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Genotoxicity Assessment of Erythritol by Using Short-term Assay

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Erythritol is a sugar alcohol that is widely used as a natural sugar substitute. Thus, the safety of its usage is very important. In the present study, short-term genotoxicity assays were conducted to evaluate the potential genotoxic effects of erythritol. According to the OECD test guidelines, the maximum test dose was 5,000 μg/plate in bacterial reverse mutation tests, 5,000 μg/ml in cell-based assays, and 5,000 mg/kg for *in vivo* testing. An Ames test did not reveal any positive results. No clastogenicity was observed in a chromosomal aberration test with CHL cells or an *in vitro* micronucleus test with L5178Y $tk^{+/-}$ cells. Erythritol induced a marginal increase of DNA damage at two high doses by 24 hr of exposure in a comet assay using L5178Y $tk^{+/-}$ cells. Additionally, *in vivo* micronucleus tests clearly demonstrated that oral administration of erythritol did not induce micronuclei formation of the bone marrow cells of male ICR mice. Taken together, our results indicate that erythritol is not mutagenic to bacterial cells and does not cause chromosomal damage in mammalian cells either *in vitro* or *in vivo*.

***Key words*:** Erythritol, Ames assay, Chromosomal aberration test, Micronucleus assay, Comet assay, Genotoxicity

INTRODUCTION

Sugar substitutes as a food additive have been used to replace sucrose (table sugar) for sweetening foods and beverages. Some sugar substitutes are natural and others are synthetic or artificial. Most artificial sweeteners are considerably sweeter than table sugar, and smaller amounts are needed to create the same level of sweetness with fewer calories. Nevertheless, the safety of artificial sweeteners has been a concern, especially for their cancer-related risks. Recently, the application and consumption of natural materials has grown quickly because natural materials are considered to be safer than artificial ones. However, a natural additive is not necessarily safe. An individual food additive consists of chemical elements combined in a particular way. Whether it is grown in a garden or manufactured, the chemical structure and composition remain the same.

Because food additives are consumed over a lifetime, they are studied, strictly regulated, and monitored in many countries. Recently, the Korean Food and Drug Administration constructed a database of food additives with their safely risks and supported some studies to collect more genotoxicity data. Erythritol is a sugar alcohol that is approved as a natural sweetener by the Korean Food and Drug Administration, which is present in some fruits and fermented foods (1). It is 60~70% as sweet as table sugar, does not affect blood sugar (2), does not cause tooth decay, and is partially absorbed by the body and then excreted in urine and feces (3). Erythritol shows a unique digestion pathway unlike sugar alcohol (4), has reported side effects (5), and requires labeling by he U.S. Food and Drug Administration (6). Nevertheless, some studies have been conducted to evaluate the genotoxicity of erythritol.

In the present study, the genotoxic profile of erythritol was investigated to evaluate its genotoxicity. Five assessment methods were used as follows: the Ames test, *in vitro* chromosomal aberration test using CHL cells, *in vitro* micronucleus test using L5178Y $tk^{+/-}$ cells, *in vitro* comet assay (a single-cell gel electrophoresis assay) by using L5178Y $tk^{+/-}$ cell, and micronucleus test by using ICR mice.

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MATERIALS AND METHODS

Test article and chemicals. Erythritol in circulation in Korea was purchased at a local marketplace. Chemical and microorganism inspection on element standard of erythritol, as a tetra-alcohol of butane, was performed by Korea Advanced Food Research Institute (Seoul, Korea) according to Korean Food Additives Code. Erythritol was dissolved in distilled water and serially diluted to the appropriate concentrations immediately before use. Most chemicals including positive controls such as 4-nitroquinoline 1-oxide (NQO) and cyclophosphamide were obtained from Sigma (St. Louis, MO). MEM medium, RPMI1640 medium, fetal bovine serum, and penicillin-streptomycin were purchased from GIBCO-Invitrogen (Carlsbad, CA). S9, which was prepared from male Sprague-Dawley rats induced with Aroclor 1254, was from Molecular Toxicology Inc. (Boone, NC) and cofactor for S9 mix was from Wako Pure Chem. Ind., Ltd. (Japan).

Bacterial reverse mutation assay. *Salmonella typhimurium* strains TA98 and TA1537 (detect frame-shift mutagens), and strains TA100, TA1535 and *Escherichia coli* WP2 *uvrA* (detect base-pair substitution mutagens) were used as tester strains. All of the tester strains were purchased from Molecular Toxicology Inc. (Boone, NC). The mutation assay was performed according to the method of Chung *et al*. (7), and Maron and Ames (8). A 0.1 ml aliquot of erythritol containing 156.3~5,000 μg per plate, 0.5 ml of S9 mix (or sodium-phosphate buffer, pH 7.4 for S9 negative group), and 0.1 ml inoculum of the tester strain were added to each tube containing 2 ml of top agar. The mixtures were poured onto the Vogel-Bonner minimal agar plates. Plates were incubated at 37°C for 48 hr. Triplicate plates were run for each assay.

In vitro chromosomal aberration assay. *In vitro* chromosomal aberration assay was performed using Chinese hamster lung fibroblast cells (CHL) (ATCC #CRL-1935), which were obtained from American Type Culture Collection (ATCC, Manassas, VA), as described by Hong *et al*. (9), and Dean and Danford (10) with minor modifications. The assay was consisted of short-term (6 hr) and continuous (24 hr) treatments. Approximately 22 hr after the start of the treatment, colcemid was added to each culture at a final concentration of 0.25 μg/ml. The slides of CHL cells were prepared following the hypotonic-methanol-glacial acetic acid-flame drying-Giemsa schedule for metaphase plate analysis. The 200 metaphases (100 metaphases from each duplicate culture) were selected and analyzed for each treatment group under 1,000 × magnification using a light microscope. The results were expressed as mean aberrant metaphases excluding gaps per 100 metaphases. A valid test required the aberration frequencies of the solvent controls to be within the historical range of the laboratory.

In vitro micronucleus test. The micronucleus assay was performed according to Kirsch-Volders *et al.* (11) with modifications (12). The L5178Y $tk^{+/-}$ cell line (ATCC #CRL-9518, subclone 3.7.2-C) used in this study was provided by ATCC. In the day before erythritol treatment, L5178Y mouse lymphoma cells were seeded at 2×10^5 cells/ml. Cells were treated with erythritol for 3 hr and harvested after a 21-hr recovery period, or treated for 24-hr and harvested immediately. The cellular suspension was centrifuged at 1,000 rpm for 5 min and cells were then resuspended in a KCl 0.075 M solution maintained at room temperature for 10 min (mild hypotonic treatment). The fixation step with methanol/acetic acid (3:1) solution was repeated twice and finally, cells were resuspended in a small volume of methanol/acetic acid and dropped on to clean slides. The slides were stained with 10% Giemsa (pH 6.8). Relative growth was used to assess cytotoxicity, and no cells with reduced cell growth > 20% were scored. Micronuclei were counted in 2000 cells with well-preserved cytoplasm. For a valid test, the negative control had to have < 5%. Mitomycin C and colchicine were used as direct-acting positive controls, and cyclophosphamide was used in the presence of S9 as an indirect-acting positive control. The identification of micronuclei was carried out according to Fenech (13).

In vitro comet assay. Exponentially growing L5178Y $tk^{+/-}$ cells were seeded at 2×10^5 cells in 12-well plates and cultured for 24 hr prior to erythritol treatment, which was carried out for either 3 hr or 24 hr with the indicated concentrations of 50, 100 and 200 μg/ml. Following erythritol treatment, cells were rinsed twice and resuspended at 2×10^5 cells/ml in ice-cold PBS. The comet assay was performed as described by Kim *et al*. (14) and manufacturer's instruction. Briefly, cell suspension (25 μl) was mixed 1:10 with 250 μl molten low melting point (LMP) agarose, and samples of 75 μl of the mixture were rapidly spread on CometSlide™ (Trevigen, Gaithersburg, MD). After gelling for 20 min at 4°C in the dark, slides were put in a tank filled with lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris base, 1% sodium lauryl sarcosinate and 1% Triton X-100) for 1 hr at 4°C in the dark. Slides were then washed three times with neutralization buffer (0.4 M Tris, pH 7.5) for 5 min and incubated in fresh alkaline buffer (0.3 M NaOH and 1 mM EDTA, pH > 13) for 30 min at room temperature to allow unwinding of DNA. Electrophoresis was then carried out at room temperature in fresh ice-cold alkaline electrophoresis buffer for 30 min (1 V/cm; 300 mA). After electrophoresis, slides were gently washed three times for 5 min in fresh neutralization buffer and exposed to 70% ethanol for 5 min. After drying at room temperature, slides were stained with 25 μl of ethidium bromide solution (20 μg/ml). Comets were examined at 200X magnification using a fluorescence microscope (excitation filter, 515~560 nm; barrier filter, 590 nm) connected to a CCD camera. Images of

25 randomly selected nuclei per slide (two slides/culture, duplicate/dose) were analyzed using image-analysis software (Komet 5.0, Kinetic Imaging, Liverpool, UK). Tail Intensity (% of tail DNA) was used as the measure of DNA damage. The results are expressed as the mean ± SD (standard deviation). Methyl methanesulfonate was the direct-acting positive control, and cyclophosphamide was the indirect-acting positive control.

In vivo micronucleus test. Approximately 6 weeks old specific pathogen free male ICR mice were obtained from Orient Co., Ltd. (Seoul, Korea). The animals were housed in polycarbonate cages. An ambient temperature of 22 ± 3°C, relative humidity of 50 ± 10%, and photoperiod of 12 hr was maintained throughout the study. All animals used in this study were cared for in accordance with the principles outlined in the "Guide for the Care and Use of Laboratory Animals", a NIH publication. Gardenia yellow was orally administered two times to groups of six mice at doses of 1,250, 2,500 and 5,000 mg/kg. Mice were killed 24 hr after the final administration, and bone marrow was prepared for evaluation with slight modifications of the method reported by Schmid (15). At least two slides of the cell suspension per animal were made. The air-dried slides were stained with May-Grunwald and Giemsa. Slides were then examined under 1,000 X magnification. Small round or oval shaped bodies, size of which ranging about 1/5 to 1/20 of the diameter of polychromatic erythrocyte (PCE), were counted as micronuclei. A total of 2,000 PCEs were scored per animal by the same observer for determining the frequencies of micronucleated polychromatic erythrocytes (MNPCEs). PCE/(PCE + NCE) ratio was calculated by counting 500 cells.

Statistical analysis. The statistical analyses for *in vitro* chromosomal aberration and *in vitro* micronucleus results were conducted using Statistical Analysis System (SAS) program according to Richardson *et al.* (16). A significant increase in micronuclei at any one concentration was determined based on a $p < 0.05$ from a one-tailed Fisher's exact test pair-wise comparison of each treatment group to control. A concentration-related response was determined based on a $p < 0.05$ from a one-tailed trend test. In CA test, pair-wise analyses of the percent aberrant cells in treated and control cultures were performed using Fisher's exact test. The result was judged as positive when there was a statistically significant and dose-related increase or a reproducible increase in the frequency of micronucleated cells (*in vitro* MN assay) or aberrant metaphases (*in vitro* CA assay). Statistically significant values that did not exceed the range of historic solvent control values were not considered positive. For statistical analysis of comet assay, the homogeneity of variances of data was tested with *Bartlett's test* ($p < 0.05$). If the variances of data were not equal, nonparametric *Kruskal-Wallis test* was used for statistical evaluations ($p < 0.05$ and 0.01). In case of Ames test, the previous results (17) reported that statistical tests tend to identify more experiments as positive than considering the linear dose-response and a 2-fold increase over the spontaneous background for significance. The OECD Test Guideline 471 (18) also suggested that biological relevance of the results should be considered first and statistical methods might be used as an aid in evaluating the test results. Thus, no statistical analysis was performed on Ames results.

RESULTS

Ames assay of erythritol. The mutagenicity of erythritol was evaluated by an Ames assay and no toxicity was observed up to a maximum dose of 5,000 μg/plate (Fig. 1). Compared with the vehicle control, there was no increase in the number of revertant colonies with any dose or strain. There were also no antibacterial effects such as a decrease in the number of colonies for any strain.

In vitro chromosomal aberration assay of erythritol. Results of the *in vitro* chromosomal aberration assay are shown in Table 1. For continuous treatment of CHL cells

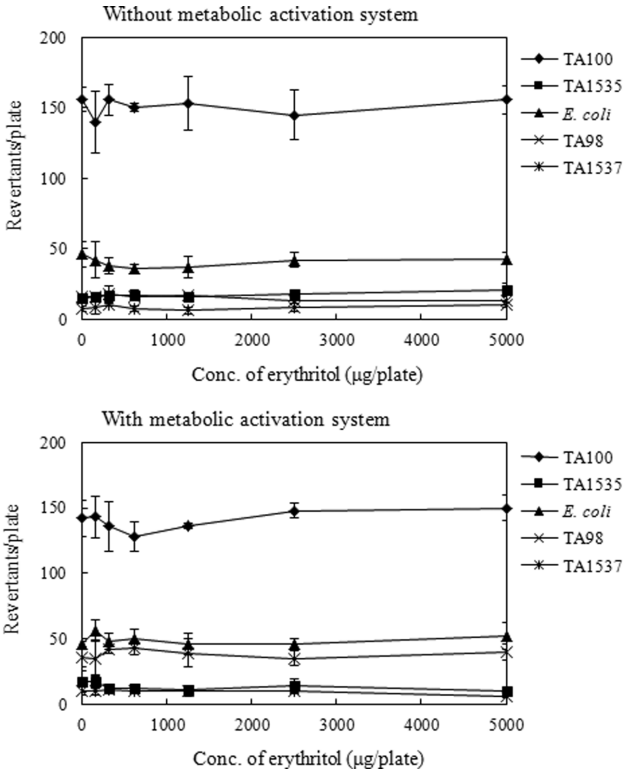

Fig. 1. Effect of erythritol on revertant colonies in the Ames assay. Five test strains (TA100, TA1537, TA98, TA1535, and *E. coli* WP2 *uvrA*) were exposed to erythritol and incubated for 48 hr. Data are expressed as the mean numbers of colonies ± SD from triplicate plates/concentration.

Table 1. *In vitro* chromosomal aberration assay for erythritol

Conc. (g/ml)	S9 mix	Times[a] (hr)	Aberrant metaphases excluding gaps (%)	Aberrant metaphases including gaps (%)
6 hr treatment				
0	+	6-18	0.5[b]	3.0
1250	+	6-18	2.0	5.0
2500	+	6-18	3.5	5.5
5000	+	6-18	1.5	4.5
CPA 5	+	6-18	23.0**[c]	26.5
6 hr treatment				
0	–	6-18	0.0	1.0
1250	–	6-18	0.0	1.5
2500	–	6-18	0.0	0.5
5000	–	6-18	0.0	1.5
MMC 0.1	–	6-18	12.0**	13.0
24 hr treatment				
0	–	24-0	0.5	1.0
1250	–	24-0	0.5	0.5
2500	–	24-0	0.0	0.5
5000	–	24-0	0.0	1.0
MMC 0.1	–	24-0	20.0**	22.0

[a]Time, Chemical treatment time-recovery time.
[b]Means of duplicate cultures; 100 metaphases were examined per culture.
[c]Fisher's exact test; ** significantly different from the control at $p < 0.01$.
Abbreviation: CPA: cyclophosphamide monohydrate, MMC: mitomycin C.

Table 2. *In vitro* micronucleus assay for erythritol

Conc. (g/ml)	S9 mix	Times[a] (hr)	Mean Micronuclei /1000 cells[b]	Relative cell count (%)[c]
3 hr treatment				
0	+	3-21	18.5	100
1250	+	3-21	30.0	75
2500	+	3-21	30.5	76
5000	+	3-21	26.0	67
CPA (5 g/ml)	+	3-21	64.5**[d]	64
3 hr treatment				
0	–	3-21	21.0	100
1250	–	3-21	27.0	85
2500	–	3-21	29.0	83
5000	–	3-21	28.0	84
MMC (0.125 g/ml)	–	3-21	155.0**	89
COL (0.2 g/ml)	–	3-21	117.0**	40
24 hr treatment				
0	–	24-0	18.5	100
1250	–	24-0	28.0	116
2500	–	24-0	26.5	112
5000	–	24-0	30.0	108
MMC (0.0625 g/ml)	–	24-0	148.0**	80
COL (0.2 g/ml)	–	24-0	58.5**	54

[a]Time, Chemical treatment time-recovery time.
[b]2000 cells were examined per culture.
[c]RCC = (Cell counts of treated flask/Cell counts of untreated flask) × 100.
[d]Fisher's exact test; ** $p < 0.01$.
Abbreviation: RCC, Relative cell count; CPA, cyclophosphamide; MMC, mitomycin C; COL, colchicine.

with erythritol at doses up to 5,000 μg/ml, the percentage of total aberrations excluding gaps of erythritol was less than 0.5%. For short-term treatment, the percentage of total aberrations excluding gaps of erythritol was less than 3.5% with or without the S9 mix. Thus, erythritol was non-clastogenic up to the highest feasible concentration that could be evaluated in the assay. As expected, a significant increase was observed in the number of aberrant metaphases in positive controls with cyclophosphamide (CPA) treatment for 6 hr + S and mitomycin C (MMC) treatment for either 6 hr-S or 24 hr-S.

In vitro micronucleus assay of erythritol. Table 2 shows the results of the *in vitro* micronucleus test that was performed at a high concentration (5,000 μg/ml) erythritol. Cytochalasin B has been previously shown to increase the level of spontaneous micronucleated cells in the L5178Y $tk^{+/-}$ cell line (19). Thus, in the present study, the *in vitro* micronucleus test was performed without cytochalasin B that is generally used to ensure the analyzed cells have completed division (20). There was a marginal increase in the number of micronucleated cells, but the difference was not statistically significant. Thus, erythritol was negative in all treatment schedules. Compared with the negative control group, the positive controls (CPA, MMC, and colchicine) induced statistically significant increases in micronucleated cell numbers.

In vitro comet assay of erythritol. Comet assays were performed using a standard 3 hr exposure time with short-term treatment in the absence or presence of the S9 mix and a 24 hr exposure time for continuous treatment at 5,000 μg/ml erythritol. Results of the alkaline comet assay with L5178Y $tk^{+/-}$ cells are shown in Table 3. Slight, but statisti-

Table 3. *In vitro* comet assay for erythritol

Conc. (g/ml)	S9 mix	% Tail DNA[a)]	Olive tail moment	Relative cell count (%)[b)]
3 hr treatment				
0	+	7.86 ± 3.91	5.51 ± 3.23	100
1250	+	8.84 ± 4.64	7.40 ± 6.48	100
2500	+	9.00 ± 5.65	6.15 ± 5.55	84.4
5000	+	8.80 ± 6.87	7.19 ± 10.86	89.3
3 hr treatment				
0	–	7.41 ± 4.55	5.14 ± 5.15	100
1250	–	8.40 ± 5.13	6.73 ± 7.07	76.7
2500	–	9.06 ± 5.25	6.93 ± 5.77	85.6
5000	–	9.97 ± 5.16*	7.81 ± 6.66	71.2
24 hr treatment				
0	–	6.46 ± 3.85	4.41 ± 3.50	100
1250	–	7.11 ± 4.95	4.29 ± 2.83	106.7
2500	–	11.81 ± 6.77**	10.61 ± 9.01	110.6
5000	–	11.89 ± 6.35**	8.59 ± 6.88	97.1
Positive controls				
CPA (10 g/ml, 3 hr)	+	19.29 ± 12.54**	11.51 ± 6.96	54.8
H_2O_2 (200 M, 30 min)	–	88.59 ± 5.17**	78.60 ± 15.37	71.1
MMS (0.1 mM, 3 hr)	–	77.78 ± 6.15**	48.54 ± 6.15	74.0

*$p < 0.05$, **$p < 0.01$, statistically significant vs. negative control according to Kruskal-Wallis test with post-test.
[a)]100 cells were examined per culture.
[b)]Viability measured by trypan blue exclusion; expressed as percentage of absorbance of control.

Table 4. *In vivo* micronucleus assay for erythritol

	Dose (mg/kg)	No. of animal	MNPCE/2000 PCEs (Mean ± S.D.)	PCE/(PCE + NCE) (Mean ± S.D.)
Erythritol	0	5	2.00 ± 1.58	0.47 ± 0.02
	1250	5	3.20 ± 0.84	0.46 ± 0.05
	2500	5	2.60 ± 0.89	0.44 ± 0.02
	5000	5	2.20 ± 1.48	0.43 ± 0.00
MMC	1	5	28.60 ± 6.47*	0.29 ± 0.03

*Significantly different from the vehicle control at $p < 0.05$ (Fisher's exact test).
Abbreviations: PCE: Polychromatic erythrocyte, NCE: Normochromatic erythrocyte, MNPCE: PCE with one or more micronuclei, MMC: mitomycin C (positive control).

cally significant, increases in the percentage of tail DNA were found sporadically. However, these marginal increases were not dose-dependent and none exceeded the maximal fold increase of 2.0. It can thus be concluded that erythritol tested negative in all treatment schedules. There were significant ($p < 0.01$) increases in both the percentage of tail moment and Olive tail moment observed in positive controls treated with CPA and MMS.

In vivo micronucleus test. Table 4 shows the incidence of micronucleated polychromatic erythrocytes (MNPCEs) per 2000 polychromatic erythrocytes (PCEs). Compared with the vehicle control, up to 5,000 mg/kg erythritol did not induce an increase MNPCE numbers. The number of MNPCEs for the vehicle (2.40 ± 1.95) and positive controls (23.20 ± 4.02, $p < 0.05$) were within the expected range for ICR mice. In addition, gardenia yellow had no remarkable effects on the mean number of PCEs per 200 erythrocytes, which is a parameter used to assess cytotoxicity.

DISCUSSION

Recently, there has been a shortage of natural sweeteners in the market because natural is considered healthier than artificial. Erythritol is a sugar alcohol with applications in food and pharmaceutical industries. In particular, erythritol is produced by microbial methods using mostly osmophilic yeasts and mutant strains of *Aureobasidium* sp. and *Pseudozyma tsukubaensis* (21). Because of the high yield and productivity in industrial scale production, erythritol serves as an inexpensive starting material for the production of other sugars. The market for erythritol and other sugar alcohols, including sorbitol, tagalose, maltitol, and xylitol, is growing quickly. The properties of erythritol such as less calories (0.2 kcal/g), a very low glycemic index, high digestive tolerance, and tooth friendly, are beneficial to patients with diabetes and obesity.

Some sugar substitutes have been associated with cancer risks as well as side effects (22). Saccharin was suggested to be involved in bladder cancer using experimental rats (23), and aspartame is associated with cancer as well as neurological and psychiatric side effects (24). However, saccharin is not classified as carcinogenic to humans because of critical interspecies differences in urine composition, despite sufficient evidence of carcinogenicity in animals (25,26). In addition, aspartame is recognized to be safe for consumption at current levels (27). Steviol, a natural extract from the Stevia plant, was also suspected to be a mutagen (28), but the safety of steviol glycoside as well as steviol oxidatives has been proved in many studies (29). Sugar alcohols, including erythritol, are also known to cause problems with the digestive tract, and only a few studies have evaluated the genotoxicity of erythritol. Kawamura *et al*. (30) reported that erythritol does not induce any mutations in reverse mutation assays by using *Salmonella typhimurium* strains and *Escherichia coli*. Erythritol is also negative in chromosome aberration tests using the Chinese hamster fibroblast cell line CHL/IU. Because both test systems are *in vitro*, there is limited extrapolation to human toxicity.

Currently, more precise and accurate test methods are available for regulatory testing. In addition to genotoxicity battery tests, the *in vitro* micronucleus assay using mammalian cells was adopted in OECD test guideline TG 487 in 2010 (31). Validation studies of the comet assay (single cell electrophoresis) *in vitro* and *in vivo* have been conducted in intra- and inter-laboratories within and between countries. Revision of the ICH recommendation for the battery of tests to evaluate genotoxicity has been released to OECD countries.

In the present study, erythritol commercially purchased as a food additive was previously analyzed to confirm the regulatory grade of the Korean Food Additive Codex. Reduced sugars, ribitol and glycerol, as well as heavy metals were not detected in the analysis. Mutagenic revertant colonies were not observed in the Ames test using bacteria, and clastogenic aberration was not detected in the chromosomal aberration test or *in vitro* micronucleus assay using mammalian cells. Furthermore, induction of micronuclei in PCEs was not observed in mice treated with oral administration twice up to the maximal dose of erythritol.

Taken together, erythritol as a natural sweetener may be not mutagenic or clastogenic. However, slight but statistically significant increases in the percentage of tail DNA were sporadically found after 24 hr of treatment with erythritol. Thus, further experiments will be required to confirm the genotoxic effects of erythritol in an appropriate *in vivo* test system.

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