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Application of the *in vivo Pig-a* gene mutation assay to test the potential genotoxicity of *p*-phenylenediamine

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ABSTRACT

Currently, it remains controversial whether *p*-phenylenediamine (PPD) is genotoxic. In this study, we evaluated the potential genotoxicity of PPD using the newly-developed *Pig-a* gene mutation assay. The results of three classical genetic toxicity tests (bacterial reverse mutation assay, mammalian cell chromosomal aberration test, and mammalian erythrocyte micronucleus test) are all positive, suggesting that PPD is potentially genotoxic. In *Pig-a* assay, Sprague-Dawley rats are orally administered with PPD for 28 consecutive days at three doses (12.5, 25, and 50 mg/kg/day). Our result shows that PPD (25 and 50 mg/kg/day) dose-dependently increases RET^{CD59-} value over controls on Day 8. RET^{CD59-} keeps increasing to the maximum on Day 15 and then decreases until Day 29. PPD also dose-dependently increase RBC^{CD59-} value on Day 15, which keeps elevating until Day 29. The time-course of RET^{CD59-} and RBC^{CD59-} induced by PPD are similar with that induced by N-ethyl-N-nitrosourea (ENU) treatment for 3 days. Our data suggests that PPD has potential genotoxic effects, and the *Pig-a* assay is sensitive to assess mutagenicity. However, further investigation of the changes of RET^{CD59-} and RBC^{CD59-} induced by hair dyes containing PPD should be detected by *Pig-a* assay in occupational exposure population to confirm the safety of PPD usage.

1. Introduction

Hair dyes are frequently used by about 30% of women and 10% of men in the USA. *p*-phenylenediamine (PPD) has been used as a precursor in hair dye products for decades. PPD is recognized to cause severe allergic dermatitis, skin erythema, and edema mediated by a delayed immune response (Smith Pease, 2003). Several epidemiologic studies have suggested that the use of PPD-containing hair dye is associated with increased incidence of malignant tumors, such as bladder cancer, hematopoietic cancer, and many others (Malvestio et al., 2010; Rauscher et al., 2004). Further studies have shown that PPD derivatives also show carcinogenic potential (Van Duuren, 1980; Shahin, 1989). For example, PPD exhibits strong mutagenicity in *Salmonella typhimurium* strain TA1538 (Garner and Nutman, 1977), and induces DNA damage in comet assays. PPD also induces p53 mutations and COX-2 upregulation in human uroepithelial cells, which probably indicate its genotoxic potential by inducing carcinogenesis (Huang et al., 2007).

The rodent *Pig-a* assay represents a new method to detect gene mutation, thus, it is widely used in evaluating the genotoxicity of pharmaceuticals and industrial agents (Bryce et al., 2008). The mutant red blood cells or reticulocytes (defined as RBC^{CD59-} or RET^{CD59-}, respectively) serve as excellent reporters for the gene mutation, based on the loss of glycosylphosphatidylinositol (GPI)-anchored proteins on cell membranes, when wild type cells are labelled with a CD59 antibody. Therefore, the frequency of RET^{CD59-} and RBC^{CD59-} can be detected via flow cytometry (Miura et al., 2008). This assay is established as a potential method for characterizing the genotoxicity and dose-response relationships of chemical substances and weak mutagens. In addition, it is very simple and convenient to quantitate mutations in comparison with other mutation assays using transgenic animals (Phonethepswath et al., 2010).

Routinely, hair dye users are exposed to PPD by direct skin contact.

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PPD is absorbed and acetylated to mono-acetyl-PPD (MAPPD) and *N,N*-diacetyl-PPD (DAPPD) by *N*-acetyltransferase 1 (NAT1) in human keratinocytes (Nohynek et al., 2015). This acetylation process is deemed as a detoxification mechanism. However, genetic polymorphisms of NAT1 may influence individual susceptibility to the toxic effects of PPD by affecting the acetylation status of PPD (Kawakubo et al.,2000). PPD, is used not only as a primary intermediate in hair dye formulations, but also as an antioxidant and accelerator for rubber vulcanization, as well as an intermediate in the production of azo dyes (Hansen et al., 1993). To date, there is no report on the genotoxicity or dose-response relationship of PPD using the novel *Pig-a* assay recommended by Organization for Economic Co-operation and Development (OECD) or China Food and Drug Administration (CFDA). To this end, we investigate the potential genotoxicity of PPD by the combination tests and assess the mutant frequency induced by accumulating doses of PPD. We also plot the dose-response curve of PPD (by oral gavage for 28 consecutive days) via *Pig-a* assay.

2. Materials and methods

2.1. Reagents and chemicals

p-phenylenediamine (PPD, CAS No.106-50-3) was purchased from Thermo Fisher (Waltham, MA, USA). Mitomycin C (MMC), methyl methanesulfonate (MMS), N-ethyl-N-nitrosourea (ENU, CAS No.759-73-9), 1,8-dihydroxyanthraquinone (1,8-DHAQ), and cyclophosphamide were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2-anlinofluorene (2-AF) was purchased from Alfa Aesar (Haver Hill, MA, USA). Sodium azide was purchased from UNI-CHEMR (Haw River, NC, USA). 2,4,7-trinitro-9-fluorenone (TN9F) was obtained from Accu Standard (New Haven, CT, USA). S9 was obtained from Molecular Toxicology Inc. (Boone, NC, USA). Anticoagulant solution, buffered salt solution and PE mouse anti-rat CD59 were purchased from BD Biosciences (Franklin Lakes, NJ, USA). Nucleic acid dye solution was obtained from Thermo Fisher (Waltham, MA, USA). Reagents used for flow cytometer (Cytomics FC 500) were purchased from Beckman Coulter (Brea, CA, USA). Reagents used for cell culture were purchased from Gibco (Grand Island, NY, USA).

2.2. Strains

Four *Salmonella typhimurium*strains are used in Ames test, *i.e.*, TA97a, TA98, TA100, and TA102 (lot numbers 4366D, 4367D, 4370D, and 4372D, respectively). Four strains were obtained from Molecular Toxicology Inc. (Boone, NC, USA) and stored as frozen stocks.

2.3. Cell culture

CHL/IU [CHL11] (ATCC®CRL-1935™), the Chinese hamster lung cell line used in mammalian cell chromosomal aberration test (CA) was purchased from ATCC (Manassas, VA, USA), with modal number 25 and cell cycle time about 16 h. Subcultures using in experiment were prepared from the CHL cells frozen stock cultures maintained in liquid nitrogen. CHL cells were plated and grew as monolayers, and cells were cultured at 37 °C in RPMI 1640 medium (containing 100 IU/ml penicillin, 100 IU/ml streptomycin and 10% FCS), which was changed every 2 days, in 37°Catmosphere containing 5% CO_2.

2.4. Animals

Male NIH mice (age, 4–5 weeks) used in mammalian erythrocyte micronucleus test (MN) and Sprague-Dawley rats (age, 6–7 weeks) used in *Pig-a* mutation assay were purchased from Guangdong Medical Laboratory Animal Center (Guangzhou, China). Animals were quarantined and acclimated for 3–7 days before experiments. Animals were supplied with food and water *ad libitum*, with circumstance temperature 20°C-26 °C, relative humidity 40%–70%, and a 12 h cycle of light/dark. Experiments were performed in accordance with Animal Care and Use Committee of Shenzhen Institute for Drug Control, Guangdong, China.

2.5. Bacterial reverse mutation assay

According to OECD Guideline 471 (Test No. 471, 1997) and the cosmetics material guidelines on Ames test (Safety and Technical Standards for Cosmetics, 2015 version, China), PPD was evaluated using four strains (TA97a, TA98, TA100, and TA102) (Tu et al., 2015). The initial dose was determined by a preliminary test in TA100 strain with or without S9 mix. A high concentration of non-cytotoxic 5 mg/plate was chosen to test, as recommended by OECD guidelines. The final doses were 0.312, 0.625, 1.25, 2.5, and 5 mg/plate, with PPD being dissolved in sterilized deionized water. The Ames assay was conducted with or without S9 mix. Sterile deionized water was used as the vehicle control. The positive reagents were selected respectively as below, without S9 mix, TN9F (0.2 μg/plate) for TA97a and TA98, sodium azide (1.5 μg/plate) for TA100, MMC (0.5 μg/plate) for TA102, whereas, with S9 mix, 2-AF (10 μg/plate) for TA97a, TA98, and TA100, 1,8-DHAQ (50 μg/plate) for TA102. Triplicates were included in each group. After incubation at 37 °C for 48 h, colonies were counted. Positive response is considered when revertant colonies for each strain increase by two-fold over vehicle control.

2.6. Mammalian cell chromosomal aberration test

The CA test was conducted according to OECD Guideline 473 (Test No. 473, 2016) and the cosmetics material guidelines (Safety and Technical Standards for Cosmetics, 2015 version, China) (Li et al., 2017). According to the results of the cytotoxicity tests, three doses were selected, 312 μg/ml (high dose), 156 μg/ml (middle dose), and 78 μg/ml (low dose). Tests were performed with or without S9 mix. PPD was prepared freshly in PBS (pH 6.0) with or without S9 mix, MMC (10 μg/ml) and CP (2 μg/ml) were selected as positive controls. CHL cells were plated in 6 well plates (23,000 cells per well) 24 h before treatment. Cells were incubated at 37 °C with atmosphere containing 5% CO_2. Media were prepared as below: (1) Without S9: test sample or control solution 0.3 ml plus complete culture medium 2.7 ml; (2) With S9: test sample or control solution 0.3 ml, S9 mix 0.3 ml, and complete culture medium 2.4 ml. Test sample was washed with PBS for 3 times after incubation for 4 h, then additional incubation was performed for 20 h. Cells were treated with colchicine (4 μg/ml) for 4 h and hypotonic solution afterwards, then harvested for observation. Slides were prepared, then air dried, and coded after cells were fixed and stained with Giemsa solution. 200 metaphase chromosome spreads were scored and recorded, including gaps, fragments, chromosome or chromatid breakage, deletions, etc. Chromosome aberration data were analyzed using SPSS 21.0 using Chi-square test.

2.7. Mammalian erythrocyte micronucleus test

Micronucleus (MN) test was performed according to OECD Guideline 474 (Test No. 474, 2016) and the cosmetics material guidelines on (Safety and Technical Standards for Cosmetics, 2015 version, China) (Li et al., 2017). Based on the LD_{50} of PPD (about 200 mg/kg as determined in our laboratory) in experimental mice, three doses were selected: 100 mg/ml (high dose), 50 mg/ml (middle dose), and 25 mg/ml (low dose). 25 male NIH mice were used and randomly assigned to five groups. Three groups of mice were administered with PPD (25, 50, or 100 mg/kg, *b.i.d.*, by oral gavages at ~24 h intervals). Cyclophosphamide (40 mg/kg) was used in the positive group. Mice were sacrificed 6 h after the second treatment. Mice sternum was removed and cleaned. The bone cells were pipetted into a clean slide and mixed with approximately 20 μL FBS. Then the mixture was smeared and dried. The smears were tested in replicates, then stained with Giemsa solution

after fixation with ice-cold methanol. MN slides were analyzed via microscopy. Polychromatic erythrocytes with micronucleus (MN-PCE) (including mono-, bi- and poly-, in 2000 PCE at least) was scored and incidence (‰) was calculated. The frequencies of MN formation were analyzed using SPSS 21.0 software with the Chi-square and Fisher's exact tests.

2.8. Pig-a mutation assay

25 male Sprague-Dawley rats were used and randomly assigned to five groups. Rats were orally administered with PPD (12.5, 25 and 50 mg/kg/day for 28 consecutive days), or purified water (as vehicle control). ENU was used as the positive control (oral gavage, 40 mg/kg/day, for 3 days). Throughout the experimental period, body weight and food intake for each rat was monitored. Peripheral blood samples (80 μL), collected from jugular vein of each rat. Blood samples were kept in heparin-coated tubes with 100 μL heparin solution, to deplete leukomonocyte for less than 2 h at room temperature before treatment (Days −1) and on Day 8, Day 15, and Day 29, respectively (Phonethepswath et al., 2010; Dertinger et al., 2011a,b).

The resulting erythrocyte-enriched samples were incubated with PE-labelled anti-CD59 antibody at 2–8 °C for 30 min. Samples were washed and resuspended in balanced salt solution with SYTO13 (to stain cell nuclei) for 30 min at 37 °C. After that, cells were processed for flow cytometry at 488 nm using a Beckman Coulter Cytomics (FC 500 MCL, with CXP software). Fluorescence in the FITC channel (FL1, nucleic acid dye) and PE channel (FL2, Anti-CD59-PE) were recorded. Approximately, 10^6 total RBCs were loaded to detect the frequency of RET^{CD59-} and the frequency of RET in total RBCs. 300, 000–400, 000 RETs were loaded to analyze the frequency of RET^{CD59-}. The ratio of RBC^{CD59-} and RET^{CD59-} were calculated in 10^6 total RBCs or RETs.

2.9. Statistical analysis

Statistical analysis was performed with SPSS 21.0 Software using one-way ANOVA with *post hoc* analysis of the Dunnett's test or Tamhane's T2 test. $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Reverse mutation assay in bacteria

As shown in Table 1 and Table 2, revertant colonies formation in positive control plates treated with several mutagenic reagents were significantly increased. In plates treated with PPD (doses of 0.312, 0.625, and 1.25 mg/plate), revertant colonies in TA98 strain with S9 mix were increased (> 2-fold) compared to the solvent control. The two high doses (2.5 and 5 mg/plate) of PPD, similar to vehicle control, did not induce revertants increased dose-dependently, maybe because of the possible mutagenicity and bacterial inhibitory effect. To sum up, at doses of 0.156, 0.312, and 0.625 mg/plate, revertant colonies increased dose-dependently; however, at t doses of 1.25, 2.5, and 5 mg/plate, the revertant colonies decreased with elevated dose. The possible reason might be the bacterial inhibitory effect, which became much more dominant with the increasing dosage. No double increase of revertants numbers were observed in other three strains (TA97a, TA100, and TA102) with or without S9 mix, and in TA98 without S9 mix at all concentrations tested. The high dose of PPD (5mg/plate) without S9 mix also produced inhibitory effect on TA97a and TA100. According to the criteria of Ames test, PPD was tested as positive inTA98 with S9 mix.

Table 2
The reverse mutation effect of PPD on four strains with S9 mix ($\bar{x}$ ± SD, n = 3).

	Dose (mg/plate)	TA97a	TA98	TA100	TA102
Vehicle control [a]	0	155 ± 7	46 ± 9	164 ± 12	349 ± 28
PPD	0.156	136 ± 10	91 ± 9	145 ± 22	340 ± 29
	0.312	143 ± 12	132 ± 5[c]	168 ± 16	375 ± 14
	0.625	136 ± 8	161 ± 25[c]	157 ± 9	362 ± 13
	1.25	156 ± 4	129 ± 20[c]	132 ± 18	365 ± 21
	2.5	147 ± 5	67 ± 14	135 ± 17	363 ± 28
	5	138 ± 12	47 ± 6	127 ± 20	358 ± 11
Positive control [b]	–	798 ± 73[c]	2149 ± 176[c]	842 ± 68[c]	1059 ± 84[c]

Note:
[a] Sterile deionized water.
[b] TA97a, TA98, TA100, 2-AF:10 μg/plate; TA102, 1,8-DHAQ:50 μg/plate.
[c] Positive response as the mean revertant colonies number increase by two-fold over vehicle control.

3.2. In vitro mammalian cell chromosomal aberration test

As presented in Table 3, PPD treatment for 4 h induced significant increase of chromosomal aberrations in CHL cells. In groups treated with different doses of PPD (final concentration 78, 156, 312 μg/ml), the percentage of chromosomal aberrations increased to 23%, 25%, and 22%, respectively. These alterations include acentric fragments, microbodies, gaps, dicentric chromosomes, chromatid and chromosome breaks. The percentage of chromosomal aberrations was significantly higher than vehicle control group (14%) without S9 mix ($p < 0.05$). The significant aberration-inducing effects, but non-linear dose response of PPD might suggest that the lowest concentration was far above the threshold to induce chromosomal aberrations. The percentage of chromosomal aberrations also increased significantly in PPD

Table 1
The reverse mutation effect of PPD on four strains without S9 mix ($\bar{\chi}$ ± SD, n = 3).

	Dose (mg/plate)	TA97a	TA98	TA100	TA102
Vehicle control [a]	0	135 ± 10	32 ± 2	140 ± 6	301 ± 18
PPD	0.156	143 ± 11	35 ± 7	170 ± 22	308 ± 21
	0.312	133 ± 10	34 ± 4	168 ± 12	309 ± 13
	0.625	134 ± 8	33 ± 6	161 ± 12	316 ± 18
	1.25	142 ± 9	43 ± 8	139 ± 14	311 ± 25
	2.5	103 ± 28	36 ± 7	124 ± 21	303 ± 18
	5	inh[d]	36 ± 6	inh[d]	301 ± 22
Positive control [b]	–	2549 ± 351[c]	2576 ± 198[c]	1364 ± 227[c]	1075 ± 106[c]

Note:
[a] Sterile deionized water.
[b] TA97a, TA98, TN9F:0.2 μg/plate; TA100, sodium azide:1.5 μg/plate; TA102, MMC:0.5 μg/plate.
[c] Positive response as the mean revertant colonies number increase by two-fold over vehicle control.
[d] inh, inhibitory effect.

Table 3
The inducing effect on chromosome aberration of PPD in CHL cells.

	Final Concentration (μg/ml)	Cell number		Aberrant cell number		Aberration rate (%)	
		-S9	+S9	-S9	+S9	-S9	+S9
Vehicle control [a]	0	200	200	28	24	14	12
PPD	78	200	200	46*	35	23*	18
	156	200	200	50*	49*	25*	24*
	312	200	200	44*	49*	22*	24*
Positive control [b]	–	200	200	58*	60*	29*	30*

*$p < 0.05$, compared to vehicle control.
Note:
[a] Phosphate buffer saline.
[b] With S9 mix, CP: 2 μg/ml; without S9 mix, MMS: 10 μg/ml.

groups at concentrations of 156 and 312 μg/ml with S9 mix ($p < 0.05$), except that the percentage of 78 μg/ml dose group slightly higher, but was not statistically significant.

3.3. Mammalian erythrocyte micronucleus test

As shown in Table 4, MN-PCE rate increased significantly ($p < 0.05$) in NIH mice after PPD treatment (by oral gavage twice at ~24 h intervals) at the two high doses of 50 and 100 mg/kg (12.6 ± 3.7‰ and 13.9 ± 3.8‰, respectively), including mono-, bi- and poly-nucleated MN-PCE, whereas MN rate was 2.5 ± 1.1‰ in vehicle control mice and 28.6 ± 5.2‰ in cyclophosphamide-treated positive mice ($p < 0.05$). The significantly increased MN-PCE rate appeared above the dose of 50 mg/kg might indicate the threshold to induce chromosomal damage at doses between the dose of 25 mg/kg and 50 mg/kg. The chromosome-damaging effect induced above the threshold dose is significant following a non-linear dose response.

3.4. Pig-a gene mutation assay performed in peripheral blood

Pig-a assay was integrated in a sub-chronic toxicity study. PPD was administered by oral gavage continuously for 28 days. All the rats grew normally, without significant difference in body weight (as shown in Table 5). Food consumption, organ weight, macroscopic examination, clinical pathology, were also not significantly different.

Time-course and dose-dependent effects of PPD (12.5, 25, 50 mg/kg/day) were shown in Fig. 1A, B and C, compared to the solvent control and the effect of ENU (40 mg/kg/day). Fig. 1A shows the frequencies of mean percentage of reticulocytes (% RETs), Fig. 1B is accumulation of RET^{CD59-}, and Fig. 1C is accumulation of RBC^{CD59-}, respectively. All RET% decreased over time (from Day 1 to Day 29). The difference in %RETs at each time point between treated animals and solvent control group is not statistically significant.

As shown in Fig. 1B, PPD (25 and 50 mg/kg/day) induced RET^{CD59-} increased dose-dependently on Day 8, Day 15, and Day 29. In Fig. 1C, PPD (25 and 50 mg/kg/day) induced RBC^{CD59-} increased dose-dependently on Day 15 and Day 29. The lowest dose (12.5 mg/kg/day) of PPD induced RBC^{CD59-} increased slightly but not statistically significant. PPD administration for 28 days produced significant genotoxic effects, and the time-course trend line of inducing RET^{CD59-} and RBC^{CD59-} was similar to that induced by ENU (40 mg/kg/day) only for 3 consecutive days. ENU induced much higher mutant frequencies at each time point, meaning more potent genotoxicity. Furthermore, the marked increase in RBC^{CD59-} induced later by PPD (50 and 100 mg/kg) and ENU (40 mg/kg/day), on Day 15 and Day 29, RBC^{CD59-} increased at a lower velocity than that of RET^{CD59-} during the early 7 days, maybe with a later coming peak value after Day 29.

Table 4
The inducing effect on MN-PCEs of PPD in erythrocytes from NIH mice.

	Dose (mg/kg)	Animal number	PCE number	MN number	MN rate (‰)
Vehicle control [a]	0	5	10000	25	2.5 ± 1.1
PPD	25	5	10000	22	2.2 ± 0.6
	50	5	10000	126	12.6 ± 3.7*
	100	5	10000	139	13.9 ± 3.8*
Positive control [b]	40(CP)	5	10000	286	28.6 ± 5.2*

*$p < 0.05$, compared to vehicle control.
Note:
[a] Sterile deionized water.
[b] CP, cyclophosphamide: 40 mg/kg.

4. Discussion

Although the safety and carcinogenicity of using oxidative hair dyes is inconclusive to date, epidemiological studies have suggested that, occupational exposure to oxidative hair dyes might increase the risk for developing carcinoma (Gago-Dominguez et al., 2001). It remains uncertain whether PPD is carcinogenic and can cause genetic damage (Nohynek et al.,2004). For example, some laboratories observed that PPD is mutagenic in *in vitro* bacterial mutagenicity tests (Chung et al., 1995; Garrigue et al., 1991), in micronuclei test using human lymphocytes (Garrigue et al., 1991), and in chromosomal aberration test using CHO cells (Chung et al., 1995) in the presence of metabolic activation. Moreover, PPD induces COX-2 protein accumulation and p53 mutation in comet assay using the urothelial cells (Huang et al., 2007). However, others find PPD is negative in rodent micronucleus (Soler-Niedziela et al., 1991), in unscheduled DNA synthesis (UDS) assays (SCCS, 2012), in Hprt locus gene mutation of L5178Y cells, and in the rat comet assay (Sasaki et al., 1997; De Boeck et al., 2015). Some studies reported that the use of hair dyes may lead to the occurrence of bladder cancers, although with some controversy (Gago-Dominguez et al., 2001).

In this study, we observed that PPD can produce consistent positive results in three genotoxic assays (Ames, CA, MN), especially significant increases in CA with or without S9 mix in CHL cells. Doses of 50–100 mg/kg PPD induce nearly half MN-PCE rate of 40 mg/kg CP-treated positive control. However, PPD has very weak positive response (> 2-fold) in *Salmonella typhimurium* TA98 with the S9 system. Our study proves the mutagenic potential of PPD in the standard battery of tests, but it seems to be a weak inducer of gene mutation.

Chromosome breakage or loss can be reliably indicated by MN result (Lajmanovich et al., 2005). For some alkylating agents at the low dose, chromosomal clastogenicity is more sensitive to prove the genotoxicity than to prove mutagenicity (Lynch et al., 2011). PPD increased the number of chromosomal aberrations (acentric fragments, microbodiesand gaps) and MN-PCE (mono-, bi- and poly-MN-PCE) that might be produced by aberrant formation of diploid cells. It seems that PPD

Table 5
The body weight of rats before and during treatment (g, $\bar{x}$ ± SD, n = 5).

	Dose (mg/kg)	Day-1	Day 7	Day 14	Day 21	Day 28
Vehicle control[a]	0	231.9 ± 15.2	272.2 ± 18.6	351.1 ± 14.8	407.9 ± 21.8	448.9 ± 24.2
PPD	12.5	237.5 ± 10.3	285.0 ± 19.8	351.5 ± 16.1	415.4 ± 18.1	449.1 ± 19.2
	25	237.1 ± 13.7	286.5 ± 17.4	357.9 ± 25.9	413.9 ± 29.8	445.8 ± 34.0
	50	236.0 ± 14.4	278.9 ± 10.4	345.5 ± 15.8	395.5 ± 22.6	432.5 ± 29.2
Positive control[b]	40 (ENU)	234.1 ± 15.3	277.7 ± 18.6	341.2 ± 20.0	393.7 ± 25.9	422.9 ± 30.9

Note:
[a] Sterile deionized water.
[b] ENU: 40 mg/kg.

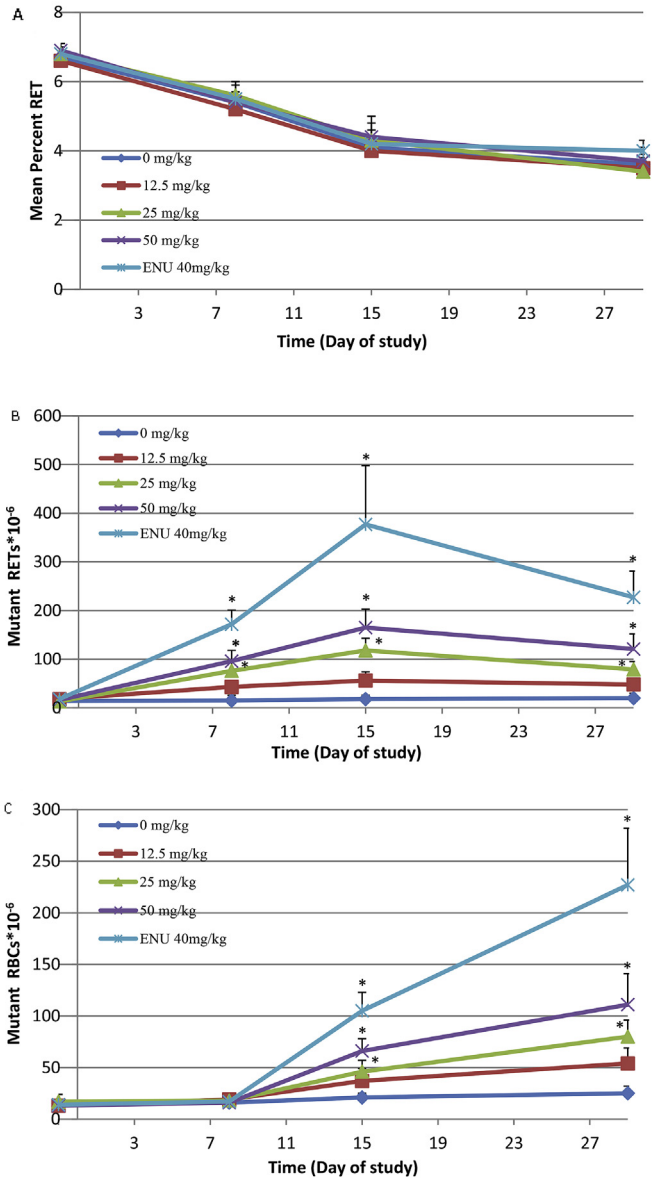

Fig. 1. Time-course and dose-dependent effect of PPD (12.5, 25, 50 mg/kg/day, for 28 days) on frequencies of %RETs, RET^{CD59-} and RBC^{CD59-}. A. Mean %RET in Sprague-Dawley rats treated with PPD or ENU (40 mg/kg/day, for 3 days). B. Induction of RET^{CD59-} by PPD or ENU. C. Induction of RBC^{CD59-} by PPD or ENU.
* Represent $p < 0.05$, as compared to solvent control.

causes chromosomal aberrations > nucleated damage > gene mutation. This indicates the possible genotoxicity of PPD. In addition, the difference in positive results with or without S9 system, may indicates the genotoxicity of PPD is associated with its metabolites. Oxidative DNA damage, which can be caused by some hair dye components, contributed to carcinogenicity. (Murata et al., 2006). PPD is an aromatic amine whose carcinogenicity is speculated to be associated with N-hydroxylation. Toxicokinetics studies showed that PPD is metabolized through N-hydroxylation in liver and through ortho-acetylation in bladder. Through these metabolic reactions, PPD is converted to highly electrophilic N-acetoxy derivatives which can integrated into DNA by covalent bonding. Through percutaneous absorption, PPD is N-acetylated by NAT1 in mammalian keratinocytess to eliminate its toxicity (Nohynek et al., 2015; Meuling et al., 2003; Gago-Dominguez et al., 2003).

As reported previously (Dobo et al., 2011), *Pig-a* gene mutation assay is a sensitive assay to assess the dose-response relationships. In the present study, PPD shows obvious mutagenicity in Sprague-Dawley rats in *Pig-a* gene mutation assay, although with lower frequency of mutant RETs and RBCs (compared with the frequency of animals receiving 40 mg/kg/day ENU). 25–50 mg/kg/day of PPD and 40 mg/kg/day ENU caused a similar time course of mutant induction. PPD and ENU both induced significant increase in RET^{CD59-} as early as Day 8, which increases to the maximal mutant frequency on Day 15RET^{CD59-}decreases on Day 29. As for RBC^{CD59-}, PPD and ENU both induced significant increase on Day 15 and kept elevating until Day 29. Dobo et al. also found RET^{CD59-} and RBC^{CD59-} increased significantly after treatment of ENU for 28 days (Dobo et al., 2011). The induction of RET^{CD59-} reaches highest level at around 4 weeks, and kept elevated or start to decrease after 4 weeks. In contrast, the frequency of RBC^{CD59-} kept increasing continuously. As Miura et al. speculated, RETs are a minority cell population of the total RBC population in rats. RETs might turn over several days and be replaced quickly. This metabolic process is speculated to be responsible for the appearance of a rapid rise and subsequent steady level of RET^{CD59-} shortly after the end of treatment. In contrast, the frequency of RBC^{CD59-} rise gradually because RBCs are highly abundant with a lifespan of about 2 months.

The cycle of bone marrow cell development is associated with the dose-dependent accumulation of RET^{CD59-} and RBC^{CD59-} frequencies. Miura et al. observed that the peak response in RET^{CD59-} frequencies appeared on Day 15 after ENU treatment, whereas the peak of RBC^{CD59-} frequencies appeared much later. This is due to the long period of erythrocyte maturation and turnover (Miura et al., 2009). When *Pig-a* gene mutation occurred at an early stage of bone marrow cell development cycle, the frequency of RBC^{CD59-} remain elevated because RBC^{CD59-} was provided incessantly flowing from bone marrow to circulation system. While when the gene mutations were mainly induced at the end stage of RBC maturation, the frequency of RBC^{CD59-} would decrease from the maximum months later. Therefore it became possible to detect weak mutagens *in vivo* by sub-chronic or chronic dosing protocols, because of not only the accumulation but also the persistence of mutants for a certain period of time. Many laboratories have also recognized the leading (RET^{CD59-}) and lagging (RBC^{CD59-}) indicators as sensitive markers of *Pig-a* gene mutation. The frequency of RET^{CD59-} and RBC^{CD59-} changes in a time- and dose-dependent manner (Dertinger et al., 2010). Our results indicated that PPD can

induce DNA damage, and produces a weak, dose-related, statistically significant increase in frequency of RET^{CD59-} and RBC^{CD59-}. The time course of mutant induced by PPD is consistent with that induced by ENU. The genotoxicity of PPD shows an additive effect which is caused by the repeated dosing.

Our results suggest that *Pig-a* assay could be considered as an appropriate *in vivo* follow-up method to verify positive results in bacterial and *in vitro* mammalian cell gene mutation assays. When there is insufficient evidence to indicate lack of relevance, *Pig-a* assay is recommended to address any indication of genotoxicity. *Pig-a* assay can be integrated into a multiple administration toxicology study. In fact, *Pig-a* assay has received intense interest as a potential assay for evaluating the *in vivo* mutagenicity of substances since 2008 (Bryce et al., 2008). Many laboratories from academic, regulatory, and industry have been committed to establishing protocols, testing the inter-laboratory reproducibility, and expanding the number of agents tested (Miura, 2014). Litron Laboratory initiated a multi-laboratory trial divided into four stages. After three stages, the trial has finished verification in a systematic manner using several potent and weak mutagens, such as ENU, dimethylbenz [a]anthracene (DMBA), N-methyl-N-nitrosourea, benzo [a]pyrene (BaP), and 4-nitroquinoline-1-oxide (4-NQO). The verification has provided good evidence for a high degree of inter-laboratory transferability and reproducibility based on the results. (Bhalli et al., 2011; Cammerer et al., 2011; Dertinger and Heflich, 2011; Dertinger et al., 2011a,b; Lynch et al., 2011; Shi et al., 2011). International Workshop on Genotoxicity Testing (IWGT) reviewed the development of the *Pig-a* assay in the context of safety assessment strategies. Those updated methods were demonstrated at Stage IV and were used to assess intra- and inter-laboratory reproducibility. IWGT also suggested the development of standard and robust-testing protocols for other species or other hematopoietic cell types in future research (Gollapudi et al., 2015). The guideline of genotoxicity testing recommended by International Conference on Harmonisation (ICH) states that *in vivo* tests have an important role in genotoxicity test strategies. ICH S2 (R1) has added another new standard test battery for genotoxicity, which includes two *in vivo* tests (ICH S2(R1), 2011). *Pig-a* assay is described as an appropriate complementary system which serves as an additional *in vivo* test to detect gene mutations (following OECD Test Guidelines No.488) (TG488:Transgenic Rodent Somatic and Germ Cell Gene Mutation Assays) (ICH M7, 2017).

Overall, *Pig-a* gene proves to be a feasible reporter in pre-clinical animal models. Further clinical studies are needed to verify its potential to make cross-species comparisons, especially in human hematopoietic cells mutation. It remains unclear whether PPD is a genotoxic carcinogen in humans, and it's necessary and urgent to conduct epidemiological investigations using large samples with the *Pig-a* assay in occupational exposure population to confirm the genotoxicity of PPD.

5. Conclusion

The mutagenic effect of PPD in *Pig-a* assay is accordant with classical genotoxicity test battery recommended by OECD and CFDA, suggesting that PPD presented potential genotoxicity. These data might be important to assess the potential carcinogenicity risk of PPD-containing hair dyes and integrate the *Pig-a* gene mutation assay into sub-chronic toxicology tests.

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