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RESEARCH PAPER

Effects of genistein aglycone in osteoporotic, ovariectomized rats: a comparison with alendronate, raloxifene and oestradiol

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Background and purpose: Genistein aglycone positively affects bone loss in postmenopausal women, but bone quality data are still lacking. To clarify this, we investigated the effects of genistein compared with alendronate, raloxifene and oestradiol in an animal model of established osteoporosis.
Experimental approach: Six months after ovariectomy, 96 ovariectomized (OVX) rats were divided into 8 equal groups, randomized to treatments (genistein aglycone (1 and 10 mg kg^{-1} s.c.); alendronate (0.003 and 0.03 mg kg^{-1} s.c.); raloxifene hydrochloride (0.05 and 0.5 mg kg^{-1} s.c.); 17-α-ethinyl oestradiol (0.003 and 0.03 mg kg^{-1} s.c.)) for 12 weeks. Untreated OVX ($n=12$) and sham OVX ($n=12$) were used as controls. At the beginning and end of treatment, bone mineral density (BMD) and bone mineral content (BMC) were assessed. At the end of the experiment, calcium, phosphorus, bone-alkaline phosphatase (b-ALP), collagen C-telopeptide (CTX), osteoprotegerin (OPG) and soluble receptor activator of nuclear factor-κB ligand (sRANKL) were assayed. Femurs were removed and tested for breaking strength and histology.
Key results: Genistein (10 mg kg^{-1}) showed a greater increase in both BMD ($P<0.0001$ vs OVX) and BMC than all the other treatments. Moreover, genistein significantly increased breaking strength, bone quality, b-ALP ($P<0.0001$ vs OVX) and OPG, and reduced CTX and sRANKL compared with the other treatments at all dose levels.
Conclusions and implications: The results strongly suggest that the genistein aglycone might be a new therapy for the management of postmenopausal osteoporosis in humans.
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Keywords: genistein; menopause; osteoporosis; alendronate; raloxifene; oestradiol

Abbreviations: b-ALP, bone-alkaline phosphatase; BMC, bone mineral content; BMD, bone mineral density; CTX, collagen C-telopeptide; ER, oestrogen receptor; OPG, osteoprotegerin; OVX, ovariectomized; sRANKL, soluble receptor activator of nuclear factor-κB ligand

Introduction

Osteoporosis is a systemic disease, characterized by reduced bone mass and structural deterioration of bone tissue. It is considered a public health issue threatening a large part of the population above 50 years of age (Hohenhaus *et al.*, 2007; Levine, 2007). Often presenting as a silent disease, it generally occurs asymptomatically and, consequently, the afflicted individuals will only be diagnosed after the occurrence of fractures. Overall, the disease increases significantly the risk of fractures and requires suitable medical treatment (Barlow, 2007).

Currently available treatments for postmenopausal osteoporosis include hormone replacement therapy, bisphosphonates, calcitonin, strontium ranelate, teriparatid and selective oestrogen receptor modulators (SERMs), such as raloxifene. Several experimental studies compared the effects of currently used therapies for osteoporosis in ovariectomized (OVX) animals (Frolik *et al.*, 1996; Sato *et al.*, 1996; Bourrin *et al.*, 2002; Helvering *et al.*, 2005), but the results were variable and different doses were used. Moreover,

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clinical practice has found that women undergoing treatment for osteoporosis require long-term dosing regimens that offer no symptomatic relief and may cause side effects. As with other chronic diseases, continuance and compliance with therapy are poor.

Hormone replacement therapy is commonly used to treat postmenopausal bone loss. Even if it is effective in reducing menopausal symptoms (Castelo-Branco *et al*., 1999; Writing Group for the Women's Health Initiative Investigators, 2002; Ahlborg *et al*., 2004), it is associated with a higher risk for breast, endometrial and in elevated levels of ovarian cancer as well as cardiovascular disease manifested as venous thromboembolism and stroke (Collaborative Group on Hormonal Factors in Breast Cancer, 1997; Million Women Study Collaborators, 2003).

Observational studies suggest that Asian women who consume diets typically high in isoflavones from soyabean have a lower rate of postmenopausal fractures than Caucasian women (Setchell and Lydeking-Olsen, 2003; Messina *et al*., 2004; McCarty, 2006). Genistein aglycone is a non-glucoside isoflavone found in low concentrations in soyabeans and in elevated amounts in certain soyabean-derived foods, whereas genistin, the glucoside form of the aglycone genistein, is much more abundant in the unprocessed soyabean.

Genistein aglycone, acting similar to a SERM, might play a preventive role against bone mass loss without the harmful oestrogenic activity on reproductive tissues. Although its mechanism of action on bone is not yet fully understood, it is likely that the positive effects of this isoflavone are a direct consequence of a greater binding affinity for the ER-β, compared with that for ER-α, causing positive effects on bone during the mineralization phase (Messina *et al*., 2004; McCarty, 2006).

We have previously shown that treatment with pure genistein aglycone increased bone mineral density (BMD) at the lumbar spine and femoral neck in postmenopausal women with no clinically significant adverse effects on the breast and uterus (Morabito *et al*., 2002; Marini *et al*., 2007). In the same cohort, genistein decreased the ratio of soluble receptor activator of nuclear factor-κB ligand (sRANKL) to osteoprotegerin (OPG), which is well correlated with changes in BMD (Crisafulli *et al*., 2004; Marini *et al*., 2008). These clinical data suggest genistein aglycone as a possible safe therapeutic alternative for the treatment of postmenopausal osteoporosis. Albertazzi (2002) has suggested that genistein aglycone would be a potentially useful, oral, bone anabolic agent.

In the light of these results, we investigated whether genistein aglycone might be a useful alternative treatment for postmenopausal osteoporosis compared with other commonly used therapies such as alendronate, raloxifene and oestradiol in an OVX animal model of established osteoporosis. Bone quality comparisons were also performed to assess each agent's effect on bone structure.

Materials and methods

Animals

All procedures complied with the standards for care and use of animal subjects as stated in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, MD, USA). The experimental protocols were reviewed and approved by the Ethics Committee of the University of Messina. A total of 108 OVX and 12 sham OVX female Sprague–Dawley rats (Charles River, Calco, Italy), aged 12 weeks and weighing about 250–275 g were purchased.

At 6 months following surgery, postmenopausal osteoporosis was assessed by BMD measurement and animals were then randomized into different experimental groups. During the experiment, animals were housed in the Animal Facility of the Department of Clinical and Experimental Medicine and Pharmacology of University of Messina, maintained under controlled environmental conditions (12-h light–dark cycle, temperature approximately 24 °C), and provided with standard food for laboratory animals and water *ad libitum*. Body weight was monitored throughout the study.

Randomization and treatments

At 6 months after ovariectomy (age: 9 months), animals were divided into 9 groups of 12 animals each (Table 1). A group of OVX rats was left untreated (untreated OVX). Both the untreated OVX and the sham OVX groups were used as controls. The different treatments (genistein aglycone (1 and 10 mg kg^{-1}); alendronate (0.003 and 0.03 mg kg^{1}); 17-α-ethinyl oestradiol (0.003 and 0.03 mg kg^{-1}); raloxifene hydrochloride (0.05 and 0.5 mg kg^{-1})) were administered subcutaneously, daily, for 12 weeks. All animals underwent BMD and bone mineral content (BMC) evaluation at baseline and after 12 weeks of treatment. At the end of the experiment (animals aged approximately 12 months), BMD and BMC were taken, blood was collected for the subsequent analysis, right femurs were removed for histology, fixed in 10% neutral-buffered formalin and stored. Left femurs were disarticulated and immediately tested for strength assessment.

Assays for BMD and BMC

BMD and the relative BMC of the femurs were measured using dual-energy X-ray absorptiometry (Hologic QDR-4500A; Hologic, Waltham, MA, USA). For basal and final measurements, animals were anaesthetized with sodium pentobarbital (50 mg kg^{-1} i.p.). During the analysis period, daily measurements were made for BMD and BMC following the manufacturer's instructions, to assess the long-term reproducibility of the measured parameters quality control (QC). A measured value of ± 1.5% was taken as acceptable. Whenever two points obtained in succession were found outside the limits of the QC curve, the procedure was repeated. The coefficient of variation for femur BMD and BMC was 1.15 and 1.10%, respectively. Moreover, accuracy of BMD and BMC final measurements were determined by duplicate scans of femurs.

Body weight, biochemical analysis and uterine assessments

Body weight (expressed in grams) was monitored at the end of the experiments. After blood collection by cardiac puncture under general anaesthesia (sodium pentobarbital 50 mg kg^{-1} i.p.), animals were killed. Uteri were removed immediately after perfusion fixation and weights were

Table 1 Flow chart of experimental protocol

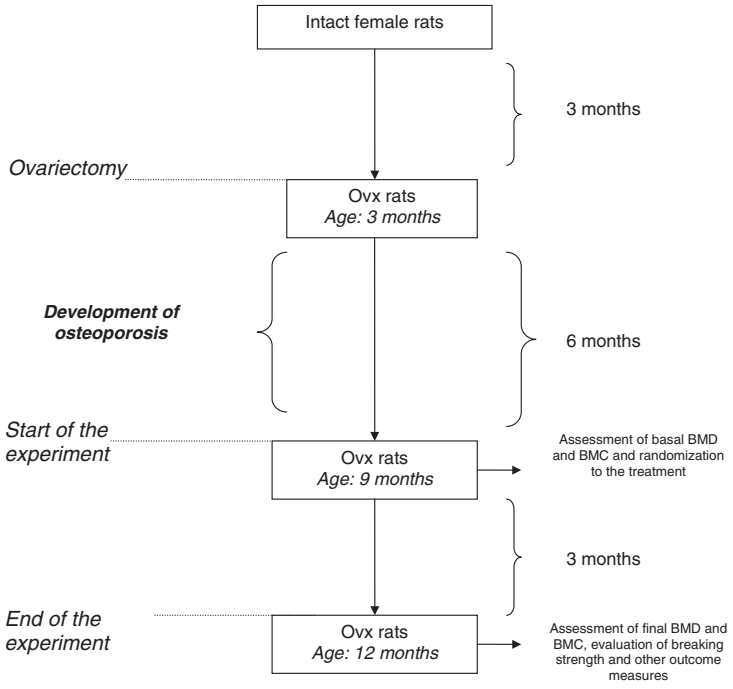

Table 2 Criteria for the evaluation of the histological score used to assess the degree of osteoporosis

Score	*Hip joint cartilage integrity*	*Structure of trabecular bone*	*Quantity of trabecular bone (% of interest area)*
0	Cartilage complete	Normal	90–100
1	Cartilage complete	Partially reduced	60–90
2	Cartilage partially complete	Markedly reduced	30–60
3	Cartilage absent	Absent	0–30

subsequently recorded. Blood was centrifuged and serum was stored immediately at −20 °C for analysis.

Sera were then collected to evaluate in duplicate, by commercially available ELISA kits, calcium, phosphorus (Wako Pure Chemical Industries Ltd, Wako, Bethesda, MD, USA), bone-alkaline phosphatase (b-ALP; IDS Ltd, UK), collagen C-telopeptide (CTX, Nordic Bioscience Diagnostics, Nordic, Herlev, Denmark), OPG (IDS Ltd) and soluble receptor activator of NF-κB ligand (sRANKL; IDS Ltd).

Femur-breaking strength

Immediately after death, the maximum load (breaking strength) tolerated by femurs was measured without knowledge of the treatments, on coded samples using a calibrated tensometer (Sans, Shenzen, China). A three-point bending strength test was performed, femurs were placed horizontally on a two-point sample holder (15 mm span) with the anterior aspect facing up, and a load was placed at the centre of the bone at the rate of 10.0 mm/min until the bone fractured. Maximum tolerated load was expressed in Newton (N).

Histology

Analysis was performed by an investigator, unaware of the treatments. For tissue collection, the leg was disarticulated at the hip, knee and ankle. For microscopic histological evaluation, femurs were removed and immediately fixed in 10% neutral-buffered formalin.

The femur was cleaned of soft tissue, placed in decalcifying solution (8% hydrochloric acid (37% v/v) and 10% formic acid (89% v/v) in phosphate-buffered saline) for about 24 h at 37 °C, dehydrated in 95% (v/v) ethanol and then embedded in paraffin. Three 5-μm-thick paraffin-embedded horizontal bone sections were cut from the proximal end of the diaphysis, stained with haematoxylin–eosin and examined by light microscopy. Femur heads (the area between the hip joint cartilage and metaphyseal cartilage) were assessed for the quality of bone and trabecular density, according to the score shown in Table 2. Cartilage integrity is considered as an additional index of bone quality, because osteoporosis is also responsible for cartilage deterioration and treatments that restore bone integrity are also able to preserve a good trophism of the cartilage indirectly.

Statistical analysis

All data are expressed as means ± s.d. The significance of difference in BMD femoral neck and BMC was assessed by a two-way repeated measures ANOVA followed by Tukey's

Table 3 Effects of alendronate, raloxifene, genistein and oestradiol on body weight, uterine weight, calcium and phosphorus serum levels

Treatment	*Body weight (g)*	*Uterine weight (g)*	*Calcium ($\mu g\,mL^{-1}$)*	*Phosphorus ($\mu g\,mL^{-1}$)*
Sham OVX	260 ± 18	0.63 ± 0.06*	102.2 ± 2.7	86.4 ± 1.8
OVX untreated	355 ± 12	0.38 ± 0.05	100 ± 3	84.7 ± 1.7
OVX + alendronate (0.003 mg kg^{-1})	352 ± 10	0.37 ± 0.05	97.7 ± 2.1	86.1 ± 1.3
OVX + alendronate (0.03 mg kg^{-1})	353 ± 8	0.38 ± 0.04	99.1 ± 1.2	85.8 ± 1.5
OVX + 17-α-ethinyl oestradiol (0.003 mg kg^{-1})	350 ± 13	0.51 ± 0.06*	98 ± 1.3	85.3 ± 1.9
OVX + 17-α-ethinyl oestradiol (0.03 mg kg^{-1})	352 ± 10	0.48 ± 0.05*	97.5 ± 1.2	84.5 ± 1.2
OVX + genistein (1 mg kg^{-1})	350 ± 14	0.38 ± 0.06	100.3 ± 2.1	85.3 ± 2
OVX + genistein (10 mg kg^{-1})	342 ± 12	0.38 ± 0.03	100.7 ± 2.5	86 ± 2.1
OVX + raloxifene hydrochloride (0.05 mg kg^{-1})	353 ± 9	0.37 ± 0.04	99.8 ± 2.4	84.3 ± 2
OVX + raloxifene hydrochloride (0.5 mg kg^{-1})	355 ± 11	0.38 ± 0.05	99.6 ± 3.1	84.9 ± 1.4

Abbreviation: OVX, ovariectomized.
*$P<0.05$ vs corresponding value in OVX-untreated group; $n=12$ for all groups.

multiple comparison test. For all other data, comparisons between different treatments were analysed by one-way ANOVA followed by Tukey's multiple comparison test. In all cases, a probability error of less than 0.05 was selected as the criterion for statistical significance. Graphs were drawn using GraphPad Prism (version 4.0 for Windows).

Drugs

Genistein aglycone was a kind gift of Primus Pharmaceuticals Inc., Scottsdale, AZ, USA; alendronate, 17-α-ethinyl oestradiol and raloxifene hydrochloride were purchased from Sigma Aldrich (Milan, Italy). All substances were prepared fresh daily and administered at a volume of 100 μL. The vehicle used to solubilize genistein aglycone, raloxifene and oestradiol was 33% DMSO in 0.9% NaCl, whereas alendronate was dissolved in a 0.9% NaCl solution.

Results

Effect of the different treatments on body weight, uterine weight, and serum calcium and phosphorus

The final body weights were significantly greater for animals in the OVX treatment and untreated groups (Table 3) compared with the sham OVX animals. There were no statistically significant differences in weights observed between any of the active treatment groups and that of the untreated OVX control group (Table 3).

The final serum calcium and phosphorus levels were not significantly different between the sham OVX and the OVX animals. Likewise, there were no changes in the blood levels of these two elements observed among the several pharmacological treatments (Table 3).

The final uterine weights were decreased in the OVX untreated control group compared with sham OVX rats. The OVX rats treated with 17-α-ethinyl oestradiol had significantly greater uterine weights compared with both untreated OVX and the treatment groups (Table 3).

Effect of the treatments on femoral BMD and BMC

At 6 months after ovariectomy, OVX animals had a significant decrease in femoral neck BMD compared with sham OVX animals ($P<0.0001$; Figure 1a) as well as in BMC ($P<0.0001$; Figure 1b). After 12 weeks of therapy, all the active treatments succeeded in increasing BMD and BMC in OVX animals. Both doses of genistein aglycone produced marked increases in BMD ($P<0.0001$ vs OVX) and BMC ($P<0.0001$ vs OVX) as shown in Figures 1a and b. Comparing results for each therapy, the higher dose of genistein aglycone showed a greater increase in both BMD and BMC than all the other treatments (Figures 1a and b). Alendronate, the most effective drug for postmenopausal osteoporosis, showed a statistically significant lower bone-building capability compared with genistein aglycone in this model ($P<0.0001$).

Effect of the treatments on bone markers

At the end of experiment, serum levels of b-ALP, a marker of bone formation, were significantly higher (Figure 2a) in the untreated OVX group than in the sham OVX group ($P<0.0001$). Genistein aglycone also significantly increased the b-ALP ($P<0.0001$ vs OVX) over all other treatments. None of the other therapeutic interventions significantly affected b-ALP at any dose level (Figure 2a).

The serum levels of the bone resorption marker CTX were significantly higher in the OVX untreated group than in the sham OVX group ($P<0.0001$). Alendronate, raloxifene hydrochloride and 17-α-ethinyl oestradiol significantly reduced CTX plasma levels at any dose levels (Figure 2b). Genistein aglycone administration also reduced the circulating levels of the resorption marker: indeed the higher dose of the isoflavone caused a greater decrease in CTX than the other treatments at any dose levels ($P<0.0001$ vs OVX).

At the end of experiment, serum levels of sRANKL were significantly higher, whereas serum OPG levels were lower in the OVX untreated group than in the sham OVX group ($P<0.0001$) (Figures 3a and b). As a consequence, the sRANKL/OPG ratio was higher in OVX rats than in sham OVX animals ($P<0.001$; Figure 3c). All the pharmacological interventions significantly increased OPG concentration, reduced sRANKL and lowered the sRANKL/OPG ratio in OVX rats. However, the effect of genistein aglycone (10 mg kg^{-1}) on the OPG system was greater than the other treatment at any dose tested (sRANKL/OPG: $P<0.001$ vs OVX) (Figures 3a–c).

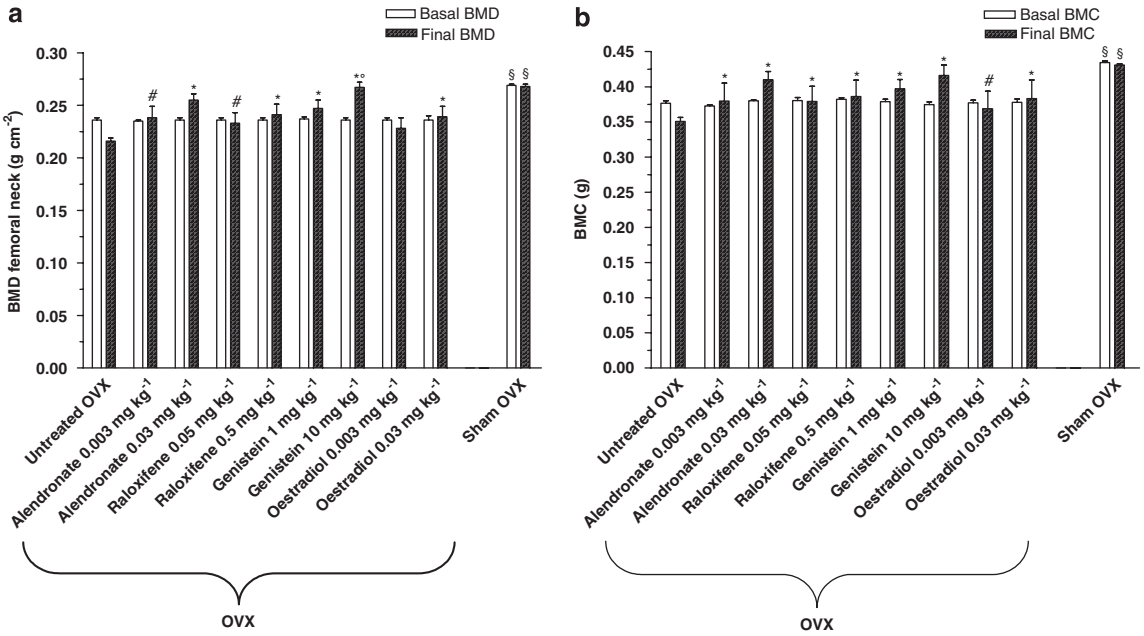

Figure 1 Effects of alendronate, raloxifene, genistein and oestradiol on femoral bone mineral density (BMD) (**a**) and bone mineral content (BMC) (**b**) in ovariectomized (OVX) rats. Data are shown as the mean ± s.d. of 12 animals. BMD: $^{*}P<0.0001$ vs untreated OVX; $^{\#}P<0.005$ vs untreated OVX; $^{\S}P<0.0001$ vs untreated OVX; $^{\circ}P<0.0001$ vs all other treatments. BMC: $^{*}P<0.0001$ vs untreated OVX; $^{\#}P=0.006$ vs untreated OVX, $^{\S}P<0.0001$ vs untreated OVX.

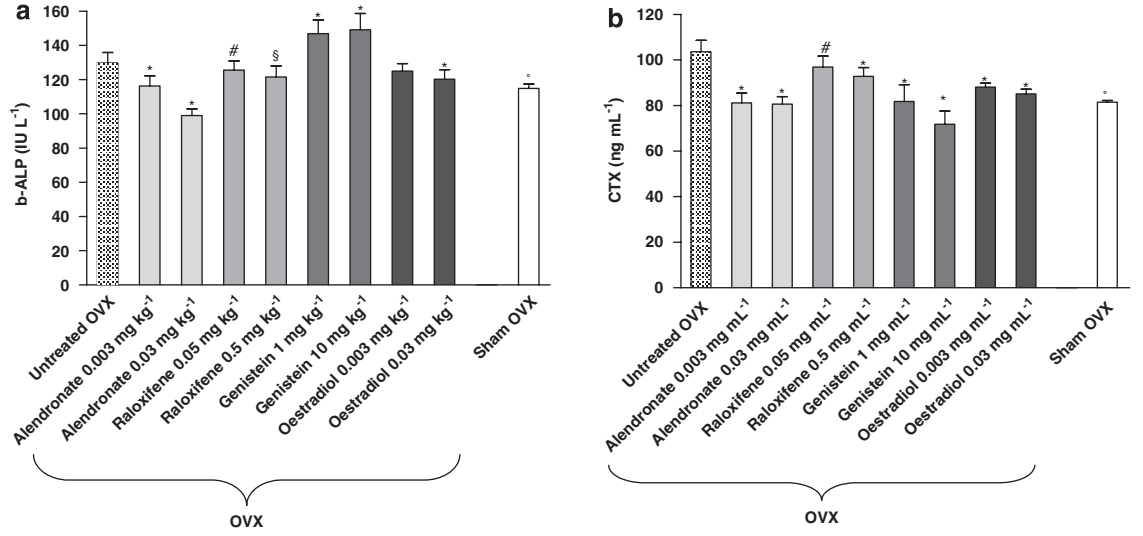

Figure 2 Effects of alendronate, raloxifene, genistein and oestradiol on serum bone-alkaline phosphatase (b-ALP) (**a**) and collagen C-telopeptide (CTX) (**b**) in ovariectomized (OVX) rats. Data are shown as the mean ± s.d. of 12 animals. b-ALP: $^{*}P<0.0001$ vs untreated OVX; $^{\#}P=0.098$ vs untreated OVX; $^{\S}P=0.005$ vs untreated OVX; $^{\circ}P<0.0001$ vs untreated OVX. CTX: $^{*}P<0.0001$ vs untreated OVX; $^{\#}P=0.008$ vs untreated OVX; $^{\circ}P<0.0001$ vs untreated OVX.

Effect of the treatments on the mechanical properties of the femur and on bone quality

In the results obtained from the three-point bending test of the femur, untreated OVX animals had significantly reduced breaking strength compared with sham OVX rats ($P<0.0001$) (Figure 4a). All the pharmacological treatments succeeded in improving the breaking strength of the femur. Genistein aglycone, however, caused a greater increase in the amount of pressure required to fracture the femur compared with other treatments at any dose level tested ($P<0.0001$; Figure 4a).

The histological score (Figure 4b) of all groups evaluated following the criteria shown in Table 1 revealed a greater effect of genistein aglycone on bone quality compared with all the other treatments at any dose level tested. Bone histology (Figures 5 and 6) revealed a marked effect of genistein aglycone in treating osteoporosis induced by ovariectomy. Histology of the femur head in animals treated

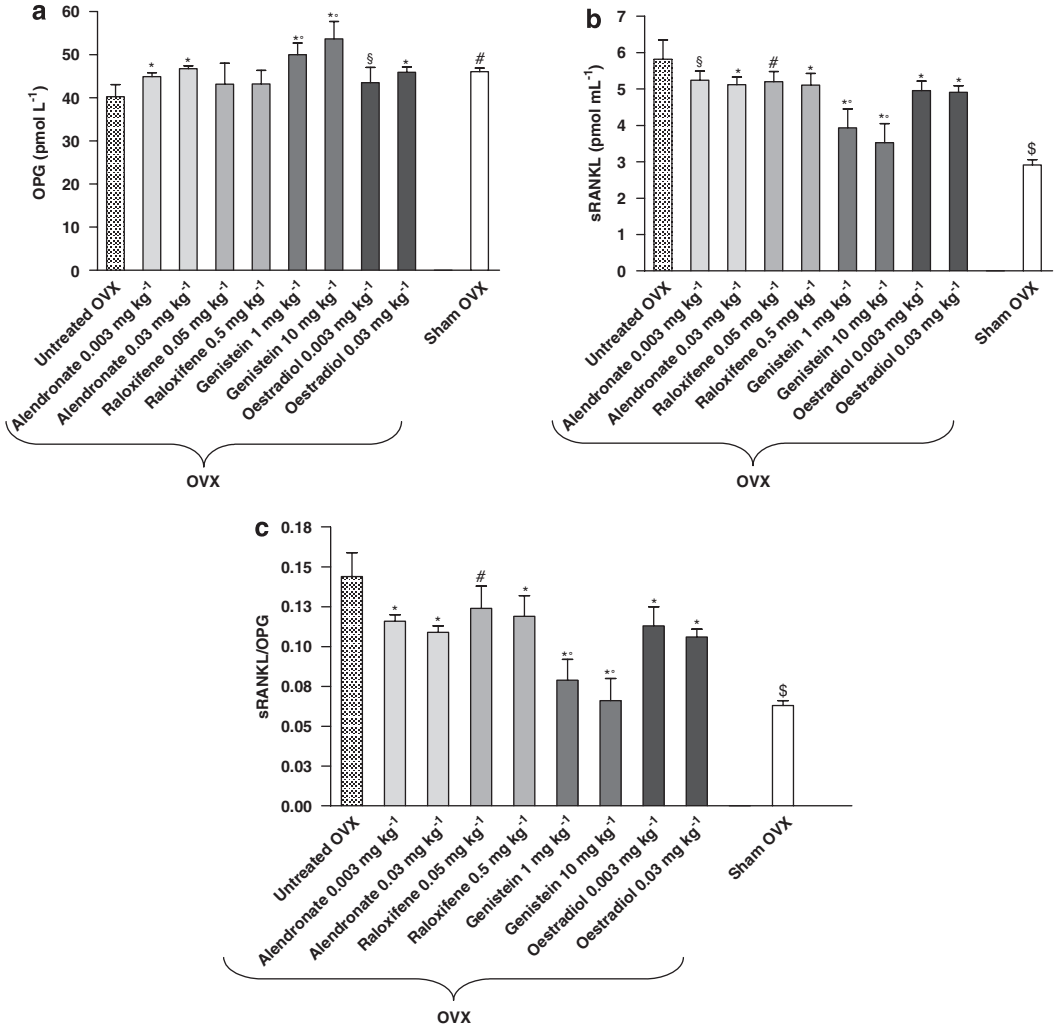

Figure 3 Effects of alendronate, raloxifene, genistein and oestradiol on serum osteoprotegerin (OPG) (**a**), soluble receptor activator of NF-κB ligand (sRANKL) (**b**) and sRANKL/OPG (**c**) in ovariectomized (OVX) rats. Data are shown as the mean ± s.d. of 12 animals. OPG: $^{*}P<0.0001$ vs untreated OVX; $^{\circ}P<0.0001$ vs all other treatments; $^{\#}P<0.0001$ vs untreated OVX. sRANKL: $^{*}P<0.0001$ vs untreated OVX; $^{\#}P=0.002$ vs untreated OVX; $^{\S}P=0.003$ vs untreated OVX; $^{\circ}P<0.0001$ vs all other treatments; $^{\$}P<0.0001$ vs untreated OVX. sRANKL/OPG: $^{*}P<0.0001$ vs untreated OVX; $^{\#}P=0.002$ vs untreated OVX; $^{\S}P=0.003$ vs untreated OVX; $^{\circ}P<0.0001$ vs all other treatments; $^{\$}P<0.0001$ vs untreated OVX.

with genistein aglycone showed a restored architecture of cortical and trabecular bone with well-organized bone matrix correlating with the enhanced resistance to fracture (Figure 4a), observed in femurs subjected to a constant load ($P<0.0001$ vs OVX).

Discussion

This study clearly shows that genistein aglycone, a well-known, but low concentration, soyabean isoflavone, was able to counteract the bone loss in an experimental model of established osteoporosis. In this context, the OVX rat is considered an appropriate model for studying human menopausal osteoporosis because of many similarities in their pathophysiological mechanisms of bone deterioration (Kalu, 1991; Wronski *et al*., 1991; Frost and Jee, 1992).

Drugs that interfere with steps in the resorptive pathway resulting in bone loss (antiresorptive agents) (Jordan *et al*., 2006) or that amplify or mimic steps in the anabolic pathway to build new and improved skeletons (anabolic or bone-forming agents) are specifically recommended to treat bone loss (Canalis *et al*., 2007). Safe anabolic agents are needed to build bone, restoring both bone structure and strength, rather than just prevention or slower progression of bone fragility, as occurs with current antiresorptive agents.

Oestrogen replacement therapy, SERMs (that is, raloxifene hydrochloride) and bisphosphonates (that is, alendronate) are widely used to oppose accelerated bone resorption in senile or postmenopausal osteopaenia/osteoporosis. All of these treatments predominantly exert antiresorptive effects by inhibiting, through several different mechanisms, the activity of osteoclasts (Jordan *et al*., 2006) rather than promoting osteoblast activity. Increasing clinical evidence suggests a role for genistein aglycone in the treatment of postmenopausal bone loss (Morabito *et al*., 2002; Marini *et al*., 2007, 2008); however, proof of efficacy in the

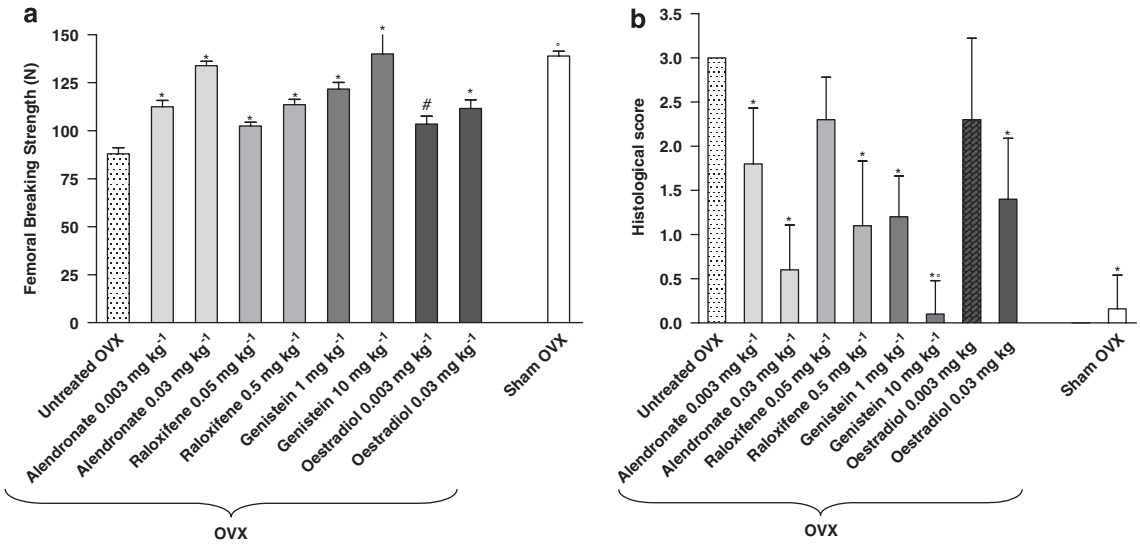

Figure 4 Effects of alendronate, raloxifene, genistein and oestradiol on femur-breaking strength (**a**) and histological score (**b**) in ovariectomized (OVX) rats. Data are shown as the mean ± s.d. of 12 animals. Femoral-breaking strength: $^{*}P<0.0001$ vs untreated OVX; $^{\#}P=0.006$ vs untreated OVX; $^{\circ}P<0.0001$ vs untreated OVX. Histological score: $^{*}P<0.0001$ vs untreated OVX; $^{\circ}P<0.05$ vs all other treatments.

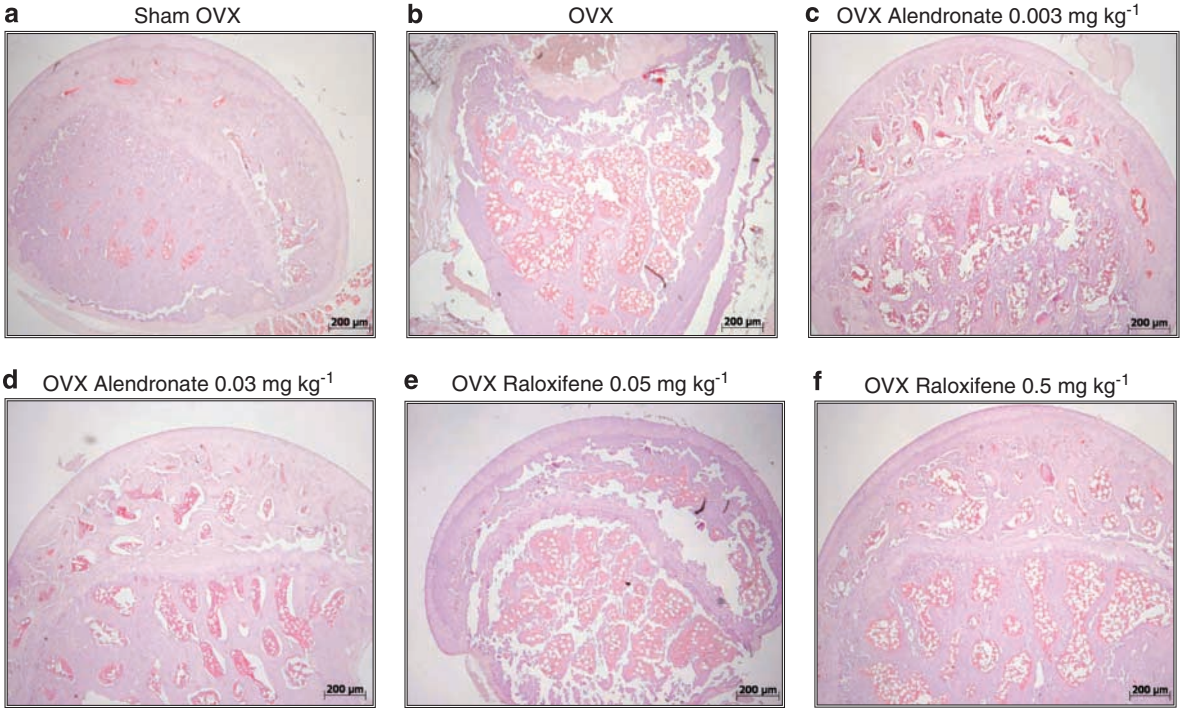

Figure 5 (**a**–**f**) Light microscopy of the cortical and trabecular structure of the femur head: effects of alendronate and raloxifene (haemotoxylin and eosin, original magnification × 5).

treatment of established osteoporosis is still lacking (Tempfer *et al*., 2007).

Our experimental data demonstrate that two doses of genistein aglycone, given by subcutaneous injection in OVX osteoporotic rats, were able to produce a marked increase in BMD and BMC in accordance with our recent observations in osteopaenic, postmenopausal women treated orally (the preferable route of administration), with 54 mg per day of aglycone genistein (Morabito *et al*., 2002; Marini *et al*., 2007, 2008), in the same range of dosing, as that used in the present experimental model. Not only did genistein aglycone increase BMD and BMC but the isoflavone also restored structure to osteoporotic bone as well or better than other well-accepted treatments. Indeed, some of the positive effects of genistein aglycone that we observed have been previously reported (Fanti *et al*., 1998; Ishimi *et al*., 2000) in both young OVX rats and mice, treated subcutaneously.

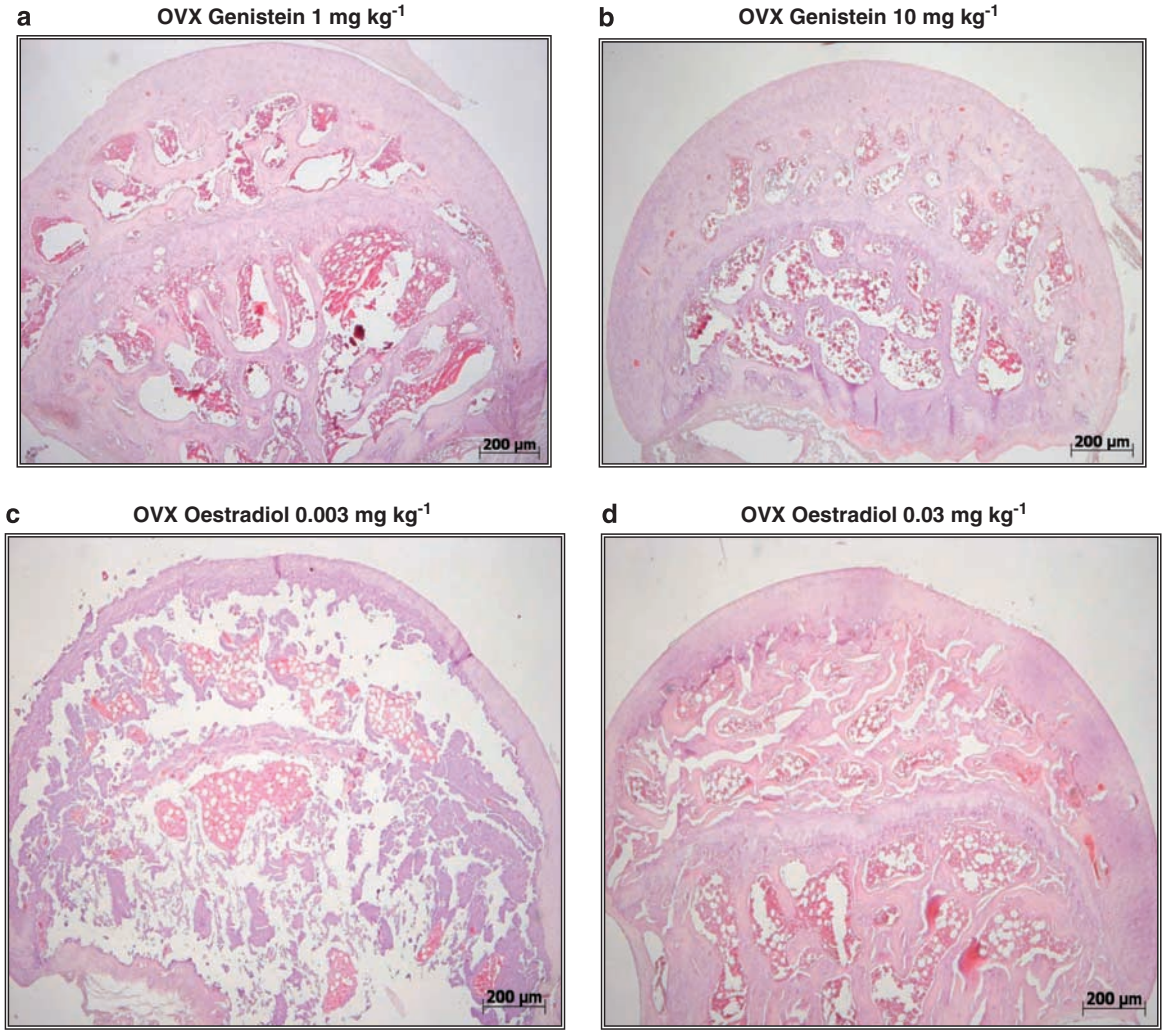

Figure 6 (**a**–**d**) Light microscopy of the cortical and trabecular structure of the femur head: effects of genistein and oestradiol (haemotoxylin and eosin stain, original magnification × 5).

Besides the changes observed in BMD, BMC and structure, genistein aglycone was more effective than alendronate, a very useful drug for the prevention of fractures in postmenopausal women with osteoporosis (Iwamoto *et al*., 2007), in inhibiting osteoclastic markers, CTX and sRANKL. In addition, genistein aglycone, at any dose tested, significantly reduced CTX and sRANKL plasma levels to a greater extent than raloxifene hydrochloride or 17-α-ethinyl oestradiol. Genistein aglycone was also the most effective treatment in terms of the osteoblastic activity markers, b-ALP, OPG and had a better sRANKL/OPG ratio. These findings show the positive and unique role of genistein aglycone in stimulating osteoblasts, while also damping osteoclasts, compared with all the other available current therapies for osteoporosis. However, the basis of the 'dual' activity is unknown.

Unlike 17β-oestradiol, which displays relatively equivalent binding on both ER subtypes, and raloxifene, which binds with greater affinity to ER-α, genistein aglycone binds selectively to ER-β over ER-α, from 7- to 48-fold, depending on the assay system employed (Kuiper *et al*., 1997, 1998; Barkhem *et al*., 1998; Hsieh *et al*., 2006). Despite a relatively low affinity for ER-α, genistein aglycone can act as a full agonist in some assay systems (Barkhem *et al*., 1998). The selective activation of ER-β by genistein aglycone is likely to be mediated by a greater capacity to recruit co-regulators of ER-β, than those of ER-α (An *et al*., 2001), through *de novo* protein synthesis in osteoblasts (Yamaguchi and Sugimoto, 2000; Yamaguchi *et al*., 2000). Osteoblasts express both receptor types and can be influenced by selective binding of oestrogenic compounds (Onoe *et al*., 1997; Valachovicova *et al*., 2004). The consequence of selective receptor modulation is enhanced transcriptional activation or repression of promoters and other genes under ER-β regulation, relative to those regulated by ER-α.

ER-β is robustly expressed in developing human bone, especially the trabecular bone that is most subject to loss following gonadal hormone deprivation (Bord *et al*., 2001). There is a greater than ninefold increase in ER-β expression in cultured human osteoblasts during bone mineralization, whereas ER-α levels remain unchanged during this process (Arts *et al*., 1997; Setchell and Lydeking-Olsen, 2003). Though experimental observations support the role for positive actions of genistein aglycone on bone that could

be related to a weak oestrogenic effect on a subtype of ER-α (Hertrampf *et al*., 2007), the accumulating evidence strongly suggests the important involvement of ER-β in bone formation and, by extension, the use of selective ER-β agonists such as genistein aglycone to treat bone loss through osteoblast stimulation. The increased b-ALP activity of genistein aglycone in comparison to other treatments in the current study is supportive of this mechanism of action. The concentration of genistein aglycone must be properly titrated, however, as some reports suggest insufficient stimulation of ER-β may occur, leading to few or even absent beneficial effects at low doses (Mäkelä *et al*., 1999; Setchell, 2001; Kostelac *et al*., 2003; Altavilla *et al*., 2004; McCarty, 2006).

Postmenopausal osteoporosis also results from an imbalance between resorption and formation associated with decreased OPG/RANKL balance (Simonet *et al*., 1997; Hofbauer and Heufelder, 2000; Li *et al*., 2000), thus indicating that the OPG/RANKL system might represent a good pharmacological target in the treatment of osteoporosis. Genistein aglycone has been shown to selectively antagonize the bone catabolic effects of parathyroid hormone in osteoblasts by reducing parathyroid hormone-induced increases in RANKL and reversing decreases in OPG expression *in vitro* (Chen and Wong, 2006). In the present paper, all the pharmacological interventions significantly increased OPG concentration, reduced sRANKL and lowered sRANKL/OPG balance in OVX rats. However, the effect of highest dose of genistein aglycone on the OPG system was greater than the other treatment at any dose tested. These data are in agreement with previous studies indicating a strong effect of genistein aglycone on the OPG/RANKL balance (Crisafulli *et al*., 2004; Marini *et al*., 2008), highly correlated with the augmented BMD in femur neck and lumbar spine.

Studies have also shown that genistein aglycone inhibits tyrosine phosphorylation in osteoclasts at the same concentrations that reduce osteoclast number *in vitro*, presumably by inducing osteoclast apoptosis (Gao and Yamaguchi, 2000). Increases in intracellular calcium signalling may also in part mediate genistein aglycone's inhibitory effects on osteoclasts, as inhibitors of the calcium-dependent signalling molecules, calmodulin and protein kinase C, antagonize the reduction in osteoclast number induced by genistein aglycone (Gao and Yamaguchi, 1999). Increases in osteoclast intracellular calcium levels induced by genistein aglycone may be mediated by direct inhibition of inward-rectifier K^+ channels independent of genistein aglycone's activity on tyrosine kinases (Okamoto *et al*., 2001).

In conclusion, genistein aglycone showed a positive effect on osteoporotic bone in the present experimental model confirmed by decreasing osteoclastic resorption and increasing osteoblastic formation markers. This putative 'uncoupling' of the bone remodelling process in bone growth may be a selective event in osteoporotic bone. Though all pharmacological treatments succeeded in improving the breaking strength of the femur, genistein aglycone caused the greatest increase in breaking strength and was supported by restored bone architecture in the femoral head of OVX-treated rats. Collectively, our results strongly suggest that genistein aglycone might be a new potential therapy for the management of postmenopausal osteoporosis in humans combining a powerful bone-forming as well as an anti-resorptive activity.

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Conflict of interest

BP Burnett, RM Levy and MA Armburster work for Primus Pharmaceuticals Inc. Scottsdale, AZ, USA. The other authors have nothing to declare.

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