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Effects Of D-003, a Mixture of High-Molecular Weight Aliphatic Acids from Sugarcane Wax, and Omega-3 Fatty Acids on Bones of Ovariectomized Rats

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SUMMARY. D-003 is a mixture of higher fatty acids purified from sugarcane wax with antiosteoporotic effects in ovariectomized (ovx) rats. Omega-3 fatty acids (Ω-3FA) have shown bone-protective effects. This study compared the effect of D-003 and Ω-3 FA on bones of ovx rats. Rats were randomized into 4 groups: one false-operated and three ovx groups: a positive control treated orally with the vehicle, one with D-003 (50 mg/kg) and other with Ω-3FA (160 mg/kg) for 3 months. Ovariectomy decreased trabecular volume, number and thickness in the fifth vertebrae, distal femur and femur neck, and increased trabecular separation, osteoclast number and surface *versus* the false-operated group. D-003 and Ω-3 FA prevented all changes induced by ovariectomy, but the effects of D-003 were significantly greater than those of Ω-3FA. Concluding, D-003 (50 mg/kg) and Ω-3FA (160 mg/kg) prevented osteoporotic changes in ovx rats, but D-003 was more effective than the Ω-3FA preparation assayed.

INTRODUCTION

Osteoporosis is a continuum process, in which multiple mechanisms converge to cause loss of bone mass and microarchitectural deterioration of skeletal structure, which leads to increased fracture risk [1]. Osteoporosis develops in both sexes, but estrogen-deficient postmenopausal women have an increased risk compared with men of same age [1]. Osteoporosis involves an imbalance of bone remodelling, in which increased bone resorption exceeds bone formation [2].

Osteoporosis 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) is recommended for subjects at risk of osteoporosis [3].

The metabolic pathway from mevalonate to cholesterol is essential for osteoclast 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 function [4]. 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 therapy [5]. 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 osteoclast activity, increasing osteoclast apoptosis and bone resorption [4,6-11].

In general, N-BP are well tolerated, although drug-related adverse events (AE) have been documented. Gastrointestinal side effects (dysphagia, esophagitis, esophageal or gastric ul-

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cers) are the most frequently AE linked with oral N-BP. On the other hand, intravenous (iv) N-BP are not associated with these symptoms, but can produce mild headache, myalgias, arthralgias, fever and flu-like symptoms [12]. In addition, a greater risk of serious atrial fibrillation with alendronate [13] and iv zoledronic acid [14] *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 osteoporosis or in individuals with Paget disease have been linked with N-BP, mainly with alendronate [15] perhaps due to the greater number of patients receiving alendronate and the longer time of this drug on the market. Since osteoporosis is a chronic and continuous disease, long-term safety of treatments is crucial and the search for new effective and safer options is justified.

Increased lipid peroxidation (LP) also predisposes to osteoporosis 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 [16]. 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 [17,18]. These agents, however, are not currently recommended to prevent or treat osteoporosis.

D-003 is a mixture of higher aliphatic primary acids purified from sugarcane wax, wherein octacosanoic, triacontanoic, dotriacontanoic, and tetratriacontanoic acids are the most abundant and C_{24} - C_{27}, C_{29}, C_{31}, C_{33}, C_{35} and C_{36} acids are at lower concentrations [19]. D-003 inhibits cholesterol synthesis prior to mevalonate formation by regulating HMG-CoA reductase activity [20] and has shown to reduce LP in experimental and clinical studies [21,22]. 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 ovariectomized (ovx) rats [23-26] and corticoid-induced osteoporosis in rats [27], 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 [28].

In turn, experimental studies have demonstrated that Omega 3 fatty acids (Ω-3FA) may produce beneficial effects on bone cells. Ω-3 FA added to diet increased bone formation rates in growing rats and reduced bone mineral loss in ovx rats. Osteoprotective effects of Ω-3FA appear to involve improved osteoblast function [29] and to reduce osteoclastic activity and alveolar bone resorption [30]. Dietary supplementation of eicosapentaenoic acid (EPA) influence both bone formation and bone resorption in normal and ovx rats [31]. Epidemiological data suggest promising applications of Ω-3FA on osteoporosis management [32], but direct evidence of any beneficial effect of Ω-3FA on human osteoporosis is still lacking.

In light of this background, this study compared the effects of D-003 and fish oil Ω-3FA 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 21 °C, humidity 55%, 12 hour light/dark cycles) for two weeks, with free access to food (rodent chow from CENPALAB) and water.

Animal handle was conducted according to the Cuban ethical regulations for the use of laboratory animals, and study conduction was consistent with the approved protocol.

Rats were ovx bilaterally or sham operated under anaesthesia with sodium pentobarbital (50 mg/kg iv).

Administration and dosage

D-003 was obtained from the Chemistry Department of the Centre of Natural Products (Havana City, Cuba), after corroborating its quality specifications.

For dosing, D-003 (Plant of Natural Products, Havana City, Cuba) suspensions in a Tween/water vehicle were prepared weekly, adjusting the concentrations according to the bodyweight gain. Ω-3 FA (Rainbow Ltd., Australia) was took out from the capsules for administration [31]. 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. Rats were randomized into 4 groups (10 rats each): a false-operated (sham or negative control) and three groups of ovx rats: one group treated orally with the vehicle (positive control) and other two groups with D-003 (50 mg/kg) and Ω-3 FA (160 mg/kg). The

dose of D-003 selected had shown to prevent bone loss and bone resorption in ovx rats [23-26] and in rats with prednisolone-induced osteoporosis [27], while Ω-3FA dose had demonstrated to produce bone protective effects in ovx rats [31].

Body weight

Body weight was recorded weekly throughout the study.

Microscopic studies

At study completion, rats were sacrificed under ether anaesthesia. Treatment effects were assessed through microscopic and morphometric studies. The right femur and fifth lumbar vertebrae were removed for the morphological study, and the following specimens were obtained from each animal: fifth lumbar vertebral body, femoral neck and distal femur. 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, while the distal femur was taken at the second 0.5 cm from the distal end of the femur [33]. The specimens were processed as reported, bones were decalcified in 0.5M disodium ethylenediaminetetraacetic acid (EDTA, pH 7.4) at 4 °C for four weeks, embedded in paraffin, sectioned and stained with haematoxylin and eosin [33].

Histomorphometric study

Morphometry was conducted as described by Parfitt *et al.* [34]. Histomorphometric changes in trabecular bone volume (TBV) and structure, such as trabecular number (Tb.N, #/#mm), thickness (Tb Th, µm), and separation (Tb Sp, 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. The calculation related to trabecular bone volume (TBV) for estimation of bone mass was performed considering BV/TV x 100, where BV is the trabecular bone area and TV the total area, as described [34]. Histomorphometric analysis was conducted using an image analysis system.

Statistical analyses

Comparisons between groups were done using the two-side Mann-Whitney U test. An α= 0.05 was *a priori* selected for the statistical significance. Statistical analyses were performed using the software Statistics for Windows (Kernel release 5.1, Statsoft, Inc.1998, Tulsa, OK, USA).

RESULTS

Ovariectomy significantly decreased the values of trabecular bone volume (TBV) when compared with sham values in the fifth vertebrae, femoral neck and distal femur. D-003 (50 mg/kg) significantly ($p<0.001$) reduced the ovariectomy-induced reduction of TBV in the three bone regions, while Ω-3FA significantly prevented such decrease in the fifth vertebrae and the femoral neck, but not in the distal femur (Table 1).

Table 2 summarizes the effects on the histomorphometric variables. Ovx rats showed reduced values of TbN and TbTh and increased TbSp compared with the sham group ($p <0.001$ *vs.* the positive control group) in the three bone regions studied, all of which were significantly prevented by D-003 (50 mg/kg) ($p <0.001$ *vs.* the positive control group). Ω-3FA reduced significantly ($p <0.01$) the histomorphometric changes induced by ovariectomy, except for the reduction of TbN, which was not significantly different from that of ovx rats.

Table 3 lists the bone resorption data. 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 mg/kg) ($p <0.001$ *versus* the positive control group for all comparisons) and by Ω-3 FA ($p<0.01$ for all comparisons).

Group	Doses (mg/kg)	5th Lumbar vertebrae	Femoral neck	Distal femur
Sham	-	33.17 ± 0.9	16.60 ± 0.65	21.07 ± 0.81
Control ovx	0	21.19 ± 1.33 +	7.91 ± 0.32 +	11.72 ± 0.57 +
D-003	50	30.46 ± 1.29 *	14.13 ± 0.62 *	18.38 ± 0.67 *
Ω-3 FA	160	22.57 ±3.03 *aa	9.81 ± 0.85 *aa	12.64 ± 1.44

Table 1. Effects of D-003 and omega-3 fatty acids (Ω-3 FA) on trabecular bone volume in bones of ovx rats + $p< 0.001$ Comparison *versus* sham group, *$p< 0.001$ Comparison *versus* positive control group. [a]: $p < 0.01$, [aa] $p < 0.001$. Comparison *versus* D003 group (Mann-Whitney U test).

Group	Doses	Tb thickness (µm)	# Tb (No/mm)	Tb separation (µm)
Femoral neck				
Sham	0	83.38 ± 0.79 *	7.29 ± 0.12 *	191.8 ± 3.42 *
Control ovx	0	55.50 ± 1.46 +	4.54 ± 0.17 +	353.8 ± 2.88 +
D-003	50 mg/kg	79.92 ± 1.48 *	6.71 ± 0.12 *	207.0 ± 4.70 *
Ω-3 FA	160 mg/kg	61.29 ± 0.77 *a	4.79 ± 0.17 a	308.9 ± 5.20 *aa
Distal fémur				
Sham	0	93.54 ± 0.79 *	1.37 ± 0.21 *	202.5 ± 1.51 *
Control ovx	0	74.58 ± 0.68 +	0.62 ± 0.21 +	371.4 ± 2.09 +
D-003	50 mg/kg	92.46 ± 0.99 *	1.29 ± 0.37 aa	205.2 ± 3.18 *
Ω-3 FA	160 mg/kg	78.92 ± 2.29 *a	0.71 ± 0.11	348.2 ± 6.83 *aa
5th Vertebrae				
Sham	0	85.63 ± 1.21 *	3.54 ± 0.17 *	217.0 ± 0.72 *
Control ovx	0	73.25 ± 1.03 +	2.08 ± 0.23 +	256.4 ± 2.97 +
D-003	50 mg/kg	85.00 ± 0.25 *	3.45 ± 0.17 *	206.1 ± 1.07 *
Ω-3 FA	160 mg/kg	78.17 ± 1.74 *a	2.50 ± 0.31 a	231.5 ± 4.06 *

Table 2. Effects of D-003 and Ω-3 FA on the trabecular bone of ovx rats: morphometric study (X ± DS). + p< 0.001 Comparison *versus* sham group, *p< 0.001 Comparison *versus* positive control group. a: p < 0.01, aa p < 0.001. Comparison *versus* D003 group (Mann-Whitney U test).

Group	Doses	Oc N (number/mm)	OcS/BS (%)
Femoral neck			
Sham	0	0.43 ± 0.17 *	5.71 ± 0.26 *
Control ovx	0	0.70 ± 0.18 +	9.1 ± 0.20 +
D-003	50 mg/kg	0.42 ± 0.02 *	5.35 ± 0.22 *
±-3 FA	160 mg/kg	0.64 ± 0.02 aa	8.00 ± 0.35 * a
Distal Femur			
Sham	0	0.91± 0.02 *	4.02 ± 0.21 *
Control ovx	0	1.81 ± 0.03 +	6.46 ± 0.16 +
D-003	50 mg/kg	0.90 ± 0.02 *	3.94 ± 0.14 *
±-3 FA	160 mg/kg	1.58 ± 0.06 * a	5.81 ± 0.27 *
5th lumbar vertebrae			
Sham	0	0.27 ± 0.02 *	1.15 ± 0.12 *
Control ovx	0	0.47 ± 0.02 +	1.57 ± 0.03 +
D-003	50 mg/kg	0.23 ± 0.01 *	0.61 ± 0.02 *
Ω-3 FA	160 mg/kg	0.40 ± 0.02 *	1.36 ± 0.07 *

Table 3. Effects of D-003 and Ω-3 FA on bones from ovx rats: bone resorption parameters (X ± DS) OcN osteoclast number, OcS/BS=osteoclast surface/bone. + p< 0.001 Comparison *versus* sham group, *p< 0.001 Comparison *versus* positive control group. a: p < 0.01, aa p < 0.001. Comparison *versus* D003 group (Mann-Whitney U test).

The effects of D-003 on histomorphometric and resorption variables were significantly (p<0.01 for all comparisons) greater than those of Ω-3 FA.

Bodyweight gain was unaffected by D-003 or Ω-3 FA treatments compared with control groups (data not shown).

DISCUSSION

This study shows that D-003 and Ω-3FA (50 and 160 mg/kg, respectively) orally administered for 3 months to ovx rats reduced the increased bone loss and bone resorption induced by ovariectomy and that at the doses tested, D-003 was more effective than Ω-3FA for preventing both the ovariectomy-induced changes of histomorphometric and resorption variables in the three bone regions analyzed.

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 women [33,35-39]. The measurement of trabecular bone loss in ovx rats using histomorphometric methods to

assess bone microarchitectural parameters has became the standard model to determine the efficacy of potential anti-osteoporotic treatments [40]. The effects of D-003 (50 mg/kg) given orally for 3 months on histomorphometric and resorption variables of ovx rats are consistent with previous results seen with a similar regimen of D-003, which were related with an increase of osteoclast apoptosis [23-26], a pivotal process for bone resorption in this model. The antiresorptive effects of D-003 does not involve an estrogenic action [41] which agrees with the inhibition of osteoporosis in rats fed by a sugar cane wax enriched diet restricted in carbohydrate and oil, normal in protein, achieved through a non estrogenic mechanism [42]. In contrast, the bone protective effects of D-003 are coherent with the inhibition of cholesterol synthesis prior to mevalonate formation induced by the regulatory effect of D-003 on HMGCoA reductase activity [20]. Moreover, the inhibition of LP produced by D-003 [21,22] could also contribute to the present results, since increased lipogenesis, hypercholesterolemia and lipid oxidation predisposes to osteoporosis development [16,43].

On the other hand, this study demonstrates that Ω-3FA (160 mg/kg) significantly attenuated the changes of histomorphometric and resorption markers induced with ovariectomy in rats. Although a decrease of bone loss in ovx rats had been reported for eicosapentanoic acid, a constituent of fish oil Ω-3FA [31], no study has referred a protective effect of Ω-3FA on histomorphometric variables of ovx rats. Osteoprotective effects of Ω-3FA, however, have been referred, which seem to involve reduced osteoclast activity and bone resorption [30] and improved osteoblast function [29]. In addition, although some pro-oxidant effect of omega has argued [44], supplementation with Ω-3FA has shown beneficial effects on LP and antioxidant enzymes in type 2 diabetic patients [45], and the study of the effect of dietary Ω-3FA supplementation on *in vivo* LP and oxidant/antioxidant status of plasma in rats has shown that it may enhance resistance to free radical attack and reduce LP, supporting the notion that dietary supplements of Ω-3FA may be effective to manage diseases in which oxidant/antioxidant defence mechanisms are decelerated [46]. An antioxidant activity of Ω-3 FA could play an important role in their bone protective effects.

The potential usefulness of Ω-3FA in the treatment of degenerative bone and joint diseases has been proposed. In fact, Ω-3FA supplementation may represent an additional therapy to the traditional pharmacological treatment of chronic inflammatory rheumatic diseases due to their anti-inflammatory properties that include production of alternative eicosanoids, reduction of inflammatory cytokines, reduction of T-lymphocytes activation and reduction of catabolic enzymes activity [47].

The antiosteoporotic effects of D-003 described in this work were greater than those of Ω-3FA despite to be given at a dose (50 mg/kg) which is about three times lower than that of Ω-3FA (160 mg/kg). We cannot conclude, however, that the bone protective efficacy of D-003 in this model is superior to that of Ω-3FA, since we did not assess the effects of different doses of both substances up to reach their ceiling effects on histomorphometric an resorptive parameters, a limitation of the present study that should be explored later.

No treatment impaired bone quality compared with positive and negative controls, consistent with the lack of D-003 related toxicity in the rat [48-50], including bone tissue, and the negligible toxicity of Ω-3FA [51].

CONCLUSIONS

D-003 (50 mg/kg) and ?-3FA (160 mg/kg) orally administered for 3 months prevented bone loss and bone resorption in the ovx rat, the effects of D-003 being moderately greater than those of Ω-3FA.

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