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Original Article

Effects of D-004, antioxidant and anti-inflammatory substances on testosterone-induced prostate hyperplasia in rats

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Key Words
Anti-inflammatory; D-004; Prostate hyperplasia; Rat

Abstract
The aim of this study was to investigate the possible contribution of the anti-inflammatory and antioxidant effects of D-004, the lipid extract of the royal palm (*Roystonea regia*) fruits, on testosterone-induced prostate hyperplasia (PH) in rats. Rats were randomized into seven groups: a negative control and six groups were injected subcutaneously with testosterone; one of the testosterone-injected groups served as positive control and five were treated with D-004 (400 mg/kg), grape seed extract (GSE) (250 mg/kg), vitamin E (VE) (250 mg/kg), ibuprofen (200 mg/kg) or celecoxib (50 mg/kg) for 14 days. Effects on prostate weight (PW), on rat prostate malondialdehyde (MDA) concentrations and myeloperoxidase (MPO) activity were assessed. The injection of testosterone increased significantly the PW, PW/bodyweight (BW) ratio, MDA concentration and MPO activity in the positive controls as compared to the negative controls. Oral administration of D-004, not of any other treatment, significantly reduced PW and PW/BW ratio as compared to the positive control. D-004, GSE and VE, not ibuprofen or celecoxib, lowered significantly MDA values (82.9, 86 and 98.8%, respectively). In contrast, significant inhibitions of MPO activity were achieved with D-004 (58.5%), GSE (56.1%), ibuprofen (73.8%) and celecoxib (61.5%), not with VE. In conclusion, oral treatment with D-004 (400 mg/kg), but not with the antioxidant and anti-inflammatory substances tested, prevented testosterone-induced PH in rats, so that the preventive effect of D-004 on this model does not seem to be associated to its antioxidant or anti-inflammatory effects.

INTRODUCTION

Benign prostate hyperplasia (BPH), a common health problem in men older than 50 years, frequently is associated to troublesome lower urinary tract symptoms (LUTS) [1]. The multifactorial etiology of BPH involves hormonal changes, mainly the increased conversion of testosterone to dihydrotestosterone (DHT) by prostate 5α-reductase enzyme activity, which contributes to prostate growth and then to the disease progression [1, 2]. On its side, the increased tone of prostate and bladder smooth muscle mediated through the α_1-adrenoreceptors (ADR) represents the main non-hormonal factor contributing to the development of the BPH/LUTS clinical entity [1, 3]. Also, several studies have shown that other processes like chronic inflammation and increased oxidative stress may play important roles in the development of BPH/LUTS [1, 4-7].

Keeping in mind this background, the pharmacological management of BPH/LUTS includes the use of 5α-reductase inhibitors, α_1-ADR antagonists and their combined therapy [3, 8]. In addition, phytotherapeutic options like extracts from *Pygeum affricanum* bark and saw palmetto fruits have been widely used to manage BPH/LUTS [9-11]. D-004, the lipid extract of the royal palm (*Roystonea regia*) fruits, contains a mixture of free fatty acids, mainly of oleic, palmitic, lauric, and myristic acids, while palmitoleic, caprylic, capric, linoleic and stearic acids are in lower concentrations [12].

Oral treatment with D-004 has been shown to prevent testosterone-, not DHT-, induced prostate hyperplasia (PH) [12, 13] in rodents; to inhibit the activity of prostate 5α-reductase *in vitro* [14], and to antagonize α_1-ADR-mediated responses *in vitro* and *in vivo* [15-17]. Moreover, antioxidant and anti-inflammatory effects of D-004 have been demonstrated previously [18-22].

Although we had demonstrated that D-004 prevented prostate enlargement and increased oxidative markers in rats with testosterone-induced PH [20, 22], we were unable to confirm if the preventive effects of D-004 on this model could be ascertained, at least partially, to its antioxidant and/or anti-inflammatory effects. In light of these facts, this study investigated the possible contribution of the anti-inflammatory and antioxidant effects of D-004 for preventing testosterone-induced prostate enlargement in rats by comparing its effects on this model with those of substances with proven antioxidant and/or anti-inflammatory effects.

MATERIALS AND METHODS

Animals

Young adult male Sprague Dawley rats (150-200 g) from the National Center for Laboratory Animals (CENPALAB, Havana, Cuba) were adapted for 7 days to the following laboratory conditions: (20-25ºC temperature, 60 ± 5% relative humidity and 12 h 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), both upgraded to the state of international guidelines on these matters. Study protocol and animal use were approved prior to study beginning by an independent animal ethics committee.

Administration and dosage

D-004 (from the Plants of Natural Products, Production Branch, National Center for Scientific Research, Havana, Cuba), grape seed extract (GSE; Extracts, Healing & Medicinal Herbs, Marrickville, NSW, Australia), vitamin E (VE; Carlson Health, Bentleigh East, VIC, Australia), ibuprofen (QUIMEFA, Havana, Cuba) and celecoxib (Pfizer, West Ryde, NSW, Australia) were all suspended in Tween-65/H_2O (2%) (vehicle). Testosterone-propionate (QUIMEFA) was diluted in soy oil.

Rats were randomized into seven groups (10 rats/group): one negative control (orally treated with the vehicle and subcutaneous (s.c.) injected with soy oil) and six testosterone-injected groups: a positive control (orally treated with the vehicle) and five treated with D-004 (400 mg/kg), GSE (250 mg/kg), VE (250 mg/kg), ibuprofen (200 mg/kg) or celecoxib (50 mg/kg), respectively. Testosterone was administered by s.c. injection (3 mg/kg/day) for 14 days [13]. All pre-treatments were given once daily by gastric gavage (5 ml/kg).

Effects on body and prostate weight

Body weight (BW) values were determined at sacrifice. After treatment completion, rats were anesthetized under ether atmosphere and sacrificed by complete bleeding from the abdominal aorta. Prostate was immediately separated and removed from bladder and weighed in a Mettler Toledo analytical balance.

Preparation of prostate homogenates

Aliquots of whole prostate tissue were taken and gently homogenized in an ice-cold bath, with an Ultra-Turrax homogenizer. Tissue samples were homogenized in 150 mmol/l Tris/HCl buffer (pH 7.4), 50 mmol/l phosphate buffer (pH 6) containing 0.5% hexadecyl trimethylammonium bromide for determining malondialdehyde (MDA) concentrations, expressed as thiobarbituric acid reactive substances (TBARS), and myeloperoxidase (MPO) activity, respectively.

Effects on malondialdehyde concentrations on prostate homogenates

Concentrations of TBARS were determined according to Ohkawa *et al* [23]. For that, the reaction mixture (prostate homogenates) was treated with 0.2 ml of 8.1% sodium dodecyl sulphate (SDS), 1.5 ml of 20% acetic acid (pH 3.5), and 1.5 ml of 0.8% thiobarbituric acid (TBA), and heated to 95ºC for 1 h. To prevent the production of TBA reactants 50 µl of 1 mmol/l butylated hydroxytoluene were added to the mixtures. After cooling, 5 ml of a n-butanol:pyridine (15:1, v/v) mixture were added, stirring vigorously with vortex, and centrifuged at 4000 rpm for 20 min. The absorbance of the organic layer was measured at 534 nm using a spectrophotometer (Genesys 10uv). TBARS concentrations were determined from a standard curve of MDA bis-(dimethyl acetal) and reported as nmol MDA/mg protein. Protein concentrations were assessed by a modified Lowry method [24].

Effects on myeloperoxidase activity on prostate homogenates

MPO activity was measured in accordance to Worthington enzyme manual [25]. In brief, prostate homogenates were sonicated for 10 seconds, frozen at –20°C and thawed at 30°C, for three times, centrifuged at 12000 rpm for 25 min at 4°C, and then the supernatant used for determining MPO activity. Then, 250 µl aliquots of the supernatant were mixed with 625 µl of 50 mmol/l phosphate buffer (pH 6) containing 0.167 mg/ml of O-dianisidine dihydrochloride and 125 µl of 0.0005% hydrogen peroxide.

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The change in absorbance at 460 nm was measured for 2 min in an UV-visible spectrophotometer. MPO activity was expressed as units (U)/g of tissue; one U being defined as the degradation of 1 µmol of peroxide/minute at 25°C and quantified as follows:

U/g tissue = ΔA min x cuvete Vol /8.3 x sample Vol x 10

-**ΔA;** min-absorbance variation

-**cuvete Vol;** final volume in the cuvete

-**sample Vol;** volume (µL) of added sample

Statistical analysis

Comparisons among groups were performed with the Kruskal Wallis test and paired comparisons with the control group with the Mann Whitney U test. The level of statistical significance was set at $P < 0.05$. All analyses were performed using statistical software for Windows (StatSoft version 6; Tulsa, OK, USA).

RESULTS

The injection of testosterone increased significantly the prostate weight (PW) and PW/BW ratio ($P < 0.001$ for both) in the positive controls as compared to the negative control group (Table 1). D-004 (400 mg/kg/day) given orally for 14 days reduced markedly (78.7%) and significantly ($P < 0.001$) the increase of PW and of PW/BW ratio with regarding to the positive control group. Oral administration of GSE, VE, ibuprofen or celecoxib, however, failed to reduce testosterone-induced prostate enlargement. No treatment modified the BW of the animals.

Prostate homogenates of positive controls exhibited increased ($P < 0.001$) MDA prostate concentrations and MPO activity as compared to the negative controls (Table 2). Oral treatment with D-004, GSE and VE, but not with ibuprofen or celecoxib, lowered markedly (82.9%, 86% and 98.8%, respectively) and significantly ($P < 0.001$) prostate MDA concentrations. In contrast, significant inhibitions ($P < 0.001$) of MPO activity were achieved with D-004 (58.5%), GSE (56.1%), ibuprofen (73.8%) and celecoxib (61.5%), but not with VE.

DISCUSSION

This study demonstrates that oral administration of D-004 for 14 days, in addition to reduce testosterone-induced prostate enlargement, lowered significantly prostate MDA concentrations (a marker of oxidative stress) and prostate MPO activity (a marker of inflammation) on this model.

Table 1. Effects of D-004, GSE, VE, ibuprofen and celecoxib on PH induced with testosterone in rats

Treatment	Dose (mg/kg)	BW (g)	PW (mg)	I (%)	PW/BW (x10 $^{-3}$)
Negative control (vehicle)	0	253.1 ± 5.46	236 ± 9.75***	--	0.94 ± 0.04***
Positive control (vehicle + testosterone)	0	255 ± 5.57	602.8 ± 37.39	--	2.35 ± 0.1
D-004 + testosterone	400	259.1 ± 7.51	366 ± 14.32***	78.7	1.41 ± 0.07***
GSE + testosterone	250	246.9 ± 4.46	564 ± 40.33	10.6	2.3 ± 0.18
VE + testosterone	250	248.3 ± 5.85	578.5 ± 25.59	6.6	2.33 ± 0.09
Ibuprofen + testosterone	200	232.7 ± 8.81	537.9 ± 24.33	17.7	2.34 ± 0.14
Celecoxib + testosterone	50	264.8 ± 7.32	600.7 ± 39.38	0.6	2.3 ± 0.2

BW: bodyweight at sacrifice; GSE: grape seed extract; I: Inhibition; PW: prostate weight; VE: vitamin E. Data presented as mean ± SEM (standard error of the mean). ***P < 0.01 compared with positive control (Mann Whitney U test).

Table 2. Effects of D-004, GSE, VE, ibuprofen and celecoxib on MDA and MPO in prostate tissue of rats with T-induced PH

Treatment	Dose (mg/kg/)	MDA (nmol /mg protein)	I (%)	MPO x 10^{-1} (U/g tissue)	I (%)
Negative control (vehicle)	0	76.3 ± 3.38 ***	-	0.77 ± 0.08 ***	-
Positive control (vehicle + testosterone)	0	153.4 ± 5.56	-	2.41 ± 0.12	-
D-004 + testosterone	400	89.5 ± 3.76 ***	82.9	1.45 ± 0.12 ***	58.5
GSE + testosterone	250	87.9 ± 2.91***	86	1.49 ± 0.09 ***	56.1
VE + testosterone	250	77.2 ± 3.08 ***	98.8	2.36 ± 0.11	3
Ibuprofen + testosterone	200	139.8 ± 1.98	17.6	1.20 ± 0.11 ***	73.8
Celecoxib + testosterone	50	141.8 ± 4.73	15.0	1.40 ± 0.18 ***	61.6

GSE: grape seed extract; I: Inhibition; PW: prostate weight; VE: vitamin E. Data presented as mean ± SEM (standard error of the mean). ***P < 0.01 compared with positive control (Mann Whitney U test).

Nevertheless, neither the antioxidant (GSE, VE) nor the anti-inflammatory (ibuprofen, celecoxib) treatments assessed in this study were able to prevent the testosterone-induced rat prostate enlargement. These facts suggest that the efficacy of D-004 on this model should be attributed to pharmacological effects others than its antioxidant and anti-inflammatory actions. Independently of their nil efficacy on testosterone-induced prostate enlargement, the antioxidant substances here assessed (GSE, VE) [26, 27] reduced significantly the generation of MDA in the rat prostate tissue, and specifically GSE also reduced MPO activity, a finding coherent with other reports [26].

On the other hand, ibuprofen and celecoxib inhibited significantly the testosterone-induced increase of prostate MPO enzyme activity, consistently with their anti-inflammatory effects [28], without modifying the prostate levels of MDA. These results confer validity to the present results, as each treatment acted on their respective targets as expected.

The lack of efficacy of the antioxidant and anti-inflammatory treatments assessed for preventing testosterone-induced prostate enlargement here found does not argue against the role of chronic inflammation and increased oxidative stress on the pathogenesis and progression of BPH [1, 4-6]. These results simply show that the efficacy of D-004 on this model does not depend of its antioxidant and anti-inflammatory effects.

Testosterone-induced PH in rodents has been associated with an increased activity of prostate 5α-reductase that reproduces partially the static component of BPH [29-31]. It is logical, therefore, to suppose that the efficacy of D-004 on this model mainly depends of its inhibitory effect on 5α-reductase prostate activity [14], consistent with the ability of some free fatty acids that compose D-004 (lauric, myristic, oleic, linoleic, palmitic acids) [32-34] for inhibiting this enzyme [12, 13].

In conclusion, oral treatment with D-004 (400 mg/kg), but not with the antioxidant and anti-inflammatory substances tested, prevented testosterone-induced PH in rats, so that the preventive effect of D-004 on this model does not seem to be associated to its antioxidant or anti-inflammatory effects.

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