Histopathological Examination of Paraphenylen Diamine Toxicity in Female Rats Liver

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***Abstract*—Para-phenylendiamine (PPD), a widely used in a variety of industrial products and in almost all hair dye formulation has been tested for its hepatotoxicity after 6 months topical application in two different dosages (0.5 and 1mg/kg/day/week) in female Wistar rats. All serum biomarker of liver toxicity aspartate transaminase , alanine transaminase and alkaline phosphatase shows a dose dependent increase in the PPD treated group of animals, as well as mean body weights and the relative liver weights were affected. The histopathological findings in group III included congestion of central and portal veins, with cellular infiltration. Disruption of blood sinusoids , necrotic of kupffer cells, as well as proliferation of bile ducts, focal necrosis of hepatic cells with deformed nuclei which appeared in pyknosis and karyolysis stages. Vacuolar cytoplasmic degeneration led to focal lysis of liver parenchyma showed in group IV. Electron microscopic changes in the group III confirmed low electron density of hepatocytes, degranulation of rough ER cisterns, replication of smooth ER, proliferation of bile canaliculi. In contrast, PPD caused various hepatic changes in group IV as nucleus polymorphism with irregular envelope. Slipper shape with cristolysis mitochondria. Necrosis of kupffer and endothelial cells nuclei with spire the membrane. Increased lysosomal numbers.**

Key words: Paraphenylendiamine, Hepatotoxicity , Hepatic enzymes, Ultra Structure.

I. INTRODUCTION

Para-Phenylendiamine (PPD) is a common ingredient in most of the hair dye preparations. It accelerates the dyeing process and may produce local as well as systemic toxic effects when applied topically and ingested**[1].** PPD is an organic compound. derivative from aniline aromatic amine, is a colourless solid when pure. It is used primarily as a fur and hair dye and as a chemical intermediate in the production of numerous substances, including dyes and polymers **[2].** PPD is commonly used in its raw form for cosmetic purposes in Africa, Middle East and Indian subcontinent while it is rarely used in the west **[3,4].**

In Sudan, PPD is mixed with henna, leaves of Lawsonia Alba, which is a non toxic herb used to decorate the hands and feet in special social events, such as wedding ceremonies **[4]**, and over a 10-year period (1995–2005), 3159 patients were reported to suffer from PPD poisoning; among these were 568 (18%) children below the age of 14 years **[5,6,7]**. A study from Morocco described 374 cases of PPD poisoning in adults and children over a 10-year period **[5]**. A report from Tunisia showed similar results**[8],** and a report from Saudi Arabia documented a suicide attempt with PPD in a 14-year old female **[9,10,11].** Similarly in India, popular hair dyes contain PPD, among other ingredients**[5,12]**.

Some product sold as henna also contains PPD Particularly black henna**[13]**. Short exposure to high level of PPD may cause severe dermatitis, eye irritation and tearing, Asthma, renal failure, vertigo tremers, convulsions and coma. Ingestion of PPD produces rapid developments of edema of face, neck, pharynx tongue and larynx with respiratory distress which often needs tracheostomy**[1]**. In the later stages Rhabdomyolysis and acute tubular necrosis with acute renal failure and hepatic failure develops, bleeding tendency (bleeding from gums), sub-conjunctival hemorrhage and bleeding from mucus membrane also occur **[13]**.

Pathological changes in mitochondria as swollen or atrophy ,cavitations within the mitochondria, considered that an important indicator of cellular damage leading to loss of functional efficiency, added that the mitochondria destruction may be due to disturbance of calcium ions Ca+2 into cells, or block the ATP synthesis and thus block the phosphorous oxidation in mitochondria, or may be due to loss of mitochondria membrane permeability or change the cellular PH **[14]**.

Few studies were identified that investigated the dermal absorption potential of PPD in humans and animals. Under exposure conditions that mimicked the intended-use conditions for such hair dyes **[15]**. Under the intended-use conditions, dermal absorption of 0.54% to 2.7% in volunteers **[16,17,18,19]** and 2.7% in monkeys **[16,18,20]** has been reported. Dermal absorption of 2.7% has been noted in human cadaver skin **[6,10]**. The degree of dermal absorption was reported as 0.93% **[18]** in excised pig skin **[19]** reported similar dermal absorption values, of 2.44% and 3.39% in vitro, in human and pig skin, respectively.

In addition tattoos are very popular in Arabian Gulf, especially in Saudi Arabia . Since, tattoo industry is not regulated, people are still getting black henna tattoos and exposing themselves to serious toxicity problems **[14]**. A very few report are available that pointed towards the hepatotoxicity by hair dye ingredients **[21]**, and chronic dermal exposure and subsequent histopathological alterations of liver tissue by PPD substance. Therefore the present study was conducted to analyze dose dependent hepatic effect of repeated topical application of Paraphenylen Diamine on hepatocytes structure and liver function of female rats.

II. MATERIALS AND METHODS

A. Chemical

P-phenylendiamine (PPD) light purple powder, purchased from commercial store in Jeddah of Saudi Arabia. CAS No : 106–50–3, Batch: 99E483**.** Molecular weight (MW): 108 and 98% purity was. C6H8N2 (free base), C6H8N2 . 2HCl (dihydrochloride),C6H8N2 . H2SO4 (sulfate) **(OECD,2010a ; Hummdi,2012)**.

Structural Formula

B. Animals and their treatment

The study was conducted according to OECD **[22,23]** on the design and conduct of chronic toxicity and the CE **[23]** recommendations on rodents housing, Wistar rats (*Rattus norvegicus albinus*) 35 weeks old female rats, (n=80), with an average body weight of about 282.67±7.10 g were obtained from the animal house of the King Fahd Center for Medical Research, King Abdul Aziz University in Jeddah of Saudi Arabia. The Animals housed under a 12 h light/12 h dark cycle in stainless steel cages and allotted randomly to four groups(n=20). Group I and II was the control groups, while groups III and IV were topical application of 0.00 5 or 0.001 ml (0.5 and 1 mg/kg/bw) respectively of PPD dissolved in double (degassed purified water) in a shaved of approximately 10% of the total body surface area of inter scapular skin for 30 minutes per day, 7 days per week, for a period of 6 months **[23,24,25]**. Since, the control groups received degassed purified water painted on their dorsal side as in other PPD treated animals. The rats were weekly weighed and observed daily for signs of toxicity and for mortality.

C. Biochemical assays

At termination of the study, the blood samples were collected 24 hours after the experiment and analyzed for aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) by using the commercial kits according to procedures**. [26]** procedures.

D. Histological study

After bleeding, the rats were sacrificed by cervical dislocation and the abdominal cavity was opened up to expose the liver which were quickly dissected out, weighted and fixed in 10% formal saline for routine histological techniques. The tissues were dehydrated in an ascending grade of alcohol (ethanol), cleared in xylene and embedded in paraffin wax. Serial sections of 2 microns thick were obtained using a rotator microtome. The deparaffinized sections were stained routinely with hematoxylin and eosin **[27]**. Photomicrographs of the desired sections were made by light microscope for further observations.

E. Electron Microscopy

Samples of liver were fixed in 2.5% glutaraldehyde and 0.25 M sodium cacodylate, post-fixed in 1% osmium tetroxide, and embedded in Spurr's epoxy. Ultrathin sections were picked up on nickel grids, stained with uranyl acetate/lead citrate **[28],** and examined in a Philips TEM 100 microscope.

F. Statistical Analysis

Mean ± standard error values were calculated for each group. Differences in the experimental data were analyzed using Student's t-test. P-values <0.05were considered to be significant. All statistical calculations were performed using SPSS 18.0 software for Windows.

III. RESULT

A. Clinical signs and mortality

Mortality and clinical signs of each groups were significantly associated with the concentration of PPD administered **table 1.**

Table 1:Clinical symptoms and the mortality recorded in groups 3 and 4 of PPD treatment rats after 24hr -6 months.

Clinical symptoms	No. of Experimental animals =20	%	No. of Experimental animals =20	%
	N0.of individual		N0.of individual	
Swelling face and neck	17	85%	19	95%
Dark discoloration of urine	17	85%	18	90%
Ataxia	13	65%	15	75%
Skin changes(dermatitis)	6	30%	10	50%
Mortality	2	10%	7	35%

B. Body and liver weights

Significant differences were observed in the terminal body weight gain among the treated and control group of animals. Both absolute and relative weight exhibits significant dose response increase after PPD treatment compared to controls **table 2.**

Table 2:Body weight and liver weight(absolute and relative) of experimental rats after 6 month topical application of PPD.

Experimental Groups					
Weights		Control (G1)	Control (G2)	Treated (G3)	Treated (G4)
Body Weigh	M	282.6667	283.5664	290.0000	295.3333
	±SEM	±7.10712	±7.00732	±6.57774	±5.95912
	P	-	-	.0126*	.000**
Absolute Liver weight	M	8.1000	8.0100	8,5000	9,6000
	±SEM	±.31623	±.32623	±.44721	±.46000
	P	-	-	.0351*	.000**
Relative liver weight	M	4.0500	4.0550	4.2500	4,8000
	±SEM	±.15811	.26311 ±	± .22360	.2300 ±
	P	-	-	.0632*	.000**

P < 0.05 , P < 0.001**

C. Effects of PPD on liver function

Table 3 showed the serum biomarker of liver toxicity (AST, ALT and ALP) associated with cell damage in the chronic phase (post – acute) **.** Shows a dose dependent increase in the PPD treated group of animals compared to their untreated control group. Serum ALP of treated animals shows fold increases over control group, While the record Serum AST, ALT decreased respectively.

Table 3: aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) of experimental animals liver after 6 month topical application of PPD.

Experimental Groups					
Kidney bioassay		Control (G1)	Control (G2)	Treated (G3)	Treated (G4)
aspartate transaminase (AST) mg/dl	M	44.2000	44.1000	41.8000	37.8000
	±SEM	±1.15758	±1.15658	±1.93391	±2.95635
	P	-	-	. 3450	.0950*
alanine transaminase (ALT) mg/dl	M	27.0000	27.1000	25.8000	22.4000
	±SEM	±3.4785	±3.4775	±2.3323	±1.5033
	P	-	-	.439	.688
alkaline phosphatase (ALP)	M	181.1000	181.0000	196.6000	198.2000
	±SEM	± 14.4256	±14.4155	± 23.1702	± 19.6198
	P	-	-	.492	.606

P < 0.05 , P < 0.001**

D. Histological Results

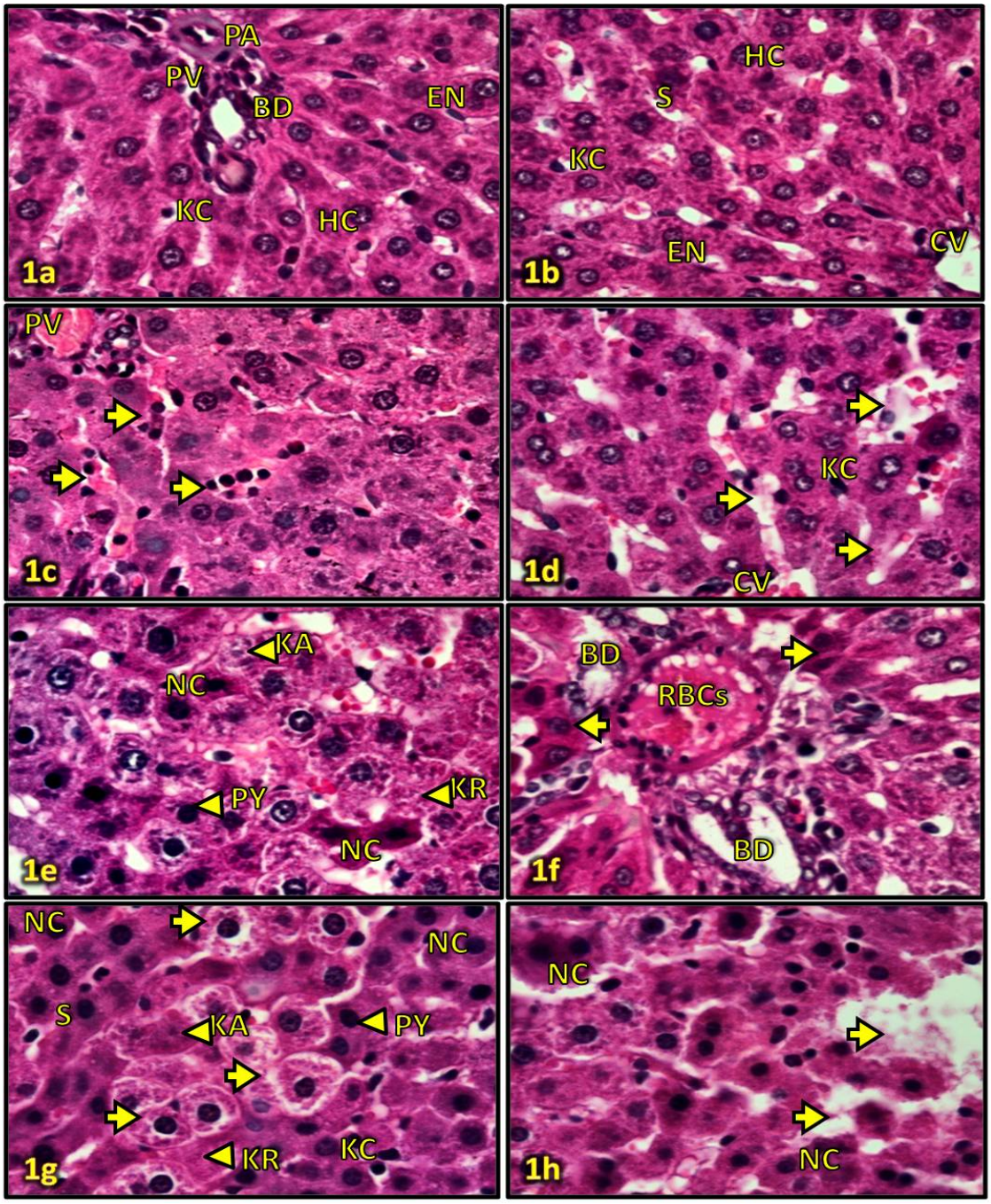
Figures (1a,b,2a,3a,b) represent the normal histological and cytological structure of liver parenchyma and hepatocytes .However, the observation of group III revealed microscopical and cytological alterations caused by PPD treatment when compared to control animals. Such as congestion of central vein, portal vein and cellular infiltration of macrophages and lymphocytes accompanied by edematous of the liver parenchyma. Focal necrosis of hepatic cells with pyknosis, karryohexsis and karyolysis nuclei, in addition to, disruption of blood sinusoids, kupffer and endothelial cells necrosis (**Figs. 1c-e**). While subcellular study confirmed mitochondrial clustered and atrophied, deformed peroxisomes, degranulation of rough ER cisterns (RER), replication of smooth ER (SER), segregation of nucleoli structure. Proliferation of bile canaliculi with dissolution of desmosomes (**Figs. 2b**).

PPD treated liver in group IV showed proliferation of bile ducts, rupture of portal vein structure with red blood cells stasis and expanded of lymphatic vessels. Necrotic some hepatocytes around the portal areas and central vein and other showed vacuolar cytoplasmic degeneration with Karyolysis nuclei. These changes led to blood sinusoids collapsed and focal lysis of liver parenchyma (**Figs. 1f-h**). On the other hand, PPD caused various hepatic ultrastructure changes, The nucleus showed necrotic or poly morphism with spire membrane, condensed euchromatin. Increased lysosomal numbers, replication of SER and RER, mitochondrial swollen, elongated with slipper shaped accompanied with cristiolysis were showed (**Figs. 2c-d**) The kupffer and endothelial cells appeared necrotic,. in addition, devastation microvilli of Disse space and deformed red blood cells in blood sinusoid were been observed (**Figs. 3c-d**).

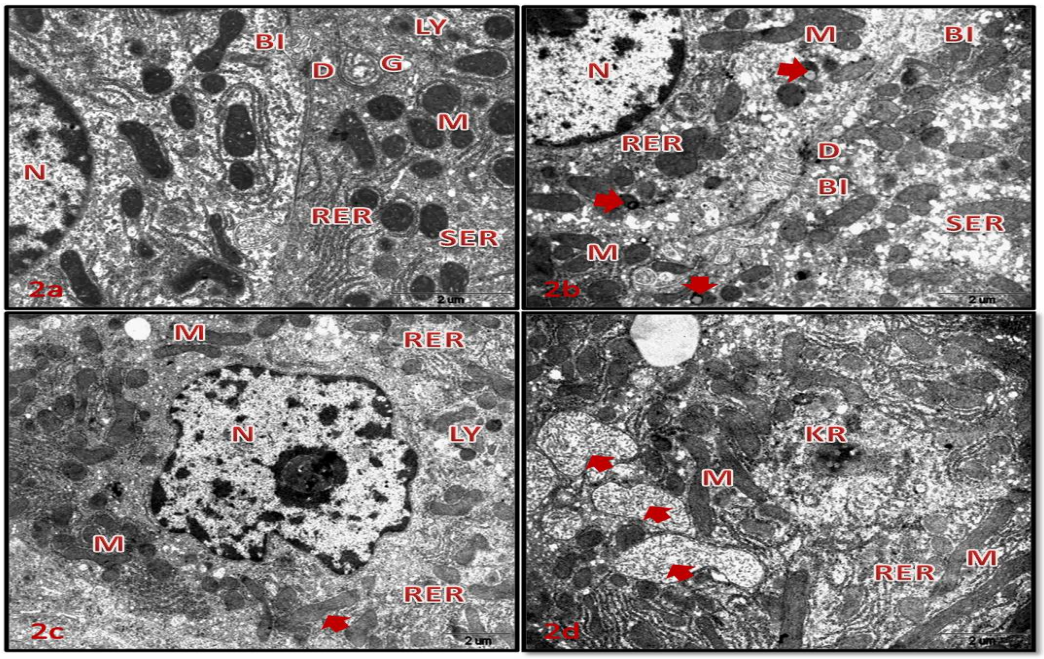
IV. DISCUSSION

It was observed morphological changes as swelling of the neck, urine color changed and dermatitis and high rate of mortality related to a test concentration of PPD on treatment animals, this is consistent with a number of previous studies which indicated that the PPD topically applied reaches the systemic circulation after absorption through the skin **[14,18,29]**. **[14]** added that the mortality rate in male rats treated with PPD is 21.1%, and 22% in cases of poisoning from hair dye, 10% -35 in female Wistar rats and 41.9% in PPD poisoning cases.

The present results indicates that the selected doses of PPD on topical exposure causes increased in absolute and related liver weight gain which may be due the congestion of blood sinusoids and central vein, and sharp rises in the serum biomarker of liver tissue, that followed a dose dependent pattern. Increased in ALP level and decreased in AST &ALT levels in treated animals confirmed by **[30]**, and **[31]** who reported that the ALP is an enzyme in the cell lining the bile duct of the liver and the ALP levels in plasma will rise with bile duct destruction suggesting that PPD led to bile ducts



Plate(1a-h): Transverse liver sections of female rats: **(a,b)**: showing the normal histological structure of hepatocytes and portal area, portal artery **(PA),** portal vein **(PV),** bile duct **(BD),** central vein **(CV),** hepatic cell **(HC)**, sinusoidal blood **(S)**, kupffer cell **(KC)** and endothelial cell **(EN)**;x400. **(c-e):** sections of 0.5 mg/kg PPD treated liver**:(c):** congestion of the portal vein **(PV)** and lymphocytes and macrophages aggregation **(arrows)** in hepatic parenchyma ;x400**.(d):** sinusoidal rupture with edematous **(arrows)** and kupffer cell **(KC)** ;x400. **(e):** focal necrosis of hepatic cells **(NC)** with pyknosis **(PY),** karyrrhexis **(KA)** or Karyolysis **(KR)** nuclei;x400. **(1f-h):** sections of 1 mg/kg PPD treated liver **:(f):** showing rupture of the portal area with red blood cells stasis **(RBCs)** and necrosis of hepatocytes around portal area **(arrows),** proliferation of bile ducts **(BD) ;** x400**. (g):** vacuolar cytoplasmic degeneration **(arrows),** necrotic cells **(NC),** blood sinusoids collapsed **(S),** nuclei pyknosis **(PY),** karyrrhexis **(KA),** Karyolysis **(KR),** and kupffer cells **(KC)** necrosis ;x400.(h): sever necrotic cells (NC) in hepatic lysis, nuclei pyknosis (arrows) ;x400.



Plate(2a-c): Electron micrographs of hepatocytes of female rats: **(a)** normal hepatocyte showing nuclei (**N**), mitochondria **(M**), rough endoplasmic reticulum (**RER**), smooth endoplasmic reticulum (**SER**), lysosomes (**LY**) Golgi bodies(**G**), bile canaliculus(**BI**) and desmosomal junction (**D**);**x10500.** (**b**): parts of two hepatocytes treated with 0.5 mg/kg PPD, showing condense the peripheral chromatin and the nuclear envelope(**N**) mitochondrial aggregated and atrophied (**M**), deformed peroxisomes **(arrows),** degranulation rough ER **(RER),** increased smooth ER **(SER)** profile, proliferation of bile canaliculus(**BI**) with deformed Desmosomes (**D**); **x10500. (c, d):** E.M. of part of Hepatocytes 1 mg/kg PPD treated: **(c):** showing irregular nuclear (**N**) envelope, condense euchromatin, elongated (**arrows**) and slipper shaped mitochondria (**M**), increased lysosomes numbers**(LY),**replication of rough ER (**RER**); **x5800.** (**d**): showing nuclear karyolysis **(KR**), mitochondrial elongated (**M**) and cristolysis (**arrows**), replication of rough ER (**RER**), ;**x7900.**

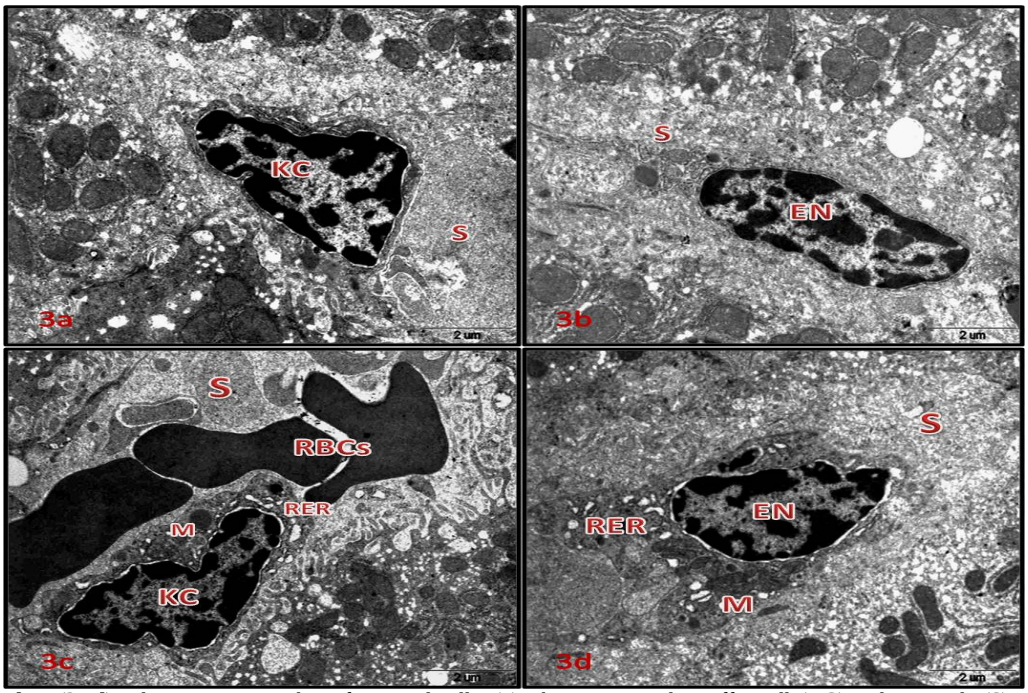


Plate (3a-d): Electron micrographs of sinusoid cells: (**a**): showing normal Kupffer cell (**KC**) and sinusoid (**S**);**x** (**7900**). **(b)**:showing normal endothelial cell (**EN**) in sinusoid cavity (**S**);**x**(**7900**). (**3c,d**): E.M. of 1mg/kg PPD treated hepatocytes:**(c)**: showing necrotic kupffer cell (**KC**), with deformed mitochondria (**M**) and (**RER**) expanded cisterns, also note: deformed red blood cells (**RBCs**) in in sinusoid cavity(**S**) ;**x7900.(d**): showing necrotic endothelial cell (**EN**)in sinusoid cavity (**S**) with atrophied mitochondria (**M**) and (**RER**) vesiculation ;**x7900.**

lesions in both rats treated groups in this study. while, AST and ALT associated with liver parenchyma cells so they are raised in acute liver damage, following the level decrease gradually in chronic diseases or toxic substance **[31,32]**,and supported the hepatocellular injury damage bile canalicular microvilli with deformation of surrounding desmosomes in recent work.

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 or indirectly from immune mediated membrane damage **[33]**. As were appeared in the present study the prolonged exposure to the toxin turned the degenerative changes into necrotic damage and tissue lysis. This result is confirmed by **[34]** who suggested that the cellular degeneration might be attributed to liberation of acid hydrolyses released from the destructed lysosomes to facilitate the process of autolysis. However, in the current work, pyknosis and karyolysis of cell nuclei may indicate the loss of functional efficiency of the cells. Similar results have been demonstrated by **[35].**It was evident that the degenerative changes appeared earlier in the cytoplasm than in the nuclei of hepatocytes. This result is consistent with the findings of **[36]** who stated that the nuclear damage is a sequence of cytoplasmic damage.

In contrast, the cytoplasmic vacuolation were showed in group IV treated rats. Similarly**[3]** and **[14]** described sever tubular necrosis in kidney PPD poisoning as vacuolar degeneration, nuclear pyknosis and cytoplasmic vacuolations brush border damage. Previously **[36]** study described the vacuolation of hepatocytes as ballooning degeneration and interpreted it as a kind of cellular defensive mechanism against injurious substances. **[37]** added these vacuoles are responsible for collecting the injurious elements and preventing them from interfering with the biological activities of these cells.

However, the present results are consistent with the **[38]** who indicated that the cell swollen may be due to inhibition of the production of energy as a result of the damage mitochondrial crista and increase the permeability of the cell plasma membrane**[39]**. Additionally, in early stages of hepatotoxicity proliferation of the smooth endoplasmic reticulum (SER) can be seen to face the adverse effects associated with an increase of detoxification enzymes, and with ongoing cellular toxicity the SER comes to occupy most of the cell, both by proliferation ,and dilatation of its cisternae with vesiculated **[37]**. Moreover, the proliferation of RER is the compensate activity for the deterioration of the cellular protein when cells are exposed to various toxins **[40]**.

Acute and chronic hepatitis is pathologically characterized by a prominent infiltration of lymphocytes into the liver **[41]** and this histological feature is predominantly found in the liver of PPD treated rats. The main encountered inflammatory cells in the recent work was macrophages and lymphocytes which predominant in intoxication, viral and protozoal diseases, cause that the lymphocytes produce anti-toxins and accelerate cell healing, whereas, the macrophages destroyed the causes of damage and injured tissues **[42]**. Furthermore, **[37]** reported that the inflammation is accompanied by lethal damage to endothelial cells, loss of tone leads to marked expansion of the blood vessels and packing of the lumen with erythrocytes.

On the contrary, the current work revealed Kupffer and endothelial cells necrosis , theses finding consistent with **[43]**study were attributed. **[44]** added the PPD induces free radicals subsequently results in lipid peroxidation **[43]**, which led to liver cells necrosis, and renal tissue necrosis **[14,42]**. However, the proliferation of bile ducts in this work is in agreement with **[45]** who stated that the cells lining the bile ducts are stem cells that activated and prolifered with necrosis or lysis of liver cells.

V. CONCLUSION

The doses (0,5 and 1 mg kg) selected in the present work for 6 months revealed liver toxicity and ultrastructure alterations resulting in impairment of liver function. Further experimental information is necessary to confirm the role and mechanism of PPD mediated hepatotoxicity.

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