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In vitro anti-resorptive activity and prevention of ovariectomy-induced osteoporosis in female Sprague–Dawley rats by ormeloxifene, a selective estrogen receptor modulator

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Abstract

Antiosteoporotic activity of ormeloxifene, a multifunctional SERM, using inhibition in parathyroid hormone (PTH) induced resorption of ^{45}Ca from prelabeled chick and rat fetal limb bones in chase cultures and modulation of certain biochemical markers of bone turnover and bone mineral density (BMD) in ovariectomized adult female rats, was investigated. Ormeloxifene concentration-dependently inhibited PTH-induced resorption of ^{45}Ca from chick fetal femora with treated/control (T/C) ratio of 0.71, 0.32 and 0.20 at 50, 100 and 200 μM concentration, in comparison to 0.49, 0.53 and 0.95 in case of CDRI-85/287 (a pure antiestrogen), tamoxifen and ethynylestradiol (100 μM), respectively. Using rat fetal limb bones, ormeloxifene (100 μM) exhibited T/C ratio of 0.67, in comparison to 1.43 with PTH alone. Heat-killed bones exhibited negligible resorption (2.9%; T/C: 0.098) in response to PTH. In adult female rats, ormeloxifene (1.25 and 12.5 mg/kg per day) inhibited ovariectomy-induced increase in serum total and bone-specific alkaline phosphatase and osteocalcin and urine calcium/creatinine ratio to almost intact control level. Ovariectomy was accompanied by marked decrease in bone mineral density of isolated femur and tibia, being maximum in femur neck (28.3%; $P < 0.01$) and midshaft (23.7%; $P < 0.01$), but only marginal (6.7%; P>0.05) in region proximal to tibio-fibular separation point. Decrease in BMD based on *T*-/*Z*-score, too, was >2.5 S.D. than mean value of normal young adult/age-matched females. This was prevented by ormeloxifene and the effect, though apparently more in females supplemented with higher dose of ormeloxifene, was not always significantly different and clear dose–response was not evident until BMD data was evaluated on *T*-/*Z*-score basis. The analysis also demonstrated much higher threshold level of tibia than femur and more so for their mid-shafts. Increase in BMD of isolated bones was also observed in ormeloxifene-treated intact females, without significantly altering biochemical markers of bone turnover or uterine weight. Findings suggest potential of ormeloxifene in management of post-menopausal osteoporosis and beneficial effect on BMD in women taking this SERM for contraception or any hormone-related clinical disorder.

Keywords: Ormeloxifene; Antiosteoporotic; ^{45}Ca resorption in vitro; Biochemical markers of bone turnover; BMD; Isolated bones

1. Introduction

Osteoporosis accompanying menopause represents a major cause of morbidity and mortality in women. Left unchecked, cumulative bone loss can potentially compromise skeleton's structural integrity resulting in painful and debilitating fractures of wrist, spine and femur [1]. This is due to estrogen deficiency-induced increased generation and activity of osteoclasts, which perforate bone trabeculae, reduce their strength and increase fracture risk [2]. Hormone replacement therapy, though effective in preventing bone loss following ovariectomy or menopause in women, is associated with increased risk of endometrial hyperplasia and carcinoma [3,4], breast cancer [5] and thromboembolic diseases [6]. Efforts are, therefore, being made to develop agents that would prevent estrogen-deficiency bone loss, but lack carcinogenic effects of estrogen on endometrium and breast, in addition to being free from other estrogen-related health hazards.

This study reports in vitro and in vivo antiresorptive activity of ormeloxifene, a multifunctional SERM, known to prevent pregnancy in women when administered in post-coital and weekly regimens. Reports of its efficacy in management of cancer breast in men and women, dysfunctional uterine bleeding (DUB), atherogenicity, dermitis, restenosis, male infertility and ovulation induction in amenorrhic

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women are also available (cf. Singh [7] for recent review). Pertinently, raloxifene, a benzothiophene reported to mimic effects of estrogen in bone and cardiovascular system and exhibit estrogen antagonistic effect in endometrial tissue [8,9], has recently been reported to increase incidence of hot flashes, deep vein thrombosis, pulmonary embolism and leg cramps similar to that associated with use of hormone replacement therapy [10]. It also induces weak, but significant, uterotrophic response (1.7-fold at 0.1 mg/kg dose) with significant increase in uterine dry weight and height, mitotic activity, vacuolization and degeneration of uterine luminal epithelial cells and number of endometrial glands in immature and ovariectomized rats [11] and a potent estrogenic effect on hypothalamo-pituitary-ovarian (H-P-O) axis [12].

2. Materials and methods

2.1. Animals and chemicals

Fertilized chicken eggs purchased from Government Poultry Farm, Lucknow on day of ovulation (day 1) were incubated at 37 °C in humidified air. Each egg was observed for embryonic growth on egg kindler and manually rotated at least once every 24 h. One-month-old (about 40 g) and 3-month-old (180–200 g) colony-bred virgin female Sprague–Dawley rats maintained under standard conditions (22 ± 1 °C) with alternate 12 h light/dark periods and free access to regular pellet diet (Lipton India Ltd., Bangalore) and tap water were used throughout this study. For in vitro antiresorptive assay, female rats were mated to males of proven fertility (day 1: day of sperm positive vaginal smear) and kept individually in plastic cages containing dry rice husk. Ormeloxifene (INN for centchroman) and CDRI-85/287 synthesized [13] at this institute were used. Tamoxifen, 17α-ethynylestradiol, BGJ_b bone culture medium, parathyroid hormone (PTH, aa 1–34, molecular weight 4117.7), bovine serum albumin (fraction V), HEPES, streptomycin, penicillin, DMSO, PPO, POPOP and methoxyethanol were purchased from Sigma Chemical Company, USA. Toluene was purchased from Qualigen Fine Chemicals, Mumbai, $^{45}CaCl_2$ (specific activity: 0.185–1.85 g Bq/mg Ca) from Amersham Pharmacia Biotech, England and kits for biochemical markers of bone turnover from Boehringer Mannheim, Germany. All other chemicals were of analytical grade.

2.2. Experimental design

2.2.1. Anti-resorptive activity in vitro

Femur bones isolated from chick embryos on day 11 post-ovulation were cleared of adhering connective tissue by carefully rotating each bone on dry Whatman (I) filter paper under stereomicroscope. Each femur was placed in a drop of PBS [14] before culturing in 300 μl of BGJ_b medium (pH 7.3) supplemented with penicillin (0.075 mg/ml), streptomycin (0.05 mg/ml), HEPES (2.382 mg/ml) and BSA (1 mg/ml) in sterile 48-/96-well plate at 37 °C under an atmosphere of 5% CO_2 in air for 24 h. Bones were transferred to BGJ_b culture medium containing $^{45}CaCl_2$ (0.5 μCi/300 μl medium) and incubated for 3 h at 37 °C under 5% CO_2 in air. Bones were then washed three times with BGJ_b medium for 3 h at 37 °C under 5% CO_2 in air. Labeled bones were transferred to BGJ_b medium containing PTH (0.4 μM) and chase cultured for 96 h in presence or absence of test agents or vehicle (ethanol/DMSO; final concentration 0.1%) in 300 μl of BGJ_b medium. Contralateral femur of each fetus served as corresponding control. Culture medium with respective treatment in each well was changed after 48 h. On termination of culture, bones were transferred to 0.1N HCl for 24 h. Radioactivity due to ^{45}Ca in spent medium collected at 48 and 96 h of culture and HCl extract was quantified by Liquid Scintillation Spectrophotometer (LKB Wallac 1282 Gamma Counter, Finland) in 10 ml of scintillation fluid (PPO: 2.00 g, POPOP: 0.05 g, toluene: 500 ml, methoxyethanol: 500 ml). In case of rat, 200 μC=i/300 μl of $^{45}CaCl_2$ (specific activity: 0.185–1.85 g Bq/mg Ca) was administered (s.c.) to each female on day 18 of pregnancy (day 1: day of sperm positive vaginal smear) and labeled humerus and radio-ulna bones isolated 48 h thereafter under sterile conditions were chase cultured (in presence of 0.05 μM PTH) as detailed in case of chick fetal bones. One set of bones was heat killed by keeping in PBS in sterile tubes in boiling water for 15 min and cultured in parallel to assess viability of bones in culture system. Bone resorbing activity was expressed as percentage of ^{45}Ca released into culture medium and effect of test agents (Table 1, Fig. 1) as percent of corresponding contralateral control [15] or T/C ratio [16] as shown below:

$$^{45}\text{Ca resorption (\%)} = \frac{^{45}\text{Ca released into the medium}}{^{45}\text{Ca released into the medium} + {}^{45}\text{Ca remaining in the bone (HCl extract)}} \times 100$$

$$\text{T/C ratio} = \frac{^{45}\text{Ca resorption in presence of PTH + test agent}}{^{45}\text{Ca resorption in presence of PTH + vehicle}}$$

2.2.2. Antiosteoporotic activity in vivo

2.2.2.1. Animals and treatment. Female rats (180–220 g) were randomized into six groups of eight each (Fig. 2) and kept in groups of four in plastic cages containing dry rice husk. Animals of four groups were bilaterally ovariectomized under light ether anesthesia and treated (po) with ormeloxifene (1.25 or 12.5 mg/kg per day), ethynylestradiol (1.25 mg/kg per day) or vehicle (gum acacia/10% ethanol in distilled water) once daily on days 1–30 post-ovariectomy (day 1: day of ovariectomy). Remaining two groups of females were sham-operated and treated similarly with vehicle or 1.25 mg/kg per day dose of ormeloxifene (which is its

Table 1
In vitro anti-resorptive activity of ormeloxifene and related compounds using ^{45}Ca prelabeled chick fetal femur bone chase cultures

Compound	Final[a] concentration (μM)	Percent ^{45}Ca resorption		Percent inhibition in ^{45}Ca resorption
		PTH[b] + vehicle[c]	PTH[b] + compound	
Ormeloxifene	50	27.20 ± 1.46	19.30 ± 0.66[d]	29
	100	21.15 ± 1.44	6.76 ± 0.38[d]	68
	200	44.30 ± 1.65	8.80 ± 1.51[d]	80
CDRI-85/287	100	46.40 ± 2.26	22.82 ± 1.60[d]	51
Tamoxifen	100	54.40 ± 2.43	28.65 ± 1.89[d]	47
17α-Ethynylestradiol	100	40.40 ± 2.61	38.30 ± 2.50	5

Values represent mean ± S.E.M. of six independent sets of bones in each treatment group.
[a] In 300 μl BGJ_b medium.
[b] 0.4 μM.
[c] Ethanol/DMSO, final concentration 0.1%.
[d] $P < 0.01$, vs. corresponding contralateral PTH + vehicle group. All other relevant comparisons were statistically not significant.

single day post-coital contraceptive dose in rat [7]) on days 1–30 post-ovariectomy. Pertinently, estrogen deficiency following ovariectomy leads to increased bone turnover and status 28 days post-ovariectomy in rats is considered to be comparable to that in post-menopausal women [17].

2.2.2.2. Urine collection. Rats were caged individually in all-glass metabolic cages fitted with steel mesh for a total period of 48 h preceding autopsy and had free access to pellet diet and tap water for first 24 h of acclimatization. During next 24 h, animals received only tap water ad libitum. Twenty-four hours fasting urine samples were collected in fresh containers, centrifuged at 2000 rpm at room temperature and stored at −20 °C until analyzed. Animals continued to receive respective treatment during this period. Urine samples were routinely collected on day 30 post-ovariectomy.

2.2.2.3. Autopsy and collection of tissues. Twenty-four hours after last treatment, about 5 ml blood samples were collected by cardiac puncture from each rat under light ether anesthesia and serum was isolated and stored at −20 °C until analyzed. Animals were then autopsied by excessive ether inhalation. Uterus of each rat was carefully excised, gently blotted, weighed and fixed in 4% formaldehyde in phosphate buffer (pH 7.4) for histology. About 5 mm pieces from middle segment of each uterus were dehydrated in ascending grades of alcohol, cleared in xylene and embedded in paraffin wax using standard procedures. Representative sections (5 μm) were stained with hematoxylin and eosin. Femur and tibia of each rat were then dissected free of adhering tissue, fixed in 70% ethanol [18] in saline and stored at −20 °C until bone mineral density (BMD) measurement.

2.2.2.4. Bone mineral density measurement. Before autopsy, whole body scan of each rat for measurement of BMD was performed on an Hologic QDR-4500A fan-beam densitometer, calibrated daily with Hologic hydroxyapatite anthropomorphic spine phantom using manufacturer provided software for small animals. Scans were performed on animals under light ether anesthesia positioned prone with care to avoid superimposition of bones with standard callimation of X-ray beam and scan speed of 1.67 mm/s (2.5 lines/mm). Tail was looped around to lie almost parallel to the animal and was included in scan window. BMD measurements of isolated bones were performed using identical regions of interest (femur: global, neck and mid-shaft; tibia: global and region about 2 mm proximal to tibio-fibular separation point) and scan speed of 1 mm/s (4 lines/mm).

2.2.2.5. Biochemical markers of bone turnover. Serum total alkaline phosphatase activity was estimated by commercially available kits (#396494; Boehringer Mannheim, Germany). Assay is based on colorimetric estimation of *p*-nitrophenol formed after breakdown of *p*-nitrophenylphosphate by alkaline phosphatase. Absorbance was read at 405 nm using a pre-programmed semiautomatic photometer (model 5010, Boehringer Mannheim, Germany).

Changes in serum bone-specific alkaline phosphatase activity were determined electrophoretically using commercially available kit (#710-A; Sigma Diagnostics, St. Louis, USA) according to the manufacturer's instructions. In the absence of a commercially available standard, serum of an adult human suffering from hepatitis was taken. The hepatic isoform of alkaline phosphatase is known to move beyond the bone-specific isoform. Briefly, 1.2% agarose gel was equilibrated with AP equilibration buffer for 1 h and 5 μl serum samples representing each treatment group were run (70 V, 3 h) with application wells kept closest to the cathode. The gel was then incubated for 1 h at 37 °C in 20 ml of the enzyme substrate 5-bromo-4-chloro-3-indolyl phosphate, containing the buffer capable of transphosphorylation to increase assay sensitivity, fixed in methanol–acetic acid for 30 min and washed with distilled water. The gels were scanned on a BioRad Densitometer (model: GS-670) using manufacturer provided software (Molecular Analyst, version 3.1) taking equal area (49 mm^2) of the scan window for each band and the optical density, automatically adjusted in

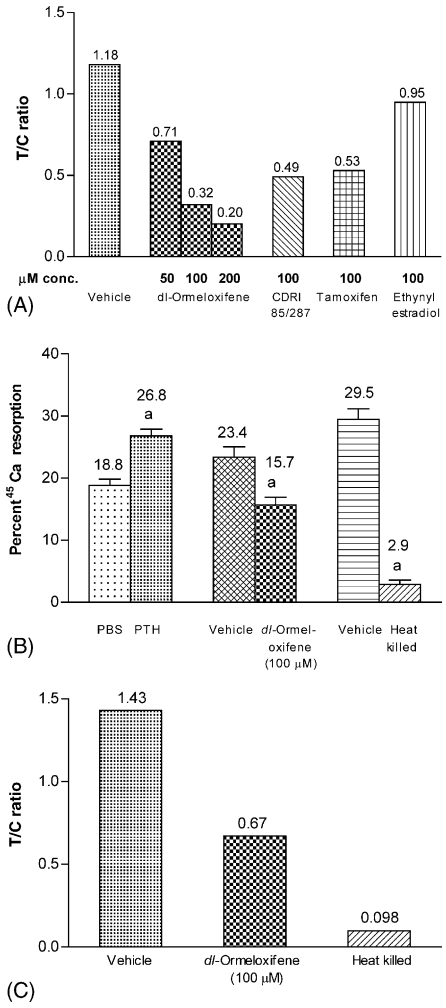

Fig. 1. PTH-induced resorption of ^{45}Ca from prelabeled chick (A) and rat (B and C) fetal limb bones in vitro. Note high (1.18) T/C ratio in PTH (0.4 μM) per se treatment group and its concentration-dependent inhibition by ormeloxifene in chick fetal femur bones (A). At 100 μM concentration, CDRI-85/287 (T/C: 0.49) and tamoxifen (T/C: 0.53) were slightly less effective than ormeloxifene (T/C: 0.32), while 17α-ethynylestradiol, the orally effective ester of estradiol-17β, was almost ineffective (T/C: 0.95). In rat fetal limb bones, there was marked increase (42.6%) in resorption (B) with T/C ratio of 1.43 (C) by PTH and its inhibition (32.9%; T/C: 0.67) by ormeloxifene. Heat killed bones did not respond to PTH (resorption: 2.9%; T/C: 0.098) during entire 96 h culture period. T/C ratio >1.0 indicates increased rate of bone resorption, while values below one indicate anti-resorptive response, except in case of heat killed bones which failed to respond to PTH. Values represent mean of experiments on six independent sets of bones in each treatment group. Vertical bars indicate standard error of mean. (a) $P < 0.01$, vs. corresponding contralateral PTH + vehicle group. All other relevant comparisons were statistically not significant.

relation to respective band area and background, was plotted (Fig. 5B).

For serum osteocalcin estimation, one step sandwich ELISA using streptavidin technology (#1822047; Boehringer Mannheim, Germany) was used. Assay quantitatively detects the most important stable metabolite, N-terminal middle fragment (N-Mid fragment; aa 1–43), of

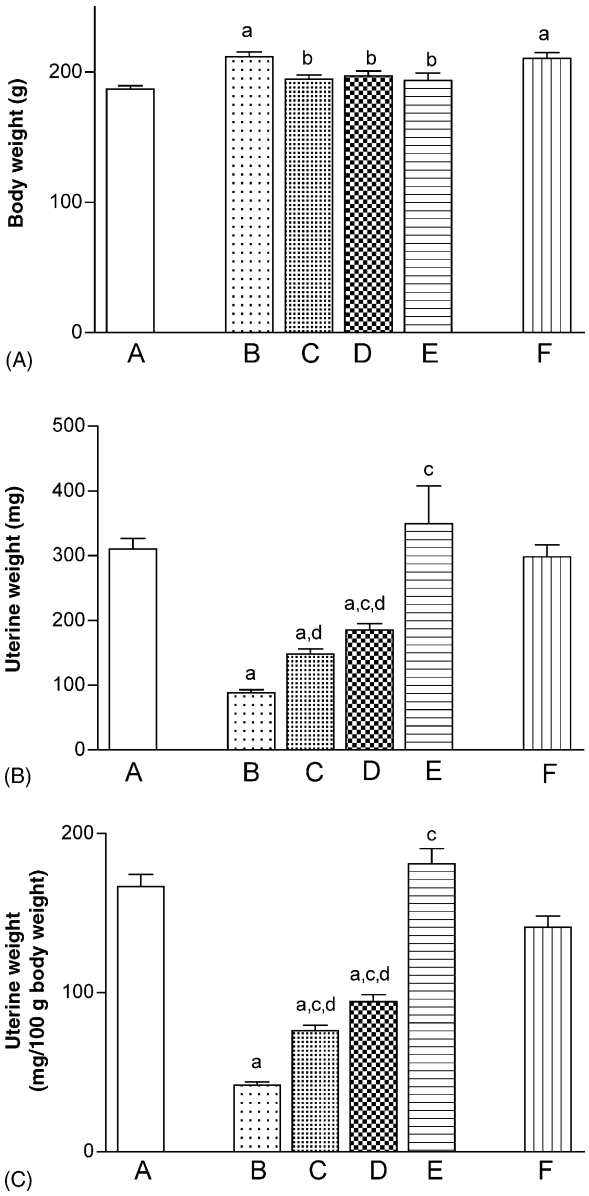

Fig. 2. Effect of ovariectomy and/or ormeloxifene (1.25 or 12.5 mg/kg per day, po for 30 days) supplementation on body weight (A) and uterine weight (B and C) in adult female rats. Note ovariectomy-induced significant increase in body weight and decrease in uterine weight. Ormeloxifene (1.25 or 12.5 mg/kg) or ethynylestradiol (1.25 mg/kg) supplementation prevented ovariectomy-induced increase in body weight and caused weak (ormeloxifene) to potent (ethynylestradiol) uterotrophic effect. In intact females, ormeloxifene (1.25 mg/kg) produced mild decrease in uterine weight. Vertical bars indicate standard error of mean. (A) Sham-intact control; (B) ovariectomized + vehicle; (C) ovariectomized + ormeloxifene (1.25 mg/kg per day); (D) ovariectomized + ormeloxifene (12.5 mg/kg per day); (E) ovariectomized + 17α-ethynylestradiol (1.25 mg/kg per day); (F) Sham-intact + ormeloxifene (1.25 mg/kg per day); (a) $P < 0.01$, vs. sham intact control group; (b) $P < 0.05$, (c) $P < 0.01$, vs. ovariectomized + vehicle treatment group; (d) $P < 0.01$, vs. ethynylestradiol treatment group. All other relevant comparisons were statistically not significant.

protease cleavage between aa 43 and 44, in addition to intact osteocalcin which is unstable under routine conditions in serum or plasma. This assay, performed routinely on day of sampling, assures that short interim time between sampling and measurements had no effect on the results. Specificity of assay is based on the fact that it employs two monoclonal antibodies specifically directed against epitopes in vicinity of N-terminal or N-side labile amino acid bridge 43–44, which do not cross-react with other endogenous osteocalcin fragments.

Assay for measurement of calcium ion content in serum and urine samples using commercial kits (#1553593; Boehringer Mannheim, Germany) was based on the principle that calcium forms a violet complex with *o*-cresolphthalein complexone in alkaline medium. Values in urine samples were represented as calcium/creatinine ratio to correct variation in individual urine volumes [19].

Urine creatinine was estimated colorimetrically using commercial kit (#977721; Boehringer Mannheim, Germany). Assay is based on measurement of rate of formation of a colored complex by creatinine with picrate in alkaline medium.

Inter- and intra-assay variations for all the biomarkers were within normal limits, i.e. ≤10%.

2.3. Statistical analysis

In vitro data on antiresorptive activity using chick and rat fetal bone culture assay was analyzed using paired *t*-test. In case of in vivo data, mean of groups were compared by analysis of variance followed by multiple comparison. Individual testing of means was done by Newman–Keuls test [20].

3. Results

3.1. Antiresorptive activity in vitro

Ormeloxifene concentration-dependently inhibited PTH-induced resorption of ^{45}Ca from chick fetal femur bones and the inhibition was of the order of 29, 68 and 80% (Table 1) with T/C ratio of 0.71, 0.32 and 0.20 (Fig. 1A) at 50, 100 and 200 μM concentration, respectively. Inhibition in ^{45}Ca resorption at 100 μM concentration of CDRI-85/287 (51%) and tamoxifen (47%) was slightly lower than that of ormeloxifene (68%). Ethynylestradiol (100 μM) was almost ineffective in preventing PTH-induced resorption with T/C ratio (0.95) close to unity.

Rat fetal limb bones (humerus + radio-ulna) exhibited basal resorption rate of 18.8%, which increased to 26.8% with T/C ratio of 1.43 following addition of PTH into the medium (Fig. 1B). Ormeloxifene (100 μM) inhibited PTH-induced ^{45}Ca resorption by 32.9% (T/C: 0.67), confirming its antiresorptive activity. PTH had no effect on heat-killed bones, as evidenced by only 2.9% resorption (T/C: 0.098; Fig. 1C) of ^{45}Ca during the entire 96 h culture period. Difference in percent resorption in response to PTH per se in individual chick/rat fetal limb bones depicts normal variation.

3.2. Antiosteoporotic activity in vivo

3.2.1. Body weight

Bilateral ovariectomy induced statistically significant ($P < 0.01$) increase in body weight in female rats (Fig. 2A). The increase (13.3%) was almost similar to that observed after 1.25 mg/kg per day dose of ormeloxifene (12.7%) administered to sham-intact females. Ormeloxifene (1.25 or 12.5 mg/kg) or ethynylestradiol (1.25 mg/kg) supplementation beginning on day of ovariectomy prevented, to almost similar extent (8.16, 7.03 and 8.59%, respectively), ovariectomy-induced increase in body weight ($P < 0.05$).

3.2.2. Uterine weight and histology

There was a marked (∼70%, $P < 0.01$; Fig. 2B and C) decrease in uterine weight following ovariectomy and a weak uterotrophic response following ormeloxifene supplementation. Increase (1.68-fold) in uterine weight after 1.25 mg/kg per day dose of ormeloxifene was statistically not significant, but became significant (1.76-fold; $P < 0.01$) when represented on mg/100 g body weight basis. Response (absolute: 2.09-fold, mg/100 g body weight: 2.18-fold) at 12.5 mg/kg per day dose, though not significantly different ($P > 0.05$) from that at 1.25 mg/kg per day dose, was slightly higher (1.24-fold) when represented in absolute or mg/100 g body weight terms, but remained lower than sham intact control rats. 17α-Ethynylestradiol (1.25 mg/kg per day), in comparison, produced marked ($P < 0.01$) increase in uterine weight when represented in absolute (3.79-fold) or mg/100 g body weight (4.18-fold) terms. Ormeloxifene (1.25 mg/kg per day) treatment to intact cyclic females for 30 days, in comparison, produced mild decrease (absolute: 4.2%; mg/100 g body weight: 15.3%; statistically not significant) in uterine weight. Histologically, uterine lumen in intact control rats was lined with low columnar epithelial cells with vacuolated cytoplasm. Uterine stroma was compact and there was infiltration of leucocytes into stroma and epithelium (Fig. 3A and B). In ovariectomized rats treated with vehicle alone, there was marked decrease in uterine diameter and luminal epithelial cell height. Endometrial stroma was compact and fibroblastic (Fig. 3E and F). Ormeloxifene treatment (1.25 mg/kg per day) to ovariectomized rats exhibited very weak estrogenic response with only slight increase in luminal epithelial cell height and subepithelial edema (Fig. 3G and H). At 12.5 mg/kg per day dose, there was a marked increase in uterine diameter and luminal epithelial cell height (Fig. 3I and J) and the effect was comparable to that seen after 1.25 mg/kg per day dose of ethynylestradiol (Fig. 3K and L), except that increase in number of glands, endometrial folding and vacuolization of luminal epithelial cells, as observed in ethynylestradiol-treated rats, were absent.

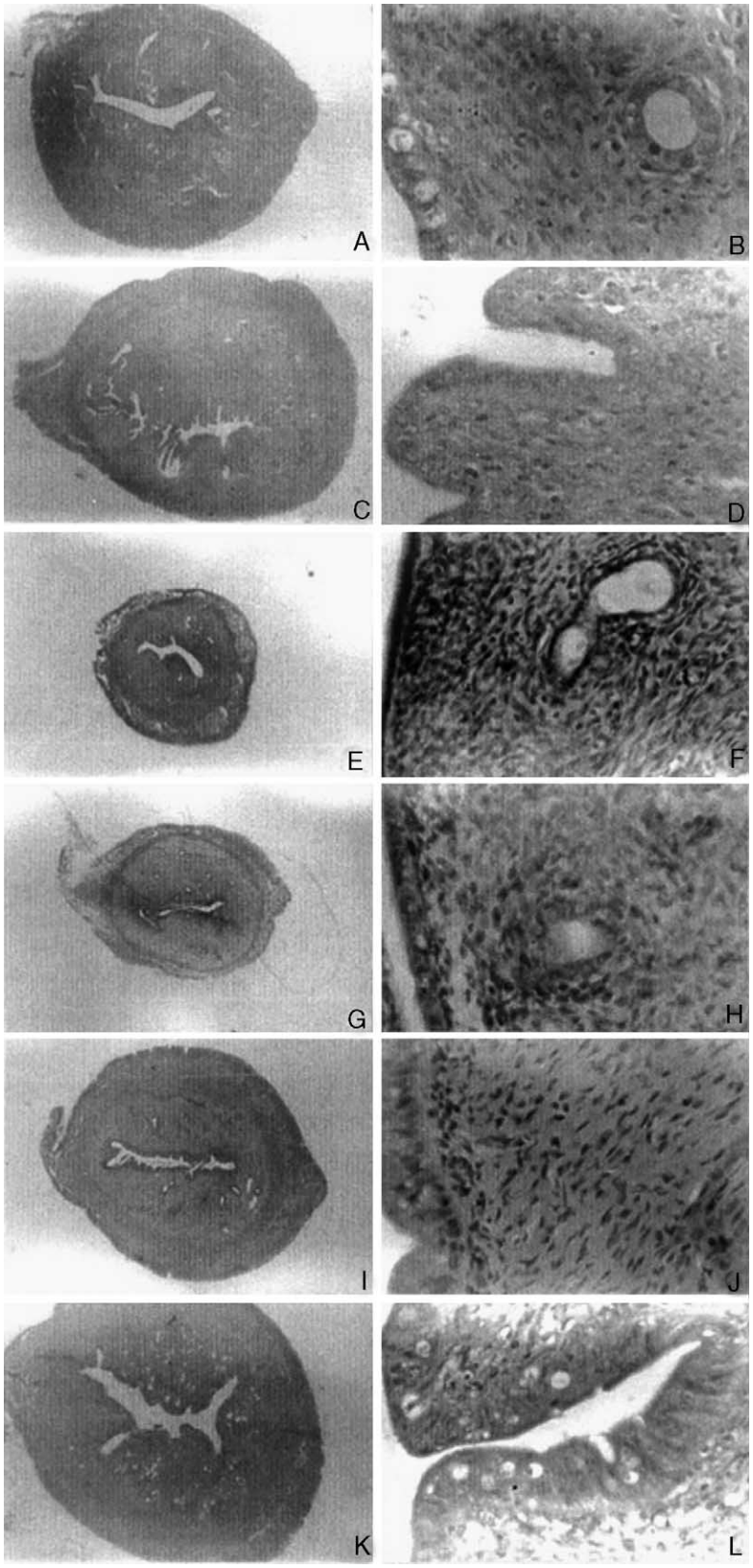

Fig. 3. Transverse sections of rat uterus. Note marked decrease in uterine diameter and luminal epithelial cell height with compact and fibroblastic endometrial stroma following ovariectomy (E and F) and weak estrogenic response with slight increase in luminal epithelial cell height and subepithelial edema following 1.25 mg/kg dose of ormeloxifene (G and H). At 12.5 mg/kg per day dose, there was marked increase in uterine diameter and luminal epithelial cell height (I and J), comparable to that after 1.25 mg/kg per day dose of ethynylestradiol (K and L). In comparison, increased endometrial folding, without markedly affecting epithelial cell height, was observed in intact rats treated with 1.25 mg/kg dose of ormeloxifene (C and D). (A, C, E, G, I and K) 60×; (B, D, F, H, J and L) 800× (approximately).

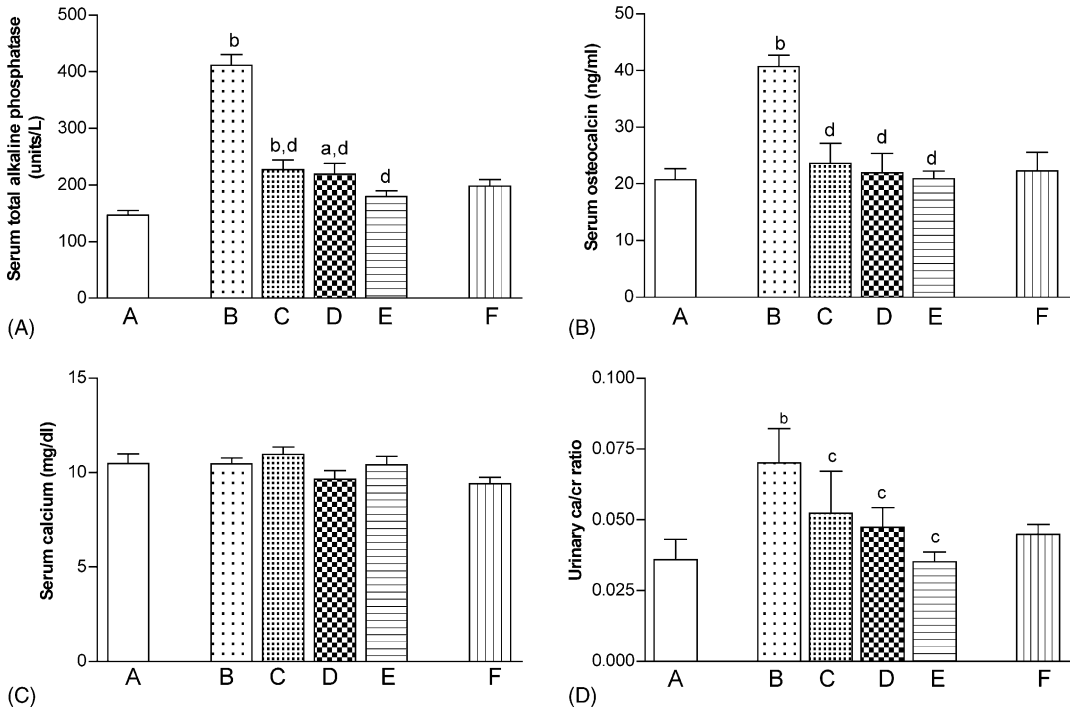

Fig. 4. Effect of ovariectomy and/or ormeloxifene (1.25 or 12.5 mg/kg) supplementation on serum and urine markers of bone turnover in adult female rats. Note marked increase in serum total alkaline phosphatase activity (A), osteocalcin concentration (B) and urine calcium/creatinine ratio (D) following ovariectomy and their decrease to almost sham-vehicle control levels following ormeloxifene or ethynylestradiol supplementation beginning on day of ovariectomy. Serum calcium concentration (C) did not show any significant change following ovariectomy or ormeloxifene/ethynylestradiol supplementation. (a) $P < 0.05$, (b) $P < 0.01$, vs. sham-intact control group; (c) $P < 0.01$, vs. ovariectomized + vehicle-treatment group. All other relevant comparisons were statistically not significant.

Increased endometrial folding, without markedly affecting epithelial cell height, was also observed in cyclic rats treated with 1.25 mg/kg dose of ormeloxifene (Fig. 3C and D).

3.2.3. Serum alkaline phosphatase and osteocalcin

There was marked increase ($P < 0.01$) in serum total alkaline phosphatase activity (2.79-fold) as well as osteocalcin concentration (1.96-fold) following ovariectomy and their decrease ($P < 0.01$, versus ovariectomized + vehicle-treatment group; Fig. 4A and B) to almost sham-control level following ormeloxifene or ethynylestradiol supplementation, except that serum total alkaline phosphatase activity still remained significantly higher in rats receiving 1.25 mg/kg per day ($P < 0.01$) or 12.5 mg/kg per day ($P < 0.05$) dose of ormeloxifene when compared to sham-intact females.

Maximum activity of bone-specific alkaline phosphatase, based on optical density adjusted in relation to respective band area and background (lane H) was observed in young (1-month-old rat; lane A, Fig. 5) female rats, representing high rate of bone turnover. The activity in adult (3-month-old) sham-operated intact females (lane B) was much lower and increased to almost the 1-month-old rat level following bilateral ovariectomy (lane D), which was prevented by supplementation with ormeloxifene (lanes E: 1.25 mg/kg per day; lane F: 12.5 mg/kg per day) or ethynylestradiol (lane G) beginning on the day of ovariectomy, the effect appeared better after ethynylestradiol than even the higher dose of ormeloxifene. There was, however, no apparent effect on total or bone-specific (lane C, Fig. 5) alkaline phosphatase activity or osteocalcin concentration following ormeloxifene treatment to intact females.

3.2.4. Serum calcium and urine calcium/creatinine ratio

Serum calcium concentration (Fig. 4C) did not show any significant change following ovariectomy or ormeloxifene/ethynylestradiol supplementation to intact or ovariectomized females.

Sham-intact rats receiving vehicle alone exhibited urine calcium/creatinine ratio of 0.0359 ± 0.0071, which increased markedly to 0.0692 ± 0.0118 ($P < 0.01$, Fig. 4D) 30 days after ovariectomy. Ormeloxifene (1.25 or 12.5 mg/kg) significantly decreased urine calcium excretion rate as evidenced by decreased calcium/creatinine ratio ($P < 0.01$, Fig. 4D), the effect being almost similar at the two doses. Ethynylestradiol, in comparison, produced an apparently greater decrease in urine calcium excretion rate (calcium/creatinine ratio: 0.0352 ± 0.0033; $P < 0.01$, versus ovariectomized + vehicle treatment group) and the levels were comparable to that of sham-intact controls.

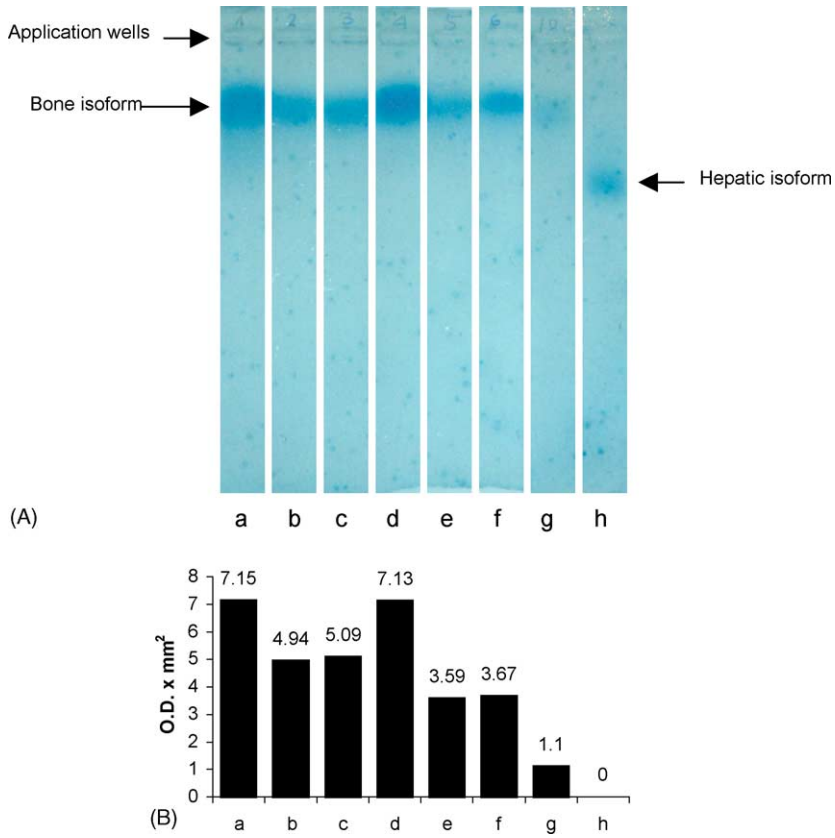

Fig. 5. Electrophoretic evaluation of bone-specific alkaline phosphatase in rat serum (A) and its densitometric analysis (B). (a) 1-month-old female rat; (b) 3-month-old adult sham-operated intact female rat; (c) sham + ormeloxifene (1.25 mg/kg per day); (d) ovariectomized + vehicle; e: ovariectomized + ormeloxifene (1.25 mg/kg per day); f: ovariectomized + ormeloxifene (12.5 mg/kg per day; g: ovariectomized + ethynylestradiol (1.25 mg/kg per day); h: hepatic isoform of alkaline phosphatase in serum of an adult human suffering from hepatitis.

3.2.5. Bone mineral density

Bone mineral density (global) of isolated femur bones was generally more than that of tibia, being maximum in femur neck and minimum in region proximal to tibio-fibular separation point (TFSP; Fig. 6). Bilateral ovariectomy was accompanied by decrease in BMD of isolated femur and tibia 30 days post-ovariectomy. Decrease was maximum in neck (28.3%, $P < 0.01$) and mid-shaft (23.7%, $P < 0.01$) regions of femur, but only marginal (6.7%, $P > 0.05$) in region proximal to TFSP (Fig. 6C, D and F). Decrease based on *T*-/*Z*-score, too, was >2.5 S.D. of normal adult/age-matched females in femur neck and mid-shaft, but not in region proximal to TFSP (Table 2).

Bilateral ovariectomy induced decrease in BMD was prevented by ormeloxifene (1.25 or 12.5 mg/kg) or ethynylestradiol (1.25 mg/kg) supplementation. Mean BMD levels were also apparently more in females supplemented with 12.5 than 1.25 mg/kg dose of ormeloxifene, but a clear dose response was not evident (Fig. 6). However, *T*-/*Z*-score analysis of the increase (over ovariectomized control levels) clearly demonstrates complete prevention of ovariectomy-induced osteoporosis in rats receiving 12.5 mg/kg per day dose of this SERM, with BMD values reaching more than (femur: neck; tibia: global, TFSP) or close to (femur: global, mid-shaft) the corresponding sham-intact control levels (Table 2). A similar effect was evident following ethynylestradiol (1.25 mg/kg per day) supplementation with mean values in all segments analyzed being more than even the corresponding sham-intact females.

At 1.25 mg/kg per day dose of ormeloxifene, however, increase in BMD reached almost the osteopenic levels with difference in mean BMD close to 1.0 S.D. of corresponding sham-intact controls only in femur (global and neck). Their mid-shafts exhibited no (tibia) or mild (femur) response. This together with comparatively mild increase in BMD of tibia (global), with slight improvement in *T*-/*Z*-score, might suggest a much higher threshold level of tibia than femur and more so for their mid-shafts.

Ormeloxifene treatment to intact females invariably increased BMD of femur as well as tibia and levels were comparable to that observed after 1.25 mg/kg per day dose of ethynylestradiol, with values reaching at least 1.0 S.D. more than even the corresponding intact control levels (Table 2).

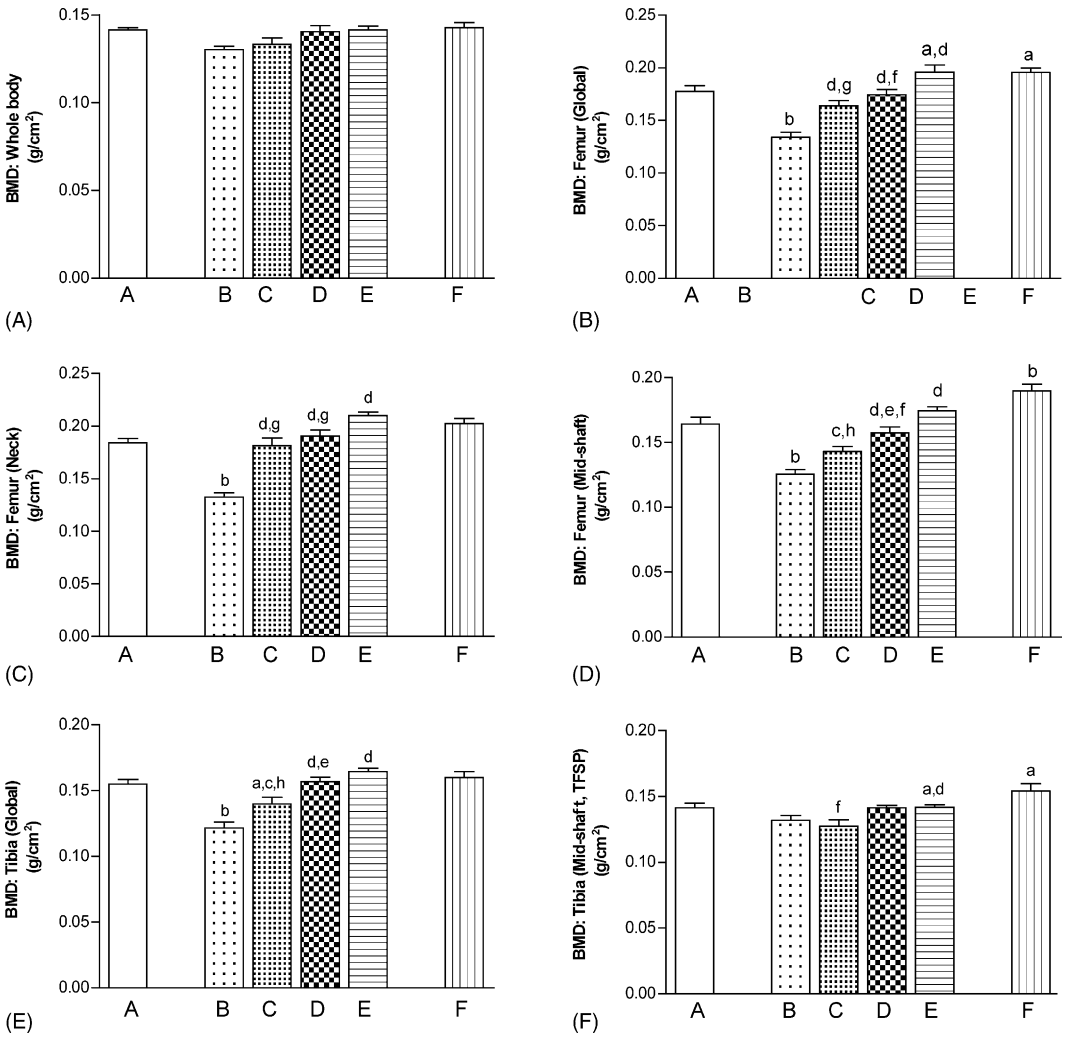

Fig. 6. Effect of ovariectomy and/or ormeloxifene (1.25 or 12.5 mg/kg, po) supplementation on BMD of whole body (A) and isolated femur and tibia bones of adult female rats. Note marked decrease in BMD of isolated femur (B) and tibia (E) bones, being maximum in neck (C; 28.3%) and mid-shaft (D; 23.7%) regions of femur, but only marginal (F; 6.7%) in region proximal to TFSP. This was prevented by ormeloxifene or ethynylestradiol administration. Effect, though apparently more in females supplemented with 12.5 mg/kg than 1.25 mg/kg dose of ormeloxifene, was not always significantly different and clear dose–response was not evident. (a) $P < 0.05$, (b) $P < 0.01$, vs. sham-intact control group; (c) $P < 0.05$, (d) $P < 0.01$, vs. ovariectomized + vehicle treatment group; (e) $P < 0.05$, vs. lower dose (1.25 mg/kg, po) of ormeloxifene-treatment group; (f) $P < 0.05$, (g) $P < 0.01$, vs. ethynylestradiol-treatment group. All other relevant comparisons were statistically not significant.

4. Discussion

Results of this study clearly demonstrate antiosteoporotic activity of ormeloxifene as evidenced by concentration-dependent inhibition in PTH-induced resorption of ^{45}Ca from prelabeled chick and rat fetal limb bones, the effect being better than that with tamoxifen or CDRI-85/287, and its ability to prevent ovariectomy-induced increase in serum total and bone-specific alkaline phosphatase and osteocalcin and urine calcium/creatinine ratio and decrease in BMD of isolated femur and tibia bones.

Based on changes in serum and urine biomarkers of bone turnover and BMD, 12.5 mg/kg per day dose of ormeloxifene appeared better in preventing ovariectomy-induced bone loss, with its effect being almost similar to that observed after 1.25 mg/kg per day dose of ethynylestradiol. However, a strict dose–response relationship and comparison with ethynylestradiol was not obvious until BMD data was evaluated on *T*-/*Z*-score basis (Table 2), which according to Munoz et al. [21] compares patient's bone mass with mean bone mass of normal young adult reference population (*T*-score) or with mean BMD of a person of same age (*Z*-score). In the present study, the above comparison, if applicable to rats, would refer both to *T*- as well as *Z*-scores, since this study was conducted simultaneously on randomized 3-month-old adult female rats, an age when they achieve maximum BMD [22]. To the best of our knowledge, this might be the first report of comparison of BMD data using *T*-/*Z*-score in any laboratory animal species. It thus appears that 1.25 mg/kg per day dose of ormeloxifene failed

Table 2
Evaluation of effect of ovariectomy and ormeloxifene or 17α-ethynylestradiol supplementation on the extent of osteoporosis based on *T*-/*Z*-score in adult female rats

Parameter	Femur			Tibia	
	Global	Neck	Mid-shaft	Global	TFSP[a]
Mean ± S.D. of sham-intact controls	0.1776 ± 0.0153	0.1843 ± 0.0113	0.1643 ± 0.0144	0.1550 ± 0.0098	0.1413 ± 0.0099
2.5 S.D. of sham-intact controls	0.0383	0.0283	0.0360	0.0245	0.0248
Sham-intact control vs. sham-intact + ormeloxifene (1.25 mg/kg per day) treatment groups					
Difference in mean BMD	+0.0178[d]	+0.0182[d]	+0.0257[d]	+0.0049[d]	+0.0128[d]
Sham-intact control vs. ovariectomized + vehicle treatment groups					
Difference in mean BMD	−0.0435[b]	−0.0521[b]	−0.0390[b]	−0.0334[b]	−0.0095
Sham-intact control vs. ovariectomized + ormeloxifene (1.25 mg/kg per day) treatment groups					
Difference in mean BMD	−0.0135	−0.0029	−0.0214[c]	−0.0152[c]	−0.0125[c]
Sham-intact control vs. ovariectomized + ormeloxifene (12.5 mg/kg per day) treatment groups					
Difference in mean BMD	−0.0031	+0.0063[d]	−0.0069	+0.0018[d]	+0.0001[d]
Sham-intact control vs. ovariectomized + ethynylestradiol (1.25 mg/kg per day) treatment groups					
Difference in mean BMD	+0.0183[d]	+0.0257[d]	+0.0102[d]	+0.0093[d]	+0.0162[d]

T-score compares patient's bone mass with mean bone mass of normal young adult reference population, while *Z*-score compares patient's BMD with mean BMD of a person of same age [21]. The above comparison, if applicable to rats, would refer both to *T*- and *Z*-scores, since this study was conducted simultaneously on randomized colony-bred 3-month-old adult female rats, an age when they achieve almost maximum BMD [22].

[a] Window about 2 mm proximal to tibio-fibular separation point (TFSP).

[b] Decrease in BMD >2.5 S.D. of corresponding sham-intact control value, a condition commonly referred to as osteoporosis [21].

[c] Decrease in BMD by >1.0 S.D., but <2.5 S.D., of the sham-intact control value, a condition commonly referred to as osteopenia [21].

[d] Increase in BMD over corresponding sham-intact control value.

to completely reverse ovariectomy-induced osteoporosis and the rats appeared to exhibit osteopenia, with decrease in BMD being >1.0 S.D., but <2.5 S.D., of the sham-intact control value [21]. This is not as clearly reflected in changes in biomarkers or mean BMD levels used in this study and routinely in laboratory animal [23] and clinical [24] studies. Further, using routine biomarkers and mean BMD levels as parameters, 1.25 mg/kg per day dose of ethynylestradiol appeared equipotent to 12.5 mg/kg per day dose of ormeloxifene. However, *T*-/*Z*-score analysis of BMD data clearly indicates 1.25 mg/kg per day dose of ethynylestradiol being far more effective with mean BMD values in all segments of isolated femur and tibia bones analyzed reaching at least 1.0 S.D. more than even corresponding sham-intact controls. This appears to be due to use of supra-physiological dose of estrogen used to draw a comparison with contraceptive and 10-fold higher doses of ormeloxifene evaluated in this study. In comparison, in rats receiving 12.5 mg/kg per day dose of ormeloxifene, whereas BMD values reached more than (femur: neck; tibia: global, TFSP) or close to (femur: global, mid-shaft) corresponding sham-intact control levels, differences were never >1.0 S.D. of sham-intact controls. Thus, while 12.5 mg/kg per day dose of this SERM appeared ideal in preventing ovariectomy-induced osteoporosis in this species, mild increase in uterine weight (109%, versus 279% after 1.25 mg/kg dose of ethynylestradiol) and associated increase in endometrial thickness and luminal epithelial cell height, almost close to that observed with ethynylestradiol, would make it unfavorable for such indications. Possibly, administration of 1.25 mg/kg per day dose of ormeloxifene over a longer period as would be required in clinical condition or titration of its dose/schedule might provide the regimen for optimal protection against post-menopausal bone loss, without significantly stimulating endometrial/breast tissue. Levormeloxifene, the L-analog of this molecule, which is a more potent receptor binder with relative binding affinity (RBA: 15.7 ± 3.1% of estradiol-17β) to rat uterine cytosol estrogen receptors (ER) [25], has recently been reported to inhibit bone loss in estrogen-deficient animal models [26]. In a double blind clinical trial, decrease in serum total alkaline phosphatase and urine excretion of C-terminal extension peptides has also been observed in post-menopausal women treated for 35 (20, 80 or 160 mg) or 56 (40 or 80 mg) days, indicating its estrogen-like bone preserving effect, but caused endometrial thickening due to fluid retention, without evidence of proliferation or hyperplasia [24]. Based on information generated in this study, it appears that DL-ormeloxifene might have better clinical acceptance in management of post-menopausal osteoporosis than levormeloxifene. It is also known to possess excellent therapeutic index and has been considered safe for chronic administration [7,27,28].

Like most antiestrogens of first generation such as tamoxifen [29] and clomiphene [18], ormeloxifene is a weak estrogen agonist [7,30]. In the present study, these as well as CDRI-85/287, a benzothiophene [31], were less effective than ormeloxifene in preventing PTH-induced ^{45}Ca resorption from prelabeled chick fetal femur bones. In an earlier study [32], tamoxifen and clomiphene (100 μM) were found to completely inhibit ^{45}Ca resorption from viable rat fetal limb bones, partial inhibition being observed at lower concentrations. Using in vitro bone slice assay [33],

ormeloxifene has been shown to inhibit osteoclastic bone resorption and osteoclast cytoplasmic spreading. However, raloxifene, also a benzothiophene reported to mimic estrogen in bone and cardiovascular system and exhibit estrogen antagonistic effect in endometrial tissue [8,9] with good clinical tolerance [34], was ineffective in this assay [33]. Raloxifene has also been recently reported to induce weak, but statistically significant, uterotrophic response (1.7-fold at 0.1 mg/kg dose) with significant increase in uterine dry weight and height, mitotic activity, vacuolization and degeneration of uterine luminal epithelial cells and number of endometrial glands in immature and ovariectomized rats [11,35] and a potent estrogenic effect on H-P-O axis as evidenced by decreased gonadotrophin secretion, hyperprolactinaemia, advanced vaginal opening, persistent presence of cornified epithelial cells in vaginal smears, anovulation, inhibition in positive feedback between estradiol and LH and infertility in neonatal rats treated with 50–500 μg doses of raloxifene on days 1–5 of age [12]. In comparison, while ormeloxifene induced 1.68-fold (statistically not significant) increase in absolute uterine weight, no evidence of vacuolization or degeneration of luminal epithelial cells or increase in number of endometrial glands was evident in this study even at 12.5 mg/kg per day dose administered for 30 consecutive days. Moreover no such effect on H-P-O axis has also been observed following subcutaneous administration of up to 500 μg dose of this SERM to male and female young ones on day 5 of post-natal life or for 7 consecutive days at day 14 of age [7]. Reports of lack of its effect on H-P-O axis when administered at contraceptive dose in rats and rhesus monkeys [7,36] are also available. Further, while ormeloxifene, because of very low toxicity, has been reported safe for chronic administration [7], raloxifene has recently been reported to increase incidence of hot flashes, deep vein thrombosis, pulmonary embolism and leg cramps similar to that associated with use of hormone replacement therapy [10].

In conclusion, this study, clearly demonstrates anti-osteoporotic activity of ormeloxifene in vitro and in vivo and the advantage that racemic mixture might have over its L-isomer in management of post-menopausal osteoporosis, in addition to providing beneficial effect on BMD in women taking this SERM for contraception or any other hormone-related clinical disorder.

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