Comparison of the effects of add-back therapy with various natural oestrogens on bone metabolism in rats administered a long-acting gonadotrophin-releasing hormone agonist

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Abstract

The hypoestrogenic state induced by gonadotrophin-releasing hormone agonist (GnRHa) has been shown to be effective in the treatment of oestrogen-dependent disorders but to induce bone loss. Adding back low doses of oestrogen in GnRHa therapy has been proposed to prevent bone loss. The purpose of this study is to assess the efficacy of add-back therapy with different natural oestrogens such as oestrone (OE1), oestradiol (OE2) and oestriol (OE3). Three-month-old female rats (250 g) were subcutaneously administered microcapsules of leuprolelin acetate in doses of 1 mg/kg of body weight every 4 weeks. GnRHa therapy lasted 16 weeks, and pellets of OE1, OE2 or OE3 (0.5 mg/pellet, 60 day release), as an add-back agent, were implanted at 8 weeks of treatment. At the end of treatment, GnRHa alone decreased bone mineral density of the femur and lumbar vertebrae, and increased serum levels of bone metabolic markers such as alkaline phosphatase and osteocalcin levels. As for cancellous bone histomorphometry, GnRHa decreased bone volume while it increased osteoid volume, osteoid surface, mineral apposition rate and bone formation rate. All the oestrogens tested prevented these changes caused by GnRHa therapy. GnRHa induced a significant increase in body weight and a marked reduction in uterine weight, which was not observed in OE1 or OE2 add-back group. Body weight and uterine weight of the OE3 add-back group were the same as those of the GnRHa group. These findings indicate that GnRHa induces high turnover bone loss which can be prevented by concomitant administration of natural oestrogens such as OE1, OE2 and OE3 to the same extent. In addition, OE3 is unique in that it is much less effective than OE1 and OE2 in blocking body weight gain and in promoting growth of uterine tissues. Because of its tissue-selective actions, OE3 could be considered as one of the most appropriate oestrogens used for GnRHa add-back therapy.

Introduction

Chronic administration of gonadotrophin-releasing hormone agonist (GnRHa) leads to inhibition of the pituitary–gonadal axis, resulting in a marked suppression of ovarian oestrogen production (Schally 1989). Accordingly, GnRHa has been widely used in the treatment of a variety of oestrogen–dependent disorders such as endometriosis (Wheeler et al. 1992) and uterine leiomyoma (Adamson 1992). However, long-term GnRHa treatment is not recommended because of concern about bone loss due to hypoestrogenism (Adashi 1994, Surrey 1995). In order to minimize bone loss caused by hypoestrogenism without undermining therapeutic efficacy of GnRHa, low dose or weak oestrogens in combination with GnRHa have been advocated, that is ‘add-back therapy’ (Adashi 1994, Surrey 1995), thus making long-term GnRHa treatment possible. To date, however, there is no agreement on an optimal GnRHa/steroid add-back regimen.

Oestriol (OE3) is a short-acting oestrogen because it is rapidly conjugated in the liver (Schiiff et al. 1980) and the duration of nuclear receptor binding is relatively short (Clark et al. 1977). A recent study has demonstrated that binding affinity of OE3 for oestrogen receptor α and β (ERα and ERβ) is remarkably lower than that of oestrone (OE1) or oestradiol (OE2) (Kuiper et al. 1997). Oestriol would, therefore, be expected to be an appropriate oestrogen used for add-back therapy. The duration of plasma OE3 elevation is 3–4 h after an oral administration (Englund et al. 1982). Notably, OE3 exerts either oestrogenically antagonistic or agonistic effects (Clark et al. 1977, Heimer 1987, Melamed et al. 1997). When given alone, it acts as an agonist, the potency of which depends on the dosage size and the frequency of administration (Heimer 1987). When given in conjunction with other more potent
were supplied by Takeda Pharmaceutical Co. (Osaka, Japan) for continuous drug release for 4 weeks after injection. Microcapsules of GnRHa leuprorelin acetate, which produces a long-acting GnRHa. This study particularly centred on cancellous bone histomorphometry in proximal tibiae and serum markers for bone metabolism to gain insight into the cellular, architectural and metabolic changes in the bone, in addition to the measurement of bone mineral density (BMD) of lumbar vertebrae (L1–L5) and femoral bone.

Materials and Methods

Chemicals

Microcapsules of GnRHa leuprorelin acetate, which provide continuous drug release for 4 weeks after injection, were supplied by Takeda Pharmaceutical Co. (Osaka, Japan). Pellets of OE1, OE2 and OE3 (0.5 mg/pellet, 60 day release) were purchased from Innovative Research Chemical Co. (St Louis, MO, USA). All other chemicals, unless otherwise mentioned, were obtained from Sigma Chemical Co. (St Louis, MO, USA).

Animals

Three-month-old female Sprague–Dawley rats, weighing 250 g, were obtained from Takasugi Experimental Animal Inc. (Saitama, Japan). The rats were in hanging wire cages, maintained at 25 °C with a 12 h light : 12 h darkness schedule, and had continuous access to food (MF Diet 1:15% calcium and 0:88% phosphorus, Oriental Yeast Co., Kanagawa, Japan) and allowed to drink water ad libitum. The guidelines for the care and use of the animals approved by the local institution were followed.

Experimental protocol

Rats were divided into five groups (six animals per group) and received the following treatments: group 1, the control, 0.2 ml injection vehicle alone was subcutaneously administered every 4 weeks; group 2, leuprorelin acetate microcapsules suspended in 0.2 ml injection vehicle were subcutaneously administered at a dose of 1 mg/kg body weight every 4 weeks; and groups 3–5, in addition to GnRHa administration, OE1, OE2 or OE3 pellets were subcutaneously implanted, respectively, on the back of animals with a trochar at the time of the third GnRHa injection. The oestrogen pellets were designed to release about 8 µg/day in rats weighing 250 g, which corresponds to clinical therapeutic dose (1–2 mg/day) of natural oestrogens in women. The treatment was continued for 16 weeks and GnRHa was injected four times in groups 2–5.

At the end of treatment, the rats were weighed and sacrificed by decapitation under ether anaesthesia, and trunk blood was collected. All the blood samples were centrifuged and serum was stored at −80 °C until assayed. Uteri were quickly removed, cleaned of surrounding tissues, and weighed. Lumbar vertebrae (L1–L5) and left femoral bone were removed, and their BMD was measured immediately. Right proximal tibiae were removed, dissected free of musculature, and fixed in 70% ethanol at room temperature for bone histomorphometry.

Determination of serum levels of oestrogens and bone metabolic markers

Serum levels of osteocalcin, OE1, OE2 and OE3 were determined by RIA. The RIA kits for osteocalcin, OE1, OE2 and OE3 were obtained from Biomedical Technologies (Stoughton, MA, USA), Diagnostic Systems Laboratories (Webster, TX, USA), Diagnostic Products Corporation (Los Angeles, CA, USA) and Amersham (Little Chalfont, UK) respectively. The detection limit of oestrogen assay was 5.0 pg/ml. Serum alkaline phosphatase (ALP) activities were determined by a standard colorimetric method, using Liquitech ALP kit (Roche Diagnostics K K, Tokyo, Japan).

Measurement of BMD

BMD was measured by dual-energy X-ray absorptiometry (DXA) using DPX-L (Lunar Co., Madison, WI, USA). Lateral lumber vertebrae (L1–L5) and antero–posterior left femoral bone were scanned to determine their BMD. The coefficients of variation for measuring BMD of lumbar vertebrae and femurs by DXA were 3% and 1% respectively.

Bone histomorphometric analysis

Tetracycline and calcien (Wako Pure Chemicals, Osaka, Japan) were subcutaneously injected to each rat at a dose of 13 and 25 mg/kg body weight on the 7th and 14th days before sacrifice respectively. After one month of the fixation in 70% ethanol, the right tibia specimens were stained with Villanueva bone stain solution for 10 days. They were dehydrated in a series of increasing concentrations of ethanol, defatted in acetone, and embedded in methyl methacrylate (MMA; Wako Pure Chemicals) mixture. Longitudinal sections of 3 µm were made with RM2065 microtome (Leica Instruments GmbH, Nussloch, Germany) and mounted on poly L-lysine coated slides.
glass slides. The sampling site was situated in the secondary spongiosa of the metaphyseal region of the proximal tibia at distances greater than 1 mm from the growth plate-metaphyseal junction to exclude the primary spongiosa. A total metaphyseal area of 0.5 mm² was sampled for each section. Photomicrographs were taken on each sampled metaphyseal area under a microscope (Nikon, Tokyo, Japan). Histomorphometric measurements were performed with Oscon A4–40 image analysis system (Pretec, Tokyo, Japan) interfaced with PC 9801 computer (NEC, Tokyo, Japan). Cancellous bone areas and surface lengths in the photomicrographs were traced with a cursor on a digitizing tablet and calculated automatically by the computer. Three different individuals made these observations three times each. Bone histomorphometric parameters were measured according to the report of ASBMR histomorphometry nomenclature committee (Parfitt et al. 1987).

**Statistical analysis**

All data are expressed as the mean ± s.e.m. Statistical analysis was performed using one-way analysis of variance (ANOVA) with post hoc Fisher’s protected least significant difference tests. The oestrogen-treated groups were compared with the control or GnRHa-treated group.

**Results**

In the control group, body weight, uterine weight and BMD of the femur and the lumbar vertebrae were 383.3 ± 11.7 g, 907.8 ± 188.1 mg, 0.27 ± 0.01 g/cm² and 0.18 ± 0.01 g/cm² respectively. The effects of GnRHa with or without oestrogens on body weight, uterine weight and BMD are shown graphically in Fig. 1. Rats given GnRHa alone exhibited a significant increase in body weight by 16.1% and a marked reduction in uterine weight by 81.8% compared with the control rats. These changes were eliminated by the concomitant treatment with OE1 or OE2. In contrast, OE3 did not prevent GnRHa-induced body weight gain and uterine atrophy.

![Figure 1](image-url) The effects of GnRHa and oestrogens on body weight, uterine weight and BMD of femur and lumbar vertebrae. Results are shown as mean percentage of untreated control ± s.e.m. (bars) of six animals. a, P < 0.05 vs GnRHa; b, P < 0.01 vs GnRHa; c, P < 0.05 vs GnRHa+OE3; d, P < 0.05 vs control.

The administration of GnRHa significantly reduced BMD of the femur and the lumbar vertebrae by 21.6% and 38.1% respectively compared with the control. The BMD of rats given either OE1 or OE2 in combination with GnRHa was significantly higher than that of those given GnRHa alone. Rats administered OE3 with GnRHa displayed BMD comparable with the control.

Serum oestrogen levels are shown in Table 1. In the control rats, serum OE2 levels range from 10.0 to 38.3 pg/ml.

**Table 1 Serum oestradiol levels (pg/ml). Values are shown as mean ± s.e.m.**

<table>
<thead>
<tr>
<th>Groups</th>
<th>OE1</th>
<th>OE2</th>
<th>OE3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>ND</td>
<td>10.0–20.2</td>
<td>ND</td>
</tr>
<tr>
<td>GnRHa</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>GnRHa+OE1</td>
<td>24.0 ± 5.7</td>
<td>33.0 ± 7.9</td>
<td>ND</td>
</tr>
<tr>
<td>GnRHa+OE2</td>
<td>ND</td>
<td>50.8 ± 11.5</td>
<td>ND</td>
</tr>
<tr>
<td>GnRHa+OE3</td>
<td>ND</td>
<td>ND</td>
<td>41.3 ± 27.0</td>
</tr>
</tbody>
</table>

ND, not detectable by RIA.

OE3 attenuated these changes in bone parameters found in measurable amounts of serum OE1 and OE2. Serum OE2 and OE3 were detected in rats treated with undetectable levels. As expected, considerable levels of BV, bone volume; TV, tissue volume; OV osteoid volume; OS, osteoid surface; BS, bone surface; ES, eroded surface.

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In the present study, we have demonstrated that OE3 has unique tissue selective effects in oestrogen-deficient animals treated with GnRHa. Oestriol produced effects similar to OE1 and OE2 on BMD, bone metabolic markers and cancellous bone turnover. Interestingly, OE3 was shown to be less oestrogenic as judged by uterine size and body weight gain in comparison with OE1 and OE2.

It remains controversial whether OE3 can effectively prevent bone loss affecting postmenopausal women (Lindsay et al. 1979, Yang et al. 1995, Minaguchi et al. 1996, Itoi et al. 1997). Yang et al. (1995) reported that 2 mg/day of oral OE3 administered for 2 years did not prevent bone loss in Chinese postmenopausal women. In another study in Scotland, postmenopausal osteoporosis was not prevented by administration of a high dose of 12 mg/day OE3 for 2 years (Lindsay et al. 1979). In contrast, a recent study conducted in Japan (Minaguchi et al. 1996) demonstrated that postmenopausal women given 2 mg/day OE3 along with 800 mg/day calcium lactate showed a significant increase in lumbar spine BMD (1-79%) after 50 weeks of treatment. The bone-preserving effect was also observed in postmenopausal women treated for 24 months with 2 mg/day OE3 plus 2.5 mg/day medroxyprogesterone acetate daily (Itoi et al. 1997). The discrepancy among these studies may be due to whether either calcium or progestin was coadministered or not.

In the present study, concomitant treatment with OE3 elicited a significant bone-protecting effect to the same extent as OE1 and OE2 in rats where hypoestrogenic state was induced by GnRHa. Although doses of OE3 administered and the species difference in the sensitivity to OE3 deserve investigation, we have presented evidence supporting bone-protecting effects of OE3 unsupplemented with other agents.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Effects of GnRHa and oestrogens on bone metabolic markers. Results are expressed as mean ± S.E.M.</th>
</tr>
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<tbody>
<tr>
<td>Groups</td>
<td>ALP (IU/l)</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>344.6 ± 16.2a</td>
</tr>
<tr>
<td>GnRHa</td>
<td>624.2 ± 61.7</td>
</tr>
<tr>
<td>GnRHa + OE1</td>
<td>284.0 ± 47.6a</td>
</tr>
<tr>
<td>GnRHa + OE2</td>
<td>467.4 ± 36.7a</td>
</tr>
<tr>
<td>GnRHa + OE3</td>
<td>311.7 ± 37.9a</td>
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</tbody>
</table>

*p < 0.05 vs GnRHa; b P < 0.05 vs control.

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Discussion

In the present study, we have demonstrated that OE3 has unique tissue selective effects in oestrogen-deficient animals treated with GnRHa. Oestriol produced effects similar to OE1 and OE2 on BMD, bone metabolic markers and cancellous bone turnover. Interestingly, OE3 was shown to be less oestrogenic as judged by uterine size and body weight gain in comparison with OE1 and OE2.

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Table 3 Results of cancellous bone histomorphometry. Results are expressed as mean ± S.E.M.

<table>
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</thead>
<tbody>
<tr>
<td>Bone volume (BV/TV, %)</td>
<td>17.0 ± 1.3b</td>
<td>5.7 ± 1.1</td>
<td>14.1 ± 2.7a</td>
<td>12.6 ± 1.9b</td>
<td>16.5 ± 1.2b</td>
</tr>
<tr>
<td>Osteoid volume (OV/BV, %)</td>
<td>3.0 ± 1.0a</td>
<td>5.5 ± 1.0</td>
<td>0.7 ± 0.2bc</td>
<td>0.8 ± 0.2bc</td>
<td>0.9 ± 0.2bc</td>
</tr>
<tr>
<td>Trabecular thickness (Th, Th, μm)</td>
<td>65.0 ± 4.0</td>
<td>48.0 ± 6.2</td>
<td>68.0 ± 7.5a</td>
<td>67.5 ± 9.5a</td>
<td>62.6 ± 6.3a</td>
</tr>
<tr>
<td>Osteoid surface (OS/BS, %)</td>
<td>24.1 ± 5.1</td>
<td>30.0 ± 4.0</td>
<td>7.9 ± 1.7bd</td>
<td>9.9 ± 2.0bc</td>
<td>9.6 ± 2.4bc</td>
</tr>
<tr>
<td>Eroded surface (ES/BS, %)</td>
<td>42.2 ± 4.1b</td>
<td>68.5 ± 3.2</td>
<td>30.6 ± 5.3b</td>
<td>33.3 ± 5.2b</td>
<td>22.1 ± 2.0bd</td>
</tr>
<tr>
<td>Mineral apposition rate (MAR, μm/day)</td>
<td>0.6 ± 0.1b</td>
<td>1.5 ± 0.3</td>
<td>0.4 ± 0.2b</td>
<td>0.5 ± 0.2b</td>
<td>0.6 ± 0.2b</td>
</tr>
<tr>
<td>Bone formation rate (BFR/BS, μm2/μm2 per day)</td>
<td>0.2 ± 0.03b</td>
<td>0.8 ± 0.3</td>
<td>0.1 ± 0.02b</td>
<td>0.1 ± 0.03b</td>
<td>0.1 ± 0.04b</td>
</tr>
</tbody>
</table>

*p < 0.05 vs GnRHa; b P < 0.01 vs GnRHa; c P < 0.05 vs control; d P < 0.01 vs control.

BV, bone volume; TV, tissue volume; OV osteoid volume; OS, osteoid surface; BS, bone surface; ES, eroded surface.
Several previous studies using rats showed oestrogen deficiency and bone loss induced by long-term administration of GnRHa such as leuprorelin and buserelin to be as effective as bilateral ovariectomy (Goulding & Gold 1989, Goulding et al. 1991, Goulding & Fisher 1992, Kurabayashi et al. 1993). Little is known about the influence of GnRHa on the synthesis and metabolism of hormones other than gonadotrophins and sex steroid hormones. However, a slight decrease in serum levels of growth hormone and insulin-like growth factor-I (IGF-I), which are known to stimulate bone metabolism, was occasionally observed in rats and mice treated with GnRH analogues (Yano et al. 1992). The mechanisms underlying such marginal unexpected effects of GnRH analogues remain unclear at present. It has been well known that bone loss caused by GnRHa treatment is associated with an increased bone turnover in women (Raven et al. 1994, Nakayama et al. 1997) and rats (Kurabayashi et al. 1993). In our study, serum OE2 levels fell to undetectable levels and BMD of the femoral bone and lumbar vertebrae decreased in GnRHa-treated rats. Regarding serum bone metabolic markers, ALP and osteocalcin, representative markers for bone formation, increased in GnRHa-treated rats. As for cancellous bone histomorphometry, GnRHa administration decreased bone volume and trabecular thickness, and increased bone resorption as determined by eroded surface and bone formation judging from increases in osteoid volume, osteoid surface, mineral apposition rate and bone formation rate. The addition of OE1, OE2 or OE3 prevented all of the bone changes caused by GnRH administration. Notably, oestrogens reduced the overall rate of bone turnover despite increasing bone volume and trabecular thickness. We reasoned that a subtle imbalance between bone formation and bone resorption, but not the status of respective bone formation and bone resorption, determines bone volume.

Most of the effects of oestrogen on reproductive tissues are believed to be mediated through oestrogen receptor (ER). The ligand interaction with the receptor, the migratory step to the nucleus and the interaction of the receptor–ligand complex with DNA have been described (Khovidhunkit & Shoback 1999, McKenna et al. 1999). There are two transcription-activating sites on the receptor, and activation of both appears to be required for full oestrogen activity. In contrast to intensive investigation into the characterization of ER and the molecular mechanism of oestrogen actions in reproductive tissues, the precise mechanism for the effects of oestrogens on the bone remains to be fully determined. Identification of ER on osteoblasts, osteoblast-like cells and osteoclasts (Eriksen et al. 1988, Komma et al. 1988, Oursler et al. 1991, Arts et al. 1997) suggests direct actions of oestrogens on the bone through ER.

It is commonly believed that OE3 is a safe oestrogen because it has little effect on endometrial tissues. It was documented that OE3 given alone does not produce endometrial hyperplasia and other abnormalities as judged by endometrial cytology and histology (Tzingounis et al. 1978, Grasso et al. 1982, Minaguchi et al. 1996). In our study, OE3 given with GnRHa successfully averted bone loss while its effect on uterine tissue was minimal as compared with OE1 and OE2. In this sense, OE3 can be viewed as one of the selective oestrogen receptor modulators (SERMs) (Cosman & Lindsay 1999). However, there have been conflicting reports on the effect of OE3 on the endometrium (Tzingounis et al. 1978, Englund & Johansson 1980, Grasso et al. 1982, Punnonen & Söderström 1983, Montoneri et al. 1987, Minaguchi et al. 1996). It appears that the uterine growth-promoting effect of OE3 depends on the route, dosage and frequency of its administration (Clark et al. 1977, Heimer 1987).

It is known that the hypoestrogenic state induced by either GnRHa or ovariectomy leads to body weight gain in rats (Ramirez 1981, Goulding & Gold 1989). In our study the bodyweight gain induced by GnRHa treatment was eliminated by OE1 and OE2, but not by OE3. Although precise mechanisms of weight gain under hypoestrogenic state are poorly understood, this could be, in part, explained by oestrogen inhibiting the activity of lipoprotein lipase (LPL), an enzyme that hydrolyses circulating triglycerides, thus permitting their uptake and storage in adipose tissues (Ramirez 1981, Price et al. 1998). Viewed in this light, it is intriguing to speculate differential effects on LPL activity between OE3 and OE1 or OE2.

Oestriol has biological activities in common with SERMs such as raloxifene, tamoxifen and clomiphene which are weak oestrogen agonists on the uterus and potent agonists on the bone (Goulding & Fisher 1991 Goulding et al. 1992, Black et al. 1994, Evans et al. 1996, Sato et al. 1996, Jimenez et al. 1997, Cosman & Lindsay 1999, Khovidhunkit & Shoback 1999). However, OE2 is effective in the management of climacteric symptoms (Tzingounis et al. 1980, Minaguchi et al. 1996), whereas the other SERMs have been reported to rather induce or deteriorate vasomotor symptoms such as hot flushes (Cosman & Lindsay 1999, Khovidhunkit & Shoback 1999). Furthermore, as stated above, OE3 did not eliminate GnRHa-induced weight gain, whereas the other SERMs can reverse hypoestrogenism-induced weight gain in rats (Goulding & Fisher 1991, Goulding et al. 1992, Black et al. 1994, Evans et al. 1996, Sato et al. 1996, Jimenez et al. 1997). Thus, it appears that tissue-specific agonistic effects of these SERMs depend on the cell type, the ligand structure, ER subtypes, ER response element–promoter context and transcriptional cofactors (Khovidhunkit & Shoback 1999, McKenna et al. 1999).

In conclusion, these results imply that concurrent administration of OE3 might be useful in long-term GnRHa treatment in light of its bone-protecting effect associated with minimal uterotrophic activity, thus offering a safer medical treatment strategy for women with
ostrogen-dependent disorders. The precise molecular mechanism of OEST actions in various ostrogen target organs including the bone remains to be identified.

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References


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