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In Vitro Testing for Genotoxicity of Indigoid Dyes by Comet Assay

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ÖZET

İndigoid boyaların genotoksik etkisinin *in vitro* comet tekniği ile değerlendirilmesi

Amaç: Doğal kaynaklı indigo ve indigoid boyalar; Bronz Çağı'ndan (M.Ö. 2000) beri bilinmektedir. Son dönemde ise indigoid boyalar sıklıkla tekstil, kozmetik, gıda ve ilaç endüstrisinde kullanılmaktadır. Bu çalışmanın amacı, *in vitro* alkali tek-hücre jel elektrofores (Comet) tekniği ile indigotin, 6-bromo indigotin, indirubin ve 6-bromo indirubin'in insan lenfositlerinde neden olabileceği olası DNA hasarını değerlendirmektir.

Yöntemler: İndigo ve indigoid boyaların sitotoksik etkisi, tripan mavisi yöntemi ile incelendi. Hücreler test bileşiklerinin 10, 25, 50 µg/mL'lik konsantrasyonları ile 37°C'de 30 dak inkübasyona bırakıldı. Test bileşiklerinin insan periferik lenfositleri üzerine genotoksik etkilerini değerlendirmek amacı ile comet tekniği kullanıldı.

Bulgular: Bu çalışma ile indigotin ve 6-bromo indigotin'in doza bağlı olarak DNA göçünü artırdığı görüldü. İndigotin ve 6-bromo indigotin'in 50 µg/mL'lik konsantrasyonu ile inkübe edilen hücrelerde DNA hasarı artmıştır ($p<0.05$).

Sonuç: Elde ettiğimiz sonuçlara göre, indigo ve indigoid boyaların yüksek konsantrasyonda genotoksik etkiye yol açabileceği ileri sürülebilir. Sonuç olarak, boyarmaddeler ile çalışan bireylerde yüksek konsantrasyonda boyaların genotoksik etkiye yol açabileceği göz önünde tutulmalıdır.

Anahtar sözcükler: İndigotin, 6-bromo indigotin, indirubin, 6-bromo indirubin, comet tekniği, DNA hasarı

ABSTRACT

In vitro testing for genotoxicity of indigoid dyes by comet assay

Objective: Indigo and indigoid dyes are natural dyes and have been known since Bronze Age (B.C. 2000). Nowadays, indigoid dyes are widely used in the industry of textile, cosmetic, food and medicine. The aim of this study was to estimate the DNA damage of indigotin, 6-bromo indigotin, indirubin and 6-bromo indirubin by *in vitro* alkaline single-cell gel electrophoresis (SCGE-Comet) in the peripheral lymphocytes.

Methods: The cytotoxic effects of indigo and indigoid dyes were assessed by trypan blue exclusion. The cells were incubated with 10, 25, 50 µg/mL of the test substances for 30 min at 37°C. Comet assay was used to evaluate the genotoxic effect of test substances on human peripheral lymphocytes.

Results: Our results revealed that indigotin and 6-bromo indigotin increased the DNA migration in a dose-dependent manner. DNA damage was higher in cells that had been incubated with 50 µg/mL indigotin and 6-bromo indigotin ($p<0.05$).

Conclusion: Our results indicate that indigo and indigoid dyes would be genotoxic at higher concentrations. It is probable that a genotoxic effect might occur due to the fact that the individuals who have worked with these dyestuffs, both in the past and today, used highly concentrated dyes.

Key words: Indigotin, 6-bromo indigotin, indirubin, 6-bromo indirubin, comet assay, DNA damage

INTRODUCTION

Indigo and indigoid dyes are natural dyes and have been known since Bronze Age (B.C. 2000) (1,2). Most natural indigoid dyes were obtained from woad (*Isatis tinctoria* L., Brassicaceae, also known as dyer's woad) and the indigo plant (*Indigofera tinctoria* L.) in temperate climates. *Isatis tinctoria* L. leaf and root extracts were shown *in vitro* and *in vivo* studies to be antibacterial and antiviral. Additionally,

woad was recommended for the treatment of wounds, ulcer and solid tumours, leukemia, haemorrhoid, snake bites, contact allergy, rheumatoid arthritis (3). Historically, woad has been used in Central Europe since antiquity as indigo dye. Indigo was chemically synthesized by Adolf von Baeyer in 1878 (2). Nowadays, indigoid dyes are widely used in the industry of textile, cosmetic, food and medicine (4). The indigoid compounds were used as astringent in the treatment of infections, ulcers, gastroenteritis and stop

bleeding (3). Moreover, indigo-related pigments (plant or animal origin) were found in historic yarns, textile fibers, printed document, icons and paintings.

Although indigo-related pigments are widely used, there are few studies about its genotoxic effects which show mutagenicity on *Salmonella typhimurium*. On the other hand, to evaluate the hazardous effects of dyes on DNA is very important to protect industry workers, who are exposed to dyes and the environment.

Alkaline single-cell gel electrophoresis (SCGE-Comet assay) is a sensitive genotoxicity assessment, which detects DNA-strand breaks and alkali-labile sites within a relatively short period of time and cost-effective manner. The comet assay has been used for the assessment of genetic damage *in vitro* and *in vivo* in a great variety of cells (5,6). It is used to assess the genotoxicity of industrial chemicals (new chemicals and existing chemicals) as well as for agrochemicals, biocides, pharmaceuticals and physical agents (7).

The aim of this study was to estimate DNA damage of indigotin, 6-bromo indigotin, indirubin and 6-bromo indirubin by *in vitro* alkaline comet assay in the peripheral lymphocytes.

MATERIALS AND METHODS

Dye Exposure

Indigotin, 6-bromo indigotin, indirubin and 6-bromo indirubin were dissolved in DMSO to obtain a 10 g/L stock solution. Immediately before use, aqueous solution of these test compounds at 1 g/L concentration was added to the lymphocytes according to the different schedules.

Approximately 5 mL of peripheral blood samples were collected from a non-smoking; healthy female donor aged 30 years in sterile disposable syringes, transferred into heparinized tubes. Unstimulated lymphocytes were isolated by Histopaque 1077 density gradient centrifugation, washed

in phosphate buffered saline (PBS), and then re-suspended in 1 mL PBS. Trypan blue exclusion was used for viability test. Human peripheral blood lymphocytes (~50.000 cells/mL) were incubated for 30 min at 37°C in Biochemical Oxygen Demand (BOD) incubator with 10, 25 and 50 µL/mL concentrations of test compounds and DMSO (10 µL/mL) as a negative control. Hydrogen peroxide (H₂O₂) was added to the prepared cells at the final concentration of 25 µL/mL as positive control. Each test compound was tested four times in each experiment along with positive and negative controls. In addition, each test compound's analysis was performed at the same time to prevent inter-test variables.

Comet assay

The alkaline comet assay was performed using an adaptation of the method of Singh et al. (8). Frosted microscope slides were covered with 0.7% normal melting agarose. At the end of incubation, prepared cells were mixed with 0.7% low melting point agarose (LMA) and the mixture was spread on microscope slides. After solidification of low-melting agarose, slides were immersed in cold lysing solution (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris, pH 10 with 1% Triton X-100 and 10% DMSO) for at least 1 h at +4°C. After the lysis, the slides were placed in a horizontal electrophoresis tank, which was filled with electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH 13); the slides were left in the solution for 20 min prior to electrophoresis to allow the unwinding of the DNA and the expression of alkali-labile damage. Subsequently, the DNA was electrophoresed for 30 min at 300 mA and 15 V. Cells were neutralized with 0.4 M Tris buffer, pH 7.5 and stained with 50 µl ethidium bromide (EtBr - 20 µg/ml). The slides were analyzed using a fluorescence microscope at 200x magnification. Analysis of randomly selected one hundred cells per slide was kept and each treatment was carried out in duplicate. Cells were scored visually into five classes according to tail size to

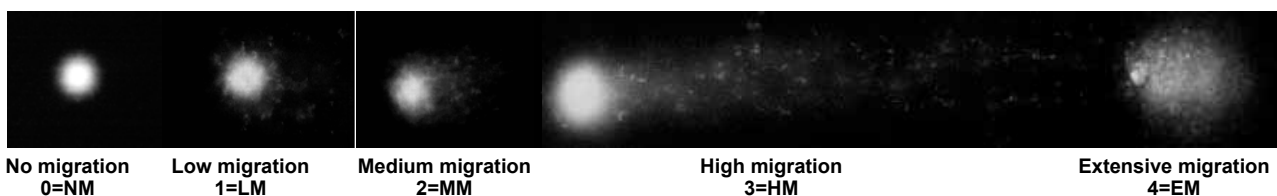


Figure 1: Representative comet images showing different levels of damage in visual scoring

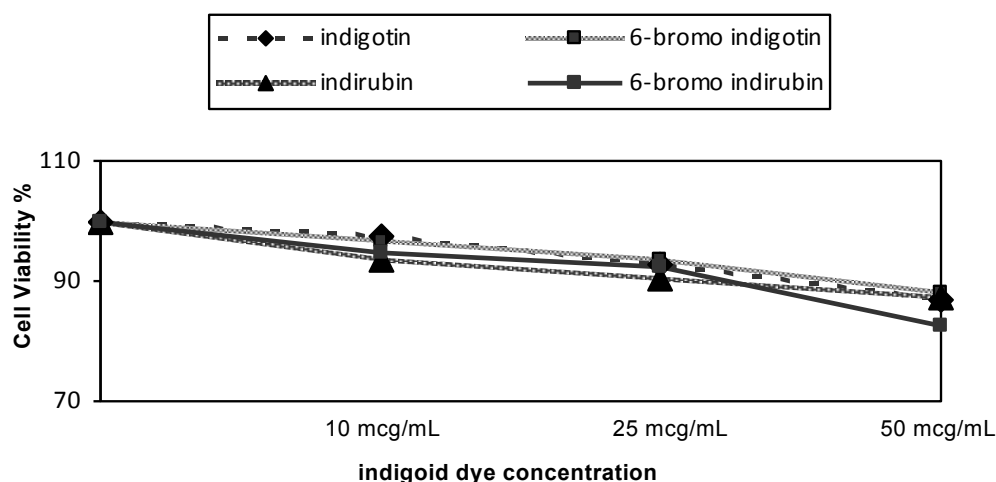


Figure 2: Cytotoxicity in lymphocytes after exposures to increasing concentrations of indigoid dyes for 30 min

facilitate the management of the data and the mean total comet scores (mean TCS) were calculated as $TCS = 0 \times \text{No Migration (NM)} + 1 \times \text{Low Migration (LM)} + 2 \times \text{Medium Migration (MM)} + 3 \times \text{High Migration (HM)} + 4 \times \text{Extensive Migration (EM)}$, which was referred by Collins (9). Undamaged cells presented an intact nucleus without a tail and damaged cells had the appearance of a comet as shown in Figure 1.

Statistical Analysis

Statistical comparison of the mean individual grade of DNA damages were analyzed by using the non-parametric Kruskal-Wallis test followed by Tukey test as a post hoc test. All

results were expressed as means \pm SD and p value less than 0.05 was defined to be statistically significant. SPSS 17 was used for statistical analysis.

RESULTS

The cytotoxicity of compounds on the cells was tested by Trypan blue exclusion. The results showed that toxicity on lymphocytes increased in the presence of indigoid dyes depending on the dose. The highest toxicity was observed with indigotin, 6-bromo indigotin, indirubin and 6-bromo indirubin at 50 $\mu\text{g/ml}$ concentration after 30 min incubation ($p < 0.05$). The toxicity of compounds at 10 and 25 $\mu\text{g/ml}$ concentration was not statistically significant ($p > 0.05$). Cell

Table 1: Cell viability percentage and total comet scores in lymphocytes after exposures of indigotin, 6-bromo indigotin, indirubin and 6-bromo indirubin at different concentrations for 30 min.

Test Compound	Concentration	Cell Viability % (Mean \pm SD)	Total Comet Score ^a (Mean \pm SD)
Indigotin	10 $\mu\text{g/mL}$	97.40 \pm 1.89	50.25 \pm 13.38
	25 $\mu\text{g/mL}$	92.59 \pm 5.95	55.75 \pm 18.28*
	50 $\mu\text{g/mL}$	87.01 \pm 5.95*	77.25 \pm 24.51*
6-bromo indigotin	10 $\mu\text{g/mL}$	96.70 \pm 4.29	47.00 \pm 24.39
	25 $\mu\text{g/mL}$	93.64 \pm 2.84	37.00 \pm 9.35
	50 $\mu\text{g/mL}$	88.02 \pm 2.84*	59.00 \pm 16.06*
Indirubin	10 $\mu\text{g/mL}$	93.35 \pm 4.55	28.25 \pm 8.14
	25 $\mu\text{g/mL}$	90.52 \pm 1.30	27.50 \pm 17.25
	50 $\mu\text{g/mL}$	87.28 \pm 6.24*	49.00 \pm 26.93
6-bromo indirubin	10 $\mu\text{g/mL}$	94.89 \pm 2.79	29.50 \pm 15.42
	25 $\mu\text{g/mL}$	92.48 \pm 4.73	26.50 \pm 3.70
	50 $\mu\text{g/mL}$	82.43 \pm 11.13*	39.50 \pm 13.03
Positive control H_2O_2	25 $\mu\text{L/mL}$	92.99 \pm 2.82	164.00 \pm 36.05
Negative control DMSO	10 $\mu\text{L/mL}$	97.02 \pm 3.95	18.00 \pm 13.29

^aTotal comet score: $0 \times \text{No Migration (NM)} + 1 \times \text{Low Migration (LM)} + 2 \times \text{Medium Migration (MM)} + 3 \times \text{High Migration (HM)} + 4 \times \text{Extensive Migration (EM)}$. *P < 0.05.

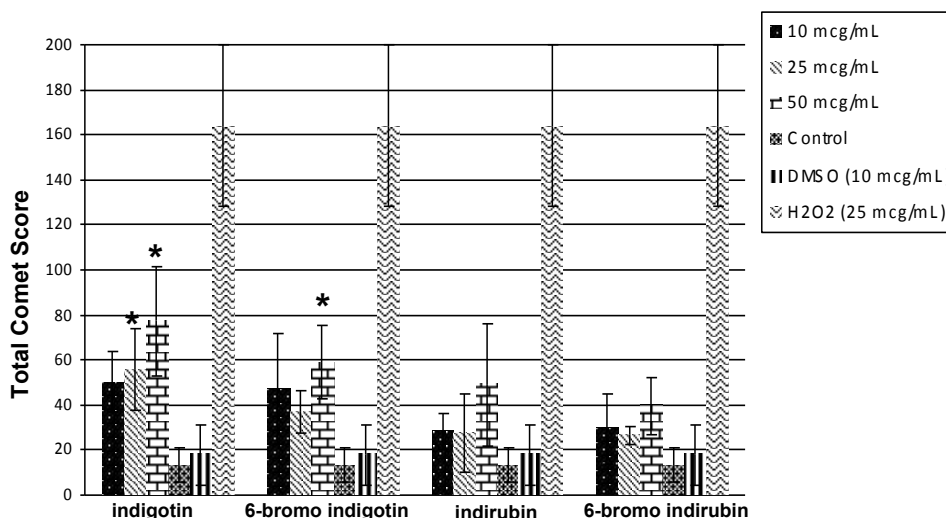


Figure 3: DNA damage levels in lymphocytes after exposures of indigotin, 6-bromo indigotin, indirubin and 6-bromo indirubin at different concentrations for 30 min (* $p < 0.05$).

viability percentage of lymphocytes after exposures to increasing concentrations of indigoid dyes for 30 min is shown in Figure 2.

Our results revealed that indigotin and 6-bromo indigotin increased the DNA migration in a dose-dependent manner. DNA damage was higher in cells which had been incubated with 50 $\mu\text{g/mL}$ indigotin and 6-bromo indigotin for 30 min ($p < 0.05$). Indirubin and 6-bromo indirubin did not induce DNA damage. Table 1 shows the cell viability percentage and total comet scores in lymphocytes after exposures of indigoid dyes. Figure 3 shows the mean total comet score distribution of DNA damage in lymphocytes after exposures of indigoid dyes at different concentrations for 30 min.

DISCUSSION

Indigoid dyes are widely used in the industry of textile, cosmetic, food and medicine (4). Medicinal uses of indigoid compounds were as astringent for treatment of infections, ulcers, gastroenteritis and stop bleeding (3). Although indigo-related pigments are used widely, the toxicity of them is little known in the literature. Labib et al. (10) reported a case of fatal poisoning in a 3-year-old child after administration of indigo for therapeutic purposes (diarrhea, vomiting and fever).

Moreover, there are few studies about its mutagenic effect in *Salmonella typhimurium*. Jongen and Alink (11) investigated the mutagenic potential of two natural and

seven synthetic commercial indigo dye products on *Salmonella typhimurium* strains TA98 and TA100. They reported that the natural products did not show mutagenic effect on TA98 and TA100. On the other hand, synthetic products were mutagenic on strain TA98 but not on strain TA100 in the presence of S9.

In another study, Rannug et al. (12) studied mutagenic effect of pure cotton, jeans fabrics extracts and synthetic indigo, indirubin, isatin on *Salmonella typhimurium* strains TA98 and TA100. Mutagenic effects on TA98 +/- S9 and TA100 +/- S9 had been seen on the extracts of both bleached and nonbleached jeans. The mutagenic effects were increased in the presence of S9. Moreover, synthetic indigo of technical grade or 98% pure showed mutagenic effects, especially on TA98 + S9. They reported that indigo might be causing a potential health risk either by revealing toxic effects of other compounds or by being a nongenotoxic carcinogen.

The genotoxicity of indigo has been reported with two studies. Hesbert et al. (13) compared the mutagenicity of natural indigo with synthetic indigo by micronucleus test in the bone marrow of male mice. They have found that natural indigo did not increase the percentage of polychromatic erythrocytes with micronuclei. Dominici et al. (14) have evaluated the genotoxicity of indigo naturalis (prepared from *Indigofera tinctoria* leaves) by cytokinesis-blocked micronucleus assay in the human metabolically active HepG2 cell line. They have reported that the

frequency of micronuclei in exposed cells was no higher than the control.

Beside these genotoxicity investigations, there is no data available about DNA damage effects of indigoid dyes by the most sensitive genotoxicity assessment comet assay. Comet assay is used to assess the genotoxicity of industrial chemicals, agrochemicals, biocides, pharmaceuticals and physical agents (7). In our study, we estimated cytotoxic effects and DNA damage of indigotin, 6-bromo indigotin, indirubin and 6-bromo indirubin by *in vitro* alkaline comet assay in the peripheral lymphocytes. The highest toxicity was observed with indigotin, 6-bromo indigotin, indirubin and 6-bromo indirubin at 50 µg/ml concentration after 30 min incubation. Our results revealed that indigotin and 6-bromo indigotin increased the DNA migration in a dose-dependent manner. DNA damage was higher in cells that

had been incubated with 50 µg/mL indigotin and 6-bromo indigotin than lower concentrations. However, it is important to mention that further studies are needed to show the genotoxic effects of indigoid dyes in dye-exposed workers by comet assay.

As a conclusion, our results indicate that indigo and indigoid dyes would be genotoxic at higher concentrations. It is probable that a genotoxic effect might occur due to the fact that the individuals who have worked with these dyestuffs, both in the past and today, used highly concentrated dyes.

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