

para-Phenylenediamine Containing Hair Dye: An Overview of Mutagenicity, Carcinogenicity and Toxicity

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Abstract

The great demand for hair dyes can be seen by the proliferation of hair salons. Their ability to impart temporary or permanent colour change to the hair satisfies the desire of consumers for beauty, fashion, and a look-younger image. *para*-Phenylenediamine (PPD) is found in more than 1000 hair dye formulations and is the most frequently used permanent hair dye component in Europe, North America, and East Asia. In addition, PPD containing permanent hair dyes account for three-quarters of global use and more than one-third of women use in Europe, North America, and East Asia. However, PPD has been banned in Germany, France and Sweden in the early 1900's as a hair dye component and the hazard of PPD on health has been discussed for the past few decades. PPD containing hair dyes have been associated with cancer and mutagenicity, with supportive evidences from both clinical and laboratory studies. Apart from that, PPD has potential toxicity which includes acute toxicity such as allergic contact dermatitis and subacute toxicity. In this article, we provide comprehensive review on the chemical ingredients of hair dyes, roles of PPD in hair dye, metabolic mechanisms of PPD through *in vivo* and *in vitro* studies, and mechanism involvement in the health effect of PPD as evidenced from both clinical and laboratory studies.

Keywords: *para*-Phenylenediamine; Hair dye; Mutagenicity; Carcinogenicity; Toxicity

Introduction

Hair dyes contain over 5000 chemical substances, in particular the aromatic amines. Hair dyes can be divided into four categories based on its chemical composition and mechanism of action; (1) permanent (oxidative) dyes, (2) temporary or semi permanent (direct) dyes, (3) metal salts and (4) natural dyes. It consists of primary intermediates (e.g., *para*-phenylenediamine, *para*-toluenediamine, *ortho*-aminophenol, and *para*-aminophenol) that are mixed with couplers (e.g., *m*-aminophenols, *m*-hydroxyphenols, resorcinol and others) and oxidizing agent to generate coloured oxidation product through chemical reaction that binds irreversibly within the hair shaft. Hydrogen peroxide is commonly used as oxidant in the dyeing process. Hydrogen peroxide causes swelling of the hair cuticle which allows the diffusion of precursors into hair cortex and catalyzes the oxidation of the precursors to large coloured molecules that are infused within the hair shaft. On the other hand, temporary hair dyes contain azo, triphenylmethane, anthraquinone, or indamines dyes, while semi-permanent dyes contain nitro-phenylenediamines, nitro-aminophenols and some azo dyes. Temporary and semi-permanent hair dyes coat on the outer cuticle or surface of hair and are removed after one to several washings [1-3]. It is estimated that in one hair dyeing process with about 4 g of amines, 40 mg or 1% of hair dye chemicals (precursors, products and side products) are absorbed through the scalp [2].

Permanent hair dyes are widely used with over 80% of market share in the United States [2,3]. In 1980's, some hair dye chemicals were banned from use due to their mutagenic and carcinogenic effects as evidenced by laboratory animal studies. However, hair dye ingredients such as *para*-phenylenediamine (PPD) and *para*-toluenediamine (PTD) are still being used to date [1]. For the past 50 years, PPD has been used commonly as a primary intermediate in the formulation of permanent hair dyes and still remains unchanged. According to a previous study, 76 out of 115 commercial oxidative hair dyes showed PPD concentrations ranging from 2.2 to 3.4% [4]. Today, the European Union cosmetic

directive regulation allows maximum PPD concentration of up to 6% in hair dyes [5]. PPD is an aromatic amine with a chemical formula C₆H₈N₂ (Figure 1). Present in the form of white crystals, PPD oxidizes in the air turning from red to brown and finally black. PPD has the ability to penetrate hair shaft and follicle and has a strong protein binding capacity thereby making it an effective hair dye chemical. Additionally, it is also used in fur and textile industries and as vulcanizing agent in the rubber industry [2].

Given its widespread use in the United States, Europe, and East Asia, safety assessment of hair dye ingredients remains a growing concern [2,3]. The possible association between permanent hair dye use and cancer risk has been examined in several cohort studies [3,6]. In a population based control study in Italy, the use of permanent hair dyes was associated with leukemia and follicular non-Hodgkin's lymphoma [7]. Additionally, Gago-Dominguez and co-workers reported that the risk of bladder cancer among women who used permanent hair dyes at least once a month and barbers with more than 10 years of working experience increases by 2.1 fold and 5 fold respectively compared to the non-users [8]. Furthermore, increased risk in cancers of the lung, colorectal, bladder, pancreas, lung, cervix and *in situ* cancer of the skin were observed among hairdressers in a follow up study from the year 1960 to 1998 [9]. Besides, bacterial test using *Salmonella thyphimurium* tester strain showed 89% of commercial permanent hair dye ingredients

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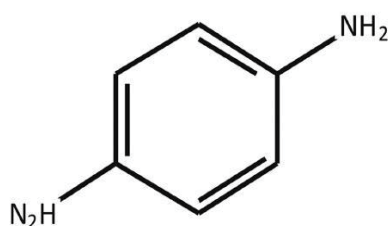


Figure 1: Chemical structure of *para*-phenylenediamine.

such as 2,4-diaminoanisole, 4-nitro-*o*-phenylenediamine, 2-nitro-*p*-phenylenediamine, PPD, and others showed various degrees of mutagenicity [10]. Although numerous studies have been done, the question on whether hair dyes possess significant risk to carcinogenesis is still an issue of debate. This is because although the existing epidemiologic data indicates the increased risk of cancer is associated with the use of hair dye, however the scientific reports that have been published are still limited [6,8,11-14].

PPD is known to cause allergic contact dermatitis, so it was banned from use in Germany, France and Sweden during the twentieth century. According to the Scientific Committee on Consumer Products (SCCP), PPD is a potent contact allergen. Skin contact during hair dyeing or tattooing process is the main route of human exposure to PPD. Occupational exposure is seen among industrial workers and regular hairdressers handling this chemical. [15,16]. Due to the popularity of hair dye use and its ability in penetrating human skin, concerns on the hair dye toxicity, mutagenicity and/or carcinogenic potential on human body need to be adequately considered.

Metabolism of PPD

Metabolism of arylamines, especially those present in the dye industry, cigarette smoking and personal care products has been extensively studied and reported in various literatures. As such, *N*-acetylation catalyzed by *N*-acetyltransferases (NATs) is found to be the major route of PPD metabolism [17]. The *N*-acetyltransferase type 1 (NAT1) found in skin and the *N*-acetyltransferase type 2 (NAT2) found mainly in the liver and guts are two isoforms known in human to catalyze the conversion of PPD to its *N*-mono- (MAPPD) and *N,N'*-diacetylated (DAPPD) metabolites [18-20]. Systemic exposure to PPD in human body causes production of skin and hepatic metabolites that can be detected in the urine. As such, these metabolites and PPD are excreted mainly through renal clearance [21-23]. In human study, five different metabolites were identified in the urine of subjects after treated with dark-shade oxidative hair dye containing (14) C-PPD. Amongst the five metabolites, MAPPD and DAPPD are found to be the major metabolites made up to around 80-95% of total metabolites found. Other metabolites include glucuronic acid conjugates are also reported [23]. In other investigations, the DAPPD is the main metabolite detected in urine of both male and women volunteers which accounted for 80% of total excretion [18,19]. It is worth mentioning that individuals with slow NAT2 acetylator phenotype are associated with an increased risk for bladder cancer. This was proven in a meta-analysis study which demonstrated increased risk for bladder cancer among cigarette smokers with slow NAT2 acetylators [24].

Mutagenicity of PPD

In vitro genotoxicity properties of PPD were tested using the Ames test, the micronucleus test in human lymphocytes and mammalian cell

mutation assay. Genotoxicity test is a short term screening study done to measure carcinogenicity and mutagenicity of various chemicals. The Ames Salmonella/microsome mutagenicity assay is a bacterial reverse mutation assay used to detect chemical substances that produce genetic damage [25-27]. PPD was shown to be strongly mutagenic to Salmonella tester strain TA1538 in the presence of rat liver S-9 preparation and weakly mutagenic to TA98 with metabolic activation. PPD when mixed with hydrogen peroxide alone gave positive results in the Ames test [28,29]. Previous studies demonstrated that the reaction between PPD and resorcinol together with an oxidizing agent (hydrogen peroxide) yielded an oxidized conjugation product (green chemical) that was mutagenic towards Salmonella tester strain TA98. This product when topically applied to shaved rats, produced urine that was mutagenic on TA98 strain of *Salmonella typhimurium* [21,28,30,31]. However, a study questioned that the activity of PPD in the Ames test might be markedly affected by the solvent dimethylsulfoxide (DMSO). It was suggested that while freshly prepared solutions in DMSO are non-mutagenic, they might become highly mutagenic when allowed standing at room temperature for 4 hours [32]. PPD also found to cause chromosomal aberrations in Chinese hamster ovary cells [33].

As an extension to the previous studies, PPD and its metabolites, MAPPD and DAPPD were tested for genotoxic properties in another study. PPD was found slightly mutagenic in *Salmonella typhimurium* strain TA98 in the presence of metabolite activation. However, its metabolites MAPPD and DAPPD was non-mutagenic in the *Salmonella typhimurium* strain TA98. Besides, PPD induced micronuclei in human peripheral blood lymphocytes both in the presence or absence of metabolite activation. However, MAPPD and DAPPD were negative in the micronucleus test. These indicates that the acetylated conversion products are the detoxified metabolites [34,35].

Contradicting to the previous results, sister-chromatid exchanges (SCE) studies found that single application of hair dye for 6 hours and 7 days did not consistently increase the SCE levels in the peripheral lymphocytes [33]. Similarly, no effect was observed on the frequency of SCE levels when the hair dye was applied 13 times at intervals of 3-5 weeks [36]. Apart from that, 13 commercial hair dye products made in China showed negative results in mutagenicity test in *Salmonella typhimurium* (strains TA98 and TA100) and the micronucleus test in mouse bone marrow polychromatic erythrocyte cells [37].

Carcinogenicity of PPD

Hairdressers and barbers were classified under occupational exposure group 2A: probably carcinogenic to humans by the International Agency for Research on Cancer. In fact, several studies have suggested that hairdressers are the high risk group for cancers. Takkouche et al. reported that hairdressers in general have increased risk of cancer compared to the general population in a meta-analysis [6]. It was found that hair dye users have increased risk of bladder cancer, non-Hodgkin's lymphoma and multiple myeloma [38-40]. On top of that, an epidemiological study before 1980s found that there was a moderate increase risk in lymphoma especially in individuals who use personal hair dye [41]. A case control study also showed that hair dye users have increased prostate and breast cancer risk [42,43]. In an *in vivo* study by Rojanapo et al. topical application and subcutaneous injection of oxidized PPD for 18 months in rats caused a statistically significant increase in the incidence of mammary gland, uterine and soft tissue tumors of both malignant and benign types [44]. Sontag demonstrated that PPD increased the incidence of liver, kidney, adrenal gland, thyroid gland, urinary bladder and lung tumors in rats [45].

Nevertheless, there are studies which are unable to show the association of carcinogenesis with hair dressers and hair dye users. Several meta-analyses have not able to show the association of increased risk of cancer among hair dressers and personal hair dye users [6,46,47]. Multiple case control studies also found no association between breast, lung, stomach, colorectal, bladder and hematopoietic cancer with hair dye users [11,12,48-52]. However, it is important to note that some of these studies suffer from limiting factors such as small population size, non-specific hair dye product, timing and intensity of hair dye exposure, thus the conclusion generated by the study should be evaluated carefully [53].

Subacute toxicity of PPD - The apoptotic effects

Apoptosis is a gene-regulated programmed cell death crucial to maintaining a balance between cell division and cell death. Execution of apoptosis ensures that cells with irreparable DNA damage and aberrant cell cycle are eliminated from the proliferating population to avoid malignant transformation. The extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway are the two main apoptosis pathways widely known to date. Defect in any step along the pathway and the failure of cells to commit apoptosis is a general hallmark for tumor progression [54,55].

As animal studies have demonstrated that increased incidence of kidney tumor in PPD-treated rats, two *in vitro* studies have investigated the apoptosis pathways in different cell lines. The first study has used Mardin-Darby canine kidney (MDCK) cells to study if PPD would induce apoptosis [55]. Research found that PPD decreased cell viability in a dose-dependent manner. Presence of apoptosis was made affirmative by several observations such as accumulation of sub-G1 peak and G0/G1-phase arrest in cell cycle, DNA fragmentation in TUNEL assay, as well as positive Annexin-V staining. It has been described that the apoptotic mechanisms of PPD might involve oxidative stress, reduction of membrane potential, p53 as well as intrinsic and extrinsic pathways [55,56]. The second study was performed in normal rat kidney proximal tubular epithelial (NRK-52E) cells. The study showed that PPD induced apoptosis via upregulation of phosphorylated stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) protein expression and downregulation of Ras and Raf protein expression in NRK-52E cells. However, Akt, Bcl-2, Bc-XL and Bad protein levels were not significantly altered when compared with the controls. In short, PPD induced apoptosis via PTK/Ras/Raf/JNK-dependent pathway and was independent of the PI3K/Akt pathway in the NRK-52E cells [54]. It is interesting to note that the concentrations of PPD used to induce apoptosis in the two studies were vastly different. Concentrations used in the second study (50, 100, 200, 300 µg/mL) were folds higher than the first study (12.5, 25, 37.5, 50 µg/mL) [54-56].

In human urothelial (UROtsa) cells, PPD was shown to induce apoptosis by inhibiting the activation of Wnt pathway through decreased phosphorylation of LRP6 and the activity of Wnt, Axin, Naked and Dvl proteins. Besides, PPD decreased the phosphorylation of IKK and IκB which subsequently inhibited the nuclear translocation of NF-κB. Moreover, PPD decreased the phosphorylation of mTOR and the activity of GbetaL and Raptor dose-dependently, while Rictor remained unchanged for all PPD treatments. Activation of Raptor protein is known to stimulate cell growth and cell proliferation while activation of Rictor contributes to the regulation of cell polarity and actin cytoskeleton. The downregulation of Raptor is correlated with the inhibition of cell proliferation by PPD. Overall the study demonstrated that PPD induced apoptosis in UROtsa cells by inhibiting the activation of NF-κB, mTOR, and Wnt signaling [57]. In another study, Huang et al.

revealed that PPD induced DNA damage and accumulation of mutant p53 and cyclooxygenase-2 proteins in SV-40 immortalized human uroepithelial (SV-HUC-1) cells [58]. Their study also found that PPD was able to induce autophagy via autophagosomes formation which increased the concentration of Beclin-1 and microtubule-associated protein light chain 3B (LC3B) via activation of extracellular signal-regulated kinase (ERK) 1/2 signaling pathway and mutant p53 in the SV-HUC-1 cells. In contrast, the MEK inhibitor (U0126) was able to suppress the autophagy through inhibition of ERK1/2, Beclin-1 and LC3B proteins expression in the SV-HUC-1 cells.

In Chang liver cells, PPD induced apoptosis via mitogen-activated protein kinase (MAPK) pathway. Results revealed that PPD activated SAPK/JNK, p38 MAPK and ERK and induced apoptosis [59]. Results from another study showed that PPD mediated oxidative stress, loss of membrane potential, and caspase 8 activation in both human and mouse melanoma cells. Under ultraviolet A exposure, PPD was photo-degraded and formed a novel photoproduct which induced the reactive oxygen species generation, single strand DNA breaks, micronuclei and cyclobutane pyrimidine dimers formation, lysosomal destabilization and cathepsin B releasing. Cathepsin B processed BID to its active form, tBID which induced the release of cytochrome C from mitochondria and subsequently induced apoptosis [60,61]. Additionally, Chye et al. found that PPD was able to induce single DNA breaks in human lymphocytes by using alkaline comet assay [62].

In vivo studies demonstrated that sub-chronic topical application of different doses (0, 1, 2 and 3 mg/kg/day) of PPD elevated oxidative stress, induced germ cell apoptosis, and decreased total sperm count causing testicular toxicity in male albino rats [63]. In another study, after intraperitoneal administration of PPD, Swiss-Albino mice melanoma tumor growth was arrested by the apoptotic activity [60].

Acute toxicity of PPD - Contact allergy and dermatitis

Several cases of mild to severe localized and systemic contact allergy and dermatitis due to the usage of PPD products such as permanent hair dyes, black henna and permanent eyelashes have been reported. Systemic contact allergy and dermatitis syndromes include conditions such as blepharconjunctivitis, acute dermatitis, erythematous, hyperpigmentation and lichenification [64-72]. It has been demonstrated that PPD in hair dye, albeit much lower than the common dosage used under diagnostic patch testing, is sufficient to elicit a moderate to strong allergic responses in PPD-allergic human subjects [73]. Factors predisposing to individual susceptibility to PPD contact dermatitis are not well defined. Supported by animal study, the immuno-stimulatory effects of PPD have been initially proposed to be induced by Bandrowski's base (BB), which is formed by cutaneous secondary oxidation products.

However, it was then discovered that elevated levels of BB were discovered in both PPD-allergic and PPD-tolerant individuals, but the formation of BB does not necessarily trigger immune response in the PPD-tolerant individuals [74]. T-cell responses, as measured by the lymphocyte transformation test, were only observed in PPD-allergic individuals. In general, increased activity of T helper 2 cytokines, including interleukin (IL)-1α, -1β, -4, -5, -6, -8, -10, and -13; interferon-γ; tumor necrosis factor-α; macrophage inflammatory proteins-1α/β; monocyte chemoattractant protein-1; and RANTES in CD4+ and CD8+ lymphocytes were observed in the allergic patients. Hence, it is likely that primary oxidation products of PPD are responsible for causing the allergy contact dermatitis. The same group later reported an alternative mechanism of PPD in which PPD selectively binds to

Cysteine-34 of human serum albumin to form a PPD hapten-protein complex to stimulate T lymphocytes in the allergic patients [75]. Nevertheless, the underlying mechanism of how PPD triggers allergic response is not well understood. More studies are still required to warrant the earlier findings.

With the spotlight highlighting cases of PPD users developed adverse effects, occupational hazards come into question. Epidemiological studies show that hairdressers have 4.4-fold chance of having sensitization towards PPD whereas black henna tattoo artists' chances are 2.3 folds. Other study has shown hairdressers have 72% tendency to obtain contact dermatitis compared to 37% in cosmetologists [71,76,77]. In Australia, similar results were seen with 157/164 hairdressers tested positive for occupational contact dermatitis. Acute contact dermatitis was seen in 71% of the volunteers whereas irritant contact dermatitis was seen in 20%. The average prevalence of contact dermatitis relating to PPD is 4.3% in Asia, 4% in Europe and 6.2% in North America [78,79]. A retrospective study in Sweden found significant increase in sensitization rate of PPD from 1992 to 2009 [80]. Strong and extremely strong patch test reactions are significantly more profound in Southern Europe. However, a 20-year study in China reported no statistical significant increase of sensitization to PPD [81]. Over the years, the prevalence of PPD sensitization has remained the same.

Children are more prone for to contact dermatitis pertaining to PPD usage, especially in black henna tattooers [82-84]. A retrospective study shows +++ reactions to PPD in children age 1 to 14 [85,86]. This is because children are more easily developing allergy. A retrospective study on 500 children showed 27% of the subjects demonstrating + or > + in the patch test. A similar study in University General Hospital Consortium of Valencia consists of 726 children reported 47.6% positive patch tests and 59% positive for atopic dermatitis. In a cohort study, adolescence to adulthood have high incident rate of contact allergy and allergic contact dermatitis, mostly towards nickel [83,87].

Conclusion

PPD containing permanent hair dyes account for three-quarters of global use and their adverse effects including allergy, mutagenicity and toxicity have been debated for more than a decade. Allergic reaction testing should be done prior to hair dyeing to minimise the chance of getting allergy and contact dermatitis. The mutagenicity test shows that while PPD is only slightly mutagenic, oxidised PPD is strongly mutagenic. On the other hand, PPD metabolites MAPPD and DAPPD are not mutagenic. There is no definite conclusion whether PPD increases risk of cancer based on epidemiological studies; but there is an increased incidence of tumor in animal studies. Moreover, results showed that PPD induced apoptosis through various pathways in a few cell lines. Taken together, PPD possesses health hazards to human body and it is not recommended to use the chemical frequently.

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