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ORIGINAL RESEARCH

The in vitro genotoxicity of benzoic acid in human peripheral blood lymphocytes

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Abstract The chromosomal aberration (CA), sister chromatid exchange (SCE) and micronucleus test (MN) were employed to investigate the in vitro effect of antimicrobial food additive benzoic acid on human chromosomes. Lymphocytes were incubated with various concentrations (50, 100, 200 and 500 μg/mL) of benzoic acid. The results of used assays showed that benzoic acid significantly increased the chromosomal aberration, sister chromatid exchange and micronucleus frequency (200 and 500 μg/mL) without changing the pH of the medium in a dose-dependent manner. Also this additive significantly decreased the mitotic index (MI) at the highest concentration for 24 h and 100, 200 and 500 μg/mL for 48 h. This decrease was dose-dependent as well. However, it did not effect the replication (RI) and nuclear division (NDI) indices.

Keywords Food additive · Benzoic acid · Sister chromatid exchanges · Chromosome aberrations · Micronucleus test · Human lymphocytes

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Abbreviations

CA	Chromosome aberration
SCE	Sister chromatid exchanges
MN	Micronucleus
BN	Binucleate
MMC	Mitomycin-C
Cyt-B	Cytochalasin B
MI	Mitotic index
RI	Replication index
CBPI	Cytokinesis-block proliferation index

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 (Mpountoukas et al. 2008). These additives are used widely for various purposes, including preservation, coloring and sweetening. Some food additives, however, have been prohibited from use because of their toxicity (Sasaki et al. 2002).

Benzoic acid (E-210) is commonly used as an antimicrobial substance in many food products, used ranged between 150 and 1,000 mg/kg, like as fruit juice, syrup, pickle, ketchup, margarine, biscuit, waffle, cake and cream for preserve these substances from yeast, mould and bacteria effects (Sarıkaya and Solak 2003).

Benzoic acid was tested as negative in several Ames tests and in one DNA damage assay with different *Salmonella typhimurium* strains in the presence or absence of metabolic activation (McCann et al. 1975; Nakamura et al. 1987; Ishidate et al. 1984; Zeiger et al. 1988). Only in one recombination assay with *Bacillus subtilis* H17 and M45 was a positive result obtained (Nonaka 1989). There was no indication of genotoxic activity in tests with mammalian cells (chromosome aberrations in Chinese hamster CHL and CHO cells, sister chromatid exchange in human lymphoblastoid cells and human lymphocytes) without metabolic activation (Oikawa et al. 1980; Tohda et al. 1980; Jansson et al. 1988). However, Ishidate et al. (1984) reported that benzoic acid was weekly positive in chromosomal aberration test in CHO cells and it significantly decreased the life period and increased the somatic mutations in *Drosophila* smart test (Sarıkaya and Solak 2003). Yılmaz et al. (2008) reported that benzoic acid significantly increased the chromosomal aberrations and decreased the mitotic index in *A. sativum* root tips.

Although epidemiological studies of food additives are important in the assessment of toxicological risk to humans, they are difficult because exposure cannot be accurately assessed. Thus, risk assessment largely depends on laboratory toxicity studies (Sasaki et al. 2002). So, the aim of this research is to examine the genotoxic and cytotoxic effects of benzoic acid used as food preservatives in human lymphocytes by using the following assays; sister chromatid exchanges (SCEs), chromosomal aberrations (CAs) and micronucleus (MN) assay in vitro.

Materials and methods

Peripheral venous blood was obtained from two healthy donors (nonsmokers, aged 24–25 years) not exposed to any drug therapy or known mutagenic agent over the past 2 years, not exposed to ionizing radiation within the previous 6 months, and with no history of chromosome fragility or recent viral infection. Blood samples (0.2 mL) were obtained by heparinized syringe and added to 2.5 mL Chromosome Medium B (Biochrom 5025) supplemented with 10 μg/mL bromodeoxyuridine. Lymphocytes were cultured for 72 h at 37 °C. Cells were treated with 50, 100, 200 and 500 μg/mL (the amount used in foods) concentrations of benzoic acid (Sigma Cat. B 3250, dissolved in distilled water) for 24 and 48 h. In addition, a negative and a positive control (mitomycin-C, Sigma Cat. No. 50-07-7, 0.10 μg/mL, dissolved in distilled water), were included for each experiment to ensure validity of the assay. For CA and SCE analysis 0.06 μg/mL colchicines (dissolved in distilled water) was present in the cultures during the last 2 h. The cells were harvested by centrifugation ($216\times g$, 10 min), and the pellet was resuspended in a hypotonic solution of 0.075 M KCI for 30 min at 37 °C. Cells were again centrifuged and fixed in cold methanol acetic acid (3:1) for 20 min. The treatment with fixative was repeated 3 times. At last, slides were made by dropping and air drying. Slides for chromosome aberrations were prepared and conventionally stained with Giemsa and for SCE analysis slides were stained according to FPG (fleuroscence plus giemsa) technique (Speit and Houpter 1985). Chromosomal aberrations were scored from 100 well spread metaphases per donor (totally 200 metaphases per concentration). Mitotic index (MI) was determined by scoring 1,000 cells from each donor. The number of SCE's was scored from a total of 50 cells (25 cells from each donor) under second metaphases for each treatment. In addition, a total of 200 cells (100 cells from each donor) were scored for the determination of the replication index (RI).

For MN analysis, human lymphocytes incubated at 37 °C for 72 and 44 h from the initiation, cytochalasin B (Sigma, Cat. No. C 6762, dissolved in DMSO) at a final concentration of 5.2 μg/mL was added to arrest cytokinesis. Benzoic acid concentrations were added 24 h after phytohaemagglutinin (PHA) stimulation. Micronuclei were scored from 1,000 binucleated cells per donor (totally 2,000 binucleated cells per concentration). Cell proliferation was evaluated using the nuclear division index (NDI).

Results

Chromosomal aberrations

Benzoic acid induced significant increase in the frequency of CAs and CA/cell at all concentrations and treatment periods when compared with negative

Table 1 Chromosome aberrations in cultured human lymphocytes treated with benzoic acid

Test substance	Treatment		Structural aberrations						Numerical aberration	Abnormal cell ± SE (%)	CA/cell ± SE
	Period (h)	Dose (μg/mL)	ctb	csb	cte	ace	dic	scu	p		
Negative control	24	0.00	1	–	–	–	–	1	–	1.00 ± 0.70	0.010 ± 0.007
Positive control	24	0.10	20	5	1	2	–	11	–	19.00 ± 2.77	0.195 ± 0.028
Benzoic acid	24	50	13	–	–	–	–	2	–	7.50 ± 1.86*	0.075 ± 0.019*
		100	11	2	1	–	4	1	–	9.50 ± 2.07**	0.095 ± 0.027**.
		200	15	2	–	–	2	4	2	12.50 ± 2.34**	0.125 ± 0.023**
		500	28	10	–	–	5	2	–	20.00 ± 2.83**	0.225 ± 0.030**
Negative control	48	0.00	4	–	–	–	1	–	–	2.50 ± 1.10	0.025 ± 0.011
Positive control	48	0.10	27	3	7	2	4	10	–	24.00 ± 3.02	0.265 ± 0.031
Benzoic acid	48	50	10	4	–	1	1	5	–	10.50 ± 2.17*	0.105 ± 0.022*
		100	14	2	–	1	2	3	–	11.50 ± 2.26**	0.115 ± 0.023**
		200	15	3	–	3	3	5	–	13.00 ± 2.38**	0.145 ± 0.025**
		500	14	6	–	2	3	10	–	17.50 ± 2.69**	0.175 ± 0.027**

200 metaphases were scored for each treatment

ctb chromatid break, *csb* chromosome break, *cte* chromatid exchange, *ace* acentric fragment, *dic* dicentric chromosome, *scu* sister chromatid union, *p* polyploidy

* Significantly different from the control $P < 0.01$ (z test)

** Significantly different from the control $P < 0.001$ (z test)

control (Table 1). The increase of the frequency in CAs and CA/cell was dose-dependent for 24 and 48 h treatments ($r = 0.95$ and 0.97, respectively for 24 h, $r = 0.85$ and 0.83, respectively for 48 h). The potency of benzoic acid on the induction of CAs was lower (except 500 μg/mL for 24 h) than caused by the positive control. Six types of structural aberrations (chromatid and chromosome breaks, chromatid exchanges, fragments, sister chromatid union and dicentric) and only one type of numerical aberration (polyploidy) were observed. Chromatid breaks and sister chromatid union were the most pronounced aberrations in all experimental groups.

Sister chromatid exchanges, cell cycle and mitotic index

In this study benzoic acid caused an increase in SCEs/cell. This increase was significant at all concentrations and treatments. These effects were dose-dependent at both 24 h ($r = 0.87$) and 48 h ($r = 0.76$) treatments. The potency of benzoic acid on the induction of SCEs/cell was lower than for the positive control. Benzoic acid decreased the replication index, especially at the highest concentration compared with negative control, however this decrease was not significant (Table 2).

Benzoic acid decreased the MI in a dose-dependent manner at 24 h treatment ($r = -0.99$). However, only 500 μg/mL concentration was significantly different from the negative control in this treatment. At 48 h treatment, 100, 200 and 500 μg/mL concentrations significantly decreased the MI in a dose-dependent manner ($r = -0.96$) (Table 2).

Lymphocytes with micronucleus

Table 3 shows that benzoic acid increased the frequency of lymphocytes with micronucleus. This increase was dose-dependent ($r = 0.79$). However, we found that the increase in the micronucleated lymphocytes were statistically significant only in 200 and 500 μg/mL concentrations. The frequencies of MN were lower than for the positive control. This chemical also decreased the cytokinesis block proliferation index (CBPI) but these results were not statistically significant.

Discussion

In vitro genotoxicity tests detect compounds that induce genetic damage directly or indirectly by various mechanisms. One of these test systems is CA which has been considered as an early warning signal for cancer development (Bonassi et al. 1995; Hagmar et al. 1998). SCE represent the interchange of DNA replication products at apparently homologous loci. These exchanges presumably involve DNA breakage and reunion (Pandita 1988). Latt and Schreck (1980) proved SCEs to be a highly sensitive indicator for assessing potential mutagens and carcinogens. MN assay detects both clastogenicity (chromosome breakage) and aneugenicity (chromosome lagging due to dysfunction of mitotic apparatus) (Albertini et al. 2000).

Benzoic acid significantly increased the chromosomal aberration, sister chromatid exchanges and micronucleus frequency (200 and 500 μg/mL) in human lymphocytes without changing of the pH of the medium. It has been reported that low pH itself can be clastogenic to different cell lines including human lymphocyte culture (Morita et al. 1992; Morita 1995). Benzoic acid also decreased the mitotic index at 500 μg/mL for 24 h and at 100, 200 and 500 μg/mL for 48 h. However, it did not affect the replication and nuclear division indices. Benzoic acid induced six types of structural aberrations. The most common aberrations are chromatid breaks which indicates benzoic acid caused DNA double strand breaks and sister chromatid union which is the breakage followed by reunion of both sister chromatids at an identical site (Murli 2003). There are many studies that showed the genotoxicity of different food additives in different cell lines (Macioszek and Kononowicz 2004; Sarıkaya and Çakır 2005; Yılmaz et al. 2008; Mpountoukas et al. 2008).

The mechanism operating in benzoic acid mediated mutation in human lymphocytes is currently unknown. However, genotoxicity may be mediated by inhibition of the activation of XRCC1, PARP-1 and DNA LIG3 proteins which are responsible for DNA repair or inhibition of OP18 stathmin activity that regulates microtubules.

It can be concluded from this study that benzoic acid is weak genotoxic agent especially in lower doses in human lymphocyte cultures when we

Table 2 Frequency of the SCE, RI and the MI in cultured human lymphocytes treated with benzoic acid

Test substance	Treatment		Min–max SCE	SCE/cell ± SE	M_1	M_2	M_3	RI ± SE	MI ± SE
	Period (h)	Dose (μg/mL)							
Negative control	24	0.00	0–7	2.80 ± 0.24	27	95	78	2.26 ± 0.048	6.10 ± 0.54
Positive control	24	0.10	6–40	16.70 ± 1.27	83	68	49	1.83 ± 0.056	4.05 ± 0.44
Benzoic acid	24	50	2–10	4.02 ± 0.24*	38	59	103	2.33 ± 0.055	5.85 ± 0.52
		100	2–12	5.68 ± 0.34*	41	67	92	2.26 ± 0.055	5.35 ± 0.50
		200	2–13	6.62 ± 0.36*	26	64	110	2.42 ± 0.050	4.85 ± 0.48
		500	4–16	7.54 ± 0.35*	40	65	95	2.28 ± 0.055	3.60 ± 0.42***
Negative control	48	0.00	0–6	2.88 ± 0.23	40	83	77	2.19 ± 0.053	6.80 ± 0.56
Positive control	48	0.10	7–43	21.84 ± 1.54	80	69	51	1.86 ± 0.056	3.85 ± 0.43
Benzoic acid	48	50	2–13	6.68 ± 0.37*	34	58	110	2.40 ± 0.054	5.60 ± 0.51
		100	3–14	6.92 ± 0.39*	50	76	74	2.12 ± 0.055	4.65 ± 0.47**
		200	2–19	6.92 ± 0.39*	43	65	92	2.25 ± 0.056	4.15 ± 0.45***
		500	5–17	8.66 ± 0.40*	72	117	11	1.69 ± 0.040	2.15 ± 0.32***

The 50 metaphases were scored for each concentration in SCE. The 200 metaphases were scored for each concentration in RI. The 2000 metaphases were scored for each concentration in MI

* Significantly different from the control $P < 0.05$ (t test)

** Significantly different from the control $P < 0.01$ (z test)

*** Significantly different from the control $P < 0.001$ (z test)

Table 3 Induction of micronuclei in cultured human lymphocytes treated with benzoic acid

Test substance	Treatment		BN cells scored	Distribution of BN cells according to the no. of MN				MN (%)	Nuclear division index (NDI)
	Period (h)	Dose (μg/mL)		(1)	(2)	(3)	(4)		
Negative control	48	0.00	2,000	4	–	–	–	0.20 ± 0.09	1.44 ± 0.38
Positive control	48	0.10	2,000	73	7	1	–	4.50 ± 0.46	1.28 ± 0.36
Benzoic acid	48	50	2,000	7	1	–	–	0.45 ± 0.15	1.49 ± 0.38
		100	2,000	10	–	–	–	0.50 ± 0.16	1.45 ± 0.38
		200	2,000	10	2	–	2	1.10 ± 0.23**	1.45 ± 0.38
		500	2,000	18	1	–	–	1.00 ± 0.22*	1.34 ± 0.36

BN binucleated, *MN* micronucleus

* Significantly different from the control $P < 0.01$ (z-test)

** Significantly different from the control $P < 0.001$ (z-test)

compare with positive control MMC. Further genotoxicity studies should be conducted especially under in vivo conditions.

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