

Effects of D-003 on Lipopolysaccharides-induced Osteonecrosis in Rabbits

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Noa, *et al.*: D-003 on LPS-induced Osteonecrosis in Rabbits

D-003, a mixture of high molecular weight acids, inhibits cholesterol synthesis prior to mevalonate and prevents osteoporosis induced by ovariectomy in rats, and both osteoporosis and osteonecrosis induced by corticoids in rats. The aim of this study was to investigate effects of D-003 on lipopolysaccharides-induced osteonecrosis in rabbits. Animals were randomized into 5 groups: a sham and four groups injected with lipopolysaccharides: one treated orally with vehicle and three with D-003 (5, 25 and 200 mg/kg, respectively) during four weeks. We assessed the effects of treatments on the incidence of osteonecrosis (number of animals with osteonecrosis lesions/animals per group), the mean numbers and areas of osteonecrosis per animal and on the mean sizes of the bone marrow fat cells. The incidence of osteonecrosis in the groups of D-003 25 and 200 mg/kg was significantly lower than in the positive controls. The reduction of osteonecrosis increased with the doses, but significant dose-dependence relationship was not achieved. D-003 significantly and dose-dependently decreased the number of osteonecrosis lesions per animal as compared to the positive controls. Likewise, the mean osteonecrosis areas in the proximal femoral and humeral bones were significantly decreased by D-003. The injection of lipopolysaccharides significantly increased the average size of bone marrow fat cells as compared to the negative controls, and such increase was significantly and markedly reduced with D-003. It is concluded that D-003 reduced the incidence, number and percent areas of osteonecrosis lesions, and the size of bone marrow fat cells, a marker of adipogenesis, in rabbits with lipopolysaccharides-induced osteonecrosis.

Key words: Bone marrow fat cells, bones, D-003, lipopolysaccharides, osteonecrosis

Osteonecrosis (ON), a bone disease characterized by the impairment of osseous blood flow, commonly affects patients in the third to fifth decade of life and may impair the quality of life of sufferers because if is not managed timely, it leads to the collapse of femur head, eventually requiring hip arthroplasty^[1].

Occurrence of ON is not related to a single precipitating event; but to a multifactorial process. ON can be post-traumatic and non-traumatic and results from both genetic predisposition and exposure to certain risk factors. The long-term use of corticosteroids, drugs commonly used to treat several inflammatory diseases, is a main risk factor for developing non-traumatic ON^[2]. Different theories of the underlying mechanisms of steroid-induced ON have been proposed, including increased size and number of bone marrow fat cells, increased

intraosseous pressure, fatty degeneration of osteocytes, fat embolism and extraosseous arterial occlusion, coagulation abnormalities and hyperlipidaemia^[3]. Animal studies have suggested that alcohol may induce adipogenesis of bone marrow stromal cells leading to ON of the femoral head^[4].

Animal models of ON, which mimic in part the features of human ON, are useful for clarifying the mechanisms of ON and to study management approaches. Indeed, models of spontaneous occurrence of ON, surgically-induced (traumatic) ON and non-traumatic ON, like corticosteroid-induced ON, have been developed^[5,6]. Also, a model of non-steroid and non-traumatic ON by use of a single- and low-dose lipopolysaccharide (LPS) injection in rabbits is useful to assess the pathogenesis of non-steroid ON in humans, and to investigate the potential value of preventive and therapeutic strategies^[7].

Currently, no drug is effective to prevent or treat ON, but bisphosphonates, lipid-lowering drugs,

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anticoagulants and vasodilators have been used due to they may reduce risk factors for ON, like lipid emboli, adipocyte hypertrophy, venous thrombosis, increased intraosseous pressure and increased bone resorption^[8-10]. Since both abnormal lipid metabolism and coagulopathy have been linked with ON, lipid-lowering agents^[11], anticoagulants and prostacyclin analogues have become strategies to treat ON^[12,13], while the use bisphosphonates is based on the reduction of osteoclasts activity^[14].

D-003 is a mixture of very long-chain aliphatic acids purified from sugarcane wax that contains C₂₄, C₂₅, C₂₆, C₂₇, C₂₈, C₂₉, C₃₀, C₃₁, C₃₂, C₃₃, C₃₄, C₃₅ and C₃₆ acids, wherein octacosanoic (C₂₈) acid is the most abundant^[15]. D-003 has been shown to inhibit cholesterol synthesis prior to mevalonate formation by regulating HMG-CoA reductase activity^[16] and to display cholesterol-lowering, antiplatelet, and antioxidant effects in experimental and clinical studies^[15].

Consistently with the inhibitory effect of D-003 on the mevalonate to cholesterol pathway^[16], oral therapy with D-003 (5–200 mg/kg) for 3 months has been shown to prevent bone loss and bone resorption in ovariectomized (ovx) rats by increasing osteoclast apoptosis^[17,18], effects persistent after 12 months of therapy^[19]. Oral treatment with D-003 (5–200 mg/kg) for 80 days prevented bone loss, bone resorption and the frequency of ON in rats treated with prednisolone^[20]. In addition, D-003 (10 mg/day) given for 6 months reduced the urinary excretion of deoxipirrylodine (DPD)/creatinine, a bone resorption marker, in postmenopausal women with low bone mineral density (BMD)^[21]. In light of this background, the aim of this study was to investigate *in vivo* effects of D-003 on LPS-induced ON in rabbits.

MATERIALS AND METHODS

Adult (defined as having the growth plate already closed) male F1 rabbits (3-3.5 kg), 30-34 weeks old, from the National Centre for Laboratory Animals Production (CENPALAB, Havana, Cuba) were adapted to laboratory conditions (temperature 21°, humidity 55%, 12 h light/dark cycles) for two weeks, with free access to food (rabbit chow from CENPALAB) and water. An independent board approved the use of the animals in the experiment, and animal handle was conducted according to the

Cuban ethical regulations for the use of laboratory animals. Study conduction was consistent with the approved protocol.

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. The identity and purity of the batch used in the study, assessed through a validated gas chromatography method^[22], was as follows (w/w): tetracosanoic (0.2%), pentacosanoic (0.4%), hexacosanoic (2.1%), heptacosanoic (2.4%), octacosanoic (36.9%), nonacosanoic (1.6%), triacontanoic (18.4%), hentriacontanoic (1.0%), dotriacontanoic (8.6%), tritriacontanoic (1.1%), tetratriacontanoic (10.6%), pentatriacontanoic (0.5%) and hexatriacontanoic (3.6%) acids. Batch purity (total content of these acids) was 87.4%.

For dosing, D-003 was suspended in a 2% Tween20/water vehicle, suspensions being prepared weekly by adjusting the concentrations to the bodyweight gain. Treatments were administered orally by gastric gavage (5 ml/kg) once a day (5-6 days/week) for four weeks.

Rabbits were randomized into 5 groups of 8 rabbits, except the negative control, composed by 6 rabbits: a sham or negative control group, not injected with LPS and treated orally with the vehicle, and four groups injected with LPS: one of them orally treated with the vehicle (positive control) and three with D-003 (5, 25 and 200 mg/kg, respectively). The lowest dose of D-003 (5 mg/kg) had been shown to lower serum cholesterol in rabbits^[15]. Also, such dose prevented bone loss and bone resorption in ovx rats^[17-19] and reduced bone loss, bone resorption and the frequency of ON in rats with corticoid-induced osteoporosis^[20].

Experimental procedure:

Body weight was recorded weekly throughout the study. The LPS used for inducing ON was extracted from a strain of *Vibrio cholerae* serology 01, biotype El Tor, serotype Ogawa and purified by gel filtration. It was kindly transfer from Vaccine Department, Biotechnology Direction, National Centre for Scientific Research (CNIC), Havana city, Cuba. LPS was reconstituted by adding 1 ml of sterile balanced salt solution. For the induction of ON, male adult rabbits were intravenously injected with a single

injection of LPS (10 µg/kg body weight), according to the procedure described by Irisa *et al.*^[8].

Microscopic studies:

At study completion, rabbits were fasted for 12 h and anesthetized with sodium pentobarbital (30 mg/kg body wt, iv) and were then killed by exsanguination via an aortectomy. Treatment effects were assessed through microscopic and morphometric studies. The femur and the humerus were removed for the morphological study, and the following specimens were obtained from each animal: whole areas of the proximal one-thirds and distal condyles of femora and humerus (total 8 regions). Since in this model ON has been reported to be present bilaterally in almost all rabbits^[8], we study only unilateral bones, choosing the right side in all animals.

The specimens were processed as reported^[23]. In brief, bones were decalcified in 0.5M disodium ethylenediaminetetraacetic acid (EDTA, pH 7.4) at 4° for four weeks, embedded in paraffin, sectioned and stained with haematoxylin and eosin. The bone samples were cut along the coronal plane in the proximal one third and axial plane in the distal part (condyle).

Histopathologic evaluation of ON:

The diagnosis of ON was determined at 4 weeks after LPS administration, as it has been documented^[8]. Whole areas of the proximal one-third and distal condyles of both the femora and humerus (totalling 8 regions) were examined histopathologically for ON. The frequency and location of ON, the size of ON areas, the number of necrotic foci and the histological features were examined.

ON was diagnosed by 2 independent researchers, who were blind to the protocol and the samples. A positive diagnosis was based on the diffuse presence of empty lacunae or pyknotic nuclei of osteocytes within the bone trabeculae, accompanied by surrounding bone marrow cell necrosis (cytolysis, karyorrhexis and/or karyolysis, fat cell necrosis with the loss of nuclei and distinct cell borders)^[8]. Rabbits with at least 1 osteonecrotic lesion in the 8 areas examined were considered to have ON, whereas those with no osteonecrotic lesions were considered ON-free. We determined both the numbers of rabbits with ON and the numbers of osteonecrotic lesions per affected rabbit (maximum 8 regions), according to Irisa *et al.*^[8].

Measurement of bone marrow fat cell sizes:

We determined the size of bone marrow fat cells with clearly defined profile in 4 randomly selected fields (up-down-left-right) of each dissected part of proximal one-thirds and distal condyles of both femora and humerus (32 fields for 8 dissected parts from each rabbit), according to Motomura *et al.*^[12]. Fat cells with necrosis were excluded.

Measurement of area percentage of ON:

For all rabbits with ON, the necrotic area was morphometrically measured in the proximal one third of either the femur or the humerus on the coronal sections at the maximal width. Necrotic rate was calculated as a percentage of necrotic area per total area examined^[8].

Statistical analyses:

Data were expressed as the mean±SD and %. The numbers of ON-positive rabbits with proximal or distal parts of the femora and humerus with ON lesions were compared using the Fisher's Exact Probability Test, the sizes of bone marrow fat cells, the numbers of ON lesions on a determined bone volume area and the area (in %) of ON were examined using the two-side Mann-Whitney U test. Statistical significance was a priori selected for $\alpha=0.05$. Dose relationships were assessed by regression analysis. Statistical analyses were performed using the software Statistics for Windows (Kernel release 5.1, Statsoft, Inc.1998, Tulsa, OK, USA).

RESULTS

Mortality:

Of 38 randomized rabbits, three (7.9%) (2 positive controls, 1 treated with D-003 200 mg/kg) died during the next 24 h after LPS injection due to circulatory disturbances.

Histopathologic features of ON:

ON was observed macroscopically as yellowish areas. Histological ON lesions demonstrated an accumulation of bone marrow cell debris (cytolysis, karyorrhexis and/or karyolysis, fat cell necrosis with the loss of nuclei and distinct cell borders), and the bone trabeculae had empty lacunae or pyknotic nuclei of osteocytes. Organized thrombi in the intraosseous small-sized arteries and arterioles were frequent in and around the necrotic tissues. Collapse could not be seen in femoral or humeral bones of LPS-treated rabbits.

Effects on the incidence of ON:

Occurrence of ON, seen in both femoral and humeral bones, was distributed mainly in the metaphyses and diaphyses and in lesser degree in epiphyses, femoral and humeral condyle as well.

The incidence of ON in the groups treated with D-003 25 mg/kg (3/8, 37.5%) and 200 mg/kg (2/8; 25%) was significantly lower ($P < 0.05$ for both doses) as compared to the positive controls (5/6; 83.3%) (Table 1). The reduction of ON was enhanced with the doses, but significant dose-dependence relationship was not achieved. Negative controls did not show ON.

Effects on the number of ON lesions and on the average necrotic extent:

Table 2 summarizes the effects on the numbers of ON lesions and on the average necrotic extent (percentage of necrotic area per total area examined) in both the femur and humerus. D-003 (5, 25 and 200 mg/kg) significantly ($P < 0.05$ for all doses) and dose-dependently decreased the numbers ON lesions as compared to the positive control group. The mean number of ON lesions in the groups treated with D-003 5, 25 and 200 mg/kg were significantly ($P < 0.05$) lower than in the positive control group.

The mean ON areas in the proximal one third of the femoral and humeral bones were significantly decreased ($P < 0.05$) by D-003 5, 25 and 200, 69.4% and 62.9%, respectively. Also, D-003 (5, 25 and 200 mg/kg) reduced significantly the percents of ON femoral areas by 81.1, 91.3 and 97.1%, respectively, whereas respective reductions of 82.3, 91.6 and 97.1% were found in the comparisons of humeral bone values ($P < 0.05$) (Table 2).

Effects on the sizes of bone marrow fat cells:

The injection of LPS significantly ($P < 0.05$) increased the average size of bone marrow fat cells as compared to the negative controls, and such increase was significantly ($P < 0.05$) and markedly reduced with D-003, so that inhibitions of 94.4% (5 mg/kg) and 100% (25 and 200 mg/kg) were obtained (Table 3).

DISCUSSION

In this study, oral treatment with D-003 (5–200 mg/kg) for 4 weeks reduced significantly the occurrence of ON, the numbers of ON lesions, percent areas of ON and the sizes of bone marrow fat

cells in rabbits with LPS-induced ON, as evidenced by significant reductions compared with the positive control group. These findings suggest that oral treatment with D-003 should prevent ON induced by low doses of LPS in rabbits.

The ON induced by a single- and low-dose of LPS injected iv in rabbits (10) is a highly reproducible model of non-traumatic-non-steroid-induced ON, characterized by the frequent development of multifocal and widespread ON lesions^[8].

The reasons leading to develop LPS-induced ON are multifactorial. As known, LPS is a constituent of the cell wall of Gram-negative bacilli that exhibit diverse biological effects, including activation of the immune system and the induction of inflammation-circulatory

TABLE 1: EFFECT OF D-003 ON THE INCIDENCE (%)^A OF ON IN LPS-INDUCED ON IN RABBITS

Groups	D003 doses (mg/kg)	Incidence (%) of ON	% Inhibition
Negative control	0	0.0 (0/6)*	
Positive control	0	83.3 (5/6)	
D-003 + LPS	5	37.5 (3/8)	55.0
D-003 + LPS	25	25.0 (2/8)*	69.9
D-003 + LPS	200	14.3 (1/7)*	82.8

LPS - lipopolysaccharides, ON - osteonecrosis, ^a(number of rabbits with ON lesions/number of rabbits per group). * $P < 0.05$, Comparisons with positive controls (Fisher Test)

TABLE 2: EFFECT OF D-003 ON THE NUMBERS AND AREAS OF ON LESIONS (X±SD) IN LPS-INDUCED ON IN RABBITS

Groups	D003 doses (mg/kg)	Numbers of ON lesions ^a	Percentage of ON areas	
			Femur	Humerus
Negative control	0	0*	0*	0*
Positive control	0	3.00±1.67	69.4±34.2	62.9±30.9
D-003 + LPS	5	0.62±0.92*	12.3±17.1*	11.9±16.4*
D-003 + LPS	25	0.37±0.74*	5.8±10.8*	5.5±10.2*
D-003 + LPS	200	0.14±0.38*	2.0±5.4*	1.8±4.7*

LPS - lipopolysaccharides, ON - osteonecrosis, X mean, SD - standard deviations, ^a number of lesions per animal/number of animals per group, * $P < 0.05$, Comparisons with positive controls, (Mann Whitney U Test)

TABLE 3: EFFECT OF D-003 ON THE SIZE OF BONE MARROW FAT CELLS IN LPS-INDUCED ON IN RABBITS

Groups	D003 doses (mg/kg)	Size of bone marrow fat cells (µm)	% I
Negative control	0	55.70±0.22*	
Positive control	0	60.17±0.33	
D-003 + LPS	5	55.95±0.33*	94.4
D-003 + LPS	25	54.79±0.35*	100
D-003 + LPS	200	53.29±0.32*	100

LPS - lipopolysaccharides, ON - osteonecrosis, * $P < 0.05$ Comparisons with positive controls (Mann Whitney U Test)

disturbance, thus participating in the development of intravascular coagulation, fat embolism, hyperlipidemia and multiple-organ dysfunction, among others. In particular, LPS activates vascular endothelial cells, platelets, monocytes/macrophages, and components to lead to their hypercoagulability and/or hypofibrinolytic states^[8].

The overall mortality rate (3/38, 7.9%) was somewhat lower than that reported by other authors (11%)^[8], but the mortality rate among positive controls (2/8, 25%) was greater. Rabbits died mainly of circulatory disturbances within 24 h after LPS injection, consistent with previous reports^[8]. Also, in our study the incidence of ON among the surviving positive controls (5/6, 83.3%) 4 weeks after the LPS injection was similar to that (77%) referred by other authors^[8]. The consistence of our results with those referred previously^[10] confers validity to our experimental conditions and to the results here reported.

The original description of this model, however, did not refer increases in the size of bone marrow fat cells^[8], but such finding has been reported and correlated to the steroid-induced ON in rabbits^[12], and in the alcohol-induced ON model^[4] which seems to be consistent with our results too. Hence, to our knowledge (Entrez PubMed, up to September 2011) this is the first report of enlarged bone marrow fat cells in rabbits with LPS-induced ON.

While all doses of D-003 (5, 25 and 200 mg/kg) significantly reduced the number of ON lesions, the ON areas and the sizes of bone marrow fat cells as compared to the positive controls, only the middle and highest doses (25 and 200 mg/kg) reduced significantly the incidence of ON. This apparent contradiction should be related with the fact that ON incidence (a categorical variable) was compared with a proportion test, for which the sample size per group (less than 10 animals per group) was relatively low for obtaining a significant difference, a situation worsened because of two positive controls died during the study. This limitation could be also responsible of the lack of a significant dose-dependence of the D-003-induced reduction of ON incidence.

The mechanisms whereby D-003 prevents the LPS-induced ON were beyond the objectives of this study, but they are consistent with the cholesterol-lowering, antiplatelet and antioxidant effects of D-003,^[15] since

lipid-lowering treatments like probucol^[12] and statins^[11] have been shown to reduce the development of ON and the size of bone marrow fat cells.

In light of these facts, the inhibitory effect of D-003 on the development of ON may be partly associated to the reduction of lipid deposition in bone marrow tissues, as manifested by the reduction of hypertrophic bone marrow adipocytes in D-003 treated animals. These results agree with previous data on rats treated with corticosteroid-induced osteoporosis and ON, in which D-003 reduced the enlargement of bone marrow fat cells induced by the corticoid^[20].

On the other hand, increased platelet activation and hypercoagulation have been linked with the development of ON^[24]. Then, antiplatelet and anticoagulant agents should be beneficial to manage ON, because they may enhance blood flow to ischemic bone areas and potentially promote revascularization. In such regard, the antiplatelet effects of D-003 previously demonstrated^[15] could have contributed to the present results.

The preventive effects of D-003 on LPS-induced ON can be also related with its antioxidant effects^[15], since *in vivo* oxidative stress has been implicated in the development of steroid-induced ON in rabbits^[25]. Lipid peroxidation may cause cytomembrane injury and induce degeneration of arterioles and arteriosclerosis, which eventually leads to ischemia in the target organ, including the femoral head. Furthermore, the direct cytotoxicity of lipid peroxidation, might further damage ischemic osteocytes, leading to irreversible injury leading, cell death and ON^[4]. In addition, the preventive effects of lovastatin and probucol on steroid-induced ON have been attributable to their antioxidant effects^[12].

Finally, since bisphosphonates have been shown to reduce ON due to the reduction of bone resorption^[14], the antiresorptive effects of D-003, which involves the increase of osteoclast apoptosis^[18], could also have contributed to the present results.

The present results encourage the research of the potential benefits of D-003 for managing ON, a disease that remains as difficult to treat^[2]. Further studies, however, are needed to confirm the present results and to demonstrate whether the effects of D-003 on ON are clinically meaningful.

This study has demonstrated that D-003 (5-200 mg/kg) administered orally for four weeks reduced the incidence, number and percent areas of ON lesions, and the size of bone marrow fat cells, a marker of adipogenesis, in rabbits with LPS-induced ON.

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