

DT56a (Femarelle) stimulates bone formation in female rats

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Objective DT56a is a natural compound for the treatment of menopausal symptoms and osteoporosis. The aim of this study was to examine the effects of long term treatment (two months) with DT56a on the skeletal tissues of intact and ovariectomised (OVX) adult rats.

Design Thirty rats were divided into two groups, in one of which the rats were ovariectomised. The rats in each group were then treated for two months with DT56a, oestrogen or vehicle.

Setting University and hospital laboratories.

Population Thirty rats.

Methods Histomorphometric measurements of trabecular bone volume (expressed as a percentage of total bone volume), trabecular and cortical thickness and growth plate width were recorded by a computerised system. In addition, creatine kinase (CK)-specific activity, as marker of oestrogen receptor activation, was measured in skeletal tissues and in the uterus.

Main outcome measures The changes in the histomorphometric measurements.

Results OVX rats developed noticeable signs of osteoporosis, namely, significant decrease in trabecular bone volume and in trabecular and cortical thickness. DT56a, like oestrogen, restored the bone structure measurements of all tested parameters in the OVX rats to the values obtained in the intact rats. In skeletal tissues, CK activity was elevated in both treatment groups. However, in the uterus DT56a did not activate oestrogen receptors while oestrogen did elevate CK activity.

Conclusions DT56a was as effective as oestrogen in reversing the bone changes caused by OVX in rats.

INTRODUCTION

Postmenopausal osteoporosis and osteoporotic fractures present a serious threat to the aging population, with incidence rates reaching epidemic levels in the Western world.¹ Several groups of products have been introduced for the treatment of osteoporosis: hormone replacement therapy (HRT), selective oestrogen-receptor modulators (SERMs), bisphosphonates, calcitonin and most recently parathyroid hormone.² Although their effectiveness has been demonstrated in large, well-designed studies, all of these products have disadvantages and side effects. With HRT, for example, there is increased risk of breast cancer, cardiovascular disease and stroke,³ with SERMs there may be an aggravation of hot flashes and vasomotor symptoms⁴ and with bisphosphonates there are gastrointestinal disturbances.^{5,6}

Because of these disadvantages, appropriate alternatives are still being sought.

On the assumption that the low rate of osteoporosis in the Asian population is at least partially attributable to the high dietary content of phytoestrogens,^{7,8} industrial groups began to develop a range of products containing phytoestrogens, mainly isoflavone (including a synthetic isoflavone), for the prevention of menopausal symptoms and osteoporosis. The results so far are inconclusive, with some studies showing a beneficial effect of soy products on bone health^{9–12} and others not.¹³ The diversity of the results might be explained by the fact that not all phytoestrogen products are alike; on the contrary, phytoestrogens contain a large group of diverse oestrogen-like components exhibiting lower affinity than that of 17 β -estradiol (E₂) for oestrogen receptors (ERs) and probably also selectivity for different types of ERs.¹⁴ Thus, different products might have differing effects on the same receptor. In a comprehensive review of the role of phytoestrogens in bone health,¹⁰ the authors conclude that 'soy protein may have a modest beneficial effect on bone. However, from the review of existing literature it is too early to state whether soy protein or its isoflavones can be substituted for oestrogen in preventing the bone loss induced by ovarian hormone deficiency'.

DT56a (Femarelle/Tofupill, Se-cure Pharmaceuticals Yavne, Israel) is a unique enzymatic isolate of the active complex in tofu. Studies have shown that it acts as a phyto-SERM.^{15,16} The effects of DT56a on bone and cartilage of ovariectomised (OVX) immature or female rats were

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Table 1. Effects of E₂ and DT56a on bone histological parameters. Values are means [SE].

Treatment	Percentage of trabecular bone volume (% TBV)	Trabecular thickness (µm)	Cortical thickness (µm)	Growth plate width (µm)
Non-OVX				
Control	62 [1.5]	23.6 [1.8]	458 [13]	177 [6]
E ₂	63 [2]	24.2 [1.1]	423 [15] [#]	217 [20] [*]
DT56a	59 [3]	27.8 [1.3]	673 [59] ^{**,#}	171 [9]
OVX				
Control	43 [2] ^{**}	30.7 [1.1] [*]	363 [15] ^{**}	117 [1] [*]
E ₂	57 [4] [*]	26.5 [1.1]	472 [24] [*]	157 [4] [*]
DT56a	53 [3] [*]	26.1 [1.1]	502 [16] [*]	176 [5] [*]

* $P < 0.05$ from the corresponding mean values in non-OVX and OVX rats.

** $P < 0.01$ from the corresponding mean values in non-OVX and OVX rats.

Difference between non-OVX + E₂ and non-OVX + DT56a.

compared with that of E₂ by measuring changes in the specific activity of the BB isozyme of creatine kinase (CK), described as a model for E₂ activation.^{16–18} When administered in multiple oral doses DT56a, like E₂, was found to stimulate CK in skeletal tissues, but unlike E₂ it did not increase CK-specific activity in the uterus. Thus, DT56a selectively stimulates ERs in skeletal tissues but not in the uterus. The SERM raloxifene was found to block CK stimulation by both DT56a and E₂ in all tissues tested, pointing to a mechanism of action that involves a common receptor or receptors.¹⁶ In a clinical study of the effects of DT56a, patients were randomly allocated to receive either the recommended standard dose (644 mg/day) or 344 mg/day with added calcium. Dual-energy X-ray absorptiometry (DEXA) scans, performed on enrolment and again after 12 months of treatment,¹⁵ showed a positive effect on bone mineral density (BMD) in the recommended dose group (an increase of 3.6% in the spine and of 2% in the hip), whereas in the low dose group BMD was reduced by 0.6% in both sites. Neither group showed any changes in sex hormone levels or in endometrial thickness after 12 weeks or 12 months of treatment.¹⁵

In this study, we compared the effects of long term daily treatment with DT56a, E₂ or vehicle on the skeletal histology and histomorphology of OVX and non-OVX rats.

METHODS

E₂ was purchased from Sigma-Aldrich (Rehovot, Israel). DT56a was provided by Se-secure Pharmaceuticals (Yavne, Israel). All other reagents were of analytical grade.

Thirty Wistar-derived, locally bred female rats, aged 25 days and weighing about 60 g at the start of the experiment, were maintained on a 14:10-hour light/dark schedule at 23°C, and provided with food pellets and water *ad libitum*. They were divided into two equal groups, in one of which the rats underwent bilateral OVX and in the other they were left intact (non-OVX). All rats were handled according to the NIH guidelines and the regulations

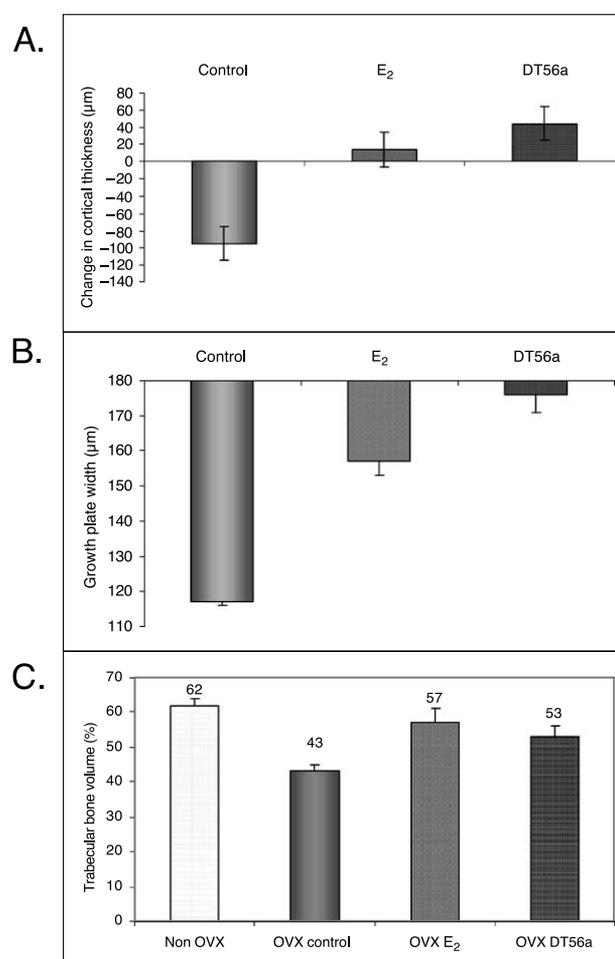


Fig. 1. The changes due to OVX in the following: (A) cortical thickness (µm): OVX resulted in a significant reduction in the cortical thickness. This reduction was not only completely prevented by DT56a treatment but even caused a slight increase in cortical thickness. (B) Growth plate width: DT56a completely restored the growth plate width. (C) Trabecular bone volume: OVX resulted in a significant reduction in the trabecular bone volume. DT56a, similarly to E₂, restored most of this reduction.

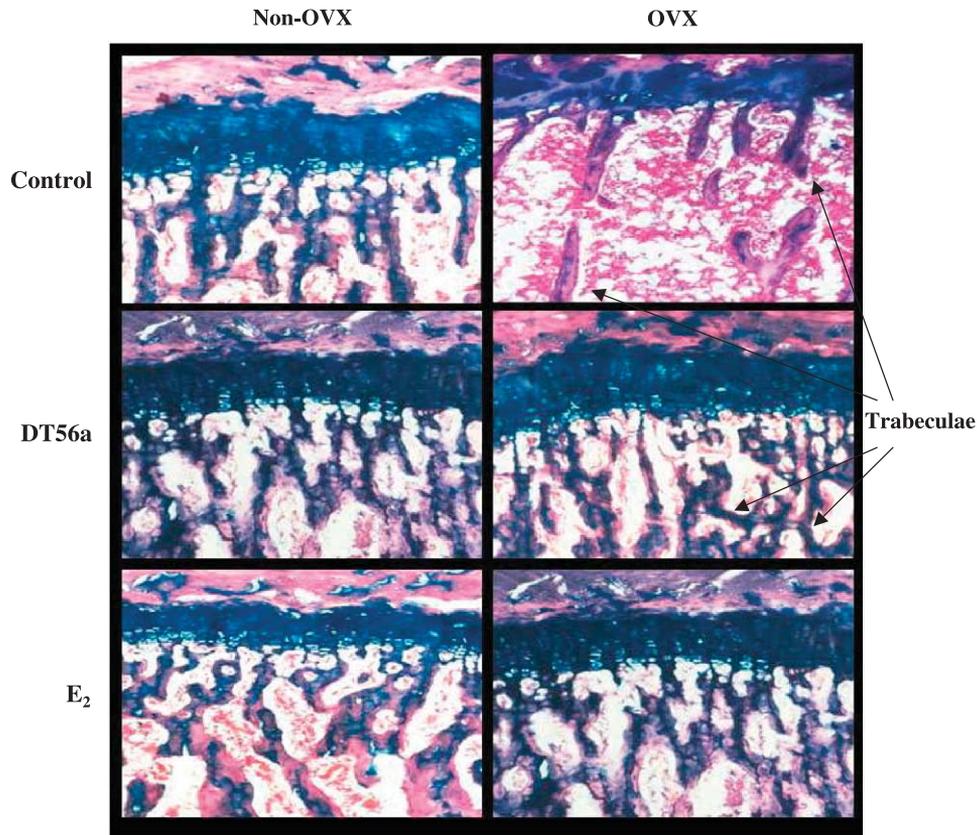


Fig. 2. Sample slides of bone histology: In the OVX rats, a significant loss in trabecular volume is noticed. Similarly to E₂, DT56a restored most of this reduction.

and studies were approved by the Committee on Experimental Animals, Tel Aviv-Sourasky Medical Center.

Rats in the non-OVX group were injected five days per week with 5 µg E₂ ($n = 5$), 650 µg DT56a or with vehicle ($n = 5$; non-OVX control). Starting two weeks after surgery, OVX rats were injected five days per week with 10 µg E₂ ($n = 5$), 1300 µg DT56a ($n = 5$) or with vehicle ($n = 5$; OVX control). The injected doses were calculated according to the animal weight and the dose-response curves, previously shown to be the effective dose.¹⁵

After eight weeks of treatment, and 24 hours after the last injection, the rats were sacrificed by cervical dislocation and organs were removed for histomorphometry and biochemical tests. Samples of whole tibiae from each treatment group of the OVX and the non-OVX rats were fixed for 48 hours in neutral buffered 4% formaldehyde in 0.1 M sodium phosphate buffer pH 7.4, decalcified (two to three weeks, room temperature) in 10% ethylene diamine tetraacetic acid (EDTA), dehydrated in graded alcohol and embedded in paraffin. Sections (6 µm thick) were stained with haematoxylin and eosin for general morphology.

Trabecular bone from each treatment group was measured in the proximal tibial metaphysis at 50 µm below the growth plate. Measurements were performed in multiple

randomised frames. The height of the growth plate, the width of primary trabeculae (spicules) underneath the growth plate and the arrangement of cells in the growth

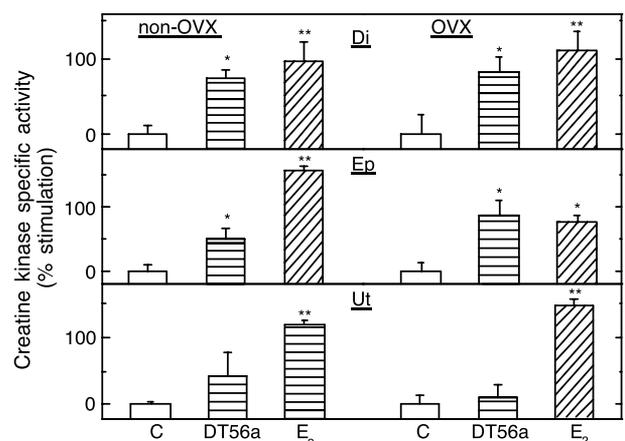


Fig. 3. Stimulation by multiple injections of E₂ or DT56a on CK-specific activity in epiphyseal cartilage (Ep), diaphyseal bone (Di) and in the uterus (Ut) of intact (non-OVX) or OVX female rats. Results are means [SEM]. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. While E₂ activated oestrogen receptors in skeletal tissues and in the uterus, in the OVX rats, DT56a stimulated CK activity only in the skeletal tissues but not in the uterus.

plate were also determined. For analysis of cortical bone, measurements were taken at 50- μm intervals on three sections of the tibial midshaft of each rat. For histomorphometry, we used a transmitted light microscope (Zeiss) linked to a CCD video camera and a computerised histomorphometric system (Galai, Migdal Ha-Emek, Israel). An ocular micrometer, magnification $\times 40$ and $\times 100$, was used for measurement. The measured parameters included trabecular bone volume (TBV; expressed as a percentage of total bone volume, and obtained by dividing the trabecular area by the tissue area), trabecular width, cortical thickness and growth plate width.

Rat bones were collected in cold isotonic extraction buffer,⁶ homogenised for a few seconds using a Polytron homogeniser (Kinematica A. G., Littau, Switzerland) and enzyme extracts obtained by centrifugation of homogenates at $14000 \times g$ for 5 minutes at 4°C in an Eppendorf microcentrifuge. CK activity was measured in a Kontron Model 922 Uvicon Spectrophotometer using a Sigma coupled assay kit (UV-47). Protein was assayed by Coomassie brilliant blue dye binding. Results are means [SEM] of $n = 5$ (15 assays from three independent experiments) and are expressed as percent stimulation of the specific activities of CK in hormone-treated and control animals.

The significance of differences between mean values obtained from the different experimental and control groups was evaluated by ANOVA. A value of $P < 0.05$ was considered significant.

RESULTS

The effects of E_2 and DT56a on bone histomorphological parameters are shown in Table 1. Despite the small sizes of the groups, the results are statistically significant because the variations between animals in each group are very small.

Vehicle-treated OVX rats (controls) were markedly osteopenic relative to control (vehicle-treated) non-OVX rats, having lost 31% of the TBV ($P < 0.01$) and 21% of the cortical thickness ($P < 0.001$), Figs 1 and 2.

OVX resulted in a 21% reduction in the cortical thickness. Treatment with E_2 completely restored this reduction ($P < 0.001$). Treatment of rats with DT56a increased the cortical thickness in the non-OVX group by 32% ($P < 0.001$) and by 38% in the OVX group ($P < 0.005$). OVX rats treated with DT56a had a thicker cortex (502 [16] μm) than the non-OVX control group 458 [13] μm (Figs 1a and 3b).

OVX caused a significant reduction in growth plate width (34%, $P < 0.05$). In the bones of non-OVX rats, the growth plate width was significantly increased by treatment with E_2 ($P < 0.01$) but was not affected by treatment with DT56a (Fig. 1b).

In OVX rats, DT56a treatment completely prevented the OVX-induced reduction of the growth plate width, restoring the growth plate to the same width as that in non-OVX controls ($P < 0.01$, Table 1), Fig. 3. Moreover, in the OVX

rats the growth plates of DT56a-treated rats were more mature in appearance than those of E_2 -treated rats, containing relatively more chondroblastic and hypertrophic cells and fewer proliferative cells.

Treatment with E_2 restored bone loss in the OVX rats by 32% compared with the control OVX rats ($P < 0.01$). Treatment of OVX rats with DT56a increased TBV, restoring the bone loss by 23% ($P < 0.01$). There was statistically significant difference between E_2 and DT56a treatment in restoring the TBV, Fig. 1c.

CK-specific activity was measured in epiphysis (Ep), diaphysis (Di) and the uterus (Ut) following the different treatments. A significant increase in CK activity was found following E_2 or DT56a treatment in both Ep and Di. However, while E_2 produced significant CK activation in the uterus, DT56a had no stimulatory effect in that site (Fig. 3). These results were found in both OVX and non-OVX rats. The basal activity of CK for non-OVX rats was in Ep 0.58 ± 0.10 $\mu\text{mol}/\text{minute}/\text{mg}$ protein, in Di 0.68 ± 0.08 $\mu\text{mol}/\text{minute}/\text{mg}$ protein and in Ut 1.47 ± 0.15 $\mu\text{mol}/\text{minute}/\text{mg}$ protein. For OVX, in Ep 0.43 ± 0.08 $\mu\text{mol}/\text{minute}/\text{mg}$ protein, in Di 0.66 ± 0.09 $\mu\text{mol}/\text{minute}/\text{mg}$ protein and in Ut 1.34 ± 0.15 $\mu\text{mol}/\text{minute}/\text{mg}$ protein.

DISCUSSION

The results of this study show that DT56a prevented the bone loss normally caused by oestrogen deficiency. Osteopenia induced by OVX in rats has been widely used as a model for studying postmenopausal osteoporosis.^{19–24} Treatment with E_2 is known to reverse the osteoporotic changes caused by OVX.^{24,25} In the present study, OVX in rats resulted in changes consistent with those previously described.^{26,27} Two months after OVX, the vehicle-treated rats in our study were profoundly osteopenic, but most of the bone loss was restored by treatment with E_2 as well as by DT56a. This positive effect was found in all the tested variables; the trabecular bone volume cortical thickness and growth plate width. The positive effects of treatment on the growth plate width of the OVX rats and the cortical thickness in the non-OVX rats were better in the DT56a-treated rats than in the E_2 -treated rats. Histologic appearance of the OVX rats treated with DT56a was similar to the non-OVX rats.

Significant elevation in CK activity, as a marker of oestrogen receptor activation, was found in skeletal tissues of both OVX and non-OVX rats. However, DT56a did not activate oestrogen receptors in the uterus. Further study is needed to determine whether DT56a exhibits oestrogenic activity in other female genital organs. CK activation in the skeletal tissues following DT56a treatment supported the results found in the histomorphometric measurements.

Thus, the present results substantiate findings from clinical studies showing that DT56a prevents postmenopausal osteoporosis,¹⁵ and from a preclinical study showing that

short term treatment with DT56a, as with E₂, increases the specific activity of CK in skeletal tissues of OVX rats.¹⁶ Studies aimed at determining the mechanism(s) of action of DT56a (membranal or genomic), as well as clinical studies, are currently under way.

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