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Does potassium sorbate induce genotoxic or mutagenic effects in lymphocytes?

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ABSTRACT

The present study evaluates the genotoxic potential of potassium sorbate (PS) in cultured and isolated human lymphocytes. To assess the damage caused by PS in humans, we designed *in vitro* experiments by measuring chromosomal aberrations (CAs), sister-chromatid exchanges (SCEs), micronucleus (MN) and comet assays. Lymphocytes were treated with negative control (sterile distilled water), positive control (MMC for cultured lymphocytes, and H_2O_2 for isolated lymphocytes) and four concentrations (125, 250, 500, and 1000 μg/ml) of PS. According to the results, PS treatment significantly increases the CAs (with or without gaps at 500 and 1000 μg/ml concentrations) and SCEs (at 250, 500, 1000 μg/ml for 24 h and 125, 250, 500, 1000 μg/ml for 48 h) compared with vehicle control. Following treatment of the isolated lymphocytes for 1 h, significant PS-induced DNA strand breaks were observed, at all concentrations. However, PS failed to significantly affect the MN assay. On the contrary, PS does not cause cell cycle delay as noted by the non-significant decrease in the cytokinesis-block proliferation index (CBPI) and replicative index (RI). Only a slight decrease was observed in the mitotic index (MI) at the highest concentration for both treatment times. From the results, PS is clearly seen to be genotoxic to the human peripheral blood lymphocytes *in vitro*.

1. Introduction

Microbial contamination reduces the shelf life of foods and increases the risk of food-borne illness. Traditional food preservation methods against microbial growth include thermal processing, drying, freezing, refrigeration, irradiation, and modified atmosphere packaging, and the addition of antimicrobial agents or salts (Quintavalla and Vicini, 2002). Currently, keen interest is being generated to identify a suitable and an acceptable chemical to reduce or eliminate microorganisms in food preservation. Investigations reveal the many food additives are being used in food preservation as antimicrobial agents, like sorbic acid and its salt, potassium sorbate (PS), in several consumer products, to inhibit yeast and mold growth and control certain bacteria (Dacosta, 1994).

Although food additives continue to cause concern in today's food supply, many studies revealed the potential genotoxic or mutagenic effects of the additives (Ishodate and Odashima, 1977; Abe and Sasaki, 1977; Nagao et al., 1977; Hasegawa et al., 1984; Mukherjee et al., 1988; Sarıkaya and Çakır, 2005; Türkoğlu, 2008). However, many food preservatives are prevalent, whose possible genotoxic effects are still unknown.

PS, used in a wide variety of foodstuffs such as cheeses, pickles, sauces, soft drinks, and fish products as an antimicrobial agent, (Chichester and Taner, 1972; WHO, 1974a,b), displayed mutagenic and/or genotoxic effects that have been assessed by several authors. Mpountoukas et al. (2008) reported that PS showed a weak genotoxic effect at two (4 and 8 mM) of the five (0.02, 0.2, 2, 4, and 8 mM) concentrations in human lymphocytes, with SCEs test. A weak genotoxic effect is consistent with other results obtained in Chinese Hamster cells at 3–4 mg/ml concentration, with CAs test (Abe and Sasaki, 1977; Ishodate and Odashima, 1977), and in Chinese Hamster V79 cells at 20 mg/ml concentration, with CAs, SCEs, and gene mutation tests (Hasegawa et al., 1984).

Contrary to the earlier findings, several studies have reported that PS is not genotoxic in 'Chinese Hamster Ovary' cells (CHO) at 10–20 mg/kg concentration with SCEs, AMES, HPRT tests (Münzner et al., 1990); Syrian Hamster embryo fibroblast cells at 120–1200 μg/ml concentration with MN and cell transformation tests (Schiffmann and Schlatter, 1992); bone marrow cells of mice at 400–1200 mg/kg bw (Jung et al., 1992); different rat organs at 2000 mg/kg concentration with comet test (Sasaki et al., 2002), and HeLa cells (Ferrand et al., 2000).

Due to the controversial results, this study aimed to further evaluate the potential genotoxic activity of PS in human peripheral blood lymphocytes. Blood offers several advantages as a test system (Lerda et al., 2005). The availability of a myriads of human cells in a few milliliters of peripheral blood can easily and repeatedly be drawn from an individual (Türkez and Geyikoğlu, 2007). The blood cells are potentially vulnerable as the highest significantly adverse effects from environmental agents reach other

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tissues through body fluids. Moreover, studies reveal that the human peripheral blood lymphocyte is an extremely sensitive indicator of both *in vivo*- and *in vitro*-induced chromosome structural changes (Lerda et al., 2005). Therefore, in this investigation, we tested PS in human lymphocytes by measuring CAs, SCEs, MN, and comet assays *in vitro*.

2. Materials and methods

2.1. Lymphocyte cultures and isolations

Experiments were performed using human peripheral blood lymphocytes from two healthy volunteers, a man and a woman. The donors fulfilled the following criteria: <30 years old, non-smokers, no medication for at least three weeks prior, and not having undergone radiological examination within three months prior. The experiments were conducted using the same blood samples, divided into two sections: CAs, SCEs and MN were evaluated in whole blood, whereas the comet assay was used to measure the PS-induced DNA strand breakage in isolated lymphocytes.

2.2. Chromosomal aberrations and sister chromatid exchange assay

For the CAs and SCEs experiments, whole blood was added to chromosome medium B (containing fetal bovine serum, heparin, antibiotics, and phytohaemagglutinin) supplemented with 10 μg/ml bromodeoxyuridine. Cultures were incubated in the dark, at 37 °C for 72 h and the cells were treated with PS different concentrations (125, 250, 500, and 1000 μg/ml) for 24 h and 48 h. Besides, a negative control (sterile distilled water) and a positive control (MMC, 0.20 μg/ml dissolved in distilled water) were also maintained throughout every experiment. Next, 0.06 μg/ml colchicine was added 2 h prior to the harvesting of the culture. PS did not change the pH of the culture medium.

To collect the cells, the cultures were centrifuged (216*g*, 15 min), treated with hypotonic KCI solution (0.075 M) for 30 min at 37 °C and then fixed in cold methanol and acetic acid in a 3:1 ratio for 20 min, at room temperature. They were then treated with a fixative, repeated thrice. Finally, metaphase spreads were prepared by dropping the concentrated cell suspension onto slides.

For the CAs assay, the slides were stained with 5% Giemsa (pH = 6.8) prepared in Sorensen buffer solution, for 20–25 min, washed with distilled water, dried at room temperature, and mounted with Depex. CAs were scored from 100 well-spread metaphases per donor. The mean frequency of abnormal cells and the number of CAs per cell (CAs/cell) were calculated. The mitotic index (MI) was determined by scoring of 1000 cells from each donor.

For the SCEs assay, the slides were stained with Giemsa following the methods of Speit and Houpter (1985). The number of SCEs was scored from a total of 50 cells (25 cells from each donor) in the second metaphase stage for each treatment. Besides, a total of 200 cells (100 cells from each donor) were scored to determine the replication index (RI). The RI was calculated using the following formula; $[1 \times M1] + [2 \times M2] + [3 \times M3]/N$ (N = number of observed cells), where M_1, M_2, and M_3 represent the number of cells undergoing the first, second, and third cell cycles, respectively (Schneider et al., 1981).

2.3. Micronucleus assay

Whole blood was incubated at 37 °C for 72 h. PS was added 24 h after phytohaemagglutinin (PHA) stimulation in chromosome medium B. The PS did not alter the pH of the culture medium. At 44 h, after the start of the culture, cyt-B was added to arrest cytokinesis (5.2 μg/ml). Cells were harvested 48 h later, treated with a hypotonic solution (0.075 M KCl for 5 min) and fixed with methanol and glacial acetic acid in a 3:1 ratio, v/v, supplemented with formaldehyde, accordingly Palus et al. (2003) with some modifications. The slides were air-dried and stained with 5% Giemsa. MN was scored from 1000 binucleated cells (BN) per donor. Next, 500 lymphocytes were scored to evaluate the percentage of cells with 1, 2, 3 or 4 nuclei from each donor. The cytokinesis-block proliferation index (CBPI) was calculated according to Surrales et al. (1995) as follows: $[1 \times N1] + [2 \times N2] + [3 \times (N3 + N4)]/N$, where $N1$–$N4$ represent the number of cells with 1–4 nuclei, respectively, and N is the total number of cells scored.

2.4. Comet assay (SCGE)

The comet assay was conducted under alkaline conditions according to Singh et al. (1988). Peripheral blood was obtained with a heparinized syringe, immediately before the conducting the test. Lymphocytes were isolated using Biocoll separating solution. Using Trypan Blue Exclusion Test the viability of cells was found to be >97%. Isolated human lymphocytes were incubated with increasing concentrations of PS for 1 h at 37 °C. Negative and positive controls (H_2O_2, 40 μM) were also included at the same temperature, and exposure time is in parallel with PS. Following incubation, the lymphocytes were centrifuged at 1348*g* for 5 min, and the supernatant was then drawn, and they were resuspended in PBS. Treated cells were suspended in low-melting point agarose (0.65%), and 75 μl of suspension was quickly layered onto slides precoated with normal-melting point agarose (0.65%), and immediately covered with a cover slip. The slides were placed on ice for 10–15 min. After solidification, the coverslip was gently removed and immersed in a cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris pH = 10, in which 10% DMSO and 1% Triton X-100 were added) at 4 °C for at least 1 h. The slides were removed and placed on a horizontal gel electrophoresis platform covered with an electrophoresis buffer (300 mM NaOH, 1 mM EDTA pH >13). The slides were left in the solution for 20 min, to facilitate unwinding of the DNA. The DNA was electrophoresed (25 V, 300 mA) for 20 min, after which the slides were removed and rinsed with neutralization buffer (0.4 M Tris, pH = 7.5). Each slide was stained with 50 μl of 20 μg/ml ethidium bromide.

The slides were examined using a fluorescent microscope (Olympus) equipped with an excitation filter 546 nm and a barrier filter of 590 nm at 400× magnification. Two slides were prepared for each concentration of PS. The tail intensities (%) of 100 comets on each slide (a total of 200 comets per concentration) were determined, using specialized Image Analysis System (“Comet Assay IV”, Perceptive Instruments Ltd., UK).

2.5. Statistical analysis

For the percentage of abnormal cells, CAs/cell, RI, MI, and MN, the results were tabulated and the experimental values were expressed as mean ± SE (standard error). The statistical significance by comparing data among the treatment groups and their vehicle controls was assessed by *z*-test.

The *t*-test was applied for SCEs and comet assay results, comparing the different treatment groups. Concentration–response relationships were determined from correlation and regression coefficients for the percentage of abnormal cell, CAs/cell, SCEs, mean MN and DNA damage.

3. Results

The results of this study indicated that PS significantly increased the CAs and CAs/cell frequency, with and without gaps at