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Research

Effects of D-004, a Lipid Extract of the *Roystonea Regia* Fruits, on Carrageenan-Induced Pleurisy in Rats

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Abstract

The inflammation constitutes an important etiological factor for the development of different diseases such as rheumatoid arthritis, atherosclerosis, osteoarthritis, bronchial asthma, benign prostatic hyperplasia and cancer. D-004, the lipid extract of the *Roystonea regia* fruits, showed anti-inflammatory effects in acute and chronic models. In this context, this study was aimed to evaluate the anti-inflammatory effects of D-004 in the carragenan acute inflammation model. Animals were randomized into six groups: one negative control group and five carrageenan-treated groups: one positive control (treated with the vehicle), three treated with D-004 (200, 400 and 800 mg/kg) and a reference acetilsalycilic acid (ASA) 150 mg/kg group. D-004 (200, 400 and 800 mg/kg) moderately and significantly reduced the pleural exudate volume (PEV) by 26.9, 30.6 and 30%, respectively. ASA, substance reference, produced a marked and significant inhibition on PEV (67.2%). D-004 moderately reduced myeloperoxidase (MPO) activitybut did not modify total protein (TP) concentration in pleural exudate, while ASA inhibited significantly MPO activity and reduced the TP concentration in pleural exudate. D-004 (200, 400 y 800 mg/kg) reduced malondyaldehide (MDA) concentrations in pleural exudate reaching 11.1, 28.2 and 52.1 % of inhibition, respectively, being significant from the dose of 400 mg/kg. In addition, D-004 significantly reduced sulphydriles groups (SHG) concentrations with 23.8, 38.1 and 47.6 % of inhibition at the doses 200, 400 and 800 mg/kg, respectively. ASA (150 mg/kg) reduced marked and significantly the increase of the concentration of MDA and SHG with 75.2 and 76.2 % of inhibition, respectively. In conclusions, D-004 significantly reduced PEV and MPO activity (neutrophil infiltration indicator), as well as significantly reduced concentrations MDA and GSH (lipid peroxidation and protein oxidation indicators, respectively) in the pleural exudate of rats with inflammation induced by carrageenan.

Keywords: D-004; Carrageenan; Inflammation; Pleurisy

Introduction

Inflammation is a molecular, cellular and vascular response of the organism against the aggression of several exogenous and/or endogenous stimuli. The inflammatory response is classified as acute or chronic according to its evolution [1,2].

The progression and development of the inflammation has been associated with the etiology of various diseases such as rheumatoid arthritis, atherosclerosis, osteoarthritis, bronchial asthma, benign prostatic hyperplasia (BPH) and cancer [3-7].

BPH, a very common health problem in the adult man over 50 years old, involves hormonal and non-hormonal changes that occur during aging [8]. Its development and progression commonly leads to lower urinary tract symptoms (LUTS) affecting quality of life [9].

At present, BPH is considered an inflammatory disease, with around 98.1 % of incidence of prostatic inflammation [10]. Its etiologic link with inflammation is based in the infiltration of the inflammatory cells and the induction of the enzyme cyclooxygenase type 2 (COX-2) in the prostate [7, 11]. In consonance with such evidences, it has been demonstrated that non-steroidal anti-inflammatory drugs (NSAIDs) are effective in the treatment of patients with BPH complaining of refractory nocturia [12] and its combined therapy with 5α-reductase inhibitors and α_1-adrenoceptor antagonists provides benefits on the BPH/LUTS clinical entity management [13]. However, although the use of NSAIDs contributes to stopping the inflammatory progression of BPH, it produces adverse effects mainly associated with the gastrointestinal tract [14] and also acute and chronic renal failure, allergic reactions and neurological

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disorders such as dizziness, headache, mental confusion and somnolence [15].

D-004, the lipid extract of the Roystonea regia fruit, consists of a reproducible mixture of fatty acids, mainly oleic, lauric, myristic and palmitic, whereas caprylic, caprylic, stearic and palmitoleic acids are in a minor proportion [16].

Previous studies have demonstrated that oral administration of D-004 prevented prostatic hyperplasia (PH) induced experimentally in rodents [17-19]. and produced anti-inflammatory effects in an acute model (xylene-induced ear oedema in mice) [20] and in a chronic inflammation model (cotton-induced granuloma in rats) [21] which is in correspondence with its action mechanism involving the dual inhibition of the activity of COX and lipoxygenase (LOX) enzymes [22].

Taking into account the contribution of chronic inflammation in the development of BPH and that D-004 presents anti-inflammatory effects in vivo, conducting studies that delve into the anti-inflammatory effects of D-004 is a topic of interest.

Carrageenan-induced pleurisy is a classical acute inflammation experimental model widely used for evaluating different drugs with potential ant-inflammatory effects [23-27]. In this model, the pleurisy is induced by the injection of carrageenan, a high-molecular-weight sulfated polysaccharide, into the pleural space of rats resulting in an immediate neutrophil movement out of the circulation into the inflamed tissue to function in the breakdown and remodeling of injured tissue. Another important occurrence is the migration and accumulation of exudates from the damaged tissues into the pleural cavity also causing a production of neutrophil-derived reactive oxygen species that promotes lipid peroxidation and protein oxidation [28-31].

In this context, this study was aimed to evaluate the anti-inflammatory effects of D-004 in a classical acute inflammation model: carragenan-induced pleurisy in rats.

Materials and Methods

Animals

Male adult Sprague Dawley rats (250-280g), from the National Centre for Laboratory Animals (CENPALAB, Havana, Cuba), were adapted for 7 days to laboratory conditions (20 or 25 0C, 60 ± 5 % relative humidity and 12 hours light/dark cycles), with free access to tap water and standard rodent chow (from CENPALAB).

Animal experiments were conducted in accordance to the Cuban guidelines of Animal Handling and the Cuban Code of Good Laboratory Practices (GLP), which follow international guidelines on this subject. Study protocol and animal use were approved prior to study beginning by an independent animal ethics committee.

Administration and dosage

D-004 batch supplied by the Plants of Natural Products, Production Branch, National Center for Scientific Research, Havana, Cuba, had the following composition: caprylic 0.2%, capric 0.5%, lauric 25.1%, myristic 10.9%, palmitic 11.3%, palmitoleic 0.2%, stearic 2.8%, oleic 42.9%, linoleic 9.5% and linolenic 0.1%). Purity (total content of these fatty acids) was 93.9%.

D-004 was suspended in Tween 65/H2O (2 %), and acetilsalycilic acid (ASA)(Farmacuba, La Havana, Cuba) was dissolved in acacia gum (1%). All treatments were given by gastric gavage through the oral route (5 mL/kg bodyweight), as single oral doses, one hour before of the induction of inflammation.

Carragenan sodium salt (BDH Chemical Ltd Poole, England), and sodium citrate (Caledon Laboratorios Ltd Georgetown, Canada), was dissolved in saline at 1 and 3.15 %, respectively.

Rats were randomized into six groups (10 rats per group): one negative control group and five carrageenan-treated groups: one positive control (treated with the vehicle), three treated with D-004 (200, 400 and 800 mg/kg) and a reference ASA 150 mg/kg group.

For the induction of pleural oedema, the rats were anaesthetized with halothane and were given 0.3 mL of an intrapleural injection of 1% carrageenan [32]. Five hours after injection the rats were anaesthetized under halothane atmosphere and sacrificed by complete bleeding from the abdominal aorta.

To obtain the exudates, an incision was made in the pleural cavity and 1 mL of sodium citrate (3.15%) was added which was homogenized whit the exudates, the fluid leakage then being collected with automatic pipettes and added in graduated plastic tubes.

Effect on volume of pleural exudates

The collected fluid was measured in graduated plastic tubes, for which the volume of added sodium citrate (1 mL) was subtracted. Blood-contaminated exudates were discarded.

The percentage of oedema inhibition was calculated as follows: Inhibition = 100- (TV/CV) x 100 (%), where TV and CV represent the volume of pleural exudates in treated and control rats, respectively.

Effect on myeloperoxidase

Myeloperoxidase (MPO) activity was measured in pleural exudate according to Worthington enzyme manual [33]. The samples were sonicated for 10 seconds, freezing and thawing at -20 to 30 °C, three times. After that, the samples were centrifuged at 12000 rpm for 25 min at 4 °C and supernatant was used for MPO determination. For that, 625mL of phosphate buffer (50 mmol/L, pH=6) containing 0.167 mg/mL O-dianisidinedihydrochloride was mixed with 250 mL of sample and

125 mL hydrogen peroxide per minute at 25°C, being quantified by the following formula:

U/mg of protein = ΔA min. x cuvete Vol. /8.3 x sample Vol. x 10

Where: ΔA min.: absorbance variation.

Vol. cuvete: cuvete final volume.

Vol. sample: volume of sample aggregated (μL).

Effect on Totals Protein

Total protein (TP) concentrations were assessed by a modification of the Lowry method [34].

Effect on Lipid Peroxidation

Lipid peroxidation was assessed through the formation of thiobarbituric acid reactive substances (TBARS) [35]. For that, 0.5 mL of exudate were added to 0.2 mL of 8.1 % SDS plus 1.5 mL of acetic acid 20 % (pH 3.5), and 1.5 mL of thiobarbituric acid (0.8 %) and heated to 95°C for 1 hours. To prevent the production of thiobarbituric acid reactants was added to the mixtures, 50 μL of butylated hydroxytoluene (1 mmol/L). After cooling, were added 5 mL of a mixture of n-butanol:piridine (15:1 v/v), a mixture was shaken and centrifuged. The absorbance of the organic layer at 532 nm was measured. Concentrations of TBARS were expressed as nmol of malondialdehyde (MDA)/mg of protein, using freshly diluted MDA-bis (dimethyl acetal) as standard.

Effect on Protein Oxidation

Sulphydriles groups (SHG) were quantified according to a modification of the Miao-Lin Hu method [36]. Briefly, 950 μL of dithionitrobenzene (10 mmol/L) was added to exudate aliquots of 50 μL, and this mixture was incubated for 20 min at 25 °C. The absorbance of the supernatant was measured at 412 nm. A blank with dithionitrobenzene was run in parallel and the total of SH groups were estimated using an absorptivity of 13 600 $cm^{-1}mol^{-1}$ and expressed in mmol.

Statistical Analysis

Data were expressed as the mean ± SE. Statistical comparisons between control and treated groups were done with the Mann-Whitney U-test. An alpha value of 0.05 was a priori established. All analyses were carried out using Statistics software for Windows (Release 6.0; StatSoft, Tulsa, OK, USA). Relation doses/effect was performed with lineal regression and correlation test using a Primer of Biostatistics program (Stanton A, Glantz; copyright (c) 1992, McGraw-Hill, Inc Version 3.01)

Results

Table 1 shows the results of effects of D-004 on volume of pleural exudate, MPO enzyme activity and concentration of TP in the pleural exudate.

Carrageenan injection into the intercostal space of the rats increased significantly the pleural exudate volume (PEV) in the positive control group compared with the negative control group. ASA (150 mg/kg),

Table 1: Effects of D-004 on PEV, MPO activity and total protein concentration in pleural exudate.

Treatment	Doses (mg/kg)	Exudate volume (mL)	I (%)	MPO (U/mg of protein)	I (%)	Total Protein (mg/mL)	I (%)
Negative Control	__	0.17 ± 0.05***	__	3.11 ± 1.03**	__	7.18 ± 1.10**	__
Positive Control	__	1.51 ± 0.10	__	69.34 ± 2.42	__	23.59 ± 2.06	__
D-004	200	1.15 ± 0.06*	26.9	62.77 ± 0.56 t	9.9	22.30 ± 1.19	7.9
D-004	400	1.10 ± 0.07**	30.6	55.55 ± 2.12 *	20.8	22.30 ± 0.93	7.9
D-004	800	1.11 ± 0.06**	30	55.60 ± 2.13*	20.8	22.26 ± 1.43	8.1
ASA	150	0.61 ± 0.13**	67.2	40.62 ± 4.50**	43.4	15.18 ± 1.64 *	51.2

I (%): inhibition percent, MPO: myeloperoxidase

* p< 0.05; ** p < 0.01; *** p< 0.001 Comparison whit positive control

(Mann Whitney U Test)

substance reference, produced a marked and significant inhibition on PEV (67.2%)

Oral administration of single doses of D-004 (200, 400 and 800 mg/kg) moderately and significantly reduced the PEV by 26.9, 30.6 and 30%, respectively. The effect achieved was not dose-dependent being 400 mg/kg the maximum effective dose since 800 mg/kg did not produce a superior inhibitory effect.

The positive control group showed an increase in MPO activity and in the TP concentration respect with the negative control group, while ASA(150 mg/kg) inhibited significantly MPO activity (43.4 %) and reduced the TP concentration (51.2 %) in pleural exudate.

Oral treatment with D-004 (200-800 mg/kg) moderately reduced MPO activitybut did not modify TP concentration in pleural exudate. The effects were significant at the dose of 400 mg/kg (20.8% of inhibition) while the dose of 200 mg/kg produced only a 9.9% inhibition that did not reach statistical significance. For its part, the highest dose tested (800 mg/kg) did not produce an additional effect (20.8% of inhibition) so that 400 mg/kg behaved as the maximum effective dose.

Table 2 shows the results of the effects of D-004on concentrations of MDA and SHG in pleural exudate. The positive control group showed an increase in the concentrations of MDA and SHG compared to the negative control group. ASA (150 mg/kg) reduced marked and significantly the increase of the concentration of MDA and SHG with 75.2 and 76.2 % of inhibition, respectively.

Treatment with D-004 (200, 400 y 800 mg/kg) reduced MDA concentrations in pleural exudate reaching 11.1, 28.2 and 52.1 % of inhibition, respectively, this effect was significant from the dose of 400 mg/kg. The maximum effect was not achieved being that the mayor dose tested (800 mg/kg)produced the highest efficacy and no higher doses were evaluated.

In addition, D-004 (200, 400 and 800 mg/kg) significantly reduced SHG concentrations with 23.8, 38.1 and 47.6 % of inhibition, respectively, without reaching the maximum effect since the major dose tested produced the highest percentage of inhibition.

The analysis of the dose/effect relationship on concentrations of MDA and SHG showed a tendency that did not reach statistical significance (p=0.06, r=0.98; p=0.07, r=0.99 on MDA and SHG, respectively).

Discussion

Single oral administration of D-004 significantly inhibited the inflammation induced by intercostal injection of carrageenan in rats (acute inflammation model).

D-004 at doses of 200, 400 and 800 mg/kg has a moderate but significant efficacy to reduce the PEV and MPO activity, while did not modify the TP concentration.

The fact that the positive control group showed an increase in PEV, MPO activity and TP concentration with respect to negative control group, and at the same time ASA, used as a reference substance, effectively inhibited these three indicators shows the validity of these results in our experimental conditions.

Carrageenan-induced pleurisy in rats is an experimental model of acute inflammation, widely used for the evaluation of substances with a potential anti-inflammatory effect, characterized by the formation of an exudate in the pleural cavity of vasogenic origin [23-27].

Table 2: Effects of D-004 on MDA and SHG concentration in pleural exudate.

Treatment	Doses (mg/kg)	MDA (nmol/ mg of protein)	I (%)	SHG (mmol/mL)	I (%)
Negative Control	—	21.39 ± 2.59**	—	0.13 ± 0.007**	—
Positive Control	—	122.81 ± 10.26	—	0.34 ± 0.016	—
D-004	200	111.55 ± 10.63	11.1	0.29 ± 0.008*	23.8
D-004	400	94.18 ± 3.57*	28.2	0.26 ± 0.007**	38.1
D-004	800	69.93 ± 4.93**	52.1	0.24 ± 0.006**	47.6
ASA	150	46.56 ± 7.37**	75.2	0.18 ± 0.018**	76.2

I (%): inhibition percent; MDA: malondialdehyde; SHG: sulphydriles groups

* p< 0.05; ** p < 0.01 Comparison whit positive control

(Mann Whitney U Test)

The enzymes involved in the metabolism of arachidonic acid, 5-LOX and COX, play an important role in the genesis and evolution of this type of edema [37]. However, the contribution of both enzymes is substantially different with a greater contribution of COX, specifically the type I isoform (COX-1) due to the effects of their products on vascular permeability [38]. In fact, vasodilation and the increase of vascular permeability with consequent plasmatic and cellular extravasation are key characteristics of the acute inflammation that occurs in this model[38,39].

Consistent with the differential contribution of these enzymes and their isoforms in the formation of carrageenan-induced pleural edema in rats, the anti-inflammatory efficacy of various agents varies according to their mechanism of action. Thus, it has been reported that NSAIDs (non-specifical inhibitors of COX) and selective COX-1 inhibitors such as ketorolac are very effective in reducing PEV and inflammatory cellular influx,while selective COX-2 inhibitors produce a moderate reduction (celecoxib and rofecocib) or do not modify (nimesulide) the PEV without affecting the cellular migration [25,38,40]. Furthermore, a new type of dual inhibitor on 5-LOX and PgE2 synthase has been reported to be effective in this model [41,42].

Previous studies have shown that D-004 presents a dual anti-inflammatory profile by inhibiting in vitro 5-LOX activity in humans PMN (100 % of inhibition) and COX, specific action on COX-2 in microsomal fraction of rat seminal vesicles (91 % of inhibition), but unmodified significantly COX-1 (11 % of inhibition) in a platelet-rich medium [43,44].

This anti-inflammatory mechanism of D-004 could constitute the basis of its moderate efficacy in this experimental model of carrageenan pleurisy, where although both enzymes are involved, COX-1 plays a crucial role. So, the inhibition on 5-LOX and COX-2 by D-004 could explain its moderate efficacy in reducing VEP (30% inhibition), while the slight inhibition of D-004 on MPO activity (≅ 21%), an indicator of neutrophil infiltration, could be directly associated with the inhibition of 5-LOX, reducing leukotriene B4 formation, a potent neutrophil chemotactic agent [37].

On the other hand, the increase of protein concentration in pleural exudates is in correspondence with the increment of vascular permeability [38]. Aspirin reduced markedly and significantly the proteins concentration, according to its effects as a classic inhibitor nonspecific of COX [45], but with a preferential ratio on COX-1 activity [46], an enzyme basically responsible for the typical increase of vascular permeability in this model. Therefore, the fact that D-004 did not modify the total protein concentration in pleural exudate is according to the absence on COX-1 activity.

Further more, D-004 produced a marked and significant inhibition of MDA and SHG concentrations in pleural exudate, indicators of lipid peroxidation and protein oxidation, respectively.

Leukocyte activation is an important source of generation and release of reactive oxygen species that cause injury to cellular structures producing lipid peroxidation and protein [47]. Migration of neutrophils to pleural exudate in the carrageenan pleurisy model in rats constitutes the fundamental source of ROS and therefore any effect that counteracts the extravasation and cellular migration to the site of the damage and/or directly inhibits the production of ROS can contribute to reduce the oxidative damage associated with the inflammatory process.

The fact that D-004 produced greater percent inhibition on lipid peroxidation and protein oxidation (52 and 47%, respectively) than on MPO activity as an indicator of neutrophil infiltration (21%) suggests that its antioxidant effects in this model are associated not only with its moderate efficacy to inhibit neutrophil infiltration, source of ROS, but to direct effects on the synthesis or catabolism of these species.

In that sense, it has been reported that the antioxidant mechanism of D-004 involves a stimulation of the endogenous antioxidant system as it increased the total antioxidant capacity of the plasma as well as the activity of the catalase enzyme [48], and a scavenger effect of the OH* [49]. In addition, a previous study showed the antioxidant effects of D-004 specifically on prostatic tissue [50].

Therefore, the antioxidant effects of D-004 constitute an additional beneficial effect in the protection of cellular and tissue structures against inflammatory damage. In congruence with these results other authors have reported the effectiveness of antioxidant agents to exert beneficial effects on different inflammatory indicators in the carrageenan pleurisy model in rats [26,27].

Overall, given the relevant role played by inflammation and oxidative stress in the progression of prostatic hyperplasia the anti-inflammatory and antioxidant effects of D-004 could be important factors that contribute to stop tthe progression of the disease as part of a mechanism multifactorial, and future studies should continue exploring in this topic.

However, although the pulmonary tissue inflammatory response may be different than the prostate's response, the fact that D-004 presents an anti-inflammatory effect per se suggest us that such effect could contribute to D-004 efficacy in the entity BPH/LUTS, which is considered an inflammatory disease.

Conclusion

In conclusions, D-004 significantly reduced PEV and MPO activity (neutrophil infiltration indicator), as well as significantly reduced concentrations MDA and GSH (lipid peroxidation and protein oxidation indicators, respectively) in the pleural exudate of rats with inflammation induced by carrageenan.

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