Original Article

Repeated Topical Application of *para*-Phenylenediamine Induces Renal Histopathological Changes in Rats

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ABSTRACT

Hemolytic anemia and rhabdomyolysis have been often reported to be an adverse effect of drug- and chemical-induced toxicity both in experimental and real-life scenario. *para*-Phenylenediamine (PPD) is a derivative of *para*-nitroaniline and has been found as an ingredient of almost all hair dye formulations in varying concentrations from 2% to 4% w/v. Earlier studies have reported that the accidental oral ingestion of PPD in humans can lead to acute renal failure because of rhabdomyolysis. In the present investigation, we have tested the chronic topical application of PPD and its effect on the renal histology of Sprague-Dawley rats. The experiment provides clear evidence that topically applied PPD induces hemolytic anemia as evident from the decrease in the total RBC count, packed cell volume, and hemoglobin content apart from rhabdomyolysis which subsequently causes acute renal failure in rats.

Key words: Hemolysis, *para*-phenylenediamine, renal dysfunction, rhabdomyolysis, topical

INTRODUCTION

para-Phenylenediamine (PPD) is a widely used chemical in almost all hair dye formulations.[1] This compound is also used as a photographic developing agent and as an intermediate in the manufacture of azo dyes, antioxidants, and accelerators for rubber vulcanization.[2] The main purpose of using PPD as a hair dye ingredient is to fasten the process of dyeing as compared to traditional henna.

PPD has been recognized as a potent contact allergen.[3] In the case of PPD, only one oxidation product is known so far. PPD may be oxidized to benzoquinone diimine, which, in turn may form the trinuclear dye *N*,*N9*-bis(4-aminophenyl)-2,5-diamino-1,4-quinone-diimine called Bandrowski's base. Krasteva *et al*.[4] reported that Bandrowski's base is responsible for the apparent contact dermatitis due to PPD. On contact with skin, PPD causes skin irritation, keratoconjunctivitis, conjunctivital swelling, and eczema of the eyelids in a sensitized individual.[5]

Drug- and chemical-induced hemolytic anemia and subsequent renal failure is one of the major toxicological impacts that affects a large number of people around the world.[6,7] The first documented case of systemic poisoning with PPD occurred from handling dye was described in 1924 concerning the owner of a hairdressing salon.[8] Systemic toxicity due to contact with PPD has been reported earlier.[9,10] Acute renal failure due to hair dye use has been often reported in clinical studies.[11,12] It has also been reported that this chemical induces rhabdomyolysis and subsequent renal failure after cutaneous absorption.[10] Systemic toxicity may occur from percutaneous absorption of henna mixed with PPD.[13]

The present study was conducted in order to investigate the relation between the subchronic topical application of

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PPD and subsequent changes in the structure of kidneys in the SpragueDawley rats.

MATERIALS AND METHODS

Chemicals

PPD (CAS: 105-60-3) was purchased from Merck, Germany.

Animals and experimental design

Twenty male Sprague–Dawley rats weighing (130±10 g) were used during the present study. The animals were maintained in accordance with NIH guidelines for care and use of laboratory animals, and the experiment protocol has been approved by Departmental Research Council of Gauhati University. The animals were housed in polypropylene cages provided with the rice husk bedding material under a constant temperature of 22±3°C and 12:12 h of L:D cycle. The animals were randomly divided into four groups (control, Group 1, Group 2, and Group 3) and due to laboratory constraints only five rats were used per group. The animals were housed in separate polypropylene cages with rice husk as the bedding material at the bottom of the cages. Each animal from Groups 1, 2, and 3 was painted on the dorsal side clipped free of fur with the test chemical dissolved in double distilled water. The control group received double distilled water only. The daily exposure of each animal to the test chemical in the control group, Groups 1, 2, and 3 was 0, 1, 2, and 3 mg/kg body weight, respectively. The animals were painted continuously for 60 days after which they were euthanized by complete exsanguinations (heart puncture) after intraperitoneal injection of sodium pentobarbital. The initial body weight at the onset of the experiment and the final body weight prior to necropsy for each animals were recorded.

Hematology and serum biochemistry

Blood was collected in EDTA tubes prior to necropsy by cardiac puncture after intraperitoneal injection of sodium pentobarbital (50 mg/kg). A fraction of blood was analyzed for total count of RBC, Hb%, hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and reticulocytes (RCs) using the standard manual technique as described elsewhere. Serum was isolated and kept at –20°C till analysis. The serum albumin, creatinine phosphokinase (CPK) and creatinine levels were evaluated as described earlier[14-16] and the absorbance of the colored reaction product was measured with a spectrophotometer.

Histological study

Both the kidneys from each animal were weighed prior to fixation and paraffin embedding for histology. Serial sections were stained in H and E and Perls' Prussian blue stains for normal microscopic observation and deposition of iron pigments, respectively. The serial sections of the kidney were evaluated by a veterinary pathologist who was unaware of the treatment protocol.

Statistical analysis

The blood parameters and serum biochemistry of the treated animals were compared with the control animals by one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test and a P value <0.05 was taken into consideration for determining significance. All statistical values were computed using SPSS 10.0 software.

RESULTS

Table 1 presents the initial and final body weight as well as absolute and relative kidney weight of the experimental animals. The body weight gain by PPD-treated animals over the 60-day experimental period was found to be 9–13% lower than their corresponding control animals; however, no statistical significance was observed for any dose of PPD applied. The absolute kidney weight was not statistically different from the controls while the relative weight of the kidneys was found to be significantly different from the control counterparts at a high dose only (3 mg/kg, $P<0.05$).

Table 2 illustrates the effect of PPD on the hematological parameters of the experimental animals. PPD causes a significant decrease in the number of RBC at 3 mg/kg doses ($P<0.05$). The packed cell volume (PCV) was

Table 1: Body weight and kidney weight (absolute and relative) of the experimental animals after 60-day chronic exposure to PPD

	Control	Group 1	Group 2	Group 3
Initial body weight (g)	139 ± 10.11	141 ± 4.32	142 ± 6.31	145 ± 2.11
Final body weight (g)	189.2 ± 4.42	171.2 ± 6.42	165.6 ± 5.93	172.4 ± 8.73
Both kidney weight (g)				
Absolute	5.26 ± 0.13	6.30 ± 0.30	5.69 ± 0.37	6.92 ± 0.63
Relative	2.78 ± 0.03	3.68 ± 0.10	3.42±0.11	4.07 ± 0.45*

Mean±SEM (n=5). *P<0.05

found to be significantly different at all doses of PPD treatment when compared to the control untreated group ($P<0.001$). The MCV was found to be elevated in the treated Group 3, but no statistical significance was observed. The hemoglobin level was found to be significantly less and reduced in the PPD-treated group compared to the control group at doses 2 mg/kg ($P<0.05$) and 3 mg/kg ($P<0.01$) and MCH values were found to be significantly different from controls at dose 3 mg/kg ($P<0.001$). RCs showed a significant increase at doses 2 and 3 mg/kg ($P<0.001$).

The microscopical examination of Wright–Giemsa-stained peripheral blood smears showed significant and progressive morphological changes in the erythrocytes of the PPD-treated animals compared to the control group [Figure 1]. Moderate anisocytosis with macrocytosis and severely hypochromic or ghost cells were observed in the high-dose group of animals [Figure 1b and d]. The blood film of the treated animals exhibited polychromasia accompanied by an increase in the number of circulating erythrocytes with Heinz bodies [Figure 1c].

The serum biomarker of kidney toxicity exhibited a dose-related alteration during the present study. After 60 days of exposure, serum creatinine level was found to significantly increase at dose 3 mg/kg ($P<0.05$) compared to the control group [Figure 2]. The serum CPK level showed a significant elevation in the entire treated group in a dose-dependent manner when compared to the control group ($P<0.05$). Similarly, serum albumin levels were found to be declining after exposure to the test chemical; however, no statistical significance was observed when compared to the control group.

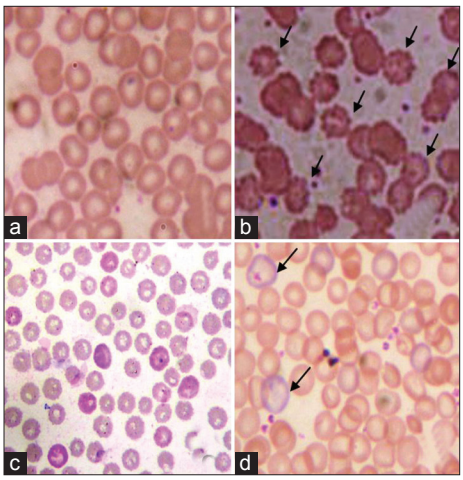

Figure 1: Comparative cytomorphology of RBC from control (a) and treated group of animals (b-d). (a) Control. (b) Echinocytic transformation of RBC after PPD treatment (arrow). (c) Polychromatic erythrocytes with Heinz bodies. (d) Ghost cells and polychromatic megalocytes (arrow)

Treatment-related histopathological changes were observed in the kidneys of all treated animals. Extensive tubular necrosis with cytoplasmic vacuolation and desquamation of the tubular epithelium from the surrounding basement membrane were observed in all treated groups [Figure 3b and c]. Tubular interstitial inflammation accompanied by the infiltration of hyperchromic leucocytes was observed in the high-dose Group 3 only (3 mg/kg; Figure 3d). No such histopathological lesions were observed in the control group [Figure 3a]. Perls' Prussian blue reaction showed an excessive deposition of iron-positive hemosiderin pigments in the renal cortex of the treated animals compared to control animals [Figure 4].

DISCUSSION

The findings of the present experimental study indicated that the repeated topical application of PPD caused a significant alteration in the hematological picture and renal histological features of the experimental animals at the selected dosage. The changes were observed even at a dose as low as 3 mg/kg/day, which is lower than the no-observed-adverse-effect level (NOAEL) of 4 mg/kg/day, prescribed for this chemical (SCCNFP/0129/99). These differences may be due to the differences in the experimental animal (rabbit) as reported by Burnett and Goldenthal[17] or the route of application which was oral in earlier studies (SCCNFP/0129/99) in contradiction to the topical application used during the present investigation.

Human exposure to PPD mainly occurs through skin contact during a hair dyeing or tattooing process and due to accidental or deliberate oral ingestion. Chronic exposure may occur in industrial workers, engaged in manufacturing dyes, or in regular hairdressers, handling hair dye

Table 2: Total count of (a) RBC, (b) packed cell volume (PCV), (c) mean cell volume (MCV), (d) hemoglobin (Hb), (e) mean corpuscular hemoglobin (MCH) and (f) reticulocytes (RCs) after 60-day chronic exposure to PPD in experimental animals

Groups	RBC ($N\times10^5/\mu l$)	PCV (%)	MCV (fl)	Hb (g/dl)	MCH (pg)	RCs (#/10^3)
Control	5.55 ± 0.85	39 ± 1.22	70.19 ± 2.20	15.27 ± 0.75	15.47 ± 0.78	27 ± 3.96
Group 1	4.05 ± 1.04*	27.8 ± 2.20	68.64 ± 5.43	14.35 ± 1.14	14.35 ± 1.14	27.8 ± 2.59
Group 2	5.04 ± 0.70*	33.4 ± 2.42	66.26 ± 4.80	13.75 ± 0.32*	13.75 ± 0.32	36 ± 2.16
Group 3	3.62 ± 0.98*	28.0 ± 3.11	77.17 ± 8.58	11.92 ± 0.42*	11.92 ± 0.42**	48.8 ± 5.08*

Mean±SEM (n = 5). *P<0.05. **P<0.001

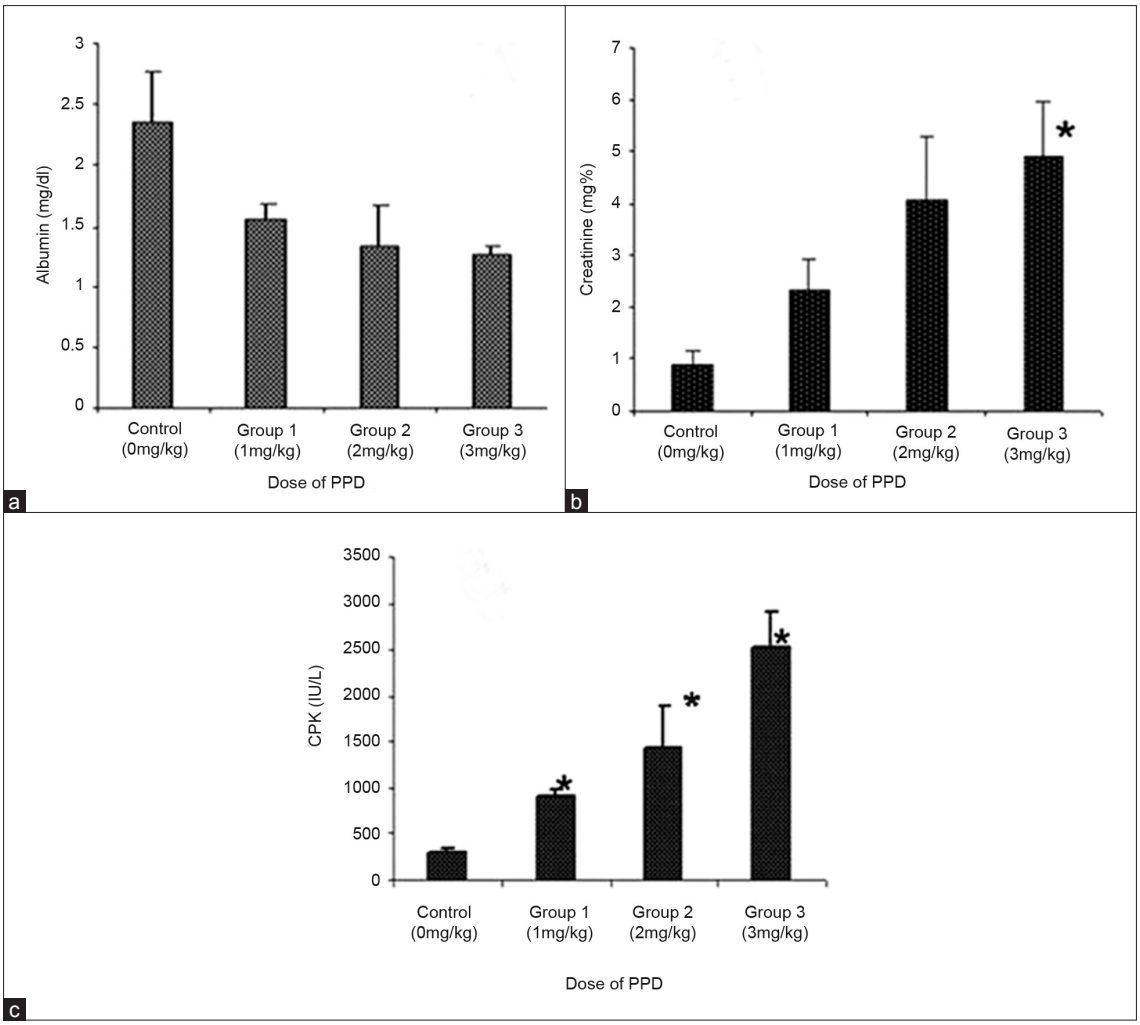

Figure 2: The effect of PPD on the (a) serum albumin (mg/dl), (b) creatinine (mg%), and (c) creatinine phosphokinase (IU/L) levels in the experimental animals after 60 days. Mean±SEM (*n*=5), statistical analysis by *t*-test. **P*<0.05, ***P*<0.001

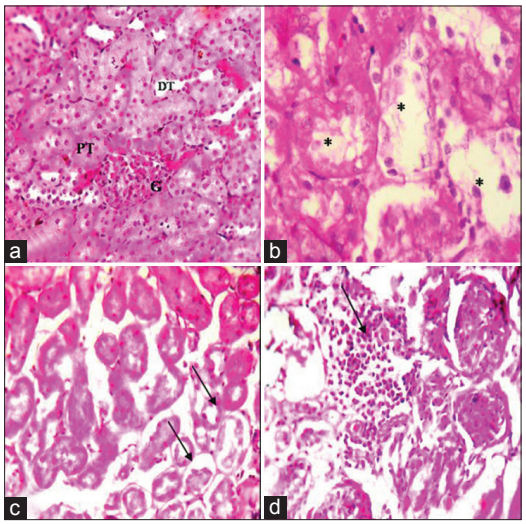

Figure 3: Kidney section from (a) control and (b-d) PPD-treated animals, respectively. Note (b) necrosis of proximal tubular cells with cytoplasmic vacuolation; (c) tubular atrophy and desquamation of the tubular epithelial layer. (d) The tubulointerstitial inflammation with hyperchromic leucocyte infiltration. H and E, original magnification ×40

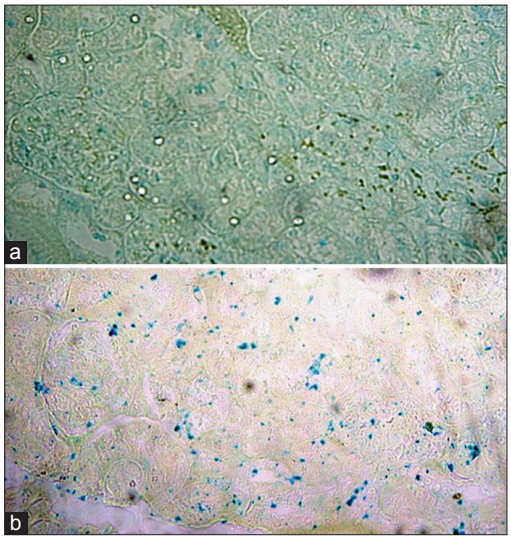

Figure 4: Kidney section from (a) control and (b) PPD-treated group of animals. Perls' Prussian blue staining, original magnification ×40. Note the increased deposition of iron pigments along the proximal tubular compartment in the PPD-treated animals compared to control animals

formulations containing this chemical, as an occupational exposure. The skin is considered as a protective barrier that serves to keep chemicals and other foreign constituents from entering and interfering with the complicated and finely tuned internal systems. But skin is not impermeable for the topically applied substances like hair dye chemicals which appear to pervade this barrier.[18] Available data also indicated that PPD can reach systemic circulation after percutaneous absorption.[18] This suggests that the changes observed during the present study may be attributed to PPD and/or its oxidized metabolites. In the case of PPD, only one oxidized product, Bandrowski base, is so far reported to be a strong allergen.[4] A number of case–control cohort studies reported the causal association of hair dye usage, leukemia, and bladder cancer risk.[19,20] Earlier we have reported that repeated topical exposure to the chemical up to 30 days caused significant histopathological changes in the liver of rats.[21] The aim of the present study was therefore to observe the effect of repeated topical exposure to PPD in the renal tissues.

Treatment-related reductions in the body weight were observed during the present study. This may be explained by the reduced food intake, secondary to the physiological disturbances caused by exposure to the test chemical. Chemical-induced stress has also been reported to activate the adrenal gland which results in the reduction of food intake, as reported by Wandhwa *et al*.[22] Changes in the absolute and relative weight of the kidneys of the treated groups, as observed during the present study, reflect the abnormalities of this vital organ and similar findings were reported during chronic progressive nephropathy by Greaves.[23]

The findings of the present study demonstrated that the repeated topical application of the chemical induced rhabdomyolysis as was evident from the sharp rise in the serum CPK level in the treated groups as compared to the control group. The elevation in the CPK level is an indication of damage to muscles and has been associated with rhabdomyolysis.[24] This observation is at par with other investigations that indicated rhabdomyolysis during PPD toxicity.[10] The exact cause/mechanism underlying renal histopathological changes in the treated groups, as observed during the present investigation, is unknown. Acute and chronic nephropathies may occur under certain physiological conditions when the kidneys are overexposed to heme proteins as may be observed during rhabdomyolysis and intravascular hemolysis.[24] The increased deposition of Perls' Prussian blue reacting iron pigments in the kidney tissues of the animals of treated groups provides a direct evidence of treatment-related accumulation of the heme protein in the renal cortex. The accumulation of hyperchromic leucocytes within the tubular interstitium also suggests the ongoing inflammatory responses within the renal tissues. Such recurrent exposure to the heme protein and subsequent tubulointerstitial inflammation in rats was also reported by Nath *et al*.[25] Another reason for the apparent nephrotoxicity may be attributed to the lipid peroxidation and subsequent damage to the subcellular organelles. The iron overload has been previously shown to increase the lipid peroxidation in the kidneys of experimental animals.[26] It is also suggested that increased heme protein accumulation may trigger the upregulation of monocyte chemoattractive protien 1 (MCP1) in the kidney tubules via nuclear factor-kB (NF-kB) and it is mainly responsible for the observed nephropathy during an iron overload.[27]

Apart from rhabdomyolysis, our data suggested that intravascular hemolysis (low Hb% conc., HCT, and RBC count) was another factor behind PPD-mediated renal nephropathy. The exact mechanism by which *para*-phenylenediamine induces hemolytic anemia and cytomorphological changes in RBC needs to be investigated. Various mechanisms have been postulated for toxicant-induced hemolysis and subsequent alteration in the cytomorphology of the red blood corpuscles. Toxicants may induce changes in the RBC shape and hemolysis either by depleting ATP or by increasing lipid peroxidation and reducing the cellular redox potential.[28] A study conducted earlier has revealed that methemoglobin formation was a major side effect of aromatic amine-induced erythrotoxicity[29] and such mechanism cannot be ruled out during the present investigation. Methemoglobin production leads to the generation of reactive oxygen species that may play a critical role in the development of hemolytic anemia.[7] The methemoglobin-induced erythrotoxicity may be enhanced due to the comparatively lower level of the methemoglobin reductase activity in the present experimental animal model system.[29] The red blood cell is an important component of the blood required for major physiological functions like carrying of oxygen to the tissues. Abnormalities in the shape or size of the RBC as observed during the present study could contribute to the decreased blood flow, loss of oxygen, and tissue damage through microvascular occlusion and it may be implicated in the present nephrotoxicity.

The skin is also capable of xenobiotic biotransformation and it has been already shown that PPD is metabolized into its acetylated derivatives when applied topically.[30] In many instances, it has been observed that the metabolized end-product is a more potent toxicant than the parent molecule itself. But in the case of PPD, the metabolized end-products are reported to be detoxified end-products.[30] However, during chronic exposure, the detoxification of PPD by the skin may become a limiting factor, thus exposing the organism to the parent PPD molecules. The observed changes in the renal histopathology as well as in the hematology of the experimental animals during the present investigation can thus be attributed to the parent PPD molecule and/or its oxidized metabolites that reach the systemic circulation escaping skin's metabolic pathways.

The rate of bioaccumulation or metabolic clearance is an important factor which may play a role in its toxicity. This rate may vary from individual to individual and therefore susceptibility to the chemical-induced damage pattern may not be uniform. Though experimental findings could not always be expected to be extrapolated to the humans but for the better safety of human health, it is always recommendable to minimize the usage of PPD-containing hair dyes.

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