British Journal of Pharmacology (2009), 156, 1287–1295

0007-1188/09
www.brjpharmacol.org

RESEARCH PAPER

Genistein aglycone reverses glucocorticoid-induced osteoporosis and increases bone breaking strength in rats: a comparative study with alendronate

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Background and purpose: Glucocorticoid-induced osteoporosis (GIO) is the leading cause of secondary osteoporosis. Clinical evidence suggests a role for genistein aglycone in the treatment of post-menopausal osteopenia although proof of efficacy in comparison with currently available treatments is still lacking. To clarify this issue, we investigated the effects of genistein on bone compared with alendronate in experimental GIO.
Experimental approach: A total of 28 female Sprague-Dawley rats were used. GIO was induced by daily injections of methylprednisolone (MP; 30 mg·kg^{-1} s.c.) for 60 days. Sham GIO animals (Sham-MP) were injected daily with the MP vehicle. At the end of the osteoporosis development period, MP rats were randomized to receive: vehicle ($n = 7$), genistein aglycone (5 mg·kg^{-1} s.c.; $n = 7$) or alendronate (0.03 mg·kg^{-1} s.c.; $n = 7$). Treatment lasted 60 days. Sham-MP animals were treated with vehicle for an additional 60 days. At the beginning and at the end of treatments, animals were examined for bone mineral density and bone mineral content. Bone-alkaline phosphatase and carboxy-terminal collagen cross links were determined; femurs were removed and tested for breaking strength and histology.
Key results: Genistein aglycone showed a greater increase in bone mineral density, bone mineral content and in breaking strength than alendronate and significantly increased bone-alkaline phosphatase (bone formation marker), reduced carboxy-terminal collagen cross links (bone resorption marker), compared with alendronate. Both treatments improved bone histology and the histological score.
Conclusion and implications: The results strongly suggest that the genistein aglycone might be an alternative therapy for the management of secondary osteoporosis.
British Journal of Pharmacology (2009) **156**, 1287–1295; doi:10.1111/j.1476-5381.2008.00100.x; published online 19 March 2009

Keywords: genistein aglycone; glucocorticoid-induced osteoporosis; alendronate; b-ALP; CTX

Abbreviations: b-ALP, bone-alkaline phosphatase; BMC, bone mineral content; BMD, bone mineral density; CTX, collagen C-telopeptides; ER, oestrogen receptor; MP, methylprednisolone; sRANKL, soluble receptor activator of nuclear factor-κB ligand

Introduction

Bone loss arising from reduced oestrogen after menopause is responsible for the majority of reported cases of osteopenia and osteoporosis in women. The majority of bone loss occurs in the first 2 years after menopause (Lindsay and Cosman, 2003). As a consequence, vertebral fractures arise earlier in women compared with femoral fractures (Riggs and Melton, 1986). Osteoporosis is found in men, but develops at a lower rate with fractures occurring later in life compared with women (van Staa *et al.*, 2005). There are also several secondary causes of osteoporosis, of which the most important is glucocorticoid-induced osteoporosis (GIO) (Devogelaer, 2006; van Staa, 2006; Woolf, 2007), a consequence of the widespread use of glucocorticoids for a variety of inflammatory conditions. Many studies have shown that glucocorticoids decrease bone mass and thereby increase the risk of fractures, particularly fractures of the ribs, spine and forearm. Studies have shown that 30–50% of all fractures occur in hospital

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Received 29 September 2008; accepted 28 October 2008

settings, usually associated with administration of high doses of glucocorticoids (Adinoff and Hollister, 1983; Tsugeno *et al*., 2002; Kanis *et al*., 2005). The risk of hip, distal forearm and proximal humeral fractures are approximately double in rheumatoid arthritis patients treated with glucocorticoids, compared with patients without glucocorticoid therapy (Kung *et al*., 1999; Dolan *et al*., 2004). In addition, approximately 20% of patients on long-term glucocorticoid treatment in outpatient settings experience fragility fractures (Kotaniemi *et al*., 1996). A recent study identified 1.6 million oral glucocorticoid prescriptions over a 10 year period in the UK (Kung *et al*., 1999). The prevalence of oral glucocorticoid use was similar between men and women and was 0.9% of the total adult population, and use increased with age.

The susceptibility to bone loss may not be the same for all disorders for which glucocorticoids are used. Patients with end-stage chronic renal failure appear to be relatively resistant to skeletal effects of high-dose glucocorticoid therapy (Kanis *et al*., 2005), whereas younger patients, as well as transplant recipients, are highly susceptible to fracture (de Nijs *et al*., 2004). Finally, genetic variations of the enzyme 11β-hydroxysteroid dehydrogenase may modulate responsiveness to glucocorticoids and thus the risk of developing osteoporosis (Tomlinson *et al*., 2000; Cooper *et al*., 2002).

A wide variety of pharmacological interventions have been shown to decrease bone loss in GIO. Proposed treatments to help maintain or increase bone density include calcium supplementation, bisphosphonates, hormone replacement therapy, vitamin D in one of its many forms (cholecalciferol, calciferol, calcitriol, calcidiol, alfacalcidol), calcitonin, parathyroid hormone, fluoride, testosterone and anabolic steroids (Adachi *et al*., 1996; Ringe *et al*., 1999; Eastell *et al*., 2000; Boutsen *et al*., 2001; Crandall, 2002; Sambrook, 2007).

Several alternative therapeutic approaches have also been considered in recent years. For example, we previously have shown that treatment with pure genistein aglycone (54 mg·day^{-1}) increased bone mineral density (BMD) at the lumbar spine and femoral neck in groups of post-menopausal women who were osteopenic or worse at baseline, with no clinically significant adverse effects on endometrium (Morabito *et al*., 2002; Crisafulli *et al*., 2004; Marini *et al*., 2007; Marini *et al*., 2008). In light of these observations, the present study aims to assess if genistein aglycone could be useful in the treatment of GIO and to assess how this compound compares in effectiveness with a commonly prescribed bisphosphonate, the therapeutic class now identified as the gold standard treatment for osteoporosis.

Methods

Animals

All procedures were evaluated and approved by the Ethics committee of the University of Messina and 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). A total of 28 female Sprague-Dawley rats (Charles River, Italy), aged 8 months and weighing about 250–275 g were used during the experiment. During the experiment, animals were housed in the Animal Facility of the Department of Clinical and Experimental Medicine and Pharmacology, 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*.

Induction of osteoporosis, randomization and treatments

One group of animals (n = 21) was injected daily with methylprednisolone (MP) (30 mg·kg^{-1} in 0.9% NaCl solution), subcutaneously for 60 days to produce GIO while a control group (n = 7) was injected daily with the MP vehicle (1 mL·kg^{-1}, subcutaneously of a 0.9 % NaCl solution) as shown in Figure 1. The latter group served as controls for the experiment (sham-MP). Following MP or vehicle administration and before randomization all animals underwent BMD and bone mineral content (BMC) evaluation to confirm the presence of GIO. MP animals were then divided into three groups of seven animals each, as summarized in Figure 1, and randomized to the following treatments for 60 days: vehicle (VEH; 1 mL·kg^{-1} subcutaneously of a 10% DMSO/in 0.9% NaCl solution; n = 7), genistein aglycone (5 mg·kg^{-1}; n = 7) or alendronate (0.03 mg·kg^{-1}; n = 7). The Sham-MP animals were also treated with vehicle for an additional 60 days.

At the end of the treatment period, BMD and BMC measurements were taken. In addition, at the time of death, serum was collected to determine bone-alkaline phosphatase (b-ALP) and collagen C-telopeptides (CTX) levels. Right femurs were removed for histological examination, fixed in 10% neutral buffered formalin and stored. Left femurs were disarticulated and immediately tested for breaking strength assessment.

BMD and BMC

Bone mineral density and the relative BMC of the femurs were measured by using dual-energy X-ray absorptiometry (DEXA, Hologic QDR-4500A, Waltham, MA, USA). For basal and final measurements, animals were kept anaesthetized with sodium pentobarbital (50 mg·kg^{-1} i.p.). The rats were positioned in the middle of the measurement table and scans obtained in the high resolution mode. All animals were evaluated by the same technician and analysed by using the same method to minimize operational errors. Boxes were drawn to limit the area of interest and the BMD and BMC of proximal femurs obtained. During the analysis period, daily measurements were made for BMD and BMC following manufacturer's instructions, in order to assess the long-term reproducibility of the measured parameters (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.

Biochemical analysis

At the end of the study, animals were killed by anaesthesia with chloral hydrate (400 mg·kg^{-1} i.p.) and blood collected by

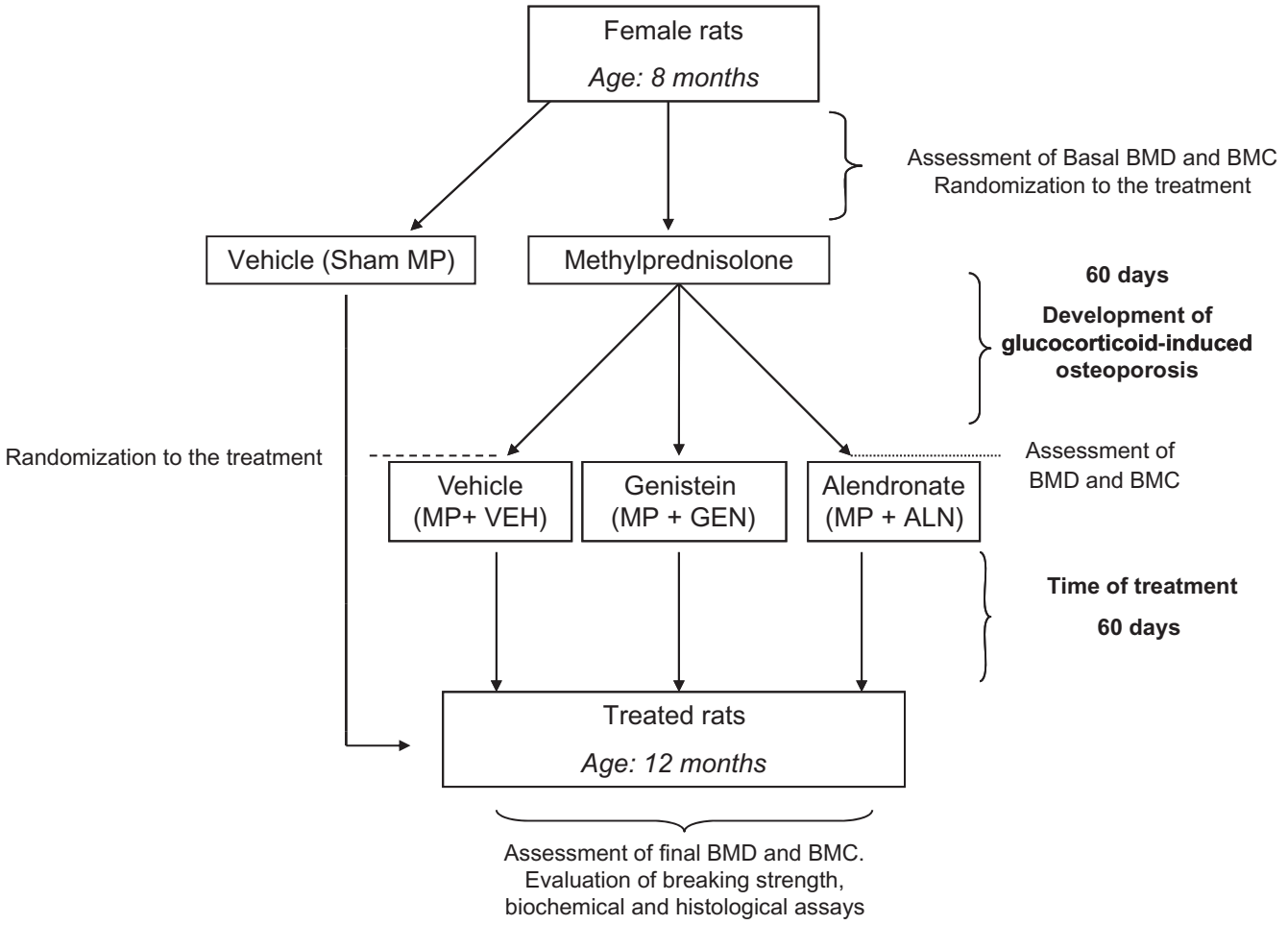

Figure 1 Flow chart of the experimental protocol. BMC, bone mineral content; BMD, bone mineral density; MP, methylprednisolone.

cardiac puncture. Blood was centrifuged and serum stored immediately at –20°C for analysis. Commercially available ELISA kits for b-ALP (IDS Ltd., UK) and CTX (Nordic Bioscience Diagnostics, DK) were then used to evaluate duplicate sera of each animal for bone formation and resorption respectively.

Femur breaking strength

Immediately after death, the maximum load (breaking strength) tolerated by femurs, expressed in Newtons (N), was measured on coded samples by using a calibrated tensometer (Sans). Femurs were placed horizontally, connected at each end by a two-point sample holder (15 mm span) with the anterior aspect facing up. The load was placed at the centre of the bone at a rate of 10.0 $mm \cdot min^{-1}$ until the bone fractured.

Histology

Histological analysis was performed by an investigator unaware of the treatments. For bone tissue collection, the leg of each animal was disarticulated at the hip, knee and ankle. Femurs were then 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 and eosin and studied by using light microscopy. Femoral heads, the area between hip-joint cartilage and metaphyseal cartilage, were scored for general bone quality and trabecular density according to the scoring system in Table 1, as already published (Bitto *et al.*, 2008). For qualitative image analysis, the metaphyseal area proximal to the growth plate, and the cortical bone below the hip-joint cartilage were used for imaging analysis. The growth plate was generally very thin in osteoporotic bones and thicker in bones from treated animals. Cartilage integrity evaluation was considered as an additional index of bone quality, because osteoporosis is in the end also responsible for cartilage deterioration, due to an enhanced osteoclastic activity as in rheumatoid arthritis (Herrak *et al.*, 2004) Thus a treatment that restores bone integrity is indirectly also able to preserve a good trophism of the cartilage. The analysis was carried out by three pathologists unaware of the treatments.

Statistical analysis

All data are expressed as means ± standard deviation (SD). The significance of difference in BMD femoral neck and BMC

Table 1 Histological scoring of osteoporotic changes

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

was assessed by a two-way repeated measures ANOVA followed by Tukey's 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 by using GraphPad Prism (version 4.0 for Windows).

Drugs

Genistein aglycone was a gift of Primus Pharmaceuticals Inc. Alendronate, and MP was purchased from Sigma Aldrich, Italy. All substances were prepared fresh daily and administered in a volume of 100 μL.

Results

Effect of the treatments on femoral BMD and BMC

Following MP administration for 60 days, glucocorticoid-treated animals showed significant decreases in femoral neck BMD (0.245 ± 0.004 g·cm^{-2}) and BMC (0.420 ± 0.003 g) compared with that for the Sham-MP animals (BMD = 0.270 ± 0.002 g·cm^{-2}; $P < 0.01$ and BMC = 0.436 ± 0.001 g; $P < 0.01$). MP rats were then randomized to different treatment groups (Figure 1).

At the end of the treatment period, both genistein aglycone and alendronate increased BMD (Figure 2A) and BMC (Figure 2B) in GIO rats to roughly the same level as in to the vehicle-treated group. There was no significant difference observed between two treatment groups (Figure 2A,B).

Effect of treatments on bone markers

At the end of the experiment, genistein aglycone significantly increased b-ALP ($P < 0.001$) compared with vehicle- and alendronate-treated groups (Figure 3A), suggesting a stimulation of osteoblast function. Serum b-ALP levels of the vehicle-treated group were found to be higher compared with the Sham-MP group ($P < 0.05$). Alendronate, known as an anti-resorptive compound, did not significantly affect serum b-ALP levels (Figure 3A).

Levels of plasma CTX were significantly higher in the vehicle-treated group compared with the Sham-MP group ($P < 0.001$). Both genistein aglycone and alendronate significantly reduced CTX plasma levels ($P < 0.001$ vs. MP + VEH). However, ANOVA analysis showed that genistein aglycone lowered CTX levels more when compared with alendronate ($P < 0.05$; Figure 3B).

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

In femoral strength tests, the vehicle-treated group had a significantly reduced breaking strength compared with Sham-MP rats ($P < 0.001$) (Figure 3C). Both genistein aglycone and alendronate improved the breaking strength of the femur. Genistein aglycone-treated animals, however, showed statistically improved femur strength compared with alendronate-treated animals ($P < 0.05$; Figure 3C).

After staining thin sections of the femoral heads, histological scoring showed that genistein aglycone reversed GIO more effectively than alendronate (Figure 3D) although both treatments were dramatically better than the vehicle control. Histological analysis revealed that the femoral heads collected from both genistein aglycone- and alendronate-treated rats had restored architecture of the cortical and trabecular structure with well-organized bone matrix (Figure 4). Histological staining and scoring both showed that genistein aglycone showed greater effects on bone structure compared with alendronate and correlated well with enhanced breaking strength of femurs subjected to a constant load.

Discussion

An increasing amount of clinical evidence suggests a role for the isoflavone genistein aglycone in the treatment of postmenopausal bone loss (Albertazzi, 2002; Morabito *et al.*, 2002; Crisafulli *et al.*, 2004; Marini *et al.*, 2007; Bitto *et al.*, 2008; Marini *et al.*, 2008). Proof of efficacy, however, in the treatment of GIO in comparison with currently available treatments is still lacking. In order to clarify this issue, we investigated the effects of genistein aglycone compared with alendronate in an experimental animal model of GIO.

The manner in which glucocorticoids induce bone loss is complex and incompletely understood (Patschan *et al.*, 2001; Canalis *et al.*, 2007), in part because there are no animal models absolutely comparable to humans. A major effect on the skeleton is a decrease in bone formation and unchanged or enhanced bone resorption (Lane, 2001). Glucocorticoid therapy results in rapid loss of BMD, which is greatest in the first year of therapy and may be as high as 30% or more in the first 3–6 months depending on dose (Eastell *et al.*, 2000; Adachi *et al.*, 2001). There is some evidence that these effects are partially reversible upon cessation of glucocorticoid treatments suggesting that bone retains the capacity to rebuild itself by virtue of having some residual osteoblast activity (Jia *et al.*, 2006). Glucocorticoids are thought to directly affect the differentiation, activity and lifespan of osteoblasts and osteocytes (O'Brien *et al.*, 2004). Glucocorticoids inhibit expression of genes important for bone formation including those responsible for the production of collagen A1, transforming growth factor-β, fibronectin- and insulin-like growth factor-1 (Iu *et al.*, 2005). The exact reason for increased bone resorption is unclear, but might include relative immobility of people sufficiently ill to require glucocorticoid therapy, intes-

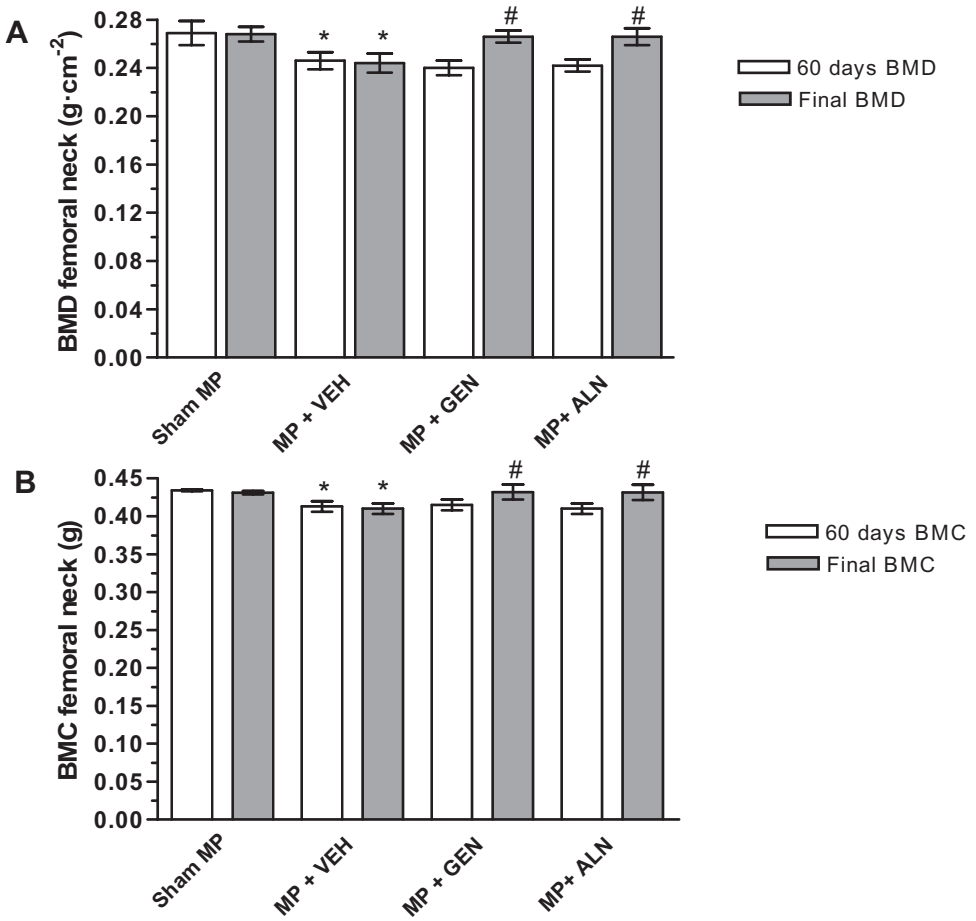

Figure 2 (A) Effects of alendronate (ALN) and genistein aglycone (GEN) on femoral bone mineral density (BMD) and (B) bone mineral content (BMC) in rats with glucocorticoid-induced osteoporosis (methylprednisolone, MP). Data are shown as the mean ± SD of seven animals. *$P <$ 0.01 versus Sham-MP; #$P <$ 0.005 versus MP + VEH (vehicle).

tinal malabsorption of calcium and gonadal hormone deficiency. The mechanisms for bone resorption in glucocorticoid therapy have not been fully established, but include activation of important and relevant kinase systems (Horsch *et al.*, 2007; Soares-Schanoski *et al.*, 2007), increased production of receptor activator of nuclear factor-κB ligand (RANKL) and reduced production of osteoprotegerin, resulting in increased osteoclast recruitment and survival (Weinstein *et al.*, 2002).

Histomorphometric analysis of biopsies from glucocorticoid-treated individuals has shown a reduction in bone formation at the cellular and tissue level, resulting in reduced bone volume and trabecular thickness (Dalle Carbonare *et al.*, 2005) and a decrease in the number of viable osteocytes (O'Brien *et al.*, 2004). There is also some evidence that glucocorticoids cause thinning of trabecular elements, in contrast to post-menopausal osteoporosis, where loss of trabeculae is more characteristic (Natsui *et al.*, 2006). Higher doses of glucocorticoids are associated with an increase in bone turnover and resorption, leading to greater bone loss and disruption of cancellous bone architecture (Tomlinson *et al.*, 2000; Cooper *et al.*, 2002). Glucocorticoids also affect many other target tissues that in turn may have an impact on skeletal metabolism. These include reduced intestinal absorption and increased renal excretion of calcium (Reid and Ibbertson, 1987; Morris *et al.*, 1990). Despite the complex pathophysiology associated with glucocorticoid treatment, the ultimate effect on bone is similar in many respects to post-menopausal osteoporosis. There is an imbalance between the amount of bone resorbed and that formed during each bone remodelling cycle and, in patients who are relatively immobilized, bone turnover is also increased.

The risk of fracture following use of glucocorticoids may not be related only to loss of bone tissue and the underlying disorder for which they are prescribed. Interestingly, the risk of fracture appears to increase rapidly upon exposure to glucocorticoids (van Staa *et al.*, 2005; Devogelaer, 2006; Woolf, 2007). Risk of fracture, however, also remains elevated after stopping treatment (Morris *et al.*, 1990). Similarly, we found in our experimental animal model of GIO that the cessation of glucocorticoid treatment was not sufficient to fully recover BMD nor to restore proper histological structure or bone resistance to rupture.

In our study, both the isoflavone, genistein aglycone, and the bisphosphonate, alendronate, succeeded in treating GIO in this experimental rat model: both increased BMD and bone breaking strength and ameliorated the histological damage caused by MP. Surprisingly, however, alendronate, a very useful drug for the prevention of fractures in post-menopausal

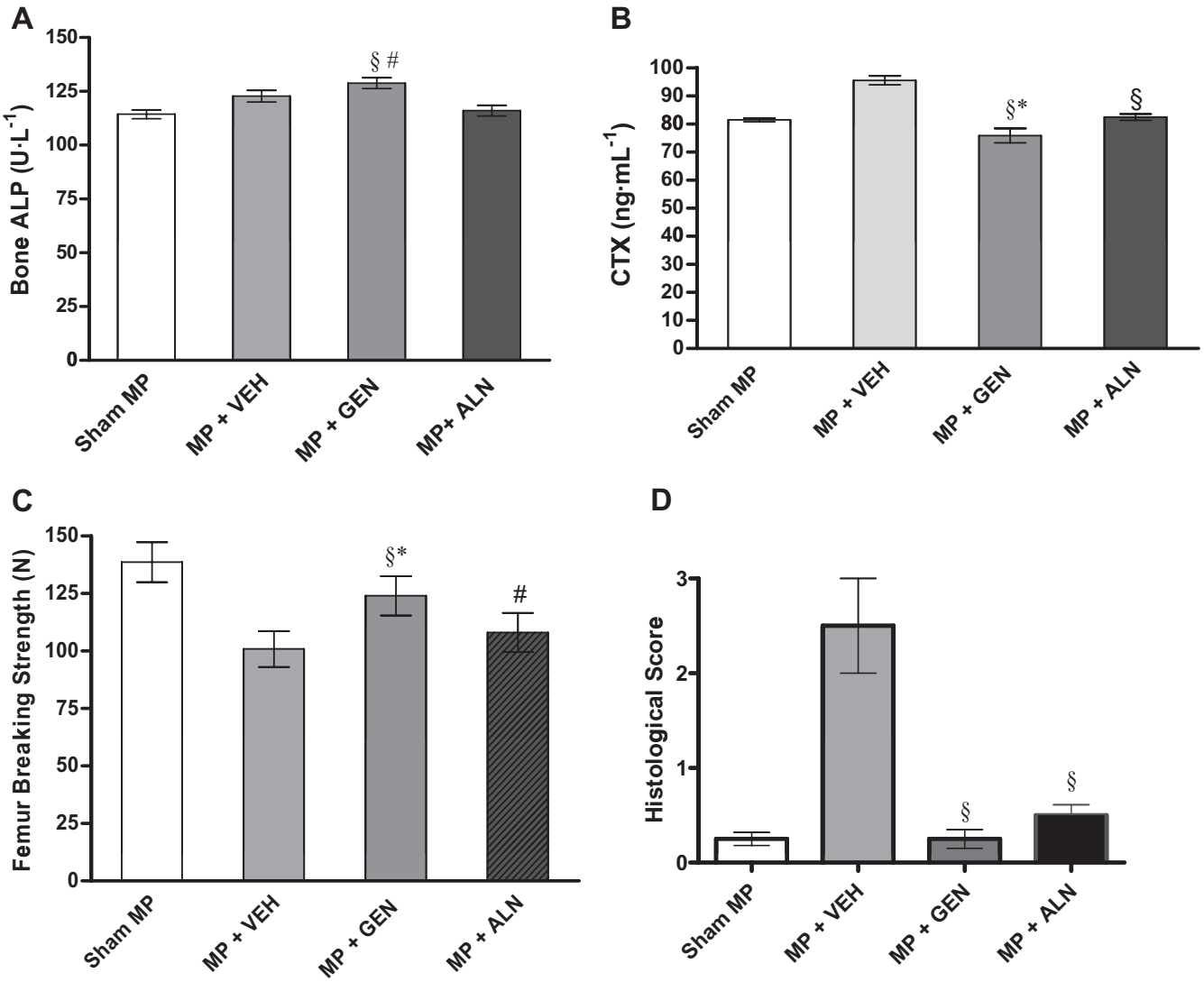

Figure 3 (A) Effects of alendronate (ALN) and genistein aglycone (GEN) on serum bone-alkaline phosphatase (b-ALP), (B) collagen C-telopeptides (CTX), (C) femur breaking strength and (D) histological score in rats with glucocorticoid-induced osteoporosis (methylprednisolone, MP). Data are shown as the mean ± SD of seven animals. b-ALP: §$P < 0.05$ versus MP + VEH (vehicle), #$P < 0.001$ versus MP + ALN. CTX: §$P < 0.001$ versus MP + VEH, *$P < 0.05$ versus MP + ALN. Femur breaking strength: §$P < 0.001$ versus MP + VEH, #$P < 0.05$ versus MP + VEH, *$P < 0.05$ versus MP + ALN. Histological score: §$P < 0.001$ versus MP + VEH.

women with osteoporosis, had a lesser effect on restoring bone quality compared with genistein aglycone. In addition, genistein aglycone induced a significant increase in serum b-ALP confirming its role as a bone forming agent. Genistein aglycone may act on *de novo* protein synthesis and on amplification of the interaction between the oestrogen receptor (ER) complex and nuclear DNA in osteoblasts. These cells express both ER-β and ER-α, but during the bone mineralization phase, ER-β is up-regulated significantly in bone tissue (Arts *et al.*, 1997). Genistein aglycone may act specifically on trabecular bone by a mechanism involving ER-β during the bone mineralization phase (Kuiper *et al.*, 1998).

The dose of genistein aglycone (5 mg·kg^{-1}) administered to our experimental animals represents the approximate human equivalent dose of 54 mg·day^{-1}, the same as was used in our recently reported clinical trial that showed that genistein aglycone is able to increase BMD and to promote bone formation also through the stimulation of the osteoprotegerin/sRANKL system in osteopenic, post-menopausal women (Marini *et al.*, 2008). Genistein aglycone shows positive histological evidence of reducing bone and cartilage erosion. It has been reported that there is a correlation between subchondral bone loss and cartilage degradation (Lajeunesse and Reboul, 2003). Enhanced femoral breaking strength in GIO animals given genistein aglycone is also further proof that genistein acts to rebuild bone via a stimulation of osteoblast function. Indeed, although all pharmacological treatments succeeded in improving the breaking strength of the femur, only genistein aglycone caused a statistically significant increase in serum b-ALP, a crucial measure of bone reconstruction. In the MP + VEH group, replacing administration of MP with subsequent vehicle administration demonstrated that there was residual osteoblast activity demonstrated by a small increase in b-ALP. No such increase was observed in

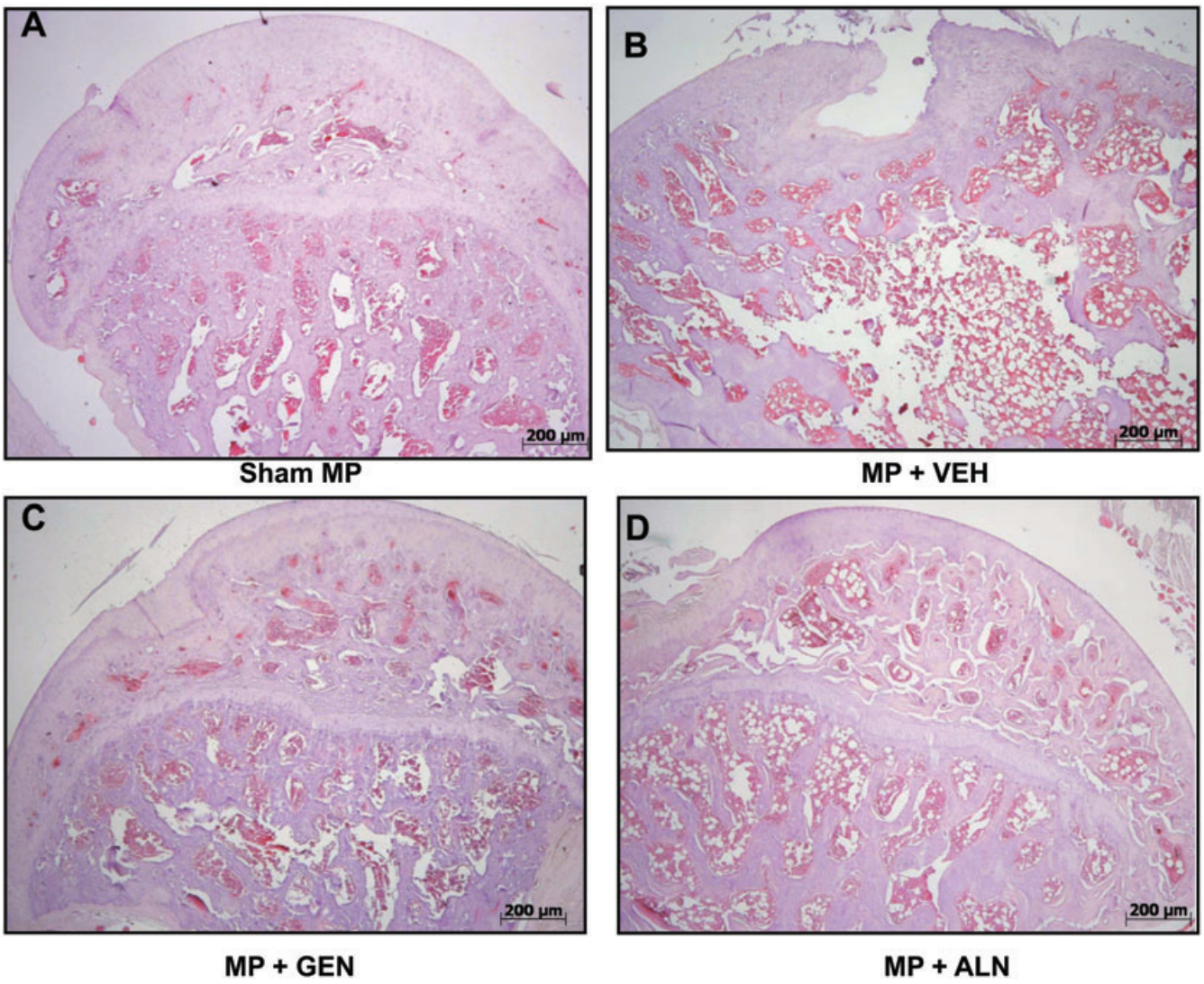

Figure 4 Light microscopy of the bone structure of the femur head taken from the different treatment groups. (haematoxylin and eosin stain; original magnification 5×). ALN, alendronate; GEN, genistein aglycone; MP, methylprednisolone; VEH, vehicle.

alendronate-treated animals suggesting a suppression of any remaining osteoblastic activity.

Genistein aglycone-treated animals showed restored and well-organized architecture of both cortical and trabecular bone matrix in femur head of osteoporotic rats. This correlates well with decreases in a critical bone resorption marker (CTX), increases in a bone formation marker (b-ALP) and enhanced resistance to fracture observed in femurs subjected to a constant load. Collectively, our results strongly suggest that the isoflavone genistein aglycone might be a new potential therapy for the management of GIO.

Usually, drugs used in management of osteoporosis have been classified as predominantly 'anti-resorptive agents' or as 'bone-forming agents'. On the basis of the present results, however, genistein aglycone might represent a therapy with bone-forming as well as an anti-resorptive activity. The only other compound shown with such activity is strontium ranelate (Seeman *et al.*, 2008). Strontium ranelate, which is only available in Europe, reduces the risk of vertebral fractures in patients with osteopenia. A recent publication, however, showed that strontium ranelate did not stimulate bone formation in ovariectomized rats (Fuchs *et al.*, 2008). Clinical studies in humans, however, are needed to truly assess the efficacy of genistein aglycone in preventing or mitigating GIO.

Statement of conflicts of interests

B Burnett, R Levy, MA Armbruster are employees of Primus Pharmaceuticals, Inc., Scottsdale, Arizona, USA.

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