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 oestril (OE₃). Three-month-old female rats (250 g) were subcutaneously administered microcapsules of leuporelin 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, eroded 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.

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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.

Oestril (OE₃) is a short-acting oestrogen because it is rapidly conjugated in the liver (Schiff *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 (ER α and ER β) is remarkably lower than that of oestrone (OE₁) or oestradiol (OE₂) (Kuiper *et al.* 1997). Oestril 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

oestrogens such as OE₂, it works as an antagonist (Melamed *et al.* 1997). Oestriol is thought to be safer than OE₁ and OE₂, especially in that it causes little, if any, change in uterine tissues such as endometrial proliferation and hyperplasia (Tzingounis *et al.* 1978, Grasso *et al.* 1982, Minaguchi *et al.* 1996). However, the efficacy of OE₃ in preventing bone loss remains to be established.

In the present study, in search of an optimal oestrogen for add-back therapy among various natural oestrogens, we compared the bone protective and uterine effects of OE₁, OE₂ and OE₃ in rats administered 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 leuporelin 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 of America (Sarasota, FL, 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 housed 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, leuporelin 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 lumbar 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 calcein (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

Figure 1 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 $\pm$ 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.

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 $\pm$ 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 $\pm$ 11.7 g, 907.8 $\pm$ 188.1 mg, 0.27 $\pm$ 0.01 g/cm² and 0.18 $\pm$ 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. 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

Table 1 Serum oestrogen levels (pg/ml). Values are shown as mean $\pm$ S.E.M.

	OE ₁	OE ₂	OE ₃
Groups			
Control	ND	10.0–20.2	ND
GnRHa	ND	ND	ND
GnRHa+OE ₁	24.0 $\pm$ 5.7	33.0 $\pm$ 7.9	ND
GnRHa+OE ₂	ND	50.8 $\pm$ 11.5	ND
GnRHa+OE ₃	ND	ND	41.3 $\pm$ 27.0

ND, not detectable by RIA.

Table 2 Effects of GnRHa and oestrogens on bone metabolic markers. Results are expressed as mean ± S.E.M.

Groups	ALP (IU/l)	Osteocalcin (ng/ml)
Control	344.6 ± 16.2 ^a	21.7 ± 1.3 ^a
GnRHa	624.2 ± 61.7	25.2 ± 1.2
GnRHa+OE ₁	284.0 ± 47.6 ^a	17.2 ± 0.8 ^{a,b}
GnRHa+OE ₂	467.4 ± 36.7 ^a	13.7 ± 1.1 ^{a,b}
GnRHa+OE ₃	311.7 ± 37.9 ^a	20.0 ± 1.6 ^a

^aP<0.05 vs GnRHa; ^bP<0.05 vs control.

20.2 pg/ml. In GnRHa-treated rats, serum OE₂ levels fell to undetectable levels. As expected, considerable levels of serum OE₂ and OE₃ were detected in rats treated with OE₂ and OE₃ respectively. OE₁-treated rats exhibited measurable amounts of serum OE₁ and OE₂.

Serum levels of ALP and osteocalcin in GnRHa-treated rats were significantly higher than those in the control rats (Table 2). These changes by GnRHa were abrogated by concomitant administration of OE₁, OE₂ or OE₃.

Data of cancellous bone histomorphometry are shown in Table 3. The bone volume of GnRHa-treated rats was diminished conspicuously, being 33.5% of the control. There was a slight decrease in trabecular thickness by GnRHa treatment. On the contrary, osteoid volume, eroded surface, mineral apposition rate and bone formation rate in GnRHa-treated rats significantly increased by 83.3%, 62.3%, 114.3% and 400.0% respectively compared with the control. There tended to be an increase in osteoid surface in GnRHa-treated rats although the difference between the GnRHa-treated group and the control was not significant. Concurrent treatment with OE₁, OE₂ or OE₃ attenuated these changes in bone parameters found in GnRHa-treated rats. A decrease in bone volume and trabecular thickness was eliminated by any oestrogen tested. It was noteworthy that osteoid volume and osteoid surface in oestrogen-treated groups were much lower than those in the control. Eroded surface was reduced by oestrogens to a lower level than that in GnRHa-treated group. Oestrinol decreased eroded surface the most

remarkably among oestrogens tested, the value being by far lower than that of the control. Mineral apposition rate and bone formation rate, which were stimulated by GnRHa treatment, were rather lower in the rats treated with OE₁, OE₂ or OE₃ than that of the control.

Discussion

In the present study, we have demonstrated that OE₃ has unique tissue selective effects in oestrogen-deficient animals treated with GnRHa. Oestrinol 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 progesterin 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 3 Results of cancellous bone histomorphometry. Results are expressed as mean ± S.E.M.

Measurement	Control	GnRHa	GnRHa+OE ₁	GnRHa+OE ₂	GnRHa+OE ₃
Bone volume (BV/TV, %)	17.0 ± 1.3 ^b	5.7 ± 1.1	14.1 ± 2.7 ^a	12.6 ± 1.9 ^a	16.5 ± 1.2 ^b
Osteoid volume (OV/BV, %)	3.0 ± 1.0 ^a	5.5 ± 1.0	0.7 ± 0.2 ^{b,c}	0.8 ± 0.3 ^{b,c}	0.9 ± 0.2 ^{b,c}
Trabecular thickness (Tb. Th, µm)	65.0 ± 4.0	48.0 ± 6.2	68.0 ± 7.5 ^a	67.5 ± 9.5 ^a	62.6 ± 4.3 ^a
Osteoid surface (OS/BS, %)	24.1 ± 5.1	30.0 ± 4.0	7.9 ± 1.7 ^{b,d}	9.9 ± 2.8 ^{b,c}	9.6 ± 2.4 ^{b,c}
Eroded surface (ES/BS, %)	42.2 ± 4.1 ^b	68.5 ± 3.2	30.6 ± 5.3 ^b	33.3 ± 5.2 ^b	22.1 ± 2.0 ^{b,d}
Mineral apposition rate (MAR, µm/day)	0.6 ± 0.1 ^b	1.5 ± 0.3	0.4 ± 0.2 ^b	0.5 ± 0.2 ^b	0.6 ± 0.2 ^b
Bone formation rate (BFR/BS, µm ³ /µm ² per day)	0.2 ± 0.03 ^b	0.8 ± 0.3	0.1 ± 0.02 ^b	0.1 ± 0.03 ^b	0.1 ± 0.04 ^b

^aP<0.05 vs GnRHa; ^bP<0.01 vs GnRHa; ^cP<0.05 vs control; ^dP<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 leuporelin 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, Komm *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, OE₃ 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

oestrogen-dependent disorders. The precise molecular mechanism of OE₃ actions in various oestrogen target organs including the bone remains to be identified.

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