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NO-donating genistein prodrug alleviates bone loss in ovariectomised rats

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Summary

Questions under study: To find a more potent alternative with less oestrogen-related side effects for hormone replacement therapy (HRT) in postmenopausal osteoporosis, we designed and synthesized a NO-releasing prodrug of genistein (NO-G) according to the structure of NO-NSAIDs. The purpose of this study was to evaluate the effects of the prodrug on bone in ovariectomised (OVX) rats.

Methods: Forty-eight adult Sprague–Dawley female rats were ovariectomised and treated with vehicle, 9mg/kg genistein and 4.5, 9 or 18 mg/kg NO-G by oral administration daily. The bioassays were performed in terms of bone mineral density (BMD), mechanical testing, bone formation markers, bone alkaline phosphatase (b-ALP) and osteocalcin (OCN) and bone resorption marker urine deoxypyridinoline (DPD). In addition, the effects of the drugs on uterus and body weight were examined.

Results: After treatment for 12 week, the BMD of whole femur and tibia in the NO-G (9 and 18 mg/kg) groups were 12.1% and 12.2% higher than that of OVX group ($P < 0.01$); the bending strength of the femur was 11.2% and 12.2% higher than OVX group ($P < 0.01$). The OVX-induced increase of serum b-ALP, OCN and urinary DPD were markedly attenuated. The prodrug showed no side effects on uterus and body weight.

Conclusions: The NO-releasing prodrug of genistein improves the bone loss in OVX rats without stimulatory effects on the uterus, which suggests that the product could potentially be used for the prevention and treatment of postmenopausal osteoporosis.

Key words: NO-donating genistein prodrug; postmenopausal osteoporosis; ovariectomised rats; bone loss; bone mineral density; mechanical strength; serum biomarkers

Introduction

Postmenopausal osteoporosis is a major health hazard in aging females associated with the climacteric period because of the decline in endogenous oestrogen production following the menopause and the prevalence of this disease is increasing. For a long time, hormone replacement therapy has been the main therapeutic measure for prevention and treatment of postmenopausal osteoporosis. However, long-term acceptance and/or compliance in hormone replacement therapy is low due to potentially malignant effects on reproductive tissues, which have been recently revealed by the findings from the Women's Health Initiative Trial [1]. Owing to the increased risks of breast cancer and cardiovascular diseases, patients look for alternatives, one of which is soy isoflavones.

Genistein is a typical soya bean isoflavone which has mild oestrogen-like biological activity, approximately 1×10^{-3} to 1×10^{-5} that of oestradiol [2]. Among Asian populations in which consumption of soy protein (rich in isoflavones) is markedly higher than Western countries, lower rates of osteoporosis and related fractures are reported [3–5]. Many studies show that genistein may exert positive effects on bone in maintaining bone mass in ovariectomised (OVX) rats. However due to its pharmacokinetic differences and unstable pharmacological actions, human and animal studies are presently focused on providing convincing data on their potency and method of action in preventing bone loss, and its possible side effects [6, 7].

For more than a century, organic nitrates

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have been used in the treatment of cardiovascular system diseases. The activity of these nitrates is mainly attributed to the release of nitric oxide (NO) [8]. NO is also produced by bone cells and may modulate the anabolic effects of oestrogen on bone homeostasis by restraining osteoclast-mediated bone resorption and stimulation of osteoblast activity. In studies of postmenopausal osteoporosis it has been found that NO metabolites decrease in postmenopausal and other amenorrhoeic women and are increased by oestrogen replacement [9–12]. Constitutive production of NO may be important in regulating osteoclastic activity. It has a direct inhibitory effect on osteoclast-mediated bone resorption [13–15]. Several recent studies have revealed a wider role of NO in bone metabolism. Recent studies have also demonstrated that administration of NO donor attenuates oestrogen deficiency bone loss in menopausal women and OVX rats. NO donor nitroglycerin can alleviate ovariectomy induced bone loss and the protective effects of oestrogens on bone are in part mediated through NO [16, 17]. The function of the eNOS gene-deficient osteoblast was restored by administration of exogenous NO donor, but their response to 17β-estradiol was blunted both in vitro and in vivo [18–20]. These findings imply that NO donors might be useful in the prevention and treatment of osteopenic conditions.

Therefore we started a project aiming at the structural modification of genistein to produce more intensive efficacy in preventing bone loss. According to the structure of NO-NSAIDs, we successfully synthesized a prodrug of genistein by esterification of 7, 4'-hydroxyls (nitrooxybutyl ester derivative of genistein, abbreviated as NO-G) [21]. The side chains may release NO slowly after biotransformation and its parent drug genistein may be liberated, synergising the treatment effect in osteopenia. The aim of the present experimental study was to evaluate the possible better beneficial effects of NO-G on bone loss than its parent drug genistein in the OVX adult rat model. Additionally, possible side effects of NO-G on body weight and uterus were investigated.

Materials and methods

Animals and chemicals

Forty eight 9-week old female Sprague Dawley rats (obtained from the laboratory animal centre of FMMU, Xi'an China, weighing on average: mean 223 SD5.6g) were housed four to a cage under standard laboratory conditions with temperature ranging between 20°C and 25ºC, relative humidity between 55 ~ 65%, and 12h light/dark cycle (lights on 08:00). All rats were allowed free access to water and a pelleted commercial diet (containing 0.97% calcium, 0.85% phosphorus and 1.05IU/g of Vitamin D3). Rats were randomly divided into six groups (n = 8 each). After one week of habituation to the facilities, animals were used for studies. All OVX rats underwent bilateral ovariectomy via a dorsal approach with a small midline dorsal skin incision, the Sham-operation rats (SH group) were subject to sham surgery exposure without removing the ovaries. The experimental protocol was approved by the Ethical Committee for Animal Care of the Fourth Military Medical University. Efforts were made to minimize animal suffering and to reduce the number of animals used.

After one week of recovery from the operation, the SH group and OVX control groups received vehicle (1% DMSO water solutions). The other four OVX groups were given genistein (9 mg/kg/day, Sigma, St. Louis, MO) and NO-G at doses of 4.5, 9 or 18 (equal molar of genistein) mg/kg/day respectively. The dose of genistein used was in accordance with a previous study in adult rats [22].

After 12 weeks of treatment, the animals were anaesthetized using intraperitoneal injection of sodium pentobarbital (45 mg/kg). Whole blood was collected by abdominal aorta puncture immediately before cervical dislocation and serum was obtained by centrifugation at 3000 rpm for 15 min, and the serum was transferred to Eppendorf tubes and stored at –70ºC until analyses were performed.

The NO-G had been synthesized in our laboratory. MW: 532.8, a light beige powder, mp: 125.5~127.5ºC. Its structure has been confirmed by ^{1}H NMR and mass spectrum [21] (fig. 1). All drugs were prepared in 1% DMSO water solution.

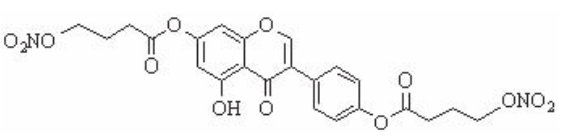


Figure 1
Chemical structure of NO-G.

Measurement of BMD

Femurs and tibias were removed at the time of necropsy, cleaned of soft tissue and stored at –70ºC until analyses were performed. BMD analyses were performed on left legs (whole femur and tibia) by dual energy X-ray absorptiometry (DEXA) (DPX-IQ 7040, Lunar Corp, USA) with small animal software 1.0.

Three-point bending test

The mechanical failure properties of femora were measured using an Instron 8501 material testing system (Instron Corp., Canton, MA, USA). Samples were hydrated in physiological saline for 4 h prior to biomechanical testing. The diaphysis in each sample was tested to failure in a three-point bending test according to a procedure described previously [23]. Briefly, samples were placed on two supports spaced 15 mm apart to a pre-load of 1N and then deformed at a rate of 1 mm/min until failure. The point of failure was defined as a successive drop in load greater than 5%.

Serum and urine analysis

Serum alkaline phosphatase (ALP, bone specific) levels and osteocalcin (OCN) are frequently used as bone formation markers to monitor drug actions [24–26]. Bone turnover markers were measured using commercially available kits as specified by the manufacturers.

B-ALP was measured by an immunoassay (Alkphase-B, Metra Biosystems Inc., Mountain View, CA, USA).

Serum OCN was measured using the sandwich ELISA Kit (Biomedical Technologies Inc., USA).

Urine samples were collected from all rats prior to the end of the experiment. An ELISA Pyrilinks-D kit (Metra Biosystems, Mountain View, CA, USA) was used to determine the urinary level of deoxypyridinoline (DPD). The urine volume was corrected for the urinary creatinine (Crt) level, which was measured with a colorimetric method using a kit from Sigma.

All assays were performed in duplicate.

Body and uterine weights

Body weights were monitored weekly throughout the experimental period using a digital portable scale (Model XP-1500, Denver Instrument). Weight gain was determined by subtracting initial body weight at the beginning of the intervention diets with final body weight immediately before necropsy. At necropsy, uteri were carefully removed and cleaned of fat tissue. Uterine weights were measured to confirm the OVX procedure, as indicated by lower uterine weights, and to monitor for any oestrogenic effects of the drug intervention.

Statistical analysis

Student-t test was used to determine the statistical difference between SH group and OVX group. Statistical differences between OVX group and treatment groups were analyzed using one-way analysis of variance (ANOVA) followed by LSD post hoc test using SPSS statistical software (SPSS, Inc., Chicago, IL). Statistical significance was set at $P < 0.05$.

Results

Bone mineral density (BMD)

The effects of 12 weeks of treatment on femoral and tibial BMD were illustrated in fig. 2. Femoral and tibia BMD were 11.5% and 12.2% lower in OVX group than in SH group respectively ($P < 0.01$). The BMD of femur and tibia in NO-G (9 and 18 mg/kg) groups were 12.1% and 12.2% higher than that in OVX controls ($P < 0.01$) and were 99.1% and 98.6 % of SH group.

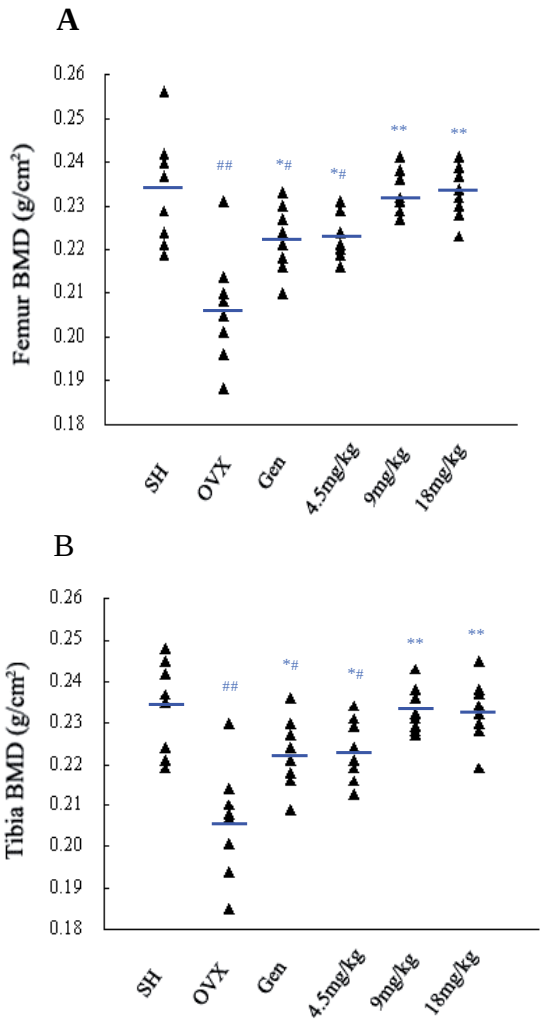


Figure 2

BMD changes after drugs administration. A). Femur BMD; B). Tibial BMD. 1w recovered from operation, rats were given different drugs: Sham-operated (SH) and OVX control (OVX) groups received vehicle (1% DMSO water solutions); other 4 groups were given 9 mg/kg/ genistein (Gen), 4.5, 9, or 18 (equal molar of Gen) mg/kg NO-G respectively. BMD were measured by dual energy X-ray absorptiometry with small animal software 1.0. After treatment for 12w, the BMD of femur and tibia in NO-G (9 and 18 mg/kg) groups were 11.5% and 12.2% higher than that of OVX controls ($P < 0.01$). The BMDs are also significantly higher in NO-G (9 and 18 mg/kg) groups compared with Gen and NO-G (4.5 mg/kg) groups ($P < 0.05$). Data are expressed as the Means with SEM of 8 rats. *p <0.05, **p <0.01 vs OVX; #p <0.05, ##p <0.01 vs SH.

Mechanical properties

OVX and all drugs administration significantly affected the failure properties of femora as evaluated with a three-point bending test (fig. 3). OVX caused a 15.4% decrease in failure torque ($P < 0.01$ vs. SH). All the OVX animals administered drugs recovered failure torque that were diminished by OVX except NO-G 4.5 mg/kg group (genistein 8.5%*, NO-G 9 and 18 mg/kg dose 11.7–12.2%**, *$P < 0.05$, **$P < 0.01$ vs. OVX).

Serum and urine biomarkers

The results of serum b-ALP, OCN and urinary DPD were shown in fig. 4.

OVX raised b-ALP to 129.4% compared with SH group ($P < 0.01$). After 12 weeks treatment, this effect was significantly suppressed by intake of NO-G at the dose of 9 and 18 mg/kg (14.5–15.0%, $P < 0.01$ vs. OVX); but not as significantly in genistein and 4.5 mg/kg NO-G groups (7.4%, 7.8%, $P < 0.05$ vs. OVX) (fig. 4A).

Serum OCN was increased by 48.6% in OVX group compared with SH group ($P < 0.01$). The OVX groups treated with genistein and 4.5 mg/kg NO-G had a 17.3% and 16.1% ($P < 0.05$) reduction in the OVX-induced increase of serum OCN, whilst the OVX plus NO-G (9 and 18 mg/kg) treated animals had a 33.3% and a 35.2% ($P < 0.01$) reduction in the OVX-induced increase of serum OCN compared with OVX group (fig. 4B)

Urinary DPD serves as a bone resorption marker in body. OVX increased DPD level by 61.3% compared with SH group ($P < 0.01$). Administration of 9 or 18 mg/kg NO-G to OVX animals significantly reduced DPD levels to 70.8% and 74.6% of OVX ($P < 0.01$) whilst there was no difference in the SH group. The effect of genistein on reducing DPD was not that significant (83.8% of OVX group, $P < 0.05$), while 4.5 mg/kg NO-G seems to have no effect on DPD (fig. 4C).

These findings suggest a more potent protective effect of NO-G (9 and 18mg/kg doses) on the OVX induced high bone turnover that is apparent in the OVX animals.

Figure 3

Mechanical property of femur.
The mechanical failure properties of the femora were conducted using an Instron 8501 material testing system. Drugs treatment recovered the failure properties that were induced by OVX except NO-G 4.5 mg/kg group. The effects of NO-G at 9 and 18 mg/kg doses are more significantly compared with Gen group. Data are expressed as the Means with SEM of 8 rats. *P <0.05, **P <0.01 vs OVX, #p <0.05, ##p <0.01 vs SH. (SH: Sham-operated; OVX: OVX control; Gen: genistein)

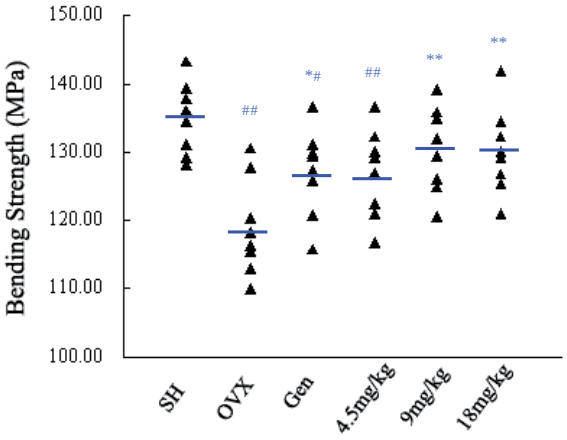
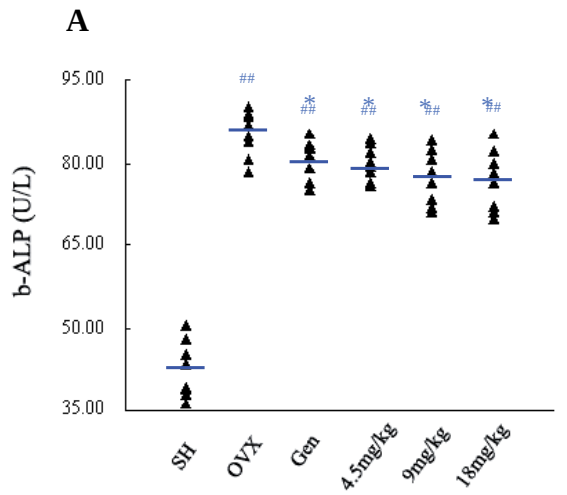
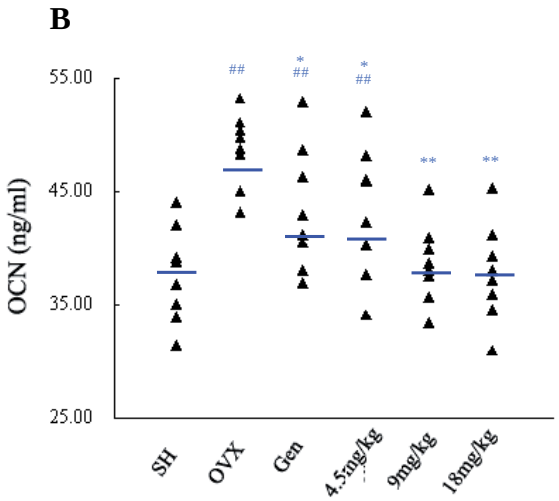
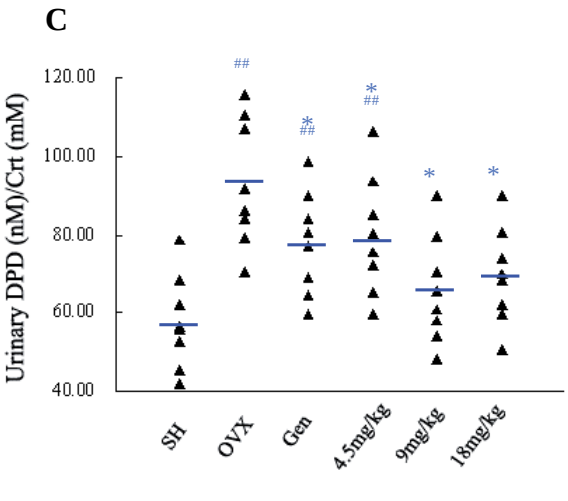


Figure 4

Serum biomarkers of bone turnover. (A) serum b-ALP; (B) serum osteocalcin (OCN); (C) Urinary DPD.
Serum markers were determined by commercially available kits as specified by the manufacturers. OVX increases serum b-ALP and OCN levels. After drugs donation, these effects have all been reversed. Compared with OVX group, Gen and NO-G (4.5 mg/kg) reduced b-ALP by 7.36% and 7.83%, OCN by 17.3% and 16.1%, NO-G (9 and 18 mg/kg) reduced b-ALP by 14.5% and 15.0%, OCN by 33.3% and 35.2% respectively. Urinary DPD level (expressed as nM of DPD per mM of creatinine to correct urine volume) in OVX rats. OVX significantly increases urinary DPD level. Administration of 9 or 18 mg/kg NO-G to OVX animals significantly reduced DPD levels to 70.8% and 74.6% of OVX (P <0.01) and has no difference with SH group. The effect of genistein was not that significant (83.8% of OVX group, P <0.05), while 4.5 mg/kg NO-G seems has no effect on DPD. Data are expressed as the Means with SEM of 8 rats. *p <0.05, **p <0.01 vs OVX; ##p <0.01 vs SH. (SH: Sham-operated; OVX: OVX control; Gen: genistein)







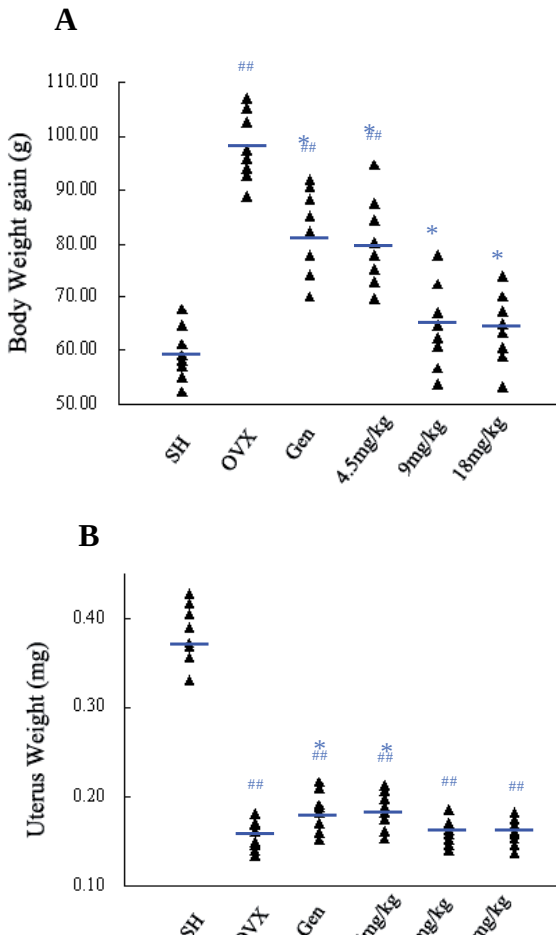


Figure 5

Body and uterus weight changes of OVX rats after 12 weeks of drugs treatment. A). Body weight gain; B). Changes of uterus weight.
OVX causes rapid weight gain. This tendency was inhibited by all drugs donation, especially NO-G at 9 and 18 mg/kg doses (61.5% and 62.6% of OVX, P <0.01). These two doses have no effects on uterus weight (116.9% and 114.3% of OVX group, P >0.05). Data are expressed as the Means with SEM of 8 rats. *p <0.05, **p <0.01 vs OVX; #p <0.05, ##p <0.01 vs SH. (SH: Sham-operated; OVX: OVX control; Gen: genistein)

Body and uterine weights

Whilst there were no significant differences between the body weights of each group at the beginning of the study, comparison of the animals' mean body weights immediately before necropsy showed a significant weight gain in OVX, OVX+genistein and OVX+4.5 mg/kg NO-G groups (P <0.05 or 0.01 vs. SH), but have no differences between SH and NO-G in the 9 or 18 mg/kg groups. This showed that NO-G at 9 or 18 mg/kg may reverse OVX induced significant body weight gain (fig. 5A).

OVX caused atrophy of uterus (40.1% of SH group, P <0.01). In our study, after 12w treatment, genistein and 4.5 mg/kg NO-G slightly increased the weight of uterus (23.4%, 26.6% heavier than OVX group, P <0.05), while NO-G at 9 or 18 mg/kg seems to have no effect on uterus (fig. 5B).

We therefore believe that the administration of NO-G at 9 or 18 mg/kg did not have any adverse effects on body and uterus weights in a 12-week period.

Discussion

NO-G is a newly synthesized prodrug intended to replace hormone replacement therapy in the prevention and treatment of osteoporosis. It binds genistein and NO together in order to improve the therapeutic effect and to avoid some possible side effects. The OVX rat model mimics changes in bone metabolism, so we used this model to evaluate the effects of NO-G on prevention and treatment of postmenopausal osteoporosis in comparison to the parent drug genistein.

Bone loss associated with increased bone turnover is a well-known consequence of OVX. In our experimental conditions, after 12 weeks of treatment, NO-G (9 and 18 mg/kg) was able to prevent this effect much better than the parent drug genistein, as shown by the values for femoral and tibial BMD (fig. 1) and the mechanical strength of the femur (fig. 2).

b-ALP and OCN were increased by OVX as a result of compensation. After treatment with the drugs, the bone turnover trended to normal level, so b-ALP and OCN were somewhat decreased. Our previous study shows a proliferation effect of NO-G on osteoblasts [21], but this was not in accordance with the in vivo experiment. This contradiction suggests that there are other mechanisms behind the bone-sparing effect of NO-G.

DPD is a product of collagen breakdown and is primarily excreted in urine. It reflects the activity of bone resorption. OVX increases bone resorption activity, drug application reverses this effect. It shows that NO-G may also inhibit bone resorption.

OVX significantly increased body weight gain and caused uterus atrophy. At the end of our experiment, NO-G at the 9 and 18 mg/kg groups showed no stimulatory effects on body and uterine weight gain indicating that NO-G may has no side effects on body weight and uterus atrophy.

In conclusion, the present study shows that daily NO-G consumption over a three month period in OVX rats may have bone-sparing effects by improving BMD and bone strength and inhibiting bone resorption. In the aforementioned results, it is shown that this prodrug contributes significantly to the prevention and treatment of the development of bone loss induced by OVX in rats without any effects on the uterus, an important factor in the treatment of osteoporosis.

This new prodrug consists of a NO-donating moiety coupled to genistein via chemical linkers. In the presence of cells or tissues, NO is released with slow kinetics and mimics the physiological levels of NO produced by constitutive NO synthase [21, 22]. Thanks to NO release, NO-G has improved pharmacological effects compared to its

parent drug genistein. Thus, although further data are required, NO-G might be a potential alternative for HRT.

The effect of low dose NO-G (4.5 mg/kg) on preventing bone loss was not very significant. This may be due to the failure of the lower dose to improve the pathological state induced by OVX. This indicates that the effect of NO-G is dose-dependent.

Additionally, the menopause is often accompanied by hot flushes and cardiovascular diseases, under which most climacteric women suffer. Most alternatives to HRT only treat a single problem and so women with multiple symptoms may need to try several approaches. Using this NO-G prodrug, we speculate that the liberated free NO may also be beneficial in the alleviation of these climacteric syndromes. This requires further studies.

Further analytical studies of this new compound are needed. We believe that NO-G may have the potential for further development as an effective anti-osteoporosis drug and we speculate that it may be used either alone or in conjunction with oestrogen.

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