Article

Toxicology and Industrial Health
29(2) 181–186

DOI: 10.1177/0748233711428642
tih.sagepub.com

Genotoxic and cytotoxic effects of storax *in vitro*

Bulent Karadeniz, Zeynep Ulker and Lokman Alpsoy

Abstract

The aim of this study is to investigate the effects of the storax balsam, which is a kind of sweet gum obtained from the *Liquidambar orientalis* Mill trees, on cell viability, cytotoxicity and genotoxicity in human lymphocyte *in vitro*. We studied the genotoxic effects of the extract of storax balsam (SE) using sister chromatid exchange (SCE) test system. Also the cytotoxic and inhibitory effects on cell proliferation of SE were evaluated using lactate *dehydrogenase (*LDH) assay and cell proliferation (WST-1) assay. The SCE frequency was increased when the cells were treated with 1.6 and 4.0 μg/mL SE concentrations ($p < 0.05$). Moreover, treatment of the cells with the same concentrations significantly depleted the cell number at 24th and 48th hours and elevated the LDH levels ($p < 0.05$) at 48th hour. These results suggest that SE can be used as an alternative antibacterial and antipathogenic agent due to its cytotoxic and genotoxic effects.

Keywords

Liquidambar orientalis, storax, genotoxicity, cytotoxicity, sister chromatid exchange (SCE)

Introduction

Liquidambar orientalis Mill (Hamamelidaceae), a medical plant has a local distribution in the southwestern coastal district of Turkey, especially in Koycegiz, Fethiye, Marmaris and Ula. The balsam is produced by injuring of the living trees after many stages. Balsam, sometimes called storax balsam and sweet gum, is frequently used as an antiseptic, topical parasiticide, expectorant and for treatment of some skin and respiratory diseases (Fernandez, 2005; Lee et al., 2009). Another usage area of storax is in perfumery and cosmetic industry as a fixative (Guenther, 1952). Up to date, several researchers reported that storax also has protective activity on many bacteria species, phytopathogenic fungi and nematode (Bayramoğlu, 2010; Kim and Seo, 2008; Lee et al., 2009; Oskay and Sarı, 2007; Sagdic et al., 2005).

Using gas chromatography–mass spectrometry analysis different research have found styrene and cinnamyl alcohol as the major components of storax (Fernandez, 2005; Kim and Seo, 2008). Although Fernandez (2005) reported that Asian storax has styrene (70.4%) as a major component, according to Lee et al. (2009) the major component of storax is trans-cinnamyl alcohol (45.07%).

Styrene was discovered in 1827 by Bonastre during experimentation with alcohol extracts of storax balsam (Tossavainen, 1978). The International Agency for Research on Cancer (IARC) has designated styrene as possibly carcinogenic to humans, with limited evidence of carcinogenicity in experimental animals and inadequate evidence in humans (group 2B; IARC, 1994). Styrene's metabolite called styrene 7,8-oxide (SO) could bind covalently to DNA and cause DNA breaks. Previous studies show that exposure to styrene in work place has been associated with increased frequency of sister chromatid exchange (SCE) (Laffon et al., 2002; Teixeira et al., 2004; Yager et al., 1993). Camurri et al. (1982) reported that increase in SCE frequency reached a threshold level at 60 ppm.

Cinnamyl compounds are a fundamental part of plant biochemistry. Trans-cinnamic acid is ubiquitous

Department of Biology, Fatih University, Istanbul, Turkey

Corresponding author:
Lokman Alpsoy, Department of Biology, Fatih University, 34500 Istanbul, Turkey.
Email: lalpsoy@fatih.edu.tr

in the plant kingdom and is required for lignin formation in plants (Goodwin and Mercer, 1972; JECFA, 2000). Cinnamyl alcohol, cinnamaldehyde and cinnamic acid undergo extensive metabolism. The alcohol is rapidly converted to the aldehyde via alcohol dehydrogenase to cinnamaldehyde, which, in turn, is converted into cinnamic acid (JECFA, 2000). Although several studies reported that cinnamyl alcohol did not increase SCE and micronuclei value (Galloway et al., 1987; Sasaki Yu et al., 1987), some researcher showed that it has mutagenic activity on animal and animal cell culture (Ishidate et al., 1984; JECFA, 2000; Kasamaki et al., 1982, 1987; Kasamaki and Urasawa, 1985).

Although storax has been used for treatment of some skin and stomach diseases, to our knowledge, genotoxic and cytotoxic effects of storax have not been evaluated using human cell culture. It is important to understand the antimicrobial activity of storax and also the use of storax in the treatment of diseases, and what will be effects of this treatment on human cells. In this study, we aimed to determine the genotoxic and cytotoxic effects of storax on human lymphocyte culture *in vitro* using SCE assay, lactate *dehydrogenase* (LDH) assay and WST-1 assay.

Materials and methods

Reagents

Roche Cytotoxicity Detection (LDH) kit and Roche Cell Proliferation Reagent (WST-1) kit were purchased from Roche (Germany). RPMI-1640, phytohaemagglutinin (PHA), foetal bovine serum, PBS, Ficoll, L-glutamine and penicillin–streptomycin (PS) were acquired from Biochrom AG (Germany). Colcemide solution, 5-bromo-2-deoksiüridin (BrdU) and Hoechst 33258 were purchased from Sigma (USA). All other chemicals were of reagent grade.

Preparation of extracts

Storax balsam was purchased from the local market; 0.5 g of balsam was dissolved in 1 mL ethanol, then 4 mL dimethyl sulfoxide was added to the solution. The extract was filtered using 0.22 μm filter and 195 mL of distilled water was added. The concentration of stock solution was 200 μg/mL. Storax extract (SE) was prepared freshly before every experiment.

Preliminary works

First we evaluated the genotoxic effects of SE's concentration (10, 20, 50, 100, 200 μg/mL), and it was found that these concentrations caused the inhibition of cell cycle on human lymphocyte. From these results, concentration of less than 10 μg/mL of SEs was determined to be 0.4, 0.8, 1.6 and 4 μg/mL.

SCE assay

Blood samples were obtained by vein puncture from three (aged 25–35) healthy, nonsmoking men volunteer donors. This study was approved by the local ethics committee. Experiments were also conformed to the guidelines of the World Medical Assembly (Declaration of Helsinki). Lymphocyte cultures were set up by adding 0.5 mL of heparinized whole blood to RPMI-1640 chromosome medium supplemented with 15% heat-inactivated foetal calf serum, 100 IU/mL streptomycin, 100 IU/mL penicillin and 1% L-glutamine. Lymphocytes were stimulated to divide by 1% PHA. CCl_4 (in concentration of 5 μM) and the extract of storax sweet gum (SE) were added to the cultures. The experiments were performed on six groups as follows:

Culture 1: control.
Culture 2: 5 μM CCl_4.
Culture 3: SE 0.4 μg/mL (SE-1).
Culture 4: SE 0.8 μg/mL (SE-2).
Culture 5: SE 1.6 μg/mL (SE-3).
Culture 6: SE 4.0 μg/mL (SE-4).

For SCE demonstration, the cultures were incubated at 37°C for 72 h, and 5-bromo-2-deoxyuridine was added at 8 mg/mL at the initiation of cultures. All cultures were maintained in darkness, and then 0.1 mg/mL of colcemide was added 1 h before harvesting to arrest the cells at metaphase. The cultures were centrifuged at 800*g* for 10 min. Cells were harvested and treated for 30 min with hypotonic solution (0.075 M KCl) and fixed in a 1:3 mixture of acetic acid/methanol (v/v). Bromodeoxyuridine-incorporated metaphase chromosomes were stained with the fluorescence plus Giemsa technique, as described by Perry and Evans (1975). In the SCE study, by selecting 20 satisfactory metaphases, the results of SCE were recorded on the evaluation table. For each treatment condition, well-spread second division metaphases containing 42–46 chromosomes

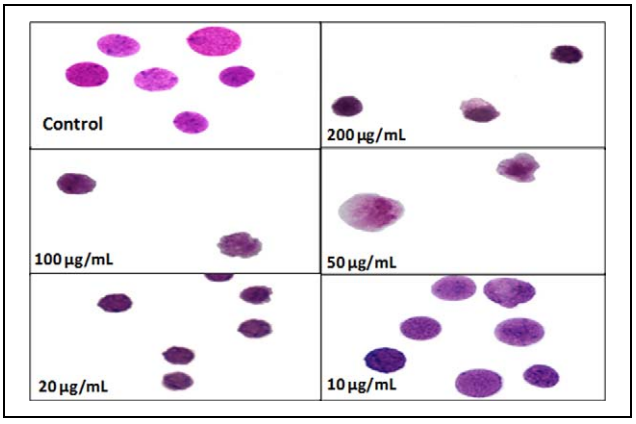

Figure 1. The views of lymphocytes with treatment 5 different SE concentrations.

in each cell were scored, and the values obtained were calculated as SCEs per cell.

Isolation and culture of PBMC

The peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples by density gradient centrifugation with Ficoll. The cells were cultured at 37°C and 5% CO_2 in RPMI-1640 supplemented with 10% foetal bovine serum.

LDH activity assay

The cytotoxic effect of SE was evaluated with the activity of LDH released from cells. Cells were seeded as 1×10^6 cell/mL equally in 96-well plates and incubated with SE at different concentrations at 37°C and 5% CO_2 for 24 and 48 h. Reaction mixture (described in the kit) was added to each well (100 µL) and incubated for 30 min at room temperature. The microplate was protected from light during this incubation period. The absorbance of the samples was measured at 490 nm with enzyme-linked immunosorbent assay (ELISA) reader (BioTek-PowerWaveXS, USA). Assays were performed in triplicate on two independent experiments.

WST-1 assay

The cell viability was assessed using WST-1 assay. Cell were seeded as 1×10^6 cell/mL equally in 96-well plate and incubated with SE at different concentrations at 37°C and 5% CO_2 for 24 and 48 h. WST-1 reagent was added in a 10 µL/well volume. Cells were incubated for 4 h in a humidified atmosphere (37°C, 5% CO_2). The absorbance of the samples was measured at 420 nm with ELISA reader (BioTek-PowerWaveXS). Assays were performed in triplicate on two independent experiments.

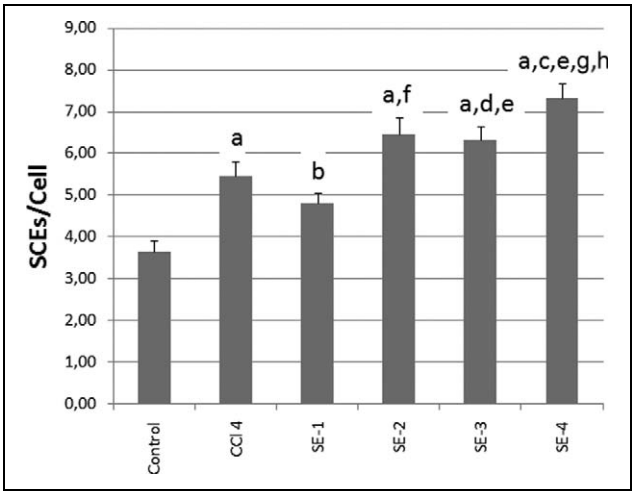

Figure 2. The values for sister chromatid exchanges (SCEs)/cells in the culture of human peripheral lymphocytes in the study groups. [a]$p < 0.001$ compared with control, [b]$p < 0.05$ compared with control, [c]$p < 0.001$ compared with CCl_4-treated group, [d]$p < 0.05$ compared with CCl_4-treated group, [e]$p < 0.001$ compared with SE-1-treated group, [f]$p < 0.05$ compared with storax balsam (SE)-1-treated group, [g]$p < 0.05$ compared with SE-2-treated group and [h]$p < 0.05$ compared with SE-3-treated group.

Statistical analysis

The statistical analysis of SCE values, LDH activity and WST-1 assay results was performed using Mann–Whitney *U* test. A value of *p* less than 0.05 was accepted as statistically significant. Results were expressed as mean ± SD. For these procedures, SPSS 15.0 version for Windows (SPSS Inc., Chicago, Illinois, USA) was used.

Results

High concentrations of SE caused the inhibition of cell cycle and any metaphase area has been found in the all SE-treated groups. Also, human lymphocyte formation was changed by SE treatment (Figure 1).

SE caused significant SCE frequency on peripheral lymphocytes, as seen in Figure 2. SCE frequency increased progressively with increased SE concentration (Figures 2 and 3). This increase was found to be statistically significant ($p < 0.001$ and $p < 0.05$). The SCE frequency in the SE-2-, -3- and -4-treated groups was higher than positive control group (5 µM CCl_4).

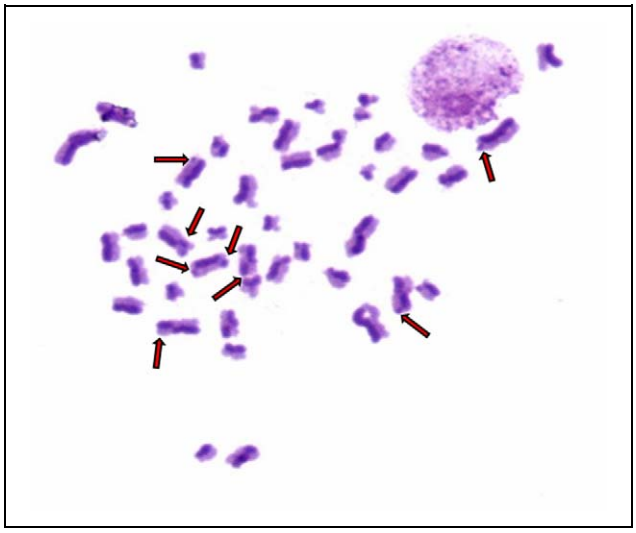

Figure 3. Representative example of storax balsam (SE)-3 treatment shows a cell with spontaneous sister chromatid exchanges.

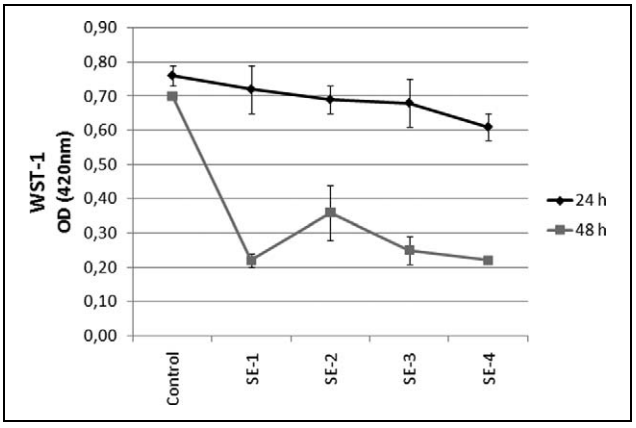

Figure 5. The cell proliferation inhibitory effects of storax balsam (SE) at different concentrations on human lymphocyte cells *in vitro*. [a]$p < 0.05$ compared with control group, [b]$p < 0.05$ compared with SE-1-treated group, [c]$p < 0.05$ compared with SE-2-treated group and [d]$p < 0.05$ compared with SE-3-treated group.

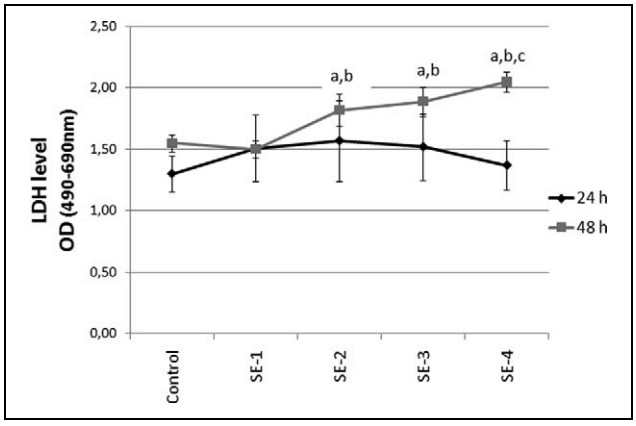

Figure 4. Cytotoxicity effects of storax balsam (SE) at different concentrations on human lymphocyte cells *in vitro*. [a]$p < 0.05$ compared with control group, [b]$p < 0.05$ compared with SE-1-treated group and [c]$p < 0.05$ compared with SE-2-treated group.

Figures 4 and 5 represent the results of the cytotoxicity in the present study, including the LDH and WST-1 assay in the control and experimental groups. Decreases in the cell proliferation and increases in the LDH level were observed after treatment with different concentrations of SEs. Statistical analysis showed a significant difference in LDH level between SE-2-, SE-3- and SE-4-treated group with control group at 48th hour. WST-1 results show that the cell proliferation reduced when the cells were treated with different SE concentrations at 24th and 48th hours ($p < 0.05$).

Discussion

L. orientalis is a medical plant, its balsam has been used as an antiseptic, topical parasiticide, expectorant and for the treatment of some skin and respiratory diseases (Fernandez, 2005; Lee et al., 2009). Previous studies reported that storax has nematidicidal, antimicrobial and antifungal activities. Storax has antibacterial activity against gram-positive and gram-negative bacteria which are resistant to some antibiotics such as nalidixic acid, penicillin G, novobiocin, imipenem, erythromycin, vancomycin, and chloramphenicol (Oskay and Sarı, 2007; Sagdic et al., 2005) and antifungal activity against three phytopathogenic fungi (Lee et al., 2009). In another study, storax showed 100% nematicidal activity at 2.0 mg/mL concentration (Kim and Seo, 2008).

According to Fernandez (2005), the major component of storax is styrene (70.4%). Concern about the potential carcinogenicity of styrene stems largely from the ability of its metabolite, SO, to bind covalently to DNA and from its activity in a variety of genotoxicity test systems (Norppa and Sorsa, 1993). SO has been classified by IARC in group 2A, probably carcinogenic to humans, with sufficient evidence of carcinogenicity in experimental animals and inadequate evidence in humans (IARC, 1994). The ability of SO and styrene to induce DNA strand breaks in mammalian cells has been well documented both *in vitro* (Bastlova et al., 1995; Vodicka et al., 1996) and *in vivo* (Maki-Paakkanen et al., 1991; Walles et al.,

1993). Artuso et al. (1995) obtained a significant styrene exposure-dependent trend for both CAs and SCE among a high-dose group (exposure range 20–326 ppm), a low-dose group (exposure range 0.5–28 ppm) and controls (Artuso et al., 1995).

Cinnamaldehyde was reported to induce chromosome aberrations at low concentrations (i.e. <15 μg/mL) in Chinese hamster fibroblasts and B241 cells tested with and without metabolic activation. In addition that the frequency of micronuclei in polychromatic erythrocytes was not increased when rats or mice were given 1100 mg/kg bodyweight or 1700 mg/kg bodyweight, respectively, of cinnamaldehyde by oral gavages. However, a dose-dependent increase in micronucleated hepatocytes was observed in both rats (1100 mg/kg bodyweight) and mice (850 and 1700 mg/kg bodyweight; Martelli et al., 1993).

Our results show that SE causes DNA damage, cytotoxic effect and the inhibition of cell proliferation *in vivo*. SE has styrene and cinnamyl alcohol which are cytotoxic and genotoxic on animal cells. Our experimental results are parallel with previous studies. The toxic effects of storax on pathogenic organism can be explained by (i) its effects on DNA damage, (ii) its effects on permeability of cell membrane and (iii) its inhibitor effects on cell proliferation.

Conclusion

Due to the toxic ability of storax on pathogenic microorganism, it can be used as an alternative agent for bacterial and fungal disease. When storax was used for the treatment of stomach diseases, it can also result in the cytotoxic and genotoxic effect on human cells.

Acknowledgements

The authors are thankful to the Fatih University, Research Project Foundation for financial support of this study.

Funding

The authors received financial support for this study from Fatih University, Research Project Foundation (Contract no: P50031102_B (1578).

References

Artuso M, Angotzi G, Bonassi S, Bonatti S, De Ferrari M, Gargano D, et al. (1995) Cytogenetic biomonitoring of styrene-exposed plastic boat builders. *Archives of Environmental Contamination and Toxicology* 29(2): 270–274.

Bastlova T, Vodicka P, Peterkova K, Hemminki K, and Lambert B (1995) Styrene oxide-induced HPRT mutations, DNA adducts and DNA strand breaks in cultured human lymphocytes. *Carcinogenesis* 16(10): 2357–2362.

Bayramoğlu EE (2010) Soaking with storax—possibility of using sığla tree (*Liquidambar orientalis* Mill. Var orientalis) storax as bactericide in the soaking float. *Jalca* 105: 62–67.

Camurri L, Codeluppi S, Scarduelli L, and Candela S (1984) Sister chromatid exchanges in workers exposed to low doses of styrene. *Basic Life Science* 29: 957–63.

Fernandez X (2005) Chemical composition of the essential oils from Turkish and Honduras Styrax. *Flavour and Fragrance Journal* 20: 70–73.

Galloway SM, Armstrong MJ, Reuben C, Colman S, Brown B, Cannon C, et al. (1987) Chromosome aberrations and sister chromatid exchanges in Chinese hamster ovary cells: evaluations of 108 chemicals. *Environmental and Molecular Mutagenesis* 10(suppl 10): 1–175.

Goodwin TW, Mercer EI (1972) *Introduction to Plant Biochemistry*. Oxford, England: Pergamon Books.

Guenther E (1952) *The Essential Oils*. Malabor, FL, New York: Krieger Publishing Co., Inc.

IARC (1994) International Agency for Research on Cancer. Some industrial chemicals. IARC Monographs on the evaluation of the carcinogenic risk of chemicals to humans. Vol. 60. Lyon.

Ishidate M Jr, Sofuni T, Yoshikawa K, Hayashi M, Nohmi T, Sawada M, et al. (1984) Primary mutagenicity screening of food additives currently used in Japan. *Food and Chemical Toxicology* 22(8): 623–636.

JECFA (2000) Cinnamyl alcohol and related flavouring agents. WHO Food Additives Series: 46. Prepared by the Fifty-fifth meeting of the Joint FAO/WHO Expert Committee on Food Additives. Geneva, Switzerland: World Health Organization.

Kasamaki A, Takahashi H, Tsumura N, Niwa J, Fujita T, and Urasawa S (1982) Genotoxicity of flavoring agents. *Mutation Research* 105(6): 387–392.

Kasamaki A, Urasawa S (1985) Transforming potency of flavoring agents in Chinese hamster cells. *The Journal of Toxicological Sciences* 10(3): 177–185.

Kasamaki A, Yasuhara T, and Urasawa S (1987) Neoplastic transformation of Chinese hamster cells in vitro after treatment with flavoring agents. *The Journal of Toxicological Sciences* 12(4): 383–396.

Kim J, Seo SM (2008) Nematicidal activity of plant essential oils and components from coriander (*Coriandrum sativum*), oriental sweetgum (*Liquidambar orientalis*), and valerian (*Valeriana wallichii*) essential oils against

pine wood nematode (*Bursaphelenchus xylophilus*). *Journal of Agricultural and Food Chemistry* 56(16): 7316–7320.

Laffon B, Pasaro E, and Mendez J (2002) Evaluation of genotoxic effects in a group of workers exposed to low levels of styrene. *Toxicology* 171(2-3): 175–186.

Lee Y S, Kim J, Lee SG, Oh E, Shin SC, and Park K (2009) Effects of plant essential oils and components from Oriental sweetgum (*Liquidambar orientalis*) on growth and morphogenesis of three phytopathogenic fungi. *Pesticide Biochemistry and Physiology* 93: 138–143.

Maki-Paakkanen J, Walles S, Osterman-Golkar S, and Norppa H (1991) Single-strand breaks, chromosome aberrations, sister-chromatid exchanges, and micronuclei in blood lymphocytes of workers exposed to styrene during the production of reinforced plastics. *Environmental and Molecular Mutagenesis* 17(1): 27–31.

Martelli A, Brambilla-Campart G, Ghia M, and Mereto E (1993) Evaluation of the carcinogenic potential of cinnamaldehyde in a battery of in vivo short-term tests. Proceedings of the Eighty-Fourth Annual Meeting of the American Association for Cancer Research. Vol. 34. Orlando, Florida, 132.

Norppa H, Sorsa M (1993) Genetic toxicity of 1,3-butadiene and styrene. *IARC Scientific Publications* (127): 185–193.

Oskay M, Sarı D (2007) Antimicrobial screening of some Turkish medicinal plants. *Pharmaceutical Biology* 45(3): 176–181.

Perry P, Evans HJ (1975) Cytological detection of mutagen-carcinogen exposure by sister chromatid exchange. *Nature* 258(5531): 121–125.

Sagdic O, Ozkan G, Ozcan M, and Ozcelik S (2005) A study on inhibitory effects of Sigla tree (*Liquidambar orientalis* Mill. var. orientalis) storax against several bacteria. *Phytotherapy Research* 19(6): 549–551.

Sasaki Yu F, Imanishi H, Ohta T, and Shirasu Y (1987) Effects of antimutagenic flavourings on SCEs induced by chemical mutagens in cultured Chinese hamster cells. *Mutation Research* 189(3): 313–318.

Teixeira JP, Gaspar J, Silva S, Torres J, Silva SN, Azevedo M C, et al. (2004) Occupational exposure to styrene: modulation of cytogenetic damage and levels of urinary metabolites of styrene by polymorphisms in genes CYP2E1, EPHX1, GSTM1, GSTT1 and GSTP1. *Toxicology* 195(2-3): 231–242.

Tossavainen A (1978) Styrene use and occupational exposure in the plastics industry. *Scandinavian Journal of Work, Environment & Health* 4(suppl 2): 7–13.

Vodicka P, Stetina R, Kumar R, Plna K, and Hemminki K (1996) 7-Alkylguanine adducts of styrene oxide determined by 32P-postlabeling in DNA and human embryonal lung fibroblasts (HEL). *Carcinogenesis* 17(4): 801–808.

Walles SA, Edling C, Anundi H, and Johanson G (1993) Exposure dependent increase in DNA single strand breaks in leucocytes from workers exposed to low concentrations of styrene. *British Journal of Industrial Medicine* 50(6): 570–574.

Yager JW, Paradisin WM, and Rappaport SM (1993) Sister-chromatid exchanges in lymphocytes are increased in relation to longitudinally measured occupational exposure to low concentrations of styrene. *Mutation Research* 319(3): 155–165.