeri were harvested. The right femur diaphysis was cleaned of soft tissue and marrow, and then snap-frozen and stored at −80 °C for gene expression. Other harvested bones were stored in phosphate - buffered saline (PBS) soaked gauze at −20 °C until analyses or embedding. All animal procedures were approved by the Institutional Animal Use and Care Committee at the University of Colorado School of Medicine. Investigators were blinded to specimen treatment status for methods described in §2.2-§2.6. For methods described in §2.7 – §2.9, specimens were randomly selected from each treatment group.

2.2. Serum chemistry

Serum biochemistry analyses were performed on blood drawn at sacrifice. Plasma creatinine concentrations were determined using kits from BioAssay System (Hayward, CA). Blood urea nitrogen (BUN), plasma phosphate, and plasma calcium levels were measured with kits from Stanbio Laboratory (Boerne, TX). Intact plasma parathyroid hormone (PTH) level was measured with kits from Immutopics (San Clemente, CA). Phosphate, calcium, and creatinine assays were performed for sixteen samples each (5/6 Nx: n = 8, sham: n = 8). BUN and PTH were assessed for twelve samples (5/6 Nx: n = 6, sham: n = 6), and eleven samples (5/6 Nx: n = 5, sham: n = 6), respectively.

2.3. qPCR

Relative gene expression was performed using quantitative real-time polymerase chain reaction (qPCR). Frozen femurs were powdered with two 30-s pulses at 2600 rpms per manufacturer's instructions (Sartorious mikro-dismembrator S). RNA was extracted and cDNA prepared as previously described by King et al. [16]. The fast SYBR green qPCR method (Bio-Rad CFX connect real-time system) was used with the primer sequences listed in Table 1. All primers were designed and then individually validated to the MIQE guidelines of amplification efficiency between 90% and 110% [17]. Conditions were set at an initiation temperature of 95 °C for 20 s, followed by 40 cycles of denaturing at 95 °C for 3 s and annealing at 60 °C for 30 s. Following this DNA amplification, a melting temperature sequence was used to determine size of amplicon. Data were analyzed using the $\Delta\Delta C_q$ method for relative fold change (Eqs. (2.3.1)–(2.3.3)) with 18 s as the reference gene. For this method, C_q is defined as the number of cycles necessary to reach the threshold cycle of target amplification, and ΔC_q is the difference between the number of cycles to threshold for the gene of interest and the reference gene. Smaller values for ΔC_q indicates the presence of more copies of RNA, because less time is necessary to reach the peak of the reverse transcription reaction. $\Delta\Delta C_q$ is the difference between ΔC_q for 5/6 Nx and sham, and its value has reverse orientation of the fold change.

$$\Delta Cq = Cq_{gene\ of\ interest} - Cq_{18s} \tag{2.3.1}$$

$$\Delta\Delta Cq = \Delta Cq_{5/6Nx} - \Delta Cq_{Sham} \tag{2.3.2}$$

$$Fold\ change = 2^{-\Delta\Delta Cq} \tag{2.3.3}$$

2.4. μCT

Left tibiae were cleaned of non-osseous tissue and stored in 70% ethanol at 4 °C. Left femurs were cleaned and fresh frozen in PBS - soaked gauze at −20 °C. The femurs were defrosted at 4 °C overnight before micro-computed tomography imaging (μCT). Microarchitecture was

Table 1
Primer sequences used for mRNA analysis.

Gene	Forward sequence	Reverse sequence
18s	CGCCGCTAGAGGTGAAATTCT	CGAACCTCCGACTTTCGTTCT
Alpl	GAGGGACGAATCTCAGGGTA	TTTCAAGGTCTCTTGGGCTT
Ibsp	CTTACCGAGCTTATGAGGATGAAT	AAATGGTAGCCAGATAAGACAG
Bglap	AGACAAGTCCCACACAGCAG	TTGGACATGAAGGCTTTGTC
Col1a1	GCTCCTCTTAGGGGGCACT	CCACGTCTCACCATTGGGG

Alpl = gene for alkaline phosphatase; *Ibsp* = gene for bone sialoprotein; *Bglap* = gene for osteocalcin; *Col1a1* = gene for type I collagen α1 chain.