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Micronucleus frequency in human lymphocytes after exposure to diphenylamine in vitro

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A B S T R A C T
Diphenylamine (DPA) is an antioxidant compound that occurs naturally in several vegetables. It is widely applied in agriculture for preservation of the quality of apples and pears, and used for controlling superficial scald, a disorder that renders fruits of a number of apple cultivars unfit for the market. Because of its anti-oxidative properties, DPA also has several industrial applications. The potential genotoxic effect of DPA on human lymphocytes has previously been investigated in only two studies, which focused on detection of chromosome aberrations and sister chromatid exchange, respectively. In the present analysis, we evaluated micronucleus (MN) formation in freshly isolated human peripheral lymphocytes exposed to different concentrations (0.625, 1.25, 2.50, 5.0 and 10.0 μg/ml) of DPA. Peripheral venous blood was collected from ten healthy subjects, and a total of 10,000 bi-nucleated cells were analyzed. Results indicated that DPA significantly increased the micronucleus frequency at concentrations of 1.25 μg/ml and higher. Significant differences in the MN frequency were also found between the lower dose (0.625 μg/ml) and all other doses tested, with the exception of 1.25 μg/ml. Our results indicate a potential cytogenetic effect of DPA on human cells in vitro and require further in vivo studies to clarify the actual genotoxicity of this compound and the consequent risks for human health.

1. Introduction
Diphenylamine (DPA) is an antioxidant widely used for preservation of the quality and prevention of post-harvest deterioration of apples and pears, in particular for controlling superficial scald, a disorder that renders fruits of a number of apple cultivars unfit for the market. Because of its anti-oxidative properties, DPA also has several industrial applications, for example as a precursor of dyes, pharmaceuticals, photographic chemicals, and as a stabilizer in nitrocellulose-containing explosives and propellants (reviewed in [1]). Moreover, DPA is a natural compound found in several vegetables, such as onions [2], baked potatoes [3], buckwheat flour [4], black and green tea [5], and dried plums [6].

Ecotoxological studies indicate that DPA and its derivatives are contaminants in soil and water, and potentially hazardous to aquatic organisms [1]. DPA treatment in short-time studies and during long-term expositions in animal experiments showed an increase of liver- and kidney-damaging effects [7–9]. Lorenzin [10] assessed the amount of pesticide residues in Italian pre-packed meals: DPA was shown to be one of the pesticides most frequently found in fruit and side dishes. Cytogenetic monitoring of human populations exposed to pesticides is generally based on the analysis of the genotoxic effects induced by chronic low doses of complex chemical mixtures. As a consequence, it is often very difficult to associate the observed genotoxic damage to specific chemical classes or compounds. On the other hand, in vitro and in vivo studies focusing on single substances provide more informative evidence about the genotoxic effects of specific pesticides.

Unfortunately, in vitro experiments with DPA are scanty. Dolara and co-workers [11,12] tested the effects of a mixture of 15 pesticides commonly found in Italian foods (including DPA at 14.4%) on human lymphocytes. Results indicated that the pesticide mixture did not induce significant variations in chromosome number or the frequency of chromatid aberrations. Conversely, a moderate but statistically significant increase of sister-chromatid exchange (SCE) at low concentrations of the pesticide mixture was observed. Subsequently, Ardito et al. [13], analyzing the effects of DPA on human lymphocytes, observed a significant increase of SCE frequency only at high concentrations.

In a FAO-WHO joint meeting [14], on the basis of negative results from studies conducted in vivo on different animals (Korolev et al., 1976; Gorecka-Turska et al., 1983; Mobil Oil Corp., 1987), it was concluded that although DPA may have genotoxic potential, it is unlikely to be a human genotoxic hazard. In the same meeting, the recommended maximum residue limit (MRL) on apples was 5 mg/kg, while the acceptable daily intake (ADI) for humans for this compound was set at 0.08 mg/kg bw [14,25].

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In this study we analyzed the effects of different DPA concentrations on human lymphocytes, by use of the micronucleus (MN) assay. Micronuclei are either a centric chromosome fragments or whole chromosomes left behind during mitotic cell division, and appear as small additional nuclei in the cytoplasm of interphase cells. In contrast to analyses of CA and SCE, which mainly reveal alterations in chromosome structure, the MN assay – in combination with centromere analysis – may allow detection of both clastogenicity (chromosome breakage) and aneugenicity (chromosome lagging due to dysfunction of the mitotic apparatus) [15–18].

The aim of the study was to determine whether DPA, at different concentrations, could induce genotoxic damage in cultured human lymphocytes. We tested 0.625, 1.25, 2.5, 5 and 10 μg/ml of DPA, where 0.625, 1.25, and 2.5 μg/ml represent the sub-multiple and 10 μg/ml the multiple of the MRL, which is the value accepted for apples (5 μg/ml).

2. Materials and methods

2.1. Chemicals, media, and enzymes

Diethylamin (DPA) (JUPAC name, N-phenylbenzenamine: CAS no 122-39-4) was obtained from Labservices, Bologna, Italy and dissolved in DMSO (CAS no. 67-68-5). Gibco RPMI 1640 cell-culture medium supplemented with l-glutamine, foetal calf serum, phytohaemagglutinin (PHA), and antibiotics were purchased from Invitrogen-Life Technologies, Milan, Italy. Cytochalasin-B and mitomycin-C (MMC) were purchased from Sigma-Aldrich, Milan, Italy. Methanol, acetic acid, Giemsa staining solution, and conventional microscope slides were purchased from Carlo Erba Reagenti, Milan, Italy. Potassium chloride (KCl) and Sörensen buffer were obtained from Merck S.p.A., Milan, Italy. Vacutainer blood-collection tubes were from Terumo Europe, Rome, Italy. Distilled water was used throughout the experiments.

2.2. Lymphocyte cultures

Peripheral venous blood was collected from ten healthy subjects (mean age ±S.E., 32.90 ±4.18 years, range 23–40 years), non-smokers, non-drinkers, not under drug therapy, and with no recent history of exposure to mutagens. Informed consent was obtained from all blood donors. The study was approved by the local ethics committee and was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

2.3. Blood-sample collection

Heparinized blood samples were obtained by venipuncture and collected in heparinised tubes, for genotoxicity testing. All blood samples were coded, cooled (4°C), and processed within 2 h after collection.

2.4. Cytokinesis-block micronucleus assay

Heparinized venous blood (0.3 ml) was cultured in 25-cm$^2$ flasks in 4.7 ml of RPMI-1640 media supplemented with 20% foetal calf serum (FCS), 2% of the mitogen phytohaemagglutinin (PHA), l-glutamine (2 mM), antibiotics (100 IU/ml penicillin, and 100 μg/ml streptomycin). The cultures were incubated for 72 h at 37°C, under 5% CO$_2$ in the air in a humidified atmosphere.

After 24 h of incubation, DPA at concentrations of 0.625 μg/ml, 1.25 μg/ml, 2.5 μg/ml, 5 μg/ml and 10 μg/ml were added to the cultures. Three control cultures were included: (1) positive control, by adding mitomycin-C (MMC, final concentration 0.01 μg/ml culture); (2) solvent control, 1% DMSO; (3) negative control, culture without DPA or DMSO.

After 44 h of incubation, cytochalasin-B was added to the cultures at a concentration of 6 μg/ml to block cytokinesis. Following a further incubation during 28 h at 37°C, the cells were collected by centrifugation and treated for three min with a pre-warmed mild hypotonic solution (75 mM KCl). After centrifugation and removal of the supernatant, the cells were fixed with a fresh mixture of methanol/acetic acid (3:1, v/v). The treatment with the fixative was repeated three times. Finally the supernatant was discarded and the pellet, suspended in a minimal volume of distilled water, was placed on the slides to detect MN by air-drying staining with 5% Giemsa (pH 6.8). Prepared in Sörensen buffer, Microscope analysis was performed at 1000× magnification with a light microscope (Dialux 20, Leica, Germany). Micronuclei were scored in 1000 bi-nucleated lymphocytes with well-preserved cytoplasm per subject (total 10,000 bi-nucleated cells per concentration), following the established criteria for evaluation of MN (Fenech et al., 2003). A total of 1000 lymphocytes per donor (total 10,000 lymphocytes) were scored to evaluate the percentage of cells with 1–4 nuclei. The cytokinesis-block proliferation index (CBPI) was calculated, according to [19].

2.5. Statistical analysis

Comparison of mean values of the percentage of cells with MN and the CBPI at different exposure conditions and their controls was done by use of the Kruskal–Wallis test. Statistical calculations were carried out with the SYSTAT software package program (version 10.0, Inc., Chicago, IL, USA). All P-values were two tailed, and P-values of 0.05 or less were considered to correspond with statistical significance for all tests carried out.

3. Results

To assess the effects of DPA on human lymphocytes, the MN frequency was investigated at different concentrations of the pesticide (Table 1). The results indicate that DPA significantly (P < 0.05) increased the MN formation compared with the negative control, at all concentrations (Table 1). When compared with the solvent control (1% DMSO), DPA significantly increased MN frequency at all concentrations with the exception of 0.625 μg/ml. Moreover, highly significant (P < 0.001) differences in MN frequency were also found between the lowest DPA dose (0.625 μg/ml) and all other concentrations except 1.25 μg/ml. The solvent-control cultures did not show any difference with the negative controls confirming that, at this low concentration (1%), DMSO has no biological effects. The cultures treated with the mutagen MMC showed a consistently increased MN formation compared with the negative and solvent controls. Finally, we observed a decrease, although not statistically significant, of the CBPI values at different DPA exposure levels.

4. Discussion

A large body of literature has been devoted to the analysis of possible toxic effects of pesticides with different animal models and cellular systems. Although various experimental data have provided convincing evidence that pesticides can exert mutagenic/genotoxic effects in vivo and in vitro, detailed information about aneugenic and clastogenic effects of exposure to specific pesticides is limited and sometimes inconsistent. Among the cytogenetic end-points (CA, SCE, MN) commonly used to investigate the genotoxicity of chemicals, the analysis of MN has often been preferred due to its reliability and sensitivity of MN as a marker of cytogenetic damage [18]. Moreover, the formation of MN after exposure to various pesticides has been reported in several test systems [18]. In this study, we chose the MN approach to evaluate the potential risk for chromosome damage in cultured human lymphocytes exposed to different concentrations of DPA. The importance of DPA in preventing superficial scald of apples, which has serious economic and logistical effects on storage, marketing and distribution of high-quality fruit, is widely recognised, but the available data on the genotoxic potential of DPA are scanty.

Our results show that DPA induces significant micronucleus formation in cells treated with concentrations ≥1.25 μg/ml, including the concentration of 5 μg/ml accepted as MRL for DPA by the WHO and European Commission. Compared with the SCE analyses performed by Ardito et al. [13], where the cytogenetic damage caused by DPA was significant only at the highest concentration tested (6 μg/ml), we observed a significant increase of MN frequency also at lower concentrations.

These discordant results confirm the higher sensitivity of the MN assay in detecting genotoxic effects related to pesticide exposure. Indeed, while the SCE analysis can only detect alterations in the chromosomal structure, formation of MN reveals changes in either chromosome number or chromosome structure, providing a method able to evaluate potential clastogenic and aneuploidy-genic exposure simultaneously (Bolognesi et al., 2003). Aneugenic chemicals induce alterations of centromeric DNA [16], with leads to failure of attaching the damaged chromosomes to the mitotic spindle [17], and subsequent formation of MN.

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Table 1

Induction of micronuclei produced by diphenylamine in human lymphocytes in vitro.

<table>
<thead>
<tr>
<th>Test substance</th>
<th>Treatment</th>
<th>BNCs scored</th>
<th>Distribution of BNCs according to the no. of MN</th>
<th>MNs</th>
<th>MN/100 cells (%) ± S.E.</th>
<th>CBPI ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative control</td>
<td>48 h, 100 μg/ml</td>
<td>10,000</td>
<td>100%</td>
<td>61</td>
<td>0.61 ± 0.14</td>
<td>2.30 ± 0.21</td>
</tr>
<tr>
<td>DPA</td>
<td></td>
<td></td>
<td></td>
<td>250</td>
<td>2.52 ± 0.13</td>
<td>1.57 ± 0.13</td>
</tr>
<tr>
<td>MMC</td>
<td></td>
<td></td>
<td></td>
<td>88</td>
<td>0.88 ± 0.19</td>
<td>1.84 ± 0.07</td>
</tr>
<tr>
<td>DMSO</td>
<td></td>
<td></td>
<td></td>
<td>109</td>
<td>1.09 ± 0.24</td>
<td>1.76 ± 0.03</td>
</tr>
<tr>
<td>DPA</td>
<td>2.50 h, 100 μg/ml</td>
<td>10,000</td>
<td>25%</td>
<td>165</td>
<td>1.65 ± 0.33</td>
<td>1.73 ± 0.03</td>
</tr>
<tr>
<td>DPA</td>
<td>5.00 h, 100 μg/ml</td>
<td>10,000</td>
<td>24%</td>
<td>170</td>
<td>1.70 ± 0.35</td>
<td>1.68 ± 0.06</td>
</tr>
<tr>
<td>DPA</td>
<td>10.00 h, 100 μg/ml</td>
<td>10,000</td>
<td>21%</td>
<td>203</td>
<td>2.03 ± 0.44</td>
<td>1.59 ± 0.12</td>
</tr>
</tbody>
</table>

BNCs, bi-nucleated cells; MMC, mitomycin-C; DPA, diphenylamine (in 1% DMSO, final concentration in culture). S.E., standard error. CBPI, cytokinesis-block proliferation index = [(1 × N₁) + (2 × N₂) + (3 × N₃ + N₄)]/N, where N₁–N₄ represent the number of cells with 1, 2, 3, and 4 nuclei, respectively, and N is the total number of cells scored.

Finally, a significant reduction of the CBPI value in cultures treated with DPA was not observed, indicating that DPA does not seem to produce effects on the proliferation/mitotic index when its concentration is equal or lower than 10 μg/ml. This result is congruent with those obtained by Arditto et al. [13] for DPA and with data about other pesticides [19,20].

In conclusion, this study shows that exposure of cultured human lymphocytes to DPA results in chromosome damage that leads to an increased frequency of MN. These positive in vitro results must be followed-up by in vivo studies to clarify the actual genotoxicity of the molecule and consequent risks for human health. To date, DPA has been tested for potential carcinogenicity in few long-term bioassays, where it has not been found carcinogenic in experimental animal models [14]. Moreover, Yoshiha and co-workers [1989] have calculated a quite high (111 mg/kg/day) NOAEL in rats.

In this perspective, it would be useful and interesting to further investigate DPA levels in tissues or blood plasma of people handling and consuming treated fruit in order to correctly assess the potential genotoxic effects of DPA for humans in vivo. In fact, the concentration in vivo will presumably be much lower and a function of the quantity consumed, plus a dilution factor due to absorption and metabolism.

Conflict of interests

The authors declare that they have no conflict of interests.

Q4 Uncited references

[21,26;28–28].

Acknowledgements

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