IN VITRO ANALYSIS OF TARTRAZINE GENOTOXICITY AND CYTOTOXICITY

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Abstract
Tartrazine (E 102) is widely used yellow food colorant. It is used in nonalcoholic and sports drinks, spicy chips, jams, jelly and chewing gum and also found in many non-food products like soaps, cosmetics, shampoo, vitamins and some drugs. Tartrazine belongs to the most important and diverse group of synthetic dyes – azo dyes. Their use often creates controversies in the public since some of them are toxic, carcinogenic, mutagenic and cause different disorders or allergic reactions. In this study we aimed to evaluate genotoxic potential of tartrazine in human lymphocytes culture and its cytotoxic potential in human lymphocytes and melanoma GR-M cell line. For testing of its genotoxic and cytotoxic potential in human lymphocyte culture, we used chromosome aberration analysis and cytokinesis-block micronucleus cytome assay. For the analysis of its cytotoxic potential in human melanoma cell culture, we applied trypan blue exclusion assay.

Key words: chromosome aberrations, micronuclei, trypan blue, human cell culture

Introduction
Synthetic food dyes are extensively used in many aspects of human life. The beginning of synthetic dyes production dates back to the middle of the 19th century and the use of natural dyes is completely overshadowed. Implementation of new analyses in the 20th century revealed significant negative and harmful impacts of certain food dyes on human health thus inducing restrictions on their use. Aside from their negative, mutagenic and carcinogenic, impact on health, synthetic dyes from household or industry also have negative impact on the environment even at very low concentrations (Forgacs et al, 2004; Hosseini et al., 2011). Establishing of appropriate mechanisms for the detection and monitoring of food dyes negative effects on human health is essential to define adequate protection and prevention measures.

Although exacerbating effects of tartrazine in chronic urticaria and asthma sufferers are long known (Lockey, 1977), tartrazine (E 102) is widely used yellow food colorant (Mittal et al., 2007, Saxena and Sharma, 2015). Tartrazine is used in nonalcoholic and sports drinks, spicy chips, jams, jelly and chewing gum, bakery goods, cereals, candies, gelatin and numerous other commodities (Saxena and Sharma, 2015). It is also found in non-food products like soaps,
cosmetics, shampoos, vitamin supplements and some drugs (Amin et al., 2010). In many countries tartrazine is used in catering as an alternative to saffron (Mehedi et al., 2009). Tartrazine belongs to the most important and diverse group of synthetic dyes – azo dyes, which includes about 3000 different compounds. They are widely used and distributed in the environment due to their convenient and inexpensive synthesis, stability and wide range of shades in comparison to natural dyes (Saratale et al., 2011). Use of azo dyes often causes controversies in the public since some of them are toxic, carcinogenic, mutagenic and cause different disorders (Saxena and Sharma, 2015) or allergic reactions in the organism (Bhatia, 2000; Ardren and Ram, 2001; Bourrier, 2006). Studies have also linked the ingestion of dyes (mostly azo dyes) in candies and drinks with hyperactivity and other disruptive behavior in children (McCann et al., 2007).

In this study we aimed to evaluate genotoxic potential of tartrazine in human lymphocytes culture and its cytotoxic potential in human lymphocytes and melanoma GR-M cell line.

Materials and methods

Lymphocyte culture

Peripheral blood was collected into heparinized vacutainers from a healthy female volunteer who signed informed consent form. Lymphocyte cultures were induced in 15-mL sterile, plastic tubes with conical base (Isolab GmbH, Wertheim Germany), which contained 5 mL of PB-MAX Karyotyping Medium (GIBCO-Life Technologies, Grand Island, NY, USA) and 400 μl of peripheral blood. Cultures were harvested after 72 hours of lymphocytes cultivation at 37°C, using standard procedure (hypotonic treatment with 0.075M KCl, triple ethanol-acetic acid fixation, followed by microscope slide preparations and staining in 5% Giemsa).

Tested substance

Tartrazine (E-102) was dissolved in dH2O and added to the cultures in the 25th hour of cultivation to the final concentrations of 2.5, 5 and 10 mM. The concentrations were determined according to literature (Mpountoukas et al., 2010). Negative controls with equivalent volume of dH2O and positive controls with 0.25 µg/mL of mytomicine C (Sigma-Aldrich Co, St Louis, MO, USA) were set up as well.

Cytokinesis-block micronucleus cytome (CBMN Cyt) assay

The analysis of genotoxic, cytotoxic and cytostatic potential of tartrazine was evaluated in human lymphocyte cultures by applying cytokinesis-block micronucleus cytome assay in vitro. In order to block cytokinesis, 4.5 μg/mL of cytochalasin B (Sigma-Aldrich Co., St Louis, MO, USA) was added to the cultures in the 45th hour of cultivation. Microscopic analysis at 400x magnification using Olympus BX51 microscope (Tokyo, Japan) included observation of 2000 binuclear cells per each treatment and controls, equally divided among two replicates (OECD, 2014). The frequencies of genotoxicity markers: micronuclei, nucleoplasmic bridges and nuclear buds, were determined according to the defined criteria (Fenech, 2007; Fenech et al., 2003). Cytotoxic and cytostatic effects were assessed by calculating nuclear division index (NDI) and nuclear division cytotoxicity index (NDCI) (Fenech, 2000). The frequencies of mononuclear, binuclear, trinuclear, and quadrinuclear cells, as well as apoptotic and necrotic cells, were registered in the total number of at least 1000 cells, counted per each treatment and controls, equally divided among two replicates.
Chromosome aberration assay

For the chromosome aberration analysis, lymphocytes were arrested in metaphase by addition of colcemid (0.18 μg/ml), 90 min before cell harvesting. 300 metaphases (46±1 chromosome) were analyzed per treatment and controls, equally divided among three replicates (OECD, 1997) at 1000× magnification. Structural chromosome aberrations were scored and registered according to the International System for Human Cytogenetic Nomenclature - ISCN (Mitelman, 1995).

Melanoma cell line

Human GR-M melanoma cell line (Culture Collections, Public Health England, London, UK, Cat. No. 95032301) was cultured in RPMI 1640 (Gibco-Invitrogen, Grand Island, NY) supplemented with L-glutamine, 10% of fetal bovine serum (FBS) and penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO) in T-25 flasks (NUNC, Rochester, NY) at 37°C, 5%CO2 atmosphere with 95% humidity. At the beginning of experiment cells were seeded at a density of 1×10⁵ cells, pre-incubated for 24 h and finally incubated with the selected concentrations of tartrazine for another 48 h.

Trypan blue exclusion assay

For the cytotoxicity analysis trypan blue exclusion assay was performed after 48h of incubation of melanoma cell cultures with tartrazine. Each treatment was carried out in triplicate. Cells were harvested by trypsinization and cell viability (%) was determined as [number of viable cells / (number of viable + non-viable cells)] x 100.

Statistical analysis

Differences in observed frequencies of genotoxic and cytotoxic parameters were calculated using proportion comparison Z-test in WINKS 4.5 Professional Software (TexaSoft, Cedar Hill, TX). Statistical significance threshold was set at 0.05.

Results and discussion

Results of tartrazin genotoxicity analysis, obtained in human lymphocytes cultures and GR-M melanoma cell line applying CBMN Cyt assay, chromosome aberration assay and trypan blue exclusion assay are presented in tables 1-3. Frequencies of genotoxicity biomarkers in CBMN Cyt assay increasewith the increase of tartrazine concentration (Table 1, Figure 1).

Table 1. Results of CBMN Cyt assay in human lymphocyte culture

| Treatment         | Genotoxicity biomarkers | Cytostasis/cytotoxicity |
|-------------------|-------------------------|                        |
|                   | MN  | NB  | NPB | NDI | NDCI |
| Negative control  | 2   | 0.5 | 0   | 1.768 | 1.752 |
| 2.5 mM            | 1ᵇ  | 0ᵇ | 1.5ᵇ | 1.816 | 1.303 |
| 5 mM              | 2ᵇ  | 0.5ᵇ | 6.5ᵃ | 1.584 | 1.573 |
| 10 mM             | 8ᵇ  | 1   | 8ᵃ   | 1.586 | 1.574 |
| Positive control  | 65.5 | 6   | 8.5  | 1.308 | 1.303 |

*Note*: MN – micronuclei; NB – nuclear buds; NPB – nucleoplasmic bridges. Genotoxicity analyzed on 1000 BN cells per culture, 2 replicate cultures per treatment; Cytostasis/cytotoxicity analyzed on 500 cells per culture, 2 replicates per treatment. (a) Significantly different against negative control; (b) Significantly different against positive control.

However, comparison with negative controls revealed no significant differences in MN and NB frequencies. In concentrations of 5 mM and 10 mM of tartrazine, significant increase in
The frequency of NPB against negative control was detected (p<0.5 for 5 mM; p=0.005 for 10 mM).

**Figure 1.** Average frequencies of genotoxicity markers in CBMN Cyt assay expressed per 1000 BN cells in human lymphocyte cultures (negative controls + treatments)

Comparison against positive control were significant for MN frequencies in all three tested concentrations (p=0.0), for NB frequencies in 2.5 mM (p=0.01) and 5 mM (p=0.03) concentrations and for NPB in the lowest tested concentration of 2.5 mM (p=0.026). Neither tartrazine nor mytomycin C induced significant differences in nuclear division indexes (NDI and NDCI) against negative control although nuclear division indexes were decreased in the highest tested concentrations and positive control (Table 1, Figure 2). However, cytotoxic effects of mytomycin C, an antitumor antibiotic, are confirmed in several oral squamous cell lines (HSC-2, HSC-3, HSC-4, Ca9-22 and NA) and human promyelocytic leukemic cell line HL-60 (Sasaki et al., 2006).

**Figure 2.** NDI and NDCI values calculated using average frequencies of analyzed cells in cytokinesis blocked human lymphocyte cultures, negative controls and treatments

Proportion comparison of observed chromosome aberrations revealed the lack of significant differences between each of three tested concentrations and negative control while positive control significantly differed for all tested concentration in chromatid- (breaks and minute fragments) and chromosome-type aberrations (breaks, double minutes) and chromosome rearrangements that were calculated separately (p=0.0). Incidence of pulverzations (pvz) and premature centromere separations (pcs) did not differ between each of three treatments or when compared with positive or negative control (p>0.05).

Previously published data regarding tartrazine genotoxicity are contradictory. Bastaki et al. (2017) dismissed tartrazine genotoxicity in vivo, while Khayyat et al. (2017) dismissed tartrazine genotoxicity in vivo, while Khayyat et al. (2017) reported tartrazine

<table>
<thead>
<tr>
<th>Treatment</th>
<th>chthb, min</th>
<th>chrb, ace, dmin</th>
<th>chre</th>
<th>pvz</th>
<th>pcs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2.5 mM</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5 mM</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10 mM</td>
<td>0.666</td>
<td>1.333</td>
<td>0</td>
<td>0</td>
<td>0.666</td>
</tr>
<tr>
<td>Positive control</td>
<td>21</td>
<td>74</td>
<td>24</td>
<td>1</td>
<td>0.333</td>
</tr>
</tbody>
</table>
potencial to induce structural and functional aberrations and genotoxic effects in vivo. Atli-Sekeroglu et al. (2017) also found that tartrazine and its metabolites have genotoxic potential on human lymphocyte cultures both with and without metabolic activation (S9) and can induce cytotoxic effects without S9 and in the highest of concentrations tested (2500 μg/ml). Tartrazin is shown to induce DNA-damage in the gastrointestinal organs at low doses, even at doses approaching the acceptable daily intake (Sasaki et al., 2002) of 7.5 mg/kg/bw/day, which is significant since many products, such as ice creams, desserts, cakes are often marketed without labeling (Elhkim et al., 2007). Study of cytotoxic and genotoxic effects of tartrazine on DNA repair in human lymphocytes demonstrated no cytotoxic effects but revealed significant genotoxic effects in all tested concentrations ranging from 0.25-64.0 mM (Soares et al., 2015), that are far below the concentrations tested in our study (2.5; 5 and 10 mM).

Figure 3. Cytotoxicity analysis using Trypan blue assay in human melanoma GR-M culture

Trypan blue exclusion assay was performed in GR-M human melanoma cell culture in order to estimate tartrazine cytotoxicity. The decrease in cell viability (%) was obvious and significant against negative control (p=0.0), contrasting negligible cytotoxic effects recorded in human lymphocytes culture by CBMN Cyt assay.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Viable</th>
<th>Nonviable</th>
<th>Viability %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>277</td>
<td>7.75</td>
<td>97.28</td>
</tr>
<tr>
<td>2.5 mM</td>
<td>122</td>
<td>10.75</td>
<td>91.90*</td>
</tr>
<tr>
<td>5 mM</td>
<td>130.5</td>
<td>7.75</td>
<td>94.394*</td>
</tr>
<tr>
<td>10 mM</td>
<td>124.25</td>
<td>12.75</td>
<td>90.693*</td>
</tr>
</tbody>
</table>

Note: (a) Significantly different against negative control.

Overall results suggest that concerns regarding tartrazine use are not unfounded, especially regarding cytotoxicity of tartrazine that should be further evaluated.

Conclusions

Obtained results and conducted statistical analysis show that tartrazine is not genotoxic in human lymphocytes in tested concentrations. Its cytotoxic effects are negligible in human lymphocyte culture but significant in GR-M melanoma cell culture. This finding suggests that future studies of tartrazine should be focused on evaluation of cytotoxic effects using human lymphocytes but also additional cell models, cytotoxicity assays in comparison against cytotoxic effects of other cytotoxicity inducers.

References


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