



## D-003, A MIXTURE OF SUGARCANE WAX ACIDS, REDUCES JOINT DAMAGE IN MONOSODIUM IODOACETATE-INDUCED OSTEOARTHRITIS IN RATS

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Accepted on: 16-08-2012; Finalized on: 29-09-2012.

### ABSTRACT

Osteoarthritis (OA) is the most common degenerative arthritis, mainly in the elderly. Non-steroidal anti-inflammatory drugs (NSAIDs) remain as the main therapeutic class to reduce the pain and functional disability in OA, but they do not prevent the damage of cartilage and produce adverse side effects that may limit their use. Then, the search for new substances to manage OA is justified. D-003 is a mixture of higher aliphatic sugarcane wax acids with antiresorptive and antioxidant effects. This study was undertaken to investigate the effects of D-003 on monosodium iodoacetate (MIA) induced OA in rats. Rats were distributed into six groups: a negative vehicle control and five groups with MIA-induced OA. One of these was a positive vehicle control, three were treated with D-003 (100, 200 and 400 mg/kg), and other with ibuprofen (30 µmol/kg). Following a single knee synovial injection of MIA, treatments were administered orally for 10 days. After sacrificed, rat knee joints removed and processed. Histology scores were assessed by evaluating changes and cartilage damage, cellular abnormalities, matrix staining, extent of inflammation, pannus formation and presence of osteoclasts. The MIA injection significantly increased the histology score as compared to the negative control group, changes that were significantly and dose-dependently decreased by D-003 (100, 200 and 400 mg/kg) as compared to the positive control. Ibuprofen 30 µmol/kg reduced significantly the inflammation, the depth and histological score of cartilage damage, but did not modify other parameters. Concluding, D-003 treatment reduced cartilage injury and associated inflammation in the model of MIA-induced OA in rats.

**Keywords:** Osteoarthritis, sugarcane wax acids, D-003, NSAIDs, monosodium iodoacetate.

### INTRODUCTION

Osteoarthritis (OA), the most common degenerative arthritis, emerges from the unbalance between the synthesis and degradation of cartilage matrix, which in turn leads to a chain of subsequent inflammatory reaction responsible for the destruction of bone and cartilage in such a way that so that the cartilage does not regenerate and may disappear almost completely.<sup>1,2</sup> The pathogenesis of OA is multifactorial. It is now clear that although cartilage degeneration is the most important underlying change of the disease, OA involves all components of the joint, so that structures like the subchondral bone, ligaments, joint capsule, synovium, periarticular muscles, menisci and sensory nerve endings are involved in the inflammatory process. Chronic inflammation, degeneration of the extracellular matrix and abnormal remodeling of the underlying bone, all take part in cartilage destruction.<sup>2,4</sup>

Although the pain that accompanies OA may be or not associated with the inflammatory process, non steroidal anti-inflammatory drugs (NSAIDs), which act by inhibiting the enzyme cyclo-oxygenase (COX), remain as the main therapeutic class to reduce the pain and functional disability in OA, but they do not prevent the damage of cartilage.<sup>5</sup> In addition, NSAIDs produce adverse side effects that may limit their use, like those of gastrointestinal nature, typical of non selective NSAIDs that inhibit both COX 1 and 2 isoforms, or the cardiovascular adverse effects associated with the use of COX-2- inhibitors.<sup>6,7</sup> Also, antiresorptive drugs, like

biophosphonates and oestrogens have shown some benefits in OA since they reduce subchondral bone lesions in elderly women with knee OA, as compared to untreated women.<sup>8</sup>

On the other hand, despite increased oxidative stress and reduced endogenous antioxidant defenses seem to contribute to the pathogenesis of OA,<sup>9</sup> the use of antioxidants is not included as part of the pharmacological armamentarium to prevent/treat this disease.

D-003 is a mixture of high molecular weight fatty acids purified from sugar cane wax whose major component is octacosanoic acid.<sup>17</sup> Oral administration of D-003 has been shown to produce cholesterol-lowering, antioxidant and bone antiresorptive effects in experimental and clinical studies.<sup>10</sup> Oral treatment with D-003 (5 to 200 mg/kg) for 3 months has been shown to reduce bone loss and augmented bone resorption in rats with ovariectomy-induced osteoporosis through by increasing osteoclast apoptosis, effect that persistz after 12 months of therapy.<sup>11-15</sup> D-003 (10 mg/day) given for 6 months reduced urinary excretion of deoxipirridoline (DPD)/creatinine), a marker of bone resorption, in postmenopausal women with low values of bone mineral density (BMD),<sup>16</sup> and given for 3 years increased spine BMD values in this population.<sup>17</sup> Also, a recent study has proven that it inhibits COX activity, mainly COX-1 isoform, *in vitro*.<sup>18</sup>



Experimental models of OA include the intra-synovial injection of monoiodoacetate (MIA) into the rat femorotibial joint space, which has been reported to share similarities with human OA.<sup>19-22</sup>

In light of the issues, this study was undertaken to investigate the effects of D-003 on MIA-induced OA in rats.

## MATERIALS AND METHODS

### Substances and chemicals

D-003 (030010110) was supplied by the Plants of Natural Products (CNIC, Havana, Cuba), and ibuprofen, the reference NSAIDs, was purchased from the Chemical Pharmaceutical Cuban Industry (Quimefa, Havana, Cuba). Quality specifications of D-003 were confirmed by gas chromatography.<sup>23</sup>

MIA was acquired from Sigma (Switzerland)

D-003 and ibuprofen were suspended in a Tween 20/H<sub>2</sub>O vehicle (2%), meanwhile MIA was dissolved in physiological saline (NaCl 20 mg/mL).

### Animals

Male Sprague Dawley rats (150 - 175g), acquired in the National Centre for Laboratory Animal Production (CENPALAB, Havana, Cuba) were adapted to laboratory conditions (temperature 20-25°C, relative humidity 60 ± 10 %, 12 hours light/darkness cycles 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.

### Treatment and experimental design

Animals were distributed into 6 groups: a negative vehicle control and five groups that received MIA injection: one positive control, treated orally with the vehicle, three treated with D-003 (100, 200 and 400 mg/kg), and other with ibuprofen (30 µmol/kg). The damage was induced by a single injection of MIA (1 mg/50 µL) into the synovial cavity of the left knee.<sup>21</sup> After the induction of the damage, rats were administered with the respective treatments once daily by gastric gavage (10 mL/kg) for 10 days.

At treatment completion, food was removed 24 hours and then the rats were sacrificed in ether atmosphere.

### Histopathological study

The left knee joint was removed and fixed overnight in 10% formalin buffered and subsequently decalcified in 0.5 M disodium EDTA (pH 7.4) dissolution at 4 °C for 4 weeks before being embedded in paraffin. Frontal sections of the medial aspect of the rat knee joints were cut. Hematoxylin and eosin staining was carried out to assess the extent of inflammatory infiltrates in the joints and

surrounding tissues. Additional sections were rehydrated in a graded series of ethanol and stained with toluidine blue to evaluate cartilage damage and osteophytes.

Cartilage damage was evaluated according to a modified Mankin score that evaluates the depth and extent of the damage.<sup>22</sup> The depth was scored from 0 to 5 (0 = normal, 1 = minimal, affecting the superficial zone only, 2 = mild invasion into the upper middle zone only, 3 = moderate invasion well into the middle zone, 4 = marked invasion into the deep zone but not to the tidemark and 5 = severe full-thickness degradation to the tidemark). The extent of tibial plateau involvement was scored as 0 = normal, 1= minimal, 2= moderate, or 3= severe.<sup>22</sup>

Later on, cartilage changes were scored on a scale of 0–6 according to the Mankin system<sup>24</sup> as follows: 0 = normal, 1 = irregular surface, including fissures into the radial layer, 2 = pannus, 3 = absence of superficial cartilage layers (≥6), 4 = slight disorganization (cellular row absent, some small superficial clusters), 5 = fissure into the calcified cartilage layer, and 6 = disorganization (chaotic structure, clusters, and osteoclasts activity). Cellular abnormalities were scored on a scale of 0–3, where 0 = normal, 1 = hypercellularity, including small superficial clusters, 2 = clusters, and 3 = hypocellularity; Matrix staining was scored on a scale of 0–4, where 0 = normal/slight reduction of staining, 1 = staining reduced in the radial layer, 2 = staining reduced in the interterritorial matrix, 3 = staining present only in the pericellular matrix, and 4 = staining absent.

Inflammation was scored on a scale of 0–4, based on the degree of cellular infiltration into the tissue, where 0 = normal (no infiltrates), 1 = minimal inflammatory cell infiltration, 2 = mild infiltration, 3 = moderate infiltration, and 4 = marked infiltration. Pannus formation in the joint tissues and synovial lining cell hyperplasia were scored from 0 to 4, where 0 = normal, 1 = minimal loss of cortical bone at a few sites, 2 = mild loss of cortical trabecular bone, 3 = moderate loss of bone at many sites, and 4 = marked loss of bone at many sites, with fragmenting and full-thickness penetration of the inflammatory process or the pannus formation into the cortical bone.

Presence of osteoclasts was also scored on a scale of 0–4, where 0 = normal (essentially no osteoclasts), 1 = few osteoclasts (lining <5% of most affected bone surfaces), 2 = some osteoclasts (lining 2–25% of most affected bone surfaces), 3 = many osteoclasts (lining 26–50% of most affected bone surfaces), and 4 = myriad osteoclasts (lining >50% of most affected bone surfaces).<sup>22</sup>

The mean of the scores for all histologic parameters was calculated. This value was designated as the histology score.<sup>22</sup>

### Statistical Analysis

The results were evaluated using Mann Whitney Test for comparisons between groups. Dose-dependent relationship was evaluated using Linear Regression Test.



The level of statistical significance was chosen at  $\alpha = 0.05$ . Data were processed with the Statistic software package for Windows (Release 6.1, StatSoft Inc, Tulsa, OK, USA).

## RESULTS AND DISCUSSION

This study shows that oral treatment with D-003 (100-400 mg/kg) was effective for reducing cartilage damage, the degree of joint inflammation and pannus formation in the joint of rats with MIA-induced knee OA, thus evidencing benefits on all the components of the joint.

The model of MIA-induced knee joint degeneration observed in the rat is suitable to assess the potential effects of any substance for preventing OA since shares many histological features with the clinical condition. The

intra-articular injection of MIA inhibits the activity of glyceraldehyde-3-phosphate dehydrogenase and hence of glycolysis, inducing death of chondrocytes in articular cartilage.<sup>24</sup>

In our study, MIA injection increased significantly cartilage damage, changes that were significantly reduced with D-003 (100, 200 and 400 mg/kg) by 41.3, 48.2 and 53.4 %, respectively, as compared to the positive control. (table 1). Ibuprofen 30  $\mu\text{mol/kg}$ , the non selective COX inhibitor used in this study as reference drug,<sup>25</sup> reduced significantly the depth and the histological score (20.7%), without modify the extent of the damage. Effects of D-003 (200 and 400 mg/kg) were significantly higher than those of ibuprofen.

**Table 1:** Effects of D-003 on the Mankin-modified histological score for cartilage damage

Treatments	Depth		Extent		Histology Score	
	X $\pm$ SD	%	X $\pm$ SD	%	X $\pm$ SD	%
Negative Control	0 <sup>+++</sup>		0 <sup>+++</sup>		0 <sup>+++</sup>	
Positive Control	4.63 $\pm$ 0.52		2.63 $\pm$ 0.52		3.63 $\pm$ 0.52	
Ibuprofen 30 $\mu\text{mol/kg}$	3.25 $\pm$ 0.71 <sup>++</sup>	<b>29.8</b>	2.50 $\pm$ 0.53	<b>4.9</b>	2.88 $\pm$ 0.44 <sup>+</sup>	<b>20.7</b>
D-003 100 mg/kg	2.63 $\pm$ 0.52 <sup>+++</sup>	<b>43.2</b>	1.63 $\pm$ 0.52 <sup>+++</sup>	<b>38.0</b>	2.13 $\pm$ 0.52 <sup>+++</sup>	<b>41.3</b>
D-003 200 mg/kg	2.38 $\pm$ 0.52 <sup>+++</sup>	<b>48.6</b>	1.38 $\pm$ 0.52 <sup>+++</sup>	<b>47.5</b>	1.88 $\pm$ 0.52 <sup>+++</sup>	<b>48.2</b>
D-003 400 mg/kg	2.13 $\pm$ 0.64 <sup>+++</sup>	<b>54.0</b>	1.25 $\pm$ 0.46 <sup>+++</sup>	<b>52.5</b>	1.69 $\pm$ 0.53 <sup>+++</sup>	<b>53.4</b>

+ p<0.05; ++ p<0.01; +++ p<0.001, Comparisons vs Positive Control. U de Mann Whitney; \* p<0.05;

\*\*p<0.01, Comparisons vs Ibuprofen. U de Mann Whitney

**Table 2:** Effects of D-003 on cartilage changes

Treatments	Structures		Cellular		Matrix Staining		Mankin score	
	X $\pm$ SD	%	X $\pm$ SD	%	X $\pm$ SD	%	X $\pm$ SD	%
Negative Control	0 <sup>++</sup>		0 <sup>++</sup>		0 <sup>++</sup>		0 <sup>++</sup>	
Positive Control	5.38 $\pm$ 0.74		2.88 $\pm$ 0.35		3.00 $\pm$ 0.00		3.75 $\pm$ 0.24	
Ibuprofen 30 $\mu\text{mol/kg}$	5.50 $\pm$ 0.53	<b>2.2</b>	2.75 $\pm$ 0.46	<b>4.5</b>	2.88 $\pm$ 0.35	<b>4.0</b>	3.71 $\pm$ 0.28	<b>1.1</b>
D-003 100 mg/kg	3.88 $\pm$ 0.64 <sup>+++</sup>	<b>27.9</b>	1.88 $\pm$ 0.64 <sup>++</sup>	<b>34.7</b>	1.88 $\pm$ 0.64 <sup>+++</sup>	<b>37.3</b>	2.54 $\pm$ 0.47 <sup>+++</sup>	<b>32.3</b>
D-003 200 mg/kg	3.13 $\pm$ 0.64 <sup>+++</sup>	<b>41.8</b>	1.38 $\pm$ 0.52 <sup>+++</sup>	<b>52.1</b>	1.50 $\pm$ 0.53 <sup>+++</sup>	<b>50.0</b>	2.00 $\pm$ 0.40 <sup>+++</sup>	<b>46.7</b>
D-003 400 mg/kg	2.75 $\pm$ 0.46 <sup>+++</sup>	<b>48.9</b>	1.25 $\pm$ 0.46 <sup>+++</sup>	<b>56.6</b>	1.38 $\pm$ 0.52 <sup>+++</sup>	<b>54.0</b>	1.79 $\pm$ 0.35 <sup>+++</sup>	<b>52.3</b>

+ p<0.01; ++ p<0.001, Comparisons vs Positive Control. U de Mann Whitney; \* p<0.05; \*\*p<0.01; \*\*\* p<0.001, Comparisons vs Ibuprofen. U de Mann Whitney; †p<0.05. Lineal Regresión Test

**Table 3:** Effects of D-003 on the inflammatory infiltrate and pannus formation (X  $\pm$  SD)

Treatments	Inflammatory infiltrate		Pannus formation		Histology Score	
	X $\pm$ SD	%	X $\pm$ SD	%	X $\pm$ SD	%
Negative Control	0 <sup>+++</sup>		0 <sup>+++</sup>		0 <sup>+++</sup>	
Positive Control	3.63 $\pm$ 0.52		3.63 $\pm$ 0.52		3.63 $\pm$ 0.44	
Ibuprofen 30 $\mu\text{mol/kg}$	2.13 $\pm$ 0.64 <sup>++</sup>	<b>41.3</b>	3.63 $\pm$ 0.52	<b>0</b>	2.88 $\pm$ 0.44 <sup>+</sup>	<b>20.7</b>
D-003 100 mg/kg	3.25 $\pm$ 0.46 <sup>**</sup>	<b>10.5</b>	2.50 $\pm$ 0.53 <sup>+++</sup>	<b>31.1</b>	2.88 $\pm$ 0.44 <sup>+</sup>	<b>20.9</b>
D-003 200 mg/kg	2.88 $\pm$ 0.35 <sup>**</sup>	<b>20.7</b>	1.88 $\pm$ 0.64 <sup>+++</sup>	<b>48.2</b>	2.38 $\pm$ 0.44 <sup>+++</sup>	<b>34.4</b>
D-003 400 mg/kg	2.88 $\pm$ 0.35 <sup>**</sup>	<b>20.7</b>	1.50 $\pm$ 0.53 <sup>+++</sup>	<b>58.7</b>	2.19 $\pm$ 0.37 <sup>**</sup>	<b>39.7</b>

\*p<0.05, \*\* p<0.01; \*\*\* p<0.001. Comparisons vs Positive Control. U de Mann Whitney; †p<0.05, ‡p<0.01; §p<0.001, Comparisons vs Ibuprofen. U de Mann Whitney; ¶p<0.05, Lineal Regresión Test

Mankin score was used to evaluate cartilage changes associated to the damage. Oral administration of D-003 (100, 200 and 400 mg/kg) prevented the MIA-induced changes of cartilage structure, reduced cellular abnormalities and preserved matrix staining, so that it reduced significantly and dose-dependently the total Mankin score by 32.3, 46.7 and 52.3%, respectively as compared to the positive controls (Table 2). Ibuprofen

had no effect on Mankin score, consistent with the fact that NSAIDs do not prevent structural changes in cartilage.<sup>26-29</sup>

Toluidine blue staining revealed a loss of proteoglycans in the positive controls, which exhibited almost complete disappearance of the cartilage or very pale staining in areas where cartilage was still present. The histologic structures of D003 treated animals, however, were



preserved, indicating that the treatment could prevent the destruction of joint proteoglycans, the major components of the extracellular matrix and those early affected in OA.<sup>30,31</sup>

D-003 (100, 200 and 400 mg/kg) significantly decreased the MIA-induced pannus formation by 31.1%, 48.2% and 58.7%, respectively, and the histological score by 20.9%, 34.4% and 39.7%, respectively, while ibuprofen did not affect pannus formation and lowered the score by 20.7%. On the other hand, ibuprofen reduced the degree of inflammatory infiltrate by 41.3%, in agreement with this ability for decreasing the formation of inflammation mediators,<sup>32,33</sup> while D-003 lowered this value only to 20.7% (table 3).

Finally, meanwhile ibuprofen did not modify the presence of osteoclasts in the knee joint, consistent with its mode of action, D-003 reduced (100, 200 and 400 mg/kg) significantly lowered this variable by 38.1%, 47.5% and 52.5%, respectively (table 4). This result is in line with the antiresorptive effect of D-003 demonstrated in experimental models of osteoporosis, associated with the increase of osteoclast apoptosis.<sup>11-15</sup>

**Table 4:** Effects of D-003 on osteoclasts occurrence

Treatments	Presence of Oc	
	X ±SD	%
Negative Control	0 <sup>+++</sup>	
Positive Control	2.63 ± 0.52	
Ibuprofen 30 µmol/kg	2.63 ± 0.52	0
D-003 100 mg/kg	1.50 ± 0.53 <sup>+++</sup>	38.1
D-003 200 mg/kg	1.38 ± 0.52 <sup>+++</sup>	47.5
D-003 400 mg/kg	1.25 ± 0.46 <sup>*</sup>	52.5

\*p<0.05, \*\*p<0.01; \*\*\*p<0.001. Comparisons vs Positive Control. U de Mann Whitney; †p<0.05, ††p<0.01; †††p<0.001, Comparisons vs Ibuprofen. U de Mann Whitney; †p<0.05, Lineal Regresión Test

The effects of D-003 in this model are in line with those previously reported in the formaldehyde-induced-OA in the rat, wherein D-003 (100 and 400 mg/kg) was able to reduce the formaldehyde-induced inflammation of rat foot and ankle.<sup>34</sup> Nevertheless, the present results demonstrate that the benefits of D-003 on osteoarthritic joints should be beyond to reduce the inflammatory component, as it also reduced cartilage damage and changes, and abnormal bone remodeling linked to osteoclasts occurrence in the joint. In such regard, although the elucidation of the possible mechanisms that explain the efficacy of D-003 in this model is beyond the scope of this study, its antioxidant and antiresorptive effects may have contributed partially to the present results, since lipid peroxidation is increased in patients with OA and antioxidant defenses are diminished in these individuals and the deficiency of antioxidants in the diet increases the incidence and progression of OA.<sup>35,36</sup>

Then, although the elucidation of the possible mechanisms that explain the efficacy of D-003 in this model is beyond the scope of this study, its antioxidant and antiresorptive effects may have contributed, at least partially, to the present results.

## CONCLUSION

Oral treatment with D-003 (100 – 400 mg/kg) was effective for reducing the cartilage injury and structural cartilage changes, pannus formation and the degree of inflammation in the model of MIA-induced OA in the rat, which suggests that it may benefit all components of osteoarthritic joint, but confirmation of this appreciation deserves further experimental and clinical research.

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