ORIGINAL ARTICLE

TERATOGENIC EFFECTS OF TOPICALLY APPLIED PARAPHENYLENEDIAMINE ON THE RAT FOETUS

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Background: Paraphenylenediamine (PPD) is an organic compound used in hair dye. It causes local and systemic side-effects. Not much is known regarding its foetotoxic effects. This study aimed at determining the teratogenic effects of topically applied PPD. **Methods:** This experimental study was carried out at IBMS, Khyber Medical University, and Peshawar Medical College, Peshawar over a period of 6 months. Twenty healthy Sprague Dawely female pregnant rats were divided into 5 groups, i.e., positive control (Group A), control (Group B), and 3 experimental groups (C, D and E). For group A, 0.1 ml of distilled water was applied topically on back of each rat. For positive control group B, 0.5 ml of dye containing 3% PPD was applied, while for group C, D and E, 1, 2 and 3 mg/Kg dose of PPD respectively was applied for 30 minutes daily. Skin was washed under tap water followed by rats mating. After successful fertilization, same doses were repeated for 20 days. Animals were sacrificed and foetuses were examined for skeletal abnormalities and hepatic changes. Skin biopsy of adult rats was examined for any changes. **Result:** Topically applied PPD resulted in significant teratogenic effects on foetal liver in a dose dependent manner. However, no significant teratogenic effect was observed on foetal skeleton (p=0.075). Topical PPD showed increased epidermal thickening and keratinization on adult rats skin. **Conclusion:** PPD has significant dermal effects on adult rats and teratogenic effects on liver of rat foetuses but no significant effects were observed on foetal skeleton.

Keywords: Paraphenylenediamine, Sprague dawley, Teratogenic, Topical application, Hair dye, Foetus

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INTRODUCTION

Paraphenylenediamine (PPD) is a chemical compound having formula $C_6H_4(NH_2)_2$.[1] PPD is an alanine derivative and is locally known as '*Kala Pathar*'.[2] The PPD is a black mineral from the banks of Nile River. The PPD is commercially available since 1990 and is still used in permanent hair dyes. It is also used in rubber chemicals, pigments, cosmetics and photographic development agent. PPD is a colourless grey or yellow crystalline solid, which oxidizes after exposure to air and turns red, brown, and finally black. In many countries of Africa, Middle East, and India PPD is mixed with Henna and traditionally applied to palm and soles. In USA more than one third of women use hair dye containing PPD. PPD is used to intensify the black colour of Henna and to reduce the time for dyeing as well.[3] N-acetylation catalysed by N-acetyltransferases (NATs) is major route of PPD metabolism. PPD is acetylated to monoacetyl-PPD (MAPPD), which is acetylated to Diacetyl-PPD (DAPPD).[4] PPD and these metabolites are mainly excreted through renal clearance.[5] Chronic exposure to PPD occurs in industrial workers manufacturing dyes, or in hairdressers using hair dyes containing PPD, as an occupational hazard.[1] Hair dye has been used to colour hair over the course of history. Hair dye can be categorized in terms of effectiveness and ingredients.[6] In terms of effectiveness hair dye can be temporary, semi-permanent and permanent. In terms of ingredients hair dye can be natural, mineral and artificial organic dye. Worldwide most commonly used hair dye is permanent hair dye. Paraphenylene diamine is used as an essential component of almost all hair dye formulations. The absorption of hair dye through skin varies from one person to other and depends upon person's health, gender, age and the moisture content of the dye.

For more than 4,000 years henna has been used as a cosmetic by Middle Eastern, Mediterranean and Asian cultures. Henna contains a burgundy dye molecule Lawsons (2-hydroxy-1,4-napthoquinone) which binds with proteins and is used in body art to dye hair, skin, nail and also silk, wool and leather.[7] The colour of natural henna is Orange/Reddish. PPD is mixed with natural henna to give it an ebony colour (Black Henna) and to reduce the time taken by natural henna to stain.[7]

PPD can cause local and systemic side-effects when applied topically or ingested orally. It is highly toxic, especially when taken orally.[8] Oral ingestion of PPD has been associated with systemic poisoning. The PPD induced systemic toxicity occurs in two phases. Acute phase occurs early some hours after ingestion. In acute phase tongue swelling, angioedema, burning and

numbness of the mouth, vomiting and airway obstruction occurs. The second phase occurs after a few days when toxic metabolites are absorbed and distributed throughout the body. Dark cola colour urine, acute injury to kidney, oliguria, anuria rhabdomyolysis, intravascular haemolysis and drowsiness can occur.[9] Oral ingestion of PPD has also been used for inducing abortions. A case study of a pregnant female who ingested unknown dose of PPD for the said purpose reported myocardial lysis in the aborted foetus.[10]

The lethal dose of PPD in humans varies from 7–10 gms, resulting in death due to cardiotoxicity and angioneurotic oedema.[8] In rats, a lethal dose of PDD ranges from 80–100 mg/Kg.[11] On topical application, PPD is metabolized in skin and its metabolites exert systemic effects. Hydrogen peroxide and superoxide radicles are produced as metabolites on long term exposure to PPD.[12] Interestingly, animal studies have suggested that orally ingested PPD fails to produce any teratogenic or foetotoxic effects.[13] However, one study which was carried out to determine the histopathological effects of PPD containing hair dye administered subcutaneously resulted in neonatal corneal changes.[14]

A number of studies showing the toxic effects of topically applied PPD on different adult organs have been reported.[14–19] Hair colouring products containing PPD account for 35% of non-Hodgkin's lymphoma in exposed women and 20% in all women.[5] Topically applied PPD induces haemolytic anaemia and rhabdomyolysis, which causes acute renal failure in rats.[15] The PPD also causes hepatotoxicity evident by an increase in serum biomarkers and on histopathology[16] and reduces the number of primary ovarian follicles in rat ovaries.[17] Sub-chronic topical application of PPD in different doses decreases the total sperm count and testicular weight.[18] Topically applied PPD causes histopathological changes in skin like proliferation of epithelial cells and increase keratinization.[19] Chronic dermal exposure to PPD resulted in toxic effects on pancreas that were evident from histopathological changes like vacuolation in cells of islets of Langerhans, irregular distorted acini, and congested blood vessels.[20] There is increased risk of lymphoma, leukaemia and bladder cancer in people using hair dye containing PPD.[21]

Most drugs and chemicals like tetracyclines, and anticoagulants cross the placenta. Many factors like molecular weight, fat solubility, polarity etc. determine the ability of a drug or chemical to cross the placenta and reach the foetus. High molecular weight compounds do not cross the placenta. Compounds having molecular weight 1,000 Daltons or more do not cross the placenta, while those compounds having molecular weight less than 600 Daltons cross the placenta.[22] PPD has molecular weight 108.14 Dalton so it can cross the placenta and reach the foetus and can cause teratogenic effects.

The effects of topically applied PPD have been studied in adults. So far, in literature not much evidence was retrieved regarding the teratogenic effects of topically applied PPD. No evidence was found whether topically applied PPD is capable of crossing placenta and inducing teratogenic effects or not. Since hair dyes containing PPD are frequently used among women, the rationale of this study was to understand the teratogenic effects of PPD on foetal liver, skeleton and maternal skin. This would lead to better guidance for the use of hair dyes containing PPD in pregnant women.

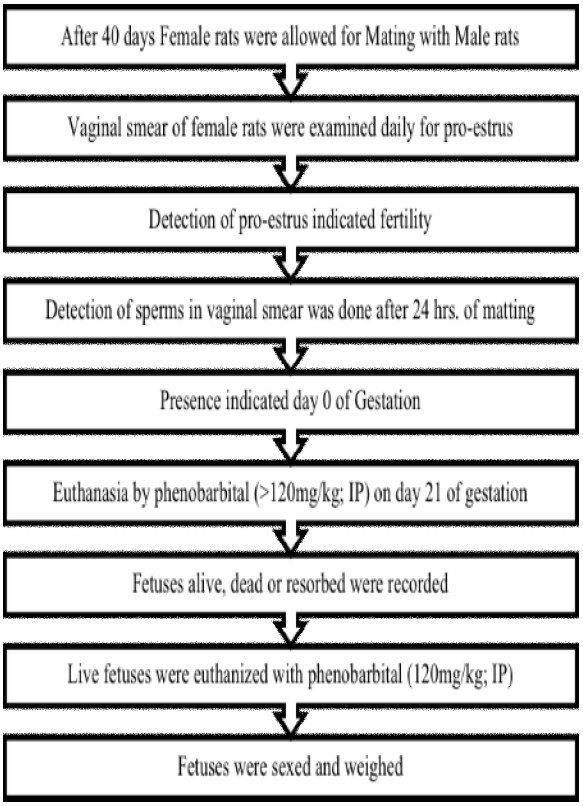
MATERIAL AND METHODS

After review and approval by Graduate Study Committee (GSC), this research proposal was approved by the Advanced Study and Research Board (ASRB) under: DIR/KMU-AS&RB/TE/001089, and Ethical Board under: DIR/KMU-EB/TE/000761. The study was carried out in the Institute of Basic Medical Sciences (IBMS) of Khyber Medical University (KMU) Peshawar, Pakistan and Peshawar Medical College Peshawar, Pakistan. This was an experimental lab-based study.

Twenty healthy Sprague Dawley female pregnant rats were taken. There were divided into five groups of four rats each as positive control, control, and three experimental groups. Sampling technique used was simple random sampling. The duration of this study was six months (June–December 2019).

The exposed surface on the dorsum of all animals were clipped free of hair two days before the topical application of PPD. PPD doses of 1, 2 and 3 mg/Kg were used for three Experimental groups C, D and E respectively. These doses were made in 1ml of distilled water and 0.1 ml of this solution was used for each dose.[15] For Group A (Control group), 0.1 ml of distal water was applied topically on exposed area of 5×6 mm^2 on the dorsal surface of each rat.[17] For Positive control group B, dye containing 3% PPD mixed with 1:1 H_2O_2, in a dose of 0.5 ml twice was applied topically.

For Group C, D and E (Experimental groups) fresh solution of 1, 2 and 3 mg/Kg dose of PPD was prepared every day in distilled water. It was applied on the exposed dorsum of each rat using plastic syringe and spreaded using spatula for 30 minutes. The skin was washed under tap water.[17] This process was repeated initially for 40 days and was then continued for 20 days after matting of rats. Total dose applied to rats over 60 days in group B was 49.5 mg, group C was 1.65 mg, group D was 3.3 mg and group E was 4.95 mg. The protocol followed to ensure mating is depicted in protocol flowchart.



Protocol flowchart

Two third of foetuses from each group were selected randomly for the examination of skeletal malformations and 1/3rd for Histopathological examination of liver.[23] Liver morphology was staged and graded on basis of Batts-Ludwig's system.[24] For isolation and examination of foetal skeleton, 2/3rd of the foetuses were fixe din 95% ethanol and defatted in 1% KOH to make tissue and skeleton visible. The skeleton was then stained with 0/0025% Alizarin red stain and 1% KOH for 24 hours. They were rinsed with water and transferred to 30% glycerine for 24 hours. Skeleton examination was carried out under dissecting microscope and foetal skeleton was observed for the skeletal abnormalities like Hemimelia, vertebral agenesis, Missing ribs, supernumerary ribs, digital defects and tail defects. For the evaluation of foetal liver, 1/3rd of foetuses were taken and their hepatic tissue was processed for Haematoxylin and Eosin staining and visualized under the light microscope.

For studying further effects of topically applied PPD we also studied on the skin sections of adult rats where PPD was applied. For skin biopsy of adult female rat, 6 mm punch biopsy was taken from the site of PPD administration and 10 micrometre cryo-sections were cut. Histological sections were fixed in 5% acetic acid in menthol. Haematoxylin and eosin staining was done to define the skin layers and structures associated with each sample for analysis of histological changes.

All the data obtained after animal experimentation was recorded in Microsoft Excel (2013). Histologic slides were analysed using image J software version 1.8.0 for determining diameter of central vein of liver and thickness of epidermis of skin. The data thus obtained was used to categorize dilation or congestion of central vein and increase or decrease in thickness of epidermis of skin. The SPSS-25 was used for all statistical analysis.

RESULTS

Number of foetuses in different groups, their male to female ratio and mean weight of foetus is shown in Table-1. The frequency of hepatic histological changes that is Periportal inflammation, Periportal necrosis, Centrilobular necrosis, Central vein dilatation and Hepatocyte hypertrophy are shown in Table-2, 3. The effects of different doses of PPD application on the skeleton of rat foetuses in different groups is shown in Table-4. In case of Periportal inflammation and necrosis the $p<0.000$ suggesting significant relationship between various PPD doses and periportal inflammation and necrosis. In case of Centrilobular necrosis, dilatation of central vein and Hepatocyte hypertrophy the p values were found to be $p= 0.03$, $p= 0.006$ and $p= 0.001$, respectively showing significant relationship between these histological changes and PPD application.

Table-1: Number of foetuses and their mean weights in each group

Groups	Number of foetuses	Male	Female	Weight in grams Mean±SD
A	21	11	10	3.39±0.18
B	24	12	12	3.68±0.30
C	33	22	11	3.78±0.63
D	24	10	14	3.5±0.18
E	30	20	10	3.51±0.30

Table-2: Frequency of periportal inflammation, periportal necrosis among foetal livers

Groups	Periportal inflammation						Periportal necrosis					
	None	Minimal	Mild	Moderate	Severe	Total	None	minimal	mild	moderate	Severe	Total
Water	7	0	0	0	0	7	7	0	0	0	0	7
Dye	0	0	0	0	8	8	2	4	1	1	0	8
1 mg/Kg	0	1	0	2	8	11	1	4	0	5	1	11
2 mg/Kg	0	0	0	1	7	8	0	1	2	2	3	8
3 mg/Kg	0	0	0	1	9	10	0	1	2	1	6	10
Total	7	1	0	4	32	44	10	10	5	9	10	44

Table-3: Frequencies of centrilobular necrosis, central vein dilatation and hepatocyte hypertrophy among foetal livers

Groups	Centrilobular Necrosis			Dilatation of central vein				Hepatocyte hypertrophy		
	Present	Absent	Total	Normal	Dilated	Congested	Total	Absent	Present	Total
Water	0	7	7	7	0	0	7	7	0	7
Dye	0	8	8	0	5	3	8	3	5	8
1 mg/Kg	6	5	11	4	6	1	11	3	8	11
2 mg/Kg	5	3	8	2	6	0	8	2	6	8
3 mg/Kg	7	3	10	4	4	2	10	0	10	10
Total	18	26	44	17	21	6	44	15	29	44

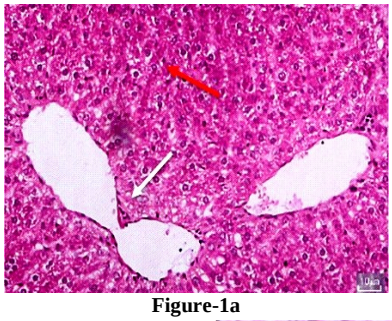


Figure-1a

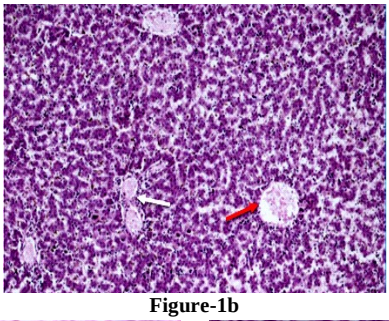


Figure-1b

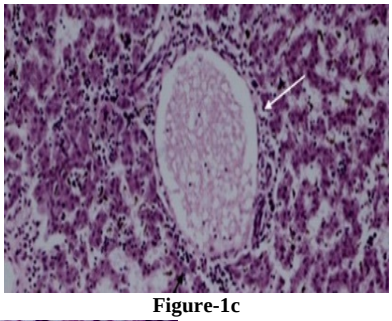


Figure-1c

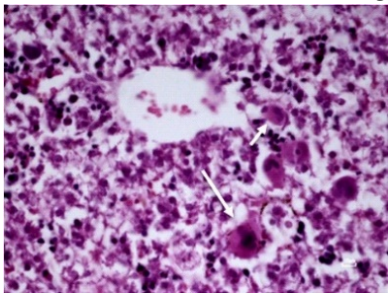


Figure-1d

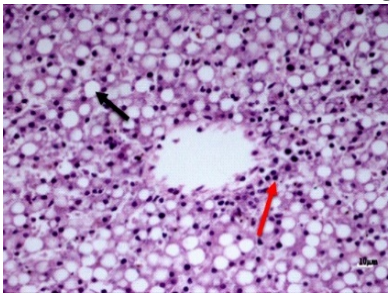


Figure-1e

Figure-1: Histology of rat foetal liver

a) normal liver histology of Control group A; white arrow is pointing toward the portal triad, red arrow points towards normal hepatic cords. b) positive control group B; white arrow showing periportal inflammation, and red pointing towards dilated central vein. c) experimental group C; white arrow showing dilated central vein, black arrows pointing hepatocyte hypertrophy with severe periportal inflammation. d) experimental group D; white arrows pointing necrotic hepatocytes. e) experimental group E; black arrow showing steatotic hepatocyte hypertrophy and red arrow pointing hepatocytes surround by infiltrative cells. A, B at 10× and C, D, E at 40×. Scale bar=10 µ

Figure-2a

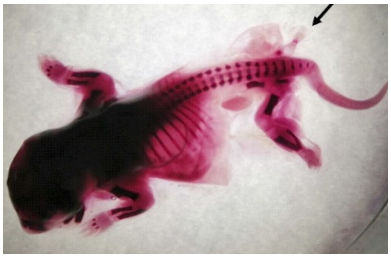


Figure-2b

Figure-2c

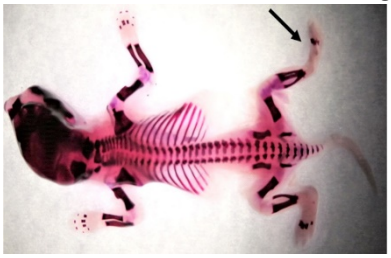


Figure-2d

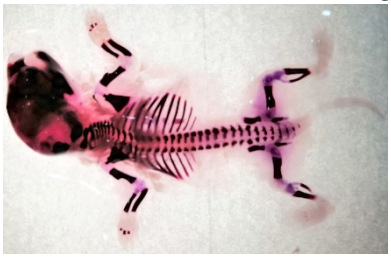


Figure-2e

Figure-2: Comparison of foetal skeleton of different groups

a) Normal foetal skeleton, b) Hemimelia (short limb) in foetal skeleton, c) Missing rib, d) Digit defect, e) Normal foetal skeleton of experimental group C

Table-4: Frequency of skeletal defects observed in each foetal group

Parameters	Animal Groups				
	A	B	C	D	E
No. of foetuses examined	14	16	22	16	20
Foetus with defects	0	0	5	2	5
Total no of defects	0	0	8	2	5
Hemimelia	0	0	0	0	1
Vertebrae agenesis	0	0	0	0	0
Ribs missing	0	0	3	0	4
Ribs supernumerary	0	0	0	0	0
Digit defects	0	0	5	2	0
Tail defects	0	0	0	0	0
Percent of foetus with defects (%)	0	0	31.25	12.5	25

As per the doses of PPD applied and frequency of defects observed, the *p* value was found to be 0.075 which was not statistically significant.

On histological examination of skin of adult rats, it was observed that topical application of different dose of PPD resulted in changes in skin usually resulting in increased keratinization and increased proliferation of epidermis resulting in increase in thickness of epidermis in all the groups except the control group.

There was a statistically significant relation between the dose of PPD, extent of keratinisation, and epidermal proliferation ($p<0.000$).

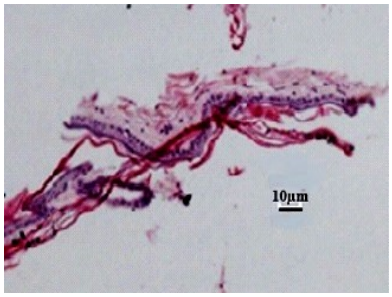


Figure-3a

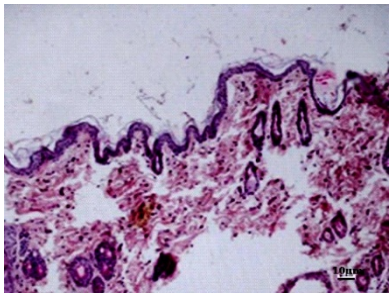
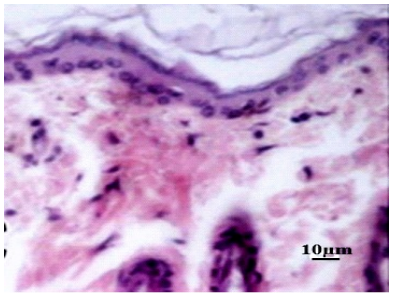


Figure-3b



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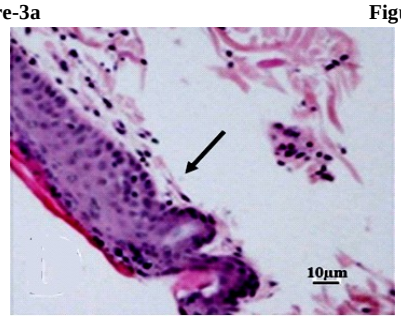

Figure-3d

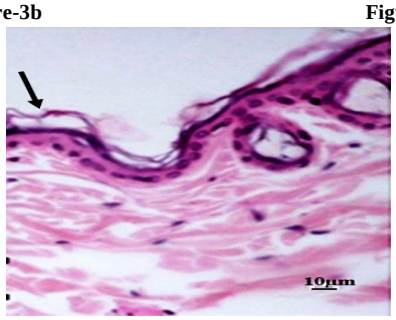

Figure-3e

Figure-3: Histologic sections of skin of adult rats of different groups

Normal skin at 10× magnification b) dye applied skin at 10× magnification c) PPD 1 mg/Kg applied skin at 10× magnification d) PPD 2 mg/Kg applied skin at 10× magnification e) PPD 3 mg/Kg applied skin at 40× magnification. Scale bar= 10 µm

DISCUSSION

This was an experimental lab-based study in which the effects of chronic application of topically applied paraphenylenediamine during gestation on foetal liver and skeleton was studied in a dose dependent manner. In addition, we looked at the local effects of topically applied PPD on the skin of adult rat. The PPD application during gestation had teratogenic effects on foetal liver. Interestingly some foetal skeletal defects were observed, however they were statistically insignificant. It was also established that chronic application of PPD leads to damaging effects on the skin of adult rat.

In this study it was found that PPD application resulted in increased incidence of all parameters of hepatotoxicity among all the study groups except for control group. Severity of hepatic injury was directly related to dose of PPD applied. These results were similar to the results found in studies of Abd-ElZaher *et al* and Bharali *et al*, where PPD induced significant increase in liver enzymes with chronic inflammation in dose dependent manner.[3,15] They studied the effects of topically applied PPD for 30 days, whereas in our study we not only examined the effects of topically applied PPD on adult rat for 60 days but also the hazardous effects of PPD on foetal liver and skeleton. We applied PPD for 40 days before matting and then 20 days after matting of the rats. Interestingly the dose of PPD applied is similar in both studies, i.e., 1, 2 and 3 mg/Kg body weight but the duration differed. They observed the changes in the adult liver parenchyma. Similar changes were observed in the foetal liver in our study. Similar inflammatory effects were found in adult rat liver after the administration of different gold nanoparticle sizes doses intraperitoneally.[25] Following Citalopram

administration in pregnant rats, inflammation of foetal liver had been observed.[26] As citalopram crosses the placenta and affects the foetus, we assume that PPD may follow the same placental transmission mechanism and cause foetal liver damage. In our study it was found that PPD had no significant teratogenic effect on foetal skeleton. Out of 88 total foetuses, skeletal defects were observed in 12 (13.6%) foetuses. Most commonly observed defects were missing ribs and digit defects followed by hemimelia. Despite occurrence of skeletal defects, no statistically significant relationship could be established with PPD exposure. This may be attributed to the limited samples size, duration of the study or the doses used in our study. How PPD have effected foetal skeletal growth needs to be studied further.

In all rats treated with PPD, epidermal thickening along chronic topical treatment of PPD in adult Sprague Dawely rats led to increased keratinization. The histopathological changes observed in our study had significant dose dependent relationship with PPD exposure. Similar results were observed by Lee *et al*[19]. Due to the production of toxic PPD metabolites in rat skin they observed decreased activity of oxygen free radical system and damaged tissue of skin suggesting that the topically applied PPD may lead to skin injury in a dose dependent manner due to oxygen free radical generation.[19] In our study also, histopathological skin changes like epidermal thickening were found to be dose dependent.

PPD is usually blended with an oxidizer to develop black colour. The allergenic hapten is produced by oxidation of PPD in epidermis or dermis. Varying degrees of skin reactions can occur due to PPD ranging from mild dermatitis, urticaria to blistering and facial edema.[27] Topically applied PPD can cause primary sensitization which results subsequently in allergic contact dermatitis. Erythema, pruritus and bullous dermatitis may manifest as skin changes.

From the above discussion it is evident that although the effects of PPD application on adult rats have been explored, the placental transmission and its fetotoxic effects need thorough consideration. Overall the study suggests that chronic topical application of PPD in doses of 1, 2 and 3 mg/Kg body weight can cause significant toxic effect on rat foetal liver and insignificant effect on rat foetal skeleton.

LIMITATIONS & RECOMMENDATIONS

We explored the histopathological features of foetal liver and gross morphological changes in foetal skeleton, the biochemical markers of hepatic injury and skeletal defects were not studied. We also did not perform chemical analysis of rat foetal blood. Further studies can be carried out to explore these aspect as it can help in measuring the dose of PPD that crossed placenta and can relate teratogenic effects with actual absorbed dose. The current study showed that application of the PPD on the skin of adult rats not only resulted in local damage to the skin but also had teratogenic effects on the liver and skeleton of the rat foetuses. Therefore, replacing hair dyes containing PPD with less harmful ones will be an urgent challenge not only from the prospective of chemical formulation but also from risk assessment prospective as well. It is necessary to take safety measures during the handling of PPD containing hair dyes for sufficient protection from its local and teratogenic effects. PPD is prohibited for use on skin because of its dangerous chemical nature. It is only allowed to be used in hair dyes when the hair dyes do not touch the scalp. Further experimental research work is required to confirm the effects of PPD mediated dermal changes in adults and its teratogenic effects on foetal liver and skeleton. Also skin at the site of application can be looked for loss of appendages such as hair follicles etc.

CONCLUSION

PPD has dose dependent statistically significant teratogenic effects on the liver of rat foetuses in terms of increased periportal inflammation and necrosis, centrilobular necrosis, dilatation of central vein and hepatocyte hypertrophy. PPD does have teratogenic effects on rat foetal skeleton in terms of missing digits, missing ribs and hemimelia but these effects were not statistically significant. PPD has statistically significant effects on the skin of adult rats in terms of histological changes, i.e., increased keratinization and proliferation of epidermis.

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