Cytogenetic study in cultured human lymphocytes treated with three commonly used preservatives

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ABSTRACT

Potassium sorbate, sodium benzoate and potassium nitrate have been tested for their genotoxic, cytostatic and cytotoxic potential in human peripheral blood cells in vitro. Potassium nitrate has shown no activity in the test system. When potassium sorbate and sodium benzoate were used at concentrations of 2.0, 0.2 and 0.02 mM no cytostatic activity was detected. However, concentrations of 4 and 8 mM have shown a weak cytostaticity. Additionally, a genotoxic activity using the SCE methodology has been observed at 8 mM of sodium benzoate and at 4 and 8 mM of potassium sorbate. No cytotoxic activity has been induced by the three preservatives. Data demonstrate that the preservatives at low concentrations can be considered as non genotoxic under conditions tested.

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1. Introduction

Food additives play a vital role in today's food supply. A food additive is any substance or mixture of substances, other than basic food components, added to food in a scientifically controlled amount. These additions can be made during production, processing, treatment, packaging, transportation or storage of food. Additives are used to preserve, blend, thicken, flavor and color foods. Since we no longer grow our own foods, additives help keep food safe, wholesome and appealing.

Preservatives are substances which are used to prolong the shelf-life of products by protecting them from deterioration caused by micro-organisms. In late 1988, the European Community adopted a directive which set out the criteria by which additives would be assessed and established the list of additives which could be used, the foods in which they could be used and any maximum levels (Council Directive 89/107/EEC). The use of preservatives is controlled by legislation across the European Union by formulating a specific directive (Council Directive 95/2/EC).

Potassium nitrate (E252) is known for its use in fireworks and gun powder or as fertilizer in plants. In terms of its use in food preservation it is a common ingredient of meat products, such as bacon, cured meats and beefs (Council Directive 95/2/EC). Additionally, it has been used in ice creams and in some toothpaste for sensitive teeth. The chemical structure of potassium nitrate, the molecular formula and molecular weight are shown in Fig. 1.

Potassium sorbate (E202) is the potassium salt of 2,4-hexadienoic acid (sorbic acid). It is used as an antimicrobial and fungi static agent in cigarettes, cheeses and fishes (Ozdemir and Demirci, 2006; Manju et al., 2007). The chemical structure of potassium sorbate is illustrated in Fig. 1.

Finally, sodium benzoate (E211) is the sodium salt of benzene carboxylic acid (benzoic acid). It is not bactericidal, but only bacteriostatic with fungi static activity (Combina et al., 1999). It is active only in acidic conditions and its use as preservative include food products such as fruit juices, pickles, salad dressings and carbonated drinks (Council Directive 95/2/EC).

The acceptable daily intake (ADI) levels recommended by the joint FAO/WHO expert committee on food additives (JECFA) for potassium nitrate, potassium sorbate and sodium benzoate is 0–3.7 mg/kg bw, 0–25 mg/kg bw and 0–5 mg/kg, respectively.

The aim of this research is to examine the genotoxic, cytostatic and cytotoxic effects of these three very commonly used food preservatives. Their activity was evaluated through the simultaneous analysis of three cytogenetic indicators: (a) the levels of SCE/cell, (b) the proliferating rate index (PRI) and (c) the mitotic index (MI), which are indices of genotoxicity, cytostaticity and cytotoxicity, respectively (Lialiaris et al., 2007).

The sister chromatid exchanges assay (SCE assay) was based on the incorporation of the thymidine DNA base analog 5-bromodeoxyuridine (5-BrdU) into the DNA of the cells that replicated twice.

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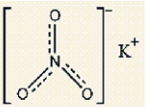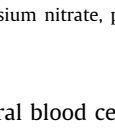*E-mail address:* lialiari@med.duth.gr (T. Lialiaris).

Fig. 1. Chemical structures of potassium nitrate, potassium sorbate and sodium benzoate.

In this study, human peripheral blood cells were used as the test system.

2. Materials and methods

Heparinized blood samples were obtained and cultured from six normal individuals (two males and four females, age 20–30). None of them was a smoker or was receiving drugs for medical or other reasons. Cultures were established by adding 11 drops of whole blood to 5 ml of chromosome medium B (Biochrom 0303 H) in universal containers. For SCEs demonstration 5 μg/ml 5-bromodeoxyuridine (BrdU, CAS No. 59-14-3, EC No. 200-415-9, ≥99.0% pure), potassium nitrate (CAS No. 7757-79-1, EC No. 231-818-8, ≥99.0% pure), potassium sorbate (CAS No. 590-00-1, EC No. 246-376-1, ≥99.0% pure) and sodium benzoate (CAS No. 532-32-1, EC No. 208-534-8, ≥99.0% pure) were added at the beginning of the culture period. The cultures were incubated at 37 °C for 72 h. Throughout, all cultures were maintained in the dark to minimize photolysis of BrdU. Metaphases were collected during the last 2 h with colchicine (CAS No. 64-86-8, EC No. 200-598-5, ≥97.0% pure) at 0.3 μg/ml. At the end of the incubation period cultures were harvested. The chromosome preparations were stained by a modified Fluorescence plus Giemsa (FPG) technique (Goto et al., 1978).

Preparations were scored blindly for cells in their first mitosis (both chromatids dark staining), second mitosis (one chromatid of each chromosome dark staining) (Fig. 2) and third and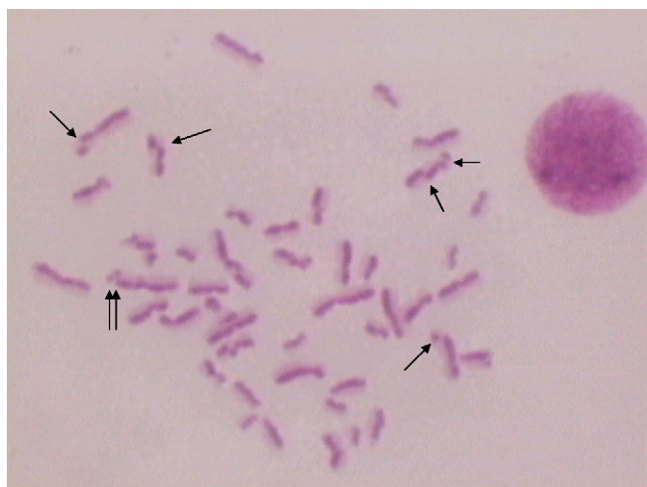 subsequent divisions (a portion of chromosomes with both chromatids light staining). Forty suitably spread second division cells from each culture were scored for SCEs. Slides had been previously coded and scoring was done blindly. Mitotic indices (MIs) for 2000–4000 activated lymphocytes were determined for all cultures. The proliferation rate index (PRI) was calculated as $PRI = (1 \cdot M_1 + 2 \cdot M_2 + 3 \cdot M_{3+})/100$ where M_1 is the percentage of cells in the first division, M_2 in the second and M_{3+} in the third and higher divisions. For PRIs, at least 100 cells were scored.

For the statistical evaluation of MI and PRI χ^2-test was used. For the statistical evaluations of SCE frequencies we needed a multiple analysis of the data. For this purpose, we used the ANOVA procedure in order to analyze total differences between groups and subsequently, we used the Duncan test for the calculations concerning pair-wise comparisons (Lialiaris et al., 1992; Maskaleris et al., 1998).

3. Results

Potassium sorbate (PS) at 8 and 4 mM (cultures 2 and 3, respectively) induced statistically more significant increase ($p < 0.01$) in SCEs/cell in comparison with control, whereas the presence of 2, 0.2 and 0.02 mM in cultures gave statistically more significant decrease ($p < 0.01$) in SCEs/cell compared with concentrations of 8 and 4mM (cultures 2 and 3, respectively) (Table 1).

Statistically more significant ($p < 0.01$) cell division delays caused by 8 and 4 mM PS. 2 mM PS gave statistically significant increase in comparison with the concentration of 8 mM. The concentrations of 0.2 and 0.02 mM gave statistically more significant increase compared to cultures 2 and 3 (concentrations of 8 and 4 mM, respectively). Finally, MIs do not have statistically significant changes in comparison with control (Table 1).

Sodium benzoate (SB) at 8 mM induced statistically more significant increase ($p < 0.01$) in SCEs/cell in comparison with control, whereas SB at 2 mM gave statistically significant decrease ($p < 0.05$) compared to culture 2 (concentration of 8 mM) (Table 2).

SB (8 and 4 mM) caused cell division delays ($p < 0.01$) compared to control. The concentrations of 2, 0.2 and 0.02 mM (cultures 4, 5 and 6, respectively) induced statistically more significant increase ($p < 0.01$) in comparison with the concentrations of 8 and 4mM (cultures 2 and 3, respectively) (Table 2).

None of the concentrations of potassium nitrate (PN) induced statistically significant ($p < 0.01$) changes of SCEs/cell in comparison with control (culture 1) (Table 3). In terms of PRIs, only the concentration of 0.02 mM gave statistically significant cell divisions delays ($p < 0.05$) in comparison with 4 mM PN (Table 3).

PN at 0.2 mM (culture 5) caused a statistically more significant decrease in MI ($p < 0.01$) compared to control. Furthermore, the same concentration gave statistically significant decrease ($p < 0.05$) in comparison with the concentrations of 4 mM (culture 3) (Table 3).

Fig. 2. Second division metaphase after FPG staining methodology (arrows show sister chromatid exchanges). Magnification ×1000.

Table 1
Cytogenetic effects of potassium sorbate (PS) in cultured lymphocytes

Treatment	MI (‰)	Mean SCEs/cell ± SEM (range)		Percent of cells in 1st, 2nd and subsequent (3rd+) divisions			PRI
				1st	2nd	3rd+	
(1) Control	28.5	6.31 ± 0.41	(1–15)	10.0	27.0	63.0	2.53
(2) PS 8 mM	22.0	9.51 ± 0.59[a]	(4–23)	17.0	42.0	41.0	2.24[a]
(3) PS 4 mM	22.5	9.10 ± 0.45[a]	(3–16)	16.0	38.0	46.0	2.30[a]
(4) PS 2 mM	23.8	6.25 ± 0.45[b,d]	(2–14)	13.5	31.5	55.0	2.42[c]
(5) PS 0.2 mM	26.0	5.65 ± 0.74[b,d]	(1–11)	14.5	24.0	61.5	2.47[b,d]
(6) PS 0.02 mM	30.0[c,e]	5.21 ± 0.55[b,d]	(2–10)	15.0	24.0	61.0	2.45[b,d]

The SCE frequency was based on 40 2nd division cells.
Results were based on two experiments with the same protocol.
A total of 200 metaphases were scored for the calculation of PRIs and 4000 activated lymphocytes were scored for MIs.
[a] $P < 0.01$ vs. line 1.
[b] $P < 0.01$ vs. line 2.
[c] $P < 0.05$ vs. line 2.
[d] $P < 0.01$ vs. line 3.
[e] $P < 0.05$ vs. line 3.

Table 2
Cytogenetic effects of sodium benzoate (SB) in cultured lymphocytes

Treatment	MI (‰)	Mean SCEs/cell ± SEM (range)	Percent of cells in 1st, 2nd and subsequent (3rd+) divisions			PRI
			1st	2nd	3rd+	
(1) Control	25.5	6.56 ± 0.43 (1–15)	14.0	36.0	50.0	2.36
(2) SB 8 mM	24.3	9.64 ± 0.73[a] (2–29)	21.0	49.0	30.0	2.09[a]
(3) SB 4 mM	25.3	7.82 ± 0.51 (2–15)	26.5	42.0	31.5	2.05[a]
(4) SB 2 mM	25.8	6.78 ± 0.54[c] (2–16)	17.0	37.0	46.0	2.29[b,d]
(5) SB 0.2 mM	25.5	6.74 ± 0.88 (0–13)	20.0	30.5	49.5	2.30[b,d]
(6) SB 0.02 mM	25.8	6.60 ± 1.02 (1–17)	18.0	31.0	51.0	2.33[b,d]

The SCE frequency was based on 40 2nd division cells.
Results were based on two experiments with the same protocol.
A total of 200 metaphases were scored for the calculation of PRIs and 4000 activated lymphocytes were scored for MIs.
[a] $P < 0.01$ vs. line 1.
[b] $P < 0.01$ vs. line 2.
[c] $P < 0.05$ vs. line 2.
[d] $P < 0.01$ vs. line 3.

Table 3
Cytogenetic effects of potassium nitrate (PN) in cultured lymphocytes

Treatment	MI (‰)	Mean SCEs/cell ± SEM (range)	Percent of cells in 1st, 2nd and subsequent (3rd+) divisions			PRI
			1st	2nd	3rd+	
(1) Control	26.5	6.45 ± 0.43 (1–15)	14.0	36.0	50.0	2.36
(2) PN 8 mM	31.5	7.48 ± 0.45 (4–17)	21.0	38.0	41.0	2.20
(3) PN 4 mM	31.8	7.24 ± 0.46 (2–14)	15.0	42.5	42.5	2.28
(4) PN 2 mM	26.5	6.96 ± 0.53 (2–17)	16.0	32.5	51.5	2.36
(5) PN 0.2 mM	24.0[a,b]	6.63 ± 0.73 (2–15)	14.0	37.0	49.0	2.35
(6) PN 0.02 mM	28.5	5.95 ± 0.66 (2–12)	16.0	36.0	48.0	2.32[b]

The SCE frequency was based on 40 2nd division cells.
Results were based on two experiments with the same protocol.
A total of 200 metaphases were scored for the calculation of PRIs and 4000 activated lymphocytes were scored for MIs.
[a] $P < 0.05$ vs. line 2.
[b] $P < 0.05$ vs. line 3.

From Fig. 3, an indication can be obtained about the cytogenetic effects of potassium sorbate and sodium benzoate in cultured human lymphocytes. PS seems to be more genotoxic than SB and that PN has only slight effects on SCEs.

4. Discussion

Sister chromatid exchanges in one of the most sensitive methods in cytogenetics for detecting potential genotoxic and clastogenic agents (Lialiaris et al., 1987; Lialiaris et al., 1990; Papachristou et al., 2006; Lialiaris et al., 2007). Thus, this study compared the induction of sister chromatid exchanges by potassium sorbate, sodium benzoate and potassium nitrate in cultured human lymphocytes.

4.1. Genotoxicity of potassium sorbate

There have been many studies on the carcinogenicity and genotoxicity of potassium sorbate and the results have been negative (Dickens et al., 1968; Jung et al., 1992; Wurgler et al., 1992; Sasaki et al., 2002). On the other hand, potassium sorbate (3–4 mg/kg) was found to cause chromosomal aberrations and sister chromatid exchanges in Chinese hamster cells (Abe and Sasaki, 1977; Ishidate and Odashima, 1977). A slight rise (1.2 times the control level) of SCEs level was observed at concentration of 20 mg/ml (Hasegawa et al., 1984).

Potassium sorbate at concentrations 120–1200 μg/ml, showed no activity in the Syrian hamster embryo fibroblast micronucleus
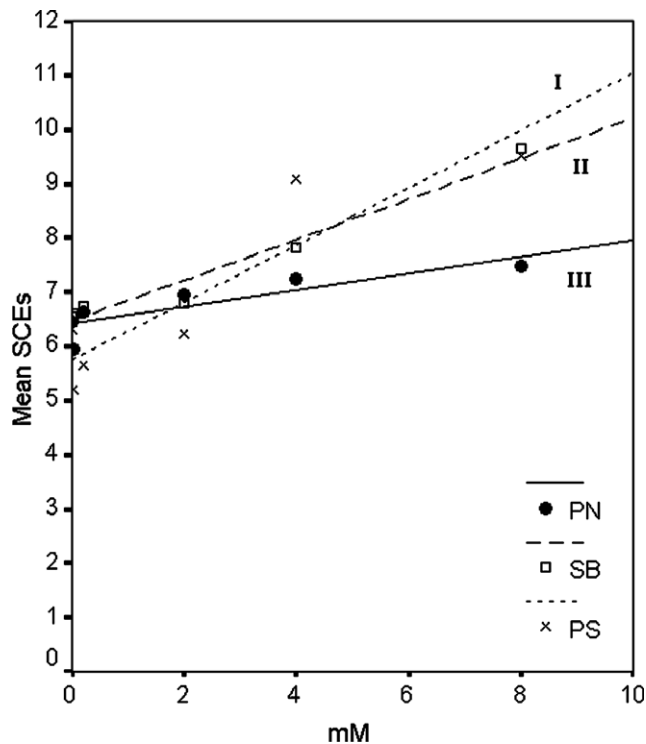

Fig. 3. Effects of potassium sorbate (PS), sodium benzoate (SB) and potassium nitrate (PN) on induction of SCEs in human lymphocytes cultures. The equation of linear regression analysis was obtained by the method of least squares for each line. I, $y = 5.75 + 0.53 \times x$ (SE = 2.90); II, $y = 6.47 + 0.37 \times x$ (SE = 3.05); III, $y = 6.42 + 0.15 \times x$ (SE = 2.80); (x = concentration (mM), y = mean SCEs/cell, SE = Standard error).

assay (Schiffman and Schlatter, 1992). Additionally, the potassium salt of sorbic acid had no genotoxic action in different genetic systems, such as the Salmonella/mammalian-microsome test, the hypoxanthine-guanine-phosphoribosyl transferase (HPRT), the SCE test with Chinese hamster ovary (CHO) cells (10–20 mg/kg), the micronucleus test on bone marrow cells of mice and the chromosome aberration (200 mg/kg) and SCE test on Chinese hamsters (Munzner et al., 1990).

Finally, 2.5 mg/kg potassium sorbate tested in cultured V79 Chinese hamster cells with regard to cell cycle alterations gave negative genotoxicity. Furthermore, 25 mM of this food additive has also been tested using the Drosophila wing somatic mutation and recombination test (SMART) with absence of positive results (Schlatter et al., 1992). Amounts of 0.15%, 1.6% and 3.7% potassium sorbate in cigarettes, did not indicate any genotoxic or cytotoxic potential of this substance (Gaworski et al., 2008).

In the present study, at the two highest tested doses, the potassium sorbate showed a genotoxic action. The level of SCEs/cell is increased 1.5 times over the control level. This weakly genotoxic effect is consistent with other results (Abe and Sasaki, 1977; Ishidate and Odashima, 1977; Hasegawa et al., 1984). Cell division delays at concentrations of 8 and 4 mM were observed indicated that potassium sorbate is a cytostatic agent at these concentrations. Finally, we can say that PS was not considered as a cytotoxic agent.

4.2. Genotoxicity of sodium benzoate

The whole activity of sodium benzoate is reviewed by Nair (2001). More specifically, the genotoxicity tests of this food additive were mostly negative, but some assays were positive. Sodium benzoate in the AMES test (3 mg/plate) and the chromosome aberrations test in Chinese hamster fibroblast cell line (2 mg/ml), was

negative (Ishidate et al., 1984). On the other hand, it was positive in chromosome aberration test carried out on Chinese hamster cells (2 mg/ml), induced chromatid breaks in a large metacentric and two medium-sized metacentric chromosomes (Ishidate and Odashima, 1977).

The SCE test in *Vicia faba* root tip cells and human lymphocytes, at a dose level of 10^{-2} M gave a significant increase of the mean number of SCEs/cell in comparison with the control (Xing and Zhang, 1990). In two different tests, *Salmonella typhimurium* assay and tryptophan reversion assay in *Escherichia coli* strain WP2, this preservative showed no evidence of carcinogenicity (Prival et al., 1991). In a recent study, 20–100 ppm of sodium benzoate tested on root tips of *Allium cepa L.*, gave significant decreases of MI at all concentrations. In the same study was established that the sodium benzoate causes inhibition of spindle formation similar to colchicine (Turkoglu, 2007).

In our results, sodium benzoate at 8 mM showed an increase in SCEs/cell (1.4 times over the control). This genotoxicity of SB is in agreement with findings of Xing and Zhang (1990). At the two largest doses tested (8 and 4 mM), a significant cell cycle delay was observed in comparison with control. This finding is an evidence of a weak cytostaticy of this compound. Similarly to PS, SB gave no evidence of cytotoxicity.

4.3. Genotoxicity of potassium nitrate

Studies in point of the genotoxicity of KNO_3 are limited. The evaluation of genotoxic effects of some food preservatives, showed that potassium nitrate has the less genotoxicity (Sarikaya and Cakir, 2005). At a range of concentrations between 25 and 100 mM in the SMART test of *Drosophila melanogaster*, potassium nitrate gave negative results at 25 mM, but weakly positive at 50, 75 and 100 mM (Sarikaya and Cakir, 2005). Additionally, 3–1000 mg/l of this food additive altered gene expression in human hepatoma HepG2 cell (Bharadwaj et al., 2005).

There are no studies on the genotoxicity of KNO_3 in human lymphocytes. The absence of genotoxicity of potassium nitrate in the tested concentrations is a finding of this study. A concentration of 8 mM, which in other additives causes significant differences compared to controls, did not give any sign of genotoxicity here.

According to PRI and MI, we should take care only of the concentration of 0.2 mM in MI test. The KNO_3 present a weak cytotoxicity, but this data was not considered as safe.

In retrospect, we can consider that SB and PS are not safe for use in concentrations of 8 and 4 mM. These substances gave clear signs of genotoxicity and cytostaticy which must be taken into account by consumers. On the other hand, the PN was considered as safe for use and we proved that this consideration is right. Finally, we should say that the best for our diet is not always the food with additives.

The usage of food preservatives demands more awareness and surveillance. Educational programs and food quality control must be take place often. So, scientists have the responsibility to enrich the public knowledge. Interspecies comparisons or in vitro and in vivo comparisons with or without metabolic activation are prospects of our future research.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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