

Research Article



Effect of D-003 and Soy Standardized Extract Rich in Daidzein on Osteoporosis Induced in Ovariectomized Rats - A Comparative Study.

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ABSTRACT

D-003 is a mixture of higher fatty acids purified from sugarcane wax with antiosteoporotic effects in ovariectomized (ovx) rats. Soy standardized extract rich in daidzein (SSE-D) have shown bone-protective effects. This study compared the effect of D-003 and SSE-D on bones of ovx rats. Rats were randomized into 6 groups: one false-operated and five ovx groups: a positive control treated orally with the vehicle, two with D-003 (50 and 200 mg/kg) and two with SSE-D (15 and 30 mg/kg) for 3 months. Ovariectomy decreased trabecular volume, number and thickness of trabeculae in the fifth vertebrae and femoral neck, and increased trabecular separation, osteoclast number and surface versus the false-operated group. D-003 and SSE-D prevented all changes induced by ovariectomy, but the effects of D-003 were significantly greater than those of SSE-D. Concluding, D-003 (50 and 200 mg/kg) and SSE-D (15 and 30 mg/kg) prevented osteoporotic changes in ovx rats, but D-003 was more effective than the SSE-D preparation assayed.

Keywords: Bones, D-003, histomorphometry, soy standardized extract, osteoporosis, ovariectomized rats.

INTRODUCTION

Osteoporosis (OP) is defined as a systemic disease in which reduced bone mass and impairment of bone microarchitecture lead to bone fragility and fracture risk in the presence of minimal trauma. Often presenting as a silent disease, it generally occurs asymptotically and, consequently, the afflicted individuals will only be diagnosed after the occurrence of fractures, being with a major frequency on hip, vertebrae and wrist. Adverse effects produced by bone fractures are associated with increase morbidity, mortality and health costs depending on fractures types, being a major increasing population health problem. By improving the public health and aging of the population, the incidence of this disorder is also increasing.¹

OP develops in both sexes, but postmenopausal women have an increased compared with men of same age due to estrogen deficiency, so, approximately 35% of women develop the clinical manifestation of the disease,²⁻⁴ which occur 15-20 years later menopause.³ The frequency increases with age in men, tending to be as in women.^{2,3}

OP involves an imbalance of bone remodelling, in which increased bone resorption exceeds bone formation.⁵ The prevention and treatment involves healthy lifestyle measures, like adequate daily intake of calcium/Vitamin D, physical activity, moderate sun exposition, stop smoking and reduced alcoholic intake. In addition, pharmacological intervention with antiosteoporotic drugs (antiresorptive, anabolic and more recently, dual action agents)⁶ is recommended for subjects at risk of OP.

The metabolic pathway from mevalonate to cholesterol is essential for osteoclasts activity and bone resorption, since it renders the intermediate isoprenoids lipids (farnesyl and geranylgeranyl diphosphates) required for

the farnesylation and geranylgeranylation of the small GTPase signalling proteins necessary for osteoclasts function⁷. A proof of the relevance of this pathway for bone resorption comes from the mode of action of Nitrogen-containing bisphosphonates (N-BP) (alendronate, risedronate, ibandronate, pamidronate, zoledronate), the mainstay of osteoporosis therapy⁸. N-BP bind to the bone surface and inhibit the farnesyl pyrophosphate synthase enzyme reducing the isoprenoids required for the prenylation of GTPases, essential step for forming the ruffled border involved in osteoclasts activity, increasing osteoclasts apoptosis and bone resorption.^{7,9-14}

In general, N-BP are well tolerated, drug-related adverse events (AE) have been documented. Gastrointestinal side effects (dysphagia, esophagitis, esophageal or gastric ulcers) are the most frequently AE linked with oral N-BP, while intravenous (iv) N-BP are not associated with these symptoms, but can produce mild headache, myalgias, arthralgias, fever and flu-like symptoms.¹⁵ In addition, a greater risk of serious atrial fibrillation with alendronate¹⁶ and iv zoledronic acid¹⁷ versus placebo have been reported recently. Also, non-healing ulceration of the jaw, usually following invasive dental procedures, and osteonecrosis of the jaw in oncology patients, postmenopausal OP or in individuals with Paget disease have been linked with N-BP, mainly with alendronate¹⁸ perhaps due to the greater number of patients receiving alendronate and the longer time of this drug on the market.

By other hand, increased lipid peroxidation (LP) also predisposes to OP by stimulating the differentiation of osteoblastic precursors in adipocytes (not in osteoblasts) in the bone, which leads to insufficient bone formation, or by increasing the osteoclasts number in bone.¹⁹



Consequently, antioxidants from natural origin, like Vitamin E and ipriflavone, have shown to prevent bone loss by inhibiting bone resorption or by increasing bone formation, respectively.^{20, 21} These agents however, are not currently recommended to prevent or treat OP.

D-003 is a mixture of higher molecular weight aliphatic primary acids purified from sugarcane wax, wherein octacosanoic (C₂₈), triacontanoic (C₃₀), dotriacontanoic (C₃₂), and tetratriacontanoic (C₃₄) acids are the most abundant and C₂₄ - C₂₇, C₂₉, C₃₁, C₃₃, C₃₅ and C₃₆ acids are at lower concentrations.²² D-003 inhibits cholesterol synthesis prior to mevalonate formation by regulating HMG-CoA reductase activity²³ and have shown to reduce LP in experimental and clinical studies.²⁴⁻²⁷ D-003 (5 – 200 mg/kg) orally given has shown to prevent, in a dose-dependent and persistent manner, the increase of bone loss and bone resorption in ovx rats²⁸⁻³¹ and corticoid-induced osteoporosis in rats,³² increasing osteoclast apoptosis. Also, D-003 (10 mg/day) for 6 months reduced the urinary excretion of deoxypyridinoline (DPD)/creatinine, a bone resorption marker, in postmenopausal women with low bone mineral density (BMD)³³ and given for 3 years produced significant increases in BMD values in the lumbar spine in this population.³⁴

Phytoestrogens are a large family of plant-derived estrogens possessing significant estrogen agonist/antagonist activity with low toxicity. These naturally occurring molecules, include the isoflavonoids (IF) (genistein, daidzein, glycitein, equol and biochanin A), the lignans, the coumestanes, the stilbens (resveratrol) and the flavonoids (quercetin and kaempferol).^{35,36}

It has been demonstrated that Daidzein administration prevents ovx-induced decrease in BMD and bone mechanical strength, and has a moderate protective effect on the microarchitecture of trabecular bone in aged Sprague-Dawley rats.³⁷

Nevertheless, several animal and *in vitro* studies indicate that phytoestrogens, mainly daidzein and genistein, prevent bone loss, human studies, however, which are of short duration (6 months) and include a small number of subjects, have demonstrated that phytoestrogens exert only moderately beneficial effects on bone.^{38,39} Based on data from the EU-funded project derived of plants (phytohealth), there is a suggestion, but no conclusive evidence, that phytoestrogens, primarily genistein and daidzein, given as soyabean-protein isolates, whole-soyabean foods or extracts, supplements or pure compounds, have a beneficial effect on bone health.^{40,41} However, it is considered that long-lasting, well-designed clinical trials are needed to prove a specific role of phytoestrogens in the prevention of osteoporosis.

In addition, the incidence of fracture has been analyzed among 24,403 post-menopausal Chinese women who underwent a soy protein or isoflavone diet for a 4.5-year period. The result showed a significant linear negative

association between soy protein or isoflavone consumption (≥ 21 mg daily) and fracture risk.⁴¹

Since OP is a chronic and continuous disease, long-term safety of treatments is crucial and the search for new effective and safer options is justified.

In light of this background, the aim of this study was to compare the effects of D-003 and SSE-D on bone loss and bone resorption in ovx rats.

MATERIALS AND METHODS

Animals

Three-month-old female Sprague-Dawley rats (225 ± 20 g) were obtained from the National Centre for Laboratory Animals Production (CENPALAB, Havana, Cuba). Animals were adapted to laboratory conditions (temperature 20-25°C, relative humidity 60 ± 10%, 12 hours light/dark cycle) for 7 days. Food and water were freely supplied. The study was conducted according to the Cuban guidelines for the care of laboratory animals and the Cuban Code of Good Laboratory Practice. An independent ethical board approved the use of rats and the study protocol.

Administration and dosage

D-003 (IF(D)47) was obtained from Production Plant of Natural Products (CNIC, Havana, Cuba), after corroborating its quality specifications. SSE-D (Batch A03736A; Arkopharma, EU), was utilized as comparison substance. D-003 and SSE-D were prepared in suspensions in Tween 20/H₂O (2%) weekly, adjusting the concentrations according to the bodyweight gain.

Body weight

Body weight was recorded weekly throughout the study.

Osteoporosis induction

The animal model of ovx rats was established as previously described. Briefly, rats were intraperitoneally anaesthetized with 7% chloral hydrate (0.4 mL/100 g body weight). Bilateral ovariectomy was performed via a dorsal approach with a small midline dorsal skin incision. Rats were randomized into 6 groups: a false-operated (sham or negative control) and five groups of ovx rats: one group treated orally with the vehicle (positive control) and other four groups with D-003 (50 and 200 mg/kg) and SSE-D (15 and 30 mg/kg). Treatments were administered orally by gastric gavage (5-10 mL/kg), once a day (5-6 days/week) for 3 months, starting from the next day after ovariectomy. The dose of D-003 selected had shown to prevent bone loss and bone resorption in ovx rats and in rats with prednisolone-induced osteoporosis,²⁸⁻³² while SSE-D doses had demonstrated to produce bone protective effects in rats.³⁷ After 3 months of treatment, the rats were euthanized by exsanguination under sodium pentobarbital anaesthesia.⁴²



Microscopic studies

Treatment effects were assessed through microscopic and morphometric studies. The right femur and fifth lumbar vertebrae were removed for the morphological study. The specimens were fixed in 10% neutral-buffered formalin, decalcified in 5% formic acid (Sigma) during 5 days.⁴³ The right femur was cut through the intertrochanteric line to create a wide and flat base for proper positioning of the femoral neck before embedding; as described,⁴⁴ dehydrated in graded ethanol, and then embedded in paraffin, sectioned and stained with haematoxylin and eosin.

Histomorphometric study

Analysis was performed by an investigator blinded to the treatment groups. Morphometry was conducted as described⁴⁵ (Parfitt et al 1987). Histomorphometric changes in structure, such as trabecular number (TbN, #/mm), thickness (TbTh, μm), and separation (TbSp, μm), osteoclast number (OcN) and surface (OcS/BS) were the primary efficacy variables. Values of histomorphometric variables were derived from primary measurements of areas and perimeters. Histomorphometric analysis was conducted using an image analysis system.

Statistical analysis

Comparisons between groups were done using the two-side Mann-Whitney U test, while those of body weight were analysed by a variance analysis and the posterior media comparisons. An $\alpha = 0.05$ was a priori selected for the statistical significance. Statistical analysis was performed using the software Statistics for Windows (Kernel release 5.1, Statsoft, Inc.1998, Tulsa, OK, USA).

RESULTS AND DISCUSSION

Table 1 summarizes the effects on the histomorphometric variables. Ovx rats showed reduced values of TbN and TbTh and increased TbSp compared with the sham group ($p < 0.001$) in the two bone regions studied.

Treatment with D-003 significantly prevented ($p < 0.001$ vs positive control) the reduction on TbTh and TbN and the increase on TbSp induced by ovariectomy without consistent differences between doses (50 and 200 mg/kg) being these effects remarkable. Also, SSE-D significantly prevented ($p < 0.001$ vs positive control) the reduction on TbTh and TbN and the increase on TbSp. Effects of EES-D 30 mg/kg were similar to those of 15 mg/kg in femoral neck, meanwhile in 5th vertebrae only in TbSp appears this difference.

Effects of D-003 50 and 200 mg/kg were significantly higher than SSE-D 15 and 30 mg/kg, in the prevention of the reduction of TbTh and TbSp in both studied structures, but in TbN results are inconsistent, being higher than SSE-D 30 mg/kg but not than 15 mg/kg in 5th vertebrae, meanwhile in femoral neck both doses of SSE-D were better than D-003.

Table 2 shows data of bone resorption markers. Both OcN and OcS/BS increased significantly in the positive controls ($p < 0.001$) with regards to the sham group, an effect prevented significantly by D-003 (50 and 200 mg/kg) ($p < 0.001$ versus the positive control group for all comparisons) and by SSE-D ($p < 0.01$ for all comparisons). Bodyweight gain was unaffected by D-003 or SSE-D treatments compared with control groups (data not shown).

Table 1: Effects of D-003 and SSE-D on the trabecular bone of ovx rats: morphometric study ($X \pm DS$).

	TbTh(μm)	% I	TbN (#/mm)	% I	TbSp (μm)	% I
T5th lumbar vertebrae b						
- Control	85.30 \pm 0.37 ^{***}		3.42 \pm 0.07 ^{***}		217.32 \pm 4.2 ^{***}	
+Control	71.93 \pm 1.09		2.15 \pm 0.21		262.22 \pm 12.23	
D003-50 mg/kg	80.18 \pm 0.89 ^{***ab}	61,71	3.50 \pm 0.15 ^{***bb}	100	214.65 \pm 1.59 ^{***aabbb}	100
D003- 200 mg/kg	80.70 \pm 0.50 ^{***aabbb}	65,59	3.42 \pm 0.17 ^{***b}	100	215.05 \pm 4.00 ^{***aabbb}	100
SSE-D-15 mg/kg	77.58 \pm 1.55 ^{***}	42,26	3.25 \pm 0.26 ^{***}	86,61	241.97 \pm 2.43 ^{***}	45,10
SSE-D-30 mg/kg	78.65 \pm 1.35 ^{***}	50,26	3.22 \pm 0.13 ^{***}	84,25	232.65 \pm 1.49 ^{***ddd}	65,86
Femoral neck						
-Control	82.85 \pm 0.66 ^{***}		6.62 \pm 0.39 ^{***}		190,27 \pm 0.88 ^{***}	
+Control	54.80 \pm 0.77		4.35 \pm 0.28		343,10 \pm 1.59	
D003- 50 mg/kg	76.15 \pm 1.48 ^{***aabbb}	76,11	5.25 \pm 0.21 ^{***aabbb}	39,65	212,35 \pm 2.40 ^{***aabbb}	100
D003- 200 mg/kg	77.20 \pm 0.75 ^{***aabbb}	79,86	5.92 \pm 0.21 ^{***bc}	69,16	201,30 \pm 1.08 ^{***aabbbc}	100
SSE-D-15 mg/kg	68.55 \pm 0.95 ^{***}	49,02	5.97 \pm 0.17 ^{***}	71,37	307,62 \pm 4.34 ^{***}	23,22
SSE-D--30 mg/kg	70.75 \pm 1.26 ^{***dd}	56,86	6.25 \pm 0.18 ^{***d}	83,70	234,97 \pm 8.01 ^{***ddd}	70,75

Trabecular thickness (TbTh), number (TbN) and separation (TbSp); ^{***} $p < 0.001$ Comparison versus positive control group. (Mann-Whitney U test); ^a $p < 0.01$; ^{aa} $p < 0.001$. Comparisons vs EESD-15 mg/kg (Mann-Whitney U test); ^b $p < 0.05$; ^{bb} $p < 0.01$; ^{bbb} $p < 0.001$. Comparisons vs EESD-30 mg/kg (Mann-Whitney U test); ^c $p < 0.001$. Comparisons D003-50 vs D003-200 mg/kg (Mann-Whitney U test); ^d $p < 0.05$; ^{dd} $p < 0.01$; ^{ddd} $p < 0.001$. Comparisons EESD-15 vs EESD-30 mg/kg (Mann-Whitney U test)



Table 2: Effects of D-003 and SSE-D on bones from ovx rats: bone resorption parameters (X ± DS)

[Efectos del D-003 y el extracto estandarizado de soya-diadzeina sobre parámetros resorptivos]

Groups	OcN (#/mm)	%I	OcS/BS (%)	%I
5th lumbar vertebrae				
Control	0.26 ± 0.01 ^{***}		1.15 ± 0.02 ^{***}	
+Control	0.46 ± 0.01		1.53 ± 0.03	
D003-50 mg/kg	0.29 ± 0.01 ^{***aaabb}	85,00	1.19 ± 0.02 ^{***aab}	89,47
D003- 200 mg/kg	0.27 ± 0.01 ^{***aaabbbc}	95,00	1.16 ± 0.02 ^{***aaabbbc}	97,37
SSE-D-15 mg/kg	0.30 ± 0.01 ^{***}	80,00	1.22 ± 0.02 ^{***}	81,58
SSE-D -30 mg/kg	0.30 ± 0.01 ^{***}	80,00	1.21 ± 0.02 [*]	84,21
Femoral neck				
-Control -	0.44 ± 0.01 ^{***}		5.61 ± 0.14 ^{***}	
+Control	0.71 ± 0.01		9.12 ± 0.08	
D003- 50 mg/kg	0.46 ± 0.01 ^{***aaabb}	92,59	6.16 ± 0.06 ^{***aaabb}	84,33
D003- 200 mg/kg	0.45 ± 0.01 ^{***aaabbbc}	96,30	5.92 ± 0.11 ^{***aaabbbc}	91,17
SSE-D -15 mg/kg	0.51 ± 0.01 ^{***}	74,07	6.52 ± 0.11 ^{***}	74,07
SSE-D -30 mg/kg	0.48 ± 0.01 ^{***dd}	85,19	6.31 ± 0.10 ^{***dd}	80,06

Osteoclast number (OcN), Osteoclast surface/bone (OcS/BS); ^{***}p< 0.001 Comparison versus positive control group. (Mann-Whitney U test); ^ap<0.05; ^{aa}p<0.01; ^{aaa}p<0.001. Comparación vs EESD-15 mg/kg (Mann-Whitney U test); ^bp<0.05; ^{bb}p<0.01; ^{bbb}p<0.001. Comparación vs EESD-30 mg/kg (Mann-Whitney U test); ^cp<0.001. Comparisons D003-50 vs D003-200 mg/kg (Mann-Whitney U test); ^dp<0.05; ^{dd}p<0.01; ^{ddd}p<0.001. Comparisons EESD-15 vs EESD-30 mg/kg (Mann-Whitney U test)

This study shows that D-003 (50 and 200 mg/kg) and SSE-D (15 and 30 mg/kg) orally administered for 3 months to ovx rats reduced the increased bone loss and bone resorption induced by ovx and at the doses tested. Overall, D-003 was more effective than SSE-D for preventing both the ovx-induced changes of histomorphometric and resorption variables in the two bone regions analyzed.

D-003 and SSE-D administration improves trabecular architecture as it increases TbTh. In addition, both treatments seem to partially restore trabecular connectivity by increasing TbN and reducing TbSp.

The demonstration of these effects on the ovx rat model is remarkable, since this model mimics the increased trabecular bone loss and resorption occurring in postmenopausal women⁴⁶⁻⁴⁸ and the measurement of trabecular bone loss in ovx rats using histomorphometric methods able to assess bone microarchitectural parameters has become the standard model to determine the efficacy of potential anti-osteoporotic treatments.⁴⁶⁻⁴⁸

The bone protective effects of D-003 on histomorphometric and resorption variables of ovx rats are coherent with the inhibition of cholesterol synthesis prior to mevalonate formation induced by the regulatory effect of D-003 on HMGCoA reductase activity²³ seen with a similar regimen of D-003, which were related with an increase of osteoclasts apoptosis^{29,30} a pivotal process for bone resorption in this model. Similar mechanism occurs with bisphosphonates⁹ and statins.⁴⁹

Since D-003 displays both cholesterol lowering and antiresorptive effects, like hormone replacement therapy

does⁵⁰ and it is well known the undesirable side effects and cautions, like the increased risk of uterine and breast cancer,^{51,52} our group studied the effects of D-003 in the uterotrophic assay orally given at 50 mg/kg, a dose able to prevent bone loss and bone resorption in ovx rats, and did not display *in vivo* estrogenic/antiestrogenic activity,⁵³ which agrees with the inhibition of OP in rats fed elicited by a sugar cane wax enriched diet, restricted in carbohydrate and oil, normal in protein, achieved through a non estrogenic mechanism.⁵⁴

Moreover, the inhibition of LP produced with D-003²⁴⁻²⁷ could also contribute to the present results, since increased lipogenesis, hypercholesterolemia and lipid oxidation predisposes to OP development.¹⁹

No impairment of bone quality was observed in treated groups compared with positive and negative controls, consistent with the negative results of short and long-term toxicity studies in the rat,⁵⁵⁻⁵⁷ showing no D-003-related toxicity, even in the bone.

Several studies indicate that phytoestrogens, mainly IF as daidzein and genistein, prevent bone loss in experimental models, including ovx rats,⁵⁸ however, human studies, which are of short duration (6 months) and include a small number of subjects, have demonstrated that phytoestrogens exert only moderately beneficial effects on bone.^{38,39}

Although its mechanism of action on bone is not yet fully understood, it is likely that the positive bone effects of IF are related with its estrogenic activity and the antioxidant property and suggest that IF might be a new potential

therapy for the management of postmenopausal osteoporosis in humans.⁵⁹

In this study SSE-D (15 and 30 mg/kg) attenuated significantly the changes of histomorphometric and resorption markers induced with ovariectomy in rats. One likely reason for this improvement in bone markers could be attributed to daidzein antioxidant effects revealed by Jiang et al, (2007)⁶⁰ which could have contributed to the preventive effects of SSE-D in the OP induced by ovx. As it has been demonstrated free radicals intervene in bone resorption, promoting osteoclastic differentiation in such a manner that bone resorption is increased with oxidative stress.⁶¹ In our model, the increased oxidative stress could be attributed to the loss of the antioxidant effects of estrogen.⁶² Enhanced osteoclastic activity/number detected in our study in the positive control group may have been responsible for increased production of reactive oxygen species [ROS]. One the most important damaging effects of ROS on tissues is lipid peroxidation. Nevertheless, we did not measured malondialdehyde (MDA), so, it is only a possible explanation for these results and is not on the scope of the study.

As daidzein has estrogenic properties it has been studied the toxicity of the female reproductive tract and authors concluded that supraphysiologic concentrations of daidzein administered via the diet did not cause significant toxicity to the female reproductive tract in rats.⁶³

CONCLUSION

D-003 (50 and 200 mg/kg) and SSE-D (15 and 30 mg/kg) orally administered for 3 months prevented bone loss and bone resorption in the ovx rat, being the effects of D-003 moderately greater than those of SSE-D.

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