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A new tool to assess the mechanical properties of bone due to collagen degradation

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ABSTRACT

Current clinical tools for evaluating fracture risk focus only on the mineral phase of bone. However, changes in the collagen matrix may affect bone mechanical properties, increasing fracture risk while remaining undetected by conventional screening methods such as dual energy x-ray absorptiometry (DXA) and quantitative ultrasound (QUS). The mechanical response tissue analyzer (MRTA) is a non-invasive, radiation-free potential clinical tool for evaluating fracture risk. The objectives of this study were two-fold: to investigate the ability of the MRTA to detect changes in mechanical properties of bone as a result of treatment with 1 M potassium hydroxide (KOH) and to evaluate the differences between male and female bone in an emu model. DXA, QUS, MRTA and three-point bending measurements were performed on *ex vivo* emu tibiae before and after KOH treatment. Male and female emu tibiae were endocortically treated with 1 M KOH solution for 1–14 days, resulting in negligible collagen loss (0.05%; by hydroxyproline assay) and overall mass loss (0.5%). Three-point bending and MRTA detected significant changes in modulus between days 1 and 14 of KOH treatment (−18%) while all values measured by DXA and QUS varied by less than 2%. This close correlation between MRTA and three-point bending results support the utility of the MRTA as a clinical tool to predict fracture risk. In addition, the significant reduction in modulus contrasted with the negligible amount of collagen removal from the bone after KOH exposure. As such, the significant changes in bone mechanical properties may be due to partial debonding between the mineral and organic matrix or *in situ* collagen degradation rather than collagen removal. In terms of sex differences, male emu tibiae had significantly decreased failure stress and increased failure strain and toughness compared to female tibiae with increasing KOH treatment time.

Introduction

Bone is a composite material composed of highly substituted, poorly crystalline mineral (apatite) and a hydrated organic matrix, consisting mainly (~90–95%) of type I collagen. The remaining 5–10% of the organic matrix components consists of various non-collagenous proteins, proteoglycans and small molecules [1]. For the purpose of this study, the organic matrix will be considered as collagen only. Historically, many investigators have sought to understand the individual contributions of the mineral and organic phases to the mechanical properties of bone. Research has commonly been focused on the mineral, and it is accepted that the non-organic component of bone is the primary contributor to its strength and stiffness [1]. In contrast, the collagen of bone is generally considered to contribute to the toughness (energy to fracture) of the tissue, mitigating the brittleness of the mineral. However, recent work suggests that collagen also contributes to bone strength [2,3]. Moreover, denaturing the collagen or 'debonding' the collagen from the mineral phase compromises the composite structure and results in correspondingly significant decreases in the modulus of elasticity, ultimate stress and toughness [4,5].

Clinically, bone mineral density (BMD) measurement is a widely used, non-invasive means of identifying individuals considered to have a high risk of fracture. However, BMD measures the bone mineral areal density only; this is only one of a number of measurable contributors to bone strength and fracture risk. Bone strength is derived from both the amount of bone tissue present (quantity), which is related to bone density and size, as well as bone quality, which comprises the bone structure and material properties, both of which are affected by bone remodeling [6]. From a clinical perspective, the BMD may not accurately predict fracture risk, as it only assays one of the relevant parameters. For example, BMD could only account for approximately 16% of the reduction in fracture risk in an alendronate treatment study [7], suggesting that other factors were significant contributors to the efficacy of the treatment. BMD alone is therefore insufficient to predict skeletal fragility. A clinical tool that can incorporate a broader assessment of bone quality, and therefore more accurately predict fracture risk, would be extremely valuable in identifying at-risk patients.

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The most common method of measuring BMD is with dual energy x-ray absorptiometry (DXA), which uses differential attenuation of x-rays to quantify the mineral component of bone. Quantitative ultrasound (QUS) is another clinical measure of bone density. As with DXA, QUS provides an indirect measurement of fracture risk; in this case, using the speed of sound (SOS) and the broadband ultrasound attenuation through bone to characterize the tissue [8]. Both DXA and QUS are in widespread clinical use. However, neither technique has been demonstrated to adequately distinguish between fracture and non-fracture populations [9]. This is unsurprising since, as discussed above, bone fragility depends not only on mineral content but also on matrix properties, architecture and geometry [6] and therefore evaluating the mineral content of bone alone may be insufficient to predict changes in bone quality [3].

An alternative technology to assess bone quality is the mechanical response tissue analyzer (MRTA). The MRTA is a radiation-free, non-invasive instrument developed by NASA to investigate the effects of space travel on the mechanical properties of astronaut bones [9]. The MRTA measures the cross-sectional bending stiffness (*EI*) of long bones on the basis of their response to low-frequency vibration. *EI* is the product of the elastic modulus, *E*, and the areal cross-sectional moment of inertia, *I* [9]. Research has shown that the measured *EI* of a long bone is predictive ($R^2 > 0.9$) of the maximum strength of the bone [10] and thus *in vivo* measurements of *EI* can be used to assess bone strength. Furthermore, previous studies suggest that the measurement of *EI* has the potential to effectively evaluate fracture risk [10–12]. Finally, studies have demonstrated the ability of the MRTA to measure *EI* differences between various populations resulting from exercise [13,14], disease state [15,16] and age [17].

MRTA measurements in humans are limited to the ulna and the tibia because the shafts of these bones are relatively close to the skin. The tibia of the emu, *Dromaius novaehollandiae*, was selected as a model for this study as its size and approximately cylindrical shape is similar to human long bone, and they are therefore suitable for assessment by the MRTA, which was designed for humans. Furthermore, sexual dimorphism is apparent in emus, as females are notably heavier, larger and have a higher basal metabolic rate compared to males [18], providing a model system to evaluate differences between male and female bones. Finally, emu tibiae may be a suitable model for long human leg bones as the bipedal nature of emus is analogous to human locomotion [19,20]; the emu model has previously been used as a model for femoral head osteonecrosis [19].

The objectives of this study were two-fold: to investigate the ability of the MRTA to detect changes in bone mechanical properties induced by changes in the collagen matrix of emu bone, and to evaluate any differences between male and female emu bone. This was achieved through bone composition analysis, KOH treatment for various durations and finally, validation of the MRTA and comparison with DXA and QUS in predicting these induced changes on the mechanical properties of emu tibial bone.

Materials and methods

Emu bone samples

Leg bones from male and female emus were obtained from slaughterhouses and farms in southern Ontario, Canada. The animals were approximately 3–5 years of age, and therefore skeletally mature [20,21]. The tibiae were carefully separated from the femora and tarsometatarsi with a scalpel. The shaft of each tibia was isolated by using a circular saw to remove the ends (15% of the total bone length from the proximal end and 10% from the distal end), resulting in bone samples 26 to 32 cm in length. The marrow and trabecular bone from the diaphysis of the tibiae were removed by drilling longitudinally through the bone shaft, after which the medullary canal was flushed with tap water. Finally, the skin and overlying tissue was carefully removed with a scalpel. The prepared bones were then individually wrapped in saline-soaked gauze and frozen at −20 °C until use.

All bone samples were allowed to thaw at room temperature for 3 h prior to analysis. Bones were divided into two groups: the first group ($n = 40$) was used for bone composition analysis and the second group ($n = 120$) was used to evaluate the effects of different degradation times on the measurements made by various bone analysis techniques. Within these two groups, the bones were further subdivided into male and female groups.

Bone composition

The four major constituents of bone—water, fat, mineral and collagen—were quantified to provide the overall composition of emu bone. One-centimeter wide sections were cut from the midshaft of each tibial shaft from male ($n = 20$) and female ($n = 20$) emus, using a circular saw. One section from each shaft was used to quantify each component.

The water content of emu bone was determined by weighing the samples before and after drying overnight in an oven at 105 °C. The percentage weight change was determined and ascribed to the water content. All other measurements were expressed with respect to the 'dry weight' of bone.

Fat was removed from the dry bone samples using 99.5% acetone (Fisher Scientific, Pittsburgh, PA). Individual bone samples were placed in plastic cassettes and immersed in acetone, while being agitated on a Vibromax Shaker (IKA Process Equipment, Wilmington, NC). After 30 min, the acetone was exchanged for fresh acetone. This was repeated five times, resulting in a three-hour extraction. The samples were then oven-dried for 30 min at 105 °C, reweighed and the percentage weight change recorded and attributed to fat content.

The mineral content in the emu bone was determined by weighing the dry samples before and after ashing overnight in a furnace (Model F46120CM Barnstead, Thermolyne Corporation, Dubuque, IA) at 800 °C. The percentage of weight remaining after ashing was attributed to mineral content.

The average initial collagen content in the emu bone was then calculated from the weight loss induced by ashing. Initial collagen content was also measured independently using a hydroxyproline assay. This colorimetric assay is an established method of determining collagen content in cartilage and soft tissue [22–24] as well as in bone [25]. Hydroxyproline was quantified according to the assay described by Reddy and Enwemeka [22], which involves four stages: digestion of the collagen, hydrolysis of the peptides, color development and microplate absorbance measurement. Hydroxyproline assay was performed on dried, defatted and demineralized bone specimens that were digested in papain (Sigma-Aldrich, Milwaukee, WI) at 65 °C for 96 h. After digestion, the bone samples were hydrolyzed in 6 N hydrochloric acid (Fisher Scientific, Pittsburgh, PA), incubated in a block heater at 110 °C for 18 h and then neutralized using sodium hydroxide (Fisher Scientific, Pittsburgh, PA). Next, the samples were prepared for colorimetric analysis with the addition of 0.05 N Chloramine-T (Sigma-Aldrich, Milwaukee, WI), 3.15 N Perchloric acid (Fisher Scientific, Pittsburgh, PA) and Ehrlich's Reagent (Sigma-Aldrich, Milwaukee, WI). The colorimetric reaction was quantified with a μ-Quant Microplate Spectrophotometer (BioTek Instruments, Winooski, VT) at 560 nm. It was assumed that 10% of the protein in emu bone collagen is hydroxyproline [22,26]. Absorbance values were plotted against the concentration of standard hydroxyproline (0–5 μg) and the quantity of hydroxyproline in the bone samples was determined from the standard curve.

KOH treatment

The endocortical lumens of whole emu tibiae were filled with 1 M potassium hydroxide (KOH) (Fisher Scientific, Pittsburgh, PA) to