# *In vitro* mutagenic evaluation of policosanol.

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Key words: policosanol, genotoxicity, sister chromatid exchanges, Ames test, micronuclei.

**RESUMEN**. Policosanol es una mezcla definida de alcoholes de alto peso molecular aislada de la cera de la caña de azúcar. El policosanol posee efectos reductores del colesterol en modelos experimentales con animales de experimentación, voluntarios sanos y pacientes hipercolesterolémicos. La ausencia de toxicidad demostrada consistentemente en los estudios toxicológicos, tanto en modelos agudos, como en los estudios subcrónicos y crónicos llevados a cabo con el policosanol, hizo posible su introducción en la práctica clínica. Por otro lado, al iniciarse la evaluación genotoxicológica, se estudió la inducción de mutaciones génicas mediante el ensayo de Ames. En este estudio, no se detectó inducción de mutaciones puntuales en *Salmonella typhimurium* a las dosis utilizadas desde 5 hasta 2 000 μg/placa. Sin embargo, dosis más elevadas deberían ser investigadas, por lo que una segunda serie experimental fue corrida, cuyos resultados se presentan en el presente trabajo. Además, se evaluaron otros eventos genéticos de especial relevancia, como el daño citogenético en linfocitos humanos *in vitro*. En este caso, también se obtuvieron resultados negativos, al no detectarse incrementos de la frecuencia de micronúcleos o intercambio de cromátidas hermanas después del tratamiento con policosanol de los linfocitos humanos. Estos resultados corroboran los hallazgos negativos previos sobre la mutagenicidad del policosanol.

**ABSTRACT.** Policosanol is a well defined mixture of long chain aliphatic alcohols isolated and purified from sugar cane (*Saccharum officinarum*) wax. Policosanol has cholesterol lowering effects in animal models, healthy volunteers and hypercholesterolemic patients. Its introduction in clinical practice was supported by toxicological studies showing absence of toxicity after acute, sub chronic and chronic treatments. When genotoxicological evaluation was initiated, gene mutation induction was studied by Ames test. Then, no point mutations were induced by policosanol on *Salmonella typhimurium* at dose levels from 5 to 2 000 μg/plate. However as higher doses should be researched, a second trial using five strains was performed and its results are presented in this paper. Additionally, different genetic end points were studied, evaluating cytogenetic damage on human lymphocyte *in vitro*. Negative results were also obtained, no increases of micronuclei or sister chromatid exchanges were observed after treatment of lymphocyte cultures. These results corroborate previous negative findings on mutagenicity of policosanol.

## INTRODUCTION

Policosanol is a well defined mixture of long chain 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 shows cholesterol lowering effects 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] In addition, policosanol inhibits cholesterol biosynthesis and enhances LDL processing in cultured human fibroblasts.[15] Acute, sub chronic and chronic toxicological studies performed on several species have shown no-drug related toxicity.[16,17]

Mutagenicity studies are an essential part in the toxicological evaluation of any synthetic chemical or naturally occurring substance. Point mutations are one of the main damages registered in mutagenicity assays. There is a great number of systems designed to this end, using bacteria, yeasts, fungi and cultured mammalian cells. Nevertheless, the use of S*almonella typhimurium* as a first step to evaluate genotoxicity of substances[18,19] is an established fact.

In consequence, a first trial was conducted to evaluate policosanol with four strains (TA 1535, 1538, 98 and 100) and doses up to 2 000 μg /plate, showing convincing negative results.[20] However, higher doses are recommended by regulatory agencies to be evaluated in this assay.[21]

Additionally, and as part of its genotoxicological evaluation, it is necessary to investigate its effects using models that represent a more complex biological level, which is a second *in vitro* method, but with mammalian cells culture where chromosomal mutations are registered. In this sense, human blood lymphocytes have been widely used because of its characteristics and advantages.[22] Several assays are suitable to identify potential mutagens and carcinogens through the induction of cytogenetic damage. Chromosomal aberrations,[22] sister chromatid exchanges[23] and micronuclei[24] are the genetic end points more frequently used.

As policosanol, is being consistently used as a cholesterol lowering product, a complete evaluation of its potential genetic hazard is needed. Taking into account previous results and recommendations done by regulatory agencies, the authors aimed to confirm them, using the maximal recommended dose 5 000 µg/plate in the five strains of *Salmonella typhimurium*. Besides, sister chromatid exchanges[23] and micronuclei[24] were used to evaluate possible cytogenetic effects of policosanol *in vitro*.

## MATERIAL AND METHODS

### Ames test using *Salmonella typhimurium*

The five strains selected were TA 1535, TA 1537, TA 1538, TA 98 and TA 100**,** all of them auxotrophic for histidine**,** which detect base pair substitutions (TA 1535 and TA 100) and frameshift mutations (TA 1537, 1538 and TA 98).

### Animals

Male Sprague Dawley rats 6 to 8 weeks old and weighing 180-200 g were obtained from Centro Nacional para la Producción de Animales de Laboratorio, Cuba.

Before obtaining microsomal fraction, animals were adapted to laboratory conditions temperature [(25 ± 2) °C and humidity 55-60 % and 12 h light dark cycles] with free access to food and water.

### Liver microsomal fraction

The liver microsomal fraction ($S_9$ mix.) was obtained from male Sprague Dawley rats. Induction of rats was made with Aroclor 1254 (a mixture of polychlorinated biphenyls). Animals were injected intraperitoneally at a dose of 500 mg/kg body weight, and sacrificed 5 d after induction, after a 12 h fast.

### Assay

All tests were done in the standard plate-incorporation assay on at least two separate days, both with and without exogenous activation.[18] Every strain was placed in nutrient broth Oxoid No. 2 for 16 h in a shaker at 37 °C . After incubation, bacteria were plated in Vogel Bonner E medium at 2 % glucose. Policosanol was used after corroborating its quality criteria. It was dissolved in 0.4 % Tween 20 for further dilution in soft agar. The resulting plate concentration was: 5, 50, 500, 2 000 and 5 000 µg/plate. Overnight bacterial culture, test chemical solution or control (0.4 %, Tween 20) and $S_9$ mix or buffer was poured on selective medium plates. Appropriate negative (solvent) controls were run in parallel with each assay. After 48-72 h of incubation, prototrophy reversion was recorded. Each value represents the average for six plates (two experimental series and three plate/dose level and controls).

### Statistical analysis

A non parametric method (Kolmogorov Smirnov test) was used that show significant differences between control and treated groups.[19] The cumulative frequency of mutant colonies across control plates was compared to the cumulative frequency for each dose level. According to this, a Bonferroni correction to the significance level was used ($p < 0.008$).

### Checking for strain characteristics and positive controls

$His^-$ character, $uvrB^-$ deletion, and rfa character were confirmed in all cases according to conventional protocols.[18] Ampicillin resistance was also checked in TA 98 and TA 100.

TA 1535, TA 1538, TA 98 and TA 100 were checked for their response to known mutagens, 2-acetylaminfluoreno (TA 1538 and TA 98) aflatoxin B1 (TA 98) and sodium azide (TA 1535 and TA 100). The two first are indirect mutagens, and then the ability of $S_9$ to convert a suitable positive control to a bacterial mutagen was also confirmed. Plate incorporation assay was used and both assays were run in parallel.

### Lymphocyte cell culture

#### Cell culture medium, reagents and cells

RPMI 1640, gentamicine, L-glutamine, colchicines were purchased (SERVA, Germany), fetal calf serum (LABIOFAM, Cuba), phytohemaglutinine (Wellcome, UK), 5-bromo-2'deoxyuridine (Sigma, USA), and Ficoll Paque (Pharmacia, Sweden).

Peripheral blood was drawn simultaneously by vein puncture from two unrelated and healthy voluntary male donors. Buffy coat was used for micronucleus assay. Lymphocytes were isolated from whole blood, according Ficoll-Paque method recommended by the manufacturer. These cells were used for sister chromatid exchanges experiments.

### Lymphocyte culture conditions

Buffy coat (300 µL) or $5 \cdot 10^6$ cells /mL were suspended in 5 mL growth medium. Its composition was as follows: RPMI 1640 supplemented with L-glutamine (2 mmol/L), gentamicine (40 µg/mL), inactivated 20 % FCS, 5-bromo-2'deoxyuridine (10 µmol/ L) (SCE experiments). The cells were stimulated with PHA (1 %) and maintained in a 5 % $CO_2$ at 37 ° C at a pH between 6.8 and 7.0 for 72 h .

### Preparation of suspensions

Thus, 0.4 % Tween 20/water was used as a vehicle. Policosanol was melted in a sand bath at 80 °C, and 0.120 mL of pure Tween 20 were added. Later on, hot water (30 mL) was added to give 10 mg/mL as final concentration. Two lower concentrations were prepared by dilution with 0.4 % Tween 20: 1 and 0.1 mg /mL . Then, 50 µL from these three suspensions were added to culture medium to reach concentrations $2.5 \cdot 10^{-4}$, $2.5 \cdot 10^{-5}$ and $2.5 \cdot 10^{-6}$. Control cultures were treated with 50 µL 0.4 % Tween 20.

### Policosanol treatment

Treatment with policosanol was started 48 h after the initiation of the cultures for micronucleus assays. Whereas, 24 h after the beginning of the cultures, policosanol was added and maintained until the end, in SCE experiences.

### Micronucleus assay

After incubation, the cells were centrifuged, the medium and the pellet was smeared onto dry slides. Staining was performed with maygrunwald and giemsa as follows; 3 min with Maygrunwald/water (1 : 1), washed with distilled water, and 3 min with a giemsa/phosphate buffer (1 : 10). The preparations were coded and 3 000 cells of each concentration treatment were examined to determine micronucleus frequency. Micronucleus size was also considered, classifying them in relation with the main nucleus (greater and smaller than 1/5 of the major nucleus).

### SCE experiments

Colchicine was present for the last two hours (0.04 µg/mL). Metaphase spreads were obtained by treating the cells with 5 mL 0.075 mol/L KCl for 30 min . After that cells were fixed with Carnoy. They were dropped onto wet and cold slides and stained.[25] Mitotic index was calculated as number of metaphases/1 000 cells, as well as

cell cycle kinetic index [(M1 + M2 + M3)/100 metaphases], and a number of exchanges in 50 second metaphases (M2).

### Positive controls

Bleomycin treatment was started 48 h after the initiation of the cultures for micronucleus assays. In SCE experiences, mytomicin C treatment was done in serum free medium during the first four hours, after that the cultures were initiated in the same conditions as in the testing of potential policosanol genotoxicity. All cultures were stopped after 72 h .

### Statistical analysis

Statistical analysis of data on micronucleus induction was made by using the Kastenbaum and Bowman test, whereas SCE frequency, CKI and MI indexes were compared through an analysis of variance.

## RESULTS AND DISCUSSION

The reversion values were obtained for every plate in the incorporation plate assay. Mean value for each strain, with and without $S_9$ mix, was calculated after different treatments for two experimental series (Table 1). Spontaneous reversion frequencies were found according those reported for the five strains.[18] Likewise, reversion rates found after treatment with direct and indirect mutagens agreed with historical controls in this laboratory for such strains. This result supports the adequate conditions of strains and $S_9$, in order to be used in the experimental series. When the statistical test was performed, no significant differences were detected between dose levels and controls, with and without exogenous metabolic activation. Results of this testing were uniformly negative. No reverse mutations were induced in *Salmonella typhimurium* TA 1535, TA 1537, TA 1538, TA 98 and TA 100 when treated with policosanol at concentrations ranging from 5 to 5 000 µg/plate, thus proving the genotoxic innocuity of policosanol under these experimental conditions and confirming previous negative results.[20]

Means for the SCE and micronucleus frequency, cell kinetics index and the mitotic index were calculated after obtaining individual results for each culture and treatment (Table 2). No citoxic evidences were detected, since mitotic and cell kinetic indexes were similar in the five treatments. No significant differences were found for parameters detecting cytogenetic damage, i.e. SCE and micronucleus frequencies.

## CONCLUSIONS

These results support those found previously in the Ames test, but using the highest dose recommended, so far. Additionally, increases of sister chromatid exchange or micronuclei frequencies were no detected, then no chromosomal damage induction on mammalian cells is reported.

**Table 1**. Reversion values after policosanol treatment in the Ames test.

| Group | Dose | TA 1535 | | TA 1537 | | TA 1538 | |
|---|---|---|---|---|---|---|---|
| | (µg/plate) | $-S_9$ | $+S_9$[a] | $-S_9$ | $+S_9$ | $-S_9$ | $+S_9$ |
| Control | | 8.6 ± 2.8 | 10.3 ± 3.4 | 16.6 ± 4.9 | 20.3 ± 4.2 | 15.0 ± 5.9 | 18.3 ± 2.1 |
| Control Tween | | 12.3 ± 4.1 | 13.8 ± 6.6 | 16.6 ± 2.9 | 17.6 ± 6.8 | 18.6 ± 2.8 | 17.8 ± 4.6 |
| 1 | 5 | 12.1 ± 3.7 | 14.0 ± 4.3 | 17.0 ± 5.9 | 6.0 ± 5.8 | 18.6 ± 4.4 | 19.6 ± 2.2 |
| 2 | 50 | 13.0 ± 4.4 | 9.0 ± 3.8 | 16.3 ± 7.2 | 18.1 ± 8.9 | 15.6 ± 1.7 | 17.0 ± 3.1 |
| 3 | 500 | 9.0 ± 1.4 | 11.0 ± 4.0 | 18.5 ± 4.9 | 21.1 ± 4.0 | 16.0 ± 5.2 | 15.0 ± 3.0 |
| 4 | 2 000 | 13.0 ± 3.5 | 10.0 ± 2.2 | 14.5 ± 6.0 | 17.3 ± 6.8 | 15.0 ± 3.3 | 14.0 ± 3.7 |
| 5 | 5 000 | 13.1 ± 2.6 | 14.5 ± 4.9 | 12.5 ± 4.0 | 17.3 ± 4.2 | 18.8 ± 2.4 | 19.5 ± 2.2 |
| 2AAF | 0.5 | – | – | – | – | 9 ± 1.2 | 52 ± 9.4 |
| AFB1 | 0.05 | – | – | – | – | – | – |
| NaAz | 3.0 | 45 ± 3.2 | – | – | – | – | – |

| Group | Dose | TA 98 | | TA 100 | |
|---|---|---|---|---|---|
| | (µg/plate) | $-S_9$ | $+S_9$ | $-S_9$ | $+S_9$ |
| Control | | 22.3 ± 2.8 | 20.0 ± 4.4 | 112.0 ± 6.0 | 124.0 ± 18.7 |
| Control Tween | | 21.0 ± 3.2 | 18.6 ± 4.0 | 117.8 ± 4.5 | 128.3 ± 8.2 |
| 1 | 5 | 18.5 ± 2.0 | 16.6 ± 2.9 | 114.8 ± 8.5 | 128.0 ± 8.0 |
| 2 | 50 | 23.1 ± 2.1 | 24.4 ± 5.9 | 135.0 ± 20.7 | 109.8 ± 11.5 |
| 3 | 500 | 25.0 ± 4.6 | 26.8 ± 4.7 | 127.6 ± 16.74 | 115.0 ± 19.1 |
| 4 | 2 000 | 25.3 ± 3.3 | 25.0 ± 5.1 | 129.0 ± 16.8 | 121.0 ± 21.8 |
| 5 | 5 000 | 18.3 ± 4.3 | 18.0 ± 4.2 | 114.3 ± 7.3 | 128.3 ± 5.8 |
| 2AAF | 0.5 | 21 ± 2.0 | 120 ± 6.9 | – | – |
| AFB1 | 0.05 | 18 ± 3.4 | 102 ± 9.4 | – | – |
| NaAz | 3.0 | – | – | 378 ± 20.1 | – |

[a] 500 mL of $S_9$ mixture /plate. Each value represents average of six plates (three plates/dose level and two experimental series). 2AAF 2-acetyl aminofluoreno. AFB1 Aflatoxin B1. NaAz Sodium azide.

**Table 2.** Mean values for SCE and micronuclei frequency and cell kinetic and proliferation indexes.

| Treatment | Dose (mol/L) | Mitotic index | Cell proliferation. Index | Number SCE/cell | MN (lymphocytes)[b] | | |
|---|---|---|---|---|---|---|---|
| | | | | | <1/5 | >1/5 | Total |
| Control[a] | | 18 | 1.525 | 7.26 ± 2.4 | 6.5 | 1.5 | 8.00 ± 5.65 |
| Tween 20 0.4 % | | 12 | 1.11 | 8.00 ± 2.7 | 10 | 3.0 | 13.0 ± 1.41 |
| Policosanol | $2.5 \cdot 10^{-6}$ | 14 | 1.47 | 7.34 ± 2.0 | 7 | 0.5 | 7.5 ± 3.53 |
| | $2.5 \cdot 10^{-5}$ | 12 | 1.46 | 8.28 ± 2.4 | 8.5 | 1.5 | 10 ± 4.24 |
| | $2.5 \cdot 10^{-4}$ | 19 | 1.51 | 7.20 ± 3.0 | 5.5 | 2.0 | 7.5 ± 0.07 |
| Bleomycin | 50 µg/mL | | | | | | 21 ± 3.8 |
| Mitomycin C | $10^{-6}$ mol/L | 8 | 0.98 | 25.0 ± 6.2 | | | |

[a] Non treated control.
[b] Frequency of micronucleated lymphocytes

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