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Single Strand DNA Breaks in Human Lymphocytes Exposed to *para*-Phenylenediamine and its Derivatives

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Abstract *Para*-Phenylenediamine (PPD), the main aromatic amines used in the hair dye formation, and its four derivatives (2-chloro-*p*-phenylenediamine, 4-chloro-*o*-phenylenediamine, 2-nitro-*p*-phenylenediamine, and 4-nitro-*o*-phenylenediamine) were examined for their potential to produce single strand DNA breaks in human lymphocytes using the alkaline comet assay. Results revealed that all the tested chemicals within the range of doses from 100 μM to 500 μM showed the genotoxicity in a dose-dependent manner after the incubation of lymphocytes with these chemicals for 2 h. In this study, we first reported that PPD and its four derivatives can elicit the type of single strand breaks in human lymphocytes.

Keywords *para*-Phenylenediamine (CAS 106-50-3) · Comet assay · Lymphocytes · Genotoxicity

para-Phenylenediamine (PPD) is the main aromatic amine used in hair dye formulations. Many azo dyes used by the industry also contain the *p*-phenylenediamine moiety (Chung et al. 1993, 1995). After azo reduction of these dyes by environmental or intestinal microorganisms, PPD may be released (Chung et al. 1993). When PPD is ingested, it will be absorbed and redistributed to target to exert its effect (Chen et al. 2006). PPD has been reported to increase the formation of liver tumors in mice (Sontag 1981). Garner and Nutman (1977) demonstrated that PPD was strongly mutagenic to Salmonella tester strain TA 1538 when tested in the presence of rat liver S-9 preparation, while this compound was weakly mutagenic to TA 98 with metabolic activation (Chung et al. 1995). The reaction products of PPD with hydrogen peroxide were positive in the Ames test, the mouse lymphoma assay and in human lymphocytes, whereas genotoxicity was inhibited by adding correct proportion of a coupler (resorcinol) (Garrigue et al. 2006). It was suggested that oxidative hair dyes containing PPD, hydrogen peroxide and couplers are non-genotoxic up to 1 h after dye development (Garrigue et al. 2006). However, the genotoxicity of this hair dye after the exposure time of 5 or 7 h at room temperature become weakly positive (Garrigue et al. 2006). Chung and Cerniglia (1992) reported that PPD is the moiety responsible for the mutagenic activity of many azo dyes. Methylation or substitution of a nitro group for an amino group in PPD did not reduce mutagenicity, but sulfonation, carboxylation, deamination or substitution of an ethyl alcohol or an acetyl group for the hydrogen in the amino group lead to a decrease in mutagenic activity (Chung et al.

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1995). Therefore, disposition of PPD and PPD-containing compounds is becoming an important issue.

Although many of PPD derivatives were found to be mutagenic with the Ames test (Shanin 1989), the Ames test only detects DNA damage of prokaryotic cells (bacterial cells) caused by genotoxic chemicals. However, the comet assay (single-cell gel electrophoresis) has been shown to be capable of analyzing DNA damage in many different eukaryotic cells in vitro (Rojas et al. 1999), and is considered as a simple, rapid, and sensitive technique for measuring single-stranded breaks in DNA (Horvathova et al. 1998). Furthermore, the DNA-damaging effects of hair dyeing in human lymphocytes (Cho et al. 2003), and PPD, the major component of hair dyeing, after metabolic activation exhibiting its mutagenicity (Chung et al. 1995) provoke our motive to prove the possibility that PPD and its derivatives without metabolic activation can cause single strand DNA breaks in human lymphocytes using alkaline comet assay.

Materials and Methods

para-Phenylenediamine (PPD; CAS 106-50-3), 4-nitro-*o*-phenylenediamine (4NPD; CAS 99-56-9), 98% pure, 2-chloro-*p*-phenylenediamine (2CPD; CAS 61702-44-1), 95% pure, 4-chloro-*o*-phenylenediamine (4CPD; CAS 95-83-0), 2-nitro-*p*-phenylenediamine (2NPD; CAS 5307-14-2), 95% pure, were purchased from Sigma-Aldrich Chemical co., (St Louis, MO). All chemicals were of the highest grade commercially available. These chemicals are free of salt and bases. Tested chemicals were freshly prepared by dissolving in Dimethyl sulfoxide (DMSO) and were kept in the dark. The final concentration of DMSO was less than 1% of the reaction mixtures.

Blood withdrawn from a female donor (health and non-smoker, aged 25) was collected into Ficoll-Hypague. The samples were then centrifuged at 200 × *g* at 25°C for 20 min. The formed lymphocyte forming a layer was directly above the Ficoll-Hypague. The isolated lymphocytes (0.3 mL) were cultured in 4.7 mL RPMI 1640 medium including 20% heat-inactivated fetal calf serum, 2% phytohemagglutin (PHA), 100 IU/mL of penicillin, 100 μg of streptomycin, and 2 mM of L-glutamine at 37°C under 5% CO_2 atmosphere.

The cell viability analysis was conducted. A volume of 0.49 mL of cell suspension treated with 50–500 μM of each tested chemical was mixed with 10 μL of 0.4% of trypan blue solution. Its viability was determined after 2 h of reaction. The cells were analyzed through microscopic observation to determine the percentage of viable cells.

The tested chemicals were dissolved in DMSO (1% as a final concentration) and diluted into 50, 100, 200, and 500 μg/mL. Lymphocytes were incubated with different concentrations of the tested chemicals at 37°C for 2 h in a dark incubator. On the other hand, the lymphocytes treated with 50 μM H_2O_2 at 37°C for 5 min and treated with 1% DMSO 37°C for 2 h were used as the positive group and the negative group, respectively. Subsequently, these lymphocytes were centrifuged at 200 *g* for 3 min at 4°C and then were mixed with low melting point agar for the comet assay. The comet assay was performed under alkaline conditions. Conventional microscope slides were dipped with a solution of 85 μL of 0.5% of normal melting point agarose (NMP) and 0.5% of low melting point agarose (LMP) in phosphate buffer solution (PBS) (pH 7.4), and allowed to dry on a flat surface at room temperature. Ten μL of cell suspension (2.5×10^5 cells/mL) were gently mixed with 75 μL of 0.5% (w/v) of LMP in PBS (pH 7.4). Seventy-five μL of this suspension was rapidly layered onto the slides pre-coated with the mixtures of 0.5% NMP and 0.5% LMP, and covered with a coverslip. The slides were maintained at 4°C for 5 min, the coverslip was removed, and cells were immersed in a freshly made lysis solution (2.5 M of NaCl, 100 mM Na_2EDTA, 10 mM Tris and 1% (v/v) of Triton X-100 at pH 10) at 4°C for 10 min. The Slides were then placed in a double row in a 260 mm wide horizontal electrophoresis tank containing 0.3 M NaOH and 1 mM Na_2EDTA for 10 min. Thereafter, the electrophoresis (30 V, 300 mA) was conducted for 15 min at 4°C. After the electrophoresis, the slides were then soaked in a cold neutralizing buffer (400 mM of Tris buffer, pH 7.5) at 4°C for 10 min. Slides were dried in methanol for 5 min, and stored in a low humidity environment before staining with silver stain. After electrophoresis and neutralization a modified version of silver staining protocols published by Nadin et al. (2001) was applied. Slides were washed twice with deionized water, and then placed at 37°C to dry gels (usually 1.5–2 h). Subsequently, the slides containing dry gels were fixed for 10 min in fix solution (15% trichloroacetic acid, 5% zinc sulphate heptahydrate, 5% glycerol), and washed twice with deionized water. Thereafter, these slides were allowed to be dried overnight at room temperature, be rehydated for 5 min in deionized water, be placed back-to-back in a horizontal staining jar, and finally be stained for 35 min in dark conditions with shaker using 100 mL of freshly prepared stain solution composed by 34 mL of vigorously mixed stock solution B (0.1% ammonium nitrate, 0.1% silver nitrate, 0.25% tungstosilicic acid, 0.15% formaldehyde, v/v) and 66 mL of stock solution A (5% sodium carbonate). After the staining, these slides were washed 2–3 times with deionized water, immersed 5 min in a stop solution (1% acetic acid), and finally air-dried. One hundreds comets on each slide were scored visually according to the relative intensity of silver in the

tail. An intensity score from class 0 (undamaged) to class 4 (severely damage) was assigned to each cell based on the procedures in Visvardis et al. (1997). Thus, the total score for the 100 comets could range from 0 to 400 because the 100 cells were observed individually in each comet assay. The extent of DNA damage was analyzed and then scored by the same experienced person, using a specific pattern when moving along the slide. The method of the observation was barred in a blind way during which the observer had no knowledge of the identity of the slide. Figure 1 illustrates examples of the visual scoring classification for lymphocytes.

A nonparametric test (Kruskel-Wallis) was used to evaluate differences in the distribution of DNA damage between the tested group and the control group. The Kendall's correlation was used to assess the level of the dose-dependent DNA damage response for all the tested chemicals. For all statistical analysis, a level of 0.05 was used as the lower bound to determine the significance of the variation.

Results and Discussion

The cytotoxicity of PPD and its derivatives in human lymphocytes was evaluated. The cell viability was greater than 94% when cells were treated with the tested chemicals at the doses ranging from 50 μM to 500 μM at 37°C for 2 h (Table 1). Since these chemicals at the doses from 50 μM to 500 μM showed insignificant toxicity to cell viability (Table 1); thus, these dosages will be applied for the comet analysis. Table 1 summarizes the results of single strand DNA breaks in human lymphocytes treated with varying concentrations of tested chemicals at 37°C for 2 h, as measured by comet assay. Results indicate that the positive group (cells pretreated with 50 μM H_2O_2) showed the maximum levels of single strand DNA breaks (275 ± 10), while the negative control (1% DMSO as solvent for each tested chemical) revealed the lowest levels of single strand DNA breaks (72 ± 14). At a concentration of 100 μM, all the tested chemicals exhibited significant single strand DNA breaks when compared to the negative control group ($p < 0.05$). These tested chemicals revealed the genotoxicity to lymphocytes in a dose-dependent manner ($p < 0.05$). Bolt and Golka (2007) reported that the PPD within the dose range of 0.1% to 0.5% may be absorbed through the skin under conditions of use of permanent hair dyes, while these absorbable doses of PPD into skin apparently exceed beyond that of PPD tested in this study.

The comet assay was used to detect the single strand DNA breaks caused by PPD and its four derivatives. Results showed the degree of single strand DNA breaks in the lymphocytes caused by these chemicals (Table 1). PPD exhibited the genotoxicity towards lymphocytes without metabolic activation (Table 1), whereas it shows the mutagenicity to TA 98 with requirement of metabolic activation (Chung et al. 1995). In the Ames test, the introduction of a nitro-group to the PPD ring greatly increased the mutagenicity, and the position of the nitro

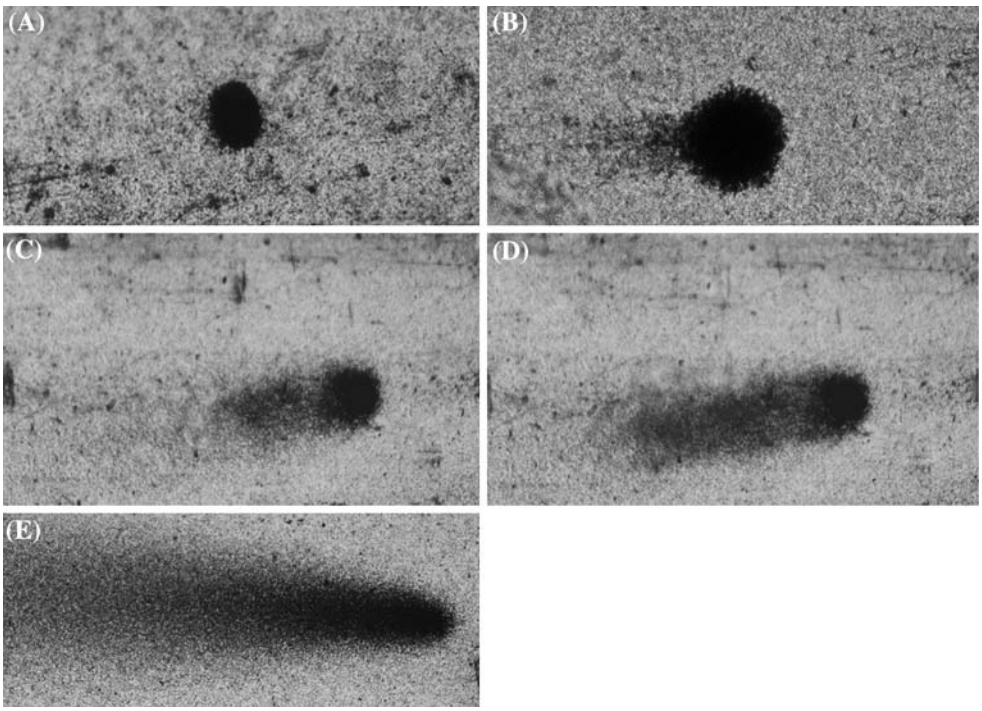

Fig. 1 Comet images of lymphocytes, illustrating the visual scoring classification. (**A**) Class O, (**B**) class 1, (**C**) class 2, (**D**) class 3, (**E**) class 4

Table 1 Responses of lymphocytes to different doses of PPD and its derivatives

Chemical[a]	Dose (μM)	Cell viability (%)	DNA damage (Arbitrary units)[b]
DMSO	–	99	72 ± 14
H_2O_2	50	93	275 ± 10*
p-Phenylenediamine			
	50	96	113 ± 15*
	100	97	146 ± 55*
	200	99	163 ± 47*
	500	97	188 ± 11*
2-Chloro-*p*-phenylenediamine			
	50	99	122 ± 14*
	100	98	127 ± 14*
	200	99	131 ± 14*
	500	98	142 ± 13*
4-Chloro-*o*-phenylenediamine			
	50	96	152 ± 24*
	100	95	193 ± 12*
	200	96	223 ± 10*
	500	94	274 ± 13*
2-Nitro-*p*-phenylenediamine			
	50	95	121 ± 28*
	100	97	127 ± 29*
	200	97	159 ± 14*
	500	96	211 ± 17*
4-Nitro-*o*-phenylenediamine			
	50	99	75 ± 11
	100	98	144 ± 14*
	200	97	175 ± 30*
	500	96	200 ± 28*

[a] All chemicals were dissolved in 1% of DMSO

[b] Mean DNA damage score in arbitrary units (±SE) were calculated from the respective values of at least three treatments (100 cells/slide, duplicate slides/treatment

*Represented for $p < 0.05$

group affected their mutagenic activities (Chung et al. 1995). However, the presence of the nitro-group did not affect potency in inducing chromosomal aberrations in Chinese hamster ovary cells (CHO-K1) (Chung et al. 1995) and in producing DNA strand breaks of human lymphocytes in this study. Since the reduction of the nitro group to produce the corresponding *N*-hydroxyamino derivatives is an important metabolic pathway (Fu 1990), and is considered to be the primary step in the conversion of amines to their ultimate mutagenic form (Kadlubar et al. 1990), an amine with a greater mutagenic activity would be expected to be oxidized more easily (Sontag 1981). However, this possibility was not supported by the measured electrochemical oxidation potentials of PPD and its derivatives (Sontag 1981).

In our study, the visual scoring was used for quantification of DNA damage based on that a good correlation between comet results by visual classification and the percentage of DNA in the tail, and the reliability of visual scoring, and the potential of combining it with silver staining in the comet assay were confirmed (Hartmann et al. 2003).

A type of single strand DNA breaks in lymphocytes treated with PPD and its derivatives was first determined using the alkaline comet assay. The level of genotoxicity of these chemicals determined by comet assay does not correlate with that by Ames test. This observation could be due to the different responses of eukaryotic and prokaryotic cells to genotoxic compounds. With the use of visual scoring and silver staining in the comet assay, the determination of these genotoxic without compounds in this study is rapid and simple. Furthermore, this method can be used fluorescence microscope and image analysis systems.

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