The evaluation of reactive textile dyes regarding their potential to cause organ-specific cyto- and geno-toxicity

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Abstract

The textile industry extensively uses synthetic chemicals such as dyes. Several studies report the deleterious effects (e.g., cell death and DNA damage) of dispersive dyes on humans. Humans can be exposed to toxic dyes by ingesting contaminated waters or dermal contact with colored garments. Thus, toxicity evaluations of textile dyes using organ-specific cell lines are relevant to estimating their hazard. Cyto- and geno-toxicity of the dyes Reactive Green 19 (RG19), Reactive Blue 2 (RB2), Reactive Blue 19 (RB19), Reactive Red 120 (RR120) and Reactive Orange 16 (RO16) were evaluated by the In Vitro MicroFlow® kit with immortalized human keratinocyte cell line (HaCaT) and immortalized human hepatic cell line (HepaRG). Concentration-dependent cytotoxicity was observed for HaCaT cells exposed to three of the five tested dyes (RB2, RB19, RO16), while in HepaRG cells, cytotoxic effects were only verified after exposure to RB19 and RO16 at the highest tested concentration (1000 µg/mL). Genotoxicity was not detected in any tested textile dyes under both test conditions (HaCaT and HepaRG). In conclusion, our data provide evidence that, although the tested reactive dyes are not genotoxic, which is in agreement with published literature, they can cause cytotoxicity in both target tissue, and the effect can be more severe in epidermal cells (HaCaT) than in liver cells (HepaRG). This differential cytotoxicity data emphasizes the need to assess the toxicity of textile dyes to the target organ specificity.

Keywords: cytotoxicity; skin cells; liver cells; flow cytometry-based micronucleus assay.

INTRODUCTION

The dyeing of fabrics is an art that began thousands of years ago, and the commercial availability of dyes is enormous (≥ 10,000 colors) (Ferraz et al., 2012). Reactive dyes are the main class of dyes used in dyeing cotton fibers. However, their application may cause undesirable levels of environmental contamination and harmful effects on living organisms, including humans (Leme et al., 2014, 2015b; Fernandes et al., 2019). The environmental problems of reactive dyes are related to their high solubility in water and nondegradable under the typical aerobic conditions of conventional and biological treatment systems (Hassan et al., 2009; Oliveira et al., 2010). Ingestion of dispersive dye-polluted waters can compromise human health by inducting DNA damages (Oliveira et al., 2006, Chequer et al., 2011; Ferraz et al., 2013; Vacchi et al., 2013, 2017; Domingues et al., 2020). Apart from the oral route of exposure, humans
can also be affected by textile dyes by dermal contact with colored garments (Leme et al., 2014, 2015b; Brüscheiler et al., 2014).

Based on the association between DNA damages and cancer development, the safety assessment of chemicals comprises genotoxicity studies (Bolognese et al., 2017). In vitro genotoxicity tests are frequently performed with transformed or permanently growing cell lines (e.g., V79 and CHO) (Corvi & Madia, 2017). However, the choice of cell type for genotoxicity testing does not consider hazard identification in different routes of exposure; and chemical toxicity can differ depending on the target organ. For instance, the textile dye Disperse Red 1 is genotoxic for human hepatoma cells (HepG2) but not for dermal equivalents (Leme et al., 2015b).

The in vitro micronucleus (MN) assay tests clastogenicity/aneugenicity, and it is an endpoint required to predict chemical genotoxicity (Corvi & Madia, 2017). Studies using flow cytometry-based MN assay demonstrated several advantages in using this method: high throughput, high number of cells capable of being evaluated, good performance in detecting chemical genotoxicity, identification of genotoxic mode of action (clastogenic and/or aneugenic effects), and detection of cytotoxic effects (Avlasevich et al., 2011; Bryce et al., 2011; Yao et al., 2013; García-Rodríguez et al., 2019). Thus, flow cytometry-based MN assay allows assessing cytotoxicity and genotoxicity, which are predictive parameters essential for characterizing chemical toxicity (García-Rodríguez et al., 2019).

In this study, the organ-specific cyto- and geno-toxicity of the textile dyes Reactive Green 19 (RG19), Reactive Blue 2 (RB2), Reactive Blue 19 (RB19), Reactive Red 120 (RR120) and Reactive Orange 16 (RO16) was investigated using In Vitro MicroFlow® kit (Litron) assay (Bryce et al., 2011; Yao et al., 2013). Three of the five tested reactive dyes (RB2 (1000 µg/mL), RB19 (500 and 1000 µg/mL), RO16 (1000 µg/mL)) were cytotoxic (i.e., significant increase in EMA-positive) for HaCaT cells (Figure 1). In contrast, in HepaRG cells, cytotoxic effects were observed only for RB19 and RO16 at the highest tested concentration (1000 µg/mL) (Figure 2).

**MATERIAL AND METHODS**

**Tested chemicals**

The textile dyes Reactive Green 19 (RG19 – CAS No. 61931-49-5, dye content 65%), Reactive Blue 2 (RB2 – CAS No. 12236-82-7, dye content 60%), Reactive Blue 19 (RB19 – CAS No. 2580-78-1, dye content ~50%), Reactive Red 120 (RR120 – CAS No. 61951-82-4, dye content 50-70%)) and Reactive Orange 16 (RO16 – CAS No. 12225-83-1, dye content ≥ 70%) (Fig. 1) were purchased from Sigma-Aldrich. Phosphate buffered saline (PBS) was used as a vehicle to prepare the working dye solutions.

*In vitro MicroFlow® kit (Litron) assay*

Cell culture. HaCaT cells (Cell line services, Germany) and HepaRG (Biopredic International, Rennes, France, acquired from Fisher Scientific) were grown in culture medium at 37°C, flushed with 5% CO2 in air. Dulbecco’s Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum (FBS) (all from Gibco®, Life Technologies, Grand Island, USA) and addition of 100 IU/mL penicillin G, 100 mg/mL streptomycin and 1 µg/mL amphotericin, was used for HaCaT cells. Williams’ E medium, supplemented with 10% fetal calf serum (FCS), 1% insulin, 1% hydrocortisone hemisuccinate and addition of 0.1% streptomycin/penicillin, was used for HepaRG.

**Chemical treatments.** Tested concentrations were determined according to Leme et al. (2015b). HaCaT and HepaRG cells, at 1×10⁴ and 2×10⁶ cells/mL, respectively, were exposed in a 96-well format to six concentrations, of the test dyes (at 31.25 to 1000 µg/mL); PBS 20%-v/v (negative control); Mitomycin C (MMC) at 2.5 to 10 µg/mL (positive control) and Vinblastine (VB) at 12.5 -50 ng/mL (positive control) for 48 and 24 h, for both cell lines, respectively. The experiments were carried out in duplicate/treatment and repeated twice.

Flow cytometry-based MN measurements. A sequential staining method was applied to the treated samples according to the instructions described in the In Vitro MicroFlow® Kit (Litron Laboratories Ltd., Rochester, New York, United States). Briefly, the medium was removed, and the treated cells were stained with the photo-activated dye ethidium monoazide (EMA). The cells were washed, then lysed, and stained with lysis solutions composed of a nonionic detergent, pan-nucleic acid dye SYTOX Green and RNase.

The plates were immediately analyzed with a BD Biosciences (San Jose, CA) FACSCanto II flow cytometer. The percentages (%) of relative survival, EMA-positive, hypodiploid nuclei and MN were determined based on the acquisition of at least 10,000 gated nuclei per well.

**Data evaluation.** The % and fold-increase calculations were made according to Bryce et al. (Bryce et al., 2011), using Excel Office 2007 (Microsoft, Seattle, WA). A result was deemed positive if the following criteria were met: (i) cytotoxicity (EMA-positive events) showed greater than 3-fold increase compared with the concurrent solvent control values; (ii) genotoxicity – not overly cytotoxic concentration (without exceeding 60% reduction of relative survival) resulting in ≥ 3-fold increase in mean % MN relative to control value.

**RESULTS AND DISCUSSION**

Three of the five tested reactive dyes (RB2 (1000 µg/mL), RB19 (500 and 1000 µg/mL), RO16 (1000 µg/mL)) were cytotoxic (i.e., significant increase in EMA-positive) for HaCaT cells (Figure 1). In contrast, in HepaRG cells, cytotoxic effects were observed only for RB19 and RO16 at the highest tested concentration (1000 µg/mL) (Figure 2).
Figure 1. Cytotoxic and genotoxic data from flow cytometry-based in vitro MN assay with HaCaT cells are graphed for the tested reactive textile dyes. The Y-axis shows fold increase values of EMA+ and MN. The YY-axis shows the percentage of Relative Survival. *≥3-Fold over the concurrent solvent control (PBS).
Figure 2. Cyto- and geno-toxicity data of flow cytometry-based in vitro MN assay with HepaRG™ cells are graphed for the tested reactive textile dyes. The Y-axis shows fold increase values of EMA+ and MN. The YY-axis showed the percentage of Relative Survival. *≥3-Fold over the concurrent solvent control (PBS).
Cytotoxic effects were correlated to organ-specific toxicity, as observed in hepatic, cardiac and nephrotoxicity. Thus, assessing cell cytotoxicity in vitro helps in the earlier prediction of toxic effects on specific organs (Lin et al., 2012). In this context, the first step is to identify differences in sensitivity for each particular cell line. Specific cytotoxicity is related to the intrinsic cellular, physiological and metabolic differences between different cell lines, emphasizing the need to study the toxicity in an organ-specific context (Yeasmin et al., 2017).

The results showed that HaCaT cells appear to be more sensitive to reactive dyes than HepaRG cells. Other studies have indicated that HaCaT cell line tends to be more sensitive than other human cell lines, such as HepG2, HPL-1D, A431 (NTP, 2019; Eremin et al., 2018). In addition, HepaRG is a metabolic competent cell line with a metabolizing rate greater than HaCaT. This may contribute to faster detoxification of these chemicals, causing a lower level of damage in the cell. Furthermore, it is important to note that RB19 and RO16 are the dyes with the lowest molecular weights among those tested, and these dyes presented the highest level of cytotoxic effects in both cell lines. Thus, our data suggest that molecular weight might be an important contributor factor to the cytotoxicity of reactive dyes, as with other types of compounds (Monnery et al., 2017; Huang et al., 2004).

Regarding the genotoxic effects, for both cell lines, genotoxicity was not verified in any of the tested dyes compared to the negative control (Figure 1-2). Studies with reactive dyes using alkaline Comet assay generally report no genotoxicity for these dyes with mammalian cell line cultures (Leme et al., 2014; Janović et al., 2017), except RG19 and RB5 that presented genotoxicity in a three-dimensional (3D) human dermal equivalent and human lung epithelial cell line (Leme et al., 2015b; Janović et al., 2017). This may occur due to the metabolic activities specific to each cell type and the test setup systems used in each study; 3D tissue-like are more likely to suffer genotoxic damages when compared to monolayer cell cultures (Mandon et al., 2019; Behravesh et al., 2005). Moreover, differences between the genotoxicity test methods (Comet assay and MN assay) may explain the divergent results for RG19 and RB5. The Comet assay can detect primary DNA damages (e.g., single and double DNA strand breaks), which are likely to suffer the rapid action of the DNA repair systems (Collins, 2014). Therefore, as the chemical can cause DNA damage by different mechanisms of action, genotoxicity testing needs to cover all types of DNA damages (DNA strand breaks, chromosomal aberrations, sister-chromatid exchanges and micronuclei) to precisely determine the genotoxic nature of a chemical (Al-Saleh et al., 2017; Tafurt-Cardona et al., 2015).

CONCLUSION

The manufacturing of textiles commonly utilizes reactive dyes for dyeing cotton and other cellulose-based fibers. Given its widespread use and different human exposure pathways, it is important to evaluate the toxicity of textile dyes considering their different context of human exposure. Although the tested reactive dyes do not cause genetic damage (genotoxicity), they can cause cytotoxic effects in keratinocytes and hepatic cells and may pose risks to human health. The cytotoxicity effects of the reactive dyes are organ-specific, and it seems that epidermal cells (HaCaT) are more prone to the cytotoxic effects of these dyes than the hepatic cells (HepaRG). In order to better understand the present results, additional mechanistic studies need to be performed to elucidate the cellular uptake of tested dyes and their mode of action, particularly those related to cytotoxicity and genotoxicity. Nevertheless, the findings of this study show the need to perform investigations on the toxicity of textile dyes, considering local toxicity in target organs relevant for human exposure.

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AUTHOR CONTRIBUTION STATEMENT

Enzo Zini Moreira Silva – Formal analysis, writing - review & editing.
Andrea Sehr - Investigation, methodology, validation, visualization, data curation, formal analysis, writing - review & editing.
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Danielle Palma de Oliveira - Conceptualization, resources, supervision, writing - review & editing.
Daniela Morais Leme - Conceptualization, formal analysis, validation, supervision, writing - review & editing, project administration.
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