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Effect of Hormone Replacement Therapy on Bone Quality in Early Postmenopausal Women

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ABSTRACT

HRT is an effective prophylaxis against postmenopausal bone loss. Infrared imaging of paired iliac crest biopsies obtained at baseline and after 2 years of HRT therapy demonstrate an effect on the mineral crystallinity and collagen cross-links that may affect bone quality.

Several studies have demonstrated that hormonal replacement therapy (HRT) is an effective prophylaxis against postmenopausal bone loss, although the underlying mechanisms are still debated. Infrared spectroscopy has been used previously for analyzing bone mineral crystallinity and three-dimensional structures of collagen and other proteins. In the present study, the technique of Fourier transform infrared microscopic imaging (FTIRI) was used to investigate the effect of estrogen on bone quality (arbitrarily defined as mineral/matrix ratio, mineral crystallinity/maturity, and relative ratio of collagen cross-links [pyridinoline/deH-DHLNL]) at the ultrastructural level, in mineralized, thin tissue sections from double (before and after administration of HRT regimen; cyclic estrogen and progestogen [norethisterone acetate]) iliac crest biopsy specimens from 10 healthy, early postmenopausal women who were not on any medication with known influence on calcium metabolism. FTIRI allows the analysis of undemineralized thin tissue sections (each image analyzes a 400 × 400 μm^2 area with a spatial resolution of ~6.3 mm). For each bone quality variable considered, the after-treatment data exhibited an increase in the mean value, signifying definite changes in bone properties at the molecular level after HRT treatment. Furthermore, these findings are consistent with suppressed osteoclastic activity. (J Bone Miner Res 2003;18:955–959)

Key words: infrared microscopic imaging, bone quality, hormone replacement therapy, osteoporosis, iliac crest biopsy

INTRODUCTION

HORMONE REPLACEMENT THERAPY (HRT) has been shown to be an effective prophylaxis against postmenopausal bone loss.[1–4] The exact mechanism of this protective effect is still the subject of debate. In a study on osteoporotic women, it was reported that the main effects of cyclical HRT on bone remodeling was a 50% reduction in turnover as reflected in a 50% decrease in activation frequency.[5] No significant changes in bone multicellular unit (BMU) balance were demonstrable in the HRT group. Women with senile osteoporosis are characterized by osteoblastic insufficiency; thus, they may not display possible effects of HRT on osteoblast function (osteoporotic women showed an increase in bone resorption rate and mean wall thickness was reduced.).[6,7] In a recent study, it was shown that that bone remodeling in early postmenopausal women is characterized by progressive osteoclastic hyperactivity, which is reduced by cyclic HRT. Furthermore, the reduction of resorptive activity at the BMU level after HRT seems to precede the reduction in activation frequency evident in studies on older postmenopausal women.[8]

Loss of bone mass (bone mineral density [BMD]) is an important feature related to osteoporotic fractures, accounting for a significant portion of osteoporotic fracture risk.[9–11] Furthermore, BMD has been shown to correlate significantly with bone strength.[12–17] On the other hand, BMD alone does not determine whether an individual will sustain a fracture.[18,19] There is a considerable overlap between

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normal and osteoporotic populations.[20] Moreover, the correlation between BMD and fracture risk has been reported to be site-specific, because it has been reported to be less consistent for vertebral than for hip fractures.[17] It is becoming clear that when assessing fracture risk, in addition to BMD, factors such as geometry and bone mass distribution, trabecular bone microarchitecture, microdamage and/or increased remodeling density, genetics, body size, environmental factors, and changes in bone tissue properties should also be taken into account.[21]

In the present study, Fourier transform infrared imaging (FTIRI) analysis was used to evaluate bone quantity (expressed as mineral:matrix ratio) and quality (expressed as mineral crystallinity and collagen cross-link ratio) in mineralized, thin tissue sections from double (before and after administration of HRT regimen) iliac crest biopsy specimens from 10 healthy, early postmenopausal women who were not on any medication with known influence on calcium metabolism. The results of the present study are in agreement with previously reported results based on histomorphometry on the same biopsy specimens[8] and are consistent with an osteoclast suppression mechanism of action for the HRT regimen.

MATERIALS AND METHODS

Double iliac crest biopsy specimens from 10 healthy (total number of biopsy specimens = 20), early postmenopausal (cessation of menstrual bleeding within 6–24 months before inclusion) women, 45–55 years of age, who were not on any medication with known influence on calcium metabolism were obtained. The first biopsy was obtained before the administration of the therapeutic regimen, and the second after 2 years on a cyclic HRT (estradiol [2 mg]/norethisterone acetate [1 mg]) regimen (Trisequence; NOVO-Nordisk A/S, Copenhagen, Denmark). These biopsy specimens were part of a larger study that has already been reported.[8] The tissues, embedded in methylmethacrylate, were cut into ~5-mm-thick sections, and the trabecular bone was analyzed by FTIRI.

FTIRI spectral images were acquired on the BioRad (Cambridge, MA, USA) "Sting-Ray" system, consisting of a step-scan interferometer interfaced to a mercury-cadmium-telluride (MCT) focal plane array detector imaged to the focal plane of an IR microscope. Interferograms were simultaneously collected from each element of the 64×64 array to provide 4096 spectra at a spectral resolution of 8 cm^{-1}. In this configuration, each spectral image corresponds to an area of 400×400 μm^2, and each spectrum to an area of $\sim 6.3 \times 6.3$ μm^2.[22–25] Background imaging spectra were acquired under identical conditions with only the BaF_2 windows on which the sections were placed.

Three spectral images were acquired per section in the area of trabecular bone. A histological slide stained with Goldner's trichrome was provided for each biopsy examined in this study. Based on these, only trabeculae devoid of resorbing surfaces (as evidenced by the lack of resorption pits) were considered. For each spectral image, three parameters were calculated. The mineral/matrix ratio based on the integrated area ratio of the phosphate (900–1200 cm^{-1}) and amide I (1585–1700 cm^{-1}) peaks corresponds to ash-weight measurements.[26] Mineral crystallinity (calculated based on the spectra absorbance ratio at 1030 and 1020 cm^{-1}[22–24]) corresponds to the c-axis crystallite size and perfection as determined from X-ray diffraction line-broadening analysis. The ratio of two of the major mineralized tissues collagen cross-links (pyridinoline [Pyr] and dehydro-dihydroxylysinonorleucine [deH-DHLNL]) was calculated based on the spectra absorbance ratio at 1660 and 1690 cm^{-1}.[25] The results were expressed both as color-coded images (Origin 6.0; Microcal Software, Northampton, MA, USA) and as histogram population distributions, where the *x* axis is the range of the parameter under investigation and the *y* axis is the number of pixels in each image with a specific value in that range. Detailed methodology and technique validation have been published elsewhere.[22–25]

The sections were received and analyzed number-coded (no prior knowledge of pairs). The values from the three images/section and for each section were averaged for each parameter monitored, and the resulting mean value and SD were treated as a single statistical unit. At the conclusion of this, the number code was provided. Data were compared in two ways: (1) the average values and SDs among the two groups (encompassing all cases analyzed) for the three parameters monitored were compared before and after 2-year treatment with HRT using the Wilcoxon signed rank test, and (2) data obtained in paired sections from the same patient before and after treatment compared using Student's paired *t*-test (SigmaStat 2.03, SPSS, and JMP 4.0 and StatView 5.01; SAS Institute, Cary, NC, USA). Statistical significance was assigned for $p < 0.01$.

RESULTS

The data for all parameters examined for all the biopsy pairs analyzed are summarized in Table 1. Values are presented as mean and SD. Values that are statistically similar ($p > 0.01$) are listed in bold. In all 10 paired cases analyzed, the mineral/matrix ratio was higher after HRT administration in a statistically significant manner. Of the 10 cases analyzed, 8 exhibited greater mineral crystallinity values after HRT treatment, whereas the other 2 were statistically similar before and after treatment, albeit in both cases the values were higher after treatment. When collagen cross-links ratio (pyr/deH-DHLNL) were compared, 9 of the 10 cases analyzed exhibited greater collagen cross-link ratio values after HRT treatment, while the other was statistically similar before and after treatment, although the values after treatment were higher. When the data between the two groups were compared in a non-paired fashion, the mineral/matrix ratio, the mineral crystallinity, and the collagen cross-links ratio (pyr/deH-DHLNL) were different after 2-year HRT treatment in a highly statistical significant manner ($p \leq 0.001$). The specific values are listed in Table 2.

Figure 1 shows typical output of the FTIRI analysis for a randomly selected pair of biopsy specimens before and after HRT treatment. Each image represents a 400×400 μm^2 area, and the size of each pixel is $\sim 6.3 \times 6.3$ μm^2. Figure 1A is the calculated spatial distribution of the mineral/matrix ratio before (top) and after (bottom) treatment. The images are presented in pseudo-color scaling (blue = minimum, red = maximum). Figure 1B is the determined spatial distribution of mineral crystallinity (bone mineral

TABLE 1. SUMMARY OF STATISTICAL COMPARISONS OF THE MINERAL:MATRIX, MINERAL CRYSTALLINITY/MATURITY, AND COLLAGEN CROSS-LINKS RATIO MEASURES AMONG THE PAIRED BIOPSIES

Biopsy number	*Mineral:matrix*	*Mineral crystallinity index*	*Collagen cross-link ratio (pyr/deH-DHLNL)*
1849 vs. 2002	1.25 ± 0.05	0.52 ± 0.12	0.85 ± 0.13
	2.19 ± 0.04	0.81 ± 0.11	1.35 ± 0.11
3674 vs. 4539	1.15 ± 0.4	0.61 ± 0.13	0.89 ± 0.02
	2.5 ± 0.3	0.97 ± 0.22	1.37 ± 0.09
4682 vs. 4771	1.1 ± 0.5	**0.8 ± 0.11**	**1.0 ± 0.1**
	2.6 ± 0.3	**0.85 ± 0.28**	**1.25 ± 0.07**
4849 vs. 7045	1.2 ± 0.4	0.73 ± 0.13	0.97 ± 0.09
	2.1 ± 0.3	0.93 ± 0.18	1.11 ± 0.04
5026 vs. 5056	1.3 ± 0.3	0.62 ± 0.13	0.91 ± 0.03
	2.21 ± 0.26	0.88 ± 0.12	1.12 ± 0.03
5027 vs. 4505	0.79 ± 0.28	**0.71 ± 0.04**	0.92 ± 0.05
	2.26 ± 0.41	**0.79 ± 0.02**	1.31 ± 0.06
5378 vs. 4865	0.617 ± 0.22	0.59 ± 0.04	0.82 ± 0.05
	1.58 ± 0.27	0.85 ± 0.12	1.2 ± 0.4
6670 vs. 6293	1.1 ± 0.4	0.7 ± 0.02	0.6 ± 0.05
	2.41 ± 0.17	0.81 ± 0.02	1.21 ± 0.06
736 vs. 2525	1.73 ± 0.35	0.61 ± 0.03	1.02 ± 0.04
	2.69 ± 0.29	0.83 ± 0.04	1.32 ± 0.02
7640 vs. 8135	1.16 ± 0.15	0.78 ± 0.01	0.81 ± 0.05
	2.53 ± 0.15	0.82 ± 0.01	1.19 ± 0.01

Statistically similar values ($p > 0.01$) are presented in bold.

TABLE 2. SUMMARY OF STATISTICAL COMPARISONS OF THE MINERAL:MATRIX, MINERAL CRYSTALLINITY/MATURITY, AND COLLAGEN CROSS-LINKS RATIO MEASURES AMONG THE TWO GROUPS (BEFORE AND AFTER HRT THERAPY)

	Mineral:matrix	*Mineral crystallinity index*	*Collagen cross-link ratio (pyr/deH-DHLNL)*
Before	1.140 ± 0.296	0.667 ± 0.09	0.879 ± 0.121
After	2.307 ± 0.321	0.854 ± 0.057	1.243 ± 0.092

crystallite size in the crystallographic c-axis) before (top) and after (bottom) HRT treatment. The scaling of the color is the same as previously. Figure 1C is the computed spatial distribution of the collagen cross-link ratio (pyr/deH-DHLNL) before (top) and after (bottom) HRT treatment. Color scaling is the same as previously.

DISCUSSION

In a randomized, double-blind, clinical prospective trial comprising 35 women treated with either HRT (cyclic estradiol/norethisterone acetate) or placebo, histomorphometric studies on paired bone biopsy specimens obtained before and after 2 years of treatment were performed.[8] The current FTIRI study expands our understanding of the accompanying changes in the mineral and matrix (collagen) properties in a randomly selected subset of these patients and shows the correspondence and complementarity between histomorphometric and spectroscopic parameters.

It has become increasingly apparent that BMD is not the sole determining factor of fracture risk.[21,27,28] Additional factors should be taken into consideration. This has led to the rise in the use of the term "bone quality." This term is a broad, multifactorial one, encompassing factors such as geometry and bone mass distribution, trabecular bone microarchitecture, microdamage and/or increased remodeling density, genetics, body size, environmental factors, and changes in bone tissue properties. Two of the bone tissue properties are mineral crystallinity/maturity (bone mineral crystallite size in the crystallographic c-axis) and collagen cross-links ratio. The role of the former becomes apparent when the effect of fluoride therapy in osteoporotic patients on bone fragility is considered. Although fluoride increases bone mass, the newly formed bone may have reduced strength.[29] Analyses of fluoride-treated bones in humans and in animal models reveal changes in the distribution, crystallinity, and crystal habit of the bone mineral crystallites.[30–32] The importance of collagen intermolecular cross-links to the mechanical performance of bone is very apparent in lathyrism,[33,34] as well as in the pyridoxine-deficient chick animal model. In this B_6-deficient chick, the mineral in the cortical bone of the deficient animals remains unaltered (both quantity and composition) compared with controls, whereas the deficient animals exhibit higher amounts of extractable collagen and altered cross-links. The B_6-deficient animals exhibited a decreased fracture load and offset yield load. These data suggest that although proper cortical bone mineralization occurred, the alterations of the collagen and collagen cross-links resulted in changes to bone mechanical performance.[35]

The histomorphometric study already reported in the literature has shown that untreated women developed a progressively more negative balance at individual BMUs, while women on HRT preserved bone balance.[8] No significant differences in wall thickness between the two

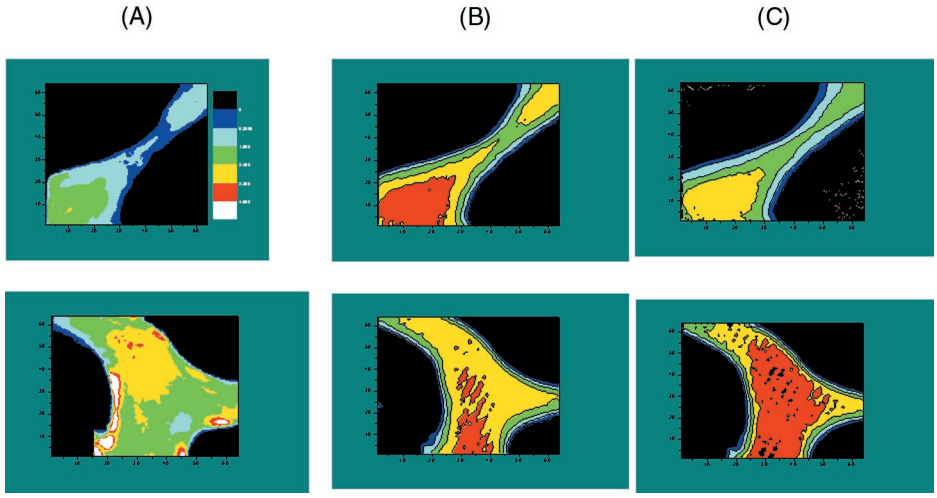

FIG. 1. Representative data of the spatial variation of the (A) mineral:matrix, (B) mineral crystallinity, and (C) collagen cross-links ratio parameters in a randomly selected pair of biopsy specimens before (top) and after (bottom) HRT treatment, in the area of trabecular bone. The data are presented as reconstructed color-coded images (each image represents a 400 × 400 μm^2 area, and each image pixel a ~6.3 × 6.3 μm^2 one; black = 0, blue = minimum, red = maximum).

groups were demonstrated, but the untreated women developed a pronounced increase morphometrically defined in erosion depth over 2 years, while the HRT group revealed no change. Furthermore, the placebo group displayed an increased osteoclastic erosion depth compared with unchanged values in the HRT group. While the placebo group revealed a slight increase in volume referent resorption rate, the HRT group revealed a pronounced decrease. No significant changes in marrow star volume (an index of trabecular perforations) were demonstrated in either group.

The FTIRI data obtained in a randomly selected subset of biopsy specimens revealed additional information about tissue properties alterations. Specifically, statistically significant increase in mineral/matrix ratios after treatment in all cases, increases in mineral crystallinity in 8 of 10 cases, and increases in collagen cross-link ratio in 9 of 10 cases, were demonstrated. These results suggest a prevalence of more mature bone[22–26,36–40] after the HRT regimen. Each of these parameters' value is known to increase with bone tissue age. Moreover, both mineral and collagen may mature even in the absence of direct cellular activity in biological environments.[41–43] Characteristics of more mature mineral and collagen include higher crystallinity/maturity and higher pyr/deH-DHLNL cross-links ratio, respectively. The results of the present study exhibit a shift in mineral crystallinity and collagen cross-links ratio toward higher values when paired biopsy specimens were compared before and after 2-year HRT treatment. This would be consistent with either diminished osteoblastic activity (less "young" tissue is being laid down thus the shift towards higher values) and/or diminished osteoclastic activity (the bone tissue is not resorbed as fast, thus stays around longer and both mineral and collagen mature causing the shift to higher values in mineral crystallinity and collagen cross-links ratio). Because, in the present study, trabeculae with bone forming and/or quiescent surfaces were analyzed, it is reasonable to assume that the observed differences observed in the mineral and collagen properties of the paired biopsy specimens are caused by suppressed osteoclastic activity. This would be in agreement with the previously published histomorphometric results on the same patients.[8]

In conclusion, this investigation show the advantages of coupling histomorphometric evaluation with infrared imaging analysis. FTIRI is a nondestructive technique from which multiple parameters concerning tissue components molecular properties can be obtained from a single section. Moreover, the spatial distribution of these parameters may be described. Analyses can provide information on changes in bone mineral and matrix properties at forming, resorptive, reversal, and quiescent surfaces with ~6.3 × 6.3 μm^2 spatial resolution.[22–25] The results of the present study show that HRT therapy has an effect on the mineral crystallinity and collagen cross-links ratio properties of bone tissue, two parameters involved in bone quality rather than quantity considerations.

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