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Ovarian hormone depletion affects cortical bone quality differently on different skeletal envelopes

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ABSTRACT

The physical properties of bone tissue are determined by the organic and mineral matrix, and are one aspect of bone quality. As such, the properties of mineral and matrix are a major contributor to bone strength, independent of bone mass. Cortical bone quality may differ regionally on the three skeletal envelopes that compose it. Each of these envelopes may be affected differently by ovarian hormone depletion. Identifying how these regions vary in their tissue adaptive response to ovarian hormones can inform our understanding of how tissue quality contributes to overall bone strength in postmenopausal women. We analyzed humeri from monkeys that were either SHAM-operated or ovariectomized. Raman microspectroscopic analysis was performed as a function of tissue age based on the presence of multiple fluorescent double labels, to determine whether bone compositional properties (mineral/matrix ratio, tissue water, glycosaminoglycan, lipid, and pyridinoline contents, and mineral maturity/crystallinity) are similar between periosteal, osteonal, and endosteal surfaces, as well as to determine the effects of ovarian hormone depletion on them. The results indicate that mineral and organic matrix characteristics, and kinetics of mineral and organic matrix modifications as a function of tissue age are different at periosteal vs. osteonal and endosteal surfaces. Ovarian hormone depletion affects the three cortical surfaces (periosteal, osteonal, endosteal) differently. While ovarian hormone depletion does not significantly affect the quality of either the osteoid or the most recently mineralized tissue, it significantly affects the rate of subsequent mineral accumulation, as well as the kinetics of organic matrix modifications, culminating in significant differences within interstitial bone. These results highlight the complexity of the cortical bone compartments, add to existing knowledge on the effects of ovarian hormone depletion on local cortical bone properties, and may contribute to a better understanding of the location specific action of drugs used in the management of postmenopausal osteoporosis.

1. Introduction

A major cause of osteoporosis is loss of ovarian hormones (especially estrogen) that are important to bone metabolism in both men and women [1,2]. They are known to regulate skeletal homeostasis through effects on all three major bone cells (osteoblasts, osteoclasts, and osteocytes) [3], and differently on the four skeletal envelopes [4]. Estrogen deficiency has been shown to modulate the effect of mechanical strain through interference with the osteogenic response to signals from strain-sensitive cells [2,5,6], potentially resulting in bone fragility. Because it affects different cell populations in different ways [2,6–11], its effects on the physical properties of the tissue matrix may vary regionally on different skeletal envelopes.

Osteoporosis is characterized by imbalanced bone remodeling due to hormonal changes coupled with decreased mobility due to aging and genetic susceptibility [2,12]. Several animal models have been used in the study of postmenopausal osteoporosis. The ovariectomized rat model is the most common due to the low cost and the ease of animal handling [13]. On the other hand, this animal model lacks the Haversian canal system present in human cortical bone, thus cortical porosity changes cannot be measured. Additionally, ovariectomy does not considerably alter cortical thickness in this animal model as endosteal resorption is balanced by periosteal formation [13]. The dog model does not suffer from these limitations as it possess Haversian systems, and the intracortical remodeling activity is similar to that in humans. On the other hand, a large part of estradiol in dogs is not of ovarian origin, so bone loss following OVX is small. Moreover, periods of luteal activity are separated by 6 months, which is most likely the culprit behind the inconsistent response of ovariectomy on the dog skeleton [13,14].

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Pigs have Haversian systems and a continuous estrous cycle, but sparse information regarding effects of ovariectomy in adult animals is available [13]. Similarly, sheep are polyestrous animals with Haversian systems, but their skeleton is subject to seasonal variations [13]. On the other hand, nonhuman primates (monkeys) do not suffer from any of these drawbacks. They are genetically close to humans, have menstrual cycles and a menopause, and their immune system is similar to humans. Although menopause occurs much later chronologically compared to humans, this can be offset through ovariectomy [13].

Features of postmenopausal osteoporosis include trabecular bone loss and vertebral fractures. On the other hand, 80% of osteoporotic fractures are non-vertebral, taking place in anatomical locations where cortical bone is predominant [15–17]. This has prompted renewed interest in the complexities of cortical bone and its role in fracture risk [15–25]. Cortical bone formation occurs at three anatomical surfaces, namely, periosteal, osteonal and endosteal, which exhibit distinct biological traits. While the first undergoes primarily modeling throughout life, the other two surfaces participate in the remodeling process, with observed age-related cortical bone loss due mostly to intracortical rather than endocortical remodeling [16]. Compared to endosteal osteoblasts, periosteal osteoblasts are characterized by greater responsiveness to osteogenic compounds such as parathyroid hormone, higher expression levels of certain proteins such as periostin, and more estrogen alpha receptors [26–31]. Periosteal bone formation rate and responsiveness to hormones and cytokines diminishes with age [26], whereas endocortical mechano-responsiveness is better conserved with advancing age [32].

In addition to the amount of bone mineral (usually expressed as bone mineral density determined by DXA, BMD), bone strength is widely accepted to be dependent on bone quality, including characteristics of the mineral crystallites and the organic matrix [33]. Moreover, it has been shown that in conditions with prevalent fragility fractures bone quality is altered at bone forming surfaces regardless of concomitant changes in BMD [34–43]. Given the importance of cortical bone properties in the determination of bone strength as well as their variability due to anatomical location and tissue age, we used Raman microspectroscopic analysis to investigate cortical bone properties in a monkey nonhuman primate model at specific tissue ages. Specifically, we analyzed the humerus from cynomolgus monkeys that had received three double fluorochrome labels (calcein, alizarin red, tetracycline) [44] to investigate (a) whether mineral/matrix ratio, mineral maturity/crystallinity, and glycosaminoglycan, lipid, tissue water, and pyridinoline content are similar at all three bone forming cortical surfaces of healthy animals; (b) whether the rates of change (kinetics) in the values of these parameters are similar as a function of tissue age and cortical forming surfaces; and (c) the effect of ovarian hormone depletion on these properties at the specific anatomical surfaces and tissue ages. Identifying differences in tissue quality on different cortical bone forming surfaces could improve our understanding of how these contribute differentially to overall changes in bone strength in aging and in postmenopausal women.

2. Materials & methods

2.1. Animals

Details of the animals from which the bone tissue was analyzed in the present study have been published elsewhere [44]. All procedures were approved by the Animal Care and Use Committee of the Bowman Gray School of Medicine at Wake Forest University and conformed to institutional and NIH guidelines. Briefly, natural habitat-derived adult female cynomolgus monkeys (*Macaca fascicularis*) were imported from Indonesia and quarantined for 3 months. Monkeys were screened radiographically to ensure absence of open growth plates and skeletal abnormalities. Double fluorochrome labels were given at 6 months (tetracycline HCl, 25 mg/kg, intravenously [iv]), 15 months (alizarin complexone, 20 mg/kg, iv), and before death at 18 months (calcein, 10 mg/kg, iv) using a 1-12-1 schedule, with death 7 days after administration of the final label [44]. In the present study, bone from SHAM-operated animals (SHAM; $N = 10$) was examined and compared to bone from ovariectomized (OVX; $N = 10$) animals.

2.2. Raman analysis

Instrumental and spectral acquisition details have been published elsewhere [41,45,46]. The variables considered in the present study are:

i. The mineral/matrix ratio was expressed as the ratio of the integrated areas of the ν_2PO_4 (410–460 cm^{-1}) to the amide III (1215–1300 cm^{-1}) bands. Unlike BMD, it accounts for both the amount of mineral and organic matrix present in the volume analyzed, correlates with ash-weight measurements [47], and has been shown in rodents to correlate better with the bending stiffness and failure moment of the humerus compared to BMD [48].
ii. Tissue water content was approximated by the ratio of the integrated areas of the spectral slice 494–509 cm^{-1} (PMMA) to Amide III band [41,43]. This ratio is expected to be zero if there is no penetration of PMMA into the voxel of analysis.
iii. The relative glycosaminoglycans (GAG) content was calculated from the ratio of the integrated areas of the CH_3 (1365–1390 cm^{-1}) band representative of glycosaminoglycans [49], to the amide III (1215–1300 cm^{-1}) band. In Raman spectroscopy, the spectral signature of proteoglycans is due to GAG chains. In bone, chondroitin 4-sulfate constitutes about 90% of the total GAG content, and the predominant proteoglycans are biglycan and decorin [50]. Proteoglycans have been shown to act as negative modulators of mineralization and bone turnover, and also to be present in micro- and nano-porous spaces such as osteocyte lacunae and canalicular network [51–56].
iv. The relative lipid content was expressed as the ratio of the integrated area of the lipids band ~1298 cm^{-1}/amide III [57].
v. The mineral maturity/crystallinity (MMC) of the bone mineral apatite crystallites was approximated from the inverse of the full width at half height (FWHH) of the ν_1PO_4 (930–980 cm^{-1}) band [58,59]. This bone quality metric describes the chemical composition of the mineral crystallites (maturity), and by extrapolation their crystallinity (size and shape) [58,59].
vi. The relative pyridinoline content was calculated from the ratio of the Raman intensity at 1660 cm^{-1}/the integrated area of the Amide I band (~1620–1700 cm^{-1}) [35,41]. Pyd is a mature, non-reducible, trivalent collagen crosslink, abundant in mineralizing type I collagen [60].

All of the utilized Raman peaks have been previously shown to be independent of tissue orientation [45,61,62], and therefore we did not differentiate measurement sites by the birefringent characteristics of the bone tissue (i.e. light vs. dark osteons).

A commonly used parameter to quantify the individual activity of osteoblasts is mineral apposition rate (MAR) based on the average distance between two fluorescent labels administered at different times. This describes the advancement of the mineralizing front in the *xy* plane within a defined time-frame. Yet, bone formation is a 3D event. Raman spectroscopic analysis determines the mineral/matrix ratio in a voxel of ~$1 \times 1 \times 1$ μm^3. Since determining the areal new bone formed based on MAR provides only partial information on the volumetric bone formation process, in the present study we normalized the individual mineral/matrix values by Raman spectroscopy with the corresponding interlabel distance (Ir.L.Th) for all three fluorescent labels available, and compared the values within each group before and after normalization.

2.3. Anatomical area selection criteria

The following areas were analyzed:

a) Osteoid: defined as a surface with evident calcein labels, 1 μm distance from the mineralizing front, and for which the Raman spectra showed the presence of organic matrix but not mineral.