Protective effect of genistein aglycone on the development of osteonecrosis of the femoral head and secondary osteoporosis induced by methylprednisolone in rats

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Abstract

Glucocorticoid (GC)-induced osteoporosis (GIO) is the most important secondary cause of bone loss. Clinical evidence suggests a role for genistein (GEN) aglycone in the prevention of osteoporosis. We investigated whether GEN could prevent GIO as well as the development of osteonecrosis in the femoral head using an experimental rat model. A total of 28 female Sprague–Dawley rats were used in the study. GIO and osteonecrosis were induced by daily s.c. injections of 30 mg/kg of methylprednisolone (MP; $n=7$). Another group of animals (MP + GEN; $n=7$) concomitantly received MP (30 mg/kg per s.c.) and GEN aglycone (5 mg/kg per i.p.) for 60 days. Control animals were administered daily with vehicle (VEH) or GEN (5 mg/kg per i.p.) only. At the beginning and end of the treatment, animals were examined for bone mineral density (BMD) and bone mineral content (BMC). After killing, serum was collected

to determine bone-alkaline phosphatase (b-ALP), carboxy-terminal collagen crosslink (CTX) and osteoprotegerin (OPG) levels. Femurs were removed and tested for breaking strength and bone histology analyzed for structural quality of the femoral neck. GEN aglycone prevented bone loss as measured by BMD and BMC. Moreover, GEN significantly increased the bone formation markers b-ALP and OPG, reduced the bone resorption marker CTX and statistically maintained comparable strength versus the VEH only group. Finally, histological scoring revealed a protective effect of GEN on bone structure statistically comparable with the VEH control animals. Results suggest that the GEN aglycone might be a preventive treatment for GIO and complications of osteonecrosis with long-term GC treatment.

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Introduction

The exact prevalence of glucocorticoid (GC)-induced osteoporosis (GIO) remains unclear. It is estimated, however, that in 30–50% of patients under chronic, long-term GC therapy, relevant bone loss occurs and 1 out of 4 also develop some degree of osteonecrosis (Mankin 1992, Cooper *et al.* 1995, Reid 1997, Lavernia *et al.* 1999). Intermittent use of GC only results in small increases in risk of fracture (de Vries *et al.* 2007). Still, a fracture caused by osteoporosis can lead to huge costs, especially in the case of hip fractures. By contrast, the prophylaxis of osteoporosis has a markedly lower cost and improves patient's quality of life (Kanis *et al.* 2007).

Bone loss induced by GCs occurs early and progresses at a fast rate becoming significant within the first 6 months (van Staa *et al.* 2002). GC therapy is responsible for bone loss

via different mechanisms that can be summarized as a decrease in bone formation and an increase in bone resorption. More specifically, GCs affect bone metabolism directly by inhibiting osteoblasts from producing new bone and decreasing osteoblast proliferation, while increasing osteoclast activity (Reid 1997, McIlwain 2003). In addition, GCs have been shown to inhibit osteoprotegerin production that further damps osteoblast function (Canalis 2003). GCs also increase apoptosis of osteocytes, which are an independent determinant of bone strength different from bone mineral density (BMD), leading to micro-architectural deterioration (Spreafico *et al.* 2008). Induction of osteocyte apoptotic pathways have been implicated in osteonecrosis as well (Weinstein *et al.* 2000). Bekler *et al.* (2007) attributes osteonecrosis to non-vascular events that induce necrotic cell death in the femoral head. The triggers for this avascular event appear to be related to tumour necrosis factor- α , RANK

ligand, and osteoprotegerin (OPG) balance in GC-treated osteoblasts, which may also affect differentiation of osteoclasts thus triggering the apoptotic events (Bejar *et al.* 2005).

GCs have other systemic effects on bone metabolism and in particular on calcium homeostasis. In fact, there is a decreased absorption from the intestine and an increased loss of calcium in the urine as a result of defective vitamin D₃ metabolism (Cosman *et al.* 1994, Patschan *et al.* 2001). Decreases in calcium and vitamin D₃ homeostasis lead to secondary hyperparathyroidism, which, in turn, produces increased bone resorption (McIlwain 2003). Finally, GCs affect bone metabolism indirectly by reducing levels of sex hormones (Vestergaard 2008).

Two types of bone are present in the skeleton: cortical (also known as compact) bone found along the shafts of the long bones (femur, tibia, radius, and ulna) and the flat bones (skull and ribs), and trabecular (also known as cancellous) bone, which is considerably finer and more delicate in appearance. Trabecular bone is found principally in the vertebrae of the spinal column and at the epiphyses of the long bones. Trabecular bone is lost more rapidly than cortical bone reflecting a greater rate of bone turnover (Eriksen *et al.* 1994). As a consequence, the lumbar spine is the first site in which GIO can be detected. A significantly increased risk of non-vertebral fractures within the first 3 months of treatment has also been observed (van Staa *et al.* 2000).

A wide variety of pharmacological interventions have been shown to decrease bone loss in GIO as well as osteonecrosis of the femoral head. Proposed treatments include bisphosphonates, hormone replacement therapy, vitamin D (cholecalciferol or calciferol) and calcium, calcitriol, calcidiol, alfacalcidol, calcitonin, fluoride, testosterone, and anabolic steroids (Adachi *et al.* 1996, Ringe *et al.* 1999, Eastell *et al.* 2000, Boutsen *et al.* 2001, Åstrand & Aspenberg 2002, Crandall 2002, Sambrook 2007). In addition, several alternative therapeutic approaches have been considered in recent years.

Genistein (GEN) aglycone is an isoflavone found in low concentrations in soybeans and elevated amounts in certain soy-derived food, whereas genistin, the glucoside form of the aglycone GEN, is much more abundant in the unprocessed soybean (Messina *et al.* 2004). We previously showed that treatment with >98% pure GEN aglycone (54 mg/day) increased BMD at the lumbar spine and femoral neck in osteopenic, postmenopausal women with no clinically significant adverse effects on the breast and uterus (Morabito *et al.* 2002, Crisafulli *et al.* 2004, Marini *et al.* 2007, 2008a,b). Furthermore, very recently it has been demonstrated that GEN at the same doses, adjusted for rat body weight and surface area, was more efficacious than alendronate or raloxifene or estradiol in treating primary osteoporosis induced by ovariectomy (Bitto *et al.* 2008) and secondary osteoporosis (Bitto *et al.* 2009). In light of these observations, this animal study presents results that suggest that GEN aglycone may be a new option for the preventive management of GIO-induced bone loss and osteonecrosis of the femoral head.

Materials and Methods

Animals

All procedures were evaluated and approved by the Ethics committee of the University of Messina and the study 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). A total of 28, 8-month-old female Sprague–Dawley rats (Charles River, Calco, Italy) weighing ~250–275 g were used in this study. 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:12 h darkness cycle, temperature ~24 °C), and provided with access to standard food for laboratory animals and water *ad libitum*.

Induction of osteoporosis, randomization, and treatments

The study protocol is summarized in Fig. 1. Briefly, a group of animals ($n=7$) was injected daily with methylprednisolone (MP; 30 mg/kg, subcutaneously) and the dissolution vehicle (VEH; 10% DMSO/in 0.9% NaCl solution) of GEN aglycone (MP) for 60 days to produce GIO and osteonecrosis of the femoral head. Another group of animals (MP+GEN; $n=7$) was dosed concomitantly with MP and GEN (5 mg/kg per i.p.) each day. A third group of animals ($n=7$) was administered with the MP VEH (1 ml/kg, subcutaneously of a 0.9% NaCl solution) daily and served as controls (VEH only), and a last group received only GEN (5 mg/kg per i.p.; GEN $n=7$). The treatment lasted 60 days.

BMD and bone mineral content

BMD and the relative bone mineral content (BMC) of the femurs were measured using dual-energy X-ray absorptiometry (Hologic QDR-4500A, Waltham, MA, USA). For basal and final measurements, animals were kept anesthetized with sodium pentobarbital (50 mg/kg i.p.). During the analysis period, daily measurements were made for BMD and BMC following the manufacturer's instructions, in order to assess the long-term reproducibility of the measured parameters (QC). A measured value of $\pm 1.5\%$ 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 under general anesthesia with chloral hydrate (400 mg/kg i.p.) after blood collection by cardiac puncture. Blood was centrifuged and

Figure 1 Flow chart of the experimental protocol.

serum stored immediately at -20°C for analysis. Commercially available ELISA kits for b-ALP (IDS Plc), CTX (Nordic Bioscience Diagnostics, Beijing, China), and OPG (IDS Ltd) were then used to evaluate sera in duplicate for each animal for bone formation and resorption markers respectively.

Histology

Analysis was performed by an investigator blinded to the treatment groups. 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% HCl from 37% (v/v) concentrate and 10% formic acid from 89% (v/v) concentrate in PBS) for ~ 24 h at 37°C , dehydrated in graded ethanol, and then embedded in paraffin. Three $5\text{-}\mu\text{m}$ -thick paraffin-embedded horizontal bone sections were cut from the proximal end of the diaphysis, dyed with a haematoxylin-eosin stain and studied using light microscopy. Femoral heads (area comprised between hip

joint cartilage and metaphyseal cartilage) were used to judge the quality of cartilage, bone, and trabecular density according to the scores reported in Table 1.

Femur-breaking strength

At the moment of killing, the maximum load (breaking strength) tolerated by femurs was measured on coded samples using a calibrated tensometer (Sans) assessed by an investigator blinded to the treatment groups. 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 center of the bone at a rate of 10.0 mm/min until the bone fractured, maximum tolerated load was expressed in Newton (N).

Drugs

GEN aglycone ($>98\%$ pure) was a kind gift of Primus Pharmaceuticals Inc., Scottsdale, AZ, USA. All substances were prepared fresh daily and administered in a volume of $100\text{ }\mu\text{l}$. Route of administration was chosen according to

Table 1 Criteria for the evaluation of the histological score used to assess the degree of osteoporosis

	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

VEH. For MP, we used only 0.9% NaCl, thus was administered subcutaneously. For GEN aglycone, we used DMSO. Thus, to avoid a poor absorption, we chose to administer it intraperitoneally.

Statistical analysis

All data are expressed as means $\pm$ S.D. The significance of differences in BMD of the femoral neck and BMC 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 analyzed by one-way ANOVA followed by Tukey's multiple comparison test. In all cases, a probability error of <0.05 was selected as the criterion for statistical significance. Graphs were drawn using GraphPad Prism (version 4.0 for Windows).

Results

Effect on femoral BMD and BMC

Following MP administration over a period of 60 days, animals had a significant decrease in BMD at the femoral neck (0.240 ± 0.003 g/cm²) compared with VEH only treated animals (0.269 ± 0.002 g/cm²; $P < 0.001$), and in BMC (0.416 ± 0.004 versus VEH only (0.431 ± 0.001 g; $P < 0.001$). At the end of the treatment period, GEN was found not only to maintain BMD (Fig. 2A) and BMC (Fig. 2B) in GC-treated animals, but also significantly increased BMD (0.267 ± 0.001 g/cm²; $P < 0.001$ versus MP) and BMC (0.432 ± 0.001 g; $P < 0.001$ versus MP)

compared with the MP group. GEN alone did not significantly improve BMD and BMC in naive animals (data not shown).

Effect on bone markers

At the end of the experiment, serum b-ALP levels were higher (Fig. 3A) in the MP group than in the VEH only group ($P < 0.001$). GEN increased the bone formation marker b-ALP even further in MP animals (135.42 ± 2.99 ; $P < 0.005$ versus MP) as well as in naive animals (130 ± 4 ; $P < 0.001$ versus VEH), confirming the positive role of the pure isoflavone in stimulating osteoblast activity (Fig. 3A). Another bone formation marker, OPG (Fig. 3B), was increased in serum from animals in the GEN and MP+GEN groups ($P = 0.018$ versus VEH and $P < 0.001$ versus MP). MP alone caused a marked decrease in OPG ($P < 0.001$ versus VEH). In addition, plasma levels of the bone resorption marker CTX were significantly higher in the MP group than in the VEH only group ($P < 0.005$) at the end of the experiment (Fig. 3C). The concomitant treatment of MP animals with GEN significantly reduced CTX plasma levels (78.78 ± 2.09 ng/ml; $P < 0.001$ versus MP) compared with VEH only animals, but especially compared with the MP group (Fig. 3C).

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

Results obtained from the three-point bending test of the femur showed that the MP+VEH group had a significant reduction in breaking strength compared with VEH only rats ($P < 0.001$; Fig. 3D). The concomitant administration of GEN, however, succeeded in preserving the breaking strength of the femur compared with the VEH only animals ($P < 0.001$; Fig. 3D). GEN alone only slightly improved bone strength in untreated animals when compared with VEH group.

Histological scores were evaluated at the end of the experiment on femoral heads, according to the criteria shown in Table 1. GC treatment significantly reduced cortical bone, deteriorated bone micro-architecture, and eroded the hip-joint cartilage shown in the MP group. There was also evidence, as shown in the histology, that the femoral heads had undergone osteonecrosis similar to that seen in other work with high MP administration (Bekler *et al.* 2007). GEN, however, prevented osteonecrosis, bone erosion and maintained a normal bone architecture comparable with the VEH group (Fig. 3E). Femoral heads collected from GEN-treated rats in the presence of MP (Fig. 4D) showed a normal shape with intact cartilage, qualitatively equivalent cortical bone, well-organized bone matrix composed of trabecular bone comparable with the VEH only (Fig. 4A), and prevented the damage shown in the MP group (Fig. 4C). GEN alone as expected stimulated bone remodeling in naive animals (Fig. 4B).

Figure 2 Effects of aglycone genistein on femoral bone mineral density (BMD) and bone mineral content (BMC) in methylprednisolone-treated rats. Data are shown as the mean $\pm$ S.D. of seven animals. * $P < 0.01$ versus Sham MP; ** $P < 0.005$ versus MP+VEH.

Figure 3 Effects of aglycone genistein on serum bone-alkaline phosphatase (b-ALP), osteoprotegerin (OPG), collagen C-telopeptides (CTX), femur-breaking strength, and histological score in methylprednisolone-treated rats. Data are shown as the mean $\pm$ s.d. of seven animals. b-ALP, * $P < 0.001$ versus VEH; *** $P < 0.005$ versus MP. OPG, # $P = 0.018$ versus VEH; * $P < 0.001$ versus VEH; *** $P < 0.001$ versus MP. CTX, * $P < 0.005$ versus VEH; *** $P < 0.001$ versus MP. Femur-breaking strength, § $P < 0.001$ versus VEH; *** $P < 0.001$ versus MP. Histological score, * $P < 0.001$ versus VEH; *** $P < 0.001$ versus MP.

Discussion

Several studies and reports show a decrease in BMD and an increased risk of fractures during GC use as well as an increase in osteonecrosis with chronic steroid use. Approximately 30% of all fractures of the hip and almost half of all fractures of the spine can be attributed to chronic, high-dose GC use in humans (van Staa *et al.* 2001). Prior and current exposure to GCs increases the risk of fractures beyond that explained by values of BMD (Civitelli & Ziambaras 2008). The main effect of GCs on bone is inhibition of osteoblastogenesis, augmented continued osteoclastogenesis and osteocyte apoptosis, leading to a decrease in bone formation, a rapid weakening of bone architecture and an increase in fracture risk (Manolagas & Weinstein 1999). Chronic administration of steroids also causes avascular necrosis via an apoptotic mechanism of osteocytes and osteoblasts (Weinstein *et al.* 2000, Bekler *et al.* 2007). Once osteonecrosis occurs, GC administration appears to also inhibit bone regeneration (Takano-Murakami *et al.* 2009).

Pharmacological intervention for prevention of GC-induced osteoporosis is needed depending on dose, expected duration of treatment, age and gender of the patient, and sometimes BMD at the start of the GC therapy. At present, calcium and vitamin D₃ supplementation are considered as important support for the prevention of GC-induced

osteoporosis (Williams *et al.* 2004). Bisphosphonates are largely used to avoid bone loss and are cost effective in certain subgroups of patients depending on age, gender, GC dose, and previous fracture history (Williams *et al.* 2004, Prinsloo & Hosking 2006). Unfortunately, calcium and vitamin D₃ supplementation may not be enough to stave off bone deterioration and bisphosphonates have safety risks associated with long-term use, such as esophageal burns and bone and muscular pain (Ettinger *et al.* 1998, Wysowski & Chang 2005). Osteonecrosis of the jaw, although rare and found primarily in cancer patients undergoing dental surgery, is a very serious and debilitating side effect of bisphosphonate use (Durie *et al.* 2005). Therefore, a safe and effective treatment for the prevention of bone loss and osteonecrosis of the femoral head in GC-treated subjects is still needed. Recent clinical and experimental evidence suggests a role for the isoflavone GEN aglycone in the treatment of primary bone loss and osteoporosis (Morabito *et al.* 2002, Crisafulli *et al.* 2004, Marini *et al.* 2007, 2008a,b, Bitto *et al.* 2008).

Based on earlier studies, we used MP to produce bone loss as well as osteonecrosis of the femoral head (Sevitt 1964, Bejar *et al.* 2005, Bekler *et al.* 2007) in the rat and we studied GEN aglycone preservative effects. One drawback of this model is its GC effect on cartilaginous structures (Peskin *et al.* 2001).

Figure 4 Light microscopy of the bone structure of the femur head. (H&E original magnification $\times 5$).

In our study, GEN succeeded in preventing GC-induced osteoporosis and osteonecrosis of the femoral head when co-administered with MP. This purified isoflavone statistically maintained BMD and BMC over the MP-treated group and showed comparable efficacy with the VEH only group. GEN co-administered with MP also statistically maintained femoral bone's resistance to rupture compared with the MP group and prevented the histological damage seen in the MP group. GEN alone or co-administered with MP caused a significant increase in b-ALP over MP and VEH only confirming its role as a bone-forming agent. MP administration caused an increase in bone remodeling as reflected by increased B-ALP and CTX levels in MP animals. In the MP+GEN group, there was a significant reduction in CTX compared with the MP group suggesting an anti-resorptive effect. There was also a small decrease in CTX levels in the VEH only group suggesting a suppressive effect of GEN on bone resorption in this rat model. The diverse mechanism of action for GEN aglycone's effect on bone metabolism is still not well understood.

GC therapy has been shown to cause apoptosis in osteoblasts and inhibit the production of osteoprotegerin required for bone formation, while RANK-L accumulates resulting in further bone resorption (Bejar *et al.* 2005). GEN has been shown to stimulate the production of osteoblasts via inhibition of a RANK-L-resorptive mechanism by producing osteoprotegerin (Viereck *et al.* 2002). This was also confirmed in the present paper where

GEN increased OPG levels in either naive or MP-treated animals. OPG is a glycoprotein secreted by osteoblasts in a differentiation-dependent manner and acts as a 'decoy receptor' (a soluble receptor that acts as antagonist) for RANK-L regulating osteoclast functions and lifespan. GEN aglycone, in human clinical trials, increases BMD and also promotes bone formation through the stimulation of the OPG/sRANK-L system in postmenopausal women with bone loss, as well as in osteoporotic rats (Morabito *et al.* 2002, Crisafulli *et al.* 2004, Marini *et al.* 2007, 2008a,b), the same phenomenon shown in osteoporotic rat models (Bitto *et al.* 2008).

GEN was shown in neonatal rats to decrease GC receptor levels in the liver (Csaba & Inczeffi-Gonda 2002). Using a reporter gene assay system in Chinese hamster ovary cells, various flavonoids, including GEN aglycone, were not found to be agonists on the GC receptor (Takeuchi *et al.* 2009). Similarly, liquiritigenin, a closely related structural polyphenol and a strong agonist of the estrogen receptor- β (ER- β) like GEN, did not activate GC receptors in transfection assays (Mersereau *et al.* 2008). Conversely, a strong GC such as dexamethasone appears not to interact with ER complexes (Wilson *et al.* 2004). Rather, GEN may act on the molecular level to inhibit transcription of GC receptors or cause their degradation. Moreover, Wallace & Cidlowski (2001) found that a proteasome-ubiquitin pathway regulates the production of GC receptors. It appears that GEN inhibits GC receptor transactivation and may also induce

a proteosomal degradation of the GC receptor complex via the p53 and ubiquitin pathways (Kinyamu & Archer 2003). Finally, GEN's tyrosine kinase inhibitor activity may play a role by limiting the subcellular nuclear transport and recycling of the GC receptors thereby inhibiting the effects of GC on bone (Yang *et al.* 1997).

It has been reported that GEN acts on *de novo* protein synthesis and on amplification of the interaction between the ER complex and nuclear DNA in osteoblasts. These cells express both ER- β and ER- α at low levels until stimulated. GEN appears to act on trabecular bone by a mechanism involving ER- β upregulation during bone mineralization phase (Kuiper *et al.* 1998). The dose that we chose to administer was the same (human equivalent) dose of GEN aglycone used in our previous clinical trials (Morabito *et al.* 2002, Crisafulli *et al.* 2004, Marini *et al.* 2007, 2008a,b).

The positive effects of GEN on prevention of GC-induced osteoporosis and osteonecrosis in the present experimental model are confirmed by the enhanced femoral breaking strength together with the preserved bone morphology observed in GEN-treated animals in the presence of MP. Normal architecture of cartilage as well as both cortical and trabecular bones with a well-organized matrix in femoral head of MP + GEN-treated rats was observed. This finding significantly correlates with the enhanced resistance to fracture observed in femurs subjected to a constant load. Furthermore, GEN alone in naive animals stimulated bone remodeling as confirmed by the biochemical markers, mechanical tests, and histology.

Collectively, our results strongly suggest that GEN aglycone might be a new potential therapy for the prevention of GC-induced osteoporosis, the most important secondary cause of osteoporosis in humans. And in the minority of cases, GEN may prevent necrotic deterioration of the femoral head. Usually, drugs used in management of osteoporosis have been classified as predominantly 'anti-resorptive agents' or as 'bone-forming agents', but, on the basis of the present results, GEN aglycone might represent the first therapy to overcome this classification combining a powerful bone-forming as well as an anti-resorptive activity.

Declaration of interest

Bruce Burnett, Robert Levy, and Mary Ann Armbruster currently work for Primus Pharmaceuticals, Inc., Scottsdale, Arizona, USA. Primus only provided us genistein aglycone. Dr Burnett, Levy, and Armbruster were involved in the design of the experiment. Dr Burnett also helped us in critical revision of the manuscript. All the other authors have nothing to declare.

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