The Biomechanical Testing for the Assessment of Bone Quality in an Experimental Model of Chronic Kidney Disease

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Abstract
Mineral metabolism disturbances are common in chronic kidney disease (CKD) and have been classified as a new clinical entity, also known as CKD-mineral and bone disorders (CKD-MBD). A decrease in the bone strength, whose clinical manifestation is a tendency for fracture, has been recognized as an important component of CKD-MBD. Because of ethical issues, measurements of the bone strength in the human body are usually limited to noninvasive techniques, such as radiography, dual-energy X-ray absorptiometry and the assays of bone turnover biomarkers. However, it has been postulated recently that the evidence concerning bone strength based solely on the determination of the bone quantity may be insufficient and that bone quality should also be examined. In this regard, an animal model of CKD can represent an experimental tool to test the effectiveness of new therapeutic strategies. Despite the many available methods that are used to diagnose metabolic bone disorders and predict fracture risk especially in small rodents with CKD, it turns out that the most appropriate are biomechanical tests, which can provide information about the structural and material properties of bone. The present review summarizes and discusses the principles for carrying out selected biomechanical tests (3-point bending test and compression test) and their application in clinical practice.

Introduction
Chronic kidney disease (CKD) is a clinical entity characterized by a gradual loss of kidney function, which is manifested by abnormal biochemistries, renal osteodystrophy and extraskeletal calcification \cite{1, 2}. Disturbances in mineral metabolism are common during the course of CKD and have been classified as a new clinical entity known as CKD-mineral and bone disorders (CKD-MBDs), which is characterized by hyperphosphatemia, secondary hyperparathyroidism and increased risk of fracture \cite{3, 4}. Some studies confirmed that CKD-MBD appears to have a considerable impact on cortical bone properties, leading to reduction in the bone mass and porosity \cite{4–6}. Moreover, the increased risk of fracture in the case of CKD is due to abnormalities in the bone quan-
tity and quality [7]. Despite the well-known pathogenesis of CKD-MBD, there is currently no effective therapeutic strategy for the treatment of these disturbances. Therefore, it is reasonable to use appropriate animal models to evaluate the effectiveness of treatment of renal osteodystrophy, which is a consequence of CKD. Various animal models, including surgically and diet-induced and/or genetic manipulation, are used to study CKD [3, 8–10]. Nevertheless, the most commonly used is a rat model with progressive renal failure model, also named as the ‘remnant kidney’ or 5/6 nephrectomy [11, 12]. It is worth noting that this classical model of subtotal nephrectomy closely mimics human CKD, and for this reason, several experimental and novel therapies have been developed using this model [11, 13]. A subtotal nephrectomy model leads to alterations in the bone quantity and quality, which can be evaluated using a variety of diagnostic techniques. Despite the many available tools that are used to diagnose bone metabolism and predict fracture risk, the most frequently applied is dual-energy X-ray absorptiometry (DEXA). This method enables the assessment of bone mass changes, characterized by bone mineral density and bone mineral content [14]. However, some studies suggested that bone mass measurement by DEXA does not always reflect bone health in the case of CKD [15–17]. Additionally, Cannata-Andiá et al. [18] did not observe strong correlation between DEXA and bone fractures in patients with CKD. Because geometric distribution and bone quantity are very important determinants of its mechanical strength [19], the skeletal integrity should be assessed by the biomechanical tests, in which the bones are examined as structures [20]. Therefore, irregular densitometry tests that are carried out on small rodents (mice, rats) with CKD should be complemented by biomechanical testing, which reflects the bone quality exactly, and is also a simple, affordable and reliable method of assessing bone health. Moreover, these methods are currently used as standards to investigate the effect of pharmacological intervention or genetic manipulation on bone properties [21]. Apart from that, Fridoni et al. [22] used the 3-point bending test to assess the effects of pulsed wave low-level laser therapy on the cortical bone in 2 experimental models of osteoporosis in rats. Despite the plurality of the methods for the assessment of the bone quality in the course of CKD, biomechanical testing is still scarce although it is able to provide reliable and important information about structural mechanical properties (ultimate load, stiffness, energy to fracture) and apparent material properties (ultimate stress, elastic modulus, toughness).

The main goal of this article is to present selected biomechanical tests (3-point bending test, compression test) that can be recommended for evaluation of bone biomechanics in rodents with CKD. Our goal is to discuss their advantages with special emphasis on how to choose working conditions in an appropriate manner to achieve the clinical relevance of biomechanical test results obtained from the investigated small rodents.

**Biomechanical Testing – General Considerations**

**Types of Biomechanical Testing**

The basis for classification of biomechanical testing is the load direction of various forces that may vary as the following 5 different types: compression, tension, torsion, curvature or shear. Each of the listed types of forces can be evaluated by an appropriate test. The first of these 3 tests are commonly used to assess the biomechanical properties of bone loaded in one direction [23]. Particularly noteworthy is an example of a bending test involving 3-point loaded directions: compression, tension and shear strength. Figure 1 shows the various types of load directions using the example of the femur. The effect of the applied strength on the bone, such as compression strength, causes shortening and extension, while a tensile strength leads to narrowing and elongation, whereas shear strength creates angular distortion [23].

**Factors Determining the Choice of an Appropriate Test**

It is worth emphasizing that biomechanical testing has limited application in in vivo conditions because it can only be applied to examine the bones that were previously isolated from the body tissue. Additionally, numerous studies confirm that animal models are applicable to study the structure and strength of the bone in many aspects (especially renal osteodystrophy and osteoporosis) of which endurance tests are widely used in small animals [24, 25].

On the other hand, the size of small animals that refers to the size of the bones is the main limiting factor for biomechanical testing. In accordance with the overall principle, the smaller the sample size, the fewer tests can be done on the bone. Therefore, from all of the strength tests listed above, only the bending test and compression test are able to measure the mechanical properties of bones in these small rodents. As it turns out, at least 5 long bones can be selected from the entire skeleton of small rodents that are proper for the whole bone fracture testing, includ-
ing the femur, the humerus, the radius and the humerus, the third metatarsal, the radius and the tibia [26, 27]. Although, these different skeletal sites can produce variations to calculate bone material properties, they are still not ideal specimens for all the biomechanical testing, mainly due to their different shapes and sizes. Moreover, the biomechanical testing performed at the different skeletal sites is characterized by various proportions of the cortical and trabecular bone. Schriefer et al. [28] showed that the radius is an ideal bone for determination of the mechanical properties of the mouse skeleton using the 3-point bending test. Nonetheless, biomechanical testing of the long bones is commonly carried out on the femur, due to its suitable size and good accessibility during dissection as well as a well-documented and validated testing protocol [29]. Furthermore, the femur is a preferred bone to be used for bending tests because of the length-to-width ratio and consistent cross-sectional shape along the entire length [30]. The biomechanical tests are performed relatively less often on selected irregular bones, the example of which is the compression test using the 3rd and/or 4th lumbar vertebrae (L3 and/or L4) from small animals. Compression tests are performed in order to assess the changes in the properties of mechanical vertebrae in a state of progressive renal osteodystrophy and osteoporosis [31], for the assessment of impact of drugs on the vertebral condition during the mentioned diseases [32, 33], as well as the impact of toxic substances on the skeletal system [34].

Selection of an appropriate test mainly depends on the kind of bone being tested (cortical or trabecular bone), the age of the bone and its anatomical location, as well as on testing conditions. The cortical bone is typically found at the diaphysis, whereas the trabecular bone is found at the epiphysis. Additionally, all vertebrae are composed of the trabecular bone. To examine the biomechanical properties of cortical bones in small rodents, the diaphysis bending test should be performed, while in the case of the trabecular bone, the compression test of the femoral neck and vertebrae are recommended. Moreover, crucial issue is the age of the animals. Sharir et al. [35] observed that during bone growth in young animals, cortex substantially remains thinner, while the cross-sectional moment of inertia may increase. Interestingly, bone modeling processes in young animals causes an increase in the stiffness, although the bone wall remains thinner [35].

Testing Conditions

Furthermore, the conditions before and during the testing are also extremely important, more specifically temperature and bone hydration. It turned out that the most desirable surrounding temperature during the examination of biomechanical properties of specimen is 37°C; however, it is not a rule. Testing at room temperature (23°C) increases the Young’s modulus of bone by about 2–4%, compared to a test at 37°C [36]. It is apparent that freshness of the bone tissue is very relevant; therefore, it is recommended to keep bones specimens hydrated. Some of the studies indicate that prior to the biomechanical testing, bones were rehydrated overnight in 0.9% NaCl at room temperature [37], while others suggest that bones were slowly thawed at room temperature on the day before testing and kept wrapped in the saline-soaked
Another important aspect in the evaluation of the biomechanical parameters of bone is the differences in the body weight of the tested animals. Bozzini et al. [36] observed that in hypophysectomized rats, which demonstrated reduction in the body weight, some structural properties of the bone were changed compared to weight-matched normal rats. Similar results were obtained by Lambert et al. [39] who compared the structural properties of the bones before and after their adjustment to the body weight, and it turned out that some of the results were significantly different. Summarizing, in order to minimize the differences of structural properties that are observed between bones in animals with CKD with different body mass, a preferable approach is the standardization of these properties by relating them to some allometric variables (the body weight of animals, the bone ash content, the mid-diaphyseal cross-sectional area) [36].

**Bending Test**

As it was mentioned previously, the bending test is commonly used to measure the mechanical properties of the whole bones from rodents and other small animals. During the bending test the whole bone is loaded in bending until fracture [26]. Generally, there are 2 varieties of the bending test, namely the 3- and 4-point bending tests. In the case of the first test, the whole bone is positioned onto 2 supports, and a single-pronged loading device is applied to the opposite surface at a point precisely in the middle of the 2 supports [26, 27, 35]. The 4-point bending test is similar, except that the load is applied by the 2 loading prongs located equidistant from the midpoint [35]. The very relevant advantage of the 3-point bending test is its plainness, while its disadvantage is creating high shear stress near the midsection of the bone [37]. However, the 4-bending test requires equal force at each loading point, which is difficult to achieve in the case of the whole bone testing [37]. Therefore, the 3-point bending test is more frequently used to evaluate the biomechanical properties of the long bones [26, 37]. Instead, during the 3-point bending test, the span between the lower loaders and the radius of the curvature of the loading surface should be properly selected depending on the species and sex of animals, for example, 5 mm for the mouse femur, 15 mm for the rat female femur and 20 mm for the rat male femur [37, 38]. Figures 2a and 2b show the principles of the 3-point 4-point bending test, respectively. Figure 2c represents the span between lower loaders and the radius of the curvature of the loading surface.

**Compression Test**

The compression test is commonly used to assess the biomechanical properties of the trabecular bone, which is present in large quantities in the vertebrae. Moreover, the
compression test performed on vertebrae specimens more closely stimulates loading conditions to which the bone is exposed [26, 37]. In the case of rodents, the compression test is performed on the whole vertebra, causing the posterior wall of the bone spinal canal to be removed, in such a way as not to damage the vertebral body (fig. 3a). However, in some cases, it is eligible to perform the compression test of the vertebral body with the intact posterior processes, what is possible to achieve using custom loading platens that make contact only with the vertebral body (fig. 3b) [37].

**Outcome of Biomechanical Testing**

Biomechanical tests measure many parameters determining the integrity of the bone. However, prior to conducting tests, it is necessary to decide which parameters are to be measured. The vast majority of testing machines requires deformation of specimen, commonly referred as displacement [27, 37]. Nonetheless, all biomechanical tests are based on significant relationship between load applied to a structure and displacement in response to the load, and these parameters are recorded throughout the test [26, 37, 39]. Figure 4a shows the load–displacement curve, which represents extrinsic properties of the bone. In order to obtain information about intrinsic properties of the bone, the load is divided by the cross-sectional area of the specimen to produce stress, and displacement is divided by the original length to the load–displacement curve to produce strain [27, 37]. As shown in figure 4b, these actions lead to obtain the stress–strain curve.

According to the load–displacement curve, the slope of the elastic region represents the extrinsic stiffness, which is closely linked with the mineralization of the bone [26, 27, 37]. Furthermore, load–displacement curve provides information about other biomechanical properties such as ultimate load (F_u – force at fracture), work to fracture (W – area under the load–displacement curve) and ultimate displacement (dl) [27, 37]. Each of the aforementioned parameters reflect different properties of the bone, namely, ultimate load (F_u) reflects the general integrity of the bone structure, work to fracture (W) is the amount of energy necessary to break the bone.
and ultimate displacement (dl) is conversely related to the brittleness of the bone [37, 40]. Despite the fact that the stress–strain curve is similar to the load–displacement curve, it refers to the material properties of the bone. The slope of the curve within its elastic region is called Young’s modulus (E), which is a value measure of the intrinsic stiffness of the material [26, 37, 40]. Additionally, an area under the stress–strain curve, also named as energy absorption or modulus of toughness, is a measure of the amount of energy needed to cause fracture [37, 40]. Moreover, the stress–strain curve distinguishes the elastic and plastic strain regions which are separated by the yield point (fig. 4b). The yield point is rarely properly defined when testing bone specimen. However, the yield point is commonly defined as the point where the stress–strain curve begins to become nonlinear [37].

Biomechanical properties of the bones should not be interpreted on the basis of a single parameter, because it can lead to inappropriate conclusions. For instance, the bones of rats with osteoporosis are very stiff, and simultaneously very brittle, which causes reduced work to fracture and increased risk of fracture. Conversely, although the bones of young rats are poorly mineralized, they can maintain ductility, resulting in increased work to fracture (the bones of young animals can absorb more energy before failure compared to adults) [26, 41]. Furthermore, the structural and material properties of bone should be taken into account in order to analyze its biomechanical properties. Parameters such as ultimate load, stiffness and energy to fracture are used to describe the structural properties of bones and characterize the tissue in its intact form. These structural properties must be understood in order to predict the behavior of a tissue in vivo [42]. On the other hand, the material properties (ultimate stress, elastic modulus, toughness) characterize the behavior of the material that comprises the tissue. Nevertheless, the results of biomechanical testing may vary depending on the analyzed disease and should be compared to a control group of animals.

Currently, there are limited data concerning application of biomechanical tests in CKD animals. It seems that it would be reasonable to apply biomechanical testing in experimental animal models of CKD as it would be an important step toward expanding the knowledge of CKD-MBD entity. Moe et al. [7] performed a 3-point bending test on the left femur in rats with autosomal dominant polycystic kidney disease, which is associated with the development of CKD. They observed a decrease in ultimate load (F_u) and work to fracture (W) and an increase in stiffness in these rats compared to normal age-matched rats. Most recent studies suggest that the biomechanical properties of bone depend strongly on the serum concentration of the parathyroid hormone (PTH) in the examined animals. Therefore, elevated serum concentration of PTH in rats with CKD causes a decrease in ultimate load (F_u), work to fracture (W), stiffness and ultimate stress (σ_u), while a slight increase was observed in Young’s modulus (E). A slight decline in ultimate load, work to fracture and simultaneously lack of changes in the stiffness, ultimate stress and slight increase in Young’s modulus (E) in rats with lowered PTH concentration [2, 4]. Furthermore, Jokihaara et al. [41] observed changes in the bone mass and volumetric bone density, whereas a lack of differences in ultimate load (F_u), structural rigidity and work to fracture (W) of femoral neck and midshaft were demonstrated between rats with CKD induced by 5/6 nephrectomy and healthy controls. In turn, Iwamoto et al. [42] using the same animal model CKD demonstrated decreased stiffness, increased work to fracture, while ultimate load was unchanged.

As it was mentioned above, the most commonly used animal model of CKD is 5/6 subtotal nephrectomy, although currently there is a limited number of studies describing the effectiveness of biomechanical tests for the diagnosis of the bone condition in rats with CKD. Accordingly, all experiments carried out on animals with CKD in order to assess the bone quality should be additionally supported by biomechanical tests.

**Conclusion**

The evaluation of the bone quality in animals with CKD should be carried out using relevant biomechanical tests (the bending and compression test), which will provide information about the structural and material properties of bone. Selection of applicable biomechanical tests appears to be the best diagnostic strategy in the case of animals with CKD. The subtotal nephrectomy model closely mimics mild-to-moderate CKD in humans. In this regard, this model of CKD can represent an experimental tool to examine the effectiveness of new therapeutic strategies. Although the biomechanical test cannot be directly used in human CKD patients, it can be used in future studies in selecting effective treatment in animal models, which can be really beneficial and effective in the treatment of patients with CKD.
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