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Evaluation of D-004 effects in the Uterotrophic Assay in Mature Ovariectomized Rats

Ariadne GUTIÉRREZ, Rafael GÁMEZ*, Miriam NOA, Rosa MÁS, Balia PARDO, Maikel VALLE, Eddy GOICOCHEA, Dayisell CURVECO, Nilda MENDOZA & Haydee GARCÍA

Center of Natural Products, National Center for Scientific Research.
Calle 198 y 19, Cubanacan, PBox 6414, Havana, Cuba

SUMMARY. D-004, a lipid extract from royal palm *(Roystonea regia)* fruits, has shown to inhibit prostate 5-α-reductase *in vitro,* and to prevent prostate hyperplasia (PH) induced with T, not with DHT, in rodents, but its potential estrogenic or antiestrogenic effects had not been studied yet. This study investigated whether D-004 produces estrogenic or antiestrogenic effects, as assessed in the uterotrophic assay in ovariectomized (ovx) rats. Rats were randomly distributed into five groups: a false-operated (sham) and four groups of ovx rats: one treated with the vehicle and three others treated with D-004, estradiol and concurrent treatment with D-004 plus estradiol, respectively. Treatments were administered for 14 days. Ovariectomy reduced significantly the uterine weight, the epithelium cell height and endometrium thickness values with respect to sham groups, effects that were significantly prevented with estradiol, but unaffected with D-004. Treatment with combined therapy produced the same effect as estradiol monotherapy. According to uterotrophic assay in ovx rats, D-004 orally given at 400 mg/kg is devoid of estrogenic/antiestrogenic activity.

RESUMEN. "Evaluación del D-004 en el Ensayo Uterotrófico en Ratas Adultas Ovarectomizadas". El D-004 es un extracto lipídico del fruto de la palma real *(Roystonea regia)* que inhibe *in vitro* la 5-α-reductasa prostática, y previene la hiperplasia prostática benigna inducida con testosterona y no por dihidrotestosterona en roedores, pero sus potenciales efectos estrogénicos o antiestrogénicos no han sido aún estudiados. Este estudio evalúa si el D-004 produce efectos estrogénicos o antiestrogénicos en roedores, empleando el ensayo uterotrófico en ratas adultas ovarectomizadas, las cuales fueron distribuidas aleatoriamente en 5 grupos experimentales, un grupo Sham o falsamente operadas y 4 grupos ovarectomizadas, los cuales recibieron el vehiculo (control), D-004, estradiol y la combinación de ambos tratamientos durante 14 días, respectivamente. La reducción en el peso del útero inducida por la ovarectomía fue prevenida por el tratamiento de estradiol, pero no por el D-004, por su parte la terapia combinada produjo el mismo efecto observado en el grupo tratado con estradiol solamente. De acuerdo a estos resultados la administración de D-004 no evidencio efectos estrogénicos ni antiestrogénicos.

INTRODUCTION

Benign prostatic hyperplasia (BPH) is the benign and uncontrolled prostate growth that leads to difficulty urinating, a very common disease in men over 50 years [1]. Although the mechanisms triggering BPH are multifactorial and involve increased α1-adrenergic smooth muscle stimulation and inflammatory processes, the hormonal changes that occur in the aging man, like the increased activity of prostate 5-α-reductase, the enzyme converting testosterone (T) in its active metabolite dihydrotestosterone (DHT), and changes on the prostate estrogen/androgens ratio seems to be pivotal [2-4]. Consequently, 5-α-reductase inhibitors and adrenergic blockers are first-line pharmacological options to treat BPH, which can induce adverse events, like impairment of men sexual function, postural hypotension, among others [5-7].

Phytotherapy, and mainly the lipid extracts of the fruits of saw palmetto *(Serenoa repens)* palm (Arecaceae) which contain fatty acids (mainly oleic, lauric, mirystic, palmitic, stearic, linoleic,), and esters, plant sterols and higher

KEY WORDS: D-004, Ovariectomized rats, Uterotrophic assays, 5-α-reductase.
PALABRAS CLAVE: D-004, Ensayo uterotrófico, Ratas ovarectomizadas, 5-α-reductasa.

*Author to whom correspondence should be addressed. *E mail:* rafael.gamez@cnic.edu.cu

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aliphatic alcohols at lower concentrations, are commonly used to treat BPH, most evidences supporting their efficacy [8-12], although some placebo-controlled studies have found negative results [13,14].

Saw palmetto lipid extracts (SPLE) have shown to prevent T-induced prostate enlargement in rat [15], and to inhibit prostate 5-α-reductase activity [16-20], although a study reported no effect on enzyme activity [21]. Components of SPLE, like lauric, myristic and oleic acids, not esterified fatty acids, alcohols or sterols, have shown also to inhibit prostate 5 α-reductase [18,19]. Likewise, the study of the effects of SPLE on androgenic receptors has shown that it inhibits moderately the binding of DHT to the androgenic receptors of cultured fibroblasts and rat prostate cytosol, and the uptake of radioactive T or DHT in several human tissues [22-24].

On the other hand, the injection of crude and partially purified extracts of *Serenoa repens* berries to immature mice produced significant, but not dose-dependent, uterine hypertrophy [25], while SPLE have shown antiestrogenic activity on the prostate tissue of BPH patients administered for 3 months up to the day before of their transvesical adenomectomy. While estrogen receptors in placebo were higher in the nuclear than in the cytosolic prostate fraction, the opposite occurred in the treated group, in which nuclear estrogen receptors were lower than in placebo, whilst cytosolic receptors remained unchanged [26].

D-004 is a lipid extract of royal palm *(Roystonea regia)* fruits that mainly contains free fatty acids, like oleic, lauric, palmitic and myristic acids, while linoleic, palmitoleic, linolenic, caprylic, capric and stearic acids are at lower concentrations. D-004 has shown to inhibit 5 α-reductase *in vitro,* [27] and to prevent T-induced prostate hyperplasia (PH) in rodents *in vivo* [28–31]. In addition, D-004 has shown to antagonize α1-adrenoreceptors mediated responses *in vitro* and *in vivo* [32,33].

No treatment-related toxicity, including genital organs as a toxicity target, has been observed in previous studies of the oral toxicity of single and repeated doses of D-004 (up to 2000 mg/kg), and the highest dose has been a non-observable-effect dose in rats [34,35].

Nevertheless, despite the unbalanced estrogen/androgen ratio contributes to BPH development, the potential estrogenic or antiestrogenic effects of D-004 had not been studied yet. Therefore, this study investigated the effects of D-004 on the uterotrophic assay in mature ovariectomized (ovx) rats [36-38].

MATERIALS AND METHODS

Animals

Three-month-old female Sprague-Dawley rats (225 ± 20 g) from the National Centre for Laboratory Animals Production (CENPALAB, Havana, Cuba) were adapted to laboratory conditions (temperature 21 °C, humidity 55%, 12 h light/dark cycles) for 7 days, with free access to food (rodent chow from CENPALAB) and water. Animal handling was conducted in accordance with the Cuban Regulations for the Use of Laboratory Animals and Ethical Principles for Animal Management. An independent institutional board approved the animal use for the study.

Rats were ovx bilaterally or sham operated under anaesthesia with sodium pentobarbital (50 mg/kg iv). For ovariectomy, the dorsolateral abdominal wall was opened at the midpoint between the costal inferior border and the iliac crest and at few mm lateral to the margin of the lumbar muscle. The ovaries were detached by incision at the junction of the oviduct and each uterine horn. The abdominal wall and skin were sutured.

Administration and dosage

D-004 was obtained from the Chemistry Department of the Centre of Natural Products (Havana City, Cuba), after corroborating its purity through a validated gas chromatography method. D-004 was suspended in Tween 65/water vehicle (2%). With the aim to make the quality control and stability of the emulsions used in pharmacological and toxicological studies, a capillary gas chromatographic analytical method was developed and validated. The method was based on the extraction of the D-004 active ingredient with n-hexane, and previous to the chromatographic analysis, a methylation process with 10% acetyl chloride in methanol was carried out. The quantitative determination of total fatty acids as methyl esters was carried out using tridecanoic acid as internal standard. Once the stability in emulsions was checked, they were prepared daily, 1-2 hours before dosing, adjusting the concentrations according to the bodyweight gain and administered orally by gastric gavage (5 mL/kg). Estradiol benzoate (Cuban Medical Pharmaceutical industry–IMEFA-, Havana City, Cuba) was dissolved in sunflower oil and injected subcutaneously (0.5 mL) for 14 days.

Rats were randomly distributed into five groups: a false-operated (sham) and four groups of ovx rats: one treated with the vehicle (positive control) and three others treated with D-004 (400 mg/kg), estradiol 30 µg/kg and concurrent treatment with D-004 (400 mg/kg) plus estradiol (30 µg/kg), respectively. Treatments started 7 days after surgery recovery and were administered for 14 days.

The dose of D-004 selected has shown to prevent T-induced PH in rodents [28-31], and the atypical HP induced *in vivo* with phenylephrine in the rat [33]. Therefore, if D-004 has a meaningful potential estrogenic effect, it should be observed with such dose.

Bodyweight control

Rats were weighed before ovariectomy, and weekly thereafter.

Uterotrophic assay and assessment of uterine hyperthrophy

Twenty-four hours after the final dose, animals were killed under an ether atmosphere, followed by cervical dislocation. The reproductive tract was excised and handled to yield the individual weights of the blotted uterus, the vagina, and the cervix, the inclusion of the cervix in the weight of the uterus allows the retention of the luminal fluid. The relation uterus/body weight was calculated.

Later each uterine horn was cut, and the luminal fluid was expressed with gentle pressure. Samples of each uterine horn were fixed in 10% buffered formaldehyde (BDH, England). Tissues were dehydrated in graded alcohol, embedded in paraffin and cut at 4 µm transverse sections, which were stained with haematoxylin and eosin (Merck, Darmstadt) and observed in an Olympus BH2 microscope.

Sections were evaluated microscopically to detect changes in the uterine epithelial cell height along the endometrial surface lining and in the endometrium thickness, both calculate as average in 5 fields/animal. The measurements were performed with an ocular graticule at the same magnification.

Statistical analyses

Comparisons between treated and positive control group with one-way analysis of variance (ANOVA). All statistical analyses were performed with the software Statistics for Windows (STATISTICA, StatSoft, Inc. (2003), version 6. www.statsoft.com.). An $\alpha = 0.05$ was *a priori* selected for significant differences.

RESULTS

Body weight

Seven days after ovariectomy and before starting the treatment, body weight significantly increased ($p<0.05$) in all ovx groups (positive control and treated groups) compared with sham group, while estradiol administered alone for the 14 days treatment period, not D-004 (400 mg/kg), prevented the increased bodyweight gain induced with ovariectomy, and the combined therapy with D-004 plus estradiol produced the same effect as estradiol alone (Table 1). Therefore, D-004 did not exhibit an estrogenic or antiestrogenic like effect on increased bodyweight gain in ovx rats.

Uterotrophic assay

The relative and absolute uterus weight significantly decreased in the positive control group with respect to the sham ($p < 0.001$), an effect significantly ($p < 0.001$) prevented with estradiol, since in this group the relative uterus weight increased compared with the positive

Groups	Body Weight values (g)		
	Day 0	Day 7 post surgery	Day 14 post treatment
Sham	173.25 ± 14.9	189.5 ± 8.2	240.75 ± 8.24
Ovariectomized groups			
Positive control	173.25 ± 13.7	208.38 ± 13.3 +	278.88 ± 13.6 +
Estradiol (30 µg/kg)	173.25 ± 12.6	206.87 ± 11.5 +	248.75 ± 14.7 [a]
D-004 400 mg/kg	173.25 ± 13.7	207.75 ± 14.8 +	275.13 ± 18.8 +
Estradiol 30 µg/kg + D-004 400 mg/kg	173.25 ± 14.4	205.63 ± 14.9 +	239.50 ± 15.6 [a]

Table 1. Effect of D-004 on body weight values (mean ± SD) of ovariectomized rats. SD standard deviation, +p<0.05 Comparison with sham group, a p<0.001, Comparison with positive control, ANOVA.

Groups	Uterus weights	
	Absolute weight (g)	**Relative weight (body weight/organ weight ratio)**
Sham	0.559 ± 0.25	0.232 ± 0.10
Ovariectomized groups		
Positive control	0.124 ± 0.04 $^{+}$	0.044 ± 0.02 $^{+}$
Estradiol (30 ±g/kg)	0.782 ± 0.25 $^{+a}$	0.317 ± 0.11 $^{+a}$
D-004 400 mg/kg	0.147 ± 0.04 $^{+}$	0.054 ± 0.02 $^{+}$
Estradiol 30 ±g/kg + D-004 400 mg/kg	0.819 ± 0.26 $^{+a}$	0.341 ± 0.10 $^{+a}$

Table 2. Effect of D-004 on relative and absolute uterus weight (mean ± SD) of ovariectomized rats. SD standard deviation, $^{+}$ p<0.001 Comparison with sham group, a p<0.001, Comparison with positive control, ANOVA.

Groups	Epithelium height (µm)	Endometrium thickness (µm)
Sham	14.85 ± 0.87	285.42 ± 0.60
Ovariectomized groups		
Positive control	9.69 ± 0.13 $^{++}$	269.15 ± 4.25 $^{+}$
Estradiol (30 ±g/kg)	14.21 ± 0.55 $^{+a}$	285.72 ± 1.71 ^{a}
D-004 400 mg/kg	9.64 ± 0.08 $^{++}$	270.21 ± 1.57 $^{+}$
Estradiol 30 ±g/kg + D-004 400 mg/kg	13.76 ± 0.35 $^{+}$	285.56 ± 2.40 ^{a}

Table 3. Effect of D-004 on the uterine height epithelium and endometrium thickness (mean ± SD) SD standard deviation, $^{+}$ p < 0.01, $^{++}$ p < 0.001 Comparison with sham group, ^{a} p < 0.001, Comparison with positive control, ANOVA.

control. D-004 administered at 400 mg/kg, however, did not prevent the uterus weight decrease, and administered concurrently did not modify the effect of estradiol alone on such variable (Table 2).

Uterine morphology

Ovariectomy reduced the epithelial cell height and the endometrium thickness with respect to the sham group (p<0.001), and a similar situation was found in the D-004-treeated group, while the groups treated with estradiol alone or co-administered with D-004 had both epithelial cell height and endometrium thickness values significantly greater (p<0.001) than the positive controls (Table 3), without differences in both estradiol treated groups.

DISCUSSION

This study demonstrates that D-004 (400 mg/kg) orally given for 14 days did not produce estrogenic/antiestrogenic effects in the uterotrophic assay in the ovx rat. These potential effects of D-004 were investigated because of two main reasons: First, SPLE, a lipid extract of *Serenoa repens* with a composition and origin somewhat similar to those of D-004, had shown to produce both estrogenic and antiestrogenic effects [25,26]. Second, the unbalance between estrogen and androgens contributes to BPH, being postulated that estrogens increase the androgen receptor level or send a signal not by attaching to the sex hormone binding globulin, already bound to the cell membrane, and activating pathways normally considered androgen responsive, instead of to the usual estrogen-binding sites [39].

The compositions of D-004 and SPLE show, although not in the same proportions, the occurrence of free fatty acids like lauric, myristic and oleic, among others, as major components, and in consequence, some common pharmacological effects. Thus, D-004, as most studies document for SPLE, [15] has shown to inhibit competitively prostate 5 α-reductase activity [27], an effect attributable to free fatty acids present in

both D-004 and SPLE, like lauric, myristic and oleic acids [18,19], and to prevent prostate enlargement induced with T in rodents [28-31]. Likewise, D-004 has displayed *in vivo* and *in vitro* antagonism of α1-adrenoreceptors, and the same has been reported for SPLE [40,41].

Nevertheless, both extracts have produced different effects too, probably due to the different proportions of each acid and/or of the presence of other components. Thus, meanwhile SPLE has shown to inhibit the binding of DHT to the androgenic receptors of rat prostate cytosol, D-004 failed to produce such effect [42].

To assess the estrogenic potential of D-004 we used the validated rat uterotrophic bioassay, in which the fall of estrogen levels is induced through eliminating the primary source of estrogens biosynthesis by surgical extirpation of the ovaries. The induced estrogen deficiency produces uterine weight decrease and morphological changes of the endothelial and stromal tissues, like the reduction of epithelial cell height and stromal thickness. Currently, this assay is recommended for evaluating the estrogenic activity of drugs [36-38].

The fact that estradiol treatment compensated all changes induced with ovariectomy supports the validity of this model in our experimental conditions, and consequently, the negative results obtained. On the other hand, although does not represent a specific outcome of this assay, the fact that rat bodyweight increased as a consequence of the estrogenic deficiency due to ovaries removal [36-38,43], and that estradiol, not D-004 (400 mg/kg), prevented the increased bodyweight gain induced with ovariectomy [36-38,43].

Although only one dose of D-004 (400 mg/kg) was assessed, since this dose has been effective in preventing PH induced with T, this negative result indicates that such effect should not be related with a potential estrogenic activity, but with the inhibition of 5-α-reductase already demonstrated [26]. On the other hand, although previous data do not support suspicious of an antiestrogenic effect of D-004, we also evaluated whether D-004 could reduce the estrogenic effect of estradiol, because antiestrogenic effects of SPLE were reported long-time ago, and such effect could lead to drug interactions clinically relevant.

The lack of estrogenic/antiestrogenic effect here found should not be attributed to an inadequate exposure to D-004 treatment, since not only the dose investigated has shown to prevent PH induced with T in rodents [18-21], but also a pharmacokinetic study demonstrated that the radioactivity of (3H)-labelled-oleic acid supplemented to D-004 at 400 mg/kg was absorbed rapidly and broadly in different tissues, concentration in the prostate being the highest [44]. Also, the present data are consistent with those of the study of the oral subchronic (90 days) oral toxicity D-004 in rat, in which doses up to 2000 mg/kg did no modify uterus weight compared with the control group [34].

CONCLUSIONS

According to uterotrophic assay in ovx rats, D-004 orally given at 400 mg/kg is devoid of estrogenic/antiestrogenic activity.

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