Research Article

Ameliorating Effects of D-002, A Mixture of Beeswax Alcohols, on Monosodium Iodoacetate-induced Osteoarthritis in Rats

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ABSTRACT

Osteoarthritis (OA) is characterized by degeneration, pain and inflammation of joint cartilage. The effects of D-002, a mixture of beeswax alcohols with anti-inflammatory action, on monosodium iodoacetate (MIA)-induced OA were investigated. Rats were distributed into a negative control group (vehicle) and five groups with MIA-induced OA and treated orally with vehicle (positive control), D-002 (50, 200 and 400 mg/kg) or ibuprofen (30 µmol/kg) for 10 days after OA induction. Joint damage was assessed using histological analysis. MIA injection increased the depth, extent and histological scores of cartilage damage. D-002 (50-200 mg/kg) reduced the MIA-induced injury on joint cartilage (loss of chondrocytes and proteoglycans, pannus formation and joint inflammation) but unaffected bone destruction. Ibuprofen reduced the inflammation degree and the depth of cartilage damage, but not the other parameters. These results indicate a protective effect of D-002 against joint damage in MIA-induced OA in rats.

Keywords: Beeswax alcohols, D-002, ibuprofen, monosodium iodoacetate, osteoarthritis.

INTRODUCTION

Osteoarthritis (OA) is the most common degenerative joint disease worldwide and its frequency increases with age causing severe pain, progressive loss of articular cartilage and physical disabilities.[1, 2]

The pathogenesis of OA depends of multiple, rather than a single cause. In such regard, it has been well established that OA is a complex, multifactorial inflammatory disease of the whole joint, whose development and progression is significantly mediated by interactions between the joint cartilage and its surrounding tissues.[3]

Non steroidal anti-inflammatory drugs (NSAIDs) are the main pharmacological option to treat OA due to the key role of chronic inflammation on OA progression.[4,5] Nevertheless, NSAIDs produce gastrointestinal, renal or/and cardiovascular adverse effects due to the inhibition of cycloxygenase (COX 1 or/and 2 activities) (non-selective NSAIDs and COX-2- inhibitors, respectively), which may limit their use.[6,7]

On the other hand, oxidative stress is involved in the progression of OA,[8] and experimental evidences have found that antioxidant substances may be effective to prevent OA.[9] Nevertheless, such evidences are still limited and antioxidants do not represent a therapeutic option to prevent or treat OA.[4]

D-002 is a mixture of high molecular weight aliphatic alcohols (tetracosanol, hexacosanol, octacosanol, triacontanol, tetratriacontanol and dotriacontanol) purified from beeswax[10] with anti-inflammatory effects demonstrated in experimental models,[11-13] and recent reports support that it inhibits both COX and 5-lipooxygenase (5-LOX) enzyme activities *in vitro*,[14] thus acting as a dual anti-inflammatory substance. Also, D-002 has been shown antioxidant effects in experimental and clinical studies.[15- 17]

The effects of D-002 on OA models, however, have not been explored before. In light of these issues, this study was aimed to investigate the effects of D-002 on the MIA-induced OA in rats, one of the best experimental models of OA, based on its similarities with human OA.[18,19]

MATERIALS AND METHODS

Substances and chemicals

D-002 (030010110), supplied by the Plants of Natural Products (CNIC, Havana, Cuba), and ibuprofen, the reference NSAIDs, purchased from the Chemical Pharmaceutical Cuban Industry (Quimefa, Havana, Cuba) were used in the experiment. MIA was acquired from Sigma (Switzerland).

D-002 and ibuprofen were suspended in a Tween 20/H_2O vehicle (2%), meanwhile MIA was dissolved in physiological saline solution (NaCl 0.9%).

Animals

Male Sprague Dawley rats (150 - 175g), acquired from the Center for Laboratory Animal Production (CENPALAB, Havana, Cuba), were adapted to laboratory conditions (temperature 20-25ºC, 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.

Treatment and experimental design

Rats were distributed into 6 groups of 8 animals each one: a negative vehicle control and 5 groups with MIA-

induced OA: a positive control treated orally with the vehicle, 4 groups treated with D-002 (50, 200 and 400 mg/kg) and other with ibuprofen (30 µmol/kg). OA was induced by a single injection of MIA (1 mg/50 µL) into the synovial cavity of the left knee (20). Treatments (vehicle, D-002 or ibuprofen) were given by gastric gavage (1 mL/100 g bodyweight) once daily (9 to 10 am) for 10 days, starting immediately after MIA injection.

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

Histopathological study

For histopathology study the left knee joint was removed and preserved in formalin for 24 hours. Then samples were decalcified in 0.5 mol/L disodium EDTA (pH 7.4) dissolution at 4 °C for 4 weeks.[21] After decalcification, the joint was sectioned in the longitudinal plane for 2 halves, and later included in paraffin, cut and stained with hematoxylin/eosin and toluidine blue to analyze the cartilage.[22]

A modified Mankin score was used to assess the depth and extent of cartilage damage. 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 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 1 (minimal), 2 (moderate), or 3 (severe).[22]

Later on, cartilage structure changes were evaluated in accordance to the overall Mankin (23) system and were scored from 0 to 6 (0 = normal, 1 = irregular surface, including fissures into the radial layer, 2 = pannus, 3 = absence of superficial cartilage layers (≥6), 4 = slight disorganization evidenced by cellular row absent, some small superficial clusters), 5 = fissure into the calcified cartilage layer, and 6 = disorganization, as *per chaotic* structure, clusters and osteoclasts activity). Cellular abnormalities in the cartilage were scored from 0 to 3 (0 = normal, 1 = hypercellularity, including small superficial clusters, 2 = clusters, and 3 = hypocellularity); and matrix staining from 0 to 4 (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 from 0 to 4, based on the degree of cellular tissue infiltration, where 0 was referred when infiltrates were seen, and 1, 2, 3 or 4 when minimal, mild, moderate or marked inflammatory cell infiltrations, respectively, were observed. Pannus formation in the joint tissues and synovial lining cell hyperplasia were scored from 0 to 4 (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).[23]

Osteoclasts presence was scored from 0 to 4, where 0 = normal (≅ no osteoclasts), 1 = few osteoclasts (lining <5% of most affected bone surfaces), 2 = some osteoclasts (lining 5–25% 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).[24]

The mean of the scores for all histological parameters was calculated, and this value was designated as the histology score.[25]

Statistical Analysis

Results were evaluated using Mann Whitney Test for comparisons between groups. 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

Positive controls knees exhibited destruction of the articular space and extensive degeneration of the cartilage, with a marked loss of chondrocytes from the femoral condyles and from the tibial plateaus, replacement of fatty by fibrotic tissue, bone destruction, and subchondral bone sclerosis, with partial replacement of the bone marrow by fibrotic tissue as compared to the negative controls. The cartilage loss, as evidenced by toluidine blue staining, was almost complete, and in the areas where it was still present, the staining was very pale, indicating loss of proteoglycans. In the D-002-treated groups, however, joint spaces and histological structures were preserved and the degenerative changes were minimal, with only few chondrocytes and lymphocytes infiltrating the cartilages.

MIA injection increased significantly the depth, extent and the histological score of cartilage damage, changes that were significantly lowered by all doses of D-002 (Table 1). D-002 (50, 200 and 400 mg/kg) reduced the histological scores by 32.8, 39.7 and 44.9%, respectively, as compared to the positive control. Ibuprofen 30 µmol/kg reduced significantly the depth and the histological score (29.8% and 20.6%, respectively), but not the extent of the damage. The effects of D-002 (200 and 400 mg/kg) were significantly greater than those of ibuprofen.

MIA injection significantly increased the abnormal structures, cellular abnormalities and matrix staining as compared to the negative controls (Table 2), while D-002 (50, 200 and 400 mg/kg) reduced significantly and in a dose-dependent manner the matrix staining (25%, 29% and 70.7%, respectively) and Mankin overall score (11.2%, 14.4% and 35.5%, respectively). Only the highest dose (400 mg/kg), however, was able to decrease significantly abnormal structures (23.2%) and cellular abnormalities

(21.9%). Cartilage changes were not prevented by ibuprofen.

Oral treatment with D-002 (50, 200 and 400 mg/kg) also ameliorated the increase of massive pannus formation (17.3%, 24.2% and 27.5%, respectively) and infiltration of mononuclear leukocytes (13.8%, 27.5% and 41.3%, respectively) induced by MIA (Table 3). Ibuprofen 30 µmol/kg reduced significantly the extension of inflammation by 41.3%, but unaffected pannus formation.

Presence of osteoclasts and bone destruction were seen in MIA-positive control rats as compared to the negative ones, changes that were not prevented neither by D-002 (50, 200 and 400 mg/kg) nor ibuprofen 30 µmol/kg (Table 4).

Table 1: Effects of D-002 on Mankin-modified histological score for cartilage damage

Treatments	Depth		Extent		Histology score	
	X ±SD	%	X ±SD	%	X ±SD	%
Negative control	0^{++}		0^{++}		0^{+++}	
Positive control	4.62 ± 0.52		2.64 ± 0.52		3.63 ± 0.51	
Ibuprofen 30 µmol/kg	$3.24 \pm 0.70^{++}$	**29.8**	2.49 ± 0.53	**4.9**	$2.87 \pm 0.43^{+}$	**20.7**
D-002 50 mg/kg	$3.00 \pm 0.76^{++}$	**35.2**	$1.88 \pm 0.64^{+}$	**28.5**	$2.44 \pm 0.68^{++}$	**32.8**
D-002 200 mg/kg	$2.75 \pm 0.71^{+++}$	**40.6**	$1.63 \pm 0.52^{+++*}$	**38.0**	$2.19 \pm 0.59^{+++*}$	**39.7**
D-002 400 mg/kg	$2.50 \pm 0.53^{+++}$	**46.0**	$1.50 \pm 0.53^{+++*}$	**43.0**	$2.00 \pm 0.53^{+++*}$	**44.9**

$^{+}$ p<0.05; $^{++}$ p< 0.01; $^{+++}$ p< 0.001, comparisons with positive control (U the Mann Whitney test),
*p<0.05; comparisons with ibuprofen (U the Mann Whitney test)

Table 2: Effects of D-002 on cartilage changes (Mankin Score)

Treatments	Structure		Cellular abnormalities		Matrix staining		Mankin Score	
	X ±SD	%	X ±SD	%	X ±SD	%		
Negative control	0^{++}		0^{++}		0^{++}		0^{+++}	
Positive control	5.39 ± 0.74		2.88 ± 0.35		3.01 ± 0.02		3.75±0.22	
Ibuprofen 30µmol/kg	5.48 ± 0.53	**2.2**	2.75 ± 0.43	**4.5**	2.87 ± 0.35	**4.0**	3.70±0.28	**1.1**
D-002 50 mg/kg	5.13 ± 0.64	**4.6**	2.63 ± 0.52	**8.7**	$2.25 \pm 0.46^{++*}$	**25.0**	$3.33 \pm 0.36^{+*}$	**11.2**
D-002 200 mg/kg	5.00 ± 0.76	**7.1**	2.50 ± 0.53	**13.2**	$2.13 \pm 0.64^{++*}$	**29.0**	$3.21 \pm 0.40^{++*}$	**14.4**
D-002 400 mg/kg	$4.13 \pm 0.64^{++}$	**23.2**	$2.25 \pm 0.71^{+}$	**21.9**	$0.88 \pm 0.64^{+++**r}$	**70.7**	$2.42 \pm 0.30^{+++**r}$	**35.5**

$^{+}$ p<0.05; $^{++}$ p< 0.01; $^{+++}$ p< 0.001, comparisons with positive controls (U the Mann Whitney test); * p<0.05; ** p< 0.001, comparisons with ibuprofen (U the Mann Whitney test); ^{r}p<0.05, linear regression test

Table 3: Effects of D-002 on the inflammatory infiltrate and pannus formation (X ±SD)

Treatment	Inflammatory infiltrate	%	Pannus formation	%	Histology score	%
Negative control	0^{+++}		0^{+++}		0^{+++}	
Positive control	3.62 ± 0.52		3.62 ± 0.52		3.62±0.44	
Ibuprofen 30 µmol/kg	$2.13 \pm 0.63^{++}$	**41.3**	3.62 ± 0.52	**0**	$2.88 \pm 0.42^{+}$	**20.7**
D-002 50 mg/kg	$3.13 \pm 0.64^{*}$	**13.8**	$3.00 \pm 0.53^{+*}$	**17.3**	$3.06 \pm 0.42^{+}$	**15.7**
D-002 200 mg/kg	$2.63 \pm 0.52^{++*}$	**27.5**	$2.75 \pm 0.46^{++*}$	**24.2**	$2.69 \pm 0.26^{++}$	**25.9**
D-002 400 mg/kg	$2.13 \pm 0.64^{++r}$	**41.3**	$2.63 \pm 0.52^{++**}$	**27.5**	$2.38 \pm 0.44^{++}$	**34.4**

$^{+}$ p<0.05; $^{++}$ p< 0.01; $^{+++}$ p< 0.001, Comparisons with the positive control (U the Mann Whitney test); * p<0.05; **p< 0.01; *** p< 0.001, Comparisons with ibuprofen (U the Mann Whitney test); ^{r}p<0.05, Linear regression test

Table 4: Effects of D-002 on osteoclasts occurrence

Treatment	Osteoclasts presence	
	X ±SD	%
Negative control	0^{++}	
Positive control	2.63 ± 0.50	
Ibuprofen 30 µmol/kg	2.63 ± 0.50	0
D-002 50 mg/kg	2.63 ± 0.52	0
D-002 200 mg/kg	2.63 ± 0.52	0
D-002 400 mg/kg	2.63 ± 0.52	0

$^{++}$ p< 0.01, Comparisons with the positive control (U the Mann Whitney test)

DISCUSSION

This study shows that oral treatment with D-002 (50- 400 mg/kg) ameliorated cartilage damage, pannus formation and joint inflammation in rats with MIA-induced knee OA.

Joint degeneration observed in this model of OA shares many histological features with the clinical condition, therefore, is suitable to assess the potential effects of any substance for preventing OA. Intra-articular injection of MIA inhibits the activity of glyceraldehyde-3-phosphate dehydrogenase and hence the extent of glycolysis, inducing the chondrocytes death in the articular cartilage.[24]

As expected, the positive MIA control, not the negative control group, exhibited evidences of cartilage damage assessed by both the Mankin and modified Mankin scores, which confers validity to the model in our experimental conditions and then to the results here presented.

D-002 (50, 200 and 400 mg/kg) prevented the MIA-induced cartilage injury, as evidenced the reduction of the modified Mankin score, which measures the depth and extent of the damage, and the overall Mankin score, which provides information on the cartilage structural changes. In particular, the reduction of matrix staining with D-002 was marked (≅70% with 400 mg/kg); which indicates that the treatment could prevent proteoglycans destruction at the joint. Also, D-002 (50, 200 and 400 mg/kg) reduced significantly pannus formation and the degree of MIA-induced joint inflammation, consistent with its anti-inflammatory effects reported previously[11-13] and with its dual inhibitory effect on COX and 5-LOX enzymes,[14] since a dual inhibition of these enzymes has been associated with a reduction on the progression of experimental OA by suppressing the synthesis of collagenase 1 and interleukin-1ß.[25]

D-002, however, unaffected the presence of osteoclasts in the joint of rats with MIA-induced OA, indicating that it is not effective for ameliorating the bone destruction associated to this model.

Ibuprofen at 30 µmol/kg, a dose similar to those used by other authors in experimental models of OA,[26] markedly reduced (41.3%) the inflammation, but did not modify structural changes and the extent of cartilage damage, pannus formation and the presence of osteoclasts in the bone, just lowering significantly, albeit moderately, the depth of cartilage damage and the modified Mankin histological score. These findings are consistent with the pharmacological profile of non-selective COX inhibitors, which decrease the formation of inflammation mediators,[5,26] but do not prevent structural changes in cartilage.[4,5]

Although this study was not focused to elucidate the mechanisms whereby D-002 should be effective in this model, the fact that it prevented significantly all indicators of cartilage injury, differently from ibuprofen, indicates that other mechanisms are involved on its effects in the MIA-induced OA in the rat, beyond its anti-inflammatory effects. Since joint damage has been associated with a raised production of free radicals,[4] and a decreased serum paraoxonase-1 activity and elevated serum lipid hydroperoxides and oxidative stress index were found in patients with knee OA,[27,28] it is rationale to suppose that the antioxidant effects of D-002[20- 22] may have contributed to the present results.

Overall, our results indicate potential advantages of D-002 to manage OA as compared to NSAIDs (ibuprofen), since D-002 acts not only on inflammation, but also on cartilage structural injury. In addition, anti-inflammatory and adverse gastrointestinal effects of NSAIDs, are due to the inhibition of COX pathway, which curtails the production of gastroprotective prostaglandins and displaces the arachidonic acid metabolism towards the LOX pathway, increasing the synthesis of gastrotoxic leukotrienes that attract leukocytes to the stomach, contributing to cause ulceration and enhance the gastrotoxicity due to the prostaglandins deficit.[29] Then, the fact that D-002 not only inhibits COX, but also 5-LOX activity,[14] reduces the gastrotoxicity derived from COX inhibition, and instead to be gastrotoxic, D-002 has been shown gastroprotective effects associated with the improved composition and increased secretion of the gastric mucus, and with its antioxidant effects on the gastric mucosa.[30-33]

These findings make a difference between the pharmacological profiles of D-002 and NSAIDs, and merit to continue research on the potential benefits of D-002 for treating OA, a disease frequent in elderly patients, with high concomitant diseases and therapies.

CONCLUSION

D-002 (50 – 400 mg/kg) was effective for preventing cartilage injury and structural cartilage changes, pannus formation and the degree of inflammation in rats with MIA induced OA, which suggests its potential usefulness to manage OA, but such hypothesis deserves conduct new experimental studies and studies in patients with OA, as well.

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