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·*Complementary Medicine*·

Ex vivo antioxidant effects of D-004, a lipid extract from *Roystonea regia* fruits, on rat prostate tissue

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Abstract

Aim: To investigate whether oral treatment with D-004, a lipid extract of the Cuban royal palm fruit, produces antioxidant effects in the prostate tissue of normal and testosterone (T)-treated rats. **Methods:** In our first experiment, normal rats were distributed into five groups: one group treated with the vehicle and four groups treated with D-004 (100, 200, 400 or 800 mg/kg). In our second experiment, rats were randomized into five groups: a negative control group and four T-injected groups. The latter were comprised of a positive control group treated with the vehicle, and three groups treated with D-004 (200, 400 or 800 mg/kg). **Results:** In normal rats, D-004 (100–800 mg/kg) inhibited significantly and dose-dependently iron-initiated malondialdehyde (MDA) accumulation in prostate homogenates (35.7%–80.0%) *vs*. the controls. D-004 (200–800 mg/kg) significantly reduced baseline MDA and carbonyl groups in prostate homogenates of normal rats to approximately 80% and 50%, respectively, and totally (100%) in T-treated rats. **Conclusion:** Oral treatment with D-004 reduced MDA and carbonyl groups dose-dependently and markedly in normal and T-injected rats. These findings show that D-004 given at doses effective to prevent prostate hyperplasia also produces antioxidant effects in the prostate tissue. *(Asian J Androl 2008 Jul; 10: 659–666)*

Keywords: D-004; *Roystonea regia*; Cuban royal palm; prostate hyperplasia; lipid peroxidation; antioxidant

1 Introduction

Benign prostate hyperplasia (BPH), a common disease in older men, is the non-malignant and uncontrolled growth of the prostate gland that could lead to urethral obstruction and to lower urinary tract symptoms (LUTS) [1].

The hormonal changes in the aging man, such as the increased conversion of testosterone (T) in dihydrotestosterone (DHT), catalyzed by prostate 5α-reductase, is pivotal in triggering BPH as increased prostate DHT concentrations promote excessive cellular growth, causing hyperplasia [2]. Therefore, prostate 5α-reductase inhibitors are widely used to treat BPH, decreasing the size of the enlarged prostate and modestly improving LUTS [3]. BPH also involves non-hormonal factors, such as the increased α1-adrenergic tone of prostate smooth muscle [4]. α1-Adrenoreceptor blockers are also indicated to treat BPH, improving mainly the symptoms [5].

However, the link between BPH and oxidative stress (OS) is limited. Cooperative function of antioxidant and redox systems against OS in male reproductive tissues

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has been reported [6]. It has been shown that OS plays a role in testes and fertility impairment [7, 8], and it might play a role in prostate cancer [9], but its influence in BPH has not been proven. However, some evidence does suggest that OS might be associated with BPH [9, 10]. Serum protein-bound sialic acid levels, a marker of prostate growth, and lipid peroxides are higher in men with BPH than in controls, but lower than in men with prostate cancer, and it has been hipothesized that OS might be linked to the extent of protein sialylation in both pathological conditions [9].

In addition, the fact that an extract from cactus flower inhibited 5α-reductase activity and displayed antioxidant effects in rat prostate homogenates [10], and that the androgenic regulation of OS during rat prostate involution and regrowth involves the antioxidant defensive enzyme system [12], suggests links between OS and BPH. Likewise, T (5 mg/kg, subcutaneously) has raised OS in rat prostate, an effect prevented by giving black tea extracts for 15 days [13]. Similar effects were found in mice injected with T and treated with diallyl sulphide for 7 days. In positive control mice, antioxidant enzyme levels lowered and lipid peroxidation (LP) markers increased in prostate and liver, and diallyl sulphide restored the T-induced antioxidant enzymes and LP in both organs [14]. Also, oral treatment with saw palmetto lipid extract (100 mg/kg) reduced prostate enlargement, lipid hydroperoxides, and glutathione peroxidase activity in rats with hyperprolactinemia-induced prostate hyperplasia (PH) [10].

D-004 is a lipid extract from Cuban royal palm (*Roystonea regia*) fruits containing a mixture of free fatty acids: oleic, lauric, palmitic, and myristic acids are the most abundant; and caprylic, capric, palmitoleic, stearic, linoleic, and linolenic acids are in lower concentrations. Mature fruits of *R. regia*, initially dried and ground, are submitted to alkaline hydrolysis and to a further selective extraction with n-hexane for obtaining this extract. D-004 competitively inhibits prostate 5α-reductase *in vitro* [15] and, given orally, prevents PH induced with T [16], not with DHT [17], in rodents. D-004 has been shown to reduce prostate enlargement and the histological changes of prostate induced with T in rats observed in the positive controls, such as the presence of irregular acinis with intraluminal projections, focal conglomerate of cells into the acinis and stroma, and stroma hyperplasia and hypertrophy of fibrocytes and smooth muscle cells, assessed with a score-chart protocol. Samples from D-004-treated rats had flattened epithelial cells, regular, unfolded acini, and delicate stroma intermingled between the acini, similar to the pattern found in negative controls [16]. In addition, D-004 antagonizes α1-adrenoreceptor-mediated responses [18, 19].

D-004 has shown to inhibit *in vitro* the iron-induced malondialdehyde (MDA) generation in prostate homogenates [20], and metal and non-metal-induced LP in rat brain and liver microsomes *ex vivo* in a dose-dependent manner [21]. Assuming a link between increased OS and BPH, the potential ability of oral treatment with D-004 to prevent prostate LP should be relevant to manage this condition, but such effect has not yet been reported.

In light of these facts, this study investigated whether oral treatment with D-004 produces "*ex vivo*" antioxidant effects in the prostate tissue of normal and T-treated rats.

2 Materials and methods

2.1 Animals

All experiments were approved by the Institutional Board of Animal Care and Use at the Center of Natural Products (National Center for Scientific Research, Havana City, Cuba). Young adult male Wistar rats (180–200 g) were housed two per cage and food (rodent chow) (EMO 1002) supplied by Centre for Laboratory Animals Production (CENPALAB, Havana City, Cuba) and tap water were available *ad libitum*. Room lights were on from 07:00 to 19:00, the room temperature was 25 ± 3ºC, and the relative humidity was 60% ± 5%.

2.2 Materials

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and the Ultrospec-Plus spectrophotometer was from Pharmacia LKB Biotechnology (Uppsala, Sweden).

2.3 Treatment methods and dosage

The batch of D-004 was obtained from the Chemistry Department of the Center of Natural Products, its composition and purity being assessed with a validated gas chromatography method. The free fatty acid composition (w/w) of the tested batch was: caprylic 0.8%, capric 1.0%, lauric 30.2%, myristic 10.4%, palmitic 7.7%, palmitoleic 0.2%, stearic 2.2%, oleic 29.7%, linoleic 9.5%, and linolenic 0.1%. The purity (as the total content of free fatty acids) was 91.8%. Results complied with sub-

stance specifications. D-004 was suspended in Tween-65/H_2O (2%). Suspensions were prepared daily, 1 h before use.

Two experiments were carried out, the first in normal rats (150–200 g), and the second in rats (200–250 g) with T-induced PH. In the first experiment, normal rats were randomized into five groups (10 rats/group) comprising a control group treated with the vehicle and four groups treated with D-004 (100, 200, 400 or 800 mg/kg), doses at which D-004 had been shown to be effective in inhibiting LP in rat plasma, liver and brain tissues [21].

In the second experiment, rats were randomized into five groups (10 rats/group) comprising a negative control group, treated with the vehicle, and four T-injected groups. The latter included a positive control group, treated with the vehicle only, and three groups treated with D-004 at 200, 400, or 800 mg/kg. In this experiment, testosterone-propionate (Cuban Medical Pharmaceutical Industry, Havana City, Cuba) was diluted in soy oil and injected subcutaneously (3 mg/kg) for 2 weeks to induce PH in the rats, as previously described, and the doses of D-004 assessed were those that had resulted in effective prevention of T-induced PH in rodents [16].

In both experiments, treatments (vehicle or D-004) were given by gastric gavage through the oral route (1 mL/rat), once daily, in the morning (08:00–10:00), 6 days a week, for 2 weeks.

Bodyweight was controlled the day before starting the treatments and weekly thereafter.

The day after treatment completion, after 12-h overnight fasting, rats were anesthetized under ether atmosphere. Prostates were immediately removed and weighed. The whole organs were taken for LP studies.

2.4 LP assays

LP in prostate homogenates was estimated by measuring baseline or iron-initiated MDA concentrations [22]. Aliquots of whole prostate tissue (200 mg) were taken and gently homogenized in 9 volumes of 150 mmol/L Tris/HCl buffer (pH 7.4), in an ice-cold bath, with an Ultra-Turrax homogenizer (model T25; Janke & Kunkel GMBH & CO.KG IKA Labortechnik, Staufen, Germany). The reaction mixture was treated with 0.2 mL of 8.1% sodium dodecyl sulfate, 1.5 mL of acetic acid 20% (pH 3.5), and 1.5 mL of thiobarbituric acid 0.8%, and heated to 95ºC for 1 h. Then 50 μL butylated hydroxytoluene (1 mmol/L) was added, samples were cooled, and 5 mL of an n-butanol:piridine (15:1 v/v) mixture was added, stirring vigorously with a vortex, and centrifuged at 1 600 × *g* for 20 min. The organic layer was taken and the optical density measured at 534 nm (final volume 1 mL, protein concentration 500 μg/mL).

MDA concentrations were determined from a standard curve of malondialdehyde bis-(dimethyl acetal) and reported as nmol MDA/mg protein. Protein concentration was assessed through a modification of the Lowry method [23]. The LP in prostate homogenates was initiated by adding a 2 μmol/L $FeCl_3$/200 μmol Adenosine 5'-Diphosphate complex and 200 μmol/L β Nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt [24]. All assays were carried out in triplicate.

2.5 Effects on protein oxidation

Protein oxidation was assayed through the dinitrophenylhydrazine (DNPH) assay [25]. In brief, prostate aliquots (200 g) were homogenized in 3 mL of phosphate buffer 50 mmol/L (pH 7.4), containing digitonin 0.1% and 40 μg/mL of a mixture of protease inhibitors and Ethylenediaminetetraacetic acid 1 mmol/L, centrifuged at 6 000 × *g* for 15 min, and the optical density of the supernatant measured at 280/260 nm to discard the presence of nucleic acids. As no value was greater than 1, the addition of streptomycin sulphate 1% to eliminate nucleic acids was not necessary. The supernatant (1 mL) was added to 4 mL DNPH 10 mmol/L dissolved in HCl 2.5 mol/L, and the mixture was stirred vigorously and placed in the dark. After 1 h, 5 mL trichloroacetic acid (10%) was added, and the mixture centrifuged at 1 000 × *g* for 15 min. The protein pellet was washed three times with a mixture of ethanol:ethyl acetate (1:1, v/v) to eliminate the excess DNPH. The protein pellet was dissolved in 2 mL guanidine 6 mol/L. Optical density measured at 450 nm, using a 22 000 mol^{-1} coefficient of molar extinction, and the concentration of carbonyl groups was reported in nmol/mg protein. The protein concentration was assessed as above [22]. Determinations were carried out in triplicate.

2.6 Statistical analysis

Data were expressed as the mean ± SE. For statistical analysis of data, the non-parametric Kruskal-Wallis test was used to compare differences among groups, and the Mann–Whitney *U*-test for paired comparisons between control and treated groups. The level of statistical significance was set at $\alpha = 0.05$, multiplicity being adjusted to $P < 0.0125$ for the results of the Kruskal-

Wallis test. All analyses were carried out using Statistics software for Windows (Release 6.0; StatSoft, Tulsa, OK, USA).

3 Results

Table 1 summarizes the effect of oral treatment with D-004 on MDA accumulation in prostate homogenates of normal rats. D-004 (100–800 mg/kg) significantly inhibited iron-initiated LP, assessed through prostate accumulation of MDA, compared with the controls, from 35.7% (100 mg/kg) to approximately 80%. The maximum effect was achieved with 400 mg/kg, as 800 mg/kg did not produce a greater inhibition.

Table 2 shows the effects of D-004 on rats with T-induced PH. D-004 (100–800 mg/kg) did not affect bodyweight gain with respect to the positive controls (data not shown). D-004 at 400 and 800 mg/kg, but not at 200 mg/kg, significantly prevented the increase of both prostate weight and prostate weight/bodyweight ratio induced with T. In this experiment, the effect of D-004 increased abruptly with the dose, as 200 mg/kg was not effective, reducing prostate enlargement by only 8.1% compared with the positive control, whereas D-400 at 400 and 800 mg/kg produced complete inhibition.

The baseline MDA values in prostate homogenates of the positive controls were greater than in the negative controls (Table 3). D-004 markedly reduced baseline MDA levels in prostate tissue of both normal and T-treated rats in a dose-dependent manner. In normal rats, the reduction induced with 200 mg/kg was not significant comapproximately 80.0% (83.6% with 400 mg/kg, 84.8% with 800 mg/kg); therefore, 400 mg/kg seems to produce the ceiling effect on this marker too. Likewise, D-004 (200–800 mg/kg) significantly lowered baseline values of MDA in prostates of T-treated rats with respect to the positive controls, although in this case the inhibition was complete (100%) and greater than in normal rats.

Table 4 lists the effects of D-004 on protein-linked carbonyl groups. D-004 (200–800 mg/kg), but not at 100 mg/kg, significantly reduced the content of carbonyl groups in prostate homogenates from normal rats. The effects were dose-dependent, but moderate, as the inhibitions achieved with 400 and 800 mg/kg were 51.6% and 54.8%, respectively. In turn, D-004 (200–800 mg/kg) significantly reduced the carbonyl groups in prostate homogenates from T-treated rats. A complete inhibition (100%) was reached with 400 mg/kg, whereas the effect of 200 mg/kg, although significant, was lower than that observed in prostate homogenates from normal rats.

Table 1. Effects of D-004 given orally on iron-induced malondialdehyde (MDA) in prostate homogenates of normal rats. Kruskal-Wallis test ($P = 0.0018$). $^{b}P < 0.05$; $^{c}P < 0.01$, compared with the control group (Mann-Whitney *U*-test). –, not applicable; TBARS, thiobarbituric acid reactant substances.

Treatment	Dose (mg/kg)	TBARS (mean ± SE) (nmol MDA/mg protein)	Inhibition (%)
Control	0	25.78 ± 8.62	–
D-004	100	16.56 ± 9.96[b]	35.7
D-004	200	10.60 ± 3.31[b]	58.8
D-004	400	5.01 ± 1.28[c]	80.5
D-004	800	5.04 ± 4.66[c]	80.4

Table 2. Effects of oral treatment with D-004 on prostate hyperplasia induced with testosterone in rats. $^{c}P < 0.01$, compared with the positive control group; $^{e}P < 0.05$, compared with the lowest dose of D-004 (Mann-Whitney *U*-test). –, not applicable.

Treatment	Dose (mg/kg)	Prostate weight (mg) (mean ± SD)	Inhibition (%)	Prostate weight /body weight (mean ± SD)
Negative control	0	356.1 ± 15.8[c]	–	1.15 ± 0.04[c]
Positive control	0	463.0 ± 20.9	–	1.50 ± 0.06
D-004	200	454.3 ± 27.7	8.1	1.49 ± 0.10
D-004	400	355.9 ± 23.2[c,e]	100	1.20 ± 0.08[c]
D-004	800	356.6 ± 19.4[c,e]	99.5	1.13 ± 0.06[c]

Table 3. Effects of D-004, given orally, on baseline malondialdehyde (MDA) levels in prostate homogenates of normal and testosterone-treated rats. [a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$, compared with the positive control group; [e]$P < 0.05$ compared with the lowest dose of D-004 (Mann-Whitney *U*-test). TBARS, thiobarbituric acid reactant substances; –, not applicable.

Treatment	Dose (mg/kg)	TBARS (mean ± SE) (nmol MDA/mg protein)	Inhibition (%)
Experiment in normal rats			
Control	0	24.17 ± 5.40	–
D-004	100	18.51 ± 3.43	23.4
D-004	200	10.60 ± 2.44[a]	56.1
D-004	400	4.02 ± 1.45[b]	83.6
D-004	800	3.66 ± 1.62[b]	84.8
Experiment in testosterone-treated rats			
Negative control	0	74.6 ± 6.05[c]	–
Positive control	0	125.7 ± 10.3	–
D-004	200	87.0 ± 11.3[a]	75.7
D-004	400	53.5 ± 8.4[c,e]	100.0
D-004	800	65.7 ± 8.4[c,e]	100.0

Table 4. Effects of D-004, given orally, on protein-linked carbonyl groups in prostate homogenates of normal and testosterone-treated rats. [a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$; [d]$P < 0.0001$ compared with the positive control group; [e]$P < 0.01$compared with the lowest dose of D-004 (Mann–Whitney *U*-test). –, not applicable.

Treatment	Dose (mg/kg)	Carbonyl groups (nmol) (mean ± SE)	Inhibition (%)
Experiment in normal rats			
Control	0	11.63 ± 1.85	–
D-004	100	8.53 ± 1.52	26.6
D-004	200	7.26 ± 2.56[a]	37.5
D-004	400	5.63 ± 1.60[b]	51.6
D-004	800	5.25 ± 1.49[b]	54.8
Experiment in testosterone-treated rats			
Negative control	0	9.94 ± 1.20[c]	–
Positive control	0	24.73 ± 1.39	–
D-004	200	21.05 ± 1.07[a]	24.8
D-004	400	7.39 ± 0.83[d,e]	100
D-004	800	8.80 ± 0.71[d,e]	100

4 Discussion

This study shows that oral treatment with D-004 at doses from 100 to 800 mg/kg given for 2 weeks produced marked and dose-dependent antioxidant effects on prostate tissue of normal rats and of rats with PH induced with T. Although the preventive effects of D-004 on PH induced with T had already been reported [26], this effect was re-assessed in this study to corroborate that the effects of D-004 on OS markers of T-treated rats were produced with the same doses of D-004 and in the same group of rats with manifested prostate enlargement.

Although D-004 has been shown to prevent LP in rat plasma, liver and brain [21], the effects on OS markers in the prostate, the presumed target of any potential benefit on PH, had not been studied. Considering this fact, and also that serum lipid peroxides have been shown to be useful to predict OS in tissues [27], we investigated here the effects of oral dosing with D-004 on OS markers in rat prostate.

The antioxidant ability of D-004 on prostate tissue was investigated in rats with normal and hypertrophied prostates, this last condition being potentially more rep-

resentative of human prostates with BPH. Although the effects on OS can be assayed by examining the extent of LP, protein oxidation, and oxidative DNA damage, here we only investigated the effects of D-004 on LP and protein oxidation in rat prostate through changes in baseline and iron-induced accumulation of MDA levels, a marker routinely used for LP [28], and the concentration of carbonyl groups, a marker of protein oxidation [28]. OS acts on many biological targets, but lipid molecules are among the most involved, and LP gives rise to a number of secondary products, MDA being the most studied marker of polyunsaturated fatty acid (PUFA) peroxidation [25]. In turn, the oxidative damage of proteins occurs concomitantly to the increase in the number of carbonyl residues that can be assayed by the stable hydrazone derivatives formed with DNPH [25].

The iron-induced MDA accumulation in prostate homogenates of normal rats reflects the tissue response to metal-elicited LP, a condition that was markedly (approximately 80%) inhibited with D-004. The effects were dose-dependent and the maximal inhibition (approximately 80%) was achieved with 400 mg/kg.

Consistent with previous data, D-004 (400 and 800 mg/kg) significantly prevented T-induced prostate enlargement without affecting bodyweight gain [26]. In a previous study, however, D-004 at 200 mg/kg had been effective in this model [17], but in the present study this dose only produced a negligible effect (8.1% inhibition). Nevertheless, doubling the dose of D-004 to 400 mg/kg produced complete inhibition (100%). This result supports the view that the antioxidant effects of D-004 on the prostate tissue of T-treated rats, reported here, were obtained in rats with evident prostate enlargement.

The increased MDA and carbonyl levels in the prostate of positive control rats indicate that T induces peroxidative reactions in lipids and proteins, and that D-004 prevented against this injury induced with T. The content of carbonyl groups in prostate homogenates of control normal rats was similar to that of the negative controls of the experiment in T-treated rats, and the MDA values of the latter were higher, probably due to the fact that the rat body weight in the second experiment was greater and baseline MDA levels in rat plasma or tissues could be affected by this factor, something observed in our historical data.

Despite the fact that D-004 was able to prevent LP and protein oxidation in prostate homogenates of both normal and T-treated rats, the antioxidant effects in T-enlarged prostates were greater than in the normal ones. In the case of baseline MDA levels, this difference was moderate, as the maximal effects in normal rats were marked (approximately 80%), whereas in T-treated rats the inhibition was complete (100%). Nevertheless, the differences regarding the reduction of carbonyl groups were actually noticeable, as the inhibition achieved in normal rats was moderate (approximately 50%), whereas the inhibition in prostates of T-treated rats was twice that in normal rats, and complete (100%). These results suggest that in conditions of increased tissue OS, as occurs in enlarged prostates, the preventive effects of D-004 on peroxidative processes are greater than in normal conditions, and that such difference is remarkable in the case of protein oxidation.

Patients with BPH have been shown to have increased blood LP and decreased antioxidant enzymes, like superoxide dismutase [29]. In light of this issue, the possibility that the antioxidant effects of D-004 on rat prostate could involve the enhancement of the antioxidant enzymes can not be discarded, but such effects were not assessed in this study. The present results, therefore, merit further research into the mechanisms of the antioxidant effects of D-004 on normal and enlarged rat prostates, including its potential effects on antioxidant enzymes.

The fractions and extracts from *Prunus africana* bark, commonly used to treat BPH, which contain high levels of myristic acid, one of the most abundant fatty acids in D-004, have been shown to potently inhibit ferrous ion-induced stimulation of LP in microsomal preparations from rabbit livers [30]. In consequence, the antioxidant effects of D-004 could be associated at least in part to this compound, although we have not found specific reports of the effects of such acid on LP in prostate tissue.

The doses of D-004 that prevented LP in prostate homogenates were similar to those reported in liver microsomes, but greater than those required to inhibit LP in brain microsomes, in which D-004 at 100 mg/kg already produced maximal inhibition [21]. Although the lipid nature of D-004 constituents could suppose a preferential distribution into the brain, a previous study found a fast and broad radioactivity distribution in rats 2 h after dosing with (^{3}H)-labelled oleic acid mixed in D-004 (400 mg/kg), the values in prostate being higher than in tissues and plasma [31], consistent with the efficacy of D-004. Consistent with these results, the magnitude of the antioxidant effect of D-004 on several markers here

studied was ≥ 80%, particularly in the hypertrophied prostate where the inhibition was complete. The doses required to inhibit OS markers in prostate tissue are greater than those required to inhibit LP in brain microsomes. This apparent discrepancy in the potency of D-004 in prostate tissue *vs.* brain tissue requires further explanation.

Previous data showed that D-004 prevents PH induced with T in rodents, with an efficacy similar to that of saw palmetto extracts [16], indicating that D-004 could be promising to treat BPH. The mechanisms involved in the effects of D-004 include the inhibition of the 5α-reductase enzyme [15] and the antagonism of responses mediated by α1-adrenergic receptors [18, 19]. The fact that D-004 given at doses that prevent PH can display antioxidant effects on prostate tissue could represent an additional benefit, but this assumption requires not only the assessment of the effects of D-004 on other parameters of OS, such as total peroxides and antioxidant enzymes, in prostate tissue, but also increased evidence of the role of OS in human BPH.

The present results, together with the negligible toxicity of D-004 indicated in toxicological studies, support the benefits in continuing studies on this substance as a potential new phytotherapeutic agent to manage BPH. However, confirmation of this assumption still requires extensive clinical research.

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