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Original Article

Bone Remodeling and Structure in Postmenopausal Women Treated with Long-Term, High-Dose Estrogen Therapy

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Abstract. Conventional hormone replacement therapy preserves bone mass predominantly by reducing bone turnover but does not exert significant anabolic skeletal effects. In contrast, high doses of estrogen have been shown to increase bone formation in animals and we have recently reported high bone mineral density values in women treated long-term with estradiol implant therapy. The aim of this study was to investigate the mechanisms by which high doses of estrogen may increase bone mass in postmenopausal women. Iliac crest biopsies were obtained from 12 women who had received long-term treatment with estradiol implants (at least 14 years), on demand, following hysterectomy and bilateral salpingo-oophorectomy. Indices of bone turnover, remodeling balance and cancellous bone structure were assessed by image analysis and compared with those of premenopausal women. Mean wall width was significantly higher in women treated with estradiol therapy than in premenopausal women (44.8 ± 4.8 vs 38.8 ± 2.8 μm; mean ± SD; $p = 0.001$) and eroded cavity area was significantly lower in the implant-treated women (3612 ± 956 vs 5418 ± 1404 μm^2; $p = 0.001$). Bone formation rate at tissue level and activation frequency were lower in the women treated with implants, although the differences were not statistically significant. Indices of cancellous bone structure were generally similar between the two groups. These results provide the first direct evidence that high-dose estrogen therapy produces anabolic skeletal effects in postmenopausal women and indicate that these are achieved by stimulation of osteoblastic activity.

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Introduction

The beneficial skeletal effects of menopausal estrogen replacement therapy are well documented [1-6]. Administration of estrogen at or after the menopause prevents bone loss and reduces fracture rate in the spine, hip and radius – effects which are believed to be mediated predominantly by inhibition of osteoclastic bone resorption. The small increase in bone mineral density during the first 1–2 years of treatment that has been observed in many studies can be attributed to the infilling of the remodeling space that occurs as resorption is inhibited and existing cavities become filled in with new bone formed by osteoblasts.

Studies in animals have shown that estrogens not only inhibit osteoclastic activity but may also have stimulatory effects on osteoblasts, resulting in increased bone formation [7,8]. Direct evidence for a similar effect in humans is lacking although indirect evidence is provided by the observation that percutaneous estrogen implant therapy, which is associated with high serum estradiol levels, results in greater increases in bone mineral density than oral or transdermal estrogen administration, with which more physiologic concentrations of serum estradiol are achieved [9–12]. However, these data are derived mainly from cross-sectional studies in which testosterone implants were also given. More recently, high bone mineral density values were reported in a

group of women receiving long-term treatment with percutaneous estradiol implants without co-administration of testosterone [13].

To explore further the skeletal changes induced by high doses of estrogen we have examined bone turnover, resorption cavity characteristics and cancellous structure in women receiving long-term percutaneous estradiol implant therapy following hysterectomy and bilateral salpingo-oophorectomy. Comparison of histomorphometric indices was made with a group of premenopausal women, based on the rationale that significant age-related bone loss had not occurred in the patient group prior to estrogen therapy and that any differences between the two groups would therefore reflect the effects of high-dose as opposed to physiologic estrogen replacement.

Patients and Methods

Patients and Controls

Twelve women, aged 52–67 years (mean 58 years), who had undergone total abdominal hysterectomy and bilateral salpingo-oophorectomy for a non-malignant indication and had received long-term estradiol implant therapy, agreed to take part in the study. These women were attending the estradiol implant clinic at the Department of Obstetrics and Gynaecology, Princess Royal, Hull, UK; all 12 consented to undergo iliac crest bone biopsy. Estradiol implants, 100 mg, had been inserted approximately 6-monthly, on demand, for at least 14 years, although for the last 2–3 years before this study the dose had been reduced to an average of 50 mg every 6 months. None of the women had received testosterone, bisphosphonates, fluoride, vitamin D or glucocorticoids, nor did any have a past or present history of illness associated with disturbances of bone or mineral metabolism.

Control values were obtained from 12 premenopausal women, aged 23–40 years (mean 31.3 years), with endometriosis, prior to treatment with gonadotropin-releasing hormone analogs [14]. None of these women had a past or present history of any illness associated with bone disease and none had taken drugs known to affect bone or mineral metabolism.

Bone Histomorphometry

Trans-iliac bone biopsies were obtained using an 8-mm internal diameter trephine under local anesthetic and mild sedation after the administration of two time-spaced oral doses of a tetracycline (demeclocycline). Informed written consent was obtained from all women and the study was approved by the Hull and East Riding research ethics committee. Biopsies were embedded in LR White medium resin (London Resin Co.). Eight micrometer undecalcified sections were stained by the von Kossa technique or with 1% toluidine blue. Histomorphometric assessment was made using a Digicad digitizing tablet and cursor with an LED point light source (Kontron) and an Olympus BHS-BH2 binocular transmitted light microscope with a BH2-DA drawing attachment (Olympus Optical UK, London). All histomorphometric data are described according to ASBMR nomenclature [15]. All measurements were made in masked fashion by the same observer (S.V.) with the exception of tetracycline-based indices in the premenopausal women; because of the lack of sufficient remaining bone tissue, fresh histologic sections could not be obtained from these biopsies and it was therefore necessary to use the values previously obtained by another observer.

Primary Measurements. Bone area/tissue area (B.Ar/T.Ar), osteoid perimeter/bone perimeter (O.Pm/B.Pm) and osteoid seam width (O.Wi) were measured on von-Kossa-stained sections on a minimum of 25 fields from three to six sections. Osteoid width was measured at four approximately equidistant points, or eight points on seams longer than 600 μm in length. A minimum of 20 seams per biopsy was measured on the same sections used for O.Pm. All seams with a width of 3 μm or more were measured.

The mean width of completed bone remodeling units (W.Wi) was measured on toluidine-blue-stained sections viewed under polarized light at $\times 156$ magnification. A minimum of 25 BMUs was measured on each biopsy from between three and eight sections. Tetracycline labeling was viewed by fluorescence microscopy on a minimum of six 15 μm unstained sections at $\times 156$ magnification. Mineralizing perimeter (Md.Pm) was calculated as follows:

$$\text{Md.Pm/B.Pm (\%)} = \text{dL.Pm} + (0.5 \times \text{sL.Pm})/\text{B.Pm}$$

where dL.Pm is the double-labeled perimeter and sL.Pm is the single-labeled perimeter.

The mean distance between double labels was measured directly at $\times 312$ magnification using the digitizing tablet and cursor. Measurements were made at approximately four equidistant points along the double labels. A minimum of 20 labels was measured for each biopsy on a minimum of six sections.

Mineral apposition rate was calculated as :

$$\text{MAR (μm/day)} = \text{L.Wi/LP}$$

where L.Wi is the inter-label width and LP is the labeling period (12 days).

Derived Indices. Adjusted apposition rate (Aj.AR), mineralization lag time (Mlt) and osteoid maturation period (Omt) were calculated as follows:

$$\text{Aj.AR (μm/day)} = \text{MAR} \times (\text{M.Pm/O.Pm\%})$$
$$\text{Mlt (days)} = \text{O.Wi/Aj.AR}$$
$$\text{Omt (days)} = \text{O.Wi/MAR}$$

The tissue-based bone formation rate (BFR/B.Pm) was calculated as follows:

$$\text{BFR/B.Pm (μm}^2\text{/μm/day)} = \text{MAR} \times (\text{M.Pm/B.Pm\%})$$

Activation frequency (Acf) was calculated as:

$$\text{Acf } (yr^{-1}) = (BFR/B.Pm)/W.Wi$$

Measurement of Resorption Cavity Characteristics. The method described by Garrahan et al. [16] was adapted for use with the digitizing tablet and light cursor for measurement. Cavities were identified on toluidine blue stained sections viewed under polarized light at ×156 magnification and measured at ×375 or ×750 magnification depending on the size of the cavity. Criteria for identification of resorption cavities included interruption of lamellae at an angle to the bone perimeter, absence of osteoid tissue and depth of greater than 3 μm. A minimum of 20 cavities was assessed for each biopsy. The following indices were obtained:

Mean eroded depth (E.De, μm)
Maximum eroded depth (E.De.Max, μm)
Eroded cavity area (E.Ar, μm^2)
Mean reconstructed cavity length (μm)
Cement line length (μm)

Strut Analysis. This method has been described in detail previously [17]. The bone section is viewed with a CCD camera mounted on a light box, allowing the whole section to appear within a single field of view (magnification ×9). Images of the whole bone section are captured on an 386DX IBM-compatible AT-based computer system containing a Virtuoso frame store (Primagraphic, UK). All analysis software was written in the 'C' language using a library of image-processing subroutines (Foster Findlay Associates, Newcastle, UK). The stored images are converted to binary images that can be interactively edited to remove minor specimen preparation artifacts. The right and left corticomedullary junctions are defined automatically using a procedure that has been described previously [18]. The upper and lower boundaries of the section are defined interactively by the operator. The upper and lower boundaries and right and left corticomedullary delineations define the 'active' regions upon which all measurements are performed.

Binary images are skeletonized to give a symmetric axis of the original bone profile. The computer automatically identifies trabecular junctions, or nodes (Nd) and the ends of trabeculae, or termini (Tm). Individual trabeculae, or struts, are defined topologically: the indices thus generated include the ratio of nodes to termini (Nd/Tm) and the terminus-to-terminus strut length (Tm.Tm), node-to-loop strut length (Nd.Lp) and node-to-terminus strut length (Nd.Tm), each of which is expressed as a percentage of the total strut length.

Statistical analysis was performed using an unpaired Student's *t*-test after log transformation of the data. Results are expressed as the mean ± SD.

Results

Details of the patient group are shown in Table 1. The time since hysterectomy and bilateral salpingo-oophorectomy was 17–23 years (mean 20 years) and the age at surgical menopause ranged between 33 and 48 years (mean 37.6 years). The mean spinal bone mineral density was 1.42 ± 0.24 g/cm^2 and the mean *T*-score was +1.73 ± 2.0; corresponding figures for femoral neck bone mineral density were 1.13 ± 0.17 g/cm^2 and +1.24 ± 1.42.

Histomorphometric indices are shown in Tables 2–4. There were no significant differences in cancellous bone area, osteoid perimeter, mineralizing perimeter or osteoid seam width between the women receiving estradiol implants and the premenopausal controls (Table 2). The mineral apposition rate was significantly lower in the implant-treated women than in the controls (0.63 ± 0.10 vs 0.89 ± 0.39 μm/day; $p = 0.0004$) and there was a trend towards lower bone formation rate and activation frequency in these women, although the differences were not statistically significant (0.030 ± 0.023 vs 0.048 ± 0.040 $\mu m^2/\mu m$/day and 0.25 ± 0.21 vs 0.47 ± 0.42 /yr^{-1}; Table 2).

Indices related to remodeling balance are shown in Table 3. In women receiving estradiol implants the mean

Table 1. Details of the patient group

Patient no.	Age (years)	Age at operation (years)	Lumbar spine BMD		Femoral neck BMD		Weight (kg)
			g/cm^2	*T*-score	g/cm^2	*T*-score	
1	58	35	1.810	5.1	1.331	2.9	67.0
2	59	36	1.330	1.1	1.390	3.4	66.0
3	57	37	1.368	1.4	1.065	0.7	67.0
4	58	37	1.477	2.3	0.969	–0.1	71.0
5	67	48	1.667	4.0	1.322	2.9	80.0
6	52	34	1.333	1.1	1.004	0.2	87.0
7	55	35	1.085	–1.0	1.106	1.0	66.5
8	63	41	1.700	4.2	1.311	2.8	80.0
9	53	36	1.115	–0.7	0.900	–0.7	76.0
10	58	39	1.553	2.9	1.145	1.4	76.5
11	55	33	1.183	–0.2	1.070	0.7	67.5
12	57	40	1.277	0.6	0.948	–0.3	62.0

Table 2. Comparison of indices of bone formation in postmenopausal women on long-term high-dose estrogen implant therapy versus control group

Indices	Estrogen implant group (n = 12)	Control group (n = 12)	Significance
Tb.Ar (%)	21.9±5.4	20.5±6.5	NS
O.Pm (%)	8.0±6.0	5.9±4.2	NS
O.Wi (μm)	6.9±2.0	9.6±5.7	NS
Md.Pm (%)	4.8±3.7	5.6±5.0	NS
MAR (μm/day)	0.63±0.11	0.89±0.39	p = 0.0004
BFR (μm^2/μm/day)	0.030±0.023	0.048±0.040	NS
Acf (yr^{-1})	0.25±0.21	0.47±0.42	NS

Table 3. Comparison of indices of remodeling balance in postmenopausal women on long-term high-dose estrogen implant therapy versus control group

Indices	Estrogen implant group (n = 12)	Control group (n = 12)	Significance
Mean eroded depth (μm)	24.2±3.6	23.0±3.5	NS
Maximum eroded depth (μm)	44.6±22.0	34.3±4.7	NS
Eroded cavity area (μm^2)	3612±956	5418±1404	p = 0.001
Reconstructed surface length (μm)	175±25.3	259±26.6	$p < 0.0001$
Cement line length (μm)	234±29.0	284±30.9	p = 0.0004
W.Wi (μm)	44.8+4.8	38.8+2.8	p = 0.001

Table 4. Indices of cancellous bone connectivity in postmenopausal women on long-term high-dose estrogen implant therapy versus control group

Indices	Estrogen implant group (n = 12)	Control group (n = 12)	Significance
Nd/Tm (%)	27.0±12.9	28.1±10.9	NS
Nd/Lp (%)	25.5±20.2	16.4±10.0	NS
Tm/Tm (%)	7.8±9.9	7.6±7.4	NS
Nd/Nd (%)	22.5±6.8	29.4±6.0	p = 0.018
Nd/Tm ratio	1.2±0.8	1.1±0.7	NS

value for wall width was significantly higher than in the control group of premenopausal women (44.8 ± 4.8 vs 38.8 ± 3.8 μm; $p = 0.001$). There were no significant differences in the mean or maximum eroded depth between the two groups. However, the eroded cavity area was significantly lower in the implant-treated women (3612 ± 956 vs 5418 ± 1404 μm^2; $p = 0.001$), as were the reconstructed surface length and cement line length (175 ± 25.3 vs 259 ± 26.6 μm; $p < 0.0001$) and 234 ± 29.0 vs 284 ± 30.9 μm; $p = 0.0004$).

The results of strut analysis are shown in Table 4. No significant differences were observed between the two groups in the node-to-terminus ratio or in the terminus-to-terminus, node-to-loop or node-to-terminus strut lengths. The node-to-node strut length was significantly lower in the women receiving implants than in the premenopausal controls (22.5 ± 6.8 vs 29.4 ± 6.0%; $p = 0.018$).

Discussion

These results provide histologic evidence that estrogen implant therapy produces anabolic skeletal effects in postmenopausal women and indicate that these are achieved by stimulation of osteoblastic activity, resulting in increased bone formation at the cellular level. The mean bone mineral density at the spine and femur in women treated with percutaneous estrogen was higher than that seen in normal premenopausal women, suggesting not only that peak bone mass had been preserved but also that estrogen replacement had further increased bone mass above this level. This contention is supported by the significantly greater thickness of completed cancellous bone structural units in the women receiving high-dose implant therapy when compared with premenopausal women. Iliac crest cancellous bone area was not significantly higher in the implant-treated women, probably reflecting the large measurement variance associated with its assessment and the relatively small number of subjects studied.

For ethical reasons it is difficult to obtain bone biopsies from normal premenopausal women and, in the present study, comparison of histomorphometric indices was made with a group of premenopausal women with endometriosis who had taken part in a previous study of the effects of gonadotropin-releasing hormone agonists [14]. Although it has been suggested, on the basis of measurements of volumetric bone density in the distal radius, that bone mass may be reduced in women with endometriosis [19], in a subsequent study of 85 women with endometriosis Lane et al. [20] reported normal spinal bone mineral density. In our group of premenopausal women, serum estradiol concentrations were normal (mean 374 pmol/l; range 232–516) and spinal bone mineral density was also normal, providing justification for their use as controls in this study.

Several other lines of evidence support the contention that estrogens, in high doses, may stimulate bone formation in the human skeleton. The use of oral contraceptives in premenopausal women has been reported in some studies to be associated with increased bone mineral density, although this finding has not been universal [21–24]. Low bone mass has been reported in a young man with estrogen resistance due to a mutation in the estrogen receptor gene [25] and in a young male with aromatase deficiency [26]; furthermore, in a young man with the latter condition, estrogen replacement was associated with a 19.8% increase in spinal bone mass after 30 months of treatment [27]. Finally, estrogen

deficiency in adolescents is associated with failure to attain peak bone mass; in a study of adolescent and young adult females, an integrated estimate of endogenous and exogenous estrogen exposure was significantly correlated with bone mineral density of the spine and wrist [28].

The measurement of wall width in cancellous bone provides an index of osteoblast cellular activity [29]; because of the long life span of the formative phase of the bone remodeling cycle and relatively low bone turnover in adult human bone, assessment of wall width after relatively short treatment periods does not accurately reflect the effects of intervention since only a small proportion of completed remodeling units will have been formed during the treatment period. Prospective studies of women treated with conventional hormone replacement therapy have shown no change [30] or a small decrease [31] in wall width after 1 or 2 years treatment respectively. In women treated with percutaneous estrogen implants (75 mg 6-monthly), Holland et al. [32] also reported no significant change in wall width after 1 year. In the present study the long treatment period in the women receiving estrogen implants insured that the majority of structural units identified were formed subsequent to the commencement of treatment.

The changes observed in indices of resorption cavity size in implant-treated women are consistent with those reported earlier in a prospective study of women treated with conventional hormone replacement therapy, in which a consistent trend toward a reduction in resorption cavity size was seen [31]. In the present study, the cavity area was significantly smaller in implant-treated women than in premenopausal controls – a difference that resulted from a smaller erosion cavity length rather than depth. The effects of estrogen on osteoclast activity are incompletely understood but inhibition of osteoclastogenesis [33] and stimulation of osteoclast apoptosis have been reported [34]. Although the relative contributions of osteoclast number and activity to erosion cavity depth and length have not been defined, it is conceivable that a reduction in the former may preferentially affect the surface extent of individual resorption cavities rather than their depth, which is likely to be determined predominantly by the activity of individual osteoclasts. This proposition is supported by the study of Delaissé et al. [35] on the effects of cysteine proteinase inhibitors on the resorptive activity of chick osteoclasts.

Accurate assessment of remodeling balance is difficult, largely because of the problems associated with measurement of completed erosion depth. The method used for the latter in the present study assesses the size of all cavities present and thus underestimates completed erosion depth by an unknown amount. Nevertheless, values for mean and maximum erosion depth were similar to those obtained in premenopausal women and this, in combination with the substantially greater values for mean wall width, provides strong evidence in favor of a positive remodeling balance.

Strut analysis of cancellous bone generally revealed similar values in the implant-treated women and premenopausal controls, indicating that connectivity had been preserved in the former group. In women with postmenopausal osteoporosis, conventional hormone replacement therapy has also been shown to preserve existing cancellous bone architecture [36]; similar effects would be predicted in the implant-treated women in the present study since the anabolic effect of estrogen was mediated via increased mean wall width rather than by de novo bone formation.

Because of the lack of availability of fresh histologic sections in the control group of premenopausal women, it was not possible for tetracycline-based measurements to be performed by the same observer. It is therefore possible that the significant differences in mineral apposition rate and its derived measurements, bone formation rate at tissue level and activation frequency, from the implant-treated women might have arisen from inter-observer variation and these data thus have to interpreted with some caution. This would also explain the apparent contradiction in the observations that mineral apposition rate was lower but wall width higher in the implant-treated women when compared with values in premenopausal women; alternatively, it is possible that mineral apposition rate had previously been higher but decreased when the dose of estradiol was reduced. Nevertheless, the values for bone formation rate and activation frequency in the implant-treated women were very similar to those measured, by the same observer, in women with postmenopausal osteoporosis after 2 years of treatment with conventional hormone replacement therapy [31], suggesting that bone turnover was relatively suppressed in the women treated with estradiol implants.

The mechanisms by which estrogens produce anabolic skeletal effects remain to be identified. Estrogen receptors have been identified on osteoblasts and osteocytes [37–39], raising the possibility that increased bone formation may result from direct stimulation of these cells. However, the estrogen receptor alpha does not appear to be required for anabolic skeletal effects of estrogen in mice [40], suggesting involvement of the estrogen receptor beta [41–43] or, alternatively, mechanisms not mediated by the estrogen receptor. In vitro, estrogen has been reported to stimulate the production of transforming growth factor beta, insulin-like growth factors and type 1 collagen [44,45], all of which are associated with increased bone formation.

Nearly all drugs currently used in the prevention and treatment of osteoporosis act predominantly by inhibition of bone turnover and resorption, and thus identification of agents that produce substantial gains in bone mass is of considerable interest. Although high doses of estrogens may be associated with increased short-term and long-term side-effects, there might be a role for a finite period of such treatment in perimenopausal women with severely reduced bone mass. The anabolic skeletal effects of estrogen may also be relevant to selective estrogen receptor modulators, which it may

be possible to administer in higher doses because of their lack of unwanted estrogenic effects outside the skeleton [46]. Finally, greater understanding of the mechanisms by which estrogen stimulates bone formation may ultimately lead to the development of novel therapeutic interventions.

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