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Articulo Investigativo

Reproductive toxicity of d-004 (a lipid extract from *roystonea regia* fruits) in rats

Toxicidad reproductiva del d-004 (un extracto lipídico de los frutos de roystonea regia) en ratas

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Abstract: D-004 is a lipid extract of Roystonea regia (royal palm) fruits that consists in a mixture of free fatty acids that prevents prostate hyperplasia induced with testosterone in rodents. D-004 has shown to inhibit the enzyme activity of prostate 5-#-reductase in vitro. The objective of this study was investigate the possible reproductive toxicity of D-004 administered to fertile Sprague Dawley rats of both sexes. Rats were randomised into four groups: a control group, treated with the vehicle and three groups with D-004 at 500, 750 or 1 000 mg/kg/d, respectively. Female rats were treated for 15 d prior mating, during mating and pregnancy, and until lactation (day 21). Male rats were treated 10 weeks before and during mating. No evidence of D-004-related toxicity was observed. Thus, maternal body weight and food consumption, litter size, survival through the weaning period and weights of male and female pups did not show differences between control and treated groups. Drug-treated and control groups offspring were comparable in both physical and sensorial development and in reproductive performance. There were no significant differences among F2 generation groups in the number of born pups, mean pup's weight and postnatal pup's survival. It is concluded that D-004 orally administered to rats of both sexes did not affect the fertility and general reproductive function, which indicates that it does not alter the lactation, growth, development and sexual maturation of the progeny.

Keywords: D-004, Roystonea regia, free fatty acids, reproductive toxicity, rats.

Resumen: El D-004 es un extracto lipídico de los frutos de Roystonea regia (palma real), consistente en una mezcla de ácidos grasos libres que previene la hiperplasia prostática inducida con testosterona en roedores y ha demostrado inhibir la actividad enzimática de la 5-#-reductasa prostática in vitro. El estudio tuvo como objetivo investigar la posible

toxicidad reproductiva del D-004 administrado a ratas Sprague Dawley de ambos sexos. Las ratas se aleatorizaron en cuatro grupos: uno control (vehículo) y tres tratados con D-004 a 500, 750 o 1 000 mg/kg/d, respectivamente. Las hembras fueron tratadas durante 15 d antes del apareamiento, durante el apareamiento, la gestación y la lactancia. Los machos fueron tratados 10 semanas antes y durante el apareamiento. No hubo evidencia de toxicidad relacionada con el D-004. Por lo tanto, el peso corporal y el consumo de alimentos materno, el tamaño de la camada, la supervivencia durante el período de destete y el peso de las crías no mostraron diferencias entre los grupos controles y los tratados. Los descendientes de grupos controles y tratados fueron comparables en desarrollo físico y sensorial y en rendimiento reproductivo. No hubo diferencias significativas entre los grupos de la generación F2 en el número de crías nacidas, el peso promedio y la supervivencia de las crías. Se concluye que el D-004 administrado por vía oral a ratas de ambos sexos, no afectó la fertilidad y la función reproductiva, lo que indica que no altera la lactancia, el crecimiento, el desarrollo y la maduración sexual de la progenie.

Palabras clave: D-004, Roystonea regia, ácidos grasos libres, toxicidad reproductiva, ratas.

INTRODUCTION

Benign prostatic hyperplasia (BPH), a progressive and non-malignant enlargement of the epithelial and stromal elements of the prostate gland. [1,2] Consequently, affected men often experience lower urinary tract symptoms (LUTS) that may impair considerably their quality of life. [3] Observational studies have shown that the incidence of BPH in the ageing men has been increasing. [4,5,6] Although BPH is a multifactorial process, that involves both hormonal and non-hormonal factors, there is wide evidence that hormonal changes occurring in the aging man plays a pivotal role in the pathogenesis of BPH. The increased conversion of testosterone in dihydrotestosterone (DHT), mediated by prostate 5α-reductase enzyme, is the main hormonal factor of BPH, since the accumulation of DHT promotes excessive prostatic cell growth, BPH also involves non-hormonal factors as the increased adrenergic tone of prostate smooth muscle, mediated through α1-adrenoceptors. [6,7,8]

Consequently with the etiological factors leading to BPH/LUTS, the two major drugs classes to treat BPH are 5α-reductase inhibitors, like finasteride and dutasteride, and a-adrenoceptor blockers, like doxazosin, terazosin, tamsulosin and alfuzosin. The combined therapy with 5α-reductase inhibitors and α1-ADR blockers provides the benefits of both therapeutic classes. [9,10,11] D-004, a lipid extract obtained from the mature fruits of the royal palm by a process that includes a first alkaline hydrolysis and a further solvent (n/hexane) extraction, contains a mixture of free fatty acids (mainly oleic, lauric, palmitic and myristic acids) wherein oleic acid is the most abundant. D-004 has shown to inhibit the activity of rat prostate 5a-reductase in vitro, [12] and prevents prostate hyperplasia induced with testosterone. [13,14,15,16] Also, D-004 has demonstrated to antagonize α1-adrenoreceptors-mediated responses both *in vitro* and *in vivo*. [17,18] Single oral dose toxicity of D-004 (2 000 mg/kg) in rats and rabbits has been practically absent, and the same was noted in

the subchronic oral studies in rats and mice treated with doses up to 2 000 mg/kg for 90 and 60 d, respectively. [19,20] In addition, D-004 has not shown evidences of cytotoxic or genotoxic potential in tests able to detect the ability of chemicals to induce gene mutations (Ames test) or to provoke chromosomal aberrations in somatic cells (Bone marrow micronucleus test). [21,22] This study was conducted to provide information on the possible reproductive toxicity in Sprague Dawley rats when administered to fertile rats of both sexes.

MATERIALS Y METHODS

Animals

Virgin female and male Sprague-Dawley rats, 100 to 120 g, were purchased at the Center of Laboratory Animals Production, (CENPALAB; Havana, Cuba). Animal handling was performed according to the Cuban Ethical Regulations for Animal Care and Cuban Code of Good Laboratory Practices for Toxicological Studies. The study protocol was approved by an independent ethical board. Animals were adapted to the laboratory conditions for seven days: controlled temperature (25 ± 2°C); humidity (50-70 %) and 12 h light/dark cycles, and bedding (processed hardwood chips) was daily changed and sterilized in autoclave. Free access to tap water and food (CENPALAB rodent chow) was allowed during the study.

Treatments and dose levels

D-004 was supplied by the Chemistry Department of the Natural Products Unit of National Centre for Scientific Research (Havana, Cuba), and assessed with a validated gas chromatography method, had the following free fatty acid composition, caprylic 0.3 %, capric 0.5 %, lauric 19.4 %, myristic 9.7 %, palmitic 10.6 %, palmitoleic 0.3 %, stearic 2.6 % and oleic 49.9 %, purity being 93.3 %. This extract was suspended in a Tween-65/water vehicle 1h before dosing. The concentrations were adjusted weekly according to bodyweight gain.

Rats were randomised into four groups: a control group, treated with the tween/water vehicle, and three treated with D-004 at 500, 750 or 1 000 mg/kg/d, respectively. The lowest dose (500 mg/kg) was slightly greater than the effective dose of D-004 (400 mg/kg) most commonly used, and the highest dose (1 000 mg/kg) is considered as an acceptable upper limit dose by International Conference on Harmonisation and Organization for Economic Cooperation and Development guidelines. [23,24] Treatments (vehicle or D-004) were administered orally through/via gastric gavage (2 mL/kg/d) (8:30-10:30 a.m.). Oral route administration was selected because it is the intended clinical route for D-004. Body weights and food consumption were measured weekly and all animals were observed daily for adverse clinical signs.

Experimental design

Female rats were treated for 15 d prior mating, during mating, and during pregnancy to lactation day 21. Male rats were treated 10 weeks before, and during mating. The effects on growth, development, reproductive performance, and fertility of the F1 generation were also assessed.

For mating, two female rats and one male rat were housed in one cage and examined daily. Gestational day 0 was defined as the day sperm was found in the vagina of the mated female. Body weights of dams were recorded on days 0; 6; 13 and 21 of gestation and the days 1; 7; 14 and 21 post-partum. Consumption of diet was monitored the same days. For apparently non-pregnant rats, the uterus was staining with ammonium sulphide to identify preimplantation death of embryos.[25] All the females were permitted natural delivery. The duration of gestation was calculated from day 0 of pregnancy. Each litter was examined after delivery to establish number of pups per litter, sex distribution and gross abnormalities of pups, stillbirths, live births and the presence of external anomalies. The day of birth was defined as day 0 of age. On postnatal day 1, live F1 pups were counted, weighed, examined and sexed. Separate weights for male and female offsprings were recorded. Pup litter weights and survival were registered on postnatal day 7, 14 and 21 and their physical and behavioral development was observed. Developmental signs as pinna unfolding, hair growth, incisor eruption, eye opening, ear opening, surface righting, geotaxis negative, air righting, auditory startle, visual placing testicular descent, pupilar reflex, corneal reflex, parpebral reflex, descent testes, vaginal opening were also examined.

The locomotor development was assessed on days 4, 7, 10 and 14 for all pups per litter. Squares entered, pivoting, crawling and coordinated walk was noted. In the same period the huddling behavior was also registered. Activity measurements such as squares entered, urination/defecation and standing on hindlegs were evaluated after postnatal day 21. Homemade activity cages (45 . 60 cm) with 12 squares were used.[26]

It was calculated the indexes of fertility (FI= pregnant females/ paired females), gestation (GI= females with live pups/pregnant females), viability (VI= no. pups surviving four days/ no. live pups at birth), and lactation (LI= no. pups surviving 21 d / no. pups retained day 4). F0 females were sacrificed by ether atmosphere on day 21 post-partum. F1 pups were also autopsied after vaginal opening, except one female and male of each litter, which were randomly selected to evaluate the reproductive potential and housed individually until sacrifice. F1 animals not selected were euthanized with an overdose of ether. Body weights were recorded once a week from weaning until breeding. When selected animals reached the sexual maturity were paired within treatment group avoiding brother-sister mating. F1 females were allowed to deliver and maternal and neonatal parameters were evaluated. The F2 pups were weighed, sexed, and examined for external malformations on day 1 postpartum. Following evaluation of reproductive potential, F1 females

that delivered and F2 pups were euthanized with an overdose of ether and discarded without further examination.

Analysis of data

The litter was taken as experimental unit and data were expressed as averages per litter and per group. [27] Data was analysed following the recommendations for toxicological studies. [28] Continuous variables such as maternal and pup body weights, consumption of diet and activity in the open field of F1 pups were analysed using a parametric analysis of variance (ANOVA) followed by Tukey' s multiple comparison test, meanwhile categorical data such as the fertility, gestation, viability, lactation indexes were compared using the Fisher's exact probability test (two tailed). Data on numbers of dead and alive foetuses, litter size, survival through the weaning period and percentage of pups showing physical and reflex development were analyzed by the Kruskal-Wallis (nonparametric) test. Linear regression from analysis of variance evaluated linear trends on continuous data, Jonckheere's test evaluated linear trends on discrete data, and the Cochran–Armitage test evaluated linear trends on proportional data.

Statistical analysis was performed using StatSoft, Inc. (2003), STATISTICA (data analysis software system), and version 6. www.statsoft.com. The prior established value for a was $\alpha = 0.05$.

RESULTS

No evidence of toxicity was observed during treatment. Mean body weights of mothers of parental generation in the treatment groups were not significantly different from the control group during gestation and lactation periods, but body weight gain in the group treated with D-004 1000 mg/kg/d was significantly less than control in the period between 0 to 6 days of gestation (Table 1). Treatment did not appear to affect food consumption (data not shown). One female in the 1 000 mg/kg group died due to gavage error. No apparent treatment- related signs of reproduction toxicity were associated with D-004 exposure. The length of gestation was unchanged by treatment, like fertility, gestation, viability and lactation indexes (Table 2).

Table 1.
Average maternal body weight gains changes (g) in D-004 rat oral reproductive toxicity study.

		D-004 (mg/kg)		
	Control	500	750	1000
Day 0 gestation	298.55 ± 23.61[a] (20)	301.50 ± 24.80 (26)	305.04 ± 26.77 (24)	309.95 ± 24.49 (22)
Gestation interval				
Days 0 - 6	27.25 ± 7.87 (20)	21.04 ± 12.86 (26)	26.71 ± 6.11 (24)	16.14 ± 13.35[b] (22)
Days 6 -13	28.95 ± 9.98 (20)	24.65± 7.23 (26)	21.88 ± 8.66 (24)	20.73 ± 14.31 (22)
Days 13-21	56.95 ± 20.41 (20)	56.12 ± 21.74 (26)	58.50 ± 15.82 (24)	64.05 ± 18.07 (22)
Days 0 - 21	113.15 ± 26.17 (20)	101.81 ± 28.74 (26)	107.08 ± 16.53 (24)	100.91 ± 27.78 (22)
Day 1 post-delivery	317.74 ± 30.08 (19)	314.12 ± 32.88 (26)	310.00 ± 26.85 (24)	314.70 ± 36.17 (20)
Lactation interval				
Days 1 - 4	9.33 ± 8.40 (18)	9.77 ± 9.53 (26)	9.36 ± 8.42 (22)	5.11 ± 14.75 (19)
Days 4 - 7	11.72 ± 12.17 (18)	11.92±11.26 (26)	9.73± 8.36 (22)	12.26 ± 10.04 (19)
Days 7 -14	15.33 ± 12.11 (18)	17.54 ± 14.41 (26)	21.09 ± 17.84 (22)	16.84 ± 13.09 (19)
Days 14-21	-17.94 ± 19.28 (18)	-11.65 ± 20.74 (26)	-10.73 ± 13.87 (22)	-8.63 ± 17.79 (19)
Days 1 - 21	18.44 ± 28.58 (18)	27.58 ± 20.31 (26)	29.45 ± 17.26 (22)	25.58 ± 24.17 (19)

a Mean ± standard deviation (number of dams); bp< 0.05, Significantly different from vehicle control (ANOVA).

Table 2.
Summary of reproduction data of F0 females in the D-004 reproductive study.

		D-004 (mg/kg)		
	Control	500	750	1 000
Length of gestation (d)	22.30 ± 0.47 [a]	22.12 ± 0.71	22.25 ± 0.61	22.55 ± 0.60
Fertility index (%)	85	82	89	89
Gestation index (%)	95	100	100	91
Viability index (%)	93	99	95	89
Lactation index (%)	100	100	100	100

a Mean ± standard deviation.

There were no differences among groups in the litter size, the number of pups born live or dead, the number of pups that survived until weaning and the pups body weights through the lactancy (Table 3). In this study a low neonatal mortality and no external abnormalities were observed. Dams and their offspring were healthy during lactation. No unusual maternal behaviour was observed. Drug-treated and control groups' offspring were comparable in physical and sensorial development and reproductive performance (Table 4). The general health and behavioural condition of offspring was good in all groups. There was no significant effect of treatment on the motor development, sensory function, open field activity and onset of reflexes (data not shown).

Table 3.
Viability and body weight of F1 offspring in the D-004 reproductive study.

	Control	D-004 (mg/kg) 500	750	1000
Born pups	11.60 ± 3.63[a] (20)	11.77 ± 4.18 (26)	13.04 ± 4.40 (24)	11.41 ± 4.87 (22)
Live pups	10.70 ± 4.37	11.58 ± 4.16	12.75 ± 4.17	10.64 ± 5.66
Dead pups	0.90 ± 2.47	0.19 ± 0.40	0.29 ± 1.04	0.77 ± 1.38
Live pups on PND 1	11.26 ± 3.66 (19)	11.62 ± 4.20 (26)	12.75 ± 4.17 (24)	11.70 ± 4,73 (20)
Live pups on PND 21	11.06 ± 3.65	11.54 ± 4.13	13.23 ± 3.48	10.89 ± 5.10
Live male pups weight (g)				
PND 1	6.87 ± 0.71	6.65 ± 0.89	6.18 ± 0.78	6.29 ± 1.05
PND 7	14,82 ± 2.66	13.40 ± 2.65	12.66 ± 2.09	12.96 ± 3.10
PND 14	28.16 ± 5.12	25.87 ± 4.93	24.34 ± 3.96	25.77 ± 5.74
PND 21	46.33 ± 8.52	42.08 ± 8.50	40.28 ± 7.97	43.24 ± 9.64
Live female pups weight (g)				
PND 1	6.55 ± 0.68	6.27 ± 0.87	5.89 ± 0.80	5.91 ± 1.00
PND 7	14.28 ± 2.39	13.34 ± 3.23	12.16 ± 2.23	12.64 ± 2.55
PND 14	27.33 ± 4.87	26.18 ± 7.07	23.49 ± 4.09	24.79 ± 4.77
PND 21	44.82 ± 7.84	42.45 ± 11.06	39.05 ± 7.87	41.89 ± 7.99

a Mean ± standard deviation (number of litters examined). PND (postnatal day).

Table 4.
Physical and reflex development on F1 pups (%).

	Control	D-004 (mg/kg) 500	750	1000
Pinna unfolding on day 4	100[a]	100	99.6	100
Hair growth on day 7	100	100	100	100
Incisor eruption on day 12	93.6	81.0	78.7	94.7
Ear opening on day 13	100	100	100	100
Eye opening on day 15	100	94.7	93.8	98,1
Surface righting on day 4	63,8	63.0	58.8	63.9
Negative geotaxis on day 10	100	98.7	100	100
Air righting on day 14	67.8	56.3	60.5	62.3
Auditory startle on day 14	100	99.7	100	100
Visual placing on day 14	99.5	97.7	97.6	100
Pupilar reflex	100	99.7	99.6	100
Corneal reflex	100	99.7	100	100
Parpebral reflex	100	99.7	100	100
Descent testes on day 25	100	100	100	100
Vaginal opening on day 37	99.0	93.7	92.4	97.5

a (Positives/examined) x 100

The fertility of the F1 generation was comparable in the control group and those treated with D-004. In turn, no treatment-related effects were apparent on F1 generation reproductive parameters such as, fertility and gestation index, litter size and duration of gestation period. (Table 5). Mean body weights gains of F1 mothers in the treatment groups had no significant different from controls during gestation, and weight gain was similarly unaffected (Table 6). Treatment did not appear to affect maternal food intake neither (data not presented). There were no significant differences among groups of F2 generation regarding to number of pups born, number of pups born live or dead, mean pup's weight and postnatal pups survival (Table 7). No offspring with malformations were found in any experimental group.

Table 5.
Summary of reproduction data of F1 females in the D-004 reproductive study.

	D-004 (mg/kg)			
	Control	500	750	1 000
Length of gestation (d)	21.88 ± 0.49 [a] (17)	21.86 ± 0.48 (21)	21.78 ± 0.43 (18)	22.00 ± 0.49 (18)
Fertility index (%)	94	81	82	95
Gestation index (%)	100	100	100	100

a Mean ± standard deviation (number of dams).

Table 6.
Average maternal body weight changes (g) during gestation of F1 females in the reproductive toxicity of D-004.

	D-004 (mg/kg)			
	Control	500	750	1 000
Day 0 gestation	342.88 ± 21.68[a]	317.55 ± 31.03	320.39 ± 29.37	321.72 ± 31.44
Gestation interval				
Days 0 - 6	20.29 ± 6.43	23.20 ± 8.63	20.50 ± 9.34	23.06 ± 7.83
Days 6 - 13	28.00 ± 7.94	25.30 ± 4.33	25.72 ± 4.79	30.44 ± 6.31
Days 13 - 21	88.29 ± 20.15	88.50 ± 17.46	89.44 ± 15.71	93.94 ± 15.82
Days 0 - 21	136.59 ± 22.89	137.00 ± 20.60	135.67 ± 23.63	147.44 ± 21.64
Day 1 post-delivery	369.47 ± 29.20	345.65 ± 33.10	349.11 ± 34.08	357.50 ± 30.76

a Mean ± standard deviation.

Table 7.
Viability and body weight of F2 offspring in the D-004 reproductive study.

	D-004 (mg/kg)			
	Control	500	750	1 000
Total born pups (females/males)	14.76 ± 3.46 [a] (17)	15.00 ± 3.61 (21)	14.39 ± 2.83 (18)	15.28 ± 4.21 (18)
Live pups	14.12 ± 3.35	14.33 ± 3.40	13.83 ± 2.71	14.89 ± 4.43
Dead pups	0.65 ± 1.00	0.67 ± 1.11	0.56 ± 0.98	0.39 ± 0.85
Live pups on PND 1	14.12 ± 3.35 (17)	14.33 ± 3.40 (21)	13.83 ± 2.71 (18)	14.89 ± 4.43 (18)
Live male pups weight on PND 1 (g)	6.35 ± 0.58	6.43 ± 0.65	6.42 ± 0.74	6.48 ± 0.55
Live female pups weight on PND 1 g)	5.85 ± 0.51	6.09 ± 0.62	5.97 ± 0.60	6.19 ± 0.35

a Mean ± standard deviation (of litters number examined). PND (postnatal day).

DISCUSSION

In the present study we report the results of the evaluation of reproductive toxicity potential of oral treatment with D-004, which has shown to inhibit competitively prostate 5 α- reductase activity in vitro, [12] an effect attributable to free fatty acids present in D-004, like lauric, myristic and oleic acids, [29] and prevents prostate enlargement induced with T in rodents. [13,14,15,16] Likewise, D-004 has displayed *in vivo* and *in vitro* antagonism of α1- adrenoreceptors. [30] Drugs used to treat BPH have been associated with adverse effects, such as gastrointestinal disturbances (nausea, diarrhoea), impairment of male sexual activity (reduced libido, erectile dysfunction, abnormal ejaculation), gynecomastia, orthostatic hypotension, dizziness, headache, asthenia and rhinitis. [7,31,32,33,34] In contrast, plant extracts used to manage BPH, have not been associated

with drug-related adverse effects. [35] Saw palmetto, that is most commonly used, has been considered to produce fewer side effects than traditional medications, [36,37] but published experimental toxicity studies are limited. [38,39] The composition of D-004 partially resembles that of the lipid extract of saw palmetto (Serenoa repens) fruits.

D-004 is a lipid extract of royal palm (*Roystonea regia*) fruits that consists in a mixture of free fatty acids, wherein oleic, lauric, palmitic, and myristic are the most abundant. Animals and humans have consumed regularly the major individual fatty acids contained in D-004 (oleic, lauric, palmitic and myristic) from the food chain, it was rationale to believe that these compounds should not be toxic for humans. An old study referred the lack of oral single dose (15-19 g/kg of body weight) toxicity of oleic, lauric, palmitic, myristic, or stearic acids in rats. [40] No-observed-adverse-effect level values higher than > 6 000 and 5 000 mg/kg have been reported for lauric acid administered orally to male rats for 18 weeks for palmitic acid administered to rats for 150 d, respectively, [41] while myristic acid did not induce a mutagenic response in either bacterial or mammalian systems in vitro, and that its use as food flavouring does not pose a health risk to humans. [42] On the other hand, the regular intake of oleic acid with the diet, as occur in the Mediterranean diet has been associated with cardiovascular benefits rather with adverse effects. [43,44] These evidences supported that the intake of D-004 should not suppose a relevant safety risk for humans.

This study demonstrates that D-004 (500-1 000 mg/kg) induced no preweaning mortality, reduce body weight, developmental delay, or behavioural changes in the FI offspring. Maternal body weight gains in the group treated with D-004 (1 000 mg/kg/d), was less than control in the period between 0 to 6 days of gestation, but such a finding is not considered an indicator of maternal toxicity. Thus, this finding appears to be a random result, within the normal fluctuation of such a parameter in such a period and species. Considering this fact, joined to the absence of any other symptoms of toxicity related to D-004 treatment, we can expect that such effect could be out of biological relevance. In addition to, the weight of each pups in this group is on the range know for this species in our conditions in previous studies. Nevertheless, we will take into account this results in furthers studies, since was found at the higher doses tested.

It is concluded that oral treatment of rats of both sexes with D-004 (500 - 1 000 mg/kg/d) affected no the fertility and general reproductive function, which indicates that it does not alter the lactation, growth, development and sexual maturation of the progeny.

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