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Hepatic Histopathological Abnormalities in Rats Treated Topically with Para-Phenylene Diamine (PPD)

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Abstract: Drug and chemical mediated hepatotoxicity for wide numbers of chemicals has been recognized. The drug mediated hepatotoxicity and its evaluation is an important aspect in the development of drugs intended for therapeutic usages as well as chemicals used as food and cosmetic additive. Para-Phenylene Diamine (PPD), a widely used chemical in almost all hair dye formulation has been tested for its hepatotoxicity after 30 days continuous topical application in three different dosages (0, 1, 2 and 3 mg kg⁻¹) in Sprague-Dawley rats. Serum biomarker (ALT, AST and ALP) of liver injury exhibit a dose dependent increases over control animals. Histopathological findings include centrilobular coagulative necrosis, periportal inflammation, fibrinous deposition, hemorrhages and increased accumulation of neutrophils within hepatic parenchyma. The PPD mediated hepatotoxicity is seems to be enhanced by increased accumulation of neutrophils.

Key words: Para phenylenediamine, hepatotoxicity, necrosis, serum enzymes, neutrophil

INTRODUCTION

Dermal exposure study of environmentally available chemicals is a new initiative in the field of toxicology research. This is particularly more relevant in case of chemicals that find its way into human system through skin. Drug and chemical induced liver injury is a serious complication, because liver is central to the metabolic disposition of virtually all drugs and foreign substances. Xenobiotics may be toxic by themselves or their metabolites play an important role in liver injury. The mechanism of hepatocytes toxicity may results either directly from the disruption of intracellular function or membrane integrity or from damages affecting endothelial or bile duct cells as seen in cholestasis or indirectly from immune mediated membrane damage.

Paraphenylene Diamine (PPD or 1, 4 diamino benzene, CAS: 106: 50: 3) is a widely used chemical in almost all hair dye formulation (Corbett and Menkart, 1973). This compound is also used as photographic developing agent and as an intermediate in the manufacture of azo dyes, antioxidants and accelerators for rubber vulcanization (Hansen *et al.*, 1993). The main purpose of using PPD as hair dye ingredients is to fasten the process of dyeing as compared to traditional henna. PPD has been recognized as potent contact allergens (Schnuch *et al.*, 1997). In 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 (BB).

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Krasteva *et al.* (1993) reported that BB is involved in contact dermatitis to PPD. On contact PPD causes skin irritation, keratoconjunctivities, conjunctivital swelling and eczema of the eyelids in sensitized individual (Sax, 1984).

A number of studies have been conducted in past regarding *in vivo* and *in vitro* percutaneous absorption of the PPD (Steiling *et al.*, 2001; Hueber-Becker *et al.*, 2004; Dressler and Appelqvist, 2006) and it clearly demonstrated that this chemical when applied topically can reach systemic circulation. This implies that topically applied PPD upon transcutaneous absorption reach systemic circulation where it could exert systemic effects. A few case studies have also been reported about drug induced hepatitis among chronic hair dye users (Yoshio *et al.*, 2003).

The present study was conducted to analyze dose dependent hepatic effect of repeated topical application of para Phenylenediamine in Sprague-Dawley rats.

MATERIALS AND METHODS

Chemicals

The test chemical Paraphenylene diamine (PPD, CAS: 106-50-3) was purchased from Merck, Germany.

Animals, Experimental Design and Treatment

The study was conducted from July, 2008 to June, 2009; in the Department of Zoology, Rajiv Gandhi University, Rono Hill, Itanagar. Twenty numbers of male Sprague-Dawley rats weighing (130±10) were during the present study. The animals were maintained in accordance with NIH guidelines for care and use of laboratory animals and experiment protocol has been approved by Institutional animal care and use committee. The animals were housed in polypropylene cages provided with rise husk bedding material under ambient temperature of 21±3°C and 12: 12 h of L: D cycle. The animals were acclimatized to the laboratory condition for one week at the commencement of the treatment protocol. The animals were randomly divided into four groups (Control, Group 1, Group 2 and Group 3) comprising five numbers of animals per group. Each animal from Group 1, Group 2 and Group 3 were painted on their dorsal side clipped free of fur with the test chemical dissolved in double distilled water while the control group received distilled water painted on their dorsal side as in other PPD treated animals. The total daily exposure per animal to the test chemical (PPD) in group 1, group 2 and group 3 were 1, 2 and 3 mg kg⁻¹ b. wt., respectively. The animals were painted for 30 continuous days with PPD solution or vehicle alone, after which they were euthanasized by complete exsanguinations (heart puncture) after intraperitoneal injection of Ketamine hydrochloride (10 mg kg⁻¹). The initial body weight at the onset of the experiment and final body weight prior to necropsy for each animals were recorded.

Biochemical Analysis

Blood was collected in EDTA tubes prior to necropsy by cardiac puncture after intraperitoneal injection of Ketamine hydrochloride (10 mg kg⁻¹). Serum was isolated and kept at -20°C till biochemical analysis. AST, ALT, ALP was measured using kits (Coral system, Goa, India) according to manufacturer's instructions within two days of serum separation and absorbance was recorded with a spectrophotometer.

Histological Study

Whole liver from each animal were weigh prior to fixation and paraffin embedding for histology. Formalin fixed central portion of the left lobe of the liver from each animal was embedded in paraffin and 5 μm thick sections were cut. The sections were stained in H and E for evaluation of liver injury or with the naphthol AS-D chloroacetate esterase technique for evaluation of neutrophil infiltration in liver as described previously (Jaeschke *et al.*, 1996). Neutrophil accumulation in the liver was quantified by counting the total number of neutrophils in 50 high power fields. Tissue hemoglobin as indicator for tissue hemorrhage was determined according to Lawson *et al.* (1998). Briefly 20% of liver homogenate was prepared in 50 mM Na-phosphate buffer (120 mM NaCl, 10 mM EDTA). After centrifugation at 8000 rpm for 10 min at 4°C, the supernatant was diluted in Dabkins reagent and following 15 min incubation, the absorbance was measured at 550 nm. The hemoglobin concentration was determined with a calibration curve and calculated as milligrams hemoglobin g^{-1} liver tissue.

Statistical Analysis

All data were presented as means \pm SEM. Multiple comparisons were performed using student's t-test. A p-value <0.05 were taken into consideration for determining significance. All statistical procedures were computed using SPSS 10.0 software.

RESULTS

The initial and terminal body weights with absolute and relative liver weight are presented in Table 1. No significant differences were observed in the terminal body weight gain among the treated and control group of animals. Both absolute and relative liver weight exhibits a dose response increase after PPD treatment. The absolute liver weight and relative liver weight for each dose of PPD applied is found to be significantly different from control untreated animals at $p<0.05$ and $p<0.001$, respectively.

All serum biomarker of liver toxicity (ALP, ALT and AST) shows a dose dependent increase (Fig. 1) in the PPD treated group of animals compared to their untreated control group. Serum ALP, AST and ALT of treated animals shows (2.3, 2.6 and 3.0), (2.7, 3 and 3.6) and (1.6, 1.8 and 2.26) fold increases over control untreated group, respectively.

The histological structure of hepatic centrilobular area (CV) and Portal Tract (PT) of the control group of animals are shown in Fig. 2A and B. Hepatocytes hypertrophy and hyperplastic portal tract was a common histopathological finding among all the PPD treated animals. At low dose (1 mg kg^{-1}) PPD causes severe periportal necrosis and inflammation characterized by increased adherence to the portal tract and infiltration of leucocytic component across portal tract has been observed (Fig. 2D). At high dose (3 mg kg^{-1}), PPD causes severe centrilobular necrosis accompanied by dialatation of the central vein (Fig. 2C). Marked fibrinous exudates surrounding central vein and infiltrating and adhering

Table 1: Final body weight and liver weight (Absolute and relative) of experimental animals after 30 days topical application of PPD

Groups	Initial body weight (g)	Final body weight (g)	Liver weight	
			Absolute (g)	Relative (%)
Control (0 mg kg^{-1})	139 \pm 10.11	168 \pm 8.03	4.675 \pm 0.211	2.78 \pm 0.033
1 (1 mg kg^{-1})	142 \pm 4.12	165 \pm 7.00	6.249 \pm 0.306*	3.78 \pm 0.037**
2 (2 mg kg^{-1})	142 \pm 6.31	159 \pm 5.69	5.447 \pm 0.262*	3.42 \pm 0.114*
3 (3 mg kg^{-1})	145 \pm 2.11	161 \pm 7.35	7.639 \pm 0.362*	4.55 \pm 0.175**

Value are presented as Means \pm SEM, n = 5 per group, * $p<0.05$ and ** $p<0.001$

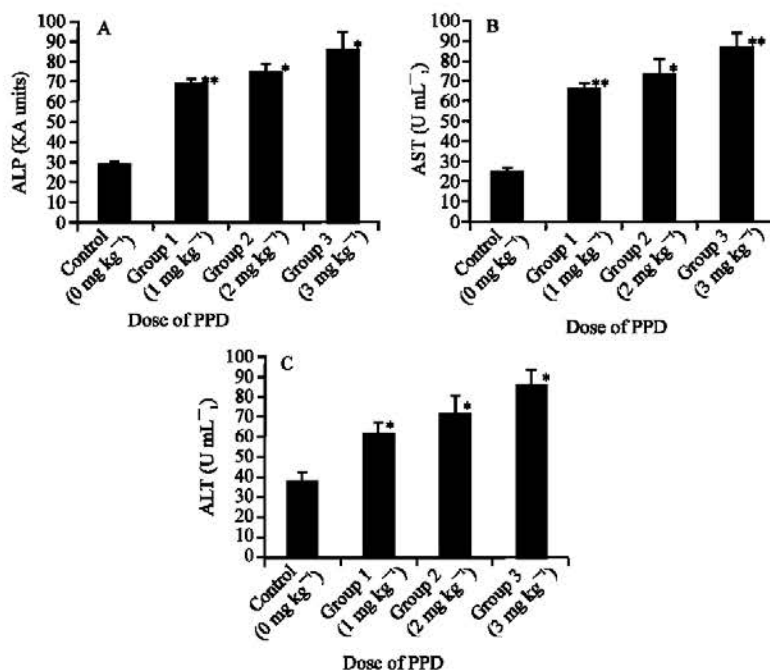


Fig. 1: Serum biomarker of liver injury (A) alkaline phosphatase (KA mL⁻¹), (B) alanine amino transferases (U mL⁻¹) and (C) ALT (U mL⁻¹) of the experimental animals after 30 days repeated topical application of PPD, value are presented as Means±SEM, n = 5 per group, *p<0.05 and **p<0.001

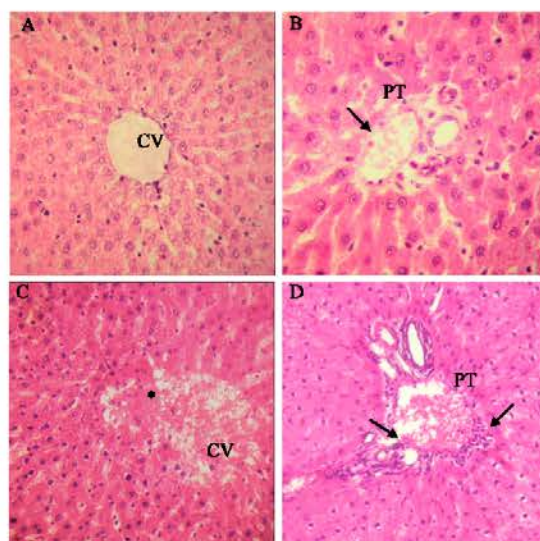


Fig. 2: (A and B) Liver section from control and (C and D) PPD treated animals. (A) Centrilobular area of control Animals compared to (C) necrotic and fibroin deposited CV area with dilated central vein from treated animals. (D) the portal tract exhibit hyperplasia and is surrounded by a leucocytic infiltration (arrows) in treated animals but not in (B) control. H and E, magnification X40, PT: Portal tract, CV: Central vein

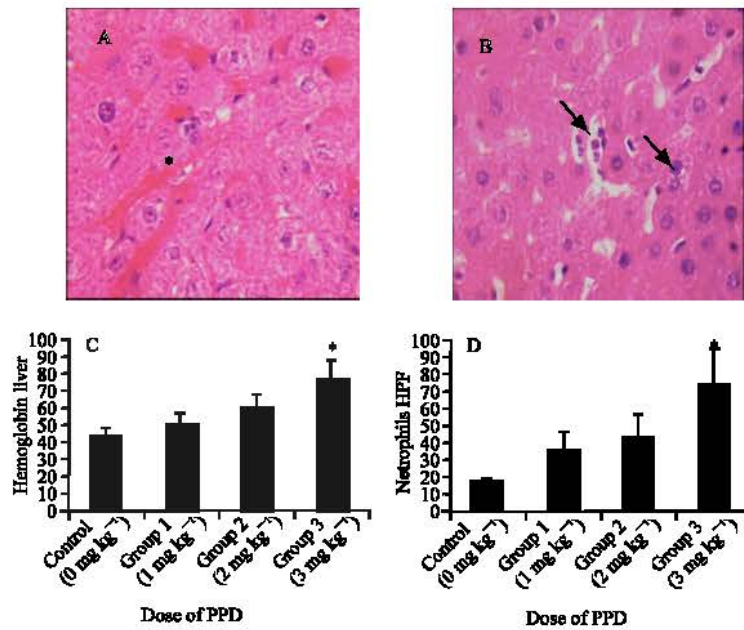


Fig. 3: Representative liver sections from PPD treated animals. (A) asterisks indicate hemorrhages, H and E, magnification X100, (B) sinusoidal dilation arrow Pointed to entrapped neutrophils surrounding a hepatocytes, H and E, magnification X100, (C) liver hemoglobin (Hb) content reflecting hemorrhages after 30 days repeated topical application of PPD, (D) hepatic neutrophils accumulation after 30 days repeated topical application of PPD indicative of neutrophils sequestration in the hepatic vasculature. Value are presented as Means±SEM, n = 5 per group, *p<0.05 and **p<0.001

leucocytes to the central vein has been observed in the PPD treated animals. The entire PPD treated animal's shows massive centrilobular and periportal hepatocytes necrosis characteristic of coagulative and ischemic necrosis (Fig. 2C, D)

Extensive hemorrhage within liver tissue and hydropic degeneration of hepatocytes were observed in all PPD treated groups of animals (Fig. 3A). Hemoglobin concentration in treated animal's increases (Fig. 3C) in a dose dependent manner and at high dose (3 mg kg⁻¹) it is significantly different (p<0.05) from control vehicle treated animals.

At high power magnification, sinusoidal widening, extensive accumulation and extravasation of neutrophil were noted (Fig. 3B) in the liver of treated animals. In control liver sections, relatively few neutrophils in sinusoids were observed compared to treated group and the neutrophil accumulation seems to be a dose dependent phenomenon although statistical significance has not been observed (Fig. 3D).

DISCUSSION

The objective of the present investigation was to test the hypothesis that repeated topical application of PPD exerts hepatotoxicity. PPD is a widely and freely available environmental chemical found in almost all hair dye formulation and human exposure mainly occurs through skin, accidental ingestion or by inhalation of powdered particles from hair

dye formulations during dying process. No report has been available regarding chronic dermal exposure and subsequent hepatotoxicity by PPD. A very few report are available that pointed towards the hepatotoxic nature of hair dye ingredients (Yoshio *et al.*, 2003). The present investigation was therefore aim at establishing the role of PPD in hair dye mediated hepatotoxicity.

The present study indicates that the selected dose of PPD on chronic topical exposure causes acute toxicity to the Liver of the experimental animals. The PPD mediated hepatotoxicity is evident from histological observation (hypertrophy, hyperplasia of portal tract, hepatocytes necrosis, hemorrhages, fibrin deposition within central vein and around the hepatic cords, fibrinous exudates as well as inflammation of the portal tract) and sharp rises in the serum biomarker of liver injury, that followed a dose dependent pattern. Further dose response acute toxicity study is required to confirm exact dose and hepatotoxic nature of PPD molecule. Acute and chronic hepatitis is pathologically characterized by a prominent infiltration of lymphocytes into the liver (Dienes *et al.*, 1987) and this histological feature is predominantly found in the liver of the PPD treated rats, while no such hepatic abnormalities were found in the control untreated group of rats.

A number of earlier report suggest that a fraction of topically applied PPD alone or in combination with an oxidizing agent reach systemic circulation after percutaneous absorption (Steiling *et al.*, 2001) and rest of the topically applied fraction are subject to metabolic conversion especially by N-Acetyltransferase, converting the parent PPD molecule into either mono or diacetylated PPD (Kawakubo *et al.*, 2000). This metabolic conversion of PPD is believed to a part of the detoxification pathway and seems to non toxic in nature (Kawakubo *et al.*, 2000). The apparent hepatotoxicity observed here in this experiment therefore mainly attributed to the smaller amount of parent PPD molecules that reach systemic circulation and thereafter hepatic circulation after percutaneous absorption. The plasma clearance rate of PPD is biphasic and plasma half life is estimated to be 24 min and 43.5 h, respectively (Rehani *et al.*, 1981). This may explain the apparent hepatic toxicity of the PPD molecule, since a little fraction of PPD absorbed through skin is possibly retained for a longer period (43.5 h) so as to produce toxicity towards hepatic parenchyma and sinusoidal and/or central vein endothelial cells. The increase in the liver hemoglobin content explains that PPD treatment cause damages to SEC or CVEC and causes extensive hemorrhages and subsequent deposition of fibrin occurs within CV and in sinusoidal compartments. Hemorrhage and fibrin deposition caused by injury or loss of SEC can result in the impairment of sinusoidal blood flow (Hirata *et al.*, 1989; Vollmar *et al.*, 1993; Yachida *et al.*, 1998) that finally leads to ischemic/hypoxic injury of hepatic parenchymal cells within affected regions (Copple *et al.*, 2002; Yee *et al.*, 2000). The SEC destruction or gap formation paves the way for neutrophil extravasation into hepatic parenchyma (Ito *et al.*, 2006). The mechanism of neutrophils extravasation is known to be dependent on some chemotactic signals like TNF- α derived from kupffer cells, CXC chemokines that include IL-8, Keratinocyte derived cytokine (KC), macrophage inflammatory protein-2 (MIP-2), Platelet-Activating Factor (PAF), from the injured hepatic parenchyma (Jaeschke and Smith, 1997; Jaeschke, 2003). In many cases it is the undergoing hepatic parenchymal apoptosis or oncotic changes that act as signal for neutrophil extravasation (Lawson *et al.*, 1998). Whether PPD causes apoptosis or oncotic changes in liver parenchyma is an area that needs further investigation. The increased dose dependent accumulation of neutrophil into hepatic parenchyma of the PPD treated rat may be due to increases in hepatocytes turnover owing to PPD treatment. Detrimental effects of hepatic neutrophil recruitment have been shown in models of ischemia-reperfusion (Jaeschke *et al.*, 1990, 1993), alcoholic hepatitis

(Bautista, 1997) and obstructive cholestasis (Gujral *et al.*, 2003). During present investigation increased neutrophil adherence to the sinusoidal endothelium and extravasation into hepatic parenchyma has been observed. The accumulated activated neutrophils undergo degranulation and generate reactive oxygen species including hypochlorite (Gujral *et al.*, 2003; Jaeschke *et al.*, 1991, 1993) that enhances hepatic injury. Our observation points towards PPD mediated SEC injury as a factor apart from hepatocytes necrosis behind apparent hepatic toxicity.

The SCCNF suggested that few data were available to allow an adequate risk assessment of PPD. Moreover, the maximum dose (3 mg kg^{-1}) selected in the present investigation for 30 days repeated toxicity study is less than that recommended by SCCNF (2000) as an NOAEL (4 mg/kg/day). Considering the results observed in the present investigation, further experimental information is necessary to confirm the role and mechanism of PPD mediated hepatotoxicity.

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