## Research Paper:

# Cytotoxic Effects of Bisphenol A as an Endocrine Disruptor on Human Lymphocytes

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## ABSTRACT

**Background:** Endocrine compounds, such as Bisphenol A (BPA), stimulate or inhibit the activities of hormones, nuclear receptors in the central nervous system, liver and other organs. They may be disposed of in the environment inadverdently around industrial sites. The aim of this study was to evaluate the cytotoxic effects of BPA on human lymphocytes in culture at varying concentrations.

**Methods:** 0.1 mL heparinized 0.2 mL peripheral blood taken from a healthy male and a female were plated in culture media under sterile conditions. To prepare the reference dose at a concentration of 0.05 mg/mL, 0.027g BPA was dissolved in 1 L dimethyl sulfoxide and the highest dose of 50 µg/mL BPA solution was prepared. After separating the stock solution, 50 µg/mL BPA was diluted to prepare 20, 10 or 5 µg/mL doses.

**Results:** After 24 h of incubation, abnormal cell±Standart Error (%)[AC±SE (%)] 1.10±1.0, chromosomal aberration/cell±Standart Error (CA/cell±SE) 0.025±0.01 was determined in control group, and AC±SE (%) 2.00±0.98 in control group. After 48 h of incubation 0.98, CA/cell±SE was found to be 0.020±0.01. After 24 and 48 h of incubation, AC±SE (%) and CA/cell±SE ratios were 30.00±3.24, 34.00±3.35 and 0.325±0.03, 0.430±0.04, respectively.

**Conclusion:** The cytotoxic effect of BPA on human lymphocytes was investigated in this study at reference concentration and lower doses. Our findings support the fact that BPA substitutes may not be sufficiently safe for widespread use as industrial chemicals.

**Keywords:** Bisphenol A, Cytotoxicity, Chromosome aberrations, Endocrine disruptors

## Introduction

Endocrine disruptors are synthetic or natural chemical substances that disrupt the normal functioning of body by imitating or inhibiting hormones when consumed [1]. They act by increasing or inhibiting the metabolism of endogenous peptidergic or steroidal hormones, or by activating or inhibiting nuclear receptors in the hypothalamus, adipose tissue, liver and other organs [2]. They may be disposed of in habitable environment as a result of industrial activities or may be found naturally in the environment [3]. These substances are thought to disrupt endocrine balance and affect many metabolic processes, such as growth, stress response, sexual development, reproductive processes, and insulin synthesis [3-5]. In recent years, both the scientific community and the general public have gained much interest in endocrine disrupting chemicals and their side effects. The basis for the interest is their widespread use, high exposure and possible health problems they may cause. Endocrine disrupting compounds include household chemicals, pesticides, e.g. Dichloro Diphenyl Trichloroethane (DDT), methoxychlor, industrial compounds, heavy metals, such as lead and mercury, flame-extinguishing chemicals (polybromobiphenyls), antibacterial soaps (triclosan), and drug coatings (phthalates) [6, 7]. Further, human exposure may occur occasionally to such natural endocrine disruptors as soybean compounds, like genistein and daidzein [8].

Bisphenol A (BPA) is produced by condensation of two moles of phenol with one mole of acetone at low pH and high temperature, and is generally referred to as 2,2– (4,4-hydroxyphenyl) propane. Bisphenol A comes as solid, crystal structure, in a creamy white color [9]. This compound in 99%-99.8% purity is used primarily as monomers in the production of polycarbanate plastics and epoxy resins [10]. Other uses of BPA are for food storage bags, water and juice bottles, plastic films covering the inner surface of cola and beer cans, beverage bottles, clothing protectors, compact discs, thermal papers, tooth fillers, optical lenses, and baby bottles in a wide range of daily applications [11, 12].

It is believed that people may be exposed to BPA throughout life, starting with intrauterine period [13]. The most common route of exposure is through to be the consumption of food and beverages from polycarbonate bottles and epoxy resin-coated packages [14]. Heating BPA-coated containers at high temperature results in the deterioration of the structure of epoxy resins and release of hydrolyzed BPA products into the foods, primarily due to prolonged use of plastic containers and the long shelf life of such packages [15]. This compound was first evaluated in 1986 by the Scientific Committee for Food (SCF) of the European Union [16]. It was used to evaluate plastic materials and its contact with foodstuff, based on Toxic Dose Index (TDI) being 0.01 mg/kg/day. Since liver detoxification enzymes are not fully developed in fetal and neonatal period yet, it is thought that the toxic effects of BPA are even more serious during this crucial time [17]. According to the report of European Food Safety Authority (EFSA), the No Observed Adverse Effect Level (NOAEL) value of BPA is 5 mg/kg/day and the TDI is 0.05 mg/kg/day [18].

In the Commission's directive #: 2002/72/EC on materials, especially those in contact with foodstuff, the specific migration limit of BPA is set at 0.6 mg/kg [19]. The arrangements made with BPA in Turkey complies with the European Union directives. The Turkish Food Codex specifies the migration limit of BPA at 0.6 mg/kg in the Communiqué on plastic materials and those in contact with foodstuff [20].

## Materials and Methods

**Lymphocyte culture:** The lymphocytes were taken from two male and female subjects, aged 24 and 25 years, respectively, who were healthy, non-alcoholic, drug-free and without a history of diseases and signed a written informed consent. All procedures were applied according to the guidelines of the International Programme on Chemical Safety (IPCS; a collaborative plan by the United Nations Environment Programme; WHO; Geneva, 1996). The samples were withdrawn as 0.1 mL of heparinized 0.2 mL of peripheral blood, and cultured in 2.5 mL tubes containing culture medium (chromosome medium B) under sterile condition. The culture tubes were then incubated at 37ºC for 72 h.

**Dose selection:** A concentration of 0.05 mg/mL represents the reference dose recommended by EFSA [18]. A 0.01 mg/mL is considered the tolerable daily intake. According to the reference dose, 0.027g of BPA was dissolved in 1L of Dimethyl Sulfoxide (DMSO) to prepare the highest dose of 50 µg/mL BPA solution. Primarily, two third of the prepared solution was used to prepare the 20 µg/mL dose of BPA. This dose was diluted in half to obtain the 10 µg/mL dose, and finally the lowest dose of 5 µg/mL was obtained by diluting the 10 µg/mL dose solution. These doses were added to the medium 24 or 48 h after initiating the culture.

Mitomycin C (MMC) at 0.10 µg/mL was added to the negative and a positive controls and to each dose and chemical groups as well. The colchicine solution was added to each tube at 0.06 µg/mL 2 h before the end of the incubation duirng the 70 h of the culture period. At the end of the incubation, tubes were centrifuged at 1200 rpm for 10 minutes and the supernatants were discarded. The bottom portion of the tubes (0.5-0.7mL) containing the cells were homogenized by vortexing. Then, the hypotonic solution (0.075 M KCl; 37ºC) was added dropwise during vortexing up to 5mL volume.

The tubes were incubated at 37ºC for 30 minutes. At the end of incubation, they were centrifuged at 1200 rpm for 10 minutes, and after discarding the supernatants, a pre-cooled fixative solution of 5:1 methanol:acetic acid was added to the tubes dropwise up to 5 mL while being vortexed. These were kept in a refrigerator for 45 minutes. Again, the tubes were centrifuged at 1200 rpm for

10 minutes and supernatant discarded and washed with the fixative solution three times. After the final washing with the fixative, the precipitate, consisting of 0.5-0.7 mL of white blood cells remained at the bottom of tubes, which were homogenized by pipetting. The suspension, which was drawn into a pasteur pipette, was previously cleaned in 1 N nitric acid ($HNO_3$) and the cells were spread in different areas of humid slides in a refrigerator in 70% ethyl alcohol, and the chromosomes were spread. These preparations were allowed to dry for 24 h at room temperature in the dark. The coverslips were then placed on the slides with entellan.

**Chromosome aberration:** Chromosome Aberration (CA) is one of the most commonly used methods in genetic studies to evaluate the effects of known or suspected genotoxic substances on chromosomes. It is use to determine the frequency of chromosomal abnormalities from peripheral blood lymphocytes in culture.

**Statistical analyses:** In this study, regression analysis was performed, using SPSS v 24 software to reveal the dose-response relationship in chromosome abnormality study of the cells. The z distribution test was used to determine whether chromosomal abnormalities in the experimental groups differed from those in the control group.

## Results

The doses administered and the observed chromosomal abnormalities are shown in Table 1. After 24 h of incubation, AC±SE (%) 1.10±1.00, CA/cell±SE 0.025±0.01 were determined in the control group. Also, AC±SE (%) 2.00±0.98 was measured in the control group after 48hr of incubation. The CA 0.98, CA/cell±SE was found to be 0.020±0.01. After 24 and 48 h of incubation, AC±SE (%) and CA/cell±SE ratios were 30.00±3.24, 34.00±3.35 and 0.32±0.03, 0.430±0.04, respectively. After 24 h of incubation, 3 chromosome fractures, 25 chromotite fractures, 3 polyploidy, 7 disenteric chromosomes, 18 sister chromotite junction and 12 chromatic changes were detected in MMC medium. After the 48 h incubation, 7 chromotite fractures, 3 polyploidy, 6 disenteric chromosomes, 42 sister chromotite junction and 31 chromotite changes were observed. These chromosamal abnormalities in MMC culture media were found to be statistically significant as compared to those in the control group ($P<0.05$). No chromosomal abnormality was observed as a result of

**Table 1.** Observed chromosomal abnormalities

| Practice | | | Chromosomal Abnormalities | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Test Article | Duration (h) | Conc'tion (ppm) | chf | cf | f | p | dic | scj | cc | AC±SE (%) | CA/Cell±SE |
| Control | 24 | 0 | - | - | - | - | - | 2 | - | 1.10±1.00 | 0.025±0.01 |
| MMC | 24 | 0.20 | 3 | 25 | - | 3 | 7 | 18 | 12 | 30.00±3.24 | 0.325±0.03 |
| BPA | 24 | 5 | - | - | - | - | - | - | - | Toxic | Toxic |
| | | 10.0 | - | - | - | - | - | - | - | Toxic | Toxic |
| | | 20.0 | - | - | - | - | - | - | - | Toxic | Toxic |
| | | 50.0 | - | - | - | - | - | - | - | Toxic | Toxic |
| Control | 48 | 0 | - | 1 | - | - | - | 3 | - | 2.00±0.98 | 0.020±0.01 |
| MMC | 48 | 0.20 | - | 7 | - | 3 | 6 | 42 | 31 | 34.00±3.35 | 0.430±0.04 |
| BPA | 48 | 5 | - | - | - | - | - | - | - | Toxic | Toxic |
| | | 10 | - | - | - | - | - | - | - | Toxic | Toxic |
| | | 20 | - | - | - | - | - | - | - | Toxic | Toxic |
| | | 50 | - | - | - | - | - | - | - | Toxic | Toxic |

h: hour; ppm: parts per million; Conc'tion: Concentration; chf: chromosome fractures; cf: chromotite fractures; f: fragment; p: polyploidy; dic: dicentric chromosome; scu: sister chromatid junction; cc: chromatid changes; MMC: Mitomycin C; AC: Abnormal Cell; SE: Standart Error; CA: Chromosomal Aberration; BPA: Bisphenol A bursting the cells at all doses of 5, 10, 20 or 50 µg/mL after either 24 or 48 h of incubation (Table 1).

## Discussion

In recent years, interest in endocrine disrupting chemicals has increased. Endocrine disruptors are frequently used in many industries because of their low production costs and their utility in a wide range of applications. Bisphenol A is an endocrine disruptor and one of the commonly used chemicals. Although the toxicity of BPA has been demonstrated by in vitro and in vivo studies, the previous findings are still inconclusive. Recently, studies on CA in which the effects of BPA on human lymphocytes were evaluated, were taken as reference due to its acceptance by EFSA (0.05mg/mL) [18]. The frequency of CA in the lymphocytes from human peripheral blood has been directly associated with increased risk of cancer and carcinogenesis [21-23]. In contrast, no cytogenetic effect has been reported, according to TDI established by European Union (EU) (2002) at a concentration of 0.01 mg/mL. This indicates that the above dose is safer for human health than the previously used dose at 0.05 mg/mL.

In this study, it was demonstrated that the doses ≤0.05 mg/mL, which were accepted as reference by EFSA, still showed cytotoxic effects. In a previous study using five different doses added to lymphocyte cultures, the ones at 0.20, 0.10 and 0.05 µg/mL caused chromosomal damage. However, the 0.01 µg/mL dose showed no cytotoxic effect [24]. In another study, BPA doses ar 0.4, 1, 4, 40, and 100 µg/mL were reported to be cytotoxic, although differing results were provided [25]. In a Chinese study, the relationship between BPA at varying concentrations in the urine and a history of recurrent abortions were investigated among 102 women with recurrent abortions compared to 162 controls [26]. This study reported that the BPA concentrations in the urine of women with recurrent abortion were higher than that in the control group. Specifically, the increased urine BPA concentrations and a history of recurrent abortion were found to be positively correlated [26].

## Conclusions

Bisphenol A, being a lipophilic compound, is migrated into foods with high heat treatment. There is also BPA exposure through drinking contaminated water and dermal contact with BPA exposed air and soil. Although BPA is largely detoxified in the liver when consumed orally, its metabolism on the contaminated skin is unknown. In addition, studies have detected BPA in blood, urine, placenta, breast milk and various tissues and organs. The toxic effects of BPA are fairly known when it enters the body mixed with foods. The cytotoxic effects of BPA on human lymphocyte have been demonstrated by this study for the first time, in which its effect was evaluated at reference and lower doses accepted by EFSA. Our findings support the conclusion that most BPA products might not be as safe as previously believed.

## Ethical Considerations

### Compliance with ethical guidelines

All procedures in this study were performed after obtaining voluntary and signed consent forms from the participants. Also, we followed the legal and regulatory requirements for human experimentation.

### Funding

This study did not receive any grant from funding agencies in the public, commercial, or non-profit sectors.

### Author's contributions

Conceptualization: Mustafa Özgür and Aslı Uçar; Methodology: Mustafa Özgür and Serkan Yilmaz; Investigation: Mustafa Özgür, Şemsi Gül Yilmaz, and Serkan Yilmaz; Writing – Original Draft: Mustafa Özgür, Aslı Uçar; Writing – Review & Editing, Funding Acquisition, All Authors; Resources: Mustafa Özgür and Şemsi Gül Yilmaz; Supervision: Aslı Uçar and Serkan Yilmaz.

### Conflict of interest

The authors declare no conflict of interest with any internal or external entity in conducting this study.

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