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Original Article

Anabolic Effect of Long-term Estrogen Replacement on Bone Collagen in Elderly Postmenopausal Women with Osteoporosis

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Abstract. Estrogen has been shown to stimulate osteoblasts in cell culture and increase bone formation in animal models. Such an anabolic effect of estrogen replacement therapy (ERT) would be beneficial to postmenopausal women with osteoporosis. Hence, we assessed the total collagen content and collagen crosslink maturity in iliac crest bone biopsy from 18 such women before and after 6 years of higher-dose ERT. These results were compared with the serum estradiol level and bone mineral density (BMD). Total collagen content of both cortical and cancellous bone increased, showing a median (95% CI) percent change of 6.7 (0.3–14.2) and 25.6 (13.5–33.8), respectively. Increase in collagen synthesis was supported by a rise in intermediate crosslinks in both cortical and cancellous bone, and mature crosslinks in cortical bone only. At the same time, BMD showed a substantial rise both at the lumbar spine and proximal femur with a median (95% CI) percent change of 28.6 (19.8–37.3) and 14.5 (8.4–20.7), respectively. Serum estradiol and BMD results correlated with cortical bone collagen levels. Our results suggest that long-term higher-dose ERT has a therapeutic role due to its anabolic effect on bone in postmenopausal women with osteoporosis.

Keywords: Anabolic effect; Collagen; Crosslinks; Estrogen; Postmenopausal osteoporosis

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Introduction

Collagen comprises 90% of the organic matrix of bone, which together with its mineral content governs its biomechanical property and functional integrity [1]. The mechanical stability and extraordinary tensile strength of collagen fibers are due to the intermolecular crosslinks, which are initially divalent but later converted to stable trivalent forms as the tissue matures [2,3]. Hence, the relative proportion of intermediate to mature crosslink provides an assessment of collagen age, and a higher concentration of intermediate crosslink is consistent with an increased rate of collagen synthesis [4]. Quantitative analytical techniques have been developed to determine both intermediate and mature crosslinks in tissue samples [5].

The collagen content of bone and its tensile strength fall with ageing [6,7], but decreases significantly more in the postmenopausal period due to estrogen deficiency [1]. The reduction in bone collagen content results in a decrease in the mechanical strength of bone consistent with postmenopausal osteoporosis. The loss of collagen matrix also explains low bone mass and disruption of trabecular architecture in these patients. Recent studies on osteoporotic bones have shown that compared with age-matched controls there is a substantial reduction in divalent crosslinks [8,9] and any perturbation in the crosslink profile is responsible for changes in bone strength [3]. Thus, an ideal treatment for patients with established osteoporosis would be one that stimulates collagen synthesis and improves crosslink profile to restore bone mass, trabecular connectivity and bone strength.

Traditionally, estrogen replacement therapy (ERT) is known to prevent bone loss by reducing the rate of bone

turnover in the early postmenopausal period [10]. However, in recent years its use has been extended to older women with established osteoporosis [11,12], even though an anabolic effect has not been demonstrated and is therefore still debatable. Estrogen has been shown to stimulate the differentiation and activity of osteoblasts in vitro [13] and it has also been shown to increase bone formation and bone mass in animal models [14,15]. Similarly, in postmenopausal women the rise in bone mineral density (BMD) with long-term ERT indirectly suggests an anabolic effect [16], as the magnitude and continuity of such a change cannot be explained simply by reduced bone turnover [17]. A higher dose of ERT via subcutaneous implant has shown an even greater increase in BMD in postmenopausal women [18]. Interestingly, with the higher circulatory level of estrogen there is a continued dose response and the rise is greatest in those with lowest BMD [19].

It is therefore likely that any anabolic effect would be manifested earlier with estradiol implants, which gives a higher circulatory hormone level. However, the only study in older osteoporotic women failed to show an increase in collagen content after 1 year of estradiol implant therapy [20]. In contrast, there was a rise in mature crosslinks, supporting the established action of ERT in suppressing bone resorption and reducing bone turnover. It may be that these older women have irreversible damage to the process of collagen synthesis or would require much longer duration of ERT to show an anabolic effect. The rise in BMD has been shown to continue as long high-dose ERT is given [21], resulting in a very high BMD in the long term [22]. We therefore conducted a longitudinal study of changes in bone collagen content and crosslinks after 6 years of estradiol implant therapy to elucidate its putative anabolic effect on osteoporotic bones in vivo. The cancellous bone is normally renewed completely every 2–3 years, while the bone turnover period is doubled with ERT [17,23]. Hence, a minimum therapy period of 4–6 years is likely to be required for any anabolic effect of estrogen to be manifested in the human skeleton.

Patients and Methods

We invited previously untreated postmenopausal women with suspected osteoporosis to participate in the study. Those with any high risk factor for osteoporosis other than ovarian failure, who had hip fracture or replacement, suffered from medical disorders or used any drugs known to affect calcium or bone metabolism were excluded. After an initial screening with dual-energy X-ray absorptiometry (DXA) we selected 24 women of white European origin who had osteoporosis according to the WHO criteria (1994). Their BMD either at lumbar spine or proximal femur was more than 2.5 standard deviations below the mean for young female adult (*T*-score <-2.5). The demographic features including age, parity, height, weight, body mass index (BMI) of these women were recorded. The interval since natural menopause was noted but those who had had a hysterectomy were considered menopausal from the onset of climacteric symptoms or from the time of surgery if ovaries were removed.

The study was approved by the hospital ethics committee and informed consent was obtained from all women before each bone biopsy. We did not get ethical approval and consent for a placebo/nontreatment control group in the long-term study, as the preventive role of ERT in postmenopausal women with osteoporosis is well established. After the initial bone biopsy all women received a 75 mg estradiol implant (Organon Laboratories, Cambridge, UK), inserted subcutaneously in the anterior abdominal wall and repeated at 6-monthly intervals. Those with an intact uterus were also given oral medroxy progesterone acetate 5 mg daily (Upjohn, Crawley, UK) for 10 days in every calendar month to protect against endometrial hyperplasia. No parallel group on oral ERT was included, as the long-term (more than 5 years) compliance with such therapy was very poor in our experience.

All women were advised to continue the HRT regimen over the long term and avoid any other treatment that may alter bone metabolism including calcium supplementation. Two women withdrew from the study in the first year and three women between the first and second year due to heavy withdrawal bleeding. None of the participants had any thromboembolic complications. All had mammograms at 3-year intervals but none developed any abnormality. After 6 years, 19 women were continuing on the same HRT regimen, and 18 of them agreed to have another biopsy. This was performed 2 months after the insertion of the last implant when the estradiol level was expected to peak and serum samples were taken on the same day to measure hormone levels. The DXA scan was also repeated to measure the changes in BMD both at the lumbar spine and the proximal femur.

Before starting therapy their mean age was 65.6 (range 56–76) years and the mean interval since menopause was 16.6 (range 10–27) years. All women had had pregnancies with a median parity of 2 (range 1–4) and none had used an oral contraceptive pill. The mean height, weight and BMI before therapy were 1.6 (range 1.5–1.7) meters, 62.8 (range 51.6–79.5) kg and 24.4 (range 19.9–29.6), which changed minimally after 6 years to 1.6 (range 1.5–1.7) meters, 63.8 (range 51.6–84.0) kg and 24.8 (range 20.3–30.7), respectively. Eight women had had hysterectomies, including two women who had also had bilateral oophorectomy, eight women had suffered from one or more osteoporotic fractures either at the spine or at the distal radius and four women had a family history of osteoporosis.

Transcortical iliac crest biopsy was performed under local anesthesia using a 7.5 mm trephine. The pre- and post-therapy biopsies were performed from the opposite sides at a standard site about 2 cm below the iliac crest summit and 2 cm behind the anterior–superior iliac spine. The bone biopsy core included both cortices and intervening cancellous area. The samples were snap

frozen in liquid nitrogen and stored at –70 °C. Specimens were analyzed in two batches, one for the pre-therapy and other for the post-therapy samples, using identical methodology. We have been using the technique and equipment for several years and have rigorously standardized the methods [5,24].

Samples were separated by dissection into cortical and cancellous bone, and then each portion was reduced to a very fine powder in a specially constructed stainless steel hammer mill cooled with liquid nitrogen. The powdered samples were extracted in phosphate-buffered 0.15 M sodium chloride, pH 7.4 at 5 °C. Centrifugation of this extract allowed flotation of any fatty material and tissue, which were removed by decanting the supernatant and re-suspending the pellet in fresh phosphate-buffered saline.

The samples were then reduced with potassium borohydrate (40:1 by weight, wet sample: KBH_4) for 1 hour at room temperature, after which the samples were centrifuged, the supernatant discarded and the pellet washed with distilled water. After a final centrifugation, the pellets were lyophilized, weighed and hydrolyzed for 24 hours in constant boiling hydrochloric acid at 110 °C. Excess acid was removed by vacuum distillation and the dried hydrolyzates were redissolved in 3 ml of the organic solvent mixture of butanol–acetic acid–water (4:1:1), from which a small aliquot was removed for collagen assay. Collagen content was measured by determination of hydroxyproline using a continuous-flow autoanalyzer (ChemLab, Essex, UK) based on a method described previously [25], and calculated assuming the hydroxyproline content to be 14% of collagen and expressed as a percentage of the dry weight.

The remainder of redissolved hydrolyzate was applied to a mini-CF1 cellulose column (Whatman, Maidenhead, UK) and eluted with the above organic solvent to remove the unbound non-crosslinking amino acids. The bound crosslinking amino acids were subsequently eluted from the column with water, lyophilized and reconstituted in 0.01 N hydrochloric acid. This sample was then analyzed by cation exchange chromatography on an AlphaPlus II automatic amino acid analyzer (Amersham Pharmacia, Little Chalfont, UK) using ninhydrin detection and configured for the identification of the immature crosslink hydroxylysino-ketonorleucine and the mature crosslinks hydroxylysyl-pyridinoline and lysyl-pyridinoline found in bone. Peak integration was accomplished using Dionex AI450 data handling and integration software (Camberley, UK). The peaks were identified from their elution positions based on those of authentic crosslink standards prepared in our laboratory [5,24].

The BMD was measured at the lumbar spine and the proximal femur using a Hologic 1000 QDR DXA scanner (Hologic, Waltham, MA). The mean coefficient of variation for the densitometer calculated with the daily use of a spinal phantom was 0.67% during the course of the study. The precision in vivo was assessed by serial scans in 10 healthy premenopausal volunteers, both during pre-therapy and 6 years after therapy measurements. The coefficients of variation were 0.98% and 0.96% at the lumbar spine and 1.21% and 1.17% at the proximal femur, respectively. There were no major repairs or alterations to the DXA scanner during the 6 years of the study. The results were presented as absolute values (gm/cm^2) but also as the number of standard deviations and percentages above or below the mean result of young female adults (*T*-score) and age-matched female population (*Z*-score).

Serum estradiol and folliclo-stimulating hormone (FSH) were measured by an automated ELISA using ES700 kits (Roche Diagnostics, Lewes, UK). The inter-assay precision for estradiol was 14.9%, 6.5% and 8.0% at serum levels of 148 pmol/l, 856 pmol/l and 2135 pmol/l respectively. The inter-assay precision for FSH was 2.9%, 2.7% and 3.0% at serum levels of 7.6 U/l, 16.7 U/l and 46.3 U/l, respectively.

The majority of bone collagen and DXA scan variables were not normally distributed and thus presented as median with interquartile range. The significance of any change in these variables was therefore assessed by Wilcoxon matched-pairs signed-ranks test. Mann–Whitney *U*-test compared the changes in these variables between different subgroups of women, such as those who received progestogens and those who did not or those with and without personal or family history of osteoporotic fractures. Spearman's correlation coefficient was used to analyze the relation between variables. The level of significance was <0.05 for all statistical tests.

Results

The results of all collagen parameters before and after therapy along with the changes are summarized in Table 1. There was a significant increase in the collagen content in both cortical and cancellous bones with a median (95% CI) percent change of 6.7 (0.3–14.2) and 25.6 (13.5–33.8) respectively. Similarly, the immature crosslink hydroxylysino-ketonorleucine increased significantly in both cortical and cancellous bones. However, the mature crosslinks hydroxylysyl-pyridinoline and lysyl-pyridinoline increased significantly only in cortical bone but not in cancellous bone. The change in any collagen variable in cortical bone did not correlate with the respective change in cancellous bone.

The changes in all collagen variables with therapy correlated inversely with their respective pre-therapy levels as follows: cortical collagen content ($p=0.001$; $r=-0.722$), cortical hydroxylysino-ketonorleucine ($p=0.138$; $r=-0.363$), cortical hydroxylysyl-pyridinoline ($p=0.005$; $r=-0.631$), cortical lysyl-pyridinoline ($p=0.006$; $r=-0.625$), cancellous collagen content ($p<0.0001$; $r=-0.819$), cancellous hydroxylysino-ketonorleucine ($p = 0.026$; $r=-0.590$), cancellous hydroxylysyl-pyridinoline ($p<0.0001$; $r=-0.922$), cancellous lysyl-pyridinoline ($p=0.001$; $r=-0.774$). However, the

Table 1. Changes in bone collagen content and crosslinks with long-term percutaneous estradiol replacement therapy

	Pre-therapy[a] (n = 18)	Post-therapy[a] (n = 18)	Median difference (interquartile range)	p value[b]
Cortical bone				
Collagen content (%)	22.55 (21.17–24.00)	24.27 (23.34–24.94)	1.04 (–0.01–2.58)	0.0079
Hydroxylysino-ketonorleucine[c]	0.07 (0.04–0.10)	0.16 (0.12–0.17)	0.08 (0.05–0.12)	0.0003
Hydroxylysyl-pyridinoline[c]	0.08 (0.06–0.10)	0.18 (0.17–0.20)	0.10 (0.06–0.13)	0.0002
Lysyl-pyridinoline[c]	0.04 (0.02–0.04)	0.11 (0.08–0.12)	0.06 (0.03–0.10)	0.0279
Cancellous bone				
Collagen content (%)	18.05 (16.32–19.45)	22.89 (21.32–23.62)	4.60 (2.13–6.12)	0.0012
Hydroxylysino-ketonorleucine [c]	0.07 (0.02–0.14)	0.18 (0.13–0.27)	0.11 (0.03–0.22)	0.0202
Hydroxylysyl-pyridinoline [c]	0.17 (0.15–0.20)	0.18 (0.16–0.20)	0.00 (–0.04–0.04)	0.9001
Lysyl-pyridinoline [c]	0.05 (0.05–0.07)	0.09 (0.08–0.11)	0.02 (–0.01–0.06)	0.1026

[a]Median (interquartile range).
[b]Wilcoxon matched-pairs signed-ranks test.
[c]Mol/mol collagen.

Table 2. Changes in bone mineral density, T-score and Z-score with long-term percutaneous estradiol replacement therapy

	Pre-therapy[a] (n = 18)	Post-therapy[a] (n = 18)	Median difference (interquartile range)	p value[b]
Lumbar spine				
BMD (gm/cm^2)	0.762 (0.639–0.856)	0.989 (0.829–1.066)	0.185 (0.118–0.262)	0.0002
T-score (SD)	–2.59 (–3.92–1.73)	–0.56 (–1.98–0.36)	1.76 (1.21–2.31)	0.0002
Z-score (SD)	–0.70 (–2.02–0.05)	1.05 (0.11–2.19)	1.94 (1.53–2.52)	0.0002
Proximal femur				
BMD (gm/cm^2)	0.760 (0.659–0.835)	0.845 (0.771–0.916)	0.089 (0.049–0.124)	0.0003
T-score (SD)	–1.49 (–2.31–0.87)	–0.94 (–1.58–0.48)	0.46 (0.22–0.74)	0.0006
Z-score (SD)	–0.48 (–1.01–0.01)	0.54 (0.12–1.04)	0.91 (0.55–1.35)	0.0003

[a]Median (interquartile range).
[b]Wilcoxon matched-pairs signed-ranks test.

degree of change in collagen content did not correlate with the changes in any crosslinks.

At the time of final bone biopsy the mean levels of serum estradiol was 1422 (range 267–3788) pmol/l and that of serum FSH was 2.58 (1–6.1) IU/l. Serum estradiol level correlated directly with post-therapy levels of cortical collagen content ($p < 0.05$; $r = 0.467$) but not with cancellous collagen content or any crosslinks of both cortical and cancellous bone. Neither age nor the interval since menopause correlated with any collagen variables. Similarly height, weight and BMI had no relationship with the collagen results.

The BMD improved in all women, both at the lumbar spine and proximal femur; the results are summarized in Table 2. The median (95% CI) percent rise of BMD at the lumbar spine was 28.6 (19.8–37.3) and that at the proximal femur was 14.5 (8.4–20.7). Both *T*- and *Z*-scores improved from osteoporotic levels before starting therapy to normal levels after therapy. The increase in BMD at the lumbar spine correlated with the increase in cortical collagen content ($p < 0.05$; $r = 0.467$). Similarly, the post-therapy lumbar spine BMD results correlated with the post-therapy cortical collagen content ($p = 0.007$; $r = 0.611$).

There were no significant differences in the bone collagen and BMD changes between those women with intact uteri who received progestogen supplements and hysterectomized women on unopposed ERT. Similarly, family or personal history of osteoporotic fracture did not alter the response to the therapy.

Discussion

This study has demonstrated for the first time prospective evidence of an increased bone collagen synthesis with long-term higher-dose ERT. Such a finding is of greater benefit in older postmenopausal women with low BMD where an anabolic effect is most desirable to reverse bone loss and trabecular connectivity. The evidence of new collagen formation was supported by the increase in intermediate crosslinks and limited change in mature crosslinks, indicating increased collagen turnover rather than just accumulation of mature collagen with increasing age or reduced bone turnover [20]. Since the collagen crosslinks are important determinants of bone mechanical strength [3], the efficacy of the therapy is likely to be more striking than would be expected from the improvement in bone mass alone.

The only other crosslink present in bone collagen is pyrrole crosslink, which also contributes to overall bone

strength. It was not measured in this study due to insufficient bone sample quantity and insensitivity of the assay available, which together were unlikely to produce meaningful results. Moreover, the main aim of this study was to evaluate whether ERT has an anabolic effect on postmenopausal osteoporotic skeleton by demonstrating new bone growth. Hence, the main focus was on the presence of increased amounts of the immature cross-links present in the newly synthesized collagen, not the effect of ERT on bone strength.

Changes in collagen parameters in the cortical bone did not parallel those changes found in the cancellous bone. The median percent change in total collagen content in cancellous bone was four times higher than that in cortical bone. The mature crosslinks increased significantly only in cortical bone but not in cancellous bone. Such differences are due to a greater remodeling rate in cancellous bone because of its larger surface area than that of cortical bone [6]. Hence, cancellous bone is lost more rapidly after menopause and responds quicker to ERT in comparison with cortical bone [26]. With a different proportion of cortical and cancellous bone [27] the skeletal response to ERT is also not uniform at different sites. Thus the greater rise in BMD at the lumbar spine than in proximal femur in this study, as also shown by others after short-term follow-up [11,12,18,19,28] was due to the varying rate of bone remodeling.

Older postmenopausal women on ERT have shown greater bone gain, which is related to their lower BMD before starting the therapy [11,18,19,29]. It has been suggested that the greater the effect of estrogen deprivation, the greater the capacity of estrogen-sensitive tissue in responding to reintroduction of estrogen [11]. This is likely to be the basis of our finding that the lower the pre-therapy levels of bone collagen parameters, the greater the respective changes with long-term ERT. The influence of age or interval since menopause on the BMD changes with ERT has been mediated through their effect on the pre-therapy BMD levels [30]. Similarly, in this study there was no independent influence of age and interval since menopause on the collagen changes with ERT. Although BMI has been shown to correlate with the treatment response to BMD changes due to the effect of mechanical stimulation on bone remodeling, there was no such influence on collagen metabolism.

The same dose of estradiol implant resulted in a wide range of serum estradiol levels, as shown earlier [18–20], indicating a variation in pharmacodymamics and possible cumulative effect after long-term use, which may explain differences in bone collagen changes between patients. Serum estradiol level has been shown to correlate with the changes and post-therapy levels of cortical bone collagen but not with those of cancellous bone collagen. Cancellous bone collagen changes are likely to have occurred earlier and hence were not proportional to serum estradiol levels after 6 years of ERT. A progressive dose-related response to ERT has also been reported with the BMD changes [19]. Serum estradiol levels increase with higher-dose ERT and the BMD changes are related to serum estradiol concentration [18,19,29]. BMD at the lumbar spine or proximal femur represents cortical bone more than cancellous bone [27], and thereby both BMD and cortical collagen content correlate with serum estradiol. Similarly, the changes and post-therapy levels of BMD correlated with respective results of cortical bone collagen but not with cancellous bone collagen.

The changes in collagen parameters with ERT in this study were actually more pronounced than was evident from the results, as these were reverse of the expected age-related reduction over 6 years [6,7]. Our results have also confirmed that the substantial rise in BMD with long-term ERT is not just due to reduced bone remodeling [10,17,23] or increased mineralization [31], but as a result of new bone formation. An increase in collagen content with ERT is certainly possible due to reduced bone resorption and a lower rate of bone turnover. However, this would be accompanied by an increase in mature and not immature crosslinks, as shown in a study after 1 year of estradiol implant therapy [20]. Certainly, this was not the case in this study as we have provided evidence of new collagen synthesis by demonstrating an increase in intermediate crosslinks, which was also proportionally greater than mature crosslinks. Hence, even with a higher dose of ERT, the initial response in reduced bone turnover [20], and an increase in bone formation is manifested only after several years.

An anabolic effect of a high dose of ERT on collagen metabolism supports its role in the treatment of postmenopausal women with established osteoporosis. Moreover, the parallel changes in bone collagen and BMD extend the role of the DXA scan in monitoring the anabolic effect of ERT. The long-term use of a physiologic or even supraphysiologic dose of ERT was perhaps the key to the anabolic effect on bone in this study. Further studies are needed to show whether long-term ERT by other routes that give a relatively lower serum estradiol level have similar anabolic effects on the skeleton.

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