Study of policosanol effects on mice germ cells

Salomé Ivonne Fernández, Alexis Rendón, Miriam Noa, Rosa Más and Abilio Laguna.

Department of Pharmacology and Toxicology, Centre of Natural Products, National Center for Scientific Research, 25th Ave. and 158 st., P.O. Box 6414, Havana, Cuba

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Palabras clave: policosanol, mutagenicidad, dominantes letales, morfología espermatozoides.
Key words: policosanol, mutagenicity, dominant lethal test, sperm morphology.

RESUMEN. Policosanol una mezcla bien definida de alcoholes de alto peso molecular, aislada y purificada de la cera de la caña de azúcar (*Saccharum officinarum*) ha demostrado tener efectos como reductor de colesterol y antiagregante plaquetario, y en la actualidad es empleado como medicamento hipocolesterolemizante. Resultados previos han demostrado que el policosanol carece de efectos genotóxicos en modelos *in vitro* en el ensayo de Ames o en cultivo de linfocitos humanos. Igualmente resultados negativos fueron obtenidos en el ensayo de micronúcleos en médula ósea de ratón. En este trabajo se presentan los resultados sobre células germinales de ratón, como parte de su evaluación toxicológica. Después de 6 ó 10 semanas de tratamiento con el policosanol, se realizó un ensayo de dominantes letales en ratones hembras o machos respectivamente, así como el ensayo de la morfología de la cabeza de los espermatozoides y el examen de los testículos en el caso de los animales machos. El conteo de los espermatozoides resultó similar en todos los grupos experimentales. No se detectaron efectos genotóxicos en las células germinales femeninas o masculinas, ni tampoco hallazgos histopatológicos a nivel de los testículos.

ABSTRACT. Policosanol, a defined mixture of higher aliphatic alcohols isolated and purified from sugar cane wax (*Saccharum officinarum*) shows cholesterol-lowering and antiplatelet effects, and is being used as hypocholesterolemic drug. Previous results have demonstrated policosanol lacks genotoxic effects on *in vitro* models *Salmonella typhimurium* or human lymphocyte cultures and *in vivo* on mouse bone marrow micronucleus test in both sexes. Here, policosanol effects on germ cells are presented, as part of its toxicological evaluation. After six or ten weeks of policosanol administration, dominant lethal test was performed on female or male mice respectively, as well as sperm morphology assay and histopathological examination of testis in case of male animals. Sperm counts were similar in all experimental groups. In addition, neither genotoxic effects on female or male germ cells, nor relevant histopathological findings were registered.

INTRODUCTION

Policosanol is a defined mixture of higher primary aliphatic alcohols isolated and purified from sugar cane (*Saccharum officinarum*) wax. Its main component is octacosanol, followed by triacontanol and hexacosanol, while the other alcohols (tetracosanol, heptacosanol, nonacosanol, dotriacontanol and tetracontanol) are minor components.[1] Policosanol reduced serum cholesterol levels in animal models,[2-4] healthy volunteers[5] and in patients with type II hypercholesterolemia[1,6-13] and inhibited the development of atherosclerotic lesions.[14]

Acute, subchronic and chronic toxicological studies performed on several species have shown no-drug related toxicity.[15,16] Moreover, negative results have been demonstrated in carcinogenicity assays performed on mice and rats.[17,18]

Policosanol was evaluated on *Salmonella typhimurium* (Ames test) to detect gene mutations. Likewise, chromosome damage induction was studied using bone marrow micronucleus test on mice, as well as *in vitro* micronuclei and sister chromatid exchange induction on human lymphocyte cell cultures. Negative results were obtained in all these assays.[19,20]

However, when a chronic administration is expected, as in the case of cholesterol lowering therapy, evaluation of toxicity and genotoxicity of any drug on rodent germ cells is recommended by regulatory agencies for safety assessment,[21] even when negative results have been previously obtained.

Thus, the aim of the present study was to determine any deleterious effect of policosanol on male or female gametes through dominant lethal test on mice and sperm morphology assay on male mice.

MATERIAL AND METHODS

Chemicals

Policosanol was supplied by the Center of Natural Products (Havana, Cuba). Its chemical purity was checked by Gas Chromatography, using a Shimadzu (CG-14ª) chromatographer with a flame ionization detector and SPB-5 capillary column. Chemical purity was above 92 %. ThioTEPA (ONCOThioTEPA) was purchased from FARMASIMES S.A. (Barcelona, Spain) Tween 20 and cyclophosphamide monohydrate were purchased from FLUKA (Buchs, Switzerland).

Policosanol was suspended in a 2 % Tween-20 water vehicle for oral administration.

Animals

Experimental assay on treated female mice

Non isogenic albine Swiss-female mice 6 to 8 weeks old and weighing 20 to 22 g obtained from

Center for Laboratory Animals (Cuba) were used. Also male mice weighing 25 to 30 g for mating were obtained from Center for Laboratory Animals. In this assay, male animals were not treated.

Experimental assay on treated male mice

Non isogenic albine Swiss-male and female mice 6 to 8 weeks old and weighing 18 to 25 g obtained from Center for Laboratory Animals (Cuba) were used.

For both assays, animals were adapted to laboratory conditions [temperature (25 ± 2) °C, humidity 55-60 %, 12 h light dark cycle] for a week and free access to water and food was allowed. They were kept in cages (6 to 8 animals each). Wood shaving bedding was changed three times a week. Early observations regarding health status were performed. A sentinel group was also run during this period. Body weight was checked weekly for both groups.

Treatment and sacrifice

The treatments were the following: control (0.9 % NaCl), vehicle control (2 % Tween 20) and policosanol at three dose levels 2.5, 25 and 250 mg/kg. Since no policosanol-related toxicity was detected in general toxicological studies (acute, subchronic and chronic) including doses 1 750 times higher than the maximal daily therapeutic dose (20 mg/d), these dose levels were selected taking into account human therapeutic dose. Thus, selected doses in the present study were approximately 8.75, 87.5 and 875 higher than this dose. Control animals as well as those treated with policosanol received volumes of 0.1 mL/10 g body weight by intragastric route. Animals received five doses/week for 6 or 10 weeks in order to treat the most part of mouse oogenesis or the whole spermatogenesis, respectively. During treatment, animal physical conditions and weight were checked.

Concomitant positive control groups were also included to confirm the sensitivity of the assays and to check scoring criteria. For this purpose ThioTEPA (2 mg/kg b.w., single dose) and cyclophosphamide (20 mg/kg b.w. five daily injections/week) were intraperitoneally injected for sperm morphology assay and dominant lethal test respectively.

Dominant lethal test on treated female mice

Mating: Immediately after six weeks of treatment, policosanol treated and control females were mated with untreated males in a 2:1 ratio (40 treated females). After mating, female mice were housed according to their respective treatment group.

Sperm morphology test

At the end of ten weeks treatment, six animals from the policosanol treated and control groups were immediately sacrificed. Both testis were collected and weighted, to be later fixed in 10 % neutral buffered formalin. Tissues were trimmed paraffin embedded, sectioned, mounted and stained with hematoxylin and eosin. Both epididymis were also processed for sperm morphology assay.

Additionally, ThioTEPA treated animals were sacrificed 35 d after single injection to see effects on treated spermatocytes.

Epididymis were dissected and processed according to Wyrobek *et al*.[22] A proteolytic enzyme, 0.25 % trypsin (50 µL) was added to the sperm suspension on the Petri dish in order to destroy connective tissue and to separate sperms (5 to 10 min). Sperm count was performed in a Newbauer chamber. One hundred sperm heads per animal were analyzed and classified as normal, amorphous, banana type or without hook.[22]

Dominant lethal test on treated male mice

Mating: Immediately after ten weeks treatment concluded, policosanol treated and control male were mated with untreated and virgin females in a 2:1 ratio (20 treated males) for two consecutive weeks. After mating, female mice were housed according to respective treatment group of their males. During the second week, males were mated again with other similar females.

Cyclophosphamide treated male animals were similarly mated, with two virgin females during the first and second week following the five daily applications period.

Sacrifice and data collection

Mated females were sacrificed by cervical dislocation 13 to 15 d after 4th day of mating. Number of pregnant females per group was registered as well as the number of implants, resorptions, corpora lutea and live fetuses were obtained for individual female animals. All these compiled data were expressed per pregnant female as is recommended by U.S. EPA working group, as well as estimated indexes.[21]

Statistical analysis

Animal weight gain was evaluated using regression and comparison analysis of the slopes among different groups. One way analysis of variance was used to test differences between testis weights.

Sperm assay data were transformed according $\sqrt{x}$. The goodness of fit test was used to determine if sample data has a normal distribution. Means were compared by applying the one way analysis of variance to transformed data, and differences among groups were determined using Duncan multiple range test, at a 5 % level of significance.

Dominant lethal data were similarly transformed and processed. Proportion test was used for analyzing pregnancy frequency.

RESULTS

In the present study, the effects of policosanol on male and female gametes and male gonads were examined.

The mean body weights were weekly calculated for treated female or male mice for each treatment group and trend curves were obtained (Figures 1 and 2, respectively). After treatment, mean body weights and trend curves were not significantly different from control groups. No evident clinical or behavioral disturbances were detected along the treatment.

In case of treated female mice, pregnancy frequency was calculated for each treatment group, as well as the number of implants, resorptions (early and late), corpora lutea and live fetuses were obtained for individual animals. Mean and standard deviations of these variables were also calculated (Table 1). No differences were appreciated between five treated groups.

Final body and testis weights as well as sperm counts were obtained for each male animal. Final mean body weights of mice at all dose levels were similar to control, as well as their relative testis weights (TW /BW) (Table 2). Likewise, as it is observed the number of cells per milliliter was unchanged by the treatments.

Histopathological examination showed an encapsulated granuloma with fibrotic thickening in a mouse treated with policosanol at 250 mg /kg . Meanwhile, unilateral testicular atrophy was detected in one control animal (0.9 % NaCl).

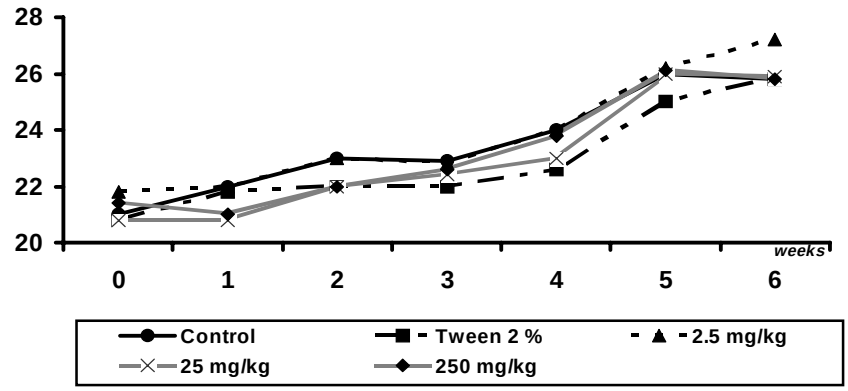

***Fig. 1**. Body weight curves of policosanol treated female mice for 6 weeks.*

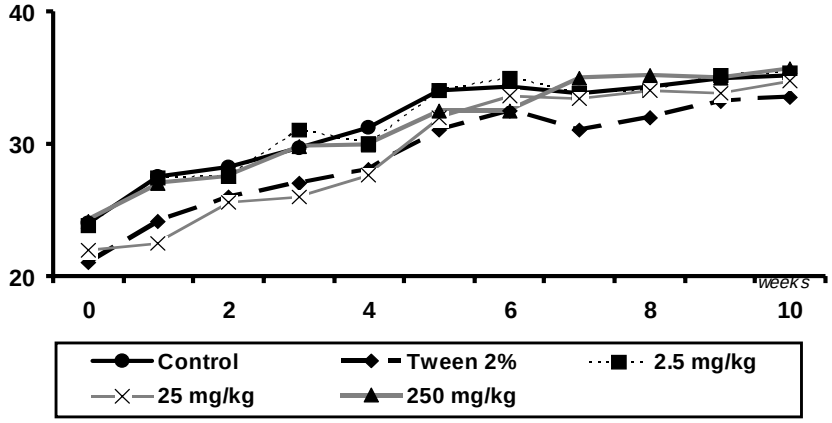

***Fig. 2**. Body weight curves of policosanol treated male mice for 10 weeks*

Sperm head morphology analysis of policosanol treated males yielded no significant differences in the frequency of abnormalities (Table 3). On the other hand, ThioTEPA was able to increase abnormal shapes in treated mice.

Also when male animals were treated, pregnancy frequency was calculated in both mating weeks for each treatment group. Means and standard deviations of the number of implants, resorptions, corpora lutea and live fetuses were calculated for each treatment group in first mating week (Table 4). As it is observed, pregnancy percent in females mated with treated males with intermediate dose (25 mg/kg) was significantly higher than in the others. Since this increase was moderate and no dose related, it can be considered as physiologically no relevant. The indexes calculated of the litters per pregnant female as well as the pregnancy frequency in the rest of policosanol treated groups were similar to those in controls.

Similarly, mean and standard deviations for the same indexes were calculated in the second mat-

Table 1. Results of dominant lethal test on female mice treated with policosanol for six weeks.

Treatment	Pregnancy	Implants	Resorptions			Corpora lutea	Live foetuses
	(%)		Early	Late	Total		
Control[a]	69.5	8.71 ± 2.0	0.89 ± 1.2	0.07 ± 0.2	0.96 ± 1.2	9.85 ± 1.7	7.76 ± 2.1
2 % Tween 20	55.0	9.80 ± 2.3	0.61 ± 0.7	0.00	0.61 ± 0.7	9.61 ± 2.3	8.66 ± 2.4
Policosanol (mg/kg)							
2.5	68	8.86 ± 2.3	1.00 ± 2.2	0.07 ± 0.2	1.06 ± 2.2	9.65 ± 2.0	7.96 ± 2.8
25.0	55.1	8.69 ± 1.5	1.08 ± 1.4	0.43 ± 0.2	1.31 ± 1.4	9.56 ± 1.6	7.56 ± 2.5
250.0	69.4	9.41 ± 2.1	0.68 ± 0.9	0.00	0.68 ± 0.9	9.93 ± 1.8	8.82 ± 2.4

[a] 0.9 % NaCl. All values are expressed as mean ± S.D.

Table 2. Effect of policosanol on testis weight and sperm count in male mice.

Treatment	Animals	Body weight	Testis weight	TW/BW[b]	Sperm count
			(g)		($\cdot 10^6$)
Control[a]	6	37.9 ± 4.2	0.26 ± 0.03	0.70 ± 0.08	67.5 ± 21.3
2 % Tween 20	6	35.1 ± 2.4	0.25 ± 0.02	0.71 ± 0.06	63.0 ± 13.5
Policosanol (mg/kg)					
2.5	6	37.3 ± 3.4	0.27 ± 0.03	0.73 ± 0.07	55.0 ± 15.0
25.0	6	36.8 ± 4.3	0.29 ± 0.04	0.80 ± 0.11	70.7 ± 26.5
250.0	6	38.8 ± 3.1	0.30 ± 0.05	0.78 ± 0.11	57.3 ± 19.1

[a] 0.9 % NaCl. [b] (TW/BW) · 100. All values are expressed as mean ± S.D.

Table 3. Sperm morphology assay in policosanol treated mice.

Treatment	Normal	Amorphous	Banana	Without hook	Abnormals
Control[a]	94.1 ± 2.7	2.7 ± 1.6	1.17 ± 0.8	2.0 ± 1.1	5.9 ± 2.7
2 % Tween 20	97.0 ± 0.7	1.5 ± 0.1	0.36 ± 0.2	1.11 ± 0.7	2.98 ± 0.7
Policosanol (mg/kg)					
2.5	95.6 ± 2.2	2.2 ± 1.6	0.41 ± 0.1	1.7 ± 0.8	4.33 ± 2.2
25.0	96.3 ± 1.9	1.5 ± 0.8	0.78 ± 0.5	1.3 ± 0.9	3.61 ± 1.9
250.0	96.3 ± 1.2	2.3 ± 1.1	0.46 ± 0.2	0.86 ± 0.4	3.68 ± 1.2
TioThepa	85.4 ± 3.0*	11.3 ± 1.6*	1.24 ± 0.49*	2.06 ± 1.0*	14.6 ± 3.0*

[a] 0.9 % NaCl. All values are expressed as mean ± S.D. * $p < 0.05$.

Table 4. Results of dominant lethal test on male mice treated with policosanol for 10 weeks. (First mating week)

Treatment	Pregnancy	Implants	Resorptions			Corpora lutea	Live foetuses
	(%)		Early	Late	Total		
Control[a]	75.0	9.8 ± 1.9	0.89 ± 0.9	0.1 ± 0.3	0.7 ± 1.1	10.3 ± 1.8	9.0 ± 2.1
2 % Tween 20	57.5	10.1 ± 2.4	0.61 ± 1.3	0.09 ± 0.3	1.0 ± 1.3	11.0 ± 1.8	9.1 ± 2.6
Policosanol (mg/kg)							
2.5	77.5	9.6 ± 1.9	0.82 ± 1.3	0.05 ± 0.3	0.9 ± 1.3	10.4 ± 2.1	8.6 ± 2.3
25.0	92.5*	9.8 ± 2.2	0.74 ± 1.1	0.08 ± 0.3	0.8 ± 1.1	10.4 ± 2.1	8.9 ± 2.6
250.0	77.5	10.1 ± 2.1	0.67 ± 1.1	0.14 ± 0.3	0.8 ± 1.1	10.6 ± 1.9	9.3 ± 2.3
Cyclophosphamide	67.5	7.0 ± 2.4*	2.82 ± 2.0*	0.0	2.82 ± 2.0*	10.1 ± 1.8	4.2 ± 1.9*

[a] 0.9 % NaCl. All values are expressed as mean ± S.D. * $p < 0.05$.

Table 5. Results of dominant lethal test on male mice treated with policosanol for 10 weeks. (Second mating week)

Treatment	Pregnancy	Implants	Resorptions			Corpora lutea	Live foetuses
	(%)		Early	Late	Total		
Control[a]	62.5	9.8 ± 1.6	1.11 ± 1.5	0.1 ± 0.4	1.2 ± 1.5	10.5 ± 1.5	8.6 ± 1.2
2 % Tween 20	60.0	9.7 ± 1.9	1.21 ± 1.4	0.09 ± 0.3	1.3 ± 1.4	10.3 ± 1.8	8.4 ± 1.9
Policosanol (mg/kg)							
2.5	67.5	10.2 ± 1.5	0.55 ± 0.8	0.07 ± 0.2	0.6 ± 0.8	10.7 ± 1.9	9.5 ± 1.8
25.0	67.5	9.7 ± 2.1	0.79 ± 0.9	0.13 ± 0.3	0.9 ± 1.1	10.4 ± 2.0	8.8 ± 2.7
250.0	67.5	9.5 ± 2.0	0.64 ± 0.9	0.03 ± 0.1	0.6 ± 0.9	10.1 ± 1.9	8.9 ± 2.0
Cyclophosphamide	60.0	7.4 ± 2.6*	3.34 ± 2.5	0.00	3.34 ± 2.5*	9.6 ± 1.1	4.0 ± 2.9*

[a] 0.9 % NaCl. All values are expressed as mean ± S.D. * $p < 0.05$.

ing week (Table 5). After calculating fertility in these animals, no differences between control and the treated groups were found. Neither the analysis of pregnancy parameters, nor the comparisons of litter size showed significant differences.

Only results obtained in female mice mated with cyclophosphamide treated male animals were significantly different from those observed in controls during first and second mating weeks.

DISCUSSION

Policosanol, a natural product isolated from sugar cane wax, has shown cholesterol lowering effects in animal models,[2-4] healthy volunteers[5] as well as in type II hypercholesterolemic patients.[6-13] Negative results have been obtained in genotoxic studies conducted so far, using prokaryotes as *S. typhimurium* or eukaryotes with *in vitro* and *in vivo* models.[15,16] As long term exposure is expected in these patients, genotoxicity studies must include testing on germinal cells. In such regard, prolonged oral administration (six or ten weeks) was used to treat different maturation stages of female and male germinal cells. Specifically, two methodologies were used with this purpose: sperm morphology and dominant lethal tests on male and female germ cells. These tests are able to detect point[22,23] or chromosome mutations,[21] respectively. Besides, sperm counts, histopathological examination and weights of testis were also regis-

tered, as general toxicological indexes for male gonads.

No policosanol related toxicity was observed in this study, indicating that this treatment does not induce chromosome damage in male or female germ cells, nor point mutations, in these experimental conditions. A positive fertility response was only seen at the intermediate dose on male treated mice, probably not drug related, since it was lacking of dose effect relationship. Otherwise, no relevant alterations in testis were registered at all, after ten weeks administration. Therefore, according these results no risk for male and female germinal cells should be expected after policosanol treatment.

CONCLUSIONS

These results add evidences about no genotoxicity of policosanol. In the present paper, previous negative results obtained on somatic cells are confirmed, but in this case on germ cells of both sexes. In summary, this work corroborates once again that policosanol is a very safe product.

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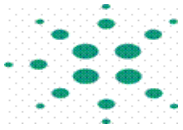

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7. SIMPOSIO INTERNACIONAL DE BIOTECNOLOGIA VEGETAL

Instituto de Biotecnología de las Plantas, Universidad Central ''Marta Abreu'' de Las Villas.

Del 17 al 20 de abril de 2006.

Temáticas

Propagación masiva de plantas. Transformación genética. Biología molecular y bioinformática. Cultivo de células y tejidos. Metabolitos secundarios. Bioseguridad y percepción pública.

Comité Organizador: Dr. Daniel Agramonte Peñalver
biotec2006@ibp.uclv.edu.cu biotvegetal2006@yahoo.es