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# The evaluation of toxicity and mutagenicity of various drinking waters in the human blood lymphocytes (HULYs) in vitro

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ABSTRACT

The aim of the study was to evaluate the toxic and mutagenic effects of bottled purified and natural spring waters for drinking. The study presents the genotoxicologic results of drinking water samples packaged in polyethylene terephthalate (PET) bottles. Genotoxic agents have the potential to interact with DNA and may cause DNA damage. Endpoints analyzed included mitotic index (MI), replication index (RI), and sister chromatid exchange (SCE). An analysis of variance test (ANOVA) was performed to evaluate the results. A significant decrease in MI and RI was observed compared with negative control cultures, respectively, ($p < 0.05$, $p < 0.01$). It is found that SCE frequency increases compared with negative control. There is no significant difference between negative control and drinking water samples and among drinking water samples for sister chromatid exchange induction ($p > 0.05$).



## 1. Introduction

Recently, the need of drinking water with quality, because of industrialization of urban areas and pollution with various compounds, has been increasing. Spring water comes from an underground formation and must flow naturally to the earth's surface or through a sanitary borehole. Purified drinking water has been gradually processed to remove chlorine and a majority of dissolved solids, such as magnesium. The source need not be named unless it is untreated public-source water. The presence of a variety of toxic compounds in drinking water is well known (Meier, 1998; Cantor, 1997; Hofer and Shuker, 2000; Rook, 1974). It is well known that food and water may be become contaminated with components of plastic containers by a diffusion process known as migration. Some of these migrants are mutagenic or carcinogenic, e.g. acetaldehyde (CASRN 70-07-0) (Eberhartinger et al., 1990; Lınssen et al., 1995), and vinyl chloride (CASRN 75-01-4) (Benfenatı et al., 1991). Polyethylene terephthalate (PET) is a relatively new packaging material prepared from terephthalic acid or its esters, and ethylene glycol. It is widely used for food and beverage bottling owing to properties (e.g. resistance, light weight, transparency and low production cost) which make it a good alternative to glass. Many studies have reported the presence of a variety of hazardous compounds in purified drinking waters. Some studies monitored the release of genotoxic substances in drinking water from PET but there is no toxicological data related with these substances affecting the public health.

In recent years attention has been drawn to abnormal sister chromatid exchange (SCE) in relation to health and disease. The SCE analysis affords an excellent parameter for monitoring DNA damage and repair aspects (Latt and Shreck, 1980). Sister chromatid exchanges may be formed in the presence of many external agents; however, the fact that formation of SCEs occurs in the absence of toxic agents leads to the conclusion that they are an integral part of DNA replication (Tucker et al., 1986). SCEs are the cytological indication of interchanges between DNA replication products. SCE analysis appears to be a very good screening test and excellent dosimeter for exposure in in vitro and in vivo short-term experiments (Painter, 1980; Tucker and Preston, 1996). The interpretation of SCE results as indicator of mutagenic effect is based on either a significant difference in the SCE frequency compared with controls or a statistically significant increase at any dose. Cytogenetic assay systems based on the detection of SCE in peripheral blood lymphocytes are widely advocated as a sensitive screening method for assessing genotoxic potential of suspected human mutagens and carcinogens. Its sensitivity and reliability have made this technique one of the most popular methods in toxicology and human biomonitoring (Çelik and Akbaş, 2005). There are at least two mechanisms to explain SCE formation: (i) one operates at replication points probably utilizing the machinery of DNA replication; (ii) the other acts only in the postreplicational DNA portion, probably in a similar fashion in a general model of crossing over in the eukaryote (Kato, 1977).

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Genotoxic agents have the potential to interact with DNA and may cause DNA damage. SCE occurs spontaneously in proliferating cells and is regarded as a manifestation of damage to the genome. It has been commonly used as a test of mutagenicity in order to evaluate cytogenetic responses to chemical exposure, and dose-response relationships for different chemicals have been reported in both in vivo and in vitro studies (Bal et al., 1998).

The present study was carried out with human peripheral blood lymphocytes (HULY) in order to investigate the ability of drinking water samples to induce DNA damage (with special reference to oxidative damage and different phases of the cell cycle).

## 2. Materials and methods

In this study, four different national drinking water brands are tested in HULYs for genetic toxicology. Two of these are natural spring water and two purified drinking water. All the bottled-drinking waters were stored under same conditions. All bottled-drinking water samples were used for genotoxicity at 8th week after bottling. The chemical contents of drinking waters samples were obtained form their commercial stickers and presented in Table 1.

### 2.1. Experimental design

#### 2.1.1. Lymphocyte cultures and sister chromatid exchange analysis in peripheral blood lymphocytes

The study was carried out by using blood samples from three healthy non-smoking male donors, aged 26 and 24 years. In three donors, results of clinical routine laboratory analyses were in normal range, and the absence of exposure to known genotoxicants was considered. All the drinking water samples were sterilized using a Sartorius filter with a 0.22-μm pore membrane. The 0.2 mL of drinking water samples was added to lymphocyte cultures under laboratory conditions. Lymphocyte cultures were prepared according to the technique of Moorhead et al. (1960) with slight modifications. Heparinized whole-blood (0.8 mL) was added to 4.5 mL of culture medium F 10 (Gibco, USA), supplemented with 20% of fetal calf serum (Gibco, USA), 0.1% mL phytohemagglutinin (Gibco), and antibiotics (10,000 IU/mL penicillin and 10,000 IU/mL streptomycin). 5-Bromo-2-deoxyuridine (9 μg/mL, BrdU, Sigma) was added to cultures at the beginning of the 72-h incubation period at 37 °C for SCE analysis. Lymphocytes were cultured in the dark for 72 h, and metaphases were blocked during the last 1.5 h with colcemid at final concentration of 0.2 μg/mL. Mitomycin C (2 μg/mL) was used as positive control. The 0.2 mL of sterile distilled water and drinking water samples were added to negative control cultures and treatment cultures, respectively. The cells were harvested by replacing the culture medium with KCl (0.075 M) in which cells were incubated for 20 min at 37 °C. The cells were fixed in Carnoy's fixative (methanol: acetic acid, 3:1 v:v) five times, and slides were kept at room temperature overnight. Air-dried slides were stained according to fluorescence-plus Giemsa method by Perry and Wolff (1974) with slight modification. The number of SCEs was counted in 100 second-metaphase cells from each of the cultures on coded slides. Thus, 100 cells were scored in blind per culture for SCEs.

#### 2.1.2. Cell proliferation kinetic (CPK) and mitotic index

The mitotic index (MI) was calculated as proportion of metaphases among the total cell population by counting a total of 1000 cells per culture. The cell proliferation kinetics was defined as the proportion of the relative frequency of first division metaphases (M1, identifiable by uniform staining of both the sister chromatids), second division metaphases (M2, identifiable by differential staining of the sister chromatids), and third and subsequent division metaphases (M3, identifiable by non-uniform pattern of staining). Replication index or proliferation index (RI) was calculated according to Ivett and Tice (1982). RI is the average number of replications completed by metaphase cells and is calculated as follows:

$$RI = 1(\%\ \text{first division metaphases}) + 2(\%\ \text{second division metaphases}) + 3(\%\ \text{subsequent division metaphases})/100$$

### 2.2. Statistical analysis

Data were compared by one-way variance analysis. Statistical analysis was performed using the SPSS for Windows 9.05 package program. *Post hoc* analysis was performed by least significant difference (LSD) test. $p < 0.05$ was considered as level of significance.

## 3. Results

Table 2 represents frequencies of SCE and the values of MI and RI in cultured HULYs treated different drinking water samples. In addition to the genotoxicologic examination, it is possible to obtain valuable data concerning mitotic activity on the same microscopic slides used for cytogenetic analysis. Values of mitotic index (MI) as a parameter used to determine possible cytotoxic or mitogenic effects are presented in Table 2.

All the drinking water samples induced slightly sister chromatid exchange frequency in cultured HULYs. There is no statistically significant difference between negative control and drinking water samples and among drinking water samples for sister chromatid exchange induction ($p > 0.05$).

A significant decrease ($p < 0.05$) in the frequency of mitosis was observed for treatment with both purified water samples and I numbered spring water compared with negative control under our experimental conditions. In RI values, significant differences were observed between negative control and both natural spring

**Table 1**
The chemical contents of drinking waters

| Group | Ca (mg/lit) | Mg (mg/lit) | Fl (mg/lit) | Cl (mg/lit) | $Cl_2$ (mg/lit) | pH | Phenolic substance (mg/lit) | Ammoniac (mg/lit) | Sulphate (mg/lit) | Nitrite (mg/lit) | Nitrate (mg/lit) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Natural spring water (I) | 25.65 | 7.05 | 0.03 | 4.9 | – | 7.48 | – | – | 2.56 | – | 1.236 |
| Natural spring water (II) | 5.6 | 1.24 | 0.21 | 4.8 | – | 6.8 | – | – | 12 | – | 6.6 |
| Purified drinking water (I) | 0.22 | 0.05 | 0.009 | 0.12 | – | 6 | – | – | 0.23 | – | 0.21 |
| Purified drinking water (II) | 8.16 | 4.86 | 0.05 | 11 | – | 5.75 | – | – | 14.12 | – | 1 |

**Table 2**
Results of frequencies of SCE and the values RI and MI of cultured human peripheral blood lymphocytes treated different drinking water samples

| Group | Donor A | | | Donor B | | | Donor C | | | Mean ± SE | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | MI | SCE | RI | MI | SCE | RI | MI | SCE | RI | MI | SCE | RI |
| Natural spring water ( I) | 5.10 | 4.86 | 2.05 | 5.12 | 4.87 | 2.00 | 5.01 | 4.95 | 1.98 | $5.07 \pm 0.03^{*}$ | $4.89 \pm 0.02$ | $2.01 \pm 0.02^{*}$ |
| Natural spring water (II) | 5.60 | 4.86 | 2.04 | 5.60 | 4.87 | 2.00 | 5.41 | 4.86 | 2.00 | $5.53 \pm 0.06$ | $4.86 \pm 0.00$ | $2.01 \pm 0.01^{*}$ |
| Purified drinking water (I) | 5.00 | 4.86 | 2.01 | 5.17 | 4.92 | 1.98 | 5.15 | 5.01 | 2.01 | $5.10 \pm 0.05^{*}$ | $4.93 \pm 0.04$ | $2.00 \pm 0.01^{**}$ |
| Purified drinking water (II) | 4.97 | 4.85 | 2.05 | 5.15 | 4.75 | 2.05 | 5.06 | 4.72 | 2.00 | $5.06 \pm 0.05^{*}$ | $4.77 \pm 0.03$ | $2.03 \pm 0.01$ |
| Positive control MMC (2 μg/mL) | 3.11 | 7.84 | 1.86 | 3.16 | 8.82 | 1.85 | 3.32 | 8.95 | 1.85 | $3.19 \pm 0.06^{***}$ | $8.53 \pm 0.35^{***}$ | $1.85 \pm 0.00^{***}$ |
| Negative control | 5.90 | 4.96 | 2.10 | 5.60 | 4.81 | 2.05 | 5.05 | 4.95 | 2.05 | $5.70 \pm 0.10$ | $4.87 \pm 0.02$ | $2.06 \pm 0.01$ |

MMC, mitomycin C; MI, mitotic index; RI, replicative index; SCE, sister chromatid exchange; SE, standard error. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ compared to negative control.

water samples and II numbered purified water samples, respectively ($p < 0.05$, $p < 0.01$). There is no significant difference among drinking water samples for RI ($p > 0.05$).

## 4. Discussion

Various screening tests have been used as an indicator of potential mutagenicity in genetic damage including the sister chromatid exchange assay. The SCE test involves exchange of DNA segments between two sister chromatids in a chromosome during replication in the cell. The increase in frequency of SCE indicates the mutagenic effect of chemical substances. SCEs are the cytological indication of interchanges between DNA replication products. SCE analysis appears to be a very good screening test and excellent dosimeter for exposure in in vitro and in vivo short-term experiments (Painter, 1980; Tucker and Preston, 1996).

Genotoxic agents have the potential to interact with DNA and may cause DNA damage. SCE occurs spontaneously in proliferating cells and is regarded as a manifestation of damage to the genome. It has been commonly used as a test of mutagenicity in order to evaluate cytogenetic responses to chemical exposure, and dose-response relationships for different chemicals have been reported in both in vivo and in vitro studies (Çelik and Akbaş, 2005; Çelik and Aras Ateş, 2006; Aydemir et al., 2005).

MI and RI are used as indicators of sufficient cell proliferation (Anderson et al., 1988; Scott et al., 1991). The genetic toxicity and mutagenicity studies concerning the bottled-drinking waters have produced controversial results depending on the assay used. Four PET bottled water drinking water samples displayed the cytotoxic effect at the HULY cultures. It is interesting to note that sign of toxicity were evident even only 8 weeks after bottling, which is reasonable shelf-life period and is well within the recommend date of expiry. Its capability to migrate from PET into water has been investigated and demonstrated by some researchers (Lınssen et al., 1995; Lo Russo et al., 1985).

In some studies, bottled-drinking waters were reported to be toxic in HULYs (Bıscardı et al., 2003) plant cells, such as Allium *cepa* root cells (Evandri et al., 2000). Bıscardı et al. (2003) studied the potential migration of genotoxic compounds in mineral water samples stored for different periods of time in PET bottles and used gas chromatography/mass spectrometry (GC/MS) for the characterization of migrant migrated form PET bottles to water.

They evaluated the potential human health hazards and in their study; they tested the mineral water stored in different conditions for Comet assay on leucocytes and found that DNA-damaging effects, suggesting a possible introduction of genotoxin through the distribution pipelines. GC/MS analysis indicated the presence in mineral water of di(2-ethylhexyl)phthalate, hepatocarcinogenic plasticizer, after 9 months of storage in PET bottles. Evandri et al. (2000) examined the two commercial non-carbonated mineral waters bottled in PET and glass and stored under different conditions, reported that the two water samples bottled in PET induced cytogenetic aberrations regardless storage conditions. Monarca et al. (1994) studied coloured PET bottles for carbonated beverages for potential migration of genotoxic compounds. They used physicochemical method and a bacterial short-term mutagenicity test and performed the GS/MS analysis in order to measure both volatile and non-volatile compounds and their residues. Some potentially genotoxic compounds (acetaldehyde, dimethyl terephthalate, terephthalic acid) were identified in these migrant compounds by GC/MS analysis.

On the other hand, a significant decrease in RI is an indicator of cytostatic effect. Our results have demonstrated that there are modifications on CPK values expressed as RI in HULYs exposed to drinking water samples bottled in PET. In the present study, the observed inhibition of cell proliferation illustrates the cytotoxicity of commercial drinking water samples. Agein Evandri et al. (2000) reported that these signs of toxicity were evident even only 8 week after bottling, which is well within the recommended expiry date and they indicated that storage conditions were very important in view of toxicity.

Therefore, it may be said that commercial drinking samples stored in PET have cytostatic effects on HULY cultures in vitro because it causes delay in the cell cycle. Among the end points describing genotoxic damage, a significant induction of SCE was not observed in all cultures treated with water samples compared with negative control cultures. No significant difference only was observed between the II numbered purified drinking water sample and negative control for RI.

In conclusion, our genotoxicological analysis showed that all the tested-drinking waters bottled in PET have cytotoxic effects and cytostatic effect on the HULYs in vitro, except at the II numbered natural spring water and II numbered purified drinking water, respectively. This genotoxicological approach is a tool to be used by health authorities to better evaluate water supplies and to monitor drinking waters before their use for human consumption.

## Conflict of interest statement

The authors declare that there are no conflicts of interest.

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